UNIVERSIDAD DE GRANADA

FACULTAD DE ODONTOLOGÍA

PROGRAMA DE DOCTORADO EN MEDICINA

CLÍNICA Y SALUD PÚBLICA

"RELEVANCIA DE LA BANDA CROMOSÓMICA 11q13 EN CARCINOGÉNESIS ORAL. UN ESTUDIO METAANALÍTICO Y EXPERIMENTAL SOBRE *CCND1*/CICLINA D1 Y *CTTN*/CORTACTIN"

TESIS DOCTORAL

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2018

Editor: Universidad de Granada. Tesis Doctorales
Autor: Pablo Ramos García
ISBN: 978-84-1306-092-7
URI: http://hdl.handle.net/10481/54755

La presente Tesis Doctoral está avalada por los siguientes artículos científicos:

- Ramos-García P, Ruiz-Ávila I, Gil-Montoya JA, Ayén Á, González-Ruiz L, Navarro-Triviño FJ, González-Moles MÁ. Relevance of chromosomal band 11q13 in oral carcinogenesis: An update of current knowledge. Oral Oncol. 2017 Sep;72:7-16. doi: 10.1016/j.oraloncology.2017.04.016.

  Factor de impacto (JCR): 4.636 - Primer decil

- Ramos-García P, Gil-Montoya JA, Scully C, Ayén A, González-Ruiz L, Navarro-Triviño FJ, González-Moles MA. An update on the implications of cyclin D1 in oral carcinogenesis. Oral Dis. 2017 Oct;23(7):897-912. doi: 10.1111/odi.12620.

  Factor de impacto (JCR): 2.310 - Primer cuartil

- Ramos-García P, González-Moles MÁ, González-Ruiz L, Ayén Á, Ruiz-Ávila I, Navarro-Triviño FJ, Gil-Montoya JA. An update of knowledge on cortactin as a metastatic driver and potential therapeutic target in oral squamous cell carcinoma. Oral Dis. 2018 Jun 7. doi: 10.1111/odi.12913

  Factor de impacto (JCR): 2.310 - Primer cuartil

- Ramos-García P, González-Moles MÁ, González-Ruiz L, Ruiz-Ávila I, Ayén Á, Gil-Montoya JA. Prognostic and clinicopathological significance of cyclin D1 expression in oral squamous cell carcinoma: A systematic review and meta-analysis. Oral Oncol. 2018 Aug;83:96-106. doi: 10.1016/j.oraloncology.2018.06.007

  Factor de impacto (JCR): 4.636 - Primer decil

- Ramos-García P, Bravo M, González-Ruiz L, González-Moles MÁ. Significance of cytoplasmic cyclin D1 expression in oral oncogenesis. Oral Dis. 2018 Mar;24(1-2):98-102. doi: 10.1111/odi.12752.

  Factor de impacto (JCR): 2.310 - Primer cuartil

- Ramos-García P, González-Moles MÁ, Ayén Á, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Gil-Montoya JA, Bravo M. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression in adjacent non-tumour epithelium in oral squamous cell carcinoma. Arch Oral Biol. 2018 Oct 9;97:12-17. doi: 10.1016/j.archoralbio.2018.10.007

  Factor de impacto (JCR): 2.050 - Primer tercil, segundo cuartil

Por los siguientes trabajos actualmente sometidos a revisión por pares:

- Ramos-García P, González-Moles MA, Ayén A, González-Ruiz L, Ruiz-Ávila I, Gil-Montoya JA. Prognostic and clinicopathological significance of CTTN/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis 2018: [In Submission]

- Ramos-García P, González-Moles MA, González-Ruiz L, Ayén A, Ruiz-Ávila I, Bravo M, Gil-Montoya JA. Prognostic relevance of tumor cyclin D1 expression in oral cancer 2018: [In Submission]

Por las siguientes comunicaciones orales y posters en reuniones científicas:

- Ramos-García P, González-Ruiz L, Ayén A, Ruiz-Ávila I, Lenouvel D, Talbaoui A, Gil-Montoya JA, González-Moles MA. Prognostic and clinicopathological significance of cyclin D1 expression in oral squamous cell carcinoma: a systematic review and meta-analysis. Formato: Póster. VII International Symposium "Advances in Oral Cancer". Bilbao, Septiembre 2018.

- Ramos-García P, Ayén A, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Talbaoui A, Bravo M, Gil-Montoya JA, González-Moles MA. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression during malignant transformation of the oral epithelium. Formato: Póster. VII International Symposium "Advances in Oral Cancer". Bilbao, Septiembre 2018.

- Ramos-García P, González-Ruiz L, Ayén A, Ruiz-Ávila I, Talbaoui A, Lenouvel D, Bravo M, Gil-Montoya JA, González-Moles MA. Significance of cytoplasmic cyclin D1 expression in oral oncogénesis. Formato: Póster. VII International Symposium "Advances in Oral Cancer". Bilbao, Septiembre 2018.

- Ramos García P, Ruiz Ávila I, Gil Montoya JA, Ayén A, González Ruiz L, Navarro Triviño FJ, González Moles MA. Implicaciones de la sobreexpresión de ciclina D1 en carcinogénesis oral. Formato: Comunicación Oral. XIV Congreso Internacional de la Sociedad Española de Medicina Oral (SEMO). Granada , Junio 2017

Y por los siguientes premios:

- Ramos-García P, Ayén A, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Talbaoui A, Bravo M, Gil-Montoya JA, González-Moles MA. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression during malignant transformation of the oral epithelium. Formato: Póster. VII International Symposium "Advances in Oral Cancer". Bilbao, Septiembre 2018.

  Premio: Research Poster Award

- Ramos García P, Ruiz Ávila I, Gil Montoya JA, Ayén A, González Ruiz L, Navarro Triviño FJ, González Moles MA. Implicaciones de la sobreexpresión de ciclina D1 en carcinogénesis oral. Formato: Comunicación Oral. XIV Congreso Internacional de la Sociedad Española de Medicina Oral (SEMO). Granada , Junio 2017

  Premio: Mejor Trabajo de Investigación

A Ángela y a mis padres

"si he podido ver un poco más lejos es

porque iba subido a hombros de gigantes"

Isaac Newton

AGRADECIMIENTOS

Quiero expresar mis más sinceros agradecimientos:

A mis directores de Tesis Doctoral. Gracias a mi maestro, Profesor Dr. Miguel Ángel González Moles, por haberme dado la oportunidad de trabajar a su lado cuando un día llegué a su despacho con la inquietud de iniciarme en el mundo de la investigación. Gracias por toda su dedicación y apoyo, por su confianza en mí, por su bien hacer, por enseñarme a crear ciencia de calidad, y a vivirla con ilusión y disfrutarla. En definitiva, gracias por ayudarme a cumplir este sueño. Gracias al Profesor Dr. José Antonio Gil Montoya por su cordialidad y cercanía, por asesorarme y guiarme siempre con tanta profesionalidad y competencia, y por todo su apoyo durante el desarrollo y crecimiento de mi carrera investigadora.

A la Dra. Isabel Ruiz Ávila, por haber puesto a nuestra disposición todos los medios técnicos y humanos propios del Servicio de Anatomía Patológica. Por todo su tiempo e inestimable ayuda, y por su cercanía y amabilidad. Sin su persona el desarrollo de la presente Tesis Doctoral no hubiera sido posible.

Al Profesor Dr. Manuel Bravo, Catedrático de Odontología Preventiva y Comunitaria de la Universidad de Granada, por su colaboración en este proyecto y aportar su conocimiento y experiencia en el apasionante mundo de la estadística. Y al Profesor Dr. Miguel Delgado, Catedrático de Medicina Preventiva de la Universidad de Jaen, por haberme enseñado los secretos del metaanálisis y por haber revisado algunos de los aspectos técnicos metaanalíticos de este proyecto.

A Daniel Lenouvel y Asmae Talbaoui, por compartir conmigo la pasión por la investigación, y porque trabajar en equipo divide el trabajo y multiplica los resultados.

A todos mis compañeros de despacho, especialmente a la Dra. Inmaculada Cabello, por todas las horas compartidas, por las dudas resueltas, y por estar ahí para todo lo que he necesitado.

A mis padres, por apoyarme en este camino, por haberme inculcado desde pequeño el valor del trabajo y la constancia como modelo para alcanzar los objetivos. Y por todos los consejos y el cariño que me transmiten desde la distancia.

A mi familia, en especial a Purificación, mi querida abuela, por representar un pilar fundamental en mi vida y ser una segunda madre para mí. Y a mi tía Celia, por compartir conmigo el entusiasmo de nuestras Tesis Doctorales.

A Jesús y Antonia, siempre dispuestos a escucharme y guiar mi camino con sus valiosos consejos. Y al pequeño de la casa, por su amistad incondicional.

Y a Ángela, por ser de vital importancia en mi desarrollo personal y profesional, por hacerme ver que no hay límites que no pueda superar con mi esfuerzo. En definitiva, por todo.

A todos, gracias.

ÍNDICE

RESUMEN

Un evento relevante en la carcinogénesis oral está constituido por la amplificación de la banda cromosómica 11q13, en la que se ubican numerosos oncogenes que frecuentemente se encuentran co-amplificados. Entre estos genes, destacan *CCND1* -que codifica a la proteína ciclina D1- y *CTTN* -que codifica a la proteína cortactin-, considerados hoy como oncogenes fundamentalmente debido a las funciones oncogénicas putativas que les han sido atribuidas, y a sus potenciales implicaciones pronósticas, lo que plantea su posible valor como futuras dianas terapéuticas en el cáncer oral. En la presente Tesis Doctoral, mediante una meticulosa revisión de la literatura se constata la relevancia de la banda cromosómica 11q13, así como las implicaciones de las alteraciones de CCND1/ciclina D1 y CTTN/cortactin tanto en estadíos tempranos de la carcinogénesis oral como en carcinomas orales de células escamosas (COCEs). Adicionalmente, mediante técnicas metaanalíticas se pudo evidenciar el importante impacto que ejercen la sobreexpresión de ciclina D1 y las alteraciones de CCND1/cortactin sobre parámetros clinicopatológicos (mayor tamaño tumoral, status N+, estadío clínico avanzado y pobre grado de diferenciación histológica) y de supervivencia de los pacientes con COCE y de cabeza y cuello, respectivamente. Además, se realizó un estudio experimental, mediante técnica inmunohistoqúima tratando de analizar las implicaciones de ciclina D1 en COCE. Se observó que la sobreexpresión de ciclina D1 nuclear está asociada a parámetros que implican un pobre pronóstico en COCE (grado de diferenciación y sobreexpresión de ki-67). La sobreexpresión concomitante de ciclina D1 en núcleo y citoplasma se asoció con una morfología invasiva de las células tumorales, quedando por dilucidar si este evento putativo es consecuencia de la ciclina D1 exclusivamente citoplasmática, o si por el contrario también depende de su expresión en el compartimento nuclear. Los resultados obtenidos también evidencian que la sobreexpresión de ciclina D1 citoplasmática se correlaciona con un pobre grado de diferenciación, con un estadío clínico avanzado, y con sobreexpresión de ki-67 y así mismo, la intensidad de expresión de ciclina D1 citoplasmática se asoció con una morfología invasiva. Se pudo también demostrar una notable expresión de ciclina D1 en el tejido epitelial no tumoral adyacente a carcinoma oral de células escamosas. Esta expresión se localizó sobre todo en el núcleo de las células epiteliales de las capas basal, parabasal y tercio medio. Los resultados también señalaron que la expresión basal y parabasal en el núcleo de células de epitelios no tumorales alejados del punto de invasión tumoral fue significativamente mayor en pacientes que desarrollaron más de 2 tumores. Observamos que la mayor expresión nuclear de ciclina D1 en los diferentes estratos del epitelio no tumoral cercano al punto de invasión se asoció también a una mayor expresión en los diferentes estratos del epitelio alejado del punto de invasión. Por último, destacó la asociación encontrada entre la expresión nuclear de ciclina D1 y la expresión de ki-67 en la capa basal del epitelio alejado del punto de invasión invasión. Como conclusión, la presente Tesis Doctoral constata la relevancia e implicaciones oncogénicas de la amplificación de la banda cromosómica 11q13 en COCE, así como de las alteraciones de *CCND1*/ciclina D1 y de

CTTN/cortactin. Nuestra revisión sistemática y metaanálisis presenta resultados consistentes que aconsejan incluir la valoración inmunohistoquímica de la sobreexpresión de ciclina D1y cortactin en la evaluación pronóstica del COCE. Además, nuestro estudio experimental mediante técnica inmunohistoquímica también soporta estas conclusiones para la valoración nuclear de ciclina D1 y demuestran que la expresión de ciclina D1 citoplasmática es un evento frecuente en COCE, que parece poseer funciones ligadas al aumento de la migración e invasión celular, también asociada a un pobre pronóstico. Por último, la sobreexpresión de ciclina D1 constituye un evento precoz en la carcinogénesis oral ligado a un cambio del patrón proliferativo fisiológico hacia un patrón de proliferación simétrica anormal. La sobreexpresión de ciclina D1, sobretodo en capas basales y parabasales de epitelios no tumorales alejados del punto de invasión, se comporta como un marcador de campos premalignos y de riesgo de desarrollo tumoral múltiple.

INTRODUCCIÓN

Concepto y tendencias estadísticas del carcinoma oral de células escamosas

El cáncer oral es una neoplasia maligna que se desarrolla en la cavidad oral. Aproximadamente el 90% de estos cánceres son carcinomas orales de células escamosas (COCE), que representa el cáncer más común de cabeza y cuello (excluyendo cáncer de piel no melanoma). El 10% restante incluye adenocarcinomas derivados de glándulas salivares menores, sarcomas, linfomas malignos y cáncer metastásico procedente de otras localizaciones anatómicas [1]. El cáncer oral (incluyendo cáncer de labio) presenta una incidencia de 300.400 nuevos casos y 145.400 muertes anuales a nivel mundial, según la última estimación publicada por el proyecto GLOBOCAN (IARC, WHO) [2]. A pesar de los avances en la prevención, diagnóstico y tratamiento, la tasa de supervivencia del COCE continúa siendo pobre, rondando aproximadamente el 40-50% a los 5 años [3].

Factores etiológicos

Tabaco. El consumo de tabaco continúa siendo el factor de riesgo más relevante en la etiopatogenia del COCE. Un metaanálisis comunicó que el riesgo de desarrollar COCE es 3.43 veces superior en fumadores que en no fumadores [4]. Aunque los cigarrillos representan mundialmente la forma predominante de consumo tabaco, diferentes tipos abundan y varían en popularidad según la región geográfica. En el sudeste asiático es una práctica común el consumo de nuez de Betel (600-1200 millones de personas) [5,6], y en los países occidentales, las modalidades principales de tabaco sin humo incluyen el snuff húmedo, el snuff seco, y el tabaco de mascar.

Alcohol. La mayoría de estudios han publicado un riesgo aumentado de desarrollar COCE al asociar el consumo de tabaco con la ingesta severa de alcohol, con estimaciones de odds ratio (OR) que oscilan entre 4.1-8.8 [7]. Además, el alcohol parece ser un factor de riesgo independiente, existiendo estudios en no fumadores en los que se observó una fuerte asociación entre el consumo de alcohol y el desarrollo de COCE [7]. Su mecanismo etiopatogénico subyacente parece obedecer a que el etanol se metaboliza por las células epiteliales y la microflora hacia acetaldehído, un conocido carcinógeno. Cabe destacar que se han identificado polimorfismos en los genes que metabolizan el alcohol (*e.g.* alcohol deshidrogenasa 1B/C y aldehído deshidrogenasa 1/2) que parecen asociarse con un riesgo aumentado de desarrollar cáncer oral [8,9]. Además, las bebidas alcohólicas pueden contener aldehído y varios carcinógenos concomitantes, tales como hidrocarburos aromáticos policíclicos y nitrosaminas [7,10].

Virus del papiloma humano (VPH). En las últimas décadas, la evidencia acumulada en estudios

epidemiológicos, clínico-patológicos y moleculares ha establecido que la infección por VPH es un importante factor etiológico en los carcinomas de células escamosas de cabeza y cuello, esencialmente en aquellos que asientan en la orofaringe [1]. En contraste, sólo una pequeña proporción de los COCEs de otras localizaciones parece estar causada por VPH [1,11]. Específicamente, el genotipo de alto riesgo VPH-16, representa la gran mayoría (aproximadamente el 90-95%) de los carcinomas de células escamosas de orofaringe HPV-positivos. Contrariamente existe una gran variabilidad en los tipos de VPH en otras sublocalizaciones de COCE [1,11].

Factores etiológicos adicionales. Numerosos factores etiológicos adicionales también podrían participar potencialmente en la etiopatogenia del COCE: microorganismos (*e.g.* Candida albicans), factores dietéticos protectores (*e.g.* carotenos, vitamina C y E, folato, flavonoides, fibra y licopeno), deficiencias vitamínicas (*e.g.* vitamina D) y de minerales (e.g. hierro), el status inmune (*e.g.* pacientes VIH positivos), contaminantes medioambientales (*e.g.* arsénico), exposiciones ocupacionales (*e.g.* metal, madera y cemento) y condiciones hereditarias (*e.g.* anemia de Fanconi, disqueratosis congénita y síndrome de Bloom) [1].

Aspectos moleculares relacionados con el desarrollo de COCE

El COCE es una enfermedad heterogénea. En los últimos años se acepta que pueden distinguirse al menos dos subclases genéticas, determinadas por su asociación con la infección por VPH, que presentan características clínicopatológicas y moleculares diferentes: tumores HPV-positivos y tumores HPV-negativos [12]. Brevemente sus características diferenciales radican esencialmente en: la incidencia progresivamente creciente de los COCEs VPH-positivos que además presentan un mejor pronóstico, una escasa asociación a mutaciones p53, ausencia de relación con el consumo de tabaco, y presencia de sexo oral como factor de riesgo, hechos que no se observan en COCEs VPH-negativos [12]. Los VPH-positivos además tienen predilección anatómica por la orofaringe [12].

Un aspecto clave en la fisiopatología del COCE es el desarrollo tumoral múltiple, que aparece en más del 30% de los pacientes con un COCE primario y es atribuible a la presencia de campos premalignos orales genéticamente alterados [13], que confieren un mal pronóstico a los pacientes con cáncer oral [13]. Aunque existen técnicas moleculares para el diagnóstico de estos campos, como el análisis de la pérdida de heterocigosidad en 3p, 9p y 17p, o el estudio del estado mutacional del gen supresor tumoral TP53 [13], estas son caras, complejas y no están universalmente disponibles para su aplicación rutinaria. El estudio de la proliferación de las

células epiteliales resulta en este contexto importante, dado su papel clave en la expansión de estos campos y en el riesgo de desarrollo tumoral múltiple [13].

La fisiopatología molecular del COCE es compleja, habiéndose identificado numerosas alteraciones moleculares subyacentes a la enfermedad [12]. En 1996 se publicó el primer modelo multipaso de progresión genética para HNSCC [14]. Este modelo trató de caracterizar la base genética de los cambios morfológicos que ocurren en el epitelio escamoso durante la carcinogénesis. La pérdida de heterocigosidad en los cromosomas 3p, 9p y 17p parecía ocurrir en la displasia, que representa el estado morfológico tisular de la carcinogénesis temprana, mientras que otras alteraciones en los cromosomas 11q, 4q y del 8 estaban típicamente presentes en los carcinomas plenamente desarrollados [14]. En general, en la carcinogénesis se produce una acumulación de cambios genéticos y epigenéticos asociados al desarrollo de cáncer, que dotan a la célula de un fenotipo maligno [15]. Estas alteraciones genéticas confieren a las células que las alberga características importantes para el desarrollo y la progresión tumoral que han sido genéricamente denominadas "hallmarks" del cáncer. Estos "hallmarks" o signos distintivos del cáncer fueron resumidos por Hanahan and Weinberg en el año 2000 y actualizados por los mismos autores en el año 2011, e incluyen potencial replicativo ilimitado, señalización proliferativa sostenida, evasión de la señalización supresora del crecimiento, resistencia a la muerte celular, angiogénesis, capacidad invasiva-metastatizante [15], evasión de la destrucción inmune y reprogramación del metabolismo energético [16]. Además, los autores sugirieron la inclusión de dos signos adicionales distintivos del cáncer que los autores denominaron "características activadoras", subyacentes al resto de hallmarks y cruciales para la adquisición de estos: la inestabilidad genómica -que acelera la adquisición de los hallmarks- y la inflamación -que promueve múltiples funciones oncogénicas- [16]. De un modo resumido, en el COCE ocurren una serie de alteraciones moleculares específicas:

Potencial replicativo ilimitado. La inmortalidad de las células cancerígenas en COCE está regulada por la disregulación de los pathways asociados al gen TP53 y al gen del retinoblastoma (Rb), que alteran el ciclo celular, probablemente en el contexto de la expresión de la transcriptasa inversa de la telomerasa (TERT) [17].

Señalización proliferativa sostenida. Entre las alteraciones oncogénicas más relevantes implicadas en la disregulación de un fenotipo más proliferativo en COCE destacan la sobreexpresión de ciclina D1 [18,19], del miembro de la familia ErbB, EGFR [20], de ras y de los miembros activados aguas abajo de la vía de señalización molecular MAPK (Raf-MEK-Erk) [21]. Estos eventos oncogénicos, de gran relevancia en el cáncer oral, potencian que las células epiteliales se vuelvan refractarias a la señalización mitogénica extracelular y se comprometan con un programa autónomo de regulación, quedando determinadas para replicar su ADN,

abandonando sus controles y permaneciendo continuamente en el ciclo celular hacia la división mitótica [12].

Evasión de la señalización supresora del crecimiento. P53 -el "Guardián del genoma" [22]- es una proteína supresora tumoral que juega un rol central en la carcinogénesis oral [23,24], regulando el arresto del ciclo celular e induciendo la apoptosis de aquellas células con el ADN dañado o activación oncogénica [25]. Los estudios más recientes mediante la tecnología Next Generation Sequencing (NGS) constatan la alta frecuencia y relevancia de las mutaciones del gen *TP53* en COCE [26]. La proteína del Retinoblastoma (Rb) es otra importante proteína supresora tumoral, que durante la fase G1 impide la progresión del ciclo celular [12]. En las células cancerígenas, a través del denominado "Rb pathway", los complejos ciclina D1-CDK4/6 promueven la fosforilación de la proteína Rb y su inactivación funcional, inhibiéndose así la supresión que esta proteína ejerce sobre el ciclo celular y promoviendo la transición G1/S [18]. El VPH contiene dos oncogenes, E6 y E7, que también inactivan a p53 y a Rb, lo que se considera el inicio de la carcinogénesis mediada por HPV [12]. Contrariamente, p16 - producto del gen supresor tumoral *CDKN2A* y miembro de la familia de proteínas inhibidoras INK4 [27,28]- previene específicamente la activación de las CDKs4/6, generalmente mediante la inhibición de su asociación con las ciclinas tipo-D [18]. Los estudios más recientes mediante la tecnología NGS constatan las frecuentes deleciones y mutaciones del gen *CDKN2A* en cáncer de cabeza y cuello [26].

Resistencia a la muerte celular. Las células cancerígenas deben evadir *muerte celular*, que esencialmente obedece a la inhibición de la señalización pro-apoptótica -p53, BAX, Noxa, Puma, Aip1, caspasas… [23,25]- y a la activación de la señalización anti-apoptótica -pathways NF-κβ y PI3K–PTEN–Akt, sobreexpresión de Bcl-2 y del factor de transcripción STAT3 y amplificación génica de *FADD,* amplificado frecuentemente junto a otros oncogenes en la banda cromosómica 11q13 [23,29,30]-.

Inducción de angiogénesis. Normalmente, los tumores producen una serie de factores neo-angiogénicos y de crecimiento, que inducen el brote angiogénico de las células endoteliales, y posibilitan el desarrollo de nuevos vasos que nutren al carcinoma. Existen numerosos inductores de angiogénesis, pero el más destacado es el factor de crecimiento endotelial vascular (VEGF) [31]. Numerosos estudios han comunicado el valor pronóstico de VEGF en el carcinoma de células escamosas de cabeza y cuello, y un metaanálisis ha corroborado que su sobreexpresión parece estar asociada a una pobre supervivencia de estos pacientes [32].

Capacidad invasiva-metastatizante. El proceso de diseminación metastásica implica una serie de pasos, incluyendo la degradación de la matriz extracelular como uno de los iniciales [33]. En este sentido, numerosos estudios han tratado de investigar la participación de las

metaloproteinasas de la matriz (MMPs), proteasas implicadas en la degradación de la matriz extracelular [34]. El análisis de las alteraciones de los genes asociados al proceso invasivo metastatizante ofrece un gran número de genes involucrados en el fenómeno de transición epitelio mesenquimal (EMT) [35]. La EMT es un proceso biológico fundamental en las células embrionarias, y muy frecuente en la células cancerígenas, caracterizado por el cambio de un fenotipo epitelial, por un fenotipo mesenquimal. La EMT está ligada fundamentalmente al aumento de la migración celular, y consecuentemente a la invasión y la metástasis. Numerosos biomarcadores asociados a EMT parecen tener importantes implicaciones en proceso invasivo metastatizante y en el pronóstico de los pacientes con COCE (E-cadherina, β-catenina, cortactin, vimentina, Snail, Twist…) [36–39].

Evasión de la destrucción inmune. El sistema inmune constituye una barrera para el desarrollo de tumores invasivos metastatizantes y, por consiguiente, las células orales neoplásicas deben adquirir mecanismos moleculares que les posibiliten la adquisición de este hallmark emergente. En este contexto, PD-L1 es un punto de control inmunitario prometedor en COCE que está siendo investigado como diana pronóstico-terapéutica, y que está revolucionando la inmunoterapia en cáncer [40].

Reprogramación del metabolismo energético. Incluso en situaciones con mucho oxígeno, algunas células cancerígenas dependen de la glucólisis y bloquean la entrada al ciclo de Krebs. Esta "glucólisis aeróbica" es denominada efecto Warburg y dota a las células cancerígenas de mayor flexibilidad metabólica [41]. Se ha comunicado que este fenómeno también ocurre en cáncer de cabeza y cuello, donde se hallaron altos niveles de lactato y transportadores de monocarboxilato, a diferencia de los metabolitos hallados en células normales [42]. Otro parámetro que está siendo muy investigado en los últimos años es la hipoxia tumoral, que ocurre cuando un tumor o una parte del mismo excede su suministro sanguíneo. La hipoxia está asociada con la transformación maligna y está considerada además como un marcador de resistencia al tratamiento quimiorradioterápico [43].

Inestabilidad genómica. Esta característica "activadora" acelera la adquisición de los hallmarks precedentes, fundamentalmente incrementando la tasa mutacional en las células [44]. En la carcinogénesis oral, las mutaciones de TP53 pueden contribuir a la ganancia de inestabilidad genómica [45]. Esto es debido a que, si la proteína p53 queda inactivada, las células con ADN dañado no serán bloqueadas durante el ciclo celular, y acumularán un mayor número de eventos oncogénicos sumatorios.

Inflamación promotora tumoral. Esta característica "activadora" también puede facilitar la adquisición de los hallmarks anteriores, suministrando a los tumores factores de crecimiento y angiogénicos. Además, las células inmunitarias presentes en el microambiente tumoral pueden

también liberar especies reactivas de oxígeno altamente mutagénicas que pueden contribuir a la ganancia de inestabilidad genómica. Se ha comunicado que COX-2 -una enzima implicada en la producción de prostaglandinas inflamatorias- se sobreexpresa en el 71% de los cánceres de cabeza y cuello [46].

A pesar de los numerosos avances, los mecanismos oncogénicos del COCE aún no han sido completamente caracterizados. El conocimiento de sus características biológicas podría conducir a nuevas terapias personalizadas en un futuro próximo.

Relevancia de la banda cromosómica 11q13 en la carcinogénesis oral

Entre las alteraciones genéticas que pueden promover el desarrollo del cáncer humano, destacan las aberraciones en el número de copias del ADN [44]. Estas aberraciones pueden observarse frecuentemente como la ganancia o la pérdida neta de un cromosoma completo (aneuploidía), o como la ganancia o la pérdida parcial de cromosomas, lo que se traduce en amplificaciones o deleciones de material genético [44,47,48]. La amplificación génica se define como el aumento del número de copias de una región restringida en un brazo cromosómico y se trata de un mecanismo prevalente en la oncogénesis, frecuentemente asociado a la sobreexpresión de los genes amplificados [48]. Actualmente se piensa que la amplificación génica se inicia con la ruptura de la doble cadena de ADN [48], evento probablemente necesario, para que la célula progrese de una forma aberrante a través del ciclo celular, y para que se superen algunos checkpoints robustos, por ejemplo, el checkpoint mediado por la función de p53 [48–50]. Se han propuesto diferentes modelos de iniciación del proceso de la amplificación génica relacionados con la ruptura inicial de la doble cadena del ADN. Las hipótesis más plausibles preconizan que dicha ruptura puede estar vinculada a sitios cromosómicos frágiles, a disfunción telomérica o a errores en la replicación del ADN [48]. Se ha propuesto un modelo de iniciación y promoción del mecanismo de amplificación génica de 11q13 específico para la carcinogénesis oral, en el que el inicio del proceso está vinculado a la ruptura de la doble hélice de ADN en sitios cromosómicos frágiles [48]. Reshmi y cols [51,52] comunicaron que en COCE la amplificación de 11q13 se produce como resultado de los denominados ciclos rotura-fusión-puente, iniciados por la rotura en el sitio frágil común cromosómico FRA11F, inducida presumiblemente por carcinógenos asociados al tabaco [48,53]. La estrecha asociación etiológica del consumo de tabaco con el desarrollo de COCE probablemente justifica la elevada frecuencia de amplificación de 11q13 en estos tumores -con una media del 46%- más del doble de la observada en el resto de cánceres humanos menos relacionados con el consumo de tabaco [30]. Una vez establecida, la amplificación se puede presentar en diferentes formas: el ADN amplificado puede organizarse como copias extracromosómicas -también conocidas como

"double minutes"- o bien intracromosómicas -formando regiones homogéneamente teñidas (HSR)-; una tercera forma de amplificación distribuye las copias de forma dispersa a lo largo del genoma [48,54,55]. Las diferentes formas de presentación pueden coexistir en una misma célula, que suele ser portadora de un número de copias superior a 5, aunque han llegado a documentarse más de 500 copias [54]. La amplificación de la banda cromosómica 11q13 frecuentemente alberga diversos genes co-amplificados y ha sido propuesta en la literatura como prototipo de la complejidad del proceso de amplificación en el cáncer [48,55,56]. Así, en la ubicación comprendida entre 11q13.2-q13.4 diversos autores [57–66] han hallado en COCE co-amplificados los genes CPT1A, MRPL21, IGHMBP2, MRGPRF (también conocido como MRGF), TPCN2, MYEOV (también conocido como OCIM), *CCND1*, ORAOV1 (también conocido como TAOS1), FGF19/4/3, ANO1 (también conocido como TMEM16A, ORAOV2, TAOS2 o DOG1), *FADD*, PPFIA1, *CTTN* (también conocido como EMS1), SHANK2 y NUMA1 (enumerados según el orden de ubicación en el cromosoma en dirección centromérica-telomérica). Estudios recientes han demostrado que los amplicones pueden albergar más de un amplicon driver -*i.e.* un gen amplificado dentro del amplicón cuya expresión confiere una ventaja a las células que lo albergan, contribuyendo al mantenimiento del fenotipo maligno- [67]. Entre los genes precedentes, *CCND1*, *CTTN* y *FADD* han sido propuestos como amplicon drivers potenciales en carcinogénesis humana y singularmente en cáncer oral [30,67]. La caracterización de un gen como amplicon driver está dificultada por el hecho de que la activación transcripcional de algunos oncogenes no está regulada únicamente por el mecanismo de amplificación [48]; por ejemplo, la amplificación de *CCND1*, ubicado en 11q13, es uno de los mecanismos fundamentales que regulan la sobreexpresión de la proteína ciclina D1 en COCE, aunque ésta se regula también por otros mecanismos tales como las vías de señalización MAPK o PI3K, a menudo alteradas en el cáncer oral, lo que dificulta la caracterización de *CCND1* como amplicon driver del amplicón 11q13 [18,30]. La amplificación de material genético tiene implicaciones diagnósticas y pronósticas, y podría ser utilizada como diana terapéutica, tratándose además de un conocido mecanismo adquirido de resistencia a fármacos [48]. Los oncogenes que integran este amplicón pueden justificar la influencia descrita de la amplificación de 11q13 sobre diferentes características clínicopatológicas del COCE, tales como afectación ganglionar, pobre grado de diferenciación tumoral o pobre supervivencia [30]. Algunas publicaciones han comunicado ganancia del número de copia o amplificación de la región cromosómica 11q13 en desórdenes orales potencialmente malignos [68–70], lo que en sí mismo parece sugerir que se trata de un evento precoz en la carcinogénesis oral. Queda por determinar si las implicaciones oncogénicas precedentes son el resultado de las acciones individuales de los oncogenes que alberga el amplicón, o si se trata del efecto cooperativo del conjunto de genes ubicados en 11q13 frecuentemente co-amplificados en COCE [30].

Implicaciones de la sobreexpresión de ciclina D1 en carcinogénesis oral

CCND1 codifica a ciclina D1, una proteína que promueve la progresión del ciclo celular durante la fase G1, favoreciendo la transición G1-S [71]. Sin duda *CCND1*/ciclina D1 han sido los componentes más estudiados de la banda cromosómica 11q13 en la carcinogénesis oral. Su función mejor caracterizada es la regulación de la proliferación celular, aunque en los últimos años le han sido atribuidas numerosas funciones determinantes en la biología celular del cáncer, como son la regulación del crecimiento celular, modulación de la actividad mitocondrial, reparación del ADN y control de la migración celular, tanto de un modo dependiente de sus compañeras de unión las CDKs4/6, como independientemente [72]. Un censo publicado concluyó que hay suficiente evidencia científica tanto de su amplificación como de su sobreexpresión en COCE [73]. La tasa de amplificación de *CCND1* en COCE varía en los diferentes estudios entre 9%-72% [18], y parece tratarse de un evento constante en el amplicon core situado en 11q13.2-q13.4 [58–61,64–66]. Además de la amplificación génica, también se han documentado otros mecanismos de activación de este oncogén entre los que se encuentran: translocaciones cromosómicas, como la que yuxtapone *CCND1* con el locus IGH (immunoglobulin heavy chain) [t(11;14)(q13;q32)] -que está considerada el hallmark genético del linfoma de células del manto-; mutaciones -Thr-286- y polimorfismos -G/A870-; alteraciones de los pathways reguladores -entre los que destacan las vías de señalización MAPK y PI3K, así como Wnt y NF-κβ-; así como sobrerregulación mediada por microRNAs [18]. *CCND1* es el gen del amplicón 11q13 al que le han sido atribuidas más implicaciones oncogénicas [30]. Se ha comunicado la implicación de *CCND1*/ciclina D1 en todas las fases de la carcinogénesis oral, incluidas sus fases iniciales [18]. Nuestro metaanálisis publicado recientemente ha hallado importantes asociaciones estadísticas al evaluar el impacto que ejerce la sobreexpresión de ciclina sobre factores clínicopatológicos y pronósticos en COCE (tamaño tumoral, afectación ganglionar, estadío clínico avanzado, pobre grado de diferenciación histológica, pobre supervivencia general y libre de enfermedad). Habitualmente el análisis de la actividad oncogénica de la sobrerregulación de ciclina D1 en COCE se ha limitado exclusivamente a su expresión nuclear, siendo pocos los grupos que han valorado los niveles de expresión citoplasmáticos de ciclina D1 en tumores diferentes a COCE, como cáncer de páncreas, vejiga, hígado, próstata, tiroides, colon y ovario [74–80]. Sin embargo, las implicaciones clinicopatológicas de la sobreexpresión citoplasmática de ciclina D1 demostradas en estos tumores humanos justifican en nuestra opinión su estudio en COCE. Además, también ha sido comunicada la asociación entre las alteraciones de *CCND1*/ciclina D1 con una peor respuesta al tratamiento radioquimioterápico, especialmente basado en cisplatino [18]. El complejo *CCND1*/ciclina D1 ha sido probablemente el más investigado como diana terapéutica en 11q13, debido fundamentalmente al mayor conocimiento que poseemos sobre sus

implicaciones clínico-patológicas en cáncer [30]. La dianización dirigida directamente hacia ciclina D1, por su localización intracelular y su carencia de actividad enzimática, puede resultar compleja [81]. Sin embargo, la evidencia creciente de la existencia de funciones de ciclina D1 independientes de CDK, como son su implicación en la migración celular [72], aconsejan considerar esta opción terapéutica aplicada al COCE [18]. En este sentido se ha comunicado la posible utilidad de diversos agentes nutracéuticos con acciones antineoplásicas en cáncer oral [82,83], entre los que destacan el té verde y la curcumina, que basan gran parte de su potencial anticancerígeno en la inhibición de ciclina D1; en concreto el té verde ha demostrado que ejerce este efecto en líneas celulares de carcinoma de células escamosas de cabeza y cuello mediante sus componentes epigallocatechin-3-gallate [84] y epicatechin gallate [85]. Sin embargo, actualmente el abordaje terapéutico más estudiado sobre ciclina D1 reside en la inhibición de las CDKs4/6; esta acción terapéutica puede lograrse mediante inhibidores globales de la acción de las CDKs, tales como flavopiridol, olomoucine o R-roscovitine, o bien mediante inhibidores selectivos de las CDK4/6, como P276-00, P1446-05 o PD0332991 (éste último -también conocido como palbociclib- ha sido aprobado recientemente por la FDA para el tratamiento del cáncer de mama) [86]. Otras alternativas terapéuticas con efectos esperados sobre los niveles de expresión de ciclina D1 que deberían tenerse en cuenta en futuras investigaciones incluyen actuar sobre la GSK-3β - una enzima que interviene en la degradación de ciclina D1-, lo que se ha ensayado con el fármaco DIF-1, que parece inhibir la proliferación en células de mamíferos; y dianizar los pathways implicados en el desarrollo del COCE en los que esté involucrada ciclina D1, tales como MAPK o PI3K. En este contexto, parece resultar de especial interés el empleo de inhibidores de mTOR -tales como ridaforolimus o everolimus- ya que la translación del RNAm de *CCND1* es mTOR dependiente [81]. Por último, como consecuencia del conocimiento actual de la resistencia al tratamiento basado en cisplatino en aquellos pacientes con COCE que sobreexpresan ciclina D1, podría resultar de especial interés el tratamiento combinado de inhibidores que actúen sobre ciclina D1 con otros agentes terapéuticos antitumorales [18]. Así, el empleo de terapia única dirigida a bloquear la ciclina D1 o los complejos ciclina D1-CDK ha ofrecido resultados más pobres que los obtenidos con la terapia combinada con otros agentes citotóxicos –por ejemplo cisplatino, 5-fluoracilo, doxoruvicin o paclitaxel- [81].

Implicaciones de la sobreexpresión de cortactin en carcinogénesis oral

El gen *CTTN* (también conocido como EMS1) codifica a la proteína cortactin, una proteína de unión a la actina F caracterizada por poseer múltiples dominios de unión que le permiten interacciones con numerosas proteínas [87,88]. El gen *CTTN* ha sido considerado el gen más

importante del amplicón 11q13 junto con *CCND1* debido a sus funciones oncogénicas y a la frecuente alteración en el número de sus copias [89], habiendose publicado que *CCND1* y *CTTN* se encuentran frecuentemente co-amplificados, *in vitro* e *in vivo* [58,59,90,91]. A través de una serie de eventos moleculares cortactin regula específicamente la migración e invasión celular en cáncer oral [39]. Para adquirir un fenotipo migratorio, las células cancerígenas deben experimentar cambios en la regulación de su citoesqueleto de actina, concomitantes con la formación de una serie de estructuras protrusivas en la membrana celular, denominadas invadopodios y lamelipodios [92,93]. Cortactin juega un rol clave uniéndose y regulando a la actina filamentosa, potenciando la polimerización y ensamblaje reticular de los monómeros de actina, estabilizando las redes de actina ramificadas y regulando su organización estructural [94]. Adicionalmente, las interacciones de cortactin con numerosas proteínas, entre las que destacan Src y el complejo Arp2/3 le confieren a cortactín un rol importante en la regulación de una variedad de eventos celulares, también relacionados con la regulación de las estructuras mencionadas con anterioridad [95]. Trabajos recientes han atribuido a cortactin funciones oncogénicas diferentes a las relacionadas con el aumento de la migración celular y la invasión, entre las que se encuentran el incremento de la angiogénesis [96,97] y de la proliferación celular [96,98], la secreción de exosomas [99] y los efectos sobre el microambiente tumoral [100]. La tasa de amplificación en COCE del gen *CTTN* oscila entre el 10-57%, según las series [58,59,63,64,90,101–103]. *CTTN* presenta consistentemente una alta expresión de RNAm y sobreexpresión tisular de su producto cortactin en aquellos tumores con amplificación del gen [58–60,90,102], lo que indica que las acciones en la oncogénesis oral de este gen son altamente dependientes de su amplificación y no de otros mecanismos de sobreexpresión [39]. La amplificación de este gen también ha sido documentada en el 20% de los desórdenes orales potencialmente malignos [103], habiéndose comunicado que la sobreexpresión de su producto es un predictor de la transformación maligna, aumentando su expresión con el grado de displasia [104]. Las alteraciones de *CTTN*/cortactín en COCE se han correlacionado en numerosos estudios con factores que implican una peor evolución de la enfermedad, como son la afectación de ganglios linfáticos cervicales [65,90,91,101,103,105,106], la presencia de extensión extracapsular [105], un patrón tumoral más infiltrativo [106], un mayor tamaño tumoral, un estadío clínico más avanzado [103,106], un pobre grado de diferenciación histológica [103], así como con una reducida supervivencia libre de enfermedad [101]. Además se ha comunicado que la expresión aberrante de cortactín se asocia en líneas celulares de carcinomas de células escamosas de cabeza y cuello positivamente con resistencia al tratamiento basado en gefitinib [107], un anticuerpo monoclonal dirigido selectivamente contra EGFR, que está siendo utilizado terapéuticamente en diferentes cánceres humanos. Finalmente, ha sido discutida la posible utilidad de cortactin como diana terapéutica en cáncer oral [39]. Las posibilidades de dianizar cortactin con fines terapéuticos derivan de observaciones en líneas

celulares y experimentación animal, aunque las conocidas funciones oncogénicas de cortactin, animan al desarrollo de futuras líneas de investigación sobre aspectos aun no explorados de esta proteína. Aunque hasta la fecha no se han diseñado moléculas inhibidoras selectivas de la proteína cortactín, la alta incidencia de sobreexpresión de cortactín en COCE justifica la implementación de futuras líneas de investigación en este sentido [39]. Otra estrategia podría basarse en la eliminación del gen *CTTN* amplificado mediante la tecnología de edición genómica CRISPR/Cas9 [108,109]. Otra posibilidad en este sentido podría basarse en la represión independiente o cooperativa de los oncogenes (*CTTN*, *CCND1* y *FADD*) que alberga el amplicon 11q13 *via* epigallocatechin-3 gallate [18,110,111]. También el empleo de fármacos dirigidos frente a EGFR (p.e. cetuximab y/o gefitinib), podría ser una opción, debido a que se ha demostrado la implicación de EGFR en algunos pathways que activan oncogénicamente a cortactín (EGFR-Src), y a la posible co-amplificación de sus respectivas bandas cromosómicas -7p11 y 11q13- [112], con coexpresión de sus productos [113]. Las conocidas implicaciones de cortactín en la resistencia adquirida al tratamiento del carcinoma de células escamosas de cabeza y cuello podría justificar la terapia combinada entre gefitinib y futuras moléculas inhibidoras de cortactín [107]. La consideración como opción terapéutica de la dianización de pathways reguladores de las funciones oncogénicas de cortactin se ha considerado recientemente [39]. Saracatinib -un agente diseñado para inhibir selectivamente la actividad de Src- no sólo parece disminuir la activación de Src, sino que también inhibe la de sus sustratos aguas abajo, bloqueando la fosforilación de cortactín en Tyr421 [114]. Además se ha podido observar que saracatinib actúa de un modo dosis dependiente, y que a altas dosis de tratamiento (0.5–1μM) disminuye los niveles de expresión de cortactín [114]. Se ha observado además, que SU6656 -otro inhibidor selectivo de Src- es capaz de suprimir la función invadopodial en carcinoma de células escamosas de cabeza y cuello, probablemente como consecuencia del bloqueo del pathway EGFR-Src-cortactín-invadopodios [115]. Otra opción plausible sería el bloqueo de los miembros del pathway MAPK que interaccionan con cortactin (i.e. MEK y Erk), a través del empleo de inhibidores de esta vía (e.g. PD98059) [115–117].

OBJETIVOS

1. Revisar la relevancia de la amplificación de la banda cromosómica 11q13 en carcinogénesis oral.

2. Revisar las implicaciones de las alteraciones de *CCND1*/ciclina D1 y *CTTN*/cortactin en carcinogénesis oral.

3. Evaluar metaanalíticamente el significado pronóstico de ciclina D1 en carcinoma oral de células escamosas.

4. Evaluar metaanalíticamente el significado pronóstico de las alteraciones de *CTTN*/cortactin en carcinoma oral de células escamosas.

5. Evaluar mediante técnica inmunohistoquímica las implicaciones pronósticas de la expresión de ciclina D1 en carcinoma oral de células escamosas y en el epitelio premaligno.

MATERIAL Y MÉTODOS

Revisión no sistemática de la literatura: Implicaciones de la amplificación de la banda cromosómica 11q13, y de las alteraciones de CCND1/ciclina D1 y CTTN/cortactin en la carcinogénesis oral.

Realizamos una revisión de la literatura científica con el fin de analizar meticulosamente las implicaciones de la amplificación de la banda cromosómica 11q13, y de las alteraciones de *CCND1*/ciclina D1 y *CTTN*/cortactin en la carcinogénesis oral. Para ello, buscamos los estudios publicados en las bases de datos PubMed (base de datos principal usada) y Web of Science (análisis bibliométrico) siendo la fecha límite superior para la revisión sobre *CCND1*/ciclina D1 el año 2016, para la revisión sobre la banda cromosómica 11q13 el año 2017 y para la revisión sobre *CTTN*/cortactin el año 2018; en ningún caso establecimos fecha límite de búsqueda inferior. Principalmente se combinaron los términos "oral squamous cell carcinoma", "11q13", "cyclin D1", "CCND1", "cortactin" y "*CTTN*". Además se condujeron numerosas búsquedas más específicas combinando los diferentes genes de la banda cromosómica 11q13 y las proteínas que estos codifican, con aspectos relevantes de los subapartados a revisar (otros tipos de cáncer, regulación molecular fisiológica, funciones biológicas, mecanismos oncogénicos de sobreexpresión, implicaciones diagnósticas o pronósticas en cáncer oral o en estadíos precoces de la carcinogénesis oral, e implicaciones terapéuticas). También se revisó la lista de referencias bibliográficas de todos los trabajos recogidos para hallar registros adicionales y publicaciones relevantes. La mayoría de los estudios fueron incluidos o excluidos de acuerdo a un exhaustivo análisis del título, abstract, año de publicación, impacto de la revista y número de citaciones recibidas. Aunque estos dos últimos criterios pueden introducir un sesgo potencial en la selección de los estudios, está bien establecido que es necesaria su aplicación cuando se maneja un elevado número de registros (y en este contexto, por ejemplo, simplemente la búsqueda: [("cyclin d1" or "ccnd1") and "cancer"] dio como resultado más de 11000 registros). Se revisaron por otra parte los ensayos clínicos finalizados o en desarrollo (Fuente:clinicaltrials.gov) y las patentes publicadas (Fuente: patentscope.wipo.int). Al tratarse de revisiones no sistemáticas, no se definió una única estrategia de búsqueda, ni se definieron criterios restrictivos de elegibilidad, generándose así un flujo de selección de estudios más sensible.

Revisión sistemática y metaanálisis de las implicaciones pronósticas y clínicopatológicas de la expresión de ciclina D1 en el carcinoma oral de células escamosas.

La presente revisión sistemática y metaanálisis se adhiere a las normas PRISMA [118], y para su elaboración se han seguido atentamente los criterios de *Cochrane Prognosis Methods Group*

[119], de *Cochrane Handbook for Systematic Reviews of Interventions* [120] y de *Centre for Reviews and Dissemination (CRD)'s guidance for undertaking reviews in health care* [121].

Protocolo. Para minimizar el riesgo de sesgo, mejorar la transparencia, la precisión y la entereza de la presente revisión sistemática y metaanálisis, se registró a priori un protocolo sobre su metodología en *PROSPERO international prospective register of systematic reviews* (www.crd.york.ac.uk/PROSPERO, registration number CRD42018081746) [122]. Para asegurar el rigor, el protocolo se condujo de acuerdo con las directrices PRISMA-P [123].

Estrategia de búsqueda. Buscamos en las bases de datos PubMed, Embase, Web of Science y Scopus estudios publicados antes de la fecha de búsqueda (límite superior=Julio 2017) y sin límite de fecha inferior. Las búsquedas se llevaron a cabo combinando términos tesauros de bases de datos (e.g. MeSH y EMTREE) y términos libres. Con el fin de maximizar la sensibilidad, nuestra estrategia de búsqueda empleada en Pubmed combinó los siguientes términos: ("cyclin d1"[MeSH Terms] OR ("cyclin"[All Fields] AND "d1"[All Fields]) OR "cyclin d1"[All Fields] OR "cyclind1"[All Fields] OR "ccnd1"[All Fields] OR "ccnd 1"[All Fields]) AND ("mouth"[MeSH Terms] OR "mouth"[All Fields] OR "oral"[All Fields]) AND ("carcinoma, squamous cell"[MeSH Terms] OR ("carcinoma"[All Fields] AND "squamous"[All Fields] AND "cell"[All Fields]) OR "squamous cell carcinoma"[All Fields]). Una estrategia de búsqueda equivalente fue adaptada a la sintaxis de cada base de datos consultada (ver protocolo).

Además, manualmente hicimos un screening en las listas de referencias de los estudios incluidos, tratando de hallar más estudios relevantes. Todas las referencias fueron gestionadas mediante el software Mendeley v.1.17.10 (Elsevier. Amsterdam, The Netherlands); las referencias duplicadas fueron eliminadas.

Criterios de elegibilidad. Los criterios de elegibilidad de los estudios fueron aplicados independientemente por dos investigadores (PRG y MAGM). Cualquier desacuerdo fue resuelto por consenso.

Criterios de inclusión. 1) Trabajos de investigación originales publicados en inglés. 2) Evaluación de la expresión de ciclina D1 empleando inmunohistoquímica (IHQ) en tejidos humanos procedentes de COCEs primarios. 3) Análisis de la asociación entre la sobreexpresión de ciclina D1 con al menos alguna de las siguientes variables clínico-patológicas y/o

pronósticas: status T, status N, grado histológico, estadío clínico, supervivencia general, supervivencia libre de enfermedad. Supervivencia general se definió como el periodo de tiempo transcurrido desde la fecha del diagnóstico o de la cirugía hasta la fecha de muerte, por cualquier causa. La supervivencia libre de enfermedad fue definida como el periodo de tiempo transcurrido desde la cirugía hasta la presencia de recurrencia locorregional o a distancia, o muerte sin recurrencia. Ante la falta de estándares de consenso internacional para la definición de "endpoints" de supervivencia, incluimos aquellos trabajos que emplearan la denominación directa de los términos precedentes de supervivencia o bien denominaciones diferentes (p.e. supervivencia libre de recurrencia) definidas en los estudios originales como en el presente trabajo. 4) En el caso de resultados derivados de una misma población de estudio, se incluyeron los comunicados más recientemente o los que aportaran más datos. Para determinar que dos trabajos correspondían a la misma población de estudio comprobamos los nombres de los autores, su afiliación, el hospital de tratamiento y el periodo de reclutamiento.

Criterios de exclusión. 1) Artículos de revisión, metaanálisis, reportes de casos, editoriales, cartas, abstracts de reuniones científicas, opiniones personales o comentarios, capítulos de libro y cualquier trabajo en idioma distinto al inglés. 2) No COCE. 3) Estudios *in vitro* o en modelos animales. 4) Empleo de técnicas diferentes a la IHQ o análisis exclusivo de las alteraciones del gen *CCND1*. 5) Trabajos que no analicen la relación con las variables clínico-patológicas y/o de supervivencia de interés. 6) Datos insuficientes para la estimación de odds ratios (OR) para el análisis de las variables clínicopatológicas. En los estudios que únicamente reportaron variables de supervivencia (time-to-event), fue criterio de exclusión la no comunicación directa de hazards ratio (HR) junto con sus intervalos de confianza (CI) al 95%, o de datos esenciales que nos permitiera su estimación estadística a través del análisis de supervivencia.

Los artículos fueron seleccionados en dos fases. En la fase I, los que parecían reunir los criterios de inclusión mediante screening de títulos y abstracts. En la fase II los artículos fueron leídos a texto completo, siendo eliminados aquellos que no reunieron las características expuestas en los criterios de inclusión.

Extracción de datos. Dos autores extrajeron independientemente los datos de los artículos seleccionados para ser trabajados a texto completo de un modo estandarizado, empleando un formulario de recogida de datos en hojas de cálculo mediante el software Excel v.2015 (Microsoft. Redmond, WA, USA). Los datos recogidos fueron revisados adicionalmente por dos autores diferentes. Las discrepancias fueron resueltas mediante consenso. Se recogió el primer autor, año de publicación, país y continente del estudio, tamaño de la muestra, localización tumoral, periodo de reclutamiento, modalidad de tratamiento, tiempo de seguimiento,

anticuerpo anti-ciclina D1 empleado, patrón de inmunopositividad intracelular evaluado (nuclear/citoplasmático), punto de corte, sobreexpresión de ciclina D1 (alta/baja), status N, T, grado histológico, estadío clínico y de supervivencia (supervivencia general y libre de enfermedad).

Evaluación de la calidad y del riesgo de sesgo. Se realizó por dos autores (PRG y MAGM) empleando Quality in Prognosis Studies (QUIPS) tool (*Cochrane Prognosis Methods Group)* [124]. Esta herramienta incluye 6 áreas importantes de sesgos potenciales en los trabajos seleccionados ([1] Study participation, [2] Study attrition, [3] Prognostic Factor Measurement, [4] Outcome Measurement, [5] Study Confounding and [6] Statistical analysis and reporting) [125]. El riesgo de sesgo se calificó como bajo, moderado o alto para cada dominio. Las discrepancias se resolvieron mediante consenso.

Análisis estadístico. La expresión de ciclina D1 fue considerada "alta" o "baja" de acuerdo a los valores de corte usados en los estudios originales. Usamos odds ratios (OR) con sus correspondientes CI al 95% como medida de asociación para determinar la correlación entre la expresión de ciclina D1 y las variables clínico-patológicas de los pacientes con COCE. Usamos hazard ratios (HR) con sus correspondientes CI al 95% como medida de asociación para estimar el impacto de la expresión de ciclina D1 sobre las variables de supervivencia (time-to-event). Si los autores publicaron HR y sus CI al 95% se extrajeron estos directamente de los artículos originales. En los estudios que publicaron HR en diferentes modelos (univariante y multivariante), se extrajeron los datos del modelo multivariante, que refleja un mayor ajuste de las variables potencialmente confundidoras. Si estos datos no aparecían en el estudio, fueron calculados por nosotros mediante los métodos de Parmar *et al* [126] y Tierney *et al* [127]. Si un estudio solo comunicó curva de supervivencia, extrajimos los datos de las curvas Kaplan-Meier mediante el software Engauge Digitizer 4.1 (open-source digitizing software desarrollado por M. Mitchell). En algunos estudios que no comunicaron HR se extrajeron estimaciones del riesgo relativo (RR) u OR ajustadas, que se asumieron como una aproximación de la misma medida [128].

En el metaanálisis, las diferentes medidas de asociación derivadas de los estudios individuales fueron agrupadas. Para la estimación de las asociaciones combinadas usamos tanto modelos de efectos fijos (métodos de Mantel-Haenszel e inverso de la varianza) como un modelo de efectos aleatorios (método de DerSimonian and Laird). Usamos forest plots para representar gráficamente el efecto general, y para su posterior análisis. Evaluamos la heterogeneidad entre

los estudios usando el test Q de Cochran basado en Chi-cuadrado [129]. Debido a la baja potencia estadística de este test, el nivel de significación se definió como $p<0.1$, en cuyo caso asumimos que existió heterogeneidad aparente y consecuentemente usamos un modelo de efectos aleatorios para calcular las estimaciones combinadas. Además, cuantificamos la proporción de heterogeneidad con el estadístico I^2 de Higgins, donde valores del 25%, 50% y 75% pueden considerarse como baja, moderada o alta heterogeneidad respectivamente [129,130].

También conducimos análisis estratificados (por continente, sitio tumoral intraoral, anticuerpo anti-ciclina D1, punto de corte y patrón de inmunotinción) con el objetivo de identificar posibles fuentes de heterogeneidad, así como para explorar las relaciones entre la sobreexpresión de ciclina D1 con las variables pronósticas y clínicopatologícas en los subgrupos precedentes.

Además, llevamos a cabo análisis de sensibilidad, evaluando la influencia de cada estudio individual sobre la estimación del efecto general, testando así la fiabilidad de los resultados combinados [131]. Para ello, repetimos secuencialmente el metaanálisis tras la omisión de un estudio en cada turno, y representamos gráficamente sus resultados. Por último, construimos funnel plots y usamos el test de regresión Egger ($p_{Egger}<0.1$) para evaluar los efectos de los estudios pequeños, tales como el sesgo de publicación [132–134]. Todos los análisis estadísticos en este metaanálisis se practicaron con el software Stata versión 14.1 (Stata Corp, College Station, TX, USA), usando comandos escritos por el usuario [135]. En el metaanálisis, consideramos estadísticamente significativo un valor $p<0.05$.

Estudio experimental mediante técnica inmunohistoquímica de las implicaciones de la expresión de ciclina D1 en la carcinogénesis oral.

Estudiamos restrospectivamente 54 pacientes que habían desarrollado 68 COCEs, con edades comprendidas entre 45-87 años (63.0 ± 12.0), bajo tratamiento en el Complejo Hospitalario de Jaen (España); 40 pacientes (74.1%) fueron hombres. La presencia de segundos tumores, cuando aparecieron, fue registrada de acuerdo a los criterios siguientes: procedencia de la superficie epitelial y no del margen quirúrgico profundo del tumor [136]; resección completa del tumor primario de acuerdo con la evaluación histopatológica convencional del margen quirúrgico [137]; presencia de al menos 2 cm de epitelio no tumoral entre los tumores, basados en hallazgos clínicos, quirúrgicos e histopatológicos [138]; y ≥6 meses de diferencia en la aparición de los tumores [139].

Todos los pacientes incluidos en el estudio firmaron un consentimiento informado durante su ingreso hospitalario en el que autorizaron la conservación y utilización posterior de sus muestras

biológicas para investigación. Una vez que el estudio fue aprobado por el comité ético del hospital, los registros hospitalarios de los pacientes fueron revisados y se recogieron los datos de las características clínico-patológicas de las lesiones. Nuestros criterios de inclusión fueron la disponibilidad de datos clínicopatológicos y de evolución de cada paciente, así como la calidad adecuada de la técnica inmunohistoquímica.

Inmunohistoquímica

Realizamos un estudio inmunohistoquímico de las proteínas ciclina D1 y Ki-67 en el servicio de Anatomía Patológica del Hospital Campus de la Salud de Granada (Parque Tecnológico de Ciencias de la Salud). Para la tinción inmunohistiquímica, se cortaron 5 secciones de 4 μm de los bloques de parafina. Se usó la técnica de la peroxidasa-antiperoxidasa, practicando el análisis inmunohistoquímico por medio el método avidina-biotina. Se empleó un sistema automatizado, usando Autostainer Link equipment (Dako, Carpinteria, CA, USA) y EnVision™ FLEX reagents (K8002; Dako, Carpinteria, CA, USA). Se siguieron rigurosamente las instrucciones del fabricante. Este sistema permite la desparafinación y rehidratación, seguidas de la recuperación del epítopo inducida por calor. La reproducibilidad del proceso se asegura cargando todo el soporte de portaobjetos, garantizando por tanto un calentamiento idéntico de todas las secciones en cada ciclo. Se usó el anticuerpo monoclonal de conejo anti-ciclina D1 humana (Clone EP12) (Dako, Carpinteria, CA, USA) y el anticuerpo primario Mib-1 frente a ki-67 (Dako, Carpintería, CA, USA), recomendados por el fabricante para este sistema automatizado. La contra-tinción se practicó usando EnVision™ Flex Hematoxylin system (K8008; Dako, Carpinteria, CA, USA), que da una tinción nuclear azul claro, seguido de un montaje permanente de las muestras en DPX. Para el control negativo, el anticuerpo primario se reemplazó por un buffer fosfato salino. Como control positivo se usó tejido de un COCE del que previamente se conocía su expresión de ciclina D1 y Ki67. Los portaobjetos fueron digitalizados usando el sistema Philips IntelliSite Ultra Fast Scanner (Philips Digital Pathology Solutions, Best, The Netherlands). La evaluación de la expresión inmunohistoquímica de las proteínas analizadas se realizó tanto en el tejido tumoral como en el epitelio no tumoral adyacente al carcinoma. Este epitelio se clasificó como cercano (n=58) o lejano (n=41) al tumor según se localizara a una distancia mayor o menor de 1 cm del punto de invasión, siguiendo criterios previamente publicados por nosotros [140]. No se ha incluido un grupo control sobre la expresión de ciclina D1 ya que ha sido comunicada reiteradamente [141–148] la constantemente negatividad para ciclina D1 del epitelio oral sano. Para la evaluación de la expresión de los marcadores se tomaron aleatoriamente 2 imágenes digitales, en epitelio cercano y lejano no tumoral adyacente al carcinoma, así como en 4 campos tumorales, cada una de un área de

0,191mm^2 -equivalente a una magnificación de 40x- usando el sistema Philips IMS viewer (Philips Digital Pathology Solutions, Best, The Netherlands). Este sistema nos permite alcanzar una alta magnificación de la imagen digital con gran definición y de un modo reproducible en todos los casos. Para la medición de la expresión en cada una de las áreas seleccionadas se empleó una técnica de recuento celular semi-automatizada [149], usando el software Adobe Photoshop CC v.2017 (San Jose, CA, USA) (Figura 1). Para minimizar los errores del recuento, se superpuso sobre cada área una cuadrícula que permitió un recuento celular ordenado y meticuloso de la expresión de los marcadores (Figura 1). Evaluamos la inmunopositividad nuclear para ciclina D1 y para Ki-67, y la inmunopositividad citoplasmática para ciclina D1, considerando positivo un marcaje de color marrón para ambos marcadores. La expresión del marcador en epitelio no tumoral adyacente a carcinoma la evaluamos dividiendo el grosor del epitelio en cuatro compartimentos: capa basal, capa parabasal (formada por aproximadamente 3 hileras celulares), tercio medio y tercio superior (Figura 1). Contamos el número de células totales, el número de células positivas y establecimos un porcentaje medio de expresión para cada área y para cada compartimento. Adicionalmente, seleccionamos de nuestra serie inicial de 68 carcinomas, aquellos que presentaron un patrón de expresión nuclear y citoplasmático de ciclina D1 (n=23). También, realizamos una evaluación de la intensidad de expresión nuclear y citoplasmática de ciclina D1, valorándola como leve, moderada o intensa. En estos casos además, se consideró la presencia de células que mostraron una morfología consistente en prolongaciones digitiformes de la membrana celular que se invaginaban entre las células tumorales adyacentes, correspondiente con estructuras protrusivas basadas en actina, denominadas lamelipodios e invadopodios [92]. Esta apariencia celular es referida en nuestro trabajo como morfología invasiva [149]. Tratamos así de investigar si existe relación estadística entre el porcentaje de células con inmunopositividad nuclear y/o citoplasmática con una morfología invasiva. Todas las evaluaciones fueron realizadas siempre por dos observadores (PRG y MAGM).

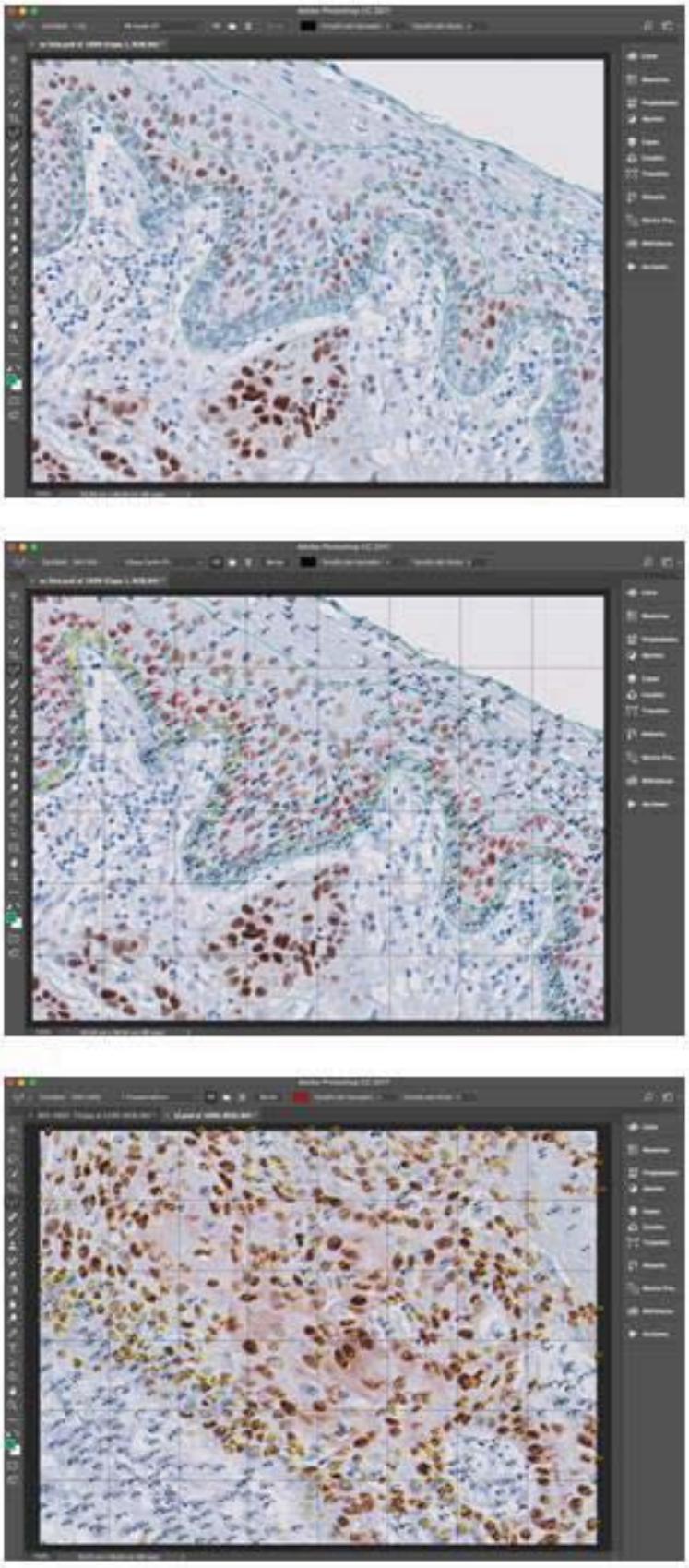

Figura 1

Métodos estadísticos aplicados a los resultados inmunohistoquímicos

La variable predictora principal fueron los niveles de expresión de ciclina D1, medidos en escala cuantitativa (porcentual). Las variables dependientes (dicotómicas/policotómicas) serán fundamentalmente las variables clínicas e histopatológicas. Para la estadística descriptiva y analítica se utilizaron los programas SPSS-Windows 15.0 (SPSS Inc., Chicago, Il) y SUDAAN 7.0 (RTI, RTP, NC), este último para el cálculo del valor p en los casos en que se cuantificaron los niveles de expresión de ciclina D1 en pacientes con múltiples COCEs (análisis cluster). Las asociaciones se midieron utilizando los tests paramétricos o no paramétricos apropiados (Chi-cuadrado, Test-T, ANOVA…) según las variables contrastadas y el cumplimiento o no de sus condiciones de aplicación, indicados en los pie de tablas.

Revisión sistemática y metaanálisis de las implicaciones pronósticas y clinicopatológicas de las alteraciones de CTTN/cortactin en el carcinoma de células escamosas de cabeza y cuello.

La presente revisión sistemática y metaanálisis se adhiere a las normas PRISMA [118], y para su elaboración se han seguido atentamente los criterios de *Cochrane Prognosis Methods Group* [119], de *Cochrane Handbook for Systematic Reviews of Interventions* [120] y de *Centre for Reviews and Dissemination (CRD)'s guidance for undertaking reviews in health care* [121].

Protocolo. Para minimizar el riesgo de sesgo, mejorar la transparencia, la precisión y la entereza de la presente revisión sistemática y metaanálisis, se registró a priori un protocolo sobre su metodología en *PROSPERO international prospective register of systematic reviews* (www.crd.york.ac.uk/PROSPERO, registration number CRD42018105228) [122]. Para asegurar el rigor, el protocolo se condujo de acuerdo con las directrices PRISMA-P [123].

Estrategia de búsqueda. Buscamos en las bases de datos PubMed, Embase, Web of Science y Scopus estudios publicados antes de la fecha de búsqueda (límite superior=Mayo 2018) y sin límite de fecha inferior. Las búsquedas se llevaron a cabo combinando términos tesauros de bases de datos (*i.e.* MeSH y EMTREE) y términos libres. Con el fin de maximizar la sensibilidad, nuestra estrategia de búsqueda empleada en PubMed combinó los siguientes términos: ("cortactin"[MeSH Terms] OR ("cortactin"[All Fields] OR "cttn"[All Fields] OR "ems1"[All Fields] OR "ems 1"[All Fields]) AND ("head"[MeSH Terms] OR "head"[All Fields] OR "neck"[MeSH Terms] OR "neck"[All Fields] OR "mouth"[MeSH Terms] OR "mouth"[All Fields] OR "oral"[All Fields] OR "pharynx"[MeSH Terms] OR pharyn*[All

Fields] OR oropharyn*[All Fields] OR nasopharyn*[All Fields] OR hypopharyn*[All Fields] OR "larynx"[MeSH Terms] OR laryn*[All Fields] OR "nose"[MeSH Terms] OR "nose"[All Fields] OR "nasal"[All Fields]) AND ("carcinoma, squamous cell"[MeSH Terms] OR ("carcinoma"[All Fields] AND "squamous"[All Fields] AND "cell"[All Fields]) OR "squamous cell carcinoma"[All Fields]). Una estrategia de búsqueda equivalente fue adaptada a la sintaxis de cada base de datos consultada (ver protocolo).

Además, manualmente hicimos un screening en las listas de referencias de los estudios incluidos, tratando de hallar más estudios relevantes. Todas las referencias extraídas como resultado del proceso de búsqueda fueron gestionadas mediante el software Mendeley v.1.17.10 (Elsevier. Amsterdam, The Netherlands); las referencias duplicadas fueron eliminadas.

Criterios de elegibilidad. Criterios de inclusión. (1) Trabajos de investigación originales publicados en inglés. (2) Evaluación de la amplificación de *CTTN* o de la sobreexpresión de cortactin en tejidos humanos procedentes de carcinomas de células escamosas de cabeza y cuello primarios. En los casos en los que se comunicó únicamente ganancia del gen, ésta fue recogida. (3) Análisis de la asociación entre las alteraciones precedentes de *CTTN*/cortactin con al menos alguna de las siguientes variables clínico-patológicas y/o pronósticas: status N, status T, estadío clínico, grado histológico, supervivencia general, supervivencia libre de enfermedad. La supervivencia general se definió como el periodo de tiempo transcurrido desde la fecha del diagnóstico o de la cirugía hasta la fecha de muerte, por la enfermedad específica o por cualquier otra causa. La supervivencia libre de enfermedad fue definido como el periodo de tiempo transcurrido desde la cirugía hasta la presencia de recurrencia locorregional o a distancia, o muerte sin recurrencia. Ante la falta de estándares de consenso internacional para la definición de "endpoints" de supervivencia, incluimos aquellos trabajos que emplearan la denominación directa de los términos precedentes (superviencia general/libre de enfermedad) o bien denominaciones diferentes (p.e. supervivencia libre de recurrencia) definidas en los estudios originales como en el presente trabajo.

Criterios de exclusión. (1) Artículos de revisión, metaanálisis, reporte de casos, editoriales, cartas, abstracts de reuniones científicas, opiniones personales o comentarios, capítulos de libro y cualquier trabajo en idioma distinto al inglés. (2) Carcinomas de otras localizaciones. (3) Análisis experimientales conducidos *in vitro* o en modelos animales. (4) Evaluación de alteraciones del gen *CTTN* distintas a la amplificación génica (p.e. polimorfismos). Evaluación de la amplificación de la banda cromosómica 11q13 mediante técnicas de mapeo y análisis de las alteraciones del conjunto de genes ubicados en este locus cromosómico, sin discriminar específicamente aquellas ligadas al gen *CTTN*. (5) Trabajos que no analicen la relación con las

variables clínico-patológicas y/o de supervivencia de interés. (6) Datos insuficientes para la estimación de odds ratios (OR) para el análisis de las variables clínico-patológicas. En los estudios que únicamente reportaron variables tiempo-evento (supervivencia general/libre de enfermedad), la no comunicación directa de los hazards ratio (HR) junto con sus intervalos de confianza (CI) al 95%, o de datos esenciales que nos permitiera su estimación estadística a través del análisis de supervivencia.

Los artículos fueron seleccionados en dos fases. En la fase I se seleccionaron los que parecían reunir los criterios de inclusión mediante el screening de títulos y abstracts. En la fase II los artículos fueron leídos y trabajados a texto completo, siendo eliminados aquellos que no reunieron las características expuestas en los criterios de inclusión y exclusión precedentes.

Extracción de datos. Dos autores extrajeron independientemente los datos de los artículos seleccionados para ser trabajados a texto completo de un modo estandarizado, empleando un formulario de recogida de datos en hojas de cálculo mediante el software Excel v.2015 (Microsoft. Redmond, WA, USA). Los datos recogidos fueron revisados adicionalmente por dos autores diferentes. Las discrepancias fueron resueltas mediante consenso. Se recogió el primer autor, año de publicación, país y continente del estudio, tamaño de la muestra, sitio tumoral, periodo de reclutamiento, modalidad de tratamiento, tiempo de seguimiento, alteración evaluada (amplificación de *CTTN* y/o sobreexpresión de cortactin), metodología aplicada y frecuencia de la alteración. En caso de análisis mediante técnica inmunohistoquímica, también recogimos el punto de corte, el anticuerpo anti-cortactin empleado y el patrón de inmunopositividad intracelular evaluado (membrana/citoplasma). También recogimos los datos necesarios para el análisis de los parámetros status N, T, grado histológico, estadío clínico, supervivencia general y libre de enfermedad.

Evaluación de la calidad y del riesgo de sesgo. Se realizó por dos autores empleando Quality in Prognosis Studies (QUIPS) tool (*Cochrane Prognosis Methods Group)* [124], desarrollada por *Cochrane Prognosis Methods Group*. Esta herramienta incluye 6 áreas importantes de sesgos potenciales en los trabajos seleccionados ([1] Study participation, [2] Study attrition, [3] Prognostic Factor Measurement, [4] Outcome Measurement, [5] Study Confounding and [6] Statistical analysis and reporting) [125]. El riesgo de sesgo se calificó como bajo, moderado o alto para cada dominio. Las discrepancias se resolvieron mediante consenso.

Análisis estadístico. La amplificación de *CTTN* fue considerada como "positiva" o "negativa" de acuerdo a metodología asumida por los autores de cada estudio. La expresión de cortactin fue considerada "alta" o "baja" de acuerdo a los valores de corte provistos por los autores de cada estudio. Cuando en un mismo estudio individual se estudió tanto la amplificación como la sobreexpresión de *CTTN*/cortactin, ambos datos fueron recogidos y analizados separadamente. Usamos odds ratios (OR) con sus correspondientes CI al 95% como medida de asociación para determinar la correlación entre las alteraciones de *CTTN*/cortactin y las variables clínico-patológicas de los pacientes con carcinoma de cabeza y cuello. Usamos hazard ratios (HR) con sus correspondientes CI al 95% como medida de asociación para estimar el impacto de las alteraciones de *CTTN*/cortactin sobre las variables tiempo-evento. Si los autores publicaron HR y sus CI al 95% se extrajeron estos directamente de los artículos originales. Ante los estudios que publicaron HR en diferentes modelos (univariante y multivariante), se extrajeron los datos del modelo multivariante, que refleja un mayor ajuste de las variables potencialmente confundidoras. Si estos datos no fueron dados por los autores explícitamente, fueron calculados por nosotros mediante los métodos de Parmar *et al* [126] y Tierney *et al* [127]. Si un estudio solo comunicó curva de supervivencia, extrajimos los datos de las curvas Kaplan-Meier mediante el software Engauge Digitizer 4.1 (open-source digitizing software desarrollado por M. Mitchell). En algunos estudios que no comunicaron HR se extrajeron estimaciones del riesgo relativo (RR) u OR ajustadas, que se asumieron como una aproximación de la misma medida [128].

En el metaanálisis, las diferentes medidas de asociación derivadas de los estudios individuales fueron agrupadas. Para la estimación de las asociaciones combinadas usamos tanto modelos de efectos fijos (métodos de Mantel-Haenszel e inverso de la varianza) como un modelo de efectos aleatorios (método de DerSimonian and Laird). Usamos forest plots para representar gráficamente el efecto general, y para su posterior análisis. Evaluamos la heterogeneidad entre los estudios usando el test Q de Cochran basado en Chi-cuadrado [129]. Debido a la baja potencia estadística de este test, el nivel de significación se definió como $p<0.1$, en cuyo caso asumimos que existió heterogeneidad aparente y consecuentemente usamos un modelo de efectos aleatorios para calcular las estimaciones combinadas. Además, cuantificamos la proporción de heterogeneidad con el estadístico I^2 de Higgins, donde valores del 25%, 50% y 75% pueden considerarse como baja, moderada o alta heterogeneidad respectivamente [129,130].

Además, llevamos a cabo análisis de sensibilidad, evaluando la influencia de cada estudio individual sobre la estimación del efecto general, testando así la fiabilidad de los resultados combinados [131]. Para ello, repetimos secuencialmente el metaanálisis tras la omisión de un estudio en cada turno, y representamos gráficamente sus resultados. También, construimos

funnel plots y usamos el test de regresión Egger ($p<0.1$) para evaluar los efectos de los estudios pequeños, tales como el sesgo de publicación [132–134].

Conducimos análisis de subgrupos (por alteración de *CTTN*/cortactin, etnicidad, sitio tumoral, anticuerpo anti-cortactin y punto de corte) con el objetivo de identificar posibles fuentes de heterogeneidad, así como para explorar las relaciones entre las alteraciones de *CTTN*/cortactin con las variables pronósticas y clínicopatologícas en los subgrupos precedentes. Por último, tratamos de analizar mediante técnicas de metarregresión si la amplificación del gen *CTTN* o la sobreexpresión de su producto cortactin poseen un valor pronóstico similar o diferente.

Todos los análisis estadísticos en este metaanálisis se practicaron con el software Stata versión 14.1 (Stata Corp, College Station, TX, USA), usando comandos escritos por el usuario [135]. En el metaanálisis, consideramos estadísticamente significativo un valor $p<0.05$.

RESULTADOS

ANÁLISIS CUALITATIVO Y CUANTITATIVO (METAANÁLISIS) DE LAS IMPLICACIONES PRONÓSTICAS Y CLÍNICOPATOLÓGICAS DE LA EXPRESIÓN DE CICLINA D1 EN EL CARCINOMA ORAL DE CÉLULAS ESCAMOSAS.

Resultados de la búsqueda de la literatura

El diagrama de flujo (Fig.1, trabajo: Ramos-García P, Ruiz-Ávila I, Gil-Montoya JA, Ayén Á, González-Ruiz L, Navarro-Triviño FJ, González-Moles MÁ. Relevance of chromosomal band 11q13 in oral carcinogenesis: An update of current knowledge. Oral Oncol. 2017 Sep;72:7-16.) muestra los resultados de la investigación de la literatura y del proceso de selección. Identificamos un total de 1786 registros, 358 en PubMed, 540 en Embase, 476 en Web of Science, 412 en Scopus y 2 registros adicionales procedentes del screening de las listas de referencias bibliográficas, antes de agosto de 2017. Tras la eliminación de registros duplicados filtramos 784 estudios potencialmente elegibles. Una vez llevado a cabo el screening de títulos y abstracts, 71 estudios seleccionados fueron evaluados a texto completo. Finalmente, tras la exclusión de los estudios que no cumplieron con los criterios de inclusión, 31 estudios fueron incluidos en la evaluación cualitativa y en el metaanálisis cuantitativo [34–64].

Características del estudio

La tabla 1 (trabajo: Ramos-García P, Ruiz-Ávila I, Gil-Montoya JA, Ayén Á, González-Ruiz L, Navarro-Triviño FJ, González-Moles MÁ. Relevance of chromosomal band 11q13 in oral carcinogenesis: An update of current knowledge. Oral Oncol. 2017 Sep;72:7-16) resume las características de los 31 estudios elegidos que incluyen 2942 pacientes con COCE. Los tamaños muestrales oscilaron entre 29 a 290 pacientes. Los estudios se realizaron en países de todos los continentes, excepto África. Se analizaron 23 estudios asiáticos y 8 de países no ásiaticos (3 en Europa, 3 en Sudamérica, 1 en Norteamérica y 1 en Australia). La expresión de ciclina D1 fue mayoritariamente investigada en el núcleo, aunque 5 estudios evaluaron la inmunotinción nuclear y citoplasmática conjuntamente. El punto de corte para la consideración de sobreexpresión de ciclina D1 varió entre estudios, siendo el 10% (rango 1-50%) el más frecuentemente seleccionado (12 estudios). Los estudios usaron diversos anticuerpos anti-ciclina D1, siendo los más frecuentes los monoclonales P2D11F11, DCS-6, SP4 y 5D4.

El presente metaanálisis evaluó el valor pronóstico de la expresión de ciclina D1 en la supervivencia general de 1524 pacientes con COCE (reclutados en 15 estudios), en la supervivencia libre de enfermedad de 831 pacientes (reclutados en 5 estudios), en el status T (T3/4 vs T1/2) de 1493 pacientes (reclutados en 15 estudios), en el status N (N+ vs N-) de 2225 de pacientes (reclutados en 21 estudios), en el estadío clínico (III/IV vs I/II) de 1526 pacientes

(reclutados en 15 estudios) y en el grado histológico (II/III vs I) de 1901 pacientes (reclutados en 20 estudios). Pudimos conducir el metaanálisis de todas las variables precedentes, debido a que todas presentaron una muestra competente en términos cuantitativos para su realización.

Análisis cualitativo

Para el análisis cualitativo se empleó *Quality In Prognosis Studie*s (QUIPS) tool, que evalúa fuentes potenciales de sesgo en seis dominios (Fig.2, trabajo: Ramos-García P, Ruiz-Ávila I, Gil-Montoya JA, Ayén Á, González-Ruiz L, Navarro-Triviño FJ, González-Moles MÁ. Relevance of chromosomal band 11q13 in oral carcinogenesis: An update of current knowledge. Oral Oncol. 2017 Sep;72:7-16.):

Study participation. El 42%, 39% y 19% de los estudios evaluados presentaron alto, moderado y bajo riesgo de sesgo, respectivamente. Los sesgos más frecuentes fueron no describir adecuadamente las características de la muestra (edad, sexo, etc), no comunicar el periodo y lugar de reclutamiento e inclusión de pacientes no representativos de la población de interés.

Study atrittion. El 58%, 26% y 16% de los estudios evaluados presentaron alto, moderado y bajo riesgo de sesgo, respectivamente. El sesgo más relevante documentado fue no comunicar la tasa de retirada (número de pacientes perdidos) durante el periodo de seguimiento. También fue frecuente la comunicación de datos insuficientes relacionados con el periodo de seguimiento, o la no comunicación del periodo de seguimiento. Ningún estudio describió el intento de recolectar información de los pacientes perdidos, las razones de pérdida de seguimiento o una descripción de las características de estos pacientes, datos esenciales para garantizar que los pacientes no perdidos durante el seguimiento representan adecuadamente a la muestra.

Prognostic factor measurement. El 29%, 29% y 42% de los estudios evaluados presentaron alto, moderado y bajo riesgo de sesgo, respectivamente. Los sesgos potenciales más relevantes fueron el uso de un método de medición de la expresión de ciclina D1 inadecuado y el empleo de puntos de corte inadecuados (p.e. el empleo de puntos de corte optimizados en base al análisis de datos, que introduce sesgos severos en los trabajos de investigación [150]). Entre los sesgos potenciales más frecuentes observamos comunicación de información insuficiente tanto de la técnica IHQ, como del sistema de medición de los niveles de expresión de ciclina D1. Eventualmente no se comunicó el patrón de inmunopositividad analizado (nuclear o nuclear/citoplasmático) o no se publicaron imágenes de la técnica IHQ.

Outcome measurement. El 42%, 42% y 16% de los estudios evaluados presentaron alto, moderado y bajo riesgo de sesgo, respectivamente. Los sesgos potenciales más frecuentes

fueron no definir los parámetros de supervivencia evaluados (esencial por la falta de consenso internacional en la nomenclatura de los endpoints de supervivencia), no comunicar el sistema de clasificación seguido (p.e. edición del AJCC/UICC TNM staging system, sujeta a cambios periódicos[151]) o el método de análisis de los diferentes parámetros clínico-patológicos.

Study confounding. El 26% y 74% de los estudios evaluados presentaron moderado y alto riesgo de sesgo, respectivamente. Este es el dominio que presentó una más baja calidad en los trabajos analizados. Los sesgos potenciales más frecuentes fueron no tener en cuenta en el diseño del estudio a los factores confundidores, y no medir todos los factores potencialmente confundidores. Ningún trabajo incluido provee a priori definiciones claras de los factores potencialmente confundidores medidos, ni tampoco posteriormente se discute sobre los factores potencialmente confundidores candidatos o sobre los principios biológicos por los que éstos factores podrían distorsionar el impacto de la sobreexpresión de ciclina D1 sobre las variables analizadas.

Statistical analysis and reporting. El 13%, 52% y 35% de los estudios evaluados presentaron bajo, moderado y alto riesgo de sesgo, respectivamente. Los sesgos potenciales detectados más relevantes fueron análisis estadístico inadecuado, comunicación errónea de datos y el análisis potencialmente sesgado de ellos derivado debido al uso de puntos de corte inadecuados. Con menor importancia -pero más frecuente- reporte selectivo y comunicación insuficiente de datos que permitan evaluar si el análisis es adecuado (p.e. curvas Kaplan-Meier, intervalos de confianza, etc).

Metaanálisis

Asociación entre la sobreexpresión de ciclina D1 y la supervivencia de los pacientes con COCE (Fig. 3A, trabajo: Ramos-García P, González-Moles MÁ, González-Ruiz L, Ruiz-Ávila I, Ayén Á, Gil-Montoya JA. Prognostic and clinicopathological significance of cyclin D1 expression in oral squamous cell carcinoma: A systematic review and meta-analysis. Oral Oncol. 2018 Aug;83:96-106).

Supervivencia general. Encontramos evidencia de heterogeneidad, de bajo grado de acuerdo a los puntos de corte de Higgins ($p=0.068$, $I^2=37.9\%$) entre los estudios incluidos. El modelo de efectos aleatorios indicó una relación estadísticamente significativa entre la sobreexpresión de ciclina D1 y una pobre supervivencia general (HR=2.00, 95% CI=1.59-2.51, $p<0.001$).

Supervivencia libre de enfermedad. No existió heterogeneidad significativa entre los estudios ($p=0.389$, $I^2=3.1\%$). El modelo de efectos fijos señaló una relación estadísticamente

significativa entre la sobreexpresión de ciclina D1 y una supervivencia libre de enfermedad disminuida (HR=1.46, 95% CI=1.13-1.87, p=0.003).

Asociación entre la sobreexpresión de ciclina D1 y las variables clínico-patológicas de los pacientes con COCE. (Fig. 3B-C, trabajo: Ramos-García P, González-Moles MÁ, González-Ruiz L, Ruiz-Ávila I, Ayén Á, Gil-Montoya JA. Prognostic and clinicopathological significance of cyclin D1 expression in oral squamous cell carcinoma: A systematic review and meta-analysis. Oral Oncol. 2018 Aug;83:96-106)

Status T. Encontramos evidencia de heterogeneidad de bajo grado (p=0.03, I^2=45%) entre los estudios. El modelo de efectos aleatorios sugirió una relación estadísticamente significativa entre la sobreexpresión de ciclina D1 y carcinomas localmente avanzados (T3/4) (OR=1.51, 95% CI=1.07-2.13, p=0.02).

Status N. Encontramos evidencia de baja heterogeneidad (p=0.01, I^2=46.7%) entre los estudios. El modelo de efectos aleatorios señaló una relación estadísticamente significativa entre la sobreexpresión de ciclina D1 y la presencia de metástasis ganglionar linfática (N+) (OR=2.16, 95% CI=1.60-2.92, p<0.001).

Estadio clínico. No existió heterogeneidad significativa entre los estudios (p=0.586, I^2=0.0%) El modelo de efectos fijos indicó una relación estadísticamente significativa entre la sobreexpresión de ciclina D1 y estadíos avanzados de la enfermedad (III/IV) (OR=1.44, 95% CI= 1.15-1.81, p=0.002).

Grado histológico. Encontramos evidencia de heterogeneidad moderada entre los estudios (p=0.004, I^2=51.8%). El modelo de efectos aleatorios señaló también una relación estadísticamente significativa entre la sobreexpresión de ciclina D1 y un grado histológico más avanzado (II/III) (OR=1.60, 95% CI=1.12-2.29, p=0.010).

Metaanálisis (análisis secundarios)

Análisis de sensibilidad

Los resultados generales no se alteraron considerablemente tras la repetición secuencial de los metaanálisis omitiendo un estudio en cada turno. Esto sugiere que las estimaciones combinadas comunicadas no son dependientes de la influencia de un estudio individual en particular (Fig.3A-C, trabajo: Ramos-García P, González-Moles MÁ, González-Ruiz L, Ruiz-Ávila I,

Ayén Á, Gil-Montoya JA. Prognostic and clinicopathological significance of cyclin D1 expression in oral squamous cell carcinoma: A systematic review and meta-analysis. Oral Oncol. 2018 Aug;83:96-106).

Análisis de los efectos de los estudios pequeños

El análisis de inspección visual de la asimetría de los funnel plots construidos (Fig.3A-C, trabajo: Ramos-García P, Ruiz-Ávila I, Gil-Montoya JA, Ayén Á, González-Ruiz L, Navarro-Triviño FJ, González-Moles MÁ. Relevance of chromosomal band 11q13 in oral carcinogenesis: An update of current knowledge. Oral Oncol. 2017 Sep;72:7-16.), así como los tests estadísticos conducidos para el mismo fin constatan la ausencia de efecto de los estudios pequeños en las variables pronósticas (supervivencia general [p_{Egger}=0.717], supervivencia libre de enfermedad [p_{Egger}=0.471]) y clínicopatológicas (estadio clínico [p_{Egger}=0.177], grado histológico [p_{Egger}=0.461]) analizadas, excepto en el status T ([p_{Egger}=0.024) y en el status N [p_{Egger}=0.015], donde no podemos descartar la existencia de sesgos, tales como el sesgo de publicación.

Análisis de subgrupos (Tabla2, trabajo: Ramos-García P, González-Moles MÁ, González-Ruiz L, Ruiz-Ávila I, Ayén Á, Gil-Montoya JA. Prognostic and clinicopathological significance of cyclin D1 expression in oral squamous cell carcinoma: A systematic review and meta-analysis. Oral Oncol. 2018 Aug;83:96-106)

En el análisis estratificado de acuerdo a la *supervivencia general*, numerosos subgrupos conservaron la asociación estadísticamente significativa (grupo asiático [HR=2.17, 95% CI=1.69-2.78, p<0.001], carcinoma lingual [OR=2.51, 95% CI=1.73-3.64, p<0.001] y subsitios mixtos (incluyen otras localizaciones intraorales diferentes a la lengua) [HR=1.86, 95% CI=1.40-2.48, p<0.001], los patrones IHQ nuclear [HR=2.03, 95% CI=1.65-2.50, p<0.001] y la combinación nuclear-citoplasmática [HR=2.26, 95% CI=1.10-4.62, p=0.03], el empleo de los anticuerpos monoclonales P2D11F11 [HR=2.42, 95% CI=1.33-4.42, p=0.004], 5D4 [HR=2.30, 95% CI=1.52-3.47, p<0.001] y otros anticuerpos variados [HR=2.72, 95% CI=1.80-4.11, p=0.01], y los puntos de corte >10% [HR=2.14, 95% CI=1.41-3.24, p<0.001] y del 10% [HR=2.02, 95% CI=1.62-2.51, p<0.001]). No encontramos una fuente potencial de heterogeneidad severa en el análisis de subgrupos. Hallamos heterogeneidad moderada en algunos subgrupos (asiáticos [p=0.08, I^2=41.1%], subsitios mixtos [p=0.03, I^2=50.2%], patrón IHQ combinado nuclear-citoplasmático [p=0.03, I^2=67.0%] y el empleo del anticuerpo monoclonal P2D11F11 [p=0.008, I^2=55.5%]).

En el análisis estratificado de acuerdo a la *supervivencia libre de enfermedad*, algunos subgrupos mantuvieron asociaciones estadísticamente significativas (asiático [HR=1.50, 95% CI=1.12-2.01, p=0.006], subsitios mixtos [HR=1.40, 95% CI=1.07-1.81, p=0.01], patrón IHQ nuclear [HR=1.71, 95% CI=1.25-2.33, p=0.001], y el punto de corte del 10% [HR=1.57, 95% CI=1.19-2.08, p=0.001]). No encontramos fuentes de heterogeneidad potenciales en el análisis de subgrupos.

En el análisis estratificado de acuerdo al *status T*, dos subgrupos conservaron la asociación estadística, los carcinomas de lengua (OR=1.93, 95% CI=1.26-2.97, p=0.02) y el punto de corte >10% (OR=3.46, 95% CI=1.77-6.78, p=<0.001). Además, encontramos fuentes importantes de heterogeneidad al estratificar por diferentes anticuerpos anti-ciclina D1 usados (clones SP4 [p=0.02, I^2=81.6%] y 5D4 [p=0.003, I2=82.4%]).

En el análisis estratificado de acuerdo al *status N*, numerosos subgrupos mantuvieron asociaciones estadísticamente significativas (por grupo étnico, asiáticos [OR=2.02, 95% CI=1.43-2.87, p<0.001] y no asiáticos [OR=3.01, 95% CI=1.83-4.94, p<0.001], por subsitio anatómico, lengua [OR=2.87, 95% CI=1.94-4.24, p<0.001] y subsitios mixtos [OR=1.88, OR=1.27-2.77, p=0.002], por patrón IHQ, el patrón nuclear [OR=2.40, 95% CI=1.66-3.47, p<0.001], por anticuerpo, los monoclonales P2D11F11 [OR=1.98, 95% CI=1.25-3.12, p=0.03] y SP4 [OR=2.62, 95% CI=1.74-3.94, <0.001] y por punto de corte, >10% [OR=3.20, 95% CI=2.12-4.84, p<0.001] y del 10% [OR=1.86, 95% CI=1.20-2.89, p=0.006]). Además, encontramos heterogeneidad significativa de grado moderado en los subgrupos subsitios mixtos (p=0.006, I^2=57.8%), patrón IHQ nuclear (p=0.004, I^2=54.4%) y punto de corte <10 (p=0.006, I2=59.2%) y con el uso del anticuerpo monoclonal 5D4 (p=0.05, I2=67.5%); y heterogeneidad severa en el empleo de otros anticuerpos anti-ciclina D1 variados (p=0.008, I^2=74.7%) y un punto de corte <10% (p=0.04, I^2=75.2%).

En el análisis estratificado de acuerdo al *estadío clínico*, numerosos subgrupos conservaron esta asociación estadística (el subgrupo asiático [OR=1.44, 95% CI=1.15-1.81, p=0.004], los carcinomas linguales [OR=1.87, 95% CI=1.23-2.83, p=0.003], el patrón IHQ nuclear [OR=1.43, 95% CI=1.10-1.86, p=0.01], los anticuerpos P2D11F11 [OR=1.91, 95% CI=1.22-2.98, p=0.004] y DCS-6 [OR=4.08, 95% CI=1.29-12.85, p=0.01], y el punto de corte 10% [OR=1.47, 95% CI=1.13-1.90]). Al igual que en el análisis global, no detectamos fuentes de heterogeneidad potenciales en ninguno de los subgrupos analizados.

En el análisis estratificado de acuerdo al *grado histológico*, algunos subgrupos mantuvieron asociaciones estadísticas (el subgrupo no asiático [OR=3.08, 95% CI=1.67-5.70, p=0.005], los subsitios mixtos [OR=1.97, 95% CI=1.22-3.20, p=0.006], el patrón IHQ nuclear [OR=1.91, 95% CI=1.49-2.45, p=0.001], los anticuerpos DCS-6 [OR=3.12, 95% CI=1.39-7.00, p=0.007] y

SP4 [OR=1.69, 95% CI=1.03-2.78, p=0.04] y el punto de corte 10% [OR=1.71, 95% CI=1.12-2.61, p=0.01]). Hallamos heterogeneidad moderada en el grupo subsitios anatómicos (en carcinoma lingual [p=0.07, I^2=47.2%] y subsitios mixtos [p=0.007, I^2=57.5%]), lo que parece indicar que no es una fuente de heterogeneidad potencial. Encontramos fuentes potenciales de heterogeneidad, de grado moderado en el subgrupo asiático [p=0.007, I^2=52.9%], y en el punto de corte del 10% [p=0.04, I2=50.2%]. También encontramos dos fuentes de heterogeneidad importante, con el empleo del anticuerpo monoclonal P2D11F11 (p=0.008, I^2=74.7%) y de un punto de corte <10% (p=0.002, I^2=84.4%).

ESTUDIO EXPERIMENTAL MEDIANTE TÉCNICA INMUNOHISTOQUÍMICA DE LAS IMPLICACIONES DE LA EXPRESIÓN DE CICLINA D1 EN CARCINOGÉNESIS ORAL.

En el presente estudio se analizaron 54 pacientes que desarrollaron un total de 68 COCEs (Tabla 1, trabajo: Ramos-García P, González-Moles MÁ, Ayén Á, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Gil-Montoya JA, Bravo M. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression in adjacent non-tumour epithelium in oral squamous cell carcinoma. Arch Oral Biol. 2018 Oct 9;97:12-17). Destaca el hecho de que 15 de los 54 pacientes (27.8%) presentaron más de 1 tumor. La tabla 2 (trabajo: Ramos-García P, González-Moles MÁ, Ayén Á, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Gil-Montoya JA, Bravo M. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression in adjacent non-tumour epithelium in oral squamous cell carcinoma. Arch Oral Biol. 2018 Oct 9;97:12-17) recoge la descripción clinicopatológica de los tumores de nuestra serie (20 tumores fueron T3 y T4 [34.5%], y 21 tumores [36.2%] N+), así como su tasa de proliferación medida a través de la expresión de ki-67.

Implicaciones pronósticas de la expresión nuclear de ciclina D1 en cáncer oral.

La expresión media de ciclina D1 nuclear de las células tumorales en todos los tumores de nuestra serie fue del 28.7% (±21.5%). Según el número de tumores por paciente, la expresión media ciclina D1 fue del 32.7% (23.7%) de las células tumorales en aquellos pacientes con un solo tumor; del 18.3% (±16.0%) en pacientes con 2 tumores, y del 27.2% (±16.3%) en pacientes con más de 2 tumores (datos no mostrados en tablas).

La tabla 3 (trabajo: Ramos-García P, González-Moles MA, González-Ruiz L, Ayén A, Ruiz-Ávila I, Bravo M, Gil-Montoya JA. Prognostic relevance of tumor cyclin D1 expression in oral cáncer 2018: [In Submission]) recoge las asociaciones estadísticas encontradas entre la expresión de ciclina D1 nuclear y las variables clinicopatológicas analizadas. Se ha encontrado una asociación estadística significativa entre la expresión nuclear de ciclina D1 y tumores con un elevado grado histológico (p=0.030). En aquellos tumores que expresaron ciclina D1 concomitantemente en núcleo y citoplasma (n=23) (Fig.1, trabajo: Ramos-García P, González-Moles MA, González-Ruiz L, Ayén A, Ruiz-Ávila I, Bravo M, Gil-Montoya JA. Prognostic relevance of tumor cyclin D1 expression in oral cáncer 2018: [In Submission]), se observó también una correlación estadística significativa entre el porcentaje de células inmunopositivas y una morfología invasiva (p=0.045). Resulta destacable la asociación encontrada entre la expresión nuclear de ciclina D1 y la expresión de ki-67 en las células tumorales (p=0.018).

Implicaciones pronósticas de la expresión citoplasmática de ciclina D1 en cáncer oral.

La tabla 3 (trabajo:Ramos-García P, Bravo M, González-Ruiz L, González-Moles MÁ. Significance of cytoplasmic cyclin D1 expression in oral oncogenesis. Oral Dis. 2018 Mar;24(1-2):98-102. doi: 10.1111/odi.12752.) muestra los resultados de la asociación estadística entre el recuento de células tumorales que expresan ciclina D1 y la intensidad de la expresión. La expresión media de ciclina D1 citoplasmática de las células tumorales de nuestra serie fue 6.4% (±16.1%) (Fig. 1B, trabajo:Ramos-García P, Bravo M, González-Ruiz L, González-Moles MÁ. Significance of cytoplasmic cyclin D1 expression in oral oncogenesis. Oral Dis. 2018 Mar;24(1-2):98-102. doi: 10.1111/odi.12752.). Se observa una mayor intensidad de la expresión de ciclina D1 en aquellos tumores con un porcentaje mayor de células ciclina D1-positivas (p=0.03). La tabla 4 (trabajo:Ramos-García P, Bravo M, González-Ruiz L, González-Moles MÁ. Significance of cytoplasmic cyclin D1 expression in oral oncogenesis. Oral Dis. 2018 Mar;24(1-2):98-102. doi: 10.1111/odi.12752.) relaciona la intensidad y el porcentaje de expresión de ciclina D1 citoplasmática con las variables clínico-patológicas. Se ha encontrado una asociación estadística significativa entre la intensidad de expresión de ciclina D1 citoplasmática y una morfología invasiva (p=0.020). Se observó también una correlación estadísticamente significativa entre el porcentaje de expresión de ciclina D1 citoplasmática y los parámetros pobre grado de diferenciación (p=0.007) y expresión de ki-67 en las células tumorales (p=0.004). Resulta destacable la asociación marginal encontrada entre el porcentaje de expresión de ciclina D1 citoplasmática y un estadío clínico avanzado. Se ha encontrado una asociación estadística entre la expresión nuclear y citoplasmática de ciclina D1 (datos no mostrados en tablas). Así, mientras el 45% de las células tumorales que presentaban expresión

nuclear intensa mostraron expresión citoplasmática leve, todos los tumores que mostraron expresión citoplasmática moderada o intensa, mostraron también expresión nuclear intensa (p=0.041). También se observó una correlación estadística significativa positiva entre el porcentaje de células que expresaron ciclina D1 nuclear y citoplasmática (r=0.64, p<0.001).

Implicaciones pronósticas de la expresión de ciclina D1 en epitelio premaligno oral.

La tabla 3 (trabajo: Ramos-García P, González-Moles MÁ, Ayén Á, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Gil-Montoya JA, Bravo M. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression in adjacent non-tumour epithelium in oral squamous cell carcinoma. Arch Oral Biol. 2018 Oct 9;97:12-17) recoge las asociaciones estadísticas encontradas entre la expresión de ciclina D1 en los epitelios no tumorales adyacentes a carcinomas múltiples y el número de tumores desarrollados. Nuestros resultados indican que la expresión basal y parabasal en el núcleo de células de epitelios no tumorales alejados del punto de invasión tumoral fue significativamente mayor en pacientes que desarrollaron más de 2 tumores (p<0.001 respectivamente) (Fig.1C, trabajo: Ramos-García P, González-Moles MÁ, Ayén Á, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Gil-Montoya JA, Bravo M. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression in adjacent non-tumour epithelium in oral squamous cell carcinoma. Arch Oral Biol. 2018 Oct 9;97:12-17).

La tabla 4 (trabajo: Ramos-García P, González-Moles MÁ, Ayén Á, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Gil-Montoya JA, Bravo M. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression in adjacent non-tumour epithelium in oral squamous cell carcinoma. Arch Oral Biol. 2018 Oct 9;97:12-17) recoge las asociaciones estadísticas encontradas en la expresión de ciclina D1 entre los epitelios no tumorales lejanos y cercanos al punto de invasión. En general se observa que la mayor expresión nuclear de ciclina D1 en los diferentes estratos del epitelio no tumoral cercano al punto de invasión se asoció también a una mayor expresión en los diferentes estratos del epitelio alejado del punto de invasión.

La tabla 5 (trabajo: Ramos-García P, González-Moles MÁ, Ayén Á, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Gil-Montoya JA, Bravo M. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression in adjacent non-tumour epithelium in oral squamous cell carcinoma. Arch Oral Biol. 2018 Oct 9;97:12-17) recoge las asociaciones estadísticas encontradas entre la expresión de ciclina D1 y la tasa de proliferación celular (expresión de ki-67) en epitelios no tumorales adyacentes a carcinomas orales. Resulta destacable la asociación encontrada entre la expresión nuclear de ciclina D1 y la expresión de ki-67 en la capa basal del epitelio alejado del punto de invasión invasión (p=0.02), así como las asociaciones de la

expresión citoplasmática de ciclina D1 en diferentes estratos de epitelios lejanos y cercanos al punto de invasión (Fig.1C, trabajo: Ramos-García P, González-Moles MÁ, Ayén Á, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Gil-Montoya JA, Bravo M. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression in adjacent non-tumour epithelium in oral squamous cell carcinoma. Arch Oral Biol. 2018 Oct 9;97:12-17) con la tasa de expresión de ki-67.

ANÁLISIS CUALITATIVO Y CUANTITATIVO (METAANÁLISIS) DE LAS IMPLICACIONES PRONÓSTICAS Y CLÍNICOPATOLÓGICAS DE LAS ALTERACIONES DE CTTN/CORTACTIN

Resultados de la búsqueda de la literatura

El diagrama de flujo (Fig.1, trabajo: Ramos-García P, González-Moles MA, Ayén A, González-Ruiz L, Ruiz-Ávila I, Gil-Montoya JA. Prognostic and clinicopathological significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis 2018: [In Submission]) muestra los resultados de la investigación de la literatura y del proceso de selección. Identificamos un total de 342 registros, 73 en PubMed, 105 en Embase, 75 en Web of Science, 89 en Scopus y 2 registros adicionales procedentes del screening de las listas de referencias bibliográficas, antes de mayo de 2018. Tras la eliminación de registros duplicados filtramos 133 estudios potencialmente elegibles. Una vez llevado a cabo el screening de títulos y abstracts, 52 estudios seleccionados fueron evaluados a texto completo. Finalmente, tras la exclusión de los estudios que no cumplieron con los criterios de inclusión, 18 estudios fueron incluidos en la evaluación cualitativa y en el metaanálisis cuantitativo [40–57].

Características del estudio

La tabla 1 (trabajo: Ramos-García P, González-Moles MA, Ayén A, González-Ruiz L, Ruiz-Ávila I, Gil-Montoya JA. Prognostic and clinicopathological significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis 2018: [In Submission]) resume las características de los 18 estudios elegidos que incluyen 1633 pacientes con HNSCC. Los tamaños muestrales de estos estudios oscilaron entre 13 y 222 pacientes. 7 estudios analizaron la amplificación de *CTTN*, 9 estudios la sobreexpresión su producto cortactin y 2 ambas alteraciones. 9 estudios evaluaron las implicaciones de *CTTN*/cortactin en carcinomas de cavidad oral, 6 en SCCs faringolaríngeos, y 3 en HNSCCs mixtos (denominamos así al subgrupo de estudios que evaluó conjuntamente a los carcinomas

precedentes o a los ubicados en diferentes localizaciones). 6 estudios fueron llevados a cabo en países asiáticos y 12 en países no ásiaticos (11 en Europa y 1 en Norteamérica). El punto de corte para la consideración inmunohistoquímica de sobreexpresión de cortactin varió entre estudios, siendo el 50% el más frecuentemente seleccionado (5 estudios), 4 estudios emplearon puntos de corte inferiores al 50% y 3 estudios no emplearon un punto de corte cuantitativo, interpretando la sobreexpresión de cortactin en base a la intensidad de la coloración. Los estudios usaron numerosos anticuerpos anti-cortactin diferentes, siendo el más frecuente el monoclonal clone 30 (5 estudios).

El presente metaanálisis evaluó el valor pronóstico de las alteraciones de *CTTN*/cortactin en el status N (N+ vs N-) de 1222 de pacientes (reclutados en 14 estudios; 2 estudios [48,49] analizaron ambas alteraciones [n=16]), en el status T (T3/4 vs T1/2) de 997 pacientes (reclutados en 10 estudios; 1 estudio [49] analizó ambas alteraciones [n=11]), en el estadío clínico (III/IV vs I/II) de 1083 pacientes (reclutados en 10 estudios; 1 estudio analizó [49] ambas alteraciones [n=11]), en el grado histológico (II/III vs I) de 1064 pacientes (reclutados en 9 estudios; 1 estudio [49] analizó ambas alteraciones [n=10]) y en la supervivencia general de 1118 pacientes con HNSCC (reclutados en 8 estudios; 1 estudio [49] analizó ambas alteraciones [n=9]). Como solamente dos estudios [47,51] investigaron el parámetro supervivencia libre de enfermedad (273 pacientes), decidimos no metaanalizar esta variable.

Análisis cualitativo

Para el análisis cualitativo se empleó *Quality In Prognosis Studie*s (QUIPS) tool, que evalúa fuentes potenciales de sesgo en seis dominios (Fig.2 trabajo: Ramos-García P, González-Moles MA, Ayén A, González-Ruiz L, Ruiz-Ávila I, Gil-Montoya JA. Prognostic and clinicopathological significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis 2018: [In Submission]):

Study participation. El 39%, 39% y 22% de los estudios evaluados presentaron alto, moderado y bajo riesgo de sesgo, respectivamente. Los sesgos más frecuentes fueron no comunicar el periodo y lugar de reclutamiento, no describir adecuadamente las características de la muestra (edad, hábitos nocivos, etc), e inclusión de pacientes no representativos de la población de interés.

Study attrition. El 11%, 11% y 78% de los estudios evaluados presentaron alto, moderado y bajo riesgo de sesgo, respectivamente. Los sesgos más frecuentes fueron la comunicación de datos insuficientes relacionados con el periodo de seguimiento, o la no comunicación del periodo de seguimiento. Ningún estudio describió el intento de recolectar información de los

pacientes perdidos, las razones de pérdida de seguimiento o una descripción de las características de estos pacientes.

Prognostic factor measurement. El 44%, 17% y 39% de los estudios evaluados presentaron alto, moderado y bajo riesgo de sesgo, respectivamente. Entre los sesgos potenciales más frecuentes observamos comunicación de información insuficiente tanto de la técnica IHQ, como del sistema de medición de los niveles de expresión de cortactin. Eventualmente no se comunicó el patrón de inmunopositividad analizado (citoplasmático o de membrana) o no se publicaron imágenes de la técnica IHQ. Menos frecuentes, aunque más relevantes fueron el uso de un método de medición de la expresión de cortactin inadecuado y el empleo de puntos de corte inadecuados.

Outcome measurement. El 33%, 33% y 33% de los estudios evaluados presentaron alto, moderado y bajo riesgo de sesgo, respectivamente. Los sesgos potenciales más frecuentes fueron no definir los parámetros de supervivencia evaluados (esencial ante la falta de consenso internacional en la nomenclatura de los endpoints de supervivencia), no comunicar el sistema de clasificación seguido (p.e. edición del AJCC/UICC TNM staging system, sujeta a cambios periódicos [58]) o el método de análisis de los diferentes parámetros clínico-patológicos.

Study confounding. El 89% y 11% de los estudios evaluados presentaron alto y moderado riesgo de sesgo, respectivamente. Como es habitual, este es el dominio que presentó una más baja calidad en los trabajos analizados. Los sesgos potenciales más frecuentes fueron no tener en cuenta en el diseño del estudio a los factores confundidores, y no medir todos los factores potencialmente confundidores. En ningún trabajo incluido se provee *a priori* definiciones claras de los factores potencialmente confundidores medidos, ni tampoco posteriormente se discute sobre los factores potencialmente confundidores candidatos o sobre los principios biológicos por los que estos factores podrían distorsionar el impacto de las alteraciones de *CTTN*/cortactin sobre las variables analizadas.

Statistical analysis and reporting. El 44%, 28% y 28% de los estudios evaluados presentaron alto, moderado y bajo riesgo de sesgo, respectivamente. Los sesgos potenciales más frecuentes fueron análisis estadístico inadecuado, comunicación insuficiente de datos que permitan evaluar si el análisis es adecuado (p.e. curvas Kaplan-Meier), comunicación errónea de datos y el análisis potencialmente sesgado de ellos derivado debido al uso de puntos de corte inadecuados. No detectamos en ningún trabajo la presencia de reporte selectivo, un sesgo que habitualmente se comete en este dominio.

Metaanálisis

Asociación entre las alteraciones de CTTN/cortactin *y las variables clínico-patológicas de los pacientes con carcinoma de células escamosas de cabeza y cuello (Fig.3, tabla 2,* trabajo: Ramos-García P, González-Moles MA, Ayén A, González-Ruiz L, Ruiz-Ávila I, Gil-Montoya JA. Prognostic and clinicopathological significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis 2018: [In Submission].

Status N. No existió heterogeneidad significativa entre los estudios (p=0.139, I^2=28.4%). El modelo de efectos fijos señaló una relación estadísticamente significativa entre las alteraciones de *CTTN*/cortactin y la presencia de metástasis ganglionar linfática (N+) (OR=2.34, 95% CI=1.83-3.00, p<0.001).

Status T. Encontramos evidencia de heterogeneidad entre los estudios, de moderado grado de acuerdo a los puntos de corte de Higgins (p=0.004, I^2=61.1%). El modelo de efectos aleatorios sugirió una relación estadísticamente significativa entre las alteraciones de *CTTN*/cortactin y carcinomas localmente avanzados (T3/4) (OR=2.65, 95% CI=1.54-4.55, p<0.001; Tabla 2).

Estadio clínico. No existió heterogeneidad significativa entre los estudios (p=0.116, I^2=35.3%; Tabla 2) El modelo de efectos fijos indicó una relación estadísticamente significativa entre las alteraciones de *CTTN*/cortactin y estadíos avanzados de la enfermedad (III/IV) (OR=2.23, 95% CI= 1.67-2.98, p<0.001).

Grado histológico. Encontramos evidencia de heterogeneidad baja entre los estudios (p=0.058, I^2=45.3%). El modelo de efectos aleatorios señaló también una relación estadísticamente significativa entre las alteraciones de *CTTN*/cortactin y un grado histológico más avanzado (II/III) (OR=2.09, 95% CI=1.36-3.23, p=0.001).

Asociación entre las alteraciones de CTTN/cortactin y la supervivencia de los pacientes con carcinoma de células escamosas de cabeza y cuello (Tabla 2, trabajo: Ramos-García P, González-Moles MA, Ayén A, González-Ruiz L, Ruiz-Ávila I, Gil-Montoya JA. Prognostic and clinicopathological significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis 2018: [In Submission]).

Supervivencia general. Encontramos evidencia de heterogeneidad entre los estudios de severo grado de acuerdo a los puntos de corte de Higgins (p<0.001, I^2=79.4%), por lo que los resultados de esta variable deben interpretarse con precaución. El modelo de efectos aleatorios indicó una relación estadísticamente significativa entre las alteraciones de *CTTN*/cortactin y una pobre OS (HR=2.39, 95% CI=1.66-3.44, p<0.001).

Supervivencia libre de enfermedad. Aunque no conducimos el metaanálisis de esta variable, debido a que sólo dos estudios cumplieron con los criterios de inclusión del presente trabajo, ambos comunicaron una asociación estadísticamente significativa entre la sobreexpresión de cortactin y una pobre supervivencia libre de enfermedad (Hofman *et al.* [47]: HR=3.00, 95% CI=1.30-7.00, p=0.01; You *et al.* [51]: HR=3.27, 95% CI=1.62-6.60, p=0.001).

Metaanálisis (análisis secundarios)

Análisis de sensibilidad

Los resultados generales no se alteraron considerablemente tras la repetición secuencial de los metaanálisis omitiendo un estudio en cada turno, y en ningún parámetro analizado se perdió la significación estadística. El estudio de Bissinger *et al.* [54] presenta la mayor influencia sobre el estimador final en los metaanálisis conducidos, esencialmente en las variables status N (omitido: OR=2.82, 95% CI=2.12-3.74) (Fig.4A, trabajo: Ramos-García P, González-Moles MA, Ayén A, González-Ruiz L, Ruiz-Ávila I, Gil-Montoya JA. Prognostic and clinicopathological significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis 2018: [In Submission]), status T (omitido: OR=3.14, 95% CI=1.94-5.10) y clinical stage (omitido: OR=2.91, 95% CI=2.07-4.10). Esto sugiere que las estimaciones combinadas comunicadas en los metaanálisis principales no son dependientes de la influencia de un estudio individual en particular. En el análisis de subgrupos, el estudio de Bissinger *et al.* [54] presumiblemente ejerce una influencia mayor y probablemente algunos de los resultados obtenidos sean conservadores e infraestimados.

Análisis de los efectos de los estudios pequeños

El análisis de inspección visual de la asimetría de los funnel plots construidos, así como los tests estadísticos conducidos para el mismo fin constatan la ausencia de efecto de los estudios pequeños en las variables analizadas (status T [p_{Egger}=0.292], clinical stage [p_{Egger}=0.106], histological grade [p_{Egger}=0.340]), excepto en el status N ([p_{Egger}=0.019) (Fig.4B, trabajo: Ramos-García P, González-Moles MA, Ayén A, González-Ruiz L, Ruiz-Ávila I, Gil-Montoya JA. Prognostic and clinicopathological significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis 2018: [In Submission]) y en el parámetro OS [p_{Egger}=0.002], donde no podemos descartar la existencia de sesgos, tales como el sesgo de publicación.

Análisis de subgrupos (Tabla 2, trabajo: Ramos-García P, González-Moles MA, Ayén A, González-Ruiz L, Ruiz-Ávila I, Gil-Montoya JA. Prognostic and clinicopathological significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis 2018: [In Submission]).

En el análisis estratificado de acuerdo al status N, numerosos subgrupos conservaron la asociación estadísticamente significativa (ambas alteraciones de *CTTN*/cortactin [amplificación: OR=2.26, 95% CI=1.52-3.37, p<0.001; sobreexpresión: OR=2.75, 95% CI=1.71-4.44, p<0.001], ambos subgrupos étnicos [asiáticos: OR=3.38, 95% CI=1.83-3.00, p<0.001; no asiáticos: OR=2.02, 95% CI=1.50-2.71, p<0.001], COCE [OR=2.78, 95% CI=1.68-4.60, p<0.001] y SCC faringolaríngeo [OR=2.54, 95% CI=1.67-3.87, p<0.001], el punto de corte 1-50% [OR=3.08, 95% CI=1.10-8.59, p=0.032], la intensidad de la tinción ≥moderada [OR=3.91, 95% CI=2.36-6.45, p<0.001], y el subgrupo de otros anticuerpos variados [que incluye el empleo de cualquier anticuerpo diferente al monoclonal clone 30] [OR=2.89, 95% CI=1.67-4.99, p<0.001]). Encontramos heterogeneidad significativa de grado leve en los subgrupos sobreexpresión de cortactin (p=0.092, I^2=41.2%) y COCE (p=0.079, I^2=43.2%); y una fuente de heterogeneidad severa en el subgrupo que empleó el anticuerpo monoclonal clone 30 (p=0.010, I^2= 78.1%).

En el análisis estratificado de acuerdo al status T, numerosos subgrupos conservaron la asociación estadísticamente significativa (ambas alteraciones de *CTTN*/cortactin [amplificación: OR=3.94, 95% CI=2.04-7.63, p<0.001; sobreexpresión: OR=2.65, 95% CI=1.54-4.55, p=0.012], el subgrupo asiático [OR=4.47, 95% CI=2.63-7.59, p<0.001], SCC faringolaríngeo [OR=2.38, 95% CI=1.47-3.84, p<0.001] y HNSCCs mixtos [incluyen aquellos estudios que evaluaron conjuntamente SCCs ubicados en distintas localizaciones] [OR=6.34, 95% CI=2.36-16.98, <0.001], la intensidad de la tinción ≥moderada [OR=2.12, 95% CI=1.16-3.87, p=0.014] y el subgrupo de otros anticuerpos variados [OR=2.89, 95% CI=1.67-4.99, p<0.001]). Encontramos heterogeneidad significativa de grado moderado en los subgrupos sobreexpresión de cortactin (p=0.004, I^2=66.8%), pacientes no asiáticos (p=0.041, I^2=54.3%), en ambos subgrupos de anticuerpos (clone 30 [p=0.038, I^2=69.3%] y en otros anticuerpos variados [p=0.067, I^2=54.4%]); y heterogeneidad severa en los subgrupos COCEs (p=0.002, I^2= 76.0%) y en el empleo de los puntos de corte >50% (p=0.007, I^2= 80.0%) y 1-50% (p=0.015, I^2=76.1%).

En el análisis estratificado de acuerdo al parámetro clinical stage, numerosos subgrupos conservaron la asociación estadísticamente significativa (ambas alteraciones de *CTTN*/cortactin [amplificación: OR=4.25, 95% CI=2.20-8.23, p<0.001; sobreexpresión: OR=1.94, 95% CI=1.18-3.18, p=0.009], ambos subgrupos étnicos [asiáticos: OR=3.26, 95% CI=1.86-5.73, p<0.001; no asiáticos: OR=1.95, 95% CI=1.40-2.74, p<0.001], SCC faringolaríngeo [OR=3.41,

95% CI=1.76-6.61, p<0.001] y HNSCCs mixtos [OR=2.14, 95% CI=1.28-3.59, p=0.004], la intensidad de la tinción ≥moderada [OR=3.85, 95% CI=1.97-7.53, p<0.001] y el subgrupo de otros anticuerpos variados [OR=2.51, 95% CI=1.57-4.02, p<0.001]). No encontramos una fuente potencial de heterogeneidad severa en el análisis de subgrupos. Encontramos heterogeneidad significativa leve en el subgrupo sobreexpresión de cortactin (p=0.098, I^2=46.1%); y moderada en el subgrupo constituido por COCEs (p=0.014, I^2=71.8%).

En el análisis estratificado de acuerdo al grado histológico, numerosos subgrupos conservaron la asociación estadísticamente significativa (ambas alteraciones de *CTTN*/cortactin [amplificación: OR=2.70, 95% CI=1.66-4.39, p<0.001; sobreexpresión: OR=1.76, 95% CI=1.01-3.08, p=0.046], el subgrupo de pacientes no asiáticos [OR=2.34, 95% CI=1.62-3.37, p<0.001], SCC faringolaríngeo [OR=2.12, 95% CI=1.23-3.41, p=0.002] y HNSCCs mixtos [OR=3.29, 95% CI=1.89-5.72, p<0.001] y el empleo de un punto de corte >50% [OR=2.65, 95% CI=1.50-4.68, p=0.001]. No encontramos una fuente potencial de heterogeneidad severa en el análisis de subgrupos. Encontramos heterogeneidad significativa leve en el subgrupo sobreexpresión de cortactin (p=0.068, I^2=49.0%); y moderada en los subgrupos de pacientes asiáticos (p=0.030, I^2=66.4%), de COCEs (p=0.079, I^2=55.9%), en el empleo de un punto de corte del 1-50% (p=0.070, I^2=69.6%) y en el subgrupo de otros anticuerpos variados (p=0.015, I^2=71.4%).

En el análisis estratificado de acuerdo al parámetro supervivencia general, numerosos subgrupos conservaron la asociación estadísticamente significativa (ambas alteraciones de *CTTN*/cortactin [amplificación: HR=2.10, 95% CI=1.40-3.16, p<0.001; sobreexpresión: HR=2.50, 95% CI=1.60-3.90, p<0.001], ambos subgrupos étnicos [asiáticos: HR=3.40, 95% CI=2.07-5.59, p<0.001; no asiáticos: HR=2.16, 95% CI=1.47-3.18, p<0.001], SCC faringolaríngeo [HR=2.76, 95% CI=1.36-5.58, p=0.005] y HNSCCs mixtos [HR=3.17, 95% CI=2.03-4.95, p<0.001], el punto de corte >50% [HR=1.76, 95% CI=1.19-2.61, p=0.005], la intensidad de la tinción ≥moderada [HR=3.45, 95% CI=2.20-5.42, p<0.001], y en ambos subgrupos de anticuerpos [clone 30: [HR=3.58, 95% CI=1.46-8.75, p=0.005; otros anticuerpos variados: HR=1.78, 95% CI=1.06-2.98, p=0.002]). Encontramos heterogeneidad significativa moderada en el subgrupo constituido por COCEs (p=0.104, I^2=62.2%) y en el empleo de otros anticuerpos variados (p=0.108, I^2=55.1%); y severa en los subgrupos sobreexpresión de cortactin (p<0.001, I^2=83.7%), pacientes no asiáticos (p<0.001, I^2=79.3%), SCCs faringolaríngeos (p<0.001, I^2=87.2%), en el empleo de un punto de corte >50% (p=0.004, I^2=77.6%) y en el subgrupo del anticuerpo clone 30 (p<0.001, I^2=90.3%).

Metarregresión

El análisis conducido de metarregresión mostró que no existen diferencias estadísticamente significativas entre el valor pronóstico de la amplificación génica de *CTTN* y la sobreexpresión de su producto cortactin en los parámetros investigados (status N, p=0.756; status T, p=0.783; clinical stage, p=0.124; grado histológico, p=0.480; OS, p=0.812).

DISCUSIÓN

Nuestra revisión sobre la relevancia de la banda cromosómica 11q13 en COCE (trabajo: Ramos-García P, Ruiz-Ávila I, Gil-Montoya JA, Ayén Á, González-Ruiz L, Navarro-Triviño FJ, González-Moles MÁ. Relevance of chromosomal band 11q13 in oral carcinogenesis: An update of current knowledge. Oral Oncol. 2017 Sep;72:7-16.) constata su implicación oncogénica en este tipo tumoral, así como su complejidad. Se ha propuesto un modelo de iniciación de la amplificación génica de 11q13 específico para el COCE, vinculado a la ruptura de la doble hélice de ADN en sitios cromosómicos frágiles [48]. Así, se comunicó [51,52] que en este tumor la amplificación de 11q13 se produce como resultado de los denominados ciclos rotura-fusión-puente, iniciados por la rotura en el sitio frágil común cromosómico FRA11F, inducida por el efecto de los carcinógenos asociados al tabaco [48,53]. La estrecha asociación etiológica del consumo de tabaco con el desarrollo del COCE parece así justificar la alta tasa de amplificación de 11q13 en la oncogénesis oral, más del doble de la observada en el resto de cánceres humanos. En este sentido, se ha documentado que la amplificación de 11q13 aparece más frecuentemente en COCEs asociados al consumo de tabaco/HPV(-) que en aquellos casos no relacionados con el tabaco/HPV(+) [63,101,152–154], lo que parece indicar además que el virus del papiloma humano no posee un papel crucial en el proceso de iniciación y establecimiento de la amplificación de 11q13 en COCE. Igualmente, tampoco parece existir una relación entre el consumo de alcohol y la amplificación de 11q13 [30]. A diferencia de otras regiones cromosómicas donde es típica la amplificación de un solo gen o de un limitado número de genes, en 11q13 es frecuente la co-amplificación de un conjunto de genes vecinos [30]. Así, en la ubicación comprendida entre 11q13.2-q13.4 numerosos estudios sobre COCE han hallado en co-amplificados a los genes *CPT1A*, *MRPL21*, *IGHMBP2*, *MRGPRF* (también conocido como *MRGF*), *TPCN2*, *MYEOV* (también conocido como *OCIM*), *CCND1*, *ORAOV1* (también conocido como *TAOS1*), *FGF19/4/3*, *ANO1* (también conocido como *TMEM16A*, *ORAOV2*, *TAOS2* o *DOG1*), *FADD*, *PPFIA1*, *CTTN* (también conocido como *EMS1*), *SHANK2* y *NUMA1* (enumerados según el orden de ubicación en el cromosoma en dirección centromérica-telomérica) [57–66]. De los genes anteriormente citados, *CTTN*, *FADD* y *CCND1*, presumiblemente, se comportan como amplicon drivers del amplicón 11q13 en COCE, como consecuencia de la constante expresión de sus productos cuando el gen se encuentra amplificado, de la relación existente entre amplificación génica y sobreexpresión proteica tisular, y de las implicaciones clínicopatológicas y pronósticas que poseen la amplificación de estos genes, tales como afectación ganglionar, pobre grado de diferenciación tumoral o peor supervivencia [30]. Algunas publicaciones han comunicado además la ganancia del número de copias o amplificación de la región cromosómica 11q13 en estadíos tempranos de la carcinogénesis oral [62–64], lo que en sí mismo parece sugerir que se trata de un evento temprano, habiéndose llegado a mapear esta amplificación en la transición de la hiperplasia a la displasia epitelial [155]. Por último, la amplificación génica es un importante mecanismo

adquirido de resistencia al tratamiento [48], y números genes amplificados en este amplicón han sido propuestos como dianas terapéuticas en cáncer, lo que sugiere que la banda cromosómica 11q13 podría poseer interesantes implicaciones emergentes en el campo de la medicina personalizada.

Nuestra revisión sobre las implicaciones de ciclina D1 -producto del gen CCND1, un gen esencial del brazo cromosómico 11q13- en la carcinogénesis oral (trabajo: Ramos-García P, Gil-Montoya JA, Scully C, Ayén A, González-Ruiz L, Navarro-Triviño FJ, González-Moles MA. An update on the implications of cyclin D1 in oral carcinogenesis. Oral Dis. 2017 Oct;23(7):897-912.) pone de manifiesto que su sobreexpresión desempeña un papel crucial en el desarrollo y evolución de esta enfermedad. La amplificación del gen *CCND1* es el principal mecanismo oncogénico de sobreexpresión de ciclina D1 en COCE [9], aunque ésta también puede depender de otros mecanismos oncogénicos como translocaciones cromosómicas (t[11;14][q13;q32], el hallmark genético del linfoma de células del manto), mutaciones (Thr-286) o la alteración de pathways frecuentemente implicados en la carcinogénesis oral (MAPK, PI3K, Wnt, NF-κβ…) [8,12]. La sobreexpresión de ciclina D1 promueve la progresión del ciclo celular durante la transición de las fases G1/S, regulando la proliferación celular [71]. En los últimos años se han comunicado funciones emergentes de ciclina D1, tales como regulación del crecimiento celular, modulación de la actividad mitocondrial, reparación del ADN y control de la migración celular [18,72,156]. Desde su descubrimiento [157], numerosas publicaciones han tratado de relacionar los niveles de expresión de ciclina D1 con el pronóstico del COCE [18,30], habiéndose asociado su sobreexpresión con parámetros que implican un pobre pronóstico (N+, status T, estadío clínico avanzado, indiferenciación tumoral, y supervivencia reducida [18]), siendo la ciclina D1, según una revisión sistemática publicada recientemente, uno de los marcadores con mayor valor potencial y mejor posicionados dentro del panel de biomarcadores candidatos aplicables a la carcinogénesis oral [158]. Sin embargo, de estas observaciones no debe deducirse que la participación oncogénica de la ciclina D1 sólo se limita a fases tumorales avanzadas. Por el contrario, múltiples estudios defienden que esta proteína se encuentra sobreexpresada en desórdenes potencialmente malignos (34,39,45,47,86,106,129–135), con una tendencia a niveles crecientes de expresión de ciclina D1 a medida que avanzamos en escalones de la carcinogénesis oral desde la displasia epitelial oral hasta COCEs localmente avanzados (121,123,124,128). La evidencia de la alta incidencia de la expresión aberrante de ciclina D1 en cáncer humano así como en COCE, y de la sensibilidad de las células cancerígenas a la inhibición de las ciclinas y las CDKs, ha impulsado su investigación como diana terapéutica potencial [18]. En este sentido, la dianización directa de ciclina D1 puede resultar compleja, por su localización intracelular y su carencia de actividad enzimática, lo que ha desalentado el diseño de agentes inhibidores anti-ciclina D1 [81]. Sin embargo, la evidencia creciente de la

existencia de funciones de ciclina D1 emergentes e independientes de la actividad CDK, fomentan la consideración de esta opción terapéutica en futuras investigaciones, aplicada al COCE [18]. En este sentido se ha comunicado que agentes como epigallocatechin-3-gallate [84] y epicatechin gallate [85] podrían ejercer un efecto anti-ciclina D1. Sin embargo, actualmente el abordaje dirigido a ciclina D1 más investigado es la inhibición de las CDKs4/6, mediante inhibidores globales de la acción de las CDKs, como flavopiridol, olomoucine o R-roscovitine, o inhibidores selectivos de las CDK4/6, como P276-00, P1446-05 o PD0332991 [86]. Otras alternativas terapéuticas que podrían ejercer un efecto indirecto sobre los niveles de expresión de ciclina D1 podrían actuar sobre moléculas implicadas en su degradación (p.e. GSK-3β, mediante el inhibidor DIF-1), o sobre los pathways reguladores de su expresión (p.e. MAPK o PI3K, a través de la molécula inhibidora QKI-5). Una reflexión final, debe hacer referencia a la resistencia al tratamiento basado en cisplatino en aquellos pacientes con COCE que sobreexpresan ciclina D1. Como consecuencia de esta creciente evidencia se ha hipotetizado que el tratamiento combinado con cisplatino y agentes terapéuticos que dianicen ciclina D1 podría mejorar el rendimiento de ambas estrategias por separado [18].

Nuestra revisión sistemática y metaanálisis sobre el significado pronóstico de la sobreexpresión de ciclina D1 en COCE (trabajo: Ramos-García P, González-Moles MÁ, González-Ruiz L, Ruiz-Ávila I, Ayén Á, Gil-Montoya JA. Prognostic and clinicopathological significance of cyclin D1 expression in oral squamous cell carcinoma: A systematic review and meta-analysis. Oral Oncol. 2018 Aug;83:96-106) sobre 31 estudios y 2942 pacientes demuestra que la sobreexpresión de ciclina D1 se correlaciona con peor supervivencia general y peor supervivencia libre de enfermedad, y se asocia con un mayor status T, status N+, estadío clínico avanzado y elevado grado histológico. Nuestra evaluación cualitativa ha demostrado que los estudios incluidos en nuestro metaanálisis, a pesar de poseer diseños experimentales similares, no se condujeron con la misma escrupulosidad. En nuestro análisis cualitativo, realizado mediante la herramienta QUIPS -Cochrane Prognosis Methods Group- [124], la mayoría de los sesgos potenciales se debieron a la falta de consideración en los estudios de los factores confundidores -dominio study confounding- y a la aplicación de análisis estadísticos inapropiados -dominio statistical analysis and reporting-. El dominio "prognostic factor measurement" albergó también numerosos sesgos potenciales esencialmente debidos al empleo de métodos de medición de la expresión de ciclina D1 y puntos de corte inapropiados. Los futuros estudios sobre el valor pronóstico de ciclina D1 en COCE deberían tener en consideración los sesgos potenciales comunicados en nuestro metaanálisis, siendo aconsejable, para mejorar y estandarizar futuros estudios, respetar los ítems de la herramienta QUIPS [124]. Los estudios incluidos en nuestro metaanálisis presentaron en general poca heterogeneidad, especialmente en lo que respecta al parámetro supervivencia general y a las variables status T y

N. La heterogeneidad fue moderada en el análisis del grado histológico y no existió en los parámetros supervivencia libre de enfermedad y estadio clínico. El análisis del impacto de la sobreexpresión de ciclina D1 sobre determinados subgrupos y la evaluación de las fuentes potenciales de heterogeneidad demostró que el subgrupo de pacientes con carcinoma lingual presentó la mayor fuerza de asociación entre la sobreexpresión de ciclina D1 y la peor evolución de la enfermedad, en comparación con otras localizaciones intraorales tanto en la supervivencia general de los pacientes (HR=2.51, 95% CI=1.73-3.64, p<0.001 vs HR=1.86, 95% CI=1.40-2.48, p<0.001), pero sobretodo en el status T (OR=1.97, 95% CI=1.26-2.97, p=0.02 vs OR=1.27, 95% CI=0.85-1.95, p=0.25), status N (OR=2.87, 95% CI=1.94-4.24, p<0.001 vs OR=1.88, 95% CI=1.27-2.77, p=0.002) y estadío clínico (OR=1.87, 95% CI=1.23-2.83, p=0.003 vs OR=1.28, 95% CI=0.97-1.69, p=0.09). Los estudios sobre COCE han señalado clásicamente al carcinoma lingual como el de peor pronóstico por razones relacionadas con la riqueza linfática de la zona, diagnóstico tardío, etc, aunque ahora, como consecuencia de nuestro metaanálisis, sabemos que este tumor también presenta aspectos moleculares diferenciales en lo que respecta a la sobreexpresión de ciclina D1 [18,159], que se comporta como un marcador pronóstico muy negativo en esta localización. Nuestro metaanálisis demuestra que los pacientes con carcinoma lingual pueden singularmente beneficiarse en su valoración pronóstica de la evaluación de la sobreexpresión de ciclina D1. También observamos que la sobreexpresión de ciclina D1 tuvo un impacto especialmente negativo en pacientes asiáticos tanto en la supervivencia general como en la supervivencia libre de enfermedad, probablemente como consecuencia del fuerte hábito de consumo tabáquico (tabaco inhalado, mascado, betel,…) presente clásicamente en esta población, lo que se comporta como la causa principal de amplificación de *CCND1*/sobreexpresión de ciclina D1 en COCE [18,30]. Liu et al [90] en este sentido han comunicado una elevada tasa de amplificación de *CCND1* en COCE de pacientes asiáticos consumidores de nuez de betel. El análisis de subgrupos finalmente demostró que en la consideración de un caso como positivo, el punto de corte de ≥10% de células tumorales ciclina D1 positivas mantuvo la mayoría de las asociaciones estadísticamente significativas comunicadas a excepción del status T; así, en nuestra opinión, este debería ser el punto de corte empleado en la valoración pronóstica de un paciente individual.

Hemos investigado también las implicaciones de la sobreexpresión nuclear y citoplasmática de ciclina D1 en COCE mediante una técnica inmunohistoquímica aplicada sobre tejido tumoral y epitelio no tumoral adyacente al carcinoma. En nuestro trabajo sobre las implicaciones de ciclina D1 en tejido tumoral hemos publicado resultados parcialmente concordantes a los comunicados en nuestro metaanálisis (trabajo: Ramos-García P, González-Moles MA, González-Ruiz L, Ayén A, Ruiz-Ávila I, Bravo M, Gil-Montoya JA. Prognostic relevance of tumor cyclin D1 expression in oral cáncer 2018: [In Submission]). Nuestro estudio demuestra la

frecuente sobreexpresión de ciclina D1 en COCE y su asociación a un pobre grado de diferenciación y a un fenotipo proliferativo (medido a través del análisis de la expresión inmunohistoquímica de ki-67), parámetros que implican un pobre pronóstico en COCE. La sobreexpresión concomitante de ciclina D1 en núcleo y citoplasma se asoció con una morfología invasiva de las células tumorales. Queda por dilucidar si este rol putativo es consecuencia de la sobreexpresión exclusivamente citoplasmática de ciclina D1, o si también depende de su expresión en el núcleo. Ciclina D1 es una proteína clave en la oncogénesis oral que actúa incrementando la proliferación celular [18,71], y a la que recientemente le han sido atribuidas numerosas funciones emergentes [72], entre las que se encuentran la regulación de la migración celular y del metabolismo mitocondrial, así como la inhibición de la diferenciación celular y de la reparación del ADN, funciones que son actualmente consideradas hallmarks del cáncer [16]. La amplificación del gen *CCND1* es el principal mecanismo oncogénico de sobreexpresión de ciclina D1 en COCE [30], aunque la alteración de pathways frecuentemente implicados en la carcinogénesis (MAPK, Wnt, NF-κβ…) también puede potenciar su activación transcripcional [18,38]. Estos mecanismos conducen a la proliferación celular incontrolada, lo que hipotéticamente se debería asociar al desarrollo de COCEs de mayor tamaño, que a su vez presentarán mayor riesgo de afectación ganglionar [12,16,18]. Sin embargo, tanto en nuestra serie como en otros estudios [145,146,160–170], la expresión de ciclina D1 nuclear no se asoció significativamente ni a un mayor tamaño tumoral [145,146,160–166] ni a la presencia de metástasis ganglionar [145,161–164,167–170]. Nuestro reciente metaanálisis [19] sobre el valor pronóstico de ciclina D1 ha identificado la influencia de su sobreexpresión sobre el status T y N especialmente sobre cáncer lingual. Pudiera ser que el carcinoma lingual, que se comporta de forma diferente al resto de los carcinomas intraorales (mayor T, N y peor supervivencia) también fuera diferente desde un punto de vista molecular, especialmente en lo referente a la sobreexpresión de ciclina D1, y ello podría justificar la discrepancia de los resultados de nuestra serie que incluye tumores de diferentes localizaciones en los que ciclina D1 podría tener una relevancia menor. También hemos podido observar una asociación estadística entre la expresión nuclear de ciclina D1 y un pobre grado de diferenciación tumoral, asociación también corroborada en nuestro metaanálisis [19]. Los mecanismos moleculares implicados en la regulación del aumento de la proliferación *via* ciclina D1, alterarían el equilibrio homeostático celular hacia un fenotipo más proliferativo [18], asociado típicamente en cáncer a un fenotipo celular inmaduro y oncogénicamente más agresivo [16]. Existen trabajos que han comunicado el rol putativo de ciclina D1 en la inhibición de la diferenciación celular en distintos linajes celulares, bien a través de la formación de complejos con sus compañeras de unión, la CDKs 4 y 6 [171,172] o de un modo independiente de la actividad CDK [173,174]. Finalmente, hemos encontrado una asociación entre los niveles de expresión de ciclina D1 y ki-67, lo que señala la importancia de ciclina D1 en la regulación de su función canónica, la proliferación celular.

En nuestro trabajo sobre las implicaciones oncogénicas de la sobreexpresión de ciclina D1 citoplasmática (trabajo: Ramos-García P, Bravo M, González-Ruiz L, González-Moles MÁ. Significance of cytoplasmic cyclin D1 expression in oral oncogenesis. Oral Dis. 2018 Mar;24(1-2):98-102) hemos podido observar que la expresión de ciclina D1 citoplasmática es un evento frecuente en COCE (33.3% de los casos de nuestra serie), que está asociado con numerosos parámetros de pobre pronóstico, entre los que se encuentran el estadio avanzado, la pobre diferenciación, la elevada expresión de ki-67, la presencia en el tumor de células con morfología invasiva, así como el status T y N. Por tanto, en nuestra opinión, la expresión citoplasmática de ciclina D1, que ha sido mayoritariamente despreciada en los estudios sobre el tema, debe ejercer algunas funciones oncogénicas, y merecería ser considerada en el análisis de la influencia de esta oncoproteína en COCE. Los mecanismos que regulan la acumulación citoplasmática de ciclina D1 no son bien entendidos. En nuestra serie observamos una estrecha asociación estadística entre la expresión citoplasmática y nuclear de ciclina D1, tanto al considerar el recuento de células positivas ($p=0.041$) como la intensidad de la expresión ($p<0.001$). Así, sugerimos que en COCE la sobreexpresión citoplasmática podría estar ligada a la amplificación masiva de los genes de la banda cromosómica 11q13, considerado actualmente como el mecanismo más relevante que regula los niveles de expresión de ciclina D1 en carcinogénesis oral [30]. Esto implicaría una masiva producción ribosómica de ciclina D1 que formaría complejos citoplasmáticos con las CDKs4/6. Aunque parte de estos complejos se van a translocar al núcleo donde ejercen sus funciones oncogénicas, en nuestra opinión, una cantidad de ciclina D1 libre o formando complejos, permanecería en el citoplasma como consecuencia de su exceso nuclear, que imposibilitaría por saturación la importación permanente al núcleo de la ciclina D1 citoplamática. Pocos grupos han cuantificado los niveles de expresión de ciclina D1 citoplasmática y han tratado de correlacionarlos con parámetros clínicopatológicos. Como se ha comentado, en nuestra serie, los tumores que expresan ciclina D1 citoplasmática se asocian a parámetros clinicopatológicos de pobre pronóstico. Algo similar ha sido comunicado en otros tumores como cáncer de páncreas, vejiga e hígado [74–76]. Aunque estos son notablemente diferentes entre sí, todos ellos, incluido el COCE, comparten una etiopatogenia altamente dependiente del tabaco, que parece ser un mecanismo clave en la amplificación de la banda cromosómica 11q13. Esto podría reforzar nuestra hipótesis de la asociación entre el patrón de expresión citoplasmático de ciclina D1 y la amplificación génica de CCDN1, lo que resulta especialmente importante en COCE, por ser éste el cáncer humano que presenta mayores tasas de amplificación de 11q13, con una media del 46% de los casos, más del doble que en el resto de cánceres humanos [30]. Contrariamente, otros cánceres cuyo patrón de expresión citoplasmático se asoció a un pronóstico clínicopatológico favorable (próstata, tiroides y colon) [77,78,80], se corresponden fundamentalmente con adenocarcinomas cuya etipotogenia no parece ser tan dependiente del tabaco y no poseen tasas de amplificación de 11q13 tan elevadas.

Se ha sugerido que la expresión de ciclina D1 citoplasmática podría estar ligada a la adquisición de funciones oncogénicas relacionadas con la migración celular via interacción con filamina A [175], con p27, con ROCKII [176], y más recientemente, mediante su interacción con paxilina [177]. Los complejos ciclina D1/Cdk4 interaccionan con la paxilina en el citoplasma. Consecuentemente, la paxilina activa aguas abajo a Rac1, que promueve un aumento de la migración celular [177]. Además, la paxilina una vez fosforilada se ha observado, junto con los complejos ciclina D1/CDK4, co-localizada en estructuras especializadas de la periferia celular compatibles con estructuras protrusivas basadas en actina [177]. Los eventos precedentes implican que la ciclina D1 citoplasmática, a través del pathway ciclina D1/CDK4/6/paxilina/Rac1 podría ejercer un rol en la regulación del aumento de la motilidad celular, que podría estar ligado presumiblemente a un aumento del potencial invasivo en COCE. Paxilina parece estar ligada a la regulación de las denominadas estructuras protrusivas basadas en actina, ubicadas en la periferia celular, que en las células cancerígenas derivan de una serie de cambios en la reorganización de su citoesqueleto de actina y que son requeridas en la adquisición de capacidades migratorias e invasivas [92,178]. Concretamente, la paxilina parece estar implicada en la regulación de los dos tipos de estructuras protrusivas basadas en actina más relevantes en la migración celular en el cáncer, los lamelipodios -estructuras esenciales para la locomoción celular- y los invadopodios -estructuras que poseen una alta actividad degradatoria de la matrix extracelular, facilitando la invasión- [92,178]. Los lamelipodios e invadopodios, morfológicamente, son compatibles con la morfología que nosotros hemos denominado invasiva y que en nuestra serie de COCEs se asoció significativamente con aquellos tumores que tienen una mayor intensidad de expresión citoplasmática de ciclina D1 y también con aquellos que presentan un mayor porcentaje de células ciclina D1 positivas. Esto quiere decir en nuestra opinión que la expresión de ciclina D1 citoplasmática podría regular la formación de estructuras protrusivas basadas en actina (lamelipodios e invadopodios), quizás aguas arriba *via* activación de paxilina, y en resumen, la migración e invasividad de las células cancerígenas.

Nuestro trabajo sobre las implicaciones de la sobreexpresión de ciclina D1 en el epitelio no tumoral adyacente a carcinomas orales, como marcador de campos premalignos y de riesgo de desarrollo tumoral múltiple (trabajo:Ramos-García P, González-Moles MÁ, Ayén Á, González-Ruiz L, Ruiz-Ávila I, Lenouvel D, Gil-Montoya JA, Bravo M. Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression in adjacent non-tumour epithelium in oral squamous cell carcinoma. Arch Oral Biol. 2018 Oct 9;97:12-17) demuestra una notable expresión de ciclina D1 en los epitelios no tumorales asociados a carcinomas invasivos. Esta expresión se localizó sobre todo en el núcleo de las células epiteliales de las capas basal, parabasal y tercio medio. Los estudios sobre el tema han reportado que en el epitelio oral sano

no aparece sobreexpresión de ciclina D1 ni amplificación del gen *CCND1* o sobreexpresión de su RNAm [141–148]. En nuestra opinión, la sobreexpresión de ciclina D1 en células de epitelios no tumorales adyacentes a carcinomas orales indica que las alteraciones del gen *CCND1* -presumiblemente su amplificación- y la subsiguiente sobreexpresión de ciclina D1 constituyen eventos oncogénicos precoces en la carcinogénesis oral [179–181]. La sobreexpresión de ciclina D1 en capas basales del epitelio oral premaligno también indica que las células basales -presumiblemente stem cells- constituyen dianas para las agresiones oncogénicas en la carcinogénesis oral. En este estudio, se ha comprobado también que la sobreexpresión de ciclina D1 se asocia significativamente a un incremento de la tasa de proliferación celular en algunos estratos de epitelios premalignos, esencialmente en la capa basal de epitelios no tumorales lejanos al punto de invasión. Las células basales normales -probablemente stem cells orales- siguen un patrón fisiológico de proliferación asimétrica caracterizado por una muy baja proliferación basal que da lugar a una población de células amplificantes transitorias de ubicación parabasal, altamente proliferativas. El acúmulo de células proliferativas en la capa basal significativamente asociado a la sobreexpresión de ciclina D1 indica que esta oncoproteína actúa induciendo un cambio del patrón proliferativo asimétrico hacia un patrón simétrico, en el cual una stem cell premaligna, en cada división da lugar a dos stem cells premalignas y no a células amplificantes transitorias, generándose una progenie clonal de stem cells que se ubican en su lugar natural, el estrato basal. Esta alteración del patrón de proliferación fisiológico, durante la transformación maligna del epitelio oral, ligado a la sobreexpresión de ciclina D1, ha sido comunicada previamente por nuestro grupo en la oncogénesis labial [182]. Nosotros también hemos comunicado previamente [183] que la actividad proliferativa incrementada de las células basales puede llegar a superar la capacidad de la capa basal para albergar la población clonal de stem cells premalignas oncogénicamente alteradas y proliferativamente muy activas, produciéndose secundariamente una ocupación progresiva de estratos más superficiales del epitelio. En nuestra opinión este razonamiento justifica la sobreexpresión de ciclina D1 encontrada en nuestro estudio tanto en capas parabasales como en el tercio medio de epitelios no tumorales adyacentes a carcinomas, acompañante a la sobreexpresión basal. Nosotros creemos que el acúmulo clonal de células que sobreexpresan ciclina D1 en estos epitelios premalignos, sobre todo en capas basales y parabasales, secundariamente a su ventaja proliferativa, reemplaza progresivamente a las células normales y genera campos premalignos que se extienden por la mucosa oral. Nuestros resultados también demuestran que la expresión de ciclina D1 en el núcleo celular de los diferentes estratos de los epitelios no tumorales adyacentes a los carcinomas de nuestra serie estaban significativamente y positivamente asociados, de tal forma que la mayor expresión de ciclina D1 en el epitelio no tumoral cercano al punto de invasión se asoció también a una mayor expresión en el epitelio alejado del punto de invasión. Esta observación señala que las funciones

oncogénicas ligadas a la alteración de ciclina D1, no sólo son precoces si no que se extienden en zonas amplias de la mucosa oral, reforzando la idea de que la expresión de ciclina D1 es un fenómeno oncogénico importante en la génesis y expansión de campos premalignos. En este sentido, un resultado interesante de nuestro estudio señala que la sobreexpresión basal y parabasal de ciclina D1 en el epitelio oral no tumoral alejado del punto de invasión se relaciona con un riesgo aumentado de desarrollar tumores múltiples (específicamente >2 tumores). En nuestra opinión esta observación obedece a que las células espiteliales, sobreexpresando ciclina D1 y proliferando activamente, desarrollan inestabilidad genómica que las convierte en una población diana para adquirir nuevos eventos oncogénicos que finalmente dotarán de capacidad invasiva a subclones ubicados en distintas zonas del campo premaligno. Por tanto, en nuestra opinión la sobreexpresión basal y suprabasal de ciclina D1 en el epitelio no tumoral alejado del punto de invasión debe ser considerada como un marcador de campos premalignos y de riesgo de desarrollo tumoral múltiple. Finalmente, hemos observado en algunos epitelios no tumorales adyacentes a COCE una expresión evidente, aunque en escasas células, de ciclina D1 citoplasmática. Esta localización de la proteína ha sido señalada por muy pocos autores [143,168] y en ningún caso se ha hecho una interpretación de sus posibles funciones. En nuestro estudio previo [149] reportamos que esta asociación de la expresión citoplasmática de ciclina D1 en células tumorales puede jugar un rol en la regulación del aumento de la motilidad celular *via* ciclina D1/CDK4/6/paxilina/Rac1 pathway. La paxilina parece estar ligada a la regulación de las estructuras protrusivas basadas en actina en la periferia celular, requeridas para la adquisición de capacidades migratorias [92,177,178]. Por tanto, en algunos epitelios premalignos, la sobreexpresión citoplasmática de ciclina D1 podría, por esta vía, colaborar también con la expansión de campos precancerosos.

Hemos investigado además las implicaciones del gen *CTTN* -que codifica a la proteína cortactin- en la carcinogénesis oral (trabajo: Ramos-García P, González-Moles MÁ, González-Ruiz L, Ayén Á, Ruiz-Ávila I, Navarro-Triviño FJ, Gil-Montoya JA. An update of knowledge on cortactin as a metastatic driver and potential therapeutic target in oral squamous cell carcinoma.Oral Dis. 2018 Jun 7). Este gen ha sido considerado junto con *CCND1* el gen más importante del amplicón 11q13, debido esencialmente a la frecuente alteración en el número de sus copias y a sus funciones oncogénicas [89]. Se ha publicado que *CCND1* y *CTTN* se encuentran frecuentemente co-amplificados *in vitro* e *in vivo* en COCE [58,59,90,91]. Cortactin es una proteína de unión a la actina F caracterizada por sus múltiples dominios de unión que le permiten interaccionar con numerosas proteínas [87,88]. A través de un conjunto de eventos moleculares, cortactin regula específicamente la migración celular en COCE [39]. Las células cancerígenas, para adquirir un fenotipo migratorio, deben experimentar cambios en la regulación de su citesqueleto concomitantes con la formación en la membrana celular de las

denominadas estructuras protrusivas basadas en actina (principalmente invadopodios y lamelipodios) [92,93]. Cortactin juega un papel central uniéndose y regulando a la actina filamentosa, potenciando la polimerización y ensamblaje reticular de los monómeros de actina, estabilizando las redes de actina ramificadas y regulando su organización estructural [94]. Deben destacarse además, las interacciones de cortactin con Src y el complejo Arp2/3, que esencialmente le permiten regular diferentes eventos celulares, también relacionados con el desarrollo de las estructuras protrusivas basadas en actina [95]. Adicionalmente, trabajos recientes le han atribuido a cortactin funciones oncogénicas emergentes, diferentes a la migración celular, entre las que se encuentran el aumento de la angiogénesis [96,97], de la proliferación celular [96,98], de la secreción de exosomas [99] y efectos en el microambiente tumoral [100]. El principal mecanismo regulador de los niveles de expresión de cortactín en COCE es la amplificación de *CTTN*, cuya tasa de oscila entre el 10-57% según las series [58,59,63,64,90,101–103]. *CTTN* presenta consistentemente una alta expresión de RNAm y sobreexpresión tisular de su producto cortactin en aquellos tumores con amplificación del gen [58–60,90,102], lo que indica que las acciones en oncogénesis oral de este gen son altamente dependientes de su amplificación y no de otros mecanismos de sobreexpresión [39]. La amplificación de *CTTN* también ha sido documentada en el 20% de los desórdenes orales potencialmente malignos [103], habiéndose comunicado que la sobreexpresión de cortactin aumenta con el grado de displasia epitelial y que podría servir como marcador en la predicción de la transformación maligna [104]. Las alteraciones de *CTTN*/cortactín en COCE, se han correlacionado con factores pronósticos, como son la metástasis ganglionar [65,90,91,101,103,105,106], la presencia de extensión extracapsular [105], un patrón tumoral más infiltrativo [106], mayor tamaño tumoral, estadío clínico más avanzado [103,106], pobre grado de diferenciación histológica [103], y peor supervivencia [101]. Además, se ha comunicado que la expresión aberrante de cortactín se asocia, en líneas celulares de carcinomas de células escamosas de cabeza y cuello positivamente con resistencia al tratamiento basado en gefitinib [107], un anticuerpo monoclonal dirigido selectivamente contra EGFR, que está siendo utilizado terapéuticamente en diferentes cánceres humanos. Por último, debe discutirse su posible utilidad como diana terapéutica en cáncer oral [39]. Las posibilidades de dianizar cortactin con fines terapéuticos derivan esencialmente de observaciones en líneas celulares y experimentación animal, aunque sus conocidas funciones oncogénicas nos han animado a hipotetizar futuras líneas de investigación sobre aspectos aún no explorados de esta proteína. Así, aunque hasta la fecha no se han diseñado moléculas inhibidoras selectivas de la proteína cortactín, futuras líneas de investigación en este sentido se justificarían debido a la alta incidencia de la sobreexpresión de cortactín en COCE [39]. Otra estrategia podría basarse en eliminar al gen *CTTN* amplificado mediante la tecnología de edición genómica CRISPR/Cas9 [108,109]. Por otra parte también, podría considerarse la inhibición independiente o cooperativa

de los oncogenes que alberga el amplicon 11q13 (*CTTN*, *CCND1* y *FADD*) *via* epigallocatechin-3 gallate [18,110,111], o contemplarse como opción terapéutica el empleo de fármacos dirigidos frente a EGFR (p.e. cetuximab y/o gefitinib), debido a las implicaciones de EGFR en pathways que activan oncogénicamente a cortactín (EGFR-Src), y a la posible co-amplificación de sus respectivas bandas cromosómicas -7p11 y 11q13- [112], con coexpresión de sus productos [113]. Además, la citada influencia de *CTTN*/cortactín sobre la resistencia adquirida al tratamiento con gefitinib podría justificar la terapia combinada entre este anticuerpo monoclonal y futuras moléculas inhibidoras de cortactín [107]. Igualmente, la dianización de pathways reguladores de las funciones oncogénicas de cortactin podría constituir una diana plausible [39]. En este sentido, Saracatinib -un agente diseñado para inhibir selectivamente la actividad de Src- no sólo parece disminuir la activación de Src, sino también la de sus sustratos aguas abajo, cortactin incluido, a través de su fosforilación en Tyr421 [114]. Además se ha comunicado que saracatinib actúa de un modo dosis dependiente, y que a altas dosis de tratamiento (0.5–1μM) disminuye directamente los niveles de expresión de cortactín [114]. Se ha podido observar adicionalmente que SU6656 -otro inhibidor selectivo de Src- actúa bloqueando la función invadopodial en carcinoma de células escamosas de cabeza y cuello, presumiblemente como consecuencia de la supresión del pathway EGFR-Src-cortactín-invadopodios [115]. Otra opción plausible podría ser la inhibición de los miembros del pathway MAPK que interaccionan con cortactin (i.e. MEK y Erk), mediante el empleo de inhibidores de esta vía (e.g. PD98059) [115–117].

Como consecuencia de las numerosas implicaciones de *CTTN*/cortactin en la carcinogénesis, decidimos investigar en mayor profundidad mediante técnicas metaanálíticas el significado pronóstico de estas alteraciones (trabajo: Ramos-García P, González-Moles MA, Ayén A, González-Ruiz L, Ruiz-Ávila I, Gil-Montoya JA. Prognostic and clinicopathological significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis 2018: [In Submission]). Consecuentemente, nuestra revisión sistemática y metaanálisis sobre 18 estudios y 1633 pacientes demuestra que las alteraciones de *CTTN*/cortactin se correlacionan con un status N+, mayor status T, estadío clínico avanzado, elevado grado histológico y peor supervivencia de los pacientes con carcinoma de células escamosas de cabeza y cuello. La acción que las alteraciones de *CTTN*/cortactin ejercen en la regulación de las ya señaladas estructuras protrusivas basadas en actina -lamelipodios e invadopodios- cuya formación y activación conducen casi irremediablemente a un aumento de la migración e invasión celular [92,93,95], debe estar ligado al desarrollo de carcinomas con status N+, asociados a una peor supervivencia de los pacientes y a un pobre pronóstico [39,158,184]. La correlación de las alteraciones de *CTTN*/cortactin con carcinomas con mayor status T y elevado grado histológico pueden obedecer a las funciones

emergentes recientemente atribuidas a cortactin [39], esencialmente aquellas relacionadas con el aumento de la proliferación celular. Clark y cols [96] demostraron una reducción significativa de la tasa de proliferación tras el knockdown de cortactin, probablemente como consecuencia de la inhibición del pathway cortactin-RhoA-Skp2 [98], que activado favorece la entrada en la fase S del ciclo celular. Presumiblemente, la correlación entre las alteraciones de *CTTN*/cortactin y HNSCCs con estadios clínicos más avanzados podría obedecer a las asociaciones precedentes con los status N+ y T avanzado. Nuestra evaluación cualitativa ha demostrado que los estudios incluidos en nuestro metaanálisis, a pesar de poseer diseños experimentales semejantes, no se condujeron con la misma escrupulosidad. En nuestro análisis cualitativo, realizado mediante la herramienta QUIPS - Cochrane Prognosis Methods Group- [124], la mayoría de los sesgos potenciales se debieron a la falta de consideración en los estudios de los factores confundidores -dominio study confounding-, a la aplicación de análisis estadísticos inadecuados -dominio statistical analysis and reporting-, y al empleo de métodos de medición inapropiados de expresión de cortactin -dominio prognostic factor measurement-. Los futuros estudios sobre el valor pronóstico de las alteraciones de *CTTN*/cortactin en carcinomas de cabeza y cuello deberían tener en consideración los sesgos potenciales comunicados en la presente revisión sistemática y metaanálisis, siendo aconsejable, para mejorar y estandarizar futuros estudios, respetar los ítems de la herramienta QUIPS [124]. Los estudios incluidos en nuestro metaanálisis presentaron en general poca heterogeneidad, especialmente en lo que respecta a los parámetros status N y estadio clínico, donde no hallamos evidencia de heterogeneidad. Esta existió significativamente en los parámetros grado histológico, status T y supervivencia general, donde fue baja, moderada y alta, respectivamente, según los puntos de corte de Higgins [129,130]. En este último parámetro (supervivencia general), los resultados del presente metaanálisis deberían ser interpretados con precaución debido a la citada heterogeneidad. El análisis del impacto de las alteraciones de *CTTN*/cortactin sobre determinados subgrupos y la evaluación de las fuentes potenciales de heterogeneidad demostró que tanto la amplificación génica como la sobreexpresión de la proteína tuvieron una asociación estadísticamente significativa con todas las variables analizadas, y en general los resultados de ambos subgrupos fueron muy similares. En los parámetros status N y supervivencia general, la sobreexpresión se asoció ligeramente a un peor pronóstico. En los parámetros status T, estadio clínico y grado histológico, la amplificación génica fue mejor predictora que la sobreexpresión. El análisis de metarregresión, así como la exploración visual de intervalos de confianza confirmó que no existieron diferencias estadísticamente significativas entre el empleo de técnicas de detección de amplificación de *CTTN* o de sobreexpresión de su producto cortactin en la valoración pronóstica de ninguno de los parámetros evaluados, lo que a nuestro modo de ver resulta muy relevante, ya que la inmunohistoquímica es actualmente rutinaria en los laboratorios de anatomía patológica. Nuestro metaanálisis también demostró que el subgrupo de pacientes asiáticos presentó la

mayor fuerza de asociación entre las alteraciones de *CTTN*/cortactin y la peor evolución de la enfermedad en todos los parámetros evaluados excepto en el grado histológico. Este impacto especialmente negativo de las alteraciones de *CTTN*/cortactin en pacientes asiáticos podría obedecer al fuerte hábito de consumo tabáquico (tabaco inhalado, mascado, betel,…) presente clásicamente en esta población, lo que se comporta como la causa principal de amplificación de la banda cromosómica 11q13 en el carcinoma de cabeza y cuello [18,30,39]. El análisis estratificado por sitio anatómico demostró que el subgrupo de pacientes con carcinomas faringolaríngeos presentó la mayor fuerza de asociación entre las alteraciones de *CTTN*/cortactin y la peor evolución de la enfermedad, en comparación con el subgrupo de COCEs, que únicamente conservó una asociación estadísticamente significativa en el status N. Por una parte, este resultado pudo depender parcialmente de la influencia del estudio de Bissinger et al (2017) -el estudio con mayor riesgo de sesgo derivado del análisis cualitativo- dando lugar a una infraestimación del estimador final del subgrupo COCE en las variables analizadas. Por otra parte, actualmente sabemos que los carcinomas de cabeza y cuello son un grupo heterogéneo de cánceres con diferencias no sólo anatómicas, sino también a nivel molecular [12]. Creemos necesario que futuros estudios investiguen el impacto que ejercen las alteraciones de *CTTN*/cortactin en carcinomas laríngeos y faríngeos por separado. Esto nos permitiría dilucidar si ambos carcinomas conservan esta asociación pronóstica, o si está más ligada a uno en particular, debido a las diferencias anatómicas y biológicas [12,185]. El anticuerpo monoclonal clone 30 ha sido el más empleado (n=5 estudios), y por ello tratamos de analizar si es preferible su uso al del resto de anticuerpos usados en los estudios (n=6 estudios). El subgrupo clone 30 perdió la significación en todos los parámetros evaluados, excepto en el parámetro de supervivencia, donde encontramos el resultado con mayor grado de heterogeneidad del presente metaanálisis. Parece razonable que los futuros estudios se centren en investigar los anticuerpos del grupo variado (*i.e.* 4F11, 4D10, H-191, G-20 o EP1922Y) que parecen asociarse a resultados menos divergentes y heterogéneos, y más precisos en la evaluación pronóstica del carcinoma de cabeza y cuello que el anticuerpo monoclonal clone 30.

CONCLUSIONES

1. La presente Tesis Doctoral constata la importancia y la complejidad de la amplificación de la banda cromosómica 11q13 en COCE en la que, a diferencia de otras regiones cromosómicas donde es típica la amplificación de un solo gen o de un limitado número de genes, es frecuente la co-amplificación de un conjunto de genes vecinos.

2. Nuestra revision narrativa evidencia la relevancia que ejercen las alteraciones de CCND1/ciclina D1 en la carcinogénesis oral, comportándose como un elemento central en la biopatología del desarrollo del COCE. Esta consideración deriva de los múltiples mecanismos oncogénicos que finalizan en su activación, no perteneciendo esta proteína a un pathway en exclusividad, lo que justifica el alto número de casos de COCEs en los que su expresión se encuentra disregulada. Por esta razón y por su posición en el ciclo celular se comporta como una de las últimas proteínas clave para disparar una proliferación celular incontrolada, asociada a un mayor incremento del riesgo en la adquisición de eventos oncogénicos sumatorios.

3. La amplificación del gen *CTTN* y la sobreexpresión de su producto cortactin son eventos frecuentes y en la carcinogénesis oral. En pacientes con COCE, las alteraciones de *CTTN*/cortactin se comportan como un biomarcador de peor pronóstico.

4. Nuestra revisión sistemática y metaanálisis demuestra que la sobreexpresión de ciclina D1 se correlaciona con peor supervivencia general y peor supervivencia libre de enfermedad, y se asocia con un mayor status T, status N+, estadío clínico avanzado y elevado grado histológico; y nuestros resultados aconsejan incluir la valoración inmunohistoquímica de la sobreexpresión de ciclina D1 en la evaluación pronóstica del COCE, esto es especialmente relevante en el carcinoma lingual, la localización intraoral más frecuente y de peor pronóstico.

5. Nuestra revisión sistemática y metaanálisis demuestra que las alteraciones de *CTTN*/cortactin se correlacionan con un status N+, mayor status T, estadío clínico avanzado, elevado grado histológico y peor supervivencia de los pacientes con carcinoma de células escamosas de cabeza y cuello; nuestros resultados aconsejan incluir la valoración de las alteraciones de *CTTN*/cortactin en la evaluación pronóstica en estos carcinomas.

6. Nuestros resultados inmunohistoquímicos soportan que la sobreexpresión de ciclina D1 constituye un evento frecuente en la carcinogénesis oral asociado a parámetros que implican un pobre pronóstico en COCE (grado de diferenciación, sobreexpresión de ki-67 y morfología invasiva).

7. La expresión de ciclina D1 citoplasmática es un evento frecuente en COCE, que parece poseer funciones ligadas al aumento de la migración e invasión celular y que está asociado con numerosos parámetros de pobre pronóstico, entre los que se encuentran el estadio avanzado, la pobre diferenciación, la elevada expresión de ki-67, la presencia en el tumor de células con

morfología invasiva, así como el status T y N. Por tanto, en nuestra opinión, la expresión citoplasmática de ciclina D1, merece ser considerada en el análisis de la influencia de esta oncoproteína en COCE.

8. La sobreexpresión de ciclina D1 constituye un evento precoz en la carcinogénesis oral ligado a un cambio del patrón proliferativo fisiológico hacia un patrón de proliferación simétrica anormal. La sobreexpresión de ciclina D1, sobretodo en capas basales y parabasales de epitelios no tumorales alejados del punto de invasión, se comporta como un marcador de campos premalignos y de riesgo de desarrollo tumoral múltiple.

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Oral Oncology 72 (2017) 7–16

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Review

Relevance of chromosomal band 11q13 in oral carcinogenesis: An update of current knowledge

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ARTICLE INFO

Article history:
Received 2 February 2017
Accepted 24 April 2017
Available online 30 June 2017

Keywords:
Oral cancer
Carcinogenesis
11q13

ABSTRACT

An important event in oral carcinogenesis is the amplification of chromosomal band 11q13, in which numerous oncogenes and some tumor-suppressor genes are localized and frequently co-amplified during the malignant transformation of oral epithelium. The objectives of this study were to review published data on the involvement of 11q13 amplification in oral cancer, to provide an update on novel concepts and terminology related to gene amplification, and to explore the composition of the 11q13 amplicon in OSCC, including its most relevant amplicon cores and potential drivers. We report on the critical oncogenes and tumor-suppressor genes in 11q13 that may play a major role in oral cancer, focusing on their functions, on the characteristics acquired by their amplification, and on their clinicopathological implications. Finally, we discuss the possible usefulness of the 11q13 region as a therapeutic target in oral cancer.

Introduction

DNA copy number aberrations [1] in the form of microscopic (>500 kbp) or submicroscopic (<500 kbp) genetic material gains or losses represent a major genetic disorder that can promote cancer development [2]. The aberrations are frequently observed as the net gain or loss of a complete chromosome (aneuploidy) or as the partial gain or loss of a chromosome, producing an amplification or deletion, respectively, of genetic material [1,3,4]. Gene amplification is defined as an increase in copy number in a restricted region of a chromosome arm [4] and is a common mechanism for oncogenesis, which is frequently associated with the overexpression of amplified genes [4]. Gene amplification also has programmed and strictly regulated physiological functions during development, as in *Drosophila* oogenesis, the development of salivary glands of *Sciara Cophrophila* larvae, or chicken myogenesis [5,6]. It is considered to begin with a double-strand DNA break [4], while the aberrant progress of the cell through its cycle would likely require the evasion of some robust checkpoints, including the p53-mediated checkpoint [4,7,8]. The most plausible hypotheses to explain the initial double-strand DNA break have related it to fragile chromosome sites, telemetric dysfunction, or DNA replication errors [4]. Once established, amplification can appear in three different forms: extrachromosomal copies, also known as "double minutes"; intrachromosomal copies, forming homogeneously stained regions (HSRs); and copies dispersed throughout the genome [4,6,9]. The different forms of presentation can coexist in the same cell, which often carries >5 copies, with some reports of >500 copies [6].

The amplification of a region frequently produces the expression of genes associated with human tumor development. The degree of causal relationship between amplification and cancer development depends on the amplified region and tumor type. Thus, the alteration proto-oncogene v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN), located in 2p24, amplified in neuroblastomata, is known to cause neuroblastoma development [9]. In contrast, the amplification of chromosome band 11q13 frequently houses various co-amplified genes and is considered a prime example of the complexity of

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http://dx.doi.org/10.1016/j.oraloncology.2017.04.016
1368-8375/

amplification in cancer [4,9,10]. The identification and quantification of amplified DNA in tumor cells permits the isolation of new candidate oncogenes involved in cell growth regulation and possibly in tumor development. Comparative genome hybridization (CGH), fluorescent "*in situ*" hybridization (FISH), Quantitative microsatellite analysis (QuMA), BAC end sequencing, and digital karyotyping are the most widely applied techniques for mapping and measuring amplification [4]. Increases in the resolution of amplification mapping techniques have led to the development of specific concepts related to amplification, including: *amplicon*, i.e., a focal DNA region with copy number increase; and *amplicon driver*, i.e., an amplified gene within an amplicon, whose expression confers advantages to the host cell and contributes to the maintenance of the malignant phenotype [11]. Recent studies demonstrated that an amplicon can house more than one amplicon driver [11,12]. However, the characterization of a gene as an amplicon driver is challenging, because the transcriptional activation of some oncogenes is not regulated by the amplification mechanism alone. For instance, *CCND1* amplification in 11q13 is an essential mechanism for regulating overexpression of cyclin D1 protein in oral squamous cell carcinoma (OSCC); however, this overexpression is also regulated by other mechanisms such as mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K) signaling pathways, which are often altered in oral cancer, hampering identification of *CCND1* as driver in the 11q13 amplicon. Another novel amplification-related concept is the *amplicon core*, i.e., the minimum unit within an amplicon that can be amplified independently from other regions in the same amplicon [11].

Genetic material amplification has diagnostic and prognostic implications and can serve as a therapeutic target, while research on *MYC*, erb-b2 receptor tyrosine kinase 2 (ERBB2), epidermal growth factor receptor (EGFR), and *CCND1* genes appears especially promising. Various publications have described gene amplification as an acquired drug-resistance mechanism. Technological advances over the past decade have achieved the genomic characterization of 11q13 region amplification, which is prevalent in numerous tumor types, including cancers of breast (15–20%), ovary (16%), bladder (11%), lung (9%), and pancreas (14–25%), melanoma (9–44%), and esophagus squamous cell (33%) and head-and-neck (30–62%) carcinomas [11]. It is of particular importance in OSCC, in which amplification of chromosome band 11q13 is highly frequent and has major clinicopathological repercussions.

The objectives of this study were: to review published data on chromosome band 11q13 amplification in human cancer, particularly in OSCC; to provide an update on key terminology and concepts; and to explore the composition of 11q13 amplicon in OSCC, including its most relevant cores and potential drivers. We report on the critical oncogenes and tumor-suppressor genes located in 11q13, which may play a major role in oral cancer, describing their functions, the characteristics conferred by their amplification, and the clinicopathological implications. Finally, we evaluate the possible usefulness of the 11q13 region as a therapeutic target in oral cancer.

11q13 amplicon. Generalities and implications in human cancer

Locus 11q13 is one of the most frequently amplified and important chromosomal regions in human cancer. The 11q13 amplicon is formed by long sequences of amplified DNA. Almost all of its genes can be simultaneously co-amplified, and it often contains neighboring co-amplified genes, making elucidation of its role in cancer highly complex [4,9,10]. The amplification values of 11q13 are frequently low (3–10 copies) [9], with the amplified DNA localized intrachromosomally as an HSR, and all copies are usually, although not always, maintained in the original chromosomal region of 11q13 [4,6,13].

11q13 amplification was first reported around three decades ago in fibroblast growth factor 3 (FGF3) gene (also known as *INT2*) in breast cancer [14] and was subsequently described in other cancers, including squamous cell carcinomas [15]. Shortly afterwards, fibroblast growth factor 4 (FGF4) gene (*HSR1*), also mapped in band 11q13, was found to be co-amplified with *FGF3* in most tumors with 11q13 amplification, including squamous cell carcinomas [16,17]. However, despite the co-amplification of *FGF3* and *FGF4*, the protein expression of these genes is frequently low or undetectable [10]. This has prompted the search for the true driver genes of 11q13, i.e., those that possess the capacity to overexpress proteins and offer the host cells an oncogenic advantage. Thus, *CCND1* gene [18], which is invariably found to be amplified and overexpressed in squamous cell cancer [19], has been proposed as 11q13 amplicon driver [11]. It encodes cyclin D1 protein, which promotes G1/S transition in the cell cycle, it is overexpressed in numerous tumors [20] and it plays a key role in OSCC development [21]. Likewise, *CTTN* gene (aka *EMS1*), which can be co-amplified alongside its neighbor CCND1 in 11q13 and overexpressed in various human cancers [22], also appears to play a major role in tumor development. Other genes mapped in 11q13-q14 (*EMSY*, p21 (RAC1) activated kinase 1 [PAK1], and GRB2 associated binding protein 2 [GAB2]) may also confer selective advantages to cells in which they are amplified [11]. It appears likely that other co-amplified genes are involved in tumor development, underscoring the complexity of 11q13 amplicon. In the late 1990s, comparative genomic hybridization (CGH) studies observed the independent amplification of genes in different loci mapped in 11q13, not only neighboring genes, and demonstrated the presence of four distinct cores within the amplicon [11]. A more precise mapping of these cores in tumor tissue has recently been achieved using high-resolution microarray-based CGH, revealing the composition and borders of the 11q13 amplicon [11]. The author reported that Core 1 (66.4–67.3 Mb) contained genes with a possible role in human cancer, e.g., RNA binding motif protein 4 (RBM4), aryl hydrocarbon receptor interacting protein (AIP), or cyclin dependent kinase 2 associated protein 2 (CDK2AP2), as did Core 2 (68.8–70.3 Mb), e.g., *CCND1*, *CTTN*, Fas associated via death domain (FADD), oral cancer overexpressed 1 (ORAOV1) or *FGF3/4*, some of which (*CCND1*, *CTTN*, and *FADD*) have been proposed as potential amplicon drivers. Core 3 (75.4–76.4 Mb) was also found to contain relevant genes, e.g., Wnt family member 11 (WNT11) and *EMSY*, and the latter is also a candidate amplicon driver. Interestingly, Core 3 houses UV radiation resistance associated (UVRAG) gene, which has been attributed with tumor-suppressor activity, because it is an essential component of the Beclin1-PI3KC3 complex, an important tumor cell growth and autophagy signaling checkpoint [23]. In other words, the amplification patterns of genes in the 11q13 amplicon do not necessarily have oncogenic functions. Finally, core 4 (77.0–79.2), which extends to region 11q14.1, includes *PAK1* and *GAB2* genes, both proposed as possible amplicon drivers. Characteristic 11q13 amplification patterns have been defined in tumors at different sites. Thus, amplification of cores 1 and 2 is less frequent in ovarian than breast cancer, in which all cores are usually amplified [24–26]. Moreover, *CCND1* amplification has been reported in adenocarcinomas, melanoma, and squamous cell carcinomas [4,21,27,28], whereas *EMSY* amplification appears to be frequent in breast adenocarcinoma but rare in squamous cell carcinomas [4,29].

The 11q13 amplicon in OSCC

The 11q13 region is one of the most frequently amplified chromosome bands in head-and-neck squamous cell carcinoma (HNSCC), particularly in OSCC. Reported copy number gains of this

chromosome band have ranged between 13 and 100% of OSCC cases [30–49] (Table 1), with a mean of 46%. However, higher amplification rates than those observed for the whole region have been reported for *CCND1*, the most widely studied gene in this band [21]. The Cancer Genome Atlas (TCGA) [50] confirmed that 11q13 amplification is a frequent and relevant event in HNCC.

It has been proposed that 11q13 gene amplification is initiated and promoted by specific mechanisms in OSCC, related to double-strand DNA breaks in fragile chromosomal sites [4]. Thus, Reshmi et al. [51,52] reported that 11q13 amplification in OSCC results from breakage–fusion–bridge cycles triggered by a break at the common chromosomal fragile site, FRA11F, possibly induced by tobacco-related carcinogens [4,53]. The close etiological association of tobacco consumption with HNSCC and OSCC development probably explains the high frequency of 11q13 amplification in these tumors, which is more than double the frequency found in human cancers less related to tobacco consumption. Among OSCC patients, 11q13 amplification has been more frequently described in human papillomavirus (HPV)-negative cases associated with tobacco consumption than in HPV-positive cases not related to tobacco [30,34,35,50,54], indicating that HPV may not play an essential role in the onset and establishment of 11q13 amplification in OSCC [21]. Likewise, no relationship has been observed between alcohol consumption and 11q13 amplification. A study by our group on the involvement of cyclin D1 in labial carcinogenesis found overexpression at all stages of labial epithelium malignization, suggesting that actinic radiation, specifically of the *CCND1* gene, is also implicated in the onset of 11q13 amplification [55].

The few studies on 11q13 amplicon cores in human squamous cell carcinomas at different sites point to a common pattern, with the amplification of cores 1 and 2 [4,11]. However, there is often a single amplicon core in OSCC, generally core 2, which is thought to be localized among 11q13.2-q13.4 sub-regions (proximal-distal ends), although its boundaries are not yet well defined (fig. 1) [31,35,37,40,43,46,56,57]. Most studies found the size of this core to be around 1.5 Mb, ranging from 0.9 to 2 Mb [35,37,40,43,46], similar to the size for core 2 in breast cancer in the study by Wilkerson [11]. However, Sugahara et al. [32] proposed two well-defined amplicon cores in oral cancer, separated by a breakpoint located between myeloma overexpressed (MYEOV) and *CCND1* genes (fig. 1), with core 1 extending from two pore segment channel 2 (TPCN2) to *MYEOV* gene (proximal-distal ends) and core 2 from *CCND1* to *CTTN* gene (proximal-distal ends).

The frequent co-amplification of 11q13 genes observed in cancer suggests the presence of a set of genes in the amplicon that would endow altered cells with growth and metastatic advantages [40]. Uchida et al. [33] reported the co-amplification of latent transforming growth factor beta binding protein 3 (LTBP3), Sjogren syndrome/scleroderma autoantigen 1 (SSSCA1), family with sequence similarity 89 member B (FAM89B), EH domain binding protein 1-like 1 (EHBP1L1), potassium two pore domain channel subfamily K member 7 (KCNK7), mitogen-activated protein kinase kinase kinase 11 (MAP3K11), and pecanex homolog 3 (PCNX3) (aka *PCNXL3*) genes in 11q13.1. Between locations 11q13.2 and q13.4, various authors [31–33,35,37,40,43,46,56,57] found carnitine palmitoyltransferase 1A (CPT1A), mitochondrial ribosomal protein L21 (MRPL21), immunoglobulin mu binding protein 2 (IGHMBP2), MAS related GPR family member F (MRGPRF) (aka *MRGF*), *TPCN2*, *MYEOV* (aka *OCIM*), *CCND1*, *ORAOV1* (aka *TAOS1*), *FGF19/4/3*, anoctamin 1 (ANO1) (aka *TMEM16A*, *ORAOV2*, *TAOS2*, or *DOG1*), *FADD*, PTPRF interacting protein alpha 1 (PPFIA1), *CTTN* (aka *EMS1*), SH3 and multiple ankyrin repeat domains 2 (SHANK2), and nuclear mitotic apparatus protein 1 (NUMA1) genes (listed in the order of their chromosomal localization in centromere-telomere direction) to be co-amplified in OSCC (fig. 1). Less frequently, the co-amplification of chromosome band 11q13 with other chromosomal regions has also been reported, and its co-amplification with 8p12 is probably the best-documented event in human carcinogenesis, mainly in breast cancer [58–60]. However, published data on 11q13 and 8p12 co-amplification in oral cancer are contradictory; thus, the most frequently observed event is the gain of 11q13 and loss of 8p [35,36,41,47,61], whereas fibroblast growth factor receptor 1 (FGFR1), one of the essential genes at 8p, is commonly amplified in HNSCC and OSCC. *FGFR1* encodes a member of the FGFR family that acts as transmembrane tyrosine kinase receptor and is associated with cell proliferation, among other functions [62]. Freier et al. [63], detected *FGFR1* amplification in 17% of OSCC cases and observed overexpression of this protein in early stages of oral carcinogenesis, while Goke et al. [64] found amplified *FGFR1* gene mRNA and expression in HNSCC cell lines. Co-amplification of genes in different chromosome bands has been reported by other

Table 1
Studies of 11q13 chromosomal band amplification in HNSCC & OSCC.

First author	Year	Tumor type	Localization	Study sample	Cases	Technique	% gain	Ref. no.
Wolff	1998	SCCs	Oral cavity	Primary tumor tissues	20	CGH	70%	49
Gebhart	1998	SCCs	Oral cavity	Primary tumor tissues	23	CGH	22%	48
Singh	2001	SCCs	Head & neck	Cell lines	12	CGH	33%	47
Huang	2002	SCCs	Oral cavity	Cell lines	30	QuMA	63%	46
Garnis	2004	SCCs	Oral cavity	Primary tumor tissues	14	CGH	50%	45
Chen	2004	SCCs	Oral cavity	Primary tumor tissues	13	CGH	69%	44
Freier	2006	SCCs	Oral cavity	Primary tumor tissues	40	CGH	60%	43
Uchida	2006	SCCs	Oral cavity	Cell lines	11	CGH	45%	42
Noutomi	2006	SCCs	Oral cavity	Primary tumor tissues	35	CGH	49%	41
Huang	2006	SCCs	Oral cavity	Primary tumor tissues	31	QuMA	32%	40
Huang	2006	SCCs	Oral cavity	Cell lines	30	QuMA	46%	40
White	2006	SCCs	Head & neck	Cell lines	52	Fish	46%	39
Martin	2007	SCCs	Oral cavity	Cell lines	11	CGH	45%	38
Freier	2010	SCCs	Oral cavity	Primary tumor tissues	40	CGH	37%	37
Freier	2010	SCCs	Head & neck	Cell lines	20	CGH	50%	36
Ambatipudi	2011	SCCS	Oral cavity	Primary tumor tissues	60	CGH	47%	35
Pathare	2011	SCCs	Oral cavity	Primary tumor tissues	97	CGH	41%	34
Uchida	2011	SCCs	Oral cavity	Primary tumor tissues	50	CGH	26%	33
Sugahara	2011	SCCs LNM(+)	Oral cavity	Primary tumor tissues	10	CGH	30%	32
Blessmann	2013	SCCs	Oral cavity	Primary tumor tissues	10	CGH	100%	31
van Kempen	2015	SCC	Oral cavity	Primary tumor tissues	164	MLPA	13%	30

SCCs, squamous cell carcinomas; LNM(+), positive for lymph node metastasis; CGH, comparative genomic hybridization; QuMA, quantitative microsatellite analysis; Fish, fluorescence *in situ* hybridization; MLPA, multiplex ligation probe amplification.

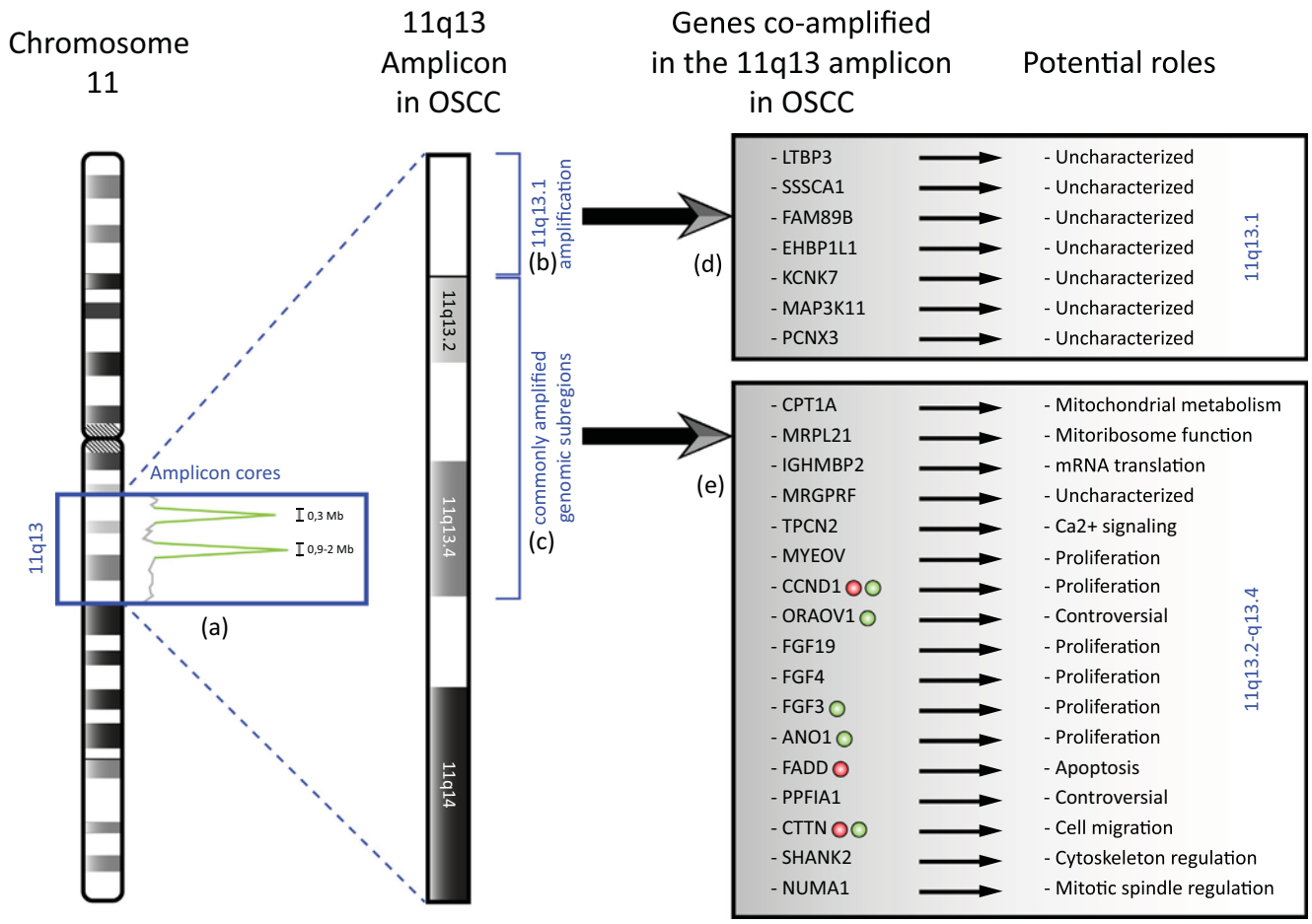

Fig. 1. Representative scheme of 11q13 chromosome band amplification in OSCC. The two amplicon cores mapped in 11q13 OSCC are indicated in green (a). The smallest amplicon core was mapped by Uchida et al. [33] in sub-region 11q13.1 (b). However, most studies mapped a single amplicon core with a size ranging between 0.9 and 2 Mb (≈1.5 Mb), located in 11q13.2-q13.4 sub-regions (c) [31,35,37,40,43,46,56,57]. In (box d), the genes co-amplified in 11q13.1 are depicted in the first column and their potential roles in OSCC in the second. In (e), the first column lists the genes co-amplified in 11q13.2-q13.4 and the second shows their oncogenic functions in OSCC. All genes in this figure are enumerated according to the order of their localization in the chromosome in a centromere-telomere direction. Genes indicated with a red dot -*CCND1*, *FADD, and CTTN*- represent the candidate amplicon drivers of this amplicon in OSCC. Genes marked with a green dot -*CCND1*, *ORAOV1*, *FGF3*, *ANO1,* and *CTTN*- may be involved in early stages of oral carcinogenesis (OPMD-related). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

researchers, including Seiwert et al. [65], who found a characteristic pattern of *EGFR* (7p12), *FGFR1* (8p12), and *CCND1* (11q13) co-amplification in HPV-negative HNSCC cases. Our study on *CCND1*/-cyclin D1 [21] described the frequency of *CCND1* and *EGFR* co-amplification and its clinicopathological implications, including its association with N + status [66] and the presence of extracapsular spread in OSCC cases [67]. The Cancer Genome Atlas (TCGA) [50] documented the statistically significant co-amplification of *CCND1*, *CTTN*, and *FADD* genes, located in 11q13, with Yes associated protein 1 (YAP1) and baculoviral IAP repeat containing 2 (BIRC2) genes, located in 11q22, while Uchida [33] and Ambatipudi [35] also found the frequent amplification of 11q13 and 11q22 in OSCC, with 11q22.1-q22.2 amplification predicting poor survival [35]. In one study, co-amplification of *FGF3/4* at 11q13 and v-myc avian myelocytomatosis viral oncogene homolog (c-Myc) at 8q24 was observed in 62% of OSCC cases [44]. We highlight the observation by Pathare et al. [34] of synergism between 11q13 gain and 18q loss, which they found to be a strong predictor of poor clinicopathological prognosis and reduced survival, despite the absence of co-amplification. The loss of 18q has also been associated with angiogenesis, disease relapse, and worse survival in HNSCC patients [68].

Numerous authors have demonstrated that 11q13 band amplification in OSCC influences clinicopathological characteristics associated with a worse prognosis [30,32,34,38,44,48,69–74]. Thus, 11q13 amplification has been related to metastasis to locoregional lymph nodes [30,32,44], while Sugahara et al. [32] found the co-amplification of *CTTN* with *TPCN2* or *MYEOV* to be a powerful predictor of N+ status and to be significantly associated with distant metastasis-free survival. Pathare et al. [34] reported a statistically significant correlation between amplification of the 11q13 region and high histological grade in OSCC, although they combined moderately and poorly differentiated carcinomas into a single group, which may act as a confounder. A correlation between 11q13 amplification and worse survival has also been described [30,38,48]. Martin et al. [38] analyzed 11 cell lines from OSCC patients and observed that only those with 11q13 amplification died from the disease (5/11). Van Kempen et al. [30] found a correlation between reduced disease-free survival (DFS) and the amplification of the 11q13 chromosomal region and of some of its genes, notably *CCND1*. With regard to the response to treatment, gene amplification is now considered to be one of the mechanisms that underlie acquired treatment resistance in various types of human cancers [4], leading to the proposal of certain

amplified genes as therapeutic targets. In this context, *CCND1* amplification or cyclin D1 overexpression has been found to reduce the response to radio- and chemotherapy, especially to cisplatin in HNSCC, including OSCC [69–74].

The 11q13 amplicon in oral potentially malignant disorders

Copy number gain or 11q13 chromosomal region amplification has been described in oral potentially malignant disorders (OPMDs) [41,75,76]. Cervigne et al. [75] found 11q13 band amplification in all degrees of epithelial dysplasia in leukoplakias that progressed to OSCC, while 11q13 gain was also reported by Noutomi et al. [41], mainly in the transition from moderate to severe epithelial dysplasia. Studies have been conducted on genes in the 11q13 amplicon that participate in early stages of oral carcinogenesis, with wide reports of *CCND1* amplification and cyclin D1 overexpression in OPMDs [21]. In addition, *FGF3* amplification has been observed in severe dysplasias and oral carcinomas *in situ* [77], while *ORAOV1* amplification was recorded in 33.3% of leukoplakias and in all grades of epithelial dysplasia studied by Xia et al. who also reported *CTTN* amplification in 20% of leukoplakias with moderate or severe dysplasia [78]. Finally, chordin like 2 (CHRDL2) amplification was reported in leukoplakias that progressed to OSCC [75]. 11q13 co-amplification with other chromosomal regions has also been reported in OPMDs, including *CCND1* co-amplification with *EGFR* in early stages of oral carcinogenesis [77,79]. Fewer data are available on the amplification of sub-regions of chromosome 11q13 in OPMDs. Amplification of chromosomal sub-region 11q13.3-q13.4 was described by Tsui et al. [77], and DNA copy number gain in chromosomal region 11q13.4 was consistently reported by Cervigne et al. [75], who also implicated *CHRDL2* amplification in the malignant transformation of oral leukoplakias and first documented this disorder in oral carcinogenesis. *CHRDL2* gene is associated with cancer; it encodes an extracellular matrix protein involved in cell differentiation whose overexpression has been found in breast, lung, and colon tumors [80].

Similar findings have also been reported in early stages of HNSCC development [81–84], including: the early detection of *FGF3* amplification, mapped in the transition from hyperplasia to dysplasia in non-tumor epithelial tissue adjacent to HNSCC [82]; *CTTN* amplification in high-grade dysplasias that progressed to invasive carcinoma in premalignant head and neck epithelial tissue [83]; and a high frequency of *ANO1* gene amplification (63%) in laryngeal dysplasias [84].

Candidate oncogenes in the 11q13 amplicon in OSCC

CCND1

CCND1 encodes cyclin D1, which promotes cell cycle progression during G1 phase and favors G1-S transition [20]. The *CCND1*/-cyclin D1 complex is the most widely studied component of chromosome band 11q13 in oral and other human cancers, including breast, lung, colon, and melanoma [85]. Besides its regulation of cell proliferation, numerous key functions in the cell biology of cancer have also been reported over recent years, including mitochondrial activity modulation, DNA repair, and cell migration control [21]. A census published in 2010 confirmed evidence of *CCND1* amplification and overexpression in OSCC [85]. The reported rate of *CCND1* amplification in OSCC ranges between 9 and 72% [21], while it has always been detected in studies of the amplicon core at 11q13.2-q13.4 [31–33,37,40,43,56]. As well as its amplification, other activation mechanisms of this oncogene have been described, including: chromosomal translocations, e.g., juxtaposition of *CCND1* with immunoglobulin heavy chain (IGH) locus [t(11;14)(q13;q32)], considered the genetic hallmark of mantle cell lymphoma; mutations, e.g., Thr-286 and G/A870 polymorphisms; regulation pathway disorders, e.g., MAPK, PI3K, Wnt and nuclear factor kappa B (NF-κB) signaling pathways; and microRNA-mediated upregulation [21,86]. Among genes in the 11q13 amplicon, *CCND1* has been the most widely implicated in cancer, with reports that *CCND1*/cyclin D1 is involved in all phases of oral carcinogenesis, including early stages [21]. Numerous studies of OSCC have associated cyclin D1 overexpression with lymph node involvement, larger tumor size, advanced clinical stage, poor differentiation, worse response to treatment, especially cisplatin, and reduced survival [21].

CTTN

The *CTTN* gene (aka *EMS1*) encodes cortactin, an F-actin binding protein with multiple binding domains that permit various protein interactions [87,88]. After EGFR signaling activation, interaction of Src with cortactin phosphorylates the latter at Tyr-421, Tyr-466, and Tyr-482, impacting on cortical actin cytoskeleton organization and cell morphology; this gives rise to protrusive structures (lamellipodia and invadopodia) at the leading edge of the cell that appear to be important for mesenchymal epithelium transition, enhancing cell migration. Cortactin also binds to and activates the actin-related protein (Arp)2/3 complex, which regulates the formation of branched actin networks and is therefore relevant in cell cytoskeleton reorganization [87]. *CTTN* amplification is a frequent event in HNSCC, ovarian, bladder, breast, and lung cancers [22]. The reported amplification rate of this gene in OSCC ranges between 10 and 57% [30,33,35,36,40,43,78,89], and high levels of cortactin mRNA and protein have been recorded in tumors with amplification [36,40,43,56,89]. Amplification of this gene has been documented in 20% of OPMDs [78], and the overexpression of its product has been described as a predictor of malignant transformation, finding an increase in its expression with more severe dysplasia [90]. However, *CTTN* amplification appears to participate in later stages of oral carcinogenesis in comparison to other 11q13 genes [78]. It is one of the genes that are persistently amplified in sub-region 11q13.2-q13.4 in OSCC [32,33,37,40,43,46,56], and its co-amplification with *CCND1*, *FGF3*, and *SHANK2* has been described in cancer [11,22,43,89]. *SHANK2* may exert a cooperative effect on tumor cell motility in OSCC invasiveness, given that both genes (*CTTN* and *SHANK2*) encode proteins associated with the cytoskeleton, which may explain the association between 11q13 band amplification and lymph node metastasis. In OSCC, *CTTN* gene and its product (cortactin) have been correlated with: cervical lymph node involvement [30,32,44,78,89,91,92]; extracapsular spread [91], more infiltrative tumor pattern [92], larger tumor size, more advanced clinical stage [78,92], poor histological differentiation [78], and reduced DFS [30]. A study of HNSCC cell lines [93] found a positive association between aberrant cortactin expression and resistance to treatment with gefitinib, a monoclonal antibody against *EGFR* that is used therapeutically in different human cancers.

FADD

The *FADD* gene encodes the Fas-associated death domain (FADD) protein, which is involved in apoptotic signaling and has more recently been attributed with functions related to cell cycle progression, innate immunity, and autophagy [94]. The main function of FADD in apoptosis regulation appears to be the activation of so-called active death receptors, e.g., Fas, DR4, or DR5, which bind with procaspases 8/10 and promote the formation of death-inducing signaling complexes. These complexes activate different procaspases and other sequential targets, e.g., caspases 3/6/7,

producing apoptosis [95]. Chen et al. [96] reported that the overexpression of *FADD* mRNA and protein in tumor cells was correlated with the increased activation of anti-apoptotic signaling *via* NF-κB, which may explain the putative role of the *FADD* gene and its product in apoptosis evasion. Its amplification has been documented in breast cancer, HNSCC, and neuroblastoma [11,97], while its reported amplification rate in OSCC ranges between 13 and 44% of cases [30,98]. Its co-amplification with neighboring genes in this chromosome band has also been reported [32,40,43,46,56,57]. A high correlation between FADD protein overexpression and gene amplification has been demonstrated in both HNSCC and OSCC, indicating the high dependence of its overexpression on this mechanism [40,56,57,97,98]. For this reason, Gibcus et al. [97] proposed *FADD* as amplicon driver of the 11q13 amplicon in head and neck carcinogenesis. A recent meta-analysis [99] positively correlated *FADD* amplification with worse survival in HNSCC. In addition, its amplification has been associated with lymph node metastasis, poor histological differentiation degree, and worse DFS in OSCC [30,98], while FADD overexpression has been positively related to lymph node metastasis and reduced survival, both in HNSCC and OSCC [97,98,100].

FGF3/4

FGF3 (aka *INT2*) and *FGF4* (aka *HST-1*) genes encode proteins from the FGF family that, alongside their receptors (FGFRs), regulate a wide variety of biological phenomena, including cell proliferation, differentiation, survival, and migration [62]. They were the first genes investigated in 11q13 and were found to be co-amplified in most tumors in which they are altered [14–17]. The frequent co-amplification of *FGF3/4* has also been reported with other neighboring genes, such as *CCND1*, *CTTN*, and *SHANK2* [43], and with genes from other chromosomal regions, e.g., *c-Myc* (8q24) [44]. Components of the FGF signaling pathway have been implicated in breast, ovarian, bladder, and gastric cancers, among others [62], and in HNSCC, being consistently amplified in OSCC [32,33,43,46], which is associated with the highest amplification rates in 11q13. There are also reports of copy number gains of around 70% in oral cancer [30,33,43,44], although the amplification is frequently not accompanied by overexpression of effector proteins in OSCC [40,43]. This appears to rule *FGF3/4* out as amplicon drivers in 11q13, although some authors have correlated amplification of these genes with lymph node involvement and reduced DFS, among other clinicopathological characteristics [30,44].

ORAOV1

ORAOV1 (aka *TAOS1*) encodes the oral cancer overexpressed 1 protein. Its functions are not well characterized but include cell cycle promotion, apoptosis evasion, angiogenesis, cell migration, and cell invasion [101–103]. *ORAOV1* amplification has reported in oral and esophageal squamous cell carcinomas, melanomas, breast, ovarian, and pancreas cancers, and neuroblastomas [11]. Reports on its amplification rate in OSCC range between 22 and 51% [33,35,45,78,104]. *ORAOV1* amplification was found in 33.3% of OPMDs in the series of Xia et al. [78] from the stage of mild dysplasia onwards and was one of the genes in 11q13 whose amplification was first observed in oral carcinogenesis. It is commonly located in the amplicon core in sub-region 11q13.2-q13-4 in OSCC [32,33,40,43,46,57]. *ORAOV1* amplification has been associated with lymph node metastasis, poor histological differentiation, and advanced clinical stage [78] [40,46,57], although Xavier et al. [104] observed a weak correlation between *ORAOV1* amplification and expression levels of its protein in OSCC.

Other candidate oncogenes

Data have also been published on other genes in chromosome band 11q13. *ANO1* (aka *TMEM16A*, *ORAOV2*, *TAOS2*, or *DOG1*) was recently mapped, finding its amplification in early stages of head and neck carcinogenesis [84]. A recent meta-analysis [99] associated this gene with worse survival in HNSCC patients. A high expression of its product (anoctamin-1 protein) has been found in OSCCs with and without *ANO1* amplification, suggesting that its expression may also be regulated by mechanisms other than overexpression [40,57]. *SHANK2*, also located in 11q13, was found to be amplified in 36% of studied OSCCs [43], in which it was co-amplified with *CCND1*, *CTTN*, and *FGF3*. Its co-amplification with *CTTN* may cooperatively modulate the motility and invasiveness of OSCC because both genes encode cytoskeleton-related proteins, which may help to explain the association between amplification of the chromosomal region 11q13 and N+ status. In addition, tumors with *SHANK2* gene amplification overexpressed its protein. There has been little research on other 11q13 genes in oral cancer, such as *TPCN2*, *FGF19*, or *MYEOV*, a gene whose amplification seems to be important in other human cancers, and none of these have been associated with clinicopathological factors in OSCC. *RIN1*, a candidate oncogene also located in 11q13.2, encodes Ras and Rab interactor 1, a putative effector of *Ras* oncogene, whose functions are poorly characterized. In relation to OSCC, only one study [105] evaluated *RIN1* amplification in 10 cell lines, finding copy number gain in 50% of cases, but the authors did not investigate the clinicopathological implications of *RIN1*, which has been associated with a poor prognosis in various human cancers [106–109]. *PPP1CA*, also located in 11q13, encodes protein phosphatase 1α (PP1α), an isoform of PP1 that appears to be involved in cell cycle and apoptosis regulation. Hsu et al. [110] observed *PPP1CA* amplification in OSCC cell lines and reported higher mRNA levels in cell lines with than without 11q13 amplification; they also found that OSCC cell growth was suppressed by small interfering RNA (siRNA)-induced knockdown of *PPP1CA*/PP1α.

Candidate tumor suppressor genes in the 11q13 amplicon in OSCC

The *PPFIA1* gene encodes Liprin-alpha-1, a member of the LAR protein-tyrosine phosphatase-interacting protein (liprin) family. Its functions are not well established but are thought to be related to the disassembly of focal adhesions at sites of cell-extracellular matrix interactions, with implications for cytoskeleton-matrix connections [111]. During the cascade of molecular phenomena and cellular interactions that enable metastasis of malignant epithelial cells, the cells must maintain an adequate relationship with the extracellular matrix [112], and greater metastasizing capacity would presumably be developed by clones acquiring a phenotype that permits anchoring. Given the action of its protein as cell-matrix unbinding element, a putative tumor-suppressor role has been proposed for the *PPFIA1* gene, which appears to act as invasion inhibitor in HNSCC, including oral cancer [113]. *PPFIA1* amplification was reported in 26% of OSCC patients and its co-amplification with *CCND1* in 92% of these cases [31], with documentation of its presence in the amplicon core in sub-region 11q13.2-q13-4 in OSCC [31,32,40,46,56,57]. There is a strong correlation between the amplification of this gene and its expression levels [40,56,57], indicating that the encoded protein may be strongly regulated by this mechanism. Accordingly, the 11q13 amplicon contains genes that can regulate the progression of oral carcinogenesis in either a negative or positive manner, as also observed in breast cancer for the *UVRAG* gene, another tumor suppressor amplified in amplicon 11q13.

The 11q13 amplicon as therapeutic target in OSCC

The *CCND1*/cyclin D1 complex is the most widely studied therapeutic target in 11q13 because there is greater knowledge of its clinicopathological implications. Cyclin D1 can be targeted in various ways: by acting on its cyclin dependent kinase (CDK) partners (CDK4/6), by acting directly on cyclin D1 or on the OSCC pathways in which cyclin D1 is involved, or by combining cyclin D1 inhibitors with other antitumor drugs [21,114]. One promising therapeutic approach is to *inhibit its CDK partners* by using global CDK action inhibitors, e.g., flavopiridol, olomoucine, or R-roscovitine, or by administering CDK4/6-selective inhibitors, e.g., P276-00, P1446-05, or PD0332991 [114]. P276-00 is a flavone with powerful selectivity towards cyclin D1-CDK4 complexes, acting in an ATP-competitive manner [115], and its antitumor activity has been reported in *in vivo* and *in vitro* HNSCC tumor models [116]. The effectiveness of P276-00 in HNSCC is currently under research in phase I/II clinical trials (NTC00824343, NCT00899054). *Directly targeting cyclin D1* is another attractive option, given increasing evidence of CDK-independent cyclin D1 functions [21]; although cyclin D1 is a complex target due to its intracellular localization and lack of enzymatic activity. The anticancer potential of green tea and curcumin appears to be based on cyclin D1 inhibition [21]. A randomized phase II clinical trial found reduced cyclin D1 expression in patients with high-risk OPMDs who responded to treatment with green tea extract [117]. Research on curcumin is under way in a clinical trial with HNSCC patients (NCT01160302), but no data have yet been published on its effectiveness. Cyclin D1 may also be directly inhibited by acting on glycogen synthase kinase 3 Beta (GSK-3β), an enzyme that participates in its degradation. This approach has been tested using differentiation-inducing factor 1 (DIF-1), a protein of the DIF family, which has been identified in *Dictyostelium discoideum* and is capable of inhibiting cell proliferation in mammal cells, although there are no clinical trials in OSCC patients [21]. *Targeting the pathways that are involved in OSCC development and include cyclin D1 as a component*, such as MAPK or PI3K, may also be a therapeutic option. The mechanistic targeting of rapamycin (mTOR) inhibitors is of special interest, because the translation of *CCND1* mRNA is mTOR-dependent [114], and it has been reported that mTOR inhibition may reduce cyclin D1 expression in OSCC *via* PI3K [21,118]. A phase I clinical trial is investigating the mTOR inhibitor ridaforolimus, which achieves a strong PI3K blockade in patients with advanced HNSCC when combined with the Notch inhibitor MK-0752 [119]. Everolimus, another m-TOR inhibitor, has also shown promising results in a phase I clinical trial with HNSCC patients [120]. Finally, there are various phase II clinical trials in HNSCC and OSCC patients using drugs that target m-TOR (NCT01195922, NCT01313390, NCT01009203, NCT01172769, NCT01016769). It is also possible to target FOXO transcription factors, which belong to the PI3K signaling pathway and are linked to cyclin D1 overexpression [121]. Overexpression of RNA-binding protein quaking 5 (QKI-5) is another potential approach, given recent *in vitro* findings of its behavior as tumor suppressor in the OSCC CAL-27 cell line, inhibiting the expression of cyclin D1 *via* MAPK [122]. Finally, the combination of cyclin D1 inhibitors and other antitumor agents may be of special interest, given evidence of resistance to cisplatin treatment in OSCC patients with cyclin D1 overexpression [21]. In fact, worse outcomes have been obtained by using single therapies to inhibit cyclin D1 or cyclin D1/CDK than by combining them with cisplatin, 5-fluoracil, doxorubicin, or paclitaxel [114]. This synergic effect appears to be more effective when the cytotoxic drug is administered first, possibly because CDK activity inhibitors are more effective when cells are synchronized or halted in specific cell cycle phases [114]. In order to achieve greater effectiveness in future clinical trials and improve the pharmacokinetics, it seems reasonable to maintain an effective dose of the inhibitor over an adequate time period, allowing the targeted cancer cells to enter a cell cycle phase that is more susceptible to the cytotoxic agents [114]. It may also be useful to combine cyclin D1 or cyclin D1/CDK inhibitors with inhibitors of tyrosine-kinase receptors, among which EGFR is the most widely studied in oral cancer [21]. The targeting of both oncoproteins is of particular interest, given the frequent co-amplification of genes encoding cyclin D1 and EGFR and the co-expression of their products in oral carcinogenesis [67,77]. Positive outcomes may be obtained by the modulation of both oncoproteins [21]. The only directed therapeutic agent approved by the FDA for administration in HNSCC patients is the EGFR inhibitor cetuximab, after a phase I clinical trial in aerodigestive tract cancers, including HNSCC (17% of patients), reported that the combination of rexinoid bexarotene with the EGFR inhibitor erlotinib, enhanced tumor growth suppression by cooperative repression of cyclin D1 [123]. This combination appears especially promising in lung cancer, for which erlotinib is a standard treatment.

Another candidate therapeutic target gene in 11q13 is *CTTN* and its product, cortactin. Yamada et al. [92] reduced cortactin expression in SAS-OSCC cell lines by silencing its mRNA and observed a significant reduction in the cells' invasive capacity, which suggests that cortactin may be a potential candidate target for gene therapy in OSCC. A study by Rothschild et al. [124] on cortactin expression downregulation by siRNA in HNSCC cell lines with amplified *CTTN* reported a significant reduction in HNSCC invasiveness and migration. Huang et al. [125] successfully used Epigallocatechin-3 gallate, a polyphenol component of green tea, to inhibit OSCC cell invasion and migration both *in vitro* and *in vivo* (athymic nude mice); the authors reported that its action mechanism was attributable to cortactin downregulation and actin cytoskeleton remodeling. Finally, Ammer et al. [126], using saracatinib, observed decreased activation of Src and its downstream substrate, the *CTTN* oncogene in HNSCC cell lines and *in vivo* models. They also verified that high doses of saracatinib reduce cortactin expression levels.

Given the relationship of *FADD*, another gene in 11q13, with apoptosis evasion, its inhibition might offer a therapeutic strategy in OSCC cases with gene amplification. Schinske et al. [127] studied the action of NSC 47147, a small molecule inhibitor of FADD kinase activity, in cell lines of lung carcinoma and colorectal adenocarcinoma and in *in vivo* models (nude mice), finding an association between the functional inactivation of FADD and reduced phosphorylation. The combination of NSC 47147 with cisplatin also improved the induction of apoptosis, with an enhanced susceptibility to the chemotherapy [127].

Clinical trials are exploring the therapeutic action of FGF signaling inhibitors in several human cancers but not the value of *FGF3/4* as a therapeutic target in oral carcinogenesis. A phase II clinical trial (NCT01377298) is investigating the action of pazopanib, an inhibitor of FGF signaling components (vascular endothelial growth factor receptors [VEGFRs] 1/2/3, platelet derived growth factor receptors [PDGFRs] α/β, c-KIT, or FGFRs1/3) in recurrent or metastatic HNSCCs, while another [128] evaluated the antitumor activity of dovitinib (TKI258), an inhibitor of FGFRs1/2/3, in breast cancer with FGFR pathway amplification, concluding that it produced a superior response in patients with *FGF3* amplification.

ANO1 has also been studied as a possible therapeutic target. Duvvuri et al. [129] reported that *ANO1* modulated cell proliferation and cell growth by activating the MAPK signaling pathway and observed that *ANO1* knockdown by shRNA inhibited tumor growth in HNSCC both *in vitro* and *in vivo*. The authors also treated cell lines UM-SCC1 and T24 with the *ANO1* inhibitor T16A-inh01 and reported cell proliferation inhibition *in vitro*.

ORAOV1 has been less well studied as a therapeutic target in cancer. However, a recent study [103] reported a downregulation in its expression by EA.hy926 endothelial cells co-cultured with OSCC, HSC-3, and SCC-2 cell lines in which *ORAOV1* silencing was conducted, associated with a marked reduction in cell proliferation, migration, and invasion. These findings suggest that *ORAOV1* may be a potential therapeutic target in OSCC.

Conclusions and final considerations

This review verifies the importance and complexity of 11q13 chromosome band amplification in OSCC. The co-amplification of a set of neighboring genes is frequent in this band, unlike other chromosomal regions, in which a single gene or a limited number of genes is typically amplified. To date, a single amplicon core has been confirmed in OSCC, located in chromosomal sub-region 11q13.2-q13.4; further studies are warranted to determine whether there is a second amplicon core in this tumor, as hypothesized in other human squamous cell carcinomas. We consider *CTTN*, *FADD*, and *CCND1* to be candidate drivers of the 11q13 amplicon in OSCC due to: the constant expression of their products when the genes are amplified, the relationship between gene amplification and protein overexpression, and the clinicopathological and prognostic implications of their amplification. Among these three genes, *CCND1*, the driver *par excellence* in the literature, may be the most difficult to designate because of the multiplicity of its regulation mechanisms, hampering confirmation of the high dependence of its expression on gene amplification. The 11q13 amplicon houses a series of oncogenes of major importance in oral cancer, including *CCND1*, *CTTN*, *FADD*, *FGF3/4*, and *ORAOV1*, as well as tumor-suppressor genes such as *PPFIA1*. Further studies are also necessary to determine whether the gene amplification process can both positively and negatively regulate disease progression. The oncogenes in this amplicon may explain the reported influence of 11q13 amplification on different clinicopathological characteristics of OSCC, including lymph node involvement, poor tumor differentiation, and low survival. However, it is yet to be determined whether these oncogenic implications result from the individual actions of these oncogenes or from the cooperative effect of 11q13 genes that are frequently co-amplified in OSCC. Gene amplification has frequently been related to resistance to treatment in human cancer, as observed for 11q13 genes in OSCC, in which associations have been found between resistance to cisplatin and *CCND1* amplification and between resistance to gefitinib and *CTTN* amplification. Further research is warranted on therapeutic strategies that target components of the 11q13 amplicon in head and neck carcinogenesis and on the effectiveness of combined therapies to simultaneously inhibit multiple genes in the amplicon.

Conflict of interest statement

None declared

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ORAL DISEASES
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Oral Diseases (2017) 23, 897–912 doi:10.1111/odi.12620

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REVIEW ARTICLE

An update on the implications of cyclin D1 in oral carcinogenesis

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Cyclin D1 promotes cell cycle progression during G1 phase, a key event in G1-S transition. The protein is encoded by gene *CCND1*, located in chromosomal band 11q13. Cyclin D1 plays key roles in cell biology, including cell proliferation and growth regulation, mitochondrial activity modulation, DNA repair, and cell migration control. *CCND1* gene and its protein cyclin D1 are frequently altered by different molecular mechanisms, including amplification, chromosomal translocations, mutations, and activation of the pathways involved in cyclin D1 expression, alterations which appear to be essential in the development of human cancers, including oral carcinoma. This is the first published review of the specific features of cyclin D1 overexpression in oral oncogenesis. Starting with the physiological regulation of cyclin D1, there is an evaluation of its functions, overexpression mechanisms, and the implications of the oncogenic activation of *CCND1*/cyclin D1 in oral squamous cell carcinoma. The potential diagnostic and prognostic value of cyclin D1 is reviewed. The influence of *CCND1*/cyclin D1 on tumor size and clinical stage is reported, and an update is provided on the utilization of cyclin D1 as therapeutic target and on the combination of cyclin D1 inhibitors with cytotoxic agents. Future research lines in this field are also proposed.

Oral Diseases (2017) **23**, 897–912

Keywords: oral carcinogenesis; cyclin D1; CCND1; oral; cancer

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Received 8 September 2016; revised 7 November 2016; accepted 1 December 2016

Introduction

Cyclins are a family of proteins whose name derives from their cyclic expression and quantitatively fluctuating pattern throughout the cell cycle (Evans *et al*, 1983). Cyclin synthesis and activation of their corresponding cyclin-dependent kinases (CDKs) in the different phases of the cell cycle sequentially coordinate DNA replication and cell division (Sherr and Roberts, 2004; Malumbres and Barbacid, 2009). D-type cyclins (D1, D2, and D3; Motokura *et al*, 1991) and their catalytic partners CDK4 and CDK6 promote progression through the restriction point (R) during G1 phase, an event essential in G1/S transition (Sherr and Roberts, 2004; Malumbres and Barbacid, 2009; Figure 1). D-type cyclins strongly depend on extracellular mitogenic signaling, which regulates their transcription, translation, and protein stability as well as some of their functions. The lability of D-type cyclins causes their degradation in the absence of mitogens, translating into the incapacity of the cell to enter the S phase (Sherr and Roberts, 2004; Kim and Diehl, 2009). Conversely, the early onset of phase S can be prevented by members of inhibitor protein families INK4 and CIP/KIP (Sherr and Roberts, 1999, 2004; Malumbres and Barbacid, 2009). Among the three D-type cyclins, overexpression of cyclin D1 is the one most frequently related to human tumorigenesis and its amplification or overexpression can lead to some hallmarks of cancer, promoting uncontrolled proliferation (Hanahan and Weinberg, 2011). Cyclin D1 expression is likely essential for the development of malignancies such as melanoma, breast, lung, colon, and oral squamous cell carcinoma (OSCC; Santarius *et al*, 2010).

Physiological regulation of cyclin D1

Greater knowledge is available on the mitogen-dependent regulation of cyclin D1 than of cyclin D2 or D3. Unlike other cyclins periodically synthesized during cell cycle progression, D-type cyclins strongly depend on extracellular mitogenic signaling. Mitogen-induced signal

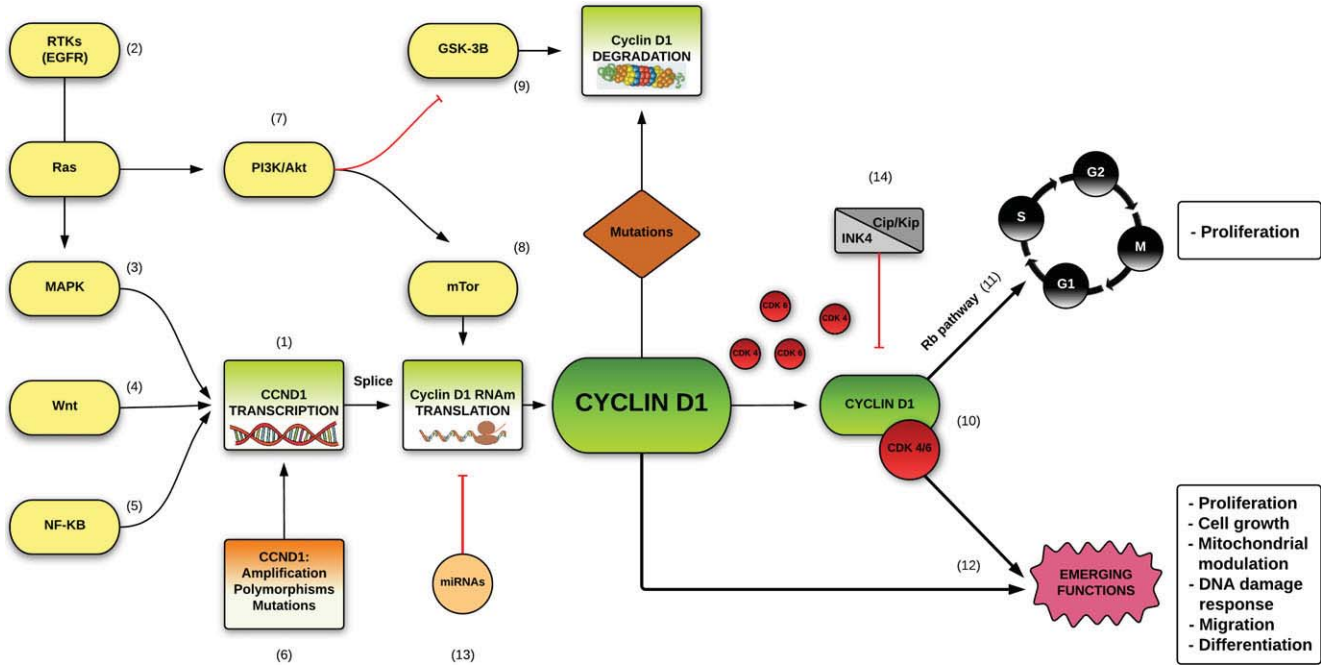


Figure 1 Simplified model for cyclin D1, including its physiological regulation and oncogenic involvement. The nuclear cyclin D1 rate is regulated by various mechanisms that can be altered, disturbing the physiological synthesis, degradation balance, and generating elevated cyclin D1 expression levels that may contribute to oral carcinogenesis. Mechanisms underlying the increased transcriptional activity of gene *CCND1* (1), with higher cyclin D1 expression levels, include the activation of tyrosine kinase receptors (TKRs) (2) such as prototypical epidermal growth factor receptor (EGFR) and of Raf-MEK-Erk (MAPK, mitogen-activated protein kinase) (3), Wnt/ β-catenin (4), and NF-$\kappa\beta$ (5) signaling pathways. *CCND1* amplification can also increase the transcriptional activity of cyclin D1, with oncogenic consequences (6). Other gene alterations such as polymorphisms and mutations (6) can also modulate cyclin D1 expression, giving rise to more aggressive isoforms (cyclin D1b) or affecting its nuclear export and proteolysis, respectively. Cyclin D1 levels can increase post-transcriptionally *via* activity of the PI3K-Akt pathway (7), which can act on cyclin D1 levels, stimulating mRNA translation *via* mTOR (8) or reducing cyclin D1 degradation *via* GSK-3β inhibition (9). In the nucleus, cyclin D1 forms complexes with CDK4/6 (10) and promotes cell proliferation *via* the retinoblastoma pathway (11), favoring G1/S transition in the cell cycle. More recently, it was reported that cyclin D1-CDK4/6 complexes and cyclin D1 cyclin-dependent kinases (CDK) independently promote multiple functions related to oral oncogenesis, including cell proliferation and growth, mitochondrial activity modulation, DNA damage repair, and cell migration or differentiation (12). It has also been observed that cyclin D1 expression levels may be reduced through the inhibitory activity of miRNAs (13) on the translation of cyclin D1 or through the inhibition of cyclin D1-CDK4/6 complexes by members of the INK4 (p16) and Cip/Kip (p21, p27, p57) families (14)

transduction pathways regulate cyclin D1 at multiple levels, increasing transcription, translation, stability of the protein, assembly with its CDK partners, and importing of cyclin D1–CDK complexes to the nucleus (Sherr and Roberts, 2004; Musgrove, 2006; Kim and Diehl, 2009). This regulation is mainly mediated by Ras and Ras-dependent signaling pathways (Musgrove, 2006; Figure 1). Mitogenic stimulation by activation of tyrosine kinase receptors (RTKs) on the cell membrane, for example, prototypical epidermal growth factor receptor (EGFR), initiates the sequential signaling cascade Ras-Raf-MEK-Erk [mitogen-activated protein kinase (MAPK) signaling pathway], which induces expression of cyclin D1 (Albanese *et al*, 1995). Cyclin D1 translation and stabilization are enhanced by the phosphatidylinositol 3-kinase (PI3K) signaling pathway (Vivanco and Sawyers, 2002), whose activation can also be Ras-mediated. Increased translation is promoted by the PI3K-Akt-mTor cascade (Muise-Helmericks *et al*, 1998), while the PI3K-Akt-GSK-3β cascade limits the degradation of cyclin D1 *via* glycogen synthase kinase 3 beta (GSK-3β) inhibition, enabling its stabilization and nuclear accumulation during G1 phase (Diehl *et al*, 1998). Functionally active GSK-3β, acting *via* cyclin D1 phosphorylation at Thr-286, regulates both its cell sublocalization, exporting it from the nucleus, and its proteasomal degradation in the cytoplasm (Diehl *et al*, 1998), where it is detected and ubiquitinated by the Skp1-Cul1-F box $SCF^{Fbx4/\alpha\beta\text{-crystalline}}$ E3 ligase and degraded by proteasome 26S (Lin *et al*, 2006). Akt can also inhibit Foxo transcription factors, capable of suppressing the expression of D-type cyclins (Schmidt *et al*, 2002), besides activating the member of the inhibitor protein family CDKs $p27^{kip1}$, among other target genes (Vivanco and Sawyers, 2002).

The molecular mechanisms reported here are the essential basis of cyclin D1 physiological regulation, but extracellular mitogen overexpression or the constitutive activation of its receptors, alongside the aberrant activation/inactivation of many of the aforementioned proteins, has been documented in OSCC. These oncogenic events culminate in the dysregulation of cyclin D1, which is overexpressed in a large proportion of these tumors (Goto *et al*, 2002; Wang *et al*, 2006b; Fang *et al*, 2011; Fu and Feng, 2015; Mishra *et al*, 2015).

Biological functions of cyclin D1

In first place, we highlight that there have been no reports on an association between specific functions of cyclin D1 and the development of oral cancer, although oncogenic

actions linked to activation of the retinoblastoma (Rb) pathway *via* the formation of cyclin D1–CDK4/6 complexes have been intensively studied in OSCC (Mori *et al*, 2005; Wang *et al*, 2006a,b; Woods *et al*, 2010; Caicedo-Granados *et al*, 2012; Ohnishi *et al*, 2014; Fu and Feng, 2015; Wang and Liu, 2015). These actions result in an aberrant increase in cell proliferation activity. New functions of cyclin D1 are emerging in *in vitro* studies of cell lines but have not yet been studied in OSCCs and warrant further research.

Functions dependent on Cyclin D1–CDK4/6 complexes

The best documented function of cyclin D1 is promotion of cell proliferation through the formation of complexes with CDK4/6. Studies of the 'retinoblastoma pathway' (Sherr and Roberts, 2004; Malumbres and Barbacid, 2009; Figure 1) show that the activation of cyclin D1–CDK4/6 complexes promotes protein Rb phosphorylation and functional inactivation, thereby inhibiting its suppression in the cell cycle and this promotes G1/S transition. Rb phosphorylation mediated by the cyclin D–CDK4/6 complex releases E2F transcription factors and promotes the activation of its target genes, changes essential for DNA synthesis. Conversely, the members of CDK-inhibitor protein families INK4 and CIP/KIP prevent the early onset of S phase (Sherr and Roberts, 1999). The INK4 family comprises INK4A (also known as p16 or CDKN2A), INK4B (p15 or CDKN2B), INK4C (p18 or CDKN2C), and INK4D (p19 or CDKN2D). The INK4 family specifically prevents CDK4 and CDK6 activation, generally by inhibiting association with D-type cyclins. The CIP/KIP family is formed by $p21^{cip1}$ (CDKN1A), $p27^{kip1}$ (CDKN1B), and $p57^{kip2}$ (CDKN1C). It binds to one of the cyclin-CDK complexes (including cyclin D–CDK4/6), although its main and most powerful inhibitory effect is exerted against cyclin E-CDK2 and cyclin A-CDK2 complexes (Sherr and Roberts, 1999, 2004; Malumbres and Barbacid, 2009). The restriction point (R), at the end of the G1 phase, determines the time at which cells become refractory to extracellular mitogen signaling and commit to autonomous regulation, remaining destined to replicate their DNA and continue in the cell cycle toward mitotic division (Sherr, 1996). This is of major importance in cancer, because oncogenic processes act on G1 phase progression regulators, so that cancer cells escape their control and tend to remain continuously in the cycle (Sherr, 1996). The levels of expression of these regulating proteins (Rb pathway) and of cyclin D1 are frequently altered in both OSCC and OSCC cell lines (Sartor *et al*, 1999; Woods *et al*, 2010). More recently, it was reported that cyclin D–CDK4/6 complexes, besides promoting cell cycle progression by modulating the function of Rb, can also phosphorylate substrates other than Rb on which different functions depend, not always in the cell cycle context. These CDK and Rb-independent functions include the following:

Regulation of proliferation. Cyclin D1–CDK complexes can regulate cell proliferation by interacting with various molecules in different tissues, including transcription factors such as small mothers against decapentaplegic 3 (Smad3), a protein that regulates the growth inhibitor response of transforming growth factor beta (TGF-β), whose *in vivo* CDK4-mediated phosphorylation at Thr 8, Thr 178, and Ser 212 inhibits its transcriptional activity and antiproliferative function. This event may contribute to tumorigenesis (Matsuura *et al*, 2004). Cyclin D1–CDK4/6 complexes also promote globin transcription factor (GATA) binding protein 4 (GATA4) degradation by *in vivo* phosphorylation at Ser 105, thereby inhibiting the differentiation of cardiomyocytes and regulating their proliferation (Nakajima *et al*, 2011). In addition, cyclin D1–CDK4 complexes phosphorylate runt-related transcription factor 2 (RUNX2) at Ser472 *in vitro* and induce its degradation, inhibiting the differentiation of chondrocytes and osteoblasts and stimulating their proliferation (Shen *et al*, 2006). It was recently reported that cyclin D1–CDK4 complexes interact with myocyte enhancer factor 2D (MEF2D), a pleiotropic transcription factor that, among other functions, targets the gene *CDKN1A*, increasing the levels of CDK-inhibiting protein $p21^{cip1}$. The *in vitro* phosphorylation of MEF2D at ser-98 and ser-110, mediated by cyclin D1–CDK4 complexes, promotes its degradation, favoring entry into S phase (Di Giorgio *et al*, 2015).

Regulation of cell growth. Cyclin D1–CDK4/6 complexes phosphorylate and inhibit tuberous sclerosis complex proteins 1 (TSC1) and 2 (TSC2), hampering the negative regulation by these proteins of mechanistic target of rapamycin (mTOR) substrates eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (S6K1), two important effectors of cell growth (Zacharek *et al*, 2005). Cyclin D1–CDK4 complexes also phosphorylate protein methylosome 50 (MEP50) at Thr5, a necessary and sufficient event to increase the intrinsic methyltransferase activity of protein arginine methyltransferase 5 (PRMT5), a protein which participates in various important cell processes (e.g., cell growth) and contributes to epigenetic regulation of cancer-associated target genes (Aggarwal *et al*, 2010).

Modulation of mitochondrial activity. The mitochondrial function coordination of cyclin D1 has been reported to be CDK4-dependent. Cyclin D1–CDK4 complexes phosphorylate nuclear respiratory factor 1 (NRF1) at Ser47 *in vivo*, repressing mitochondrial function and aerobic glycolysis and potentiating cytosolic glycolysis (Wang *et al*, 2006a). A change toward cytosolic glycolysis is known to occur during tumor progression (Wang *et al*, 2006a). Inhibited mitochondrial activity and enhanced cytosolic glycolysis have been reported in breast tumors induced by cyclin D1 (Pestell, 2013). It therefore appears that cyclin D1, through this function, can promote a mechanism that contributes to tumor maintenance.

DNA damage repair. Various publications have described the interaction of cyclin D1–CDK4 complexes with breast cancer 1 (BRCA1), a tumor suppressor protein critical for

genome stability that facilitates DNA damage repair. Cyclin D1–CDK4 complex-mediated phosphorylation of BRCA1 at Ser632 may inhibit DNA repair by this protein (Kehn *et al*, 2007) which may be important in breast and ovarian cancers, in which cyclin D1 is frequently overexpressed.

Cell migration. There have also been reports on the interaction between cyclin D1–CDK4/6 complexes and the protein filamin A, which promotes the phosphorylation of this protein at Ser2152 and Ser1459, participating in cell remodeling, reducing adhesion, and promoting cell migration (Zhong *et al*, 2010). This finding may explain the association between cyclin D1 overexpression and lymph node metastasis rate observed in OSCC. Although the relationship between cyclin D1 overexpression and filamin A has not been described in OSCC, it has been reported in esophageal cancer (Fan *et al*, 2013).

Sequestration of p21/p27. A well-documented function of cyclin D1 is the cyclin D–CDK4/6 complex-mediated sequestration of $p21^{cip1}$ and $p27^{kip1}$. In response to continuous mitogenic stimulation, cyclin D–CDK complexes act stoichiometrically to bind to and sequester these proteins, lowering their inhibitor threshold and indirectly favoring the activation of cyclin E-CDK2 complexes (Sherr and Roberts, 1999). This interaction with CIP/KIP proteins is essential for the coordination of CDK activity during cell cycle G1 phase and the regulation of G1/S transition. As with cyclin D1 overexpression, the low expression of proteins $p27^{kip1}$ and $p21^{cip1}$ is frequently observed in numerous human tumor types. A recent meta-analysis correlated low $p27^{kip1}$ expression with a poor prognosis in OSCC (Gao *et al*, 2013), whereas the prognostic significance of $p21^{cip1}$ expression is controversial, and contradictory results have been published on its overexpression and loss of expression (Kudo *et al*, 1999; Gonzalez-Moles *et al*, 2004). This important function of cyclin D1 represents an additional mechanism that may contribute to OSCC development.

CDK-independent functions

Cyclin D1 develops CDK-independent functions that may also be involved in oral carcinogenesis. Cyclin D1 has been reported to CDK independently participate in transcriptional regulation and gene expression (Coqueret, 2002; Pestell, 2013), likely by direct interaction with transcription factors, chromatin-modifying enzymes, or gene activation promoter regions, indirectly regulating cell proliferation, differentiation, and growth, among other processes (Coqueret, 2002; Pestell, 2013). Cyclin D1 interacts with numerous transcription factors, including nuclear receptors [e.g., peroxisome proliferator-activated receptor-γ (PPARγ), thyroid hormone receptor (TR), androgen receptor (AR), and estrogen receptor-α (ERα)], and also with its co-activators [steroid receptor activator (SRC1), general receptor for phosphoinositides 1 (GRIP-1), and amplified in breast 1 (AIB-1)]; other transcription factors with which cyclin D1 interacts are the proteins signal transducer and activator of transcription 3 (STAT3), v-myb, dentin matrix acidic phosphoprotein (DMP-1), and basic helix-loop-helix, BETA2/NeuroD. Cyclin D1 also binds to chromatin-modifying enzymes, including histone acetyltransferases (HATs) [E1A binding protein p300 (p300), CREB binding protein (CBP), and p300/CBP-associated factor (P/CAF)], histone deacetylases (HDACs), and histone methyltransferases (HMTs) [Su (Var) 39H1 (SUV39)] (Coqueret, 2002; Pestell, 2013). A recent *in vivo* study reported that cyclin D1 acts directly on the promoters of numerous genes during normal mouse development, suggesting the possibility that cyclin D1 may potentially be capable of regulating oncogenic activation (Bienvenu *et al*, 2010).

Cyclin D1 can CDK independently intervene in cell migration. It has been attributed with a central role in promoting the migration of epithelial cells by direct interaction with $p27^{kip1}$ (Li *et al*, 2006a). There is evidence that $p27^{kip1}$ participates in cell migration, besides inhibiting CDK activity. Although the mechanism that regulates this process is controversial, it may induce actin cytoskeleton rearrangement by ras homolog family member A (RhoA) inhibition or in a ras-related C3 botulinum toxin substrate (Rac)-dependent manner. Li *et al* (2006a) described three molecular mechanisms by which cyclin D1 could promote migration through interaction with $p27^{kip1}$. First, using siRNA for $p27^{kip1}$, they found that cyclin D1 requires the presence of this protein to induce cell migration; second, they found that the domains of cyclin D1 required to induce migration depend on binding to $p27^{kip1}$; and third, they demonstrated that cyclin D1 induces $p27^{kip1}$ abundance by phosphorylation at Ser10, which endows the protein with stability, and by repression of Skp2, one of the SCF E3 ligase complex components that degrades to $p27^{kip1}$. It has also been reported that cyclin D1 promotes cell migration by forming complexes with rho-associated coiled-coil containing protein kinase 2 (ROCKII), inhibiting Rho/ROCK signaling (Li *et al*, 2006b). There has been little research on the interaction between Rho family members and cyclin D1 in OSCC, but the study by Li *et al* (2006b) appears to confirm that cyclin D1 may promote cell migration *via* Rho/ROCK signaling.

The direct interaction of cyclin D1 with $p21^{cip1}$ contributes toward its emerging DNA repair function (Li *et al*, 2010). Cyclin D1 interacts with proteins that participate in DNA damage response (DDR), BRCA2-dependently binding to BRCA2 and RAD51 recombinase (RAD51) and facilitating the localization and stabilization of RAD51 at DNA damage sites, thereby assisting homologous recombination-mediated DNA repair (Jirawatnotai *et al*, 2012). Furthermore, the induction of DDR by cyclin D1 and the formation of RAD51 foci require $p21^{cip1}$ (Li *et al*, 2010).

Mechanisms of cyclin D1 overexpression in OSCC

The *amplification* of oncogene *CCND1* is one of the most frequent copy-number alterations in human cancer. A recent census evidenced its amplification and overexpression in OSCC (Santarius *et al*, 2010). The reported frequency of *CCND1* amplification in OSCC has ranged

widely between 9% and 72% (Rousseau *et al*, 2001; Goto *et al*, 2002; Miyamoto *et al*, 2003; Wong *et al*, 2003; Niméus *et al*, 2004; Myo *et al*, 2005; Freier *et al*, 2006; Liu *et al*, 2009; Kaminagakura *et al*, 2011; Mahdey *et al*, 2011; Hanken *et al*, 2014; van Kempen *et al*, 2015; Table 1). The wide variations in *CCND1* amplification rates may in fact be real and linked to geographic differences in etiologic factors for oral cancer, as in the case of some other genes. The initial mechanism underlying *CCND1* amplification is DNA breakage linked to fragile chromosomal sites largely associated with tobacco consumption. Thus, one of these sites, FRA11F, was found to be readily affected by tobacco and associated with the initiation of 11q13 amplification (Reshmi *et al*, 2007). In our view, another possible explanation of the variation in published *CCND1* amplification rates may be the varied sensitivities of the different techniques used for their measurement. A recent meta-analysis associated *CCND1* amplification with the clinicopathological prognosis, finding a strong correlation with lymph node metastasis (Noorlag *et al*, 2015). Chromosome band 11q13 is frequently amplified in OSCC and is a key event in tumor onset and progression. *CCND1* is a crucial oncogene of this chromosomal region and is often co-amplified along with other genes of the same region, and it has been proposed that they cooperatively contribute to OSCC pathogeny. *CTTN* gene (also known as EMS1) encodes a protein cortactin, which promotes cytoskeleton rearrangement. *CTTN* has been considered the most important gene of the 11q13 amplicon, alongside *CCND1*, given its biological and oncogenic functions and the frequently observed copy-number alterations (Schuuring, 1995). The frequent co-amplification of *CCND1* and *CTTN* has been observed *in vivo* and *in vitro* (Chen *et al*, 2004; Freier *et al*, 2006; Huang *et al*, 2006a; Liu *et al*, 2009). The co-amplification of *CCND1* and SH3 and multiple ankyrin (SHANK2) has also been reported (Freier *et al*, 2006). Freier *et al* (2006) proposed that *CTTN* and *SHANK2* genes, which encode cytoskeleton-associated proteins, may have a cooperative effect on tumor cell motility and invasiveness in OSCC, which might play a role in the strong association between cyclin D1 overexpression and lymph node metastasis. Other candidate genes located at 11q13 may also be co-amplified alongside *CCND1* and participate in OSCC pathogenesis, including fibroblast growth factors 3 (FGF3) and 4 (FGF4; Chen *et al*, 2004), protein phosphatase 1 catalytic subunit alpha (PPP1CA; Hsu *et al*, 2006), or oral cancer overexpressed 1 (ORAOV1, also known as TAOS1; Huang *et al*, 2002). In relation to the influence of HPV infection of cyclin D1 expression, published results suggest that HPV exerts oncogenic effects in OSCC that are not linked to *CCND1*/cyclin D1 overexpression. The Cancer Genome Atlas (Cancer Genome Atlas Network, 2015) and other studies (van Kempen *et al*, 2015) found that the amplification of 11q13, in which *CCND1* is localized, is an essential event in smoking-related HNSCC not associated with HPV.

Translocations that juxtapose *CCND1* with the immunoglobulin heavy chain (IGH) locus [t(11;14)(q13;q32)] are a genetic hallmark of mantle cell lymphoma (MCL) and Cyclin D1 overexpression *via* this mechanism is present in virtually all (>90%) cases of MCL (Bertoni *et al*, 2006).

Although various *polymorphisms* have been identified at *CCND1* locus, G/A870 polymorphism has received the greatest attention in OSCC and in oral potentially malignant disorders (Wong *et al*, 2003; Huang *et al*, 2006b; Knudsen *et al*, 2006; Sathyan *et al*, 2008; Murali *et al*, 2015). A870 allele encodes cyclin D1b, an alternatively spliced cyclin D1 isoform associated with increased cancer risk (Knudsen *et al*, 2006) and attributed with greater oncogenic potency in comparison with 'cyclin D1a' (full-length canonical cyclin D1 protein). Although the expression of cyclin D1b has not been studied in oral cancer, some authors (Holley *et al*, 2001; Sathyan *et al*, 2008) observed expression of transcript b of *CCND1* in OSCC, likely indicating that the more oncogenically active form of cyclin D1 (D1b) is also expressed in oral cancer. Wang *et al* (2014) addressed in a meta-analysis the relationship between G/A870 polymorphism and oral cancer. No global association was observed with higher oral cancer risk, but G/A870 polymorphism was related to a higher cancer risk in Asian populations. Further research is required to evaluate the true oncogenic implications of G/A870 polymorphism and cyclin D1b isoform in OSCC. A second polymorphism, in the 3′ untranslated region (3′UTR) of *CCND1* (C/G1722), has been studied in OSCC (Holley *et al*, 2001; Sathyan *et al*, 2008) and reported to modulate cyclin D1 expression in this disease (Sathyan *et al*, 2008).

Table 1 Cyclin D1 deregulation in oral cancer

Mechanism of deregulation	*Tumor type*	*Tumor site*	*Frequency*	*Refs*[a]
Amplification and overexpression				
CCND1 amplification	OSCC	Oral cavity	9–72%	Miyamoto *et al* (2003)
Cyclin D1 overexpression	OSCC	Oral cavity	32–75%	Mineta *et al* (2000)
Chromosomal rearrangement				
CCND1/IGH translocation t(11;14)(q13.3;q32)	MCL	Oral cavity	100%	Guggisberg and Jordan (2010)
CCND1/IGH translocation t(11;14)(q13.3;q32)	PBL	Oral cavity	2%	Boy *et al* (2011)
Splicing variants and transcript aberrations				
Cyclin D1b overexpression	OSCC	Oral cavity	No studies	
Mutations				
Cyclin D1 Thr-286[b]	OSCC	Oral cavity	No studies	

OSCC, oral squamous cell carcinoma; IGH, immunoglobulin heavy chain; MCL, mantle cell lymphoma; PBL, plasmablastic lymphoma.
[a]Key authors on cyclin D1 dysregulation mechanisms.
[b]Affecting nuclear export and proteolysis.

Mutations may also be involved in the aberrant overexpression of cyclin D1 in OSCC. Mori *et al* (2005) cultured tongue squamous cell carcinoma (TSCC) cell lines transfected with human mutated cyclin D1 and analyzed their response to the differentiation-inducing factor-1 (DIF-1)-mediated proliferation inhibition effect. They found that mutated cyclin D1 was highly resistant to degradation mediated by DIF-1-induced GSK-3B. However, there has been no research on cyclin D1 mutations or on their possible effects in oral cancer patients. In esophageal cancer, thr-286 mutation significantly increased the oncogenic potential of cyclin D1 through an alteration in the nuclear export and proteolysis of the protein, which was stabilized and constitutively remained in the nucleus (Benzeno *et al*, 2006).

Oncogenic activation of pathways involved in cyclin D1 expression is a plausible mechanism, because the overexpression of cyclin D1 is much more frequent than can be explained by CCDN1 amplification, polymorphisms, and mutational events. The oncogenic activation of different mitogen signaling pathway intermediaries, including RTKs, Ras-Raf-MEK-ERK, or PI3K, and of other important signaling pathways, such as Wnt or NF-$\kappa\beta$ (Guttridge *et al*, 1999; Tetsu and McCormick, 1999; Caicedo-Granados *et al*, 2012; González-moles *et al*, 2014), may explain the abundance of cyclin D1 in OSCC. These pathways can increase the transcriptional activity of *CCND1* and also have post-transcriptional effects on protein translation (*via* PI3K-mTor) and protein stabilization (*via* PI3K-GSK-3β).

MicroRNAs target can be *CCND1* or other genes that encode *CCND1* transcriptional activity regulating proteins. This has been little studied in OSCC (Jia *et al*, 2013, 2015; Chi, 2015; Wang and Liu, 2015). Chi (2015) reported a markedly downregulation of miR-194 expression in OSCC and OSCC cell lines, finding that it could suppress the PI3K/Akt pathway by direct acyl glycerol kinase (AGK) inhibition, downregulating cyclin D1 expression and reducing cell proliferation. Jia *et al* (2015) found that low miR-375 expression in TSCC and TSCC cell lines was associated with a poor prognosis. They also demonstrated that its overexpression inhibited cell proliferation *via* sp1 transcription factor (Sp1) inhibition, resulting in cyclin D1 downregulation. In a previous investigation (Jia *et al*, 2013), the same group had observed that reduced miR195 expression in TSCC was associated with a poor prognosis and that its overexpression reduced cyclin D1 and Bcl-2 expression in TSCC lines, with cell cycle inhibition and apoptosis promotion. A more recent study by Wang and Liu (2015) described miR-188 downregulation in OSCC. They also demonstrated *in vitro* that miR-188 transfection in the Detroit 562 cell line suppressed cell proliferation and cell cycle progression *via* cyclin D1 downregulation mediated by inhibition of the oncoprotein homeobox protein SIX1 (SIX1), whose gene was a direct target of the transfected miRNA.

Diagnostic and prognostic implications of cyclin D1 in oral cancer

As noted above, translocations that juxtapose *CCND1* with the *IGH* locus [t(11;14)(q13;q32)] are a genetic hallmark of MCL, and cyclin D1 overexpression via this mechanism is present in virtually all (>90%) cases of MCL (Bertoni *et al*, 2006). To date, ten cases of oral cavity MCL have been reported (Rajkumar *et al*, 2015), less than half with a cytogenetic analysis, which always confirmed translocation (Guggisberg and Jordan, 2010; Kyo *et al*, 2010; Scheller *et al*, 2011; Table 1). In the largest cohort of oral cavity, plasmablastic lymphoma (PBL) patients published to date Boy *et al* (2011) evaluated t(11;14)(q13;q32) and obtained a positive t(11;14)(q13;q32) result in only 1 of 41 cases (2.4%); they observed an increased number of *CCND1* copies in 17 of these cases (41.4%), of which 7 (17%) were amplifications. Hence, this overexpression mechanism does not appear to be as relevant in oral cavity PBL as in MCL.

Numerous publications have investigated the association between cyclin D1 expression and clinicopathological and prognostic factors in OSCC. Cyclin D1 overexpression has frequently been correlated with parameters that imply a poor prognosis, including lymph node involvement, larger tumor size, advanced clinical stage, poor differentiation, reduced survival, and lack of response to treatment.

Tumor size and clinical stage

Higher levels of cyclin D1 expression have been found in OSCCs of larger size (Carlos de Vicente *et al*, 2002; Das *et al*, 2011; Gupta *et al*, 2014; Guimarães *et al*, 2015). In their meta-analysis, Zhao *et al* (Zhao *et al*, 2014) reported a significant correlation between cyclin D1 overexpression and higher T stage. Lymph node metastasis and cyclin D1 overexpression have also been related to tumor thickness or invasion depth (Wang *et al*, 2006b). However, the oncogenic role of cyclin D1 is not limited to advanced tumor stages. Many authors found this protein to be overexpressed in potentially malignant disorders (Castle *et al*, 1999; Sartor *et al*, 1999; Rousseau *et al*, 2001; Huang *et al*, 2006b; Tsui *et al*, 2009; Ramakrishna *et al*, 2013; Mishra *et al*, 2015), with a tendency for higher cyclin D1 expression with each stage of oral carcinogenesis from oral dysplasia to locally advanced OSCC (Carlos de Vicente *et al*, 2002; Das *et al*, 2011; Gupta *et al*, 2014; Guimarães *et al*, 2015). Indeed, cyclin D1 overexpression was positively correlated with more advanced clinical stages of OSCC (Huang *et al*, 2012; Gupta *et al*, 2014), as corroborated by the meta-analysis of Zhao *et al* (Zhao *et al*, 2014), consistent with the correlation of this overexpression with larger tumor size and lymph node invasion.

Lymph node metastasis. A key focus for researchers has been the role of cyclin D1 in loco-regional lymph node metastasis. Many authors found a significant correlation between cyclin D1 overexpression or gene amplification and N stage (Mineta *et al*, 2000; Carlos de Vicente *et al*, 2002; Myo *et al*, 2005; Wang *et al*, 2006b; Das *et al*, 2011; Mahdey *et al*, 2011; Huang *et al*, 2012; Gupta *et al*, 2014; van Kempen *et al*, 2015), and this association has been corroborated by two meta-analyses (Zhao *et al*, 2014; Noorlag *et al*, 2015). Extracapsular spread, one of the worst prognostic features in oral cancer, has been positively correlated with *CCND1* copy-number aberrations in primary tumors ($P = 0.011$; Michikawa *et al*, 2011). Fluorescence *in situ* hybridization (FISH) in specimens

obtained by fine needle aspiration in patients with stage I and II OSCC found that presence of cyclin D1 gene numerical aberrations (*CCND1* amplification/chromosome 11 polysomy) was strongly correlated with lymph node metastasis and a more diffuse invasion pattern (Myo *et al*, 2005). Positive correlations between *CCND1* amplification and a diffuse tumor invasion pattern have been reported in OSCC (Miyamoto *et al*, 2003). These observations suggest that the more frequent lymph node involvement in OSCCs with cyclin D1 overexpression/amplification may be related to the most unfavorable invasion behaviors of these carcinomas.

Differentiation grade

The association of cyclin D1 overexpression with tumor differentiation grade is controversial. A positive association has been reported between cyclin D1 overexpression and worse histological grade (Angadi and Krishnapillai, 2007; Mishra and Das, 2009; Zhao *et al*, 2014), but some studies grouped moderately and poorly differentiated carcinomas together (Lam *et al*, 2000; Miyamoto *et al*, 2003; Huang *et al*, 2012), a potential confounding factor. Thus, Perisanidis *et al* (2012) and Das *et al* (2011) found an association between cyclin D1 overexpression and moderately differentiated carcinomas. However, Saawarn *et al* (2012) observed an elevated cyclin D1 overexpression in well-differentiated tumors, apparently inconsistent with the cell proliferation promoter function of cyclin D1 but possibly by other functions with which it has recently been attributed, especially in regard to the regulation of cell differentiation. In this context, Woods *et al* (2010) demonstrated that cyclin D1 increased the proliferation of oral keratinocyte cell lines but could not block their differentiation. This suggests that cyclin D1 may be capable of directly regulating the transcription of genes involved in oral keratinocyte cell differentiation, perhaps CDK independently. This proposition is supported by the finding (Ohnishi *et al*, 2014) that cyclin D1 participates not only in cell proliferation but also in cell differentiation and prevention of cell death in OSCC. Immunoexpression of cyclin D1 and ki-67 analyzed by double immunostaining showed that both proteins were simultaneously co-expressed in the basal and suprabasal layers of oral epithelial cells (Ohnishi *et al*, 2014); however, they detected numerous cyclin D1-positive and ki-67-negative cells in the center of tumor nests, occupied by differentiated cells that expressed keratin. Further studies are required to evaluate whether cyclin D1 directly participates in oral keratinocyte differentiation and whether it can modulate OSCC histological differentiation toward a less aggressive grade with a better prognosis.

Overall survival and disease-free survival

Numerous studies on OSCC have found positive correlations between cyclin D1 overexpression and poor patient survival (Mineta *et al*, 2000; Goto *et al*, 2002; Feng *et al*, 2011; Kaminagakura *et al*, 2011; Huang *et al*, 2012; Zhong *et al*, 2013; Hanken *et al*, 2014), and *CCND1* gene amplification and cyclin D1 overexpression has been significantly associated with worse overall survival (OS) in oral and head and neck cancer patients (Miyamoto *et al*, 2003; Feng *et al*, 2011; Mahdey *et al*, 2011; Hanken *et al*, 2014). A positive association has also been reported between cyclin D1 overexpression in OSCC and reduced disease-free survival (DFS; Sathyan *et al*, 2008; Feng *et al*, 2011; Kaminagakura *et al*, 2011; Huang *et al*, 2012; Zhong *et al*, 2013), although this was not supported in a recent meta-analysis (Zhao *et al*, 2014). A statistically significant positive association has also been observed between *CCND1* gene amplification and worse DFS in OSCC patients (Miyamoto *et al*, 2003; van Kempen *et al*, 2015). Lin *et al* (2012) found no relationship between A870G polymorphism and cancer development in a Taiwanese population with OSCC; but found the *CCND1* + 870G allele to be an independent risk factor for a poor DFS. In their multivariate analysis, Sathyan *et al* (2008) proposed that A870G 'GG' and C1722G 'CC' genotypes were predictors of a poor DFS, and found that the combination of genotype A870G and cyclin D1 expression was an independent predictor of survival.

Other associations of cyclin D1 overexpression with possible prognostic implications

Studies on the role of cyclin D1 in head and neck oncogenesis found marked differences in protein and gene expression as a function of the tumor localization (Takes *et al*, 1998; Niméus *et al*, 2004; Hanken *et al*, 2014). A higher tendency to cyclin D1 expression alterations was found in pharyngeal, laryngeal, and oropharyngeal tumors, with *CCND1* gene amplification being found in 63% of pharyngeal carcinomas (Hanken *et al*, 2014). Gene amplification and protein overexpression are significantly more frequent in these aforementioned sites than in the oral cavity (Takes *et al*, 1998; Niméus *et al*, 2004; Hanken *et al*, 2014). Differences in cyclin D1 expression rate have also been found among distinct intra-oral tumor localizations, and significantly higher *CCND1* gene overexpression (Huang *et al*, 2012) and amplification (Mahdey *et al*, 2011) were found in cancer of the tongue *versus* other localizations, including the buccal mucosa (Trivedi *et al*, 2011).

Various authors have analyzed cyclin D1 expression levels in OSCC patients as a function of age, but with contradictory results. Kaminagakura *et al* (2011) found significantly higher cyclin D1 overexpression and *CCND1* amplification in OSCC patients aged 40 years or younger than in those aged 50 years or older. In contrast, Das *et al* (2011) reported significantly higher cyclin D1 expression levels in patients with tobacco-related OSCC over the age of 50 years in comparison with younger patients. However, Castle *et al* (1999) observed no age-related differences in cyclin D1 expression.

The possible relationship between cyclin D1 expression levels and tobacco habits has been investigated in OSCC patients, especially in South-East Asia, although many studies of highly heterogeneous populations with varied habits have been performed worldwide. In general, cyclin D1 overexpression is often correlated with tobacco consumption, suggesting that cyclin D1 alteration plays a major role as a tobacco-related oncogenic molecular mechanism (Xu *et al*, 1998; Wong *et al*, 2003; Liu *et al*, 2009; Mishra and Das, 2009; Das *et al*, 2011; Basnaker *et al*,

2014). Most studies have found cyclin D1 overexpression in 31–74% of tumors from consumers of both smoked and smoke-free tobacco (Xu *et al*, 1998; Mishra and Das, 2009; Das *et al*, 2011; Basnaker *et al*, 2014), indicating that the effect on cyclin D1 expression does not vary according to the type of tobacco use (Xu *et al*, 1998; Basnaker *et al*, 2014) or geographic setting (Xu *et al*, 1998; Mishra and Das, 2009; Das *et al*, 2011; Basnaker *et al*, 2014). Some studies found cyclin D1 overexpression in 80% of oral mucosal samples with a healthy clinical appearance, in smokers *versus* controls, and epithelial dysplasia was observed in 65% of these mucosas (Basnaker *et al*, 2014). Thus, cyclin D1 likely has an early influence on oral carcinogenesis and may play a role in field cancerization (Basnaker *et al*, 2014). Cyclin D1 overexpression has also been related to higher *cyclin D1* mRNA levels protein in the OSCC than in the healthy oral mucosa of patients who smoked or chewed tobacco (Mishra and Das, 2009), and *CCND1* gene amplification was found in 22% (Wong *et al*, 2003) and 50% (Liu *et al*, 2009) of tumors. Gene amplification was detected in 28% of clinically healthy oral mucosa from smokers (Liu *et al*, 2009), supporting the proposition that cyclin D1 is involved in oral carcinogenesis at an early stage. However, the association between G870A polymorphism and a smoking habit has not been demonstrated (Wong *et al*, 2003). With regard to alcohol however, no relationship has been found between its consumption and cyclin D1 disorders at any level (protein, mRNA, or *CCND1* gene; Mineta *et al*, 2000; Huang *et al*, 2012; Guimarães *et al*, 2015).

Given the important prognostic role of cyclin D1 in OSCC, application of immunohistochemistry can be recommended as a routine, simple, and inexpensive technique that faithfully reflects the oncogenic involvement of cyclin D1, regardless of the molecular mechanisms underlying its upregulation.

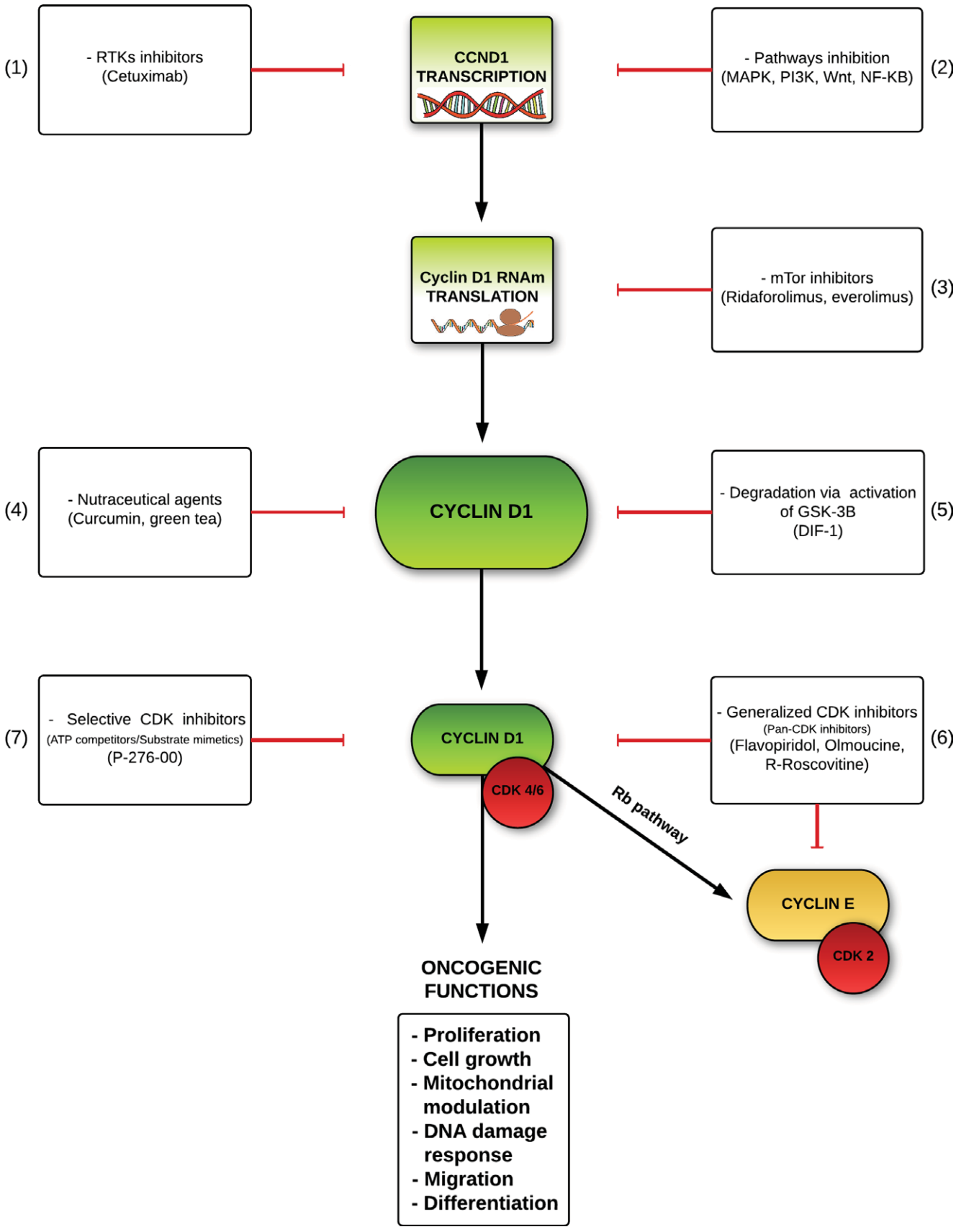
Cyclin D1 as a therapeutic target in OSCC

Evidence on the high incidence of aberrant cyclin D1 expression in numerous human cancers, including OSCC, and on the susceptibility of cancer cells to cyclin and CDK inhibition has increased the interest in therapeutic implications. Cyclin D1 appears to play a key role in oral carcinogenesis and may therefore be potentially useful as a therapeutic target, in accordance with the concept of oncogenic addiction proposed by Weinstein and Joe (2006). This term is used to describe the appreciable and frequent dependence of cancer cells on certain oncoproteins/oncogenes, which allow them to maintain constant proliferation and to survive. The primary value of this concept is that the deactivation of an isolated oncoprotein can be sufficient to block the proliferation and survival of cancer cells, even in cases of multiple summative oncogenic events. The high frequency of alterations in cyclin D1 expression in oral cancer (Table 1) and the different mechanisms that can trigger its expression suggest that intervention against aberrant expression levels may be of value. The susceptibility of cancer cells to the inhibition of cyclin D1 and of CDK activity has increased interest in its therapeutic potential (Musgrove *et al*, 2011). Thus, it is well documented that inhibition of cyclin D1 of its CDK partners or of its main overexpression mechanisms inhibits cell proliferation in OSCC (Masuda *et al*, 2001; Mukhopadhyay *et al*, 2002; Mori *et al*, 2005; Lim *et al*, 2006; Zhou *et al*, 2009; Fang *et al*, 2011; Kaminagakura *et al*, 2011; Jia *et al*, 2013, 2015; Mishra *et al*, 2013; Xiao *et al*, 2014; Chi, 2015; Fu and Feng, 2015; Wang and Liu, 2015).

Possible therapeutic approaches to cyclin D1 include action on CDKs, direct action on cyclin D1, action on cyclin D1-mediated pathways involved in OSCC development, or the administration of drugs that act on cyclin D1 in combination with other antitumor drugs (Figure 2). The effectiveness of therapy targeting cyclin D1 or CDK4/6 may be greater in patients selected according to the results of genome and/or proteome studies and thus having more targeted therapy for OSCC.

Generalized therapeutic CDK inhibition (Musgrove *et al*, 2011) by pan-CDK inhibitors, including flavopiridol, olomoucine, or R-roscovitine, lacks specificity and demonstrates only modest therapeutic effectiveness, inhibiting CDK1/2/3 and frequently also CDK7/9, which have transcriptional functions besides those related to cell cycle control. These pan inhibitors also act against CDK10/11, which has antiproliferative functions (Malumbres and Barbacid, 2009; Musgrove *et al*, 2011). A second generation of CDK inhibitors has been developed, such as ATP competitors P1446-05 or PD0332991, which block the kinase site and selectively inhibit CDK4/6 with greater potency (IC_{50} of ~10 nM or less). After demonstrating evident antitumor activity, these drugs are currently undergoing promising clinical trials (Musgrove *et al*, 2011). Another way to selectively inhibit CDK activity would be to design peptides capable of targeting cyclin D1–CDK4/6 complexes by mimicking their substrate (Musgrove *et al*, 2011). However, it is not known whether such selective inhibition exerts a greater antitumor effect in comparison with the generalized blocking of CDK activity. Various clinical trials on these inhibitors are in progress, but they have not yet been studied in OSCC: Data have been published on the antitumoral activity of flavone P276-00 in

Figure 2 Cyclin D1 as therapeutic target in oral cancer. There are various ways in which cyclin D1 could be a therapeutic target, affecting the different steps involved in its overexpression. Thus, it would be possible to target tyrosine kinase receptors, mainly epidermal growth factor receptor (EGFR), using cetuximab or other drugs (1), and to inhibit pathways involved in *CCND1* transcriptional activation (2), such as the Wnt/canonical, NF-$\kappa\beta$, MAPK, and PI3K pathways. This last pathway is associated with mTor, on which clinical trials are under way using ridaforolimus and everolimus, among others; the targeting of mTor may also modulate *CCND1* mRNA translation (3). Direct cyclin D1 inhibition may be achieved with nutraceutical agents such as curcumin or green tea (4), which enhance its degradation and reduce its biosynthesis, or by stimulating cyclin D1 degradation through the potentiation of GSK-3β with differentiation-inducing factor-1 (DIF-1) (5). Generalized (6) cyclin D1-cyclin-dependent kinases (CDK) complex inhibition has been attempted with Pan-CDK inhibitors, such as flavopiridol, olomoucine, or R-roscovitine. A further advantage of Pan-CDK inhibitors is that they inhibit other complexes involved in the Rb pathway, such as cyclin E-CDK2 (6). In oral cancer, the selective inhibition of cyclin D1-CDK4/6 complexes has been tested using the ATP-competitive drug P276-00 (7)


(1)
- RTKs inhibitors (Cetuximab)
CCND1 TRANSCRIPTION
- Pathways inhibition (MAPK, PI3K, Wnt, NF-KB)
(2)
Cyclin D1 RNAm TRANSLATION
- mTor inhibitors (Ridaforolimus, everolimus)
(3)
(4)
- Nutraceutical agents (Curcumin, green tea)
CYCLIN D1
- Degradation via activation of GSK-3B (DIF-1)
(5)
(7)
- Selective CDK inhibitors (ATP competitors/Substrate mimetics) (P-276-00)
CYCLIN D1
CDK 4/6
- Generalized CDK inhibitors (Pan-CDK inhibitors) (Flavopiridol, Olmoucine, R-Roscovitine)
(6)
Rb pathway
CYCLIN E
CDK 2
ONCOGENIC FUNCTIONS
- Proliferation
- Cell growth
- Mitochondrial modulation
- DNA damage response
- Migration
- Differentiation

in vivo and *in vitro* tumor models of HNSCC (Mishra *et al*, 2013). These indicate that this flavone can selectively inhibit cyclin D1–CDK4 complex activity in an ATP-competitive manner, demonstrating a 40-fold higher selectivity for cyclin D1–CDK4 than for cyclin E-CDK2 complexes (Joshi *et al*, 2007). This potent inhibitor is currently being evaluated in HNSCC patients in stage I/II clinical trials (NTC00824343, NCT00899054) and it appears to be a promising chemotherapeutic agent.

Directly targeting cyclin D1 may be a challenging task, given its intracellular localization and lack of enzymatic activity. However, increasing evidence of the CDK-independent functions of cyclin D1, for example, cell migration involvement, suggests that it may be a potentially viable therapeutic option in OSCC. There have been reports on the possible usefulness of various nutraceutical agents, notably green tea and curcumin, whose anticancer potential appears to be based on cyclin D1 inhibition. Green tea was reported to exert action against HNSCC, which was attributed to its components epigallocatechin-3-gallate (Masuda *et al*, 2001) and epicatechin gallate (Lim *et al*, 2006), but scientific evidence on the effects of these substances remains inadequate. In a randomized phase II clinical trial of green tea extract in patients with high-risk potentially malignant oral disorders, reduced cyclin D1 expression levels were found in patients with a better clinical response to treatment (Tsao *et al*, 2009). Curcumin, a phytochemical derived from the *Curcuma longa* rhizome, was found to inhibit proliferation of SCC-9 OSCC cells *via* cyclin D1 downregulation and to negatively act on various mechanisms that contribute to its overexpression, including GSK-3β upregulation or inhibition of the Wnt/β-catenin molecular signaling pathway (Xiao *et al*, 2014). Mukhopadhyay *et al* (2002) observed that curcumin inhibits the proliferation of various human cancer cell lines (e.g., HNSCC JMAR and TU167), attributing the marked downregulation of cyclin D1 to curcumin-induced protein degradation by proteasomes and to suppression of biosynthesis, with reduced *cyclin D1* mRNA expression levels. Curcumin therefore appears to be an inhibitor of cyclin D1 expression inhibitor in OSCC and is undergoing a clinical trial in HNSCC patients (NCT01160302). Another viable strategy for direct cyclin D1 inhibition may be based on its degradation by GSK-3β. Thus, it has been confirmed that DIF-1 (protein of the differentiation-inducing factor [DIF] family, identified in Dictyostelium discoideum, capable of inhibiting cell proliferation in mammal cells) induced cyclin D1 degradation in TSCC cell lines by promoting GSK-3β-mediated phosphorylation of Thr-286 (Mori *et al*, 2005). DIF family members with demonstrated capacity to inhibit cell proliferation and induce differentiation may be useful in the design of a novel therapeutic approach, but no clinical trials appear currently to be underway in OSCC patients.

Targeting the different pathways in OSCC may also be an effective way to indirectly deal with aberrant cyclin D1 levels. Increasing attention is being paid to mTOR as an attractive therapeutic target in OSCC (Simpson *et al*, 2015). Therapeutic targeting of cyclin D1 by mTOR inhibitors has appeal (Musgrove *et al*, 2011) given that *CCND1* mRNA translation is mTOR-dependent. In a recent phase I clinical trial, the mTOR inhibitor ridaforolimus was combined with Notch MK-0752 inhibitor to achieve a strong blockade of the PI3K molecular signaling pathway in patients with advanced solid tumors; the best response to treatment being in HNSCCs (Piha-Paul *et al*, 2015). The administration of everolimus, another mTOR inhibitor, has also shown good results in a phase I clinical trial in HNSCC patients (Saba *et al*, 2014). Various phase II clinical on mTOR-targeting drugs (NCT01195922, NCT01313390, NCT01009203, NCT01172769, NCT01016769), trials are under way in OSCC and HNSCC patients). Targeting the pathways involved in OSCC development may be of special interest when components of these pathways and the protein are both altered in patients. We highlight the inhibition of PI3K and MAPK pathways in this context. Blockade of mTOR or dysregulation of the Akt-FOXO3a signaling axis (Fang *et al*, 2011) could reduce cyclin D1 expression levels in OSCC *via* PI3K (Simpson *et al*, 2015). The upregulation of RNA-binding protein quaking 5 (QKI-5) represents another possible therapeutic approach, because it has recently been reported to act as *in vitro* tumor suppressor in the CAL-27 OSCC cell line, inhibiting cyclin D1 expression *via* MAPK (Fu and Feng, 2015). Even more indirectly, RTKs are also possible therapeutic targets that could theoretically change aberrant cyclin D1 expression levels by inhibiting the different pathways that produce its overexpression. This is an interesting proposal, given the well-known participation of EGFR in oral cancer. The EGFR inhibitors cetuximab (NCT00003809), afatinib (NCT01427478), and lapatinib (NCT00424255), have demonstrated effectiveness in various clinical trials and are undergoing phase III trials.

Influence of cyclin D1/CCND1 overexpression on the response to treatment

The effect of cyclin D1 overexpression or *CCND1* gene amplification on the response to radiotherapy and/or chemotherapy has also been widely studied (Henriksson *et al*, 2006; Zhang *et al*, 2006; Zhou *et al*, 2009; Feng *et al*, 2011; Zhong *et al*, 2013; Gupta *et al*, 2014). Most studies analyzed the response to cisplatin, but there were contradictory results. Molecular analyses demonstrated that *CCND1* gene amplification confers significant resistance to cisplatin (Henriksson *et al*, 2006), as also observed in the cisplatin-resistant cell line (Tca/cisplatin) obtained from a TSCC line (Tca8113), which showed marked *CCND1* gene upregulation (Zhang *et al*, 2006). The same research group demonstrated that gene silencing by transfection of various cyclin D1 short hairpin RNAs (shRNAs), inhibiting cyclin D1 expression, enhances the sensitivity of tumors to cisplatin, increasing apoptosis and reducing NF-$\kappa\beta$-mediated oncogenic activity (Zhou *et al*, 2009). In a more recent study by Gupta *et al* (2014) of patients with locally advanced OSCC treated with cisplatin, paclitaxel, and radiotherapy, those with higher cyclin D1 expression had a worse prognosis and response to treatment. A clinical trial in patients with locally advanced HNSCC (stage III/IV), mainly OSCC (89.7% of cases), found that patients with tumors that overexpressed cyclin D1 had a poor response to cisplatin chemotherapy

and a worse survival and shorter disease-free period (DFP; Feng *et al*, 2011). The authors proposed cyclin D1 status as a marker of response to treatment. However, discrepant results were observed (Zhong *et al*, 2013) in a randomized prospective phase III trial on the prognostic and predictive value of cyclin D1 in pretreatment biopsy specimens from patients with locally advanced OSCC who underwent surgery and postoperative radiotherapy, preceded or not by induction with docetaxel, cisplatin, and 5-fluorouracil (TPF). Low cyclin D1 expression significantly predicted a worse prognosis in this trial, but cyclin D1 levels did not predict the response to treatment. In fact, significantly higher OS and metastasis-free survival were found in TPF-treated N2 patients whose tumors had a high cyclin D1 expression. Only one study analyzed the influence of cyclin D1 overexpression on the effectiveness of chemotherapy with drugs other than cisplatin (mitomycin C, 5-fluorouracil + radiotherapy, and radical surgery) and found no differences in response among patients with different cyclin D1 expression levels (Perisanidis *et al*, 2012). Fewer data are available on the response to radiotherapy. Shintani *et al* (2001) evaluated the relationship between cyclin D1 expression and radiosensitivity in OSCC cell lines and in OSCC patients undergoing preoperative radiotherapy and found that OSCCs with elevated cyclin D1 expression were significantly more sensitive to radiotherapy than those with reduced expression. This may be explained by the greater sensitivity to radiotherapy of tumors with a higher proliferation rate.

Musgrove *et al* (2011) reported that the application of a single therapy to block cyclin D1 or D1–CDK has achieved worse outcomes in comparison with this therapy in combination with other cytotoxic drugs, for example, cyclin D1 inhibitors in synergy with cytotoxic drugs such as cisplatin, fluorouracil, doxorubicin or paclitaxel. This synergic effect appears to be more effective when the cytotoxic agent is administered before cyclin D1/CDK inhibition. According to Musgrove *et al* (2011), this may be because CDK activity inhibitors are more effective when cells are synchronized or detained in specific cell cycle phases. The same authors also suggest that the effective dose of these inhibitors should be maintained for a longer time period in future trials, thereby allowing target cancer cells to enter a cell cycle phase that is more susceptible to cytotoxic agents. Another possible therapeutic target in combination with cyclin D1 is focused on action on tyrosine kinase receptors, of which EGFR is the most widely studied. Treatment based on the targeting of both oncoproteins is of particular interest, given the frequent co-amplification of the genes that encode cyclin D1 and EGFR in OSCC and the frequent co-expression of their products (Tsui *et al*, 2009; Michikawa *et al*, 2011), suggesting that positive outcomes may be obtained through the dual modulation of the two oncoproteins. The EGFR inhibitors cetuximab (NCT00003809), afatinib (NCT01427478), and lapatinib (NCT00424255), have demonstrated effectiveness in various clinical trials and are undergoing phase III trials. Cetuximab is currently the only targeted therapeutic agent approved by the FDA for the treatment of patients with head and neck cancer. *In vitro* studies and a phase I clinical trial in aerodigestive tract cancers, including HNSCCs (17% of patients), showed that the combination of rexinoid bexarotene and the EGFR inhibitor erlotinib enhanced the suppression of tumor growth by cooperative repression of cyclin D1 (Dragnev *et al*, 2005). This combined therapy is considered especially promising in lung cancer, for which erlotinib has become part of the standard treatment.

Reflections and future research lines

This review evidences the role in oral carcinogenesis of aberrant cyclin D1 expression, which behaved as marker of a worse prognosis in numerous studies. Less attention has been paid to the role of D-type cyclins in oral carcinogenesis. Researchers in cancer molecular biology have focused on cyclin D1, whereas cyclins D2 and 3 appear largely to have been ignored. We also highlight the lack of data in OSCC on the cyclin 'D1b' isoform, which has been attributed with even more relevant oncogenic characteristics than does cyclin D1 itself (full-length canonical cyclin D1 protein). Following this review, a first conclusion would be the need to evaluate the importance of the expression of cyclin D1b and other D-type cyclins in order to increase knowledge on their oncogenic potential in OSCC.

Cyclin D1 appears to behave as a central element in the bio-pathology of OSCC development. Cyclin D1 activation results from multiple oncogenic mechanisms that are not exclusive to any single pathway explaining the large number of OSCC cases in which its expression is dysregulated. Given the position of Cyclin D1 in the cell cycle, it is one of the last key proteins to trigger uncontrolled cell proliferation, causing genome instability increasing the risk of acquiring summative oncogenic events. Given the importance of cyclin D1, the question arises as to the best method for analyzing alterations in its expression in order to verify its role in carcinogenesis and its involvement in oral cancer development in specific patients. Cyclin D1 upregulation is attributable to various upstream oncogenic events of varied importance, including: CCND1 gene amplification, polymorphisms and mutations; aberrant activation of pathways related to physiological mitogenic signaling (PI3K, MAPK); activation of pathway Wnt/canonical or NF-$\kappa\beta$ pathways; oncogenic alterations of Rb pathway proteins; and miRNA activity. These all result in cyclin D1 overexpression in the cell nucleus, where it exerts oncogenic action. One robust method for its analysis is the immunohistochemical study of cyclin D1 overexpression in the cell nucleus in comparison with its expression in healthy tissue (Figure 3). Immunohistochemical analysis not only quantifies the number and percentage of cells expressing this protein but also reveals the topographic involvement of cyclin D1 in premalignant epithelium and tumor tissue, which could yield important diagnostic and prognostic information. This proposition is supported by the varied frequency among different tumors or OSCC localizations of the activation of oncogenic mechanisms that upregulate cyclin D1. Thus, various authors (Mahdey *et al*, 2011; Trivedi *et al*, 2011; Huang *et al*, 2012) have reported that upstream cyclin D1 upregulating mechanisms (amplification, ...) are more

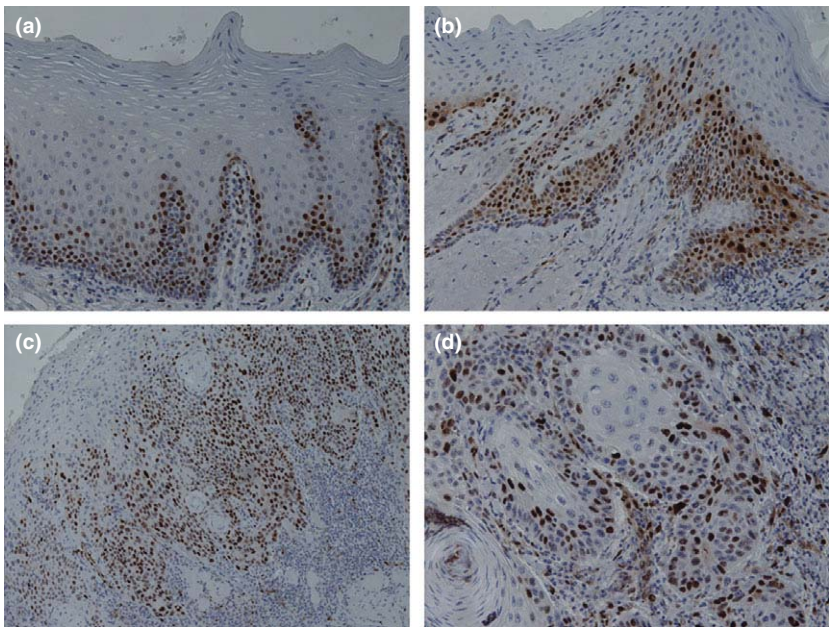


Figure 3 Immunohistochemical expression of cyclin D1 in malignant and premalignant epithelia. Immunohistochemistry yields information on the differential topography of cyclin D1 expression at both intracellular and epithelial level. The images show: (**a**) High expression of cyclin D1 in parabasal layer of hyperplastic oral epithelium (40×); (**b**) marked nuclear expression in basal and parabasal layers of a dysplastic oral epithelium (40×) alongside an elevated cytoplasmic expression whose significance has not yet been fully elucidated, although it may reflect the amplification of gene *CCND1* with intense overexpression of cyclin D1, which may not only translocate to the nucleus but also accumulate in the cytoplasm (see reference Garcia *et al*, 2016); (**c**) high nuclear expression of cyclin D1 in carcinoma *in situ* (20×); and (**d**) peripheral expression in tumor nests with central negativity in basal and parabasal layers of a well-differentiated squamous cell carcinoma (40×)

frequently activated in OSCC of the tongue versus other sites, although buccal mucosa carcinomas with *CCND1* gene disorders are as aggressive as tongue carcinomas (Mahdey *et al*, 2011).

The most important oncogenic activity of cyclin D1 is the promotion of uncontrolled cell proliferation, although there have also been more recent reports on its participation in other oncogenic mechanisms, largely related to increased cell migration. However, no research data are available on cell motility in relation to cyclin D1 upregulation in OSCC. The high frequency of lymph node metastasis in OSCC patients with cyclin D1 overexpression suggests that cyclin D1 regulates cell migration and participates in metastatic development in OSCC as in other tumors. Various putative molecular mechanisms may underlie this function of cyclin D1, including: the interaction of cyclin D1–CDK4/6 complexes with proteins involved in cell motility (e.g., filamin A); the physical association of cyclin D1 with $p27^{kip1}$, which has been related to high motility in cell lines; the association of cyclin D1 with oncoproteins that enhance cell motility, including ROCKII (Rho/ROCK signaling); and the presence of *CCND1* neighbor genes in chromosome band 11q13, whose alteration is one of the most representative oncogenic events in OSCC. These genes are frequently co-amplified and have functions related to cytoskeleton remodeling that could cooperatively contribute with *CCND1* cell migration. Finally, Flores *et al* (2016) recently described the co-activation in OSCC of cyclin D1 and vimentin, a key protein in epithelial–mesenchymal transition and associated with gains in epithelial cell motility. Both proteins could be modulated by eukaryotic translation elongation factor 1 delta (EEF1D), a component of the elongation factor 1 complex, which is involved in protein synthesis and also overexpressed in OSCC.

The phase of oral oncogenesis in which the expression of *CCND1*/cyclin D1 exerts its effect is not well understood. Some authors have described this occurring in the final stages of oral carcinogenesis, close to invasion (Callender *et al*, 1994), while other researchers have described oncogenic actions of cyclin D1 during *in situ* carcinoma development (Califano *et al*, 1996; Forastiere *et al*, 2001; Argiris *et al*, 2008). However, the present review of the literature reveals cyclin D1 involvement throughout oral oncogenesis, including its initial phases (Castle *et al*, 1999; Sartor *et al*, 1999; Rousseau *et al*, 2001; Huang *et al*, 2006b; Tsui *et al*, 2009; Ramakrishna *et al*, 2013; Mishra *et al*, 2015). This appears to be a consequence of the oncogenic activation of cyclin D1 *via* multiple mechanisms that act during different stages of carcinogenesis stages. Further research is warranted on the precise timing of cyclin D1 disorders in the multistep process of oral carcinogenesis.

Finally, given the increasing evidence of resistance to cisplatin treatment in OSCC patients who overexpress cyclin D1, we hypothesize that the combination of cisplatin with therapeutic agents that target cyclin D1 may improve the response to treatment of these patients, ideally selected after immunohistochemistry studies.

Author contributions

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Conflicts of interest

None to declare.

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Received: 9 February 2018 | Revised: 15 May 2018 | Accepted: 5 June 2018

DOI: 10.1111/odi.12913

REVIEW ARTICLE

WILEY ORAL DISEASES

An update of knowledge on cortactin as a metastatic driver and potential therapeutic target in oral squamous cell carcinoma

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Abstract

Cortactin is a protein encoded by the *CTTN* gene, localized on chromosome band 11q13. As a result of the amplification of this band, an important event in oral carcinogenesis, CTTN is also usually amplified, promoting the frequent overexpression of cortactin. Cortactin enhances cell migration in oral cancer, playing a key role in the regulation of filamentous actin and of protrusive structures (invadopodia and lamellipodia) on the cell membrane that are necessary for the acquisition of a migratory phenotype. We also analyze a series of emerging functions that cortactin may exert in oral cancer (cell proliferation, angiogenesis, regulation of exosomes, and interactions with the tumor microenvironment). We review its molecular structure, its most important interactions (with Src, Arp2/3 complex, and SH3-binding partners), the regulation of its functions, and its specific oncogenic role in oral cancer. We explore the mechanisms of its overexpression in cancer, mainly related to genetic amplification. We analyze the prognostic implications of the oncogenic activation of cortactin in potentially malignant disorders and in head and neck cancer, where it appears to be relevant in the development of lymph node metastasis. Finally, we discuss its usefulness as a therapeutic target and suggest future research lines.

KEYWORDS
11q13, carcinogenesis, cortactin, CTTN, oral cancer

1 | INTRODUCTION

Cortactin is a protein encoded by the *CTTN* gene (*aka EMS1*), located in chromosome band 11q13, and it was originally identified as a substrate of the nonreceptor tyrosine kinase Src (Kanner, Reynolds, Vines, & Parsons, 1990). Its name derives from its vicinity to cytoskeletal structures of cortical actin (Wu, Reynolds, Kanner, Vines, & Parsons, 1991). Cortactin has been characterized as a filamentous actin (F-actin)-binding protein, playing an essential part in F-actin regulation. However, multiple cell functions have recently been proposed, including the direct regulation of cell motility and migration, cell cycle progression, and angiogenesis (Daly, 2004; Weed & Parsons, 2001), all consistent with the possible role of cortactin in human carcinogenesis. The amplification of region 11q13 is a frequent disorder in cancer, notably in head and neck squamous cell carcinomas (HNSCCs) (Ramos-García, Ruiz-Ávila, et al., 2017). Amplification of the *CTTN* gene has frequently been reported in HNSCC, particularly in oral squamous cell carcinoma (OSCC), and its co-amplification with *CCND1* (which encodes cyclin D1 protein) and other neighboring genes located at 11q13 is well documented

(Ramos-García, Ruiz-Ávila, et al., 2017). Alteration of the *CTTN*/cortactin complex appears to be frequent in cancer and is associated with a more aggressive oncogenic phenotype. Specifically, overexpression of this complex appears to specifically promote cell migration and lymph node metastasis in OSCC. This review provides an update on knowledge of the amplification of *CTTN* and overexpression of its product cortactin in OSCC, focusing on its molecular structure, protein interactions, and on emerging oncogenic functions, among others. The aim was to offer new perspectives on the usefulness of cortactin as a therapeutic target in human cancer, especially in HNSCC. Finally, future research lines are discussed.

2 | CORTACTIN STRUCTURE

Cortactin has multiple binding domains that enable numerous interactions (Figure 1, Table 1). The molecular classification of cortactin has been of major interest since its discovery because of the different functions that derive from these interactions. The multidomain structure of cortactin comprises 84-94 amino-terminal amino acid residues (Wu et al., 1991). A series of acid residues found between amino acids 15 and 35 of cortactin is designated the N-terminal amino acid (NTA) domain (Weed et al., 2000). This domain is characteristic of a group of proteins known as actin nucleation-promoting

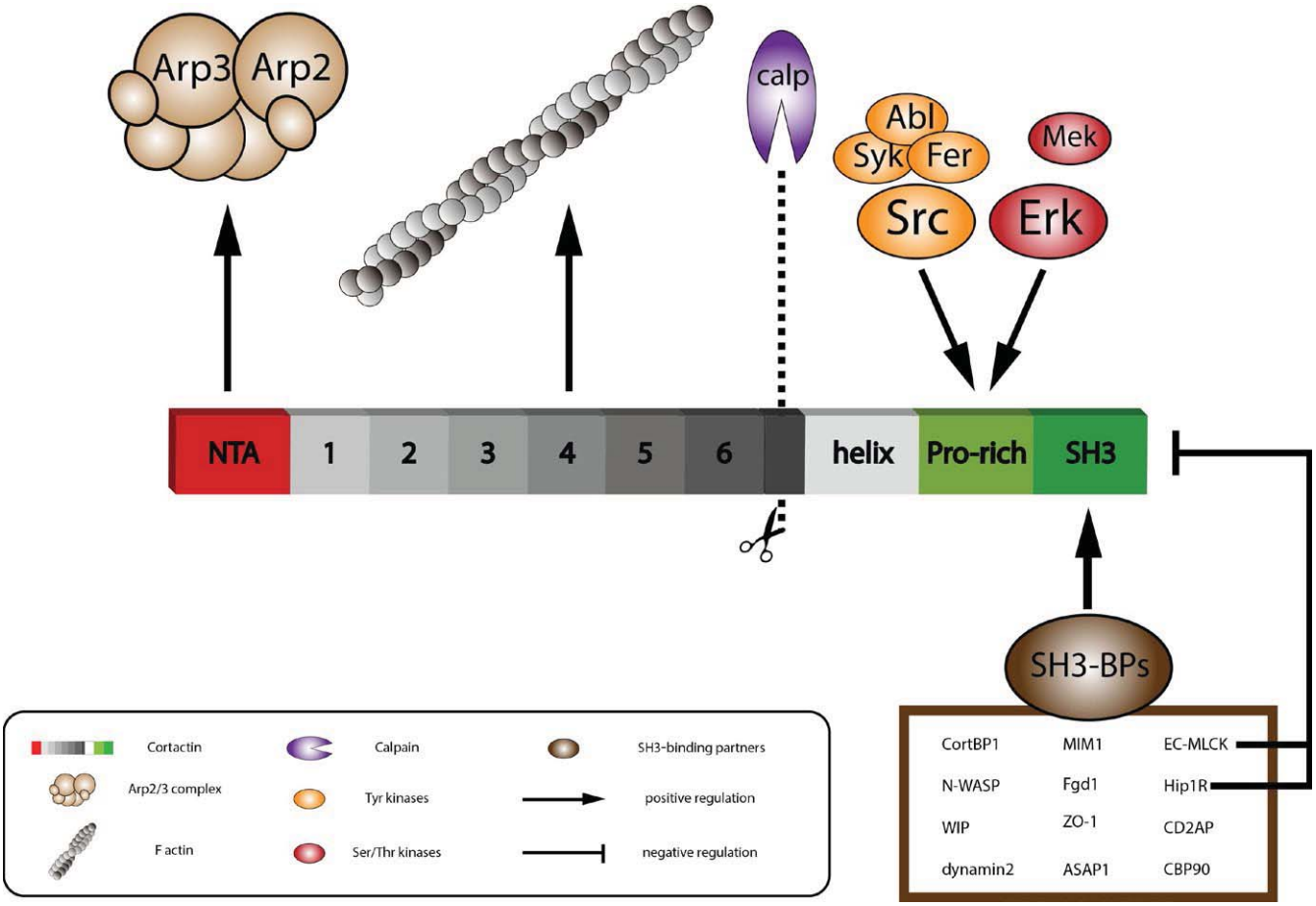

FIGURE 1 Cortactin structure. Graphic representation of the most important interaction sites with other proteins. Cortactin has multiple binding domains that enable interaction with numerous proteins to exert its functions. Its amino-terminal end includes a series of acid residues known as the N-terminal amino acid (NTA) domain (Weed et al., 2000), site of interaction between cortactin and the Arp2/3 complex (Weed et al., 2000). Adjacent to the NTA domain is another domain designated repeat region (or tandem repeat domain), which includes six complete repeated copies of 37 amino acids followed by a final incomplete copy of 20 amino acids (Sparks et al., 1996). This domain allows cortactin to directly bind to F-actin through the fourth repeat. Adjacent to the repeat region is an α-helicoidal domain (helix) formed by 48-52 amino acids with unknown function, although it has been hypothesized that the region between the last incomplete copy of 20 amino acids of the previous domain and the helix may be involved in cortactin proteolysis, likely regulated by calpain2 (Perrin et al., 2006). Another region adjacent to the α-helicoidal domain is proline-rich (Pro-Rich), of variable length, with abundant tyrosine, serine, and threonine residues (Wu et al., 1991). Post-translational modifications *via* this domain are mediated by numerous regulating molecules that phosphorylate cortactin, largely in its tyrosine and serine residues. Among these regulators, we highlight proteins with tyrosine kinase activity, notably Src, and with serine/threonine kinase activity, mainly Erk. Finally, a domain at the terminal carboxyl end designated Src homology 3 (SH3) shares homology with the SH3 domains of the Src family of kinases and is frequently observed in proteins involved in cytoskeletal regulation (Sparks et al., 1996; Wu et al., 1991). Cortactin interacts with numerous proteins *via* this domain, including cortbp1, N-WASP (*aka* WASL), or dynamin2, which positively regulate cortactin-derived functions; and EC-MLCK and Hip1R, which negatively regulate them

TABLE 1 Cortactin-binding partners, binding sites, and downstream effects

Cortactin-binding protein[a]	Binding site	Localization of the interaction	Downstream effect	References
Arp2/3 complex	NTA	Cytoplasm and cell periphery	Nucleation and stabilization of actin.	Weed et al. (2000)
p120 catenin	N-terminal region	Cell–cell junction	Assembly of focal adhesions and lamellipodial persistence.	Boguslavsky et al. (2007)
F-actin	Repeat region	Cell cytoskeleton	Stabilization of branched actin.	Wu et al. (1991)
HDAC6	Repeat region	Cytoplasm	Deacetylation of cortactin, enhancing its binding to actin F.	Zhang et al. (2007)
SIRT1	Repeat region	Cytoplasm and nucleus	Deacetylation of cortactin, increasing cell migration.	Zhang, Zhang, et al., 2009
p300	Repeat region?	Cytoplasm	Acetylation of cortactin, reducing its binding to actin F.	Zhang, Zhang, et al., 2009
Calpain	Helix	Cytoplasm and cell periphery	Proteolysis of cortactin.	Perrin et al. (2006)
Src	Pro-Rich (Y-421,-466,-482)	Cytoplasm and cell periphery	Actin-based protrusive structures.	Brown and Cooper (1996)
Fer	Pro-Rich (Y-421,-466,-482)	Cytoplasm	Recruitment of SH2-domain proteins.	Kim and Wong (1998)
Syk	?	Cell–cell junction	Reduction in cell motility.	Zhang, Shrikhande et al. (2009)
NCK1	Pro-Rich (Y-421,-466)	Cytoplasm and cell periphery	Regulation of connection of cortactin with WASL and WIP.	Oser et al. (2010)
Abl	Pro-Rich (Y-421,-466,-482)	Cytoplasm and membrane	Actin-based protrusive structures.	Boyle et al. (2007)
Arg	Pro-Rich (Y-421,-466,-482)	Cytoplasm and membrane	Actin-based protrusive structures.	Boyle et al. (2007)
MEK	?	Cytoplasm	Increase in cell motility.	Campbell et al. (1999)
Erk	Pro-Rich (S-405,-418)	Cytoplasm	Formation of actin-based protrusive structures and increase in cortactin–WASL association.	Martinez-Quiles et al. (2004)
PKD	Pro-Rich (S-298)	Lamellipodia	Activation of Arp2/3 complex.	Eiseler et al. (2010)
PAK-1	Pro-Rich (S-405,-418)	Cell periphery	Increase in cortactin–WASL association.	Grassart et al. (2010)
PAK-3	Pro-Rich (S-113)	Perinuclear region, cytoplasm and cell periphery	Regulation of actin polymerization and branching.	Webb et al. (2006)
CortBP1/*SHANK2*	SH3	Cytoplasm and membrane	Organization of transmembrane protein complexes	Kreienkamp (2002)
WASL	SH3	Cell periphery	Activation of Arp2/3 complex.	Kowalski et al. (2005)
WIPF1	SH3	Cell periphery	Activation of Arp2/3 complex	Kinley et al. (2003)
Dynamin2	SH3	Cell periphery	Actin-based protrusive structures.	McNiven et al. (2000)
Fgd1	SH3	Cytoplasm	Activation of Arp2/3 complex.	Hou et al. (2003)
MIM1	SH3	Cytoplasm and membrane	Activation of Arp2/3 complex	Lin et al. (2005)
EC-MLCK	SH3	Cell cytoskeleton	Inhibition of Arp2/3 complex activity.	Dudek et al. (2002)
Hip1R	SH3	Clathrin-coated pits	Inhibition of actin polymerization in endocytosis.	Le Clainche et al. (2007)

(Continues)

TABLE 1 (Continued)

Cortactin-binding protein[a]	Binding site	Localization of the interaction	Downstream effect	References
ZO-1	SH3	Tight junctions	Connection between cell–cell adhesion and actin cytoskeleton.	Katsube et al. (2004)
ASAP1	SH3	Invadopodia	Tumor Invasion.	Onodera et al. (2005)
CD2AP	SH3	Membrane ruffles	Connection between endocytosis and actin cytoskeleton.	Lynch et al. (2003)
CBP90	SH3	Cytosol, membrane, and synaptic vesicles	Regulation of actin cytoskeleton in brain tissue	Ohoka and Takai (1998)

Note. [a]Full forms of abbreviations given in cases in the text.

factors (NPFs), including Wiskott–Aldrich syndrome (WAS, aka WASP), Wiskott–Aldrich syndrome like (WASL, aka N-WASP), and WAS protein family member 1 (WASF1, aka WAVE) (Higgs & Pollard, 2001; Weed et al., 2000). The NTA domain is highly preserved in these proteins, allowing them to interact with the Arp2/3 complex and regulate F-actin polymerization (Higgs & Pollard, 2001; Weed et al., 2000), as described in more detail in the next section. Adjacent to the NTA domain is another domain designated repeat region (or tandem repeat domain), which includes six complete repeated copies of 37 amino acids followed by a final incomplete copy of 20 amino acids (Sparks, Hoffman, McConnell, Fowlkes, & Kay, 1996). This domain allows cortactin to directly bind to F-actin through the fourth repeat of the 37 amino acids (Weed et al., 2000). The repeat region is followed by an α-helicoidal domain of 48-52 amino acids with unknown function, although its involvement in cortactin proteolysis has been proposed (Perrin, Amann, & Huttenlocher, 2006), likely regulated by calpain 2 (Perrin et al., 2006). Another region adjacent to the α-helicoidal domain is proline-rich, of variable length, with abundant tyrosine, serine, and threonine residues (Wu et al., 1991). Post-translational modifications through this domain are mediated by numerous regulating molecules, notably Src, which phosphorylate cortactin, largely in its tyrosine and serine residues. Finally, a domain at the terminal carboxyl end designated Src homology 3 (SH3) shares homology with the SH3 domains of the Src family of kinases and is frequently observed in proteins involved in cytoskeletal regulation (Sparks et al., 1996; Wu et al., 1991).

Cortactin shares structural and functional similarities with hematopoietic lineage cell-specific protein 1 (HS1), which is only expressed in hematopoietic lineages (Kitamura, Kaneko, Miyagoe, Ariyasu, & Watanabe, 1989). With regard to their molecular structure, HS1 possesses the same domains as cortactin but has only 3.5 repeats in its repeat region (Kitamura et al., 1989). Functionally, HS1 regulates the dynamics of actin and stabilizes branched actin filaments (Babich & Burkhardt, 2013). At the pathological level, HS1 appears to favor the development of leukemia and worsen its prognosis (Butrym, Majewski, Dzietczenia, Kuliczkowski, & Mazur, 2012). To date, only the *CTTN* gene has been identified as responsible for encoding cortactin in its canonical form (full-length canonical cortactin protein), which can be expressed in most cell types (Wu & Montone, 1998). Two additional isoforms have been identified, cortactin-B and cortactin-C, which result from the alternative splicing of *CTTN* mRNA (Ohoka & Takai, 1998). These isoforms differ from the canonical form in the tandem repeat domain, which includes five and four repeats, respectively, rather than the six reported for the canonical form. Isoforms identified in humans have been designated as splice variant 1 (SV1)-cortactin and SV2-cortactin, respectively (van Rossum et al., 2003). Although their functions remain poorly characterized, they are known to have less affinity for F-actin in comparison with the canonical form of cortactin (Ohoka & Takai, 1998; van Rossum et al., 2003).

3 | FUNCTION OF CORTACTIN IN CELL MIGRATION

3.1 | Cortactin, an F-actin-binding protein promoter of actin nucleation

Cortactin is related to increased cell motility and invasiveness, which directly depend on its cell localization. Cortactin translocates from perinuclear cytoplasmic localizations to the cell periphery as a consequence of tyrosine kinase (epidermal growth factor receptor [EGFR] and platelet-derived growth factor receptor beta [PDGFRB]) and integrin signaling (Ozawa, Kashiwada, Takahashi, & Sobue, 1995; Weed, Du, & Parsons, 1998). At the cell periphery, it activates the Rac family small GTPase 1 (RAC1) and cell division cycle 42 (Cdc-42), proteins that belong to the Rho family of small GTPases (Clark, King, Brugge, Symons, & Hynes, 1998; Weed et al., 1998). RAC1 and Cdc-42 bind to and activate F-actin nucleation-promoting factors (NPFs) downstream, including WAS, WASL, and WASF1 (Higgs & Pollard, 2001), which in turn activate the actin-related protein (Arp) 2/3 complex (Hall, 1998). This induces a rapid polymerization of cortical F-actin, which organizes into branched networks, a phenomenon known as actin nucleation. Cortactin can also directly bind to the Arp2/3 complex, stimulating actin nucleation (Uruno et al., 2001; Weaver et al., 2001; Weed et al., 2000) and, more importantly, stabilizing filament branchpoints (Weaver et al., 2001). The Arp2/3

complex comprises seven subunits: two actin-related proteins, Arp2 and Arp3, and five polypeptides, ARPC1–5 (Higgs & Pollard, 2001). Binding of the cortactin–Arp2/3 complex takes place through a motif of three amino acids (DDW) located in the NTA domain of cortactin (Weed et al., 2000) and bound to the Arp3 subunit (Weaver et al., 2002). However, the affinity of cortactin for this complex is fivefold lower than the affinity of WAS or WASL proteins (Uruno et al., 2001; Weed et al., 2000). Nevertheless, pioneer studies have reported that synergy between cortactin and WASL compensates for the lower affinity of cortactin for the Arp2/3 complex (Weaver et al., 2001). The binding of cortactin to F-actin and to the Arp3 subunit of the complex appears to be synchronized with the binding of WASL to subunits Arp2, Arp3, and ARPC1 (via a WASL domain designated VCA). It has been reported that stimulation of the Arp2/3 complex induced by WASL synergistically enhances cortactin in both polymerization and actin filament ramification. However, WASL–cortactin interactions and their effects remain controversial. The same research group (Weaver et al., 2002) subsequently reported that the VCA domain of WASL not only synergistically cooperates in activating the Arp2/3 complex but also competes with cortactin for binding to the Arp3 subunit of the complex; they observed that it can displace cortactin, completely blocking its binding with the Arp2/3 complex via subunit Arp3. More recent studies observed that cortactin can also exert a synergistic impact on WASL, binding to it and inducing its activation (Kowalski et al., 2005). According to the most recently proposed model, there is a clear synergy between these proteins, with the activation of both increasing the polymerization rate of actin around 3.5-fold more than WASL alone (Helgeson & Nolen, 2013). However, cortactin would displace WASL from nascent sites of actin ramification (Helgeson & Nolen, 2013; Siton et al., 2011), thereby accelerating the process and stimulating actin nucleation. Hence, cortactin and WASL activate the Arp2/3 complex in various ways, and the precise regulation mechanisms of the branched actin networks in vivo remain poorly understood. The association of cortactin with the Arp2/3 complex protects and stabilizes branched actin from spontaneous disassembly. In contrast, coronin 1B, a member of the coronin family of actin binding proteins that regulate cell motility in different contexts, appears to antagonize cortactin and directly bind to the Arp2/3 complex, inhibiting cortactin-mediated actin nucleation and destabilizing branched actin networks (Cai, Makhov, Schafer, & Bear, 2008). Experiments have demonstrated the incapacity of cortactin lacking its fourth repeat to bind to F-actin (Weed et al., 2000). Through its fourth repeat, cortactin can also bind to phosphatidylinositol 4,5-bisphosphate (PIP2), a membrane phospholipid, which probably also collaborates in cortactin–F-actin binding (He et al., 1998). Cortactin-mediated cortical actin nucleation entails dynamic changes in the cell cytoskeleton, which promote the formation of lamellipodia, an actin-based structure involved in cell motility gain (Bryce et al., 2005). Tumors are known to have a characteristic disorder of F-actin microfilament architecture, increasing their invasiveness (Guck et al., 2005), which may be related to the translocation of cortactin from its perinuclear localization to the cell periphery.

3.2 | Cortactin SH3 domain as cell migration regulator

The cortactin SH3 C-terminal domain behaves as a binding site for numerous proteins through which cortactin regulates multiple processes. The first protein identified as binding to the cortactin SH3 domain was designated cortactin-binding protein 1 (CortBP1) and characterized as an alternative splice variant of *SHANK2* (Du, Weed, Xiong, Marshall, & Parsons, 1998). The interaction of CortBP1 with cortactin may be involved in the organization of transmembrane protein complexes. Hence, cortactin provides an indirect connection between specific transmembrane receptors and the underlying actin cytoskeleton (Kreienkamp, 2002). Cortactin also binds *via* its SH3 domain to proteins that promote actin polymerization mediated by the Arp2/3 complex, including WASL and the WAS/WASL interacting protein family member 1 (WIPF1, *aka* WIP). Direct interaction with WASL may be an additional pathway to those described above for actin polymerization regulation (Kowalski et al., 2005). WIPF1 is a protein identified by its interaction with WASL (Kinley et al., 2003; Ramesh, Antón, Hartwig, & Geha, 1997). WIPF1 and cortactin are colocated in the cell periphery (Kinley et al., 2003), and WIP concentration-dependently increases the effectiveness of cortactin-mediated Arp2/3 complex activation and hence actin polymerization. The co-expression of both proteins also enhances the formation of actin-based protrusive structures, important for cell migration (Kinley et al., 2003).

Cortactin SH3 domain may also interact with dynamin2, which belongs to the dynamin family of GTPases, mainly involved in endocytic and secretor pathways (McNiven et al., 2000). The intracellular dynamic behaviors of Dynamin2 and cortactin are highly similar. Thus, when PDGF is stimulated, it translocates to protrusive structures of the cell periphery and activates cell migration, for which cortactin likely behaves as a functional requirement (McNiven et al., 2000). Finally, dynamin2 can also regulate actin polymerization, enhancing Arp2/3 complex activity (Schafer et al., 2002).

FYVE, RhoGEF, and the PH domain containing 1 (Fgd1), a protein characterized by its role in faciogenital dysplasia, contain a direct binding domain with cortactin SH3. Cortactin–Fgd1 interaction also appears to enhance Arp2/3 complex activation and actin polymerization (Hou et al., 2003; Kim, Hou, Gorski, & Cooper, 2004). Fgd1 contains a domain designated RhoGEF, through which it appears to regulate the formation of actin-based protrusive structures (Hou et al., 2003).

Other SH3-binding proteins are as follows: missing in metastasis 1 (MIM1), which activates the Arp2/3 complex by this pathway (Lin et al., 2005); endothelial cell myosin light chain kinase (EC-MLCK), which regulates cell contractile strength by actin and myosin coordination (Dudek, Birukov, Zhan, & Garcia, 2002) and negatively regulates actin polymerization, as does huntingtin-interacting protein 1 related (Hip1R), another SH3-binding protein (Le Clainche et al., 2007); ZO-1, a member of the membrane-associated guanylate kinase homologs [MAGUKs] protein family involved in the regulation of cell–cell binding and cytoskeleton components, also binds

to cortactin via SH3 and recruits it to specialized adhesion structures (e.g., invadopodia) (Katsube, Togashi, Hashimoto, Ogiu, & Tsuji, 2004; Katsube et al., 1998). This interaction appears to serve as a connection between cell–cell adhesion phenomena and the underlying actin cytoskeleton. ArfGAP with SH3 domain, ankyrin repeat, and PH domain 1 (ASAP1 aka AMAP1), a GTP-binding protein of the Arf family, was observed to bind in invadopodia to the cortactin SH3 domain, forming a trimeric complex with paxillin protein, and its blockade inhibited cell invasion in vitro and in vivo (Onodera et al., 2005); CD2-associated protein (CD2AP) (another actin cytoskeleton-regulating molecule) was reported to bind to cortactin via SH3, forming a complex that appears to connect the cytoskeleton of actin with the endocytosis and traffic of internalized epidermal growth factor receptor (EGFR) (Lynch et al., 2003). Cortactin-binding protein 2 (CBP90 aka CortBP2) (a specific brain tissue protein) also binds to cortactin SH3 domain and appears to play a role in regulating the cytoskeleton of actin in brain tissue (Ohoka & Takai, 1998).

3.3 | Cortactin regulation of cell–cell interactions via cortactin in cell migration

Cortactin may also promote cell migration by regulating cell–cell interactions. As noted above, interaction has been described between cortactin and ZO-1, a protein that regulates tight junctions in cell–cell binding (Katsube et al., 1998, 2004). This interaction was observed in colorectal cancer cells and found to be crucial for disease progression, being a marker of lymph node metastasis (Hirakawa, Shibata, & Nakayama, 2009). Interaction has also been reported between E-cadherin (classic cadherin essential for the regulation of cell–cell adhesion processes) and cortactin at adherens junctions, stabilizing E-cadherin–β-catenin complexes (Sroka et al., 2016). In addition, interaction between cortactin and p120 catenin (a protein of the armadillo family that binds to the juxtamembrane domain of classic cadherins) was found to regulate both the assembly of focal adhesions and the lamellipodial persistence of cancer cells (Boguslavsky et al., 2007). The assembly relationships of malignant epithelial cells with the extracellular matrix and cell–cell interactions are crucial in the cascade of molecular phenomena and cell interactions that lead to metastasis (Fidler, 2003), and only clones that acquire a phenotype enabling anchorage would presumably develop increased metastatic capacity. In OSCC, a cohesive invasive pattern based on the formation of tumor nests is of major importance [Bryne, 1998]). The interaction of cortactin with molecules that are critical for cell–cell adhesion in cancer cells may be related to lamellipodial stability, which requires the formation of new adhesions, and this appears to be a plausible mechanism for cell migration regulation.

3.4 | Post-translational modifications of cortactin in cell migration

Post-translational modifications of cortactin may also regulate its functions associated with cell migration. Thus, cortactin has been identified as a substrate of Src tyrosine kinase activity (Kanner et al., 1990) and exerts an impact on the organization of cortical actin cytoskeleton via this Src pathway (Brown & Cooper, 1996). Src can be found in an inactive conformational state, with no kinase activity, or it can be activated by various cell stimuli, largely growth factors such as epidermal growth factor [EGF], fibroblast growth factor 1 [FGF1], colony-stimulating factor 1 [CSF1], PDGF, thromboxane A2, and cell–cell/cell–extracellular matrix adhesion events such as $\alpha_5\beta_1$ integrin, $\alpha_v\beta_3$ integrin, $\alpha_{IIB}\beta_3$ integrin, syndecan 3, CD44, and intercellular adhesion molecule 1 [ICAM-1]. Once activated, Src phosphorylates cortactin through the recombinant SH2 domain of Src (Okamura & Resh, 1995). This direct Src–cortactin interaction can be prevented by different ligands that contain the binding sequence to the SH2 domain of Src (pTyr-Glu-Glu-Ile [pYEEI]) (Nam et al., 2004) and behave as Src–cortactin pathway inhibitors. Src phosphorylates cortactin in tyrosine residues Tyr-421,-466, and -482 (Huang, Liu, Haudenschild, & Zhan, 1998). Mutation of these tyrosine residues may also hamper Src-mediated cortactin phosphorylation. Tyr-421 is probably the first phosphorylated residue, stabilizing the Src–cortactin complex and facilitating the consequent phosphorylation of tyr-466 and -482 (Huang et al., 1998). Src–cortactin interaction also gives rise to "phospho-dependent binding sites," which permit assembly with other molecular signaling complexes that exert different actions (Okamura & Resh, 1995; Parsons & Parsons, 1997). Recent studies suggest that cortactin phosphorylation by Src and other kinases may be functionally related to cell migration, regulating reorganization downstream of the cortical actin cytoskeleton and lamellipodia and invadopodia projections (MacGrath & Koleske, 2012). Cortactin tyrosine phosphorylation may also enhance the recruitment of adaptor proteins such as NCK adaptor protein 1 (NCK1) (Oser et al., 2010; Tehrani, Tomasevic, Weed, Sakowicz, & Cooper, 2007), which would regulate the connection of cortactin with WASL and WIP and therefore induce a greater activation of the Arp2/3 complex and potentiate its functions in the regulation of actin-based protrusive structures (Huang et al., 1998; Kowalski et al., 2005). However, according to a previously published model (S-Y switch model), tyrosine phosphorylation mediated by Src (Y421, Y466, and Y482) would inhibit the phosphorylation of serine residues of cortactin, regulated by Erk (S405 and S418), thereby inhibiting the binding and activation of the cortactin–WASL complex (Lua & Low, 2005; Martinez-Quiles, Ho, Kirschner, Ramesh, & Geha, 2004). Although Src appears to be one of the most important cortactin activators in cell migration regulation (Kelley, Hayes, Ammer, Martin, & Weed, 2010; Oser et al., 2010), the molecular mechanisms derived from this interaction have yet to be elucidated.

FER tyrosine kinase (Fer) is a nonreceptor tyrosine kinase of the FPS/FES subfamily, which, like Src, includes an SH2 domain among its structural characteristics (Kim & Wong, 1998). Fer activation mediated by growth factors PDGF and CSF1 leads to the tyrosine phosphorylation of cortactin (Kim & Wong, 1998) in the same tyrosine residues as those targeted by Src. Spleen-associated tyrosine kinase (Syk), another kinase that appears to participate in hematopoiesis and also has an SH2 domain, interacts with cortactin during platelet maturation and activation (Gallet et al., 1999). A study of

Syc–cortactin interaction in breast cancer cell lines reported an association between the kinase activity of Syc and the reduced motility of epithelial cancer cells, and the authors therefore attributed Syc with tumor suppressor qualities (Zhang, Shrikhande, Alicie, Zhou, & Geahlen, 2009). Cortactin is also a substrate of Abl tyrosine kinase (Abl) and Abl-related gene (*aka* Arg), members of the ABL tyrosine kinase family, and when induced by PDGF they phosphorylate cortactin in the same three tyrosine residues that are targets of Src: Tyr-421,-466, and -482. This interaction may regulate the downstream reorganization of cortical actin cytoskeleton and the formation of protrusive structures, related to a gain in cell motility (Boyle, Michaud, Schweitzer, Predki, & Koleske, 2007).

Cortactin is also phosphorylated by various serine/threonine kinases, including members of the mitogen-activated protein kinase (MAPK) signaling pathway. Cortactin is phosphorylated by MEK, a component of the MAPK pathway, in the HNSCC UMSCC2 cell line, and this interaction can be prevented by the MEK PD98059-specific inhibitor (Campbell, Sutherland, & Daly, 1999; van Damme, Brok, Schuuring-Scholtes, & Schuuring, 1997; Hayes et al., 2013). It has also been reported that Erk, another member of the MAPK pathway, phosphorylates cortactin in ser-405 and ser-418, which appears to be associated with increased cell migration, the regulation of cortactin translocation from cytoplasm to cell periphery, and the formation of actin-based protrusive structures (Campbell et al., 1999; van Damme et al., 1997; Hayes et al., 2013). Erk-mediated cortactin phosphorylation can also enhance the association of cortactin with WASL, translating into activation of the Arp2/3 complex and its derivative roles, also involved in cell migration (Martinez-Quiles et al., 2004).

Other molecules that do not belong to the MAPK pathway have been involved in the phosphorylation of cortactin serine/threonine residues. Thus, protein kinase D (PKD), a macromolecule involved in the regulation of cell shape, adhesion, and migration, appears to phosphorylate cortactin in ser-298 (Eiseler, Hausser, De Kimpe, Van Lint, & Pfizenmaier, 2010). By this mechanism, PKD behaves upstream as a regulator of increased Arp2/3 complex activation, thereby enhancing cell migration. p21 (RAC1) activated kinase 1 and 3 (PAK-1 and -3), members of the PAK family of serine/threonine kinase proteins that are involved in cell shape and polarity, among other functions, and appear to be putative cortactin regulators. PAK-1, located at chromosome band 11q13 and frequently amplified in breast cancer (Ramos-García, Ruiz-Ávila, et al., 2017), phosphorylates cortactin in ser-405 and -418 (Grassart et al., 2010) and consequently favors the association of cortactin with WASL, increasing cell migration. PAK-3 phosphorylates cortactin in ser-113, and this event is associated with cortactin translocation to the cell periphery and increased cell migration-related actions (Webb et al., 2006).

It has been proposed that cortactin may be an acetylation and deacetylation substrate at post-translational level. Zhang et al. (Zhang et al., 2007) observed that histone deacetylase 6 (HDAC6), a member of the HDAC superfamily involved in cell motility regulation, appears to bind to and hypoacetylate cortactin, enhancing its

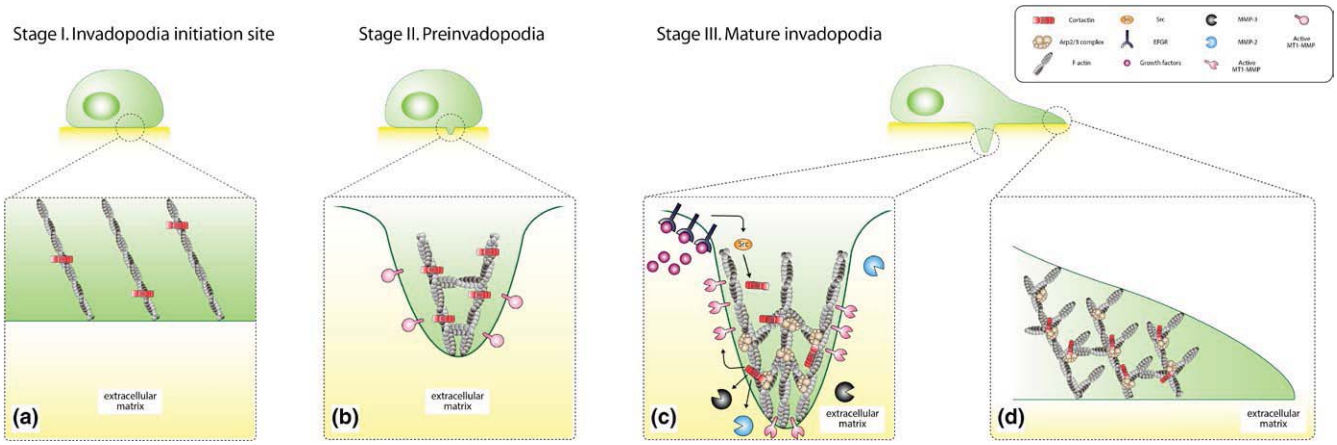

FIGURE 2 Cortactin-mediated functional regulation of actin-based protrusive structures. (a) Stage I. At the invadapodial initiation site, cortactin first accumulates in areas where the cell membrane is in contact with the extracellular matrix, forming aggregates with F-actin (Artym et al., 2006). (b) Stage II. In the preinvadopodia stage, there is an increase in the concentration of cortactin and F-actin, responsible for the recruitment of MT1-MMP (inactive) to invadopodial initiation sites. The definitive characteristic of this stage is MT1-MMP accumulation (Artym et al., 2006). (c) Stage III. In the mature invadopodia stage, the amount of MT1-MMP in invadopodia increases, and this metalloproteinase acquires degradation capacity. Cortactin overaccumulation in mature invadopodia also regulates the increased expression of active MMP-2 and -3, which are located in the extracellular matrix. Mature invadopodia formation is largely regulated by EGFR and by the tyrosine kinase activity of Src (EGFR-Src pathway), and in turn depends on the nucleation of Arp2/3 complex actin, which promotes the assembly of branched actin networks (Artym et al., 2006). (d) Lamellipodia are cytological structures crucial for cell locomotion (Yilmaz & Christofori, 2009). They are thin and flat protrusions of the sheetlike plasmatic membrane formed by a network of intertwined orthogonal actin filaments, whose organization and function result from actin nucleation activity mediated by the Arp2/3 complex (Condeelis, 2001; Yilmaz & Christofori, 2009), although their kinetic-migration characteristics are better known than the biology of their formation. In lamellipodia, cortactin selectively binds to and stabilizes cortical F-actin, enhancing its polymerization and regulating its reticular assembly. These mechanisms appear to be essential to maintain this actin-based protrusive structure

capacity to bind to F-actin and therefore cell migration. The same research group found that sirtuin 1 (SIRT1), another HDAC member, also deacetylated cortactin, stimulating cell migration in the OV2008 line (Zhang, Zhang, et al., 2009), and this effect was reversed by EX-527, an SIRT1 inhibitor. P300, an acetyltransferase histone, acetylates cortactin and has been considered a tumor suppressor gene, whose mutations and deletions in different human cancers, including OSCC, promote cortactin deacetylation and induce oncogenic action (Iyer, Ozdag, & Caldas, 2004). Zhang, Zhang, et al., (2009)proposed that cortactin acetylation by p300 is an antitumor mechanism that is lost in cells with mutations or deletions of this gene (p300-null cells), leading to increased cell migration.

3.5 | Role of cortactin in the regulation of actin-based protrusive structures

The acquirement of migratory and invasive properties by cancer cells requires changes in their actin cytoskeleton cells to form a series of protrusive membrane structures: invadopodia and lamellipodia (Yilmaz & Christofori, 2009). As noted above, the localization of cortactin in the cortical actin cytoskeleton, its binding to F-actin, its interaction with Arp2/3 complex and other proteins *via* its SH3 domain, and its post-translational modifications give actin a role in events that are essential for the regulation of these protrusive structures.

Cortactin affects invadopodial formation and function (Figure 2). Invadopodia are actin- and phosphotyrosine-rich membrane projections that extend and couple by matrix metalloproteinase (MMP) sequester toward the extracellular matrix, for which they have a high degradation capacity, facilitating cell invasion (Weaver, 2006; Yilmaz & Christofori, 2009). Cortactin is essential for the formation and function of invadopodia, regulating their initiation and structural organization, also stabilizing the F-actin in the central invadopodia core (Artym, Zhang, Seillier-Moiseiwitsch, Yamada, & Mueller, 2006). siRNA-mediated cortactin knockdown was found to prevent invadopodia formation and extracellular matrix degradation in several cell lines, including Src-transformed fibroblasts, breast cancer, melanoma, and HNSCC (Ammer & Weed, 2008). In a four-stage stepwise model of the molecular events involved in invadopodial formation and function (Artym et al., 2006), cortactin initially accumulates in areas in which the cell membrane is in contact with the extracellular matrix and aggregates with F-actin (stage 1: invadopodia initiation site) (Figure 2a), acting as an adaptor for the recruitment and organization of other essential proteins of these invadopodial structures. There is a progressive increase in the amounts of cortactin and F-actin (stage 2: preinvadopodia) (Figure 2b), responsible for the recruitment of membrane type 1 metalloprotease (MT1-MMP) at invadopodial initiation sites. MT1-MMP is a membrane-bound MMP with an essential role in cell migration through the extracellular matrix in different biological processes, including embryogenesis, wound healing, and cancer cell invasion; it initiates the extracellular matrix degradation activity that defines stage 3 (mature invadopodia) (Figure 2c). They are formed by an external adhesive ring rich in integrin β2 and β3 adhesion receptors, which regulate the cell–matrix connection, and by an actin-rich central core regulated by cortactin, Src, Arp2/3 complex, actin nucleation promotion factors (NPFs), and talin and paxillin, among other proteins (Block et al., 2008; Yilmaz & Christofori, 2009). The tyrosine kinase activity of Src is necessary for invadopodia formation and is regulated upstream by growth factors *via* EGFR-Src (Weaver, 2006). Invadopodia formation also appears to depend on the nucleation of Arp2/3 complex actin, which promotes the assembly of branched actin networks, by interactions with WASL, WASF1, and cortactin (Weaver, 2006). The final stage (Stage 4: late invadopodia) is marked by the dissolution of invadopodia cortactin and F-actin and the preservation of high concentrations of MT1-MMP, which continue to degrade the focal matrix (Artym et al., 2006). It has been reported that cortactin overexpression by invadopodia in HNSCC cell lines may also regulate the increased expression of MMP-2 and MMP-3, which are both involved in extracellular matrix degradation in physiological and pathological processes (Clark, Whigham, Yarbrough, & Weaver, 2007).

Lamellipodia are crucial for cell locomotion (Yilmaz & Christofori, 2009) (Figure 2d). They are thin and flat protrusions of the sheetlike plasmatic membrane formed by a network of intertwined orthogonal actin filaments, whose organization results from actin nucleation activity mediated by the Arp2/3 complex (Yilmaz & Christofori, 2009). Underlying lamellipodia and separating them from the cell body are lamellae, thicker than lamellipodia and rich in tropomyosin and myosin II (Ponti, Machacek, Gupton, Waterman-Storer, & Danuser, 2004). The cell acquires migratory capacity in response to external stimuli (Figure 3), related to a series of cell phenomena triggered by lamellipodium protrusion, which begins with actin filament polymerization and elongation, mediated by cortactin, Arp2/3 complex, and cofilin, a protein that regulates actin filament length (Condeelis, 2001). Besides actin filament elongation, there is an adherence of new actin monomers that generate protrusive forces to move the cell membrane forward (Ponti et al., 2004). The cortactin/Arp2/3 complex can include vinculin, a cytoskeletal protein that provides stability for lamellipodium anchoring (Ammer & Weed, 2008; DeMali, Barlow, & Burridge, 2002). A second stage required for cells to acquire migratory capacity is adhesion of the lamellipodium/lamella complex to the extracellular substrate through “focal contacts.” Focal contacts bound to lamellipodia are induced by Rac1 and are less stable than those established by lamellae, which are induced by Rho (Ridley et al., 2003); they establish new substrate binding sites that provide the lamella with new adhesion points (Small & Resch, 2005). It has been demonstrated that the absence of focal contacts connected to the lamella induces the folding of lamellipodia toward the cell body, generating membrane ruffles (Ammer & Weed, 2008). The cell migration process concludes with the separation of posterior adhesions, retraction of the posterior cell portion, and advance of the cell body toward focal contacts of the lamellipodium/lamella complex (Condeelis, 2001; Ponti et al., 2004).

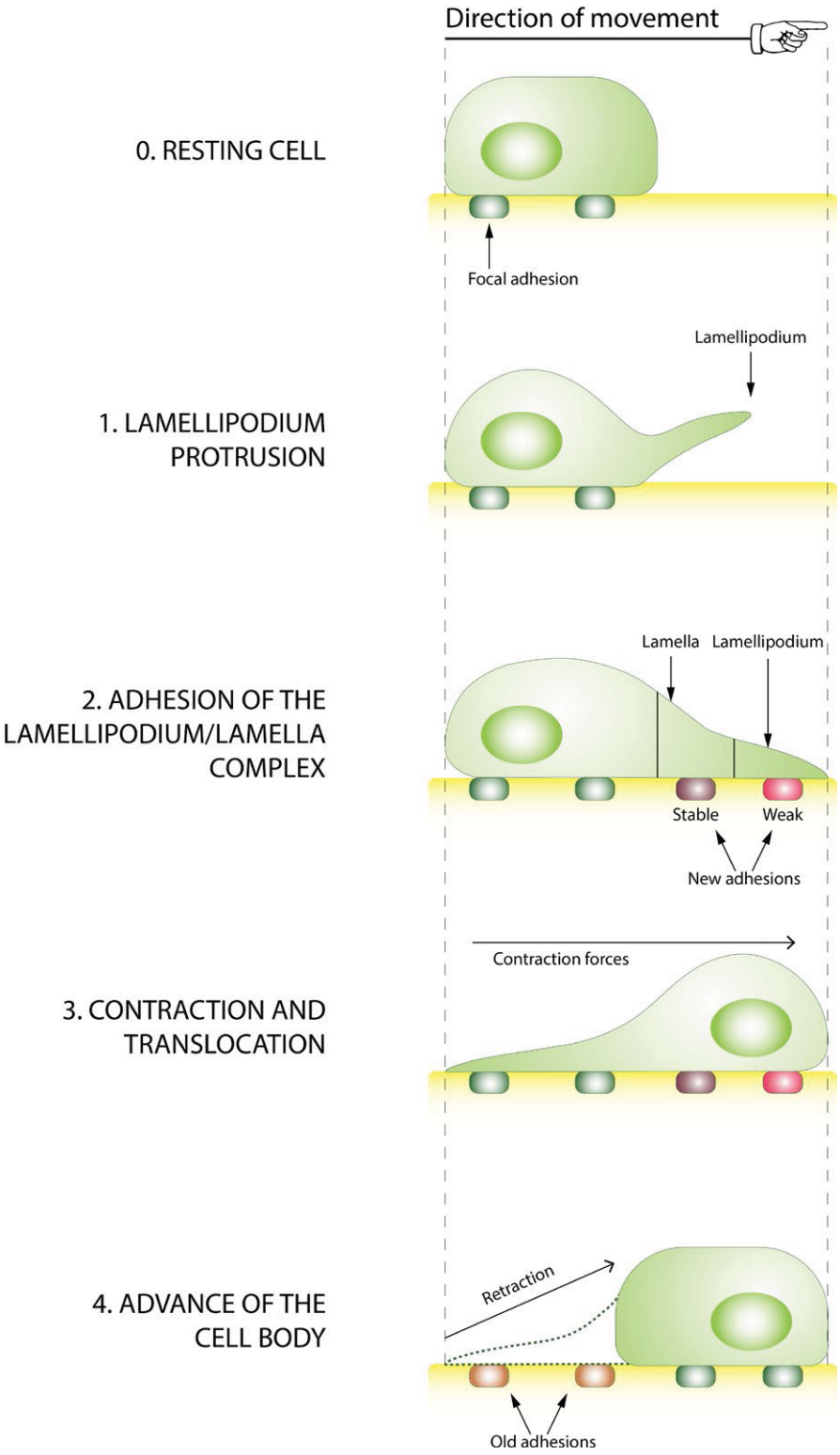

FIGURE 3 Lamellipodium-regulated cell migration stages. The cancer cell acquires migratory capacity in response to external stimuli, related to cell phenomena that are dependent on lamellipodia (a) (Condeelis, 2001; Ponti et al., 2004). Elongation or protrusion of lamellipodia is essential for effective cell migration (b) (Condeelis, 2001; Ponti et al., 2004), and the lamellipodium/lamella complex must adhere to the extracellular substrate. The cell body is separated from the lamellipodium by the lamella, which is thicker (Ponti et al., 2004) and forms more stable adhesions in comparison to the lamellipodium (Ridley et al., 2003; Small & Resch, 2005). The adhesion of both structures is necessary for the cancer cell to acquire migratory capacity (c). The nucleus and cell body translocate forward through contraction forces (d) (Condeelis, 2001; Ponti et al., 2004). Finally, this process concludes with the loss of posterior adhesions and retraction of the posterior cell portion, allowing the cell body to advance (e) (Condeelis, 2001; Ponti et al., 2004)

4 | SPECIFIC FUNCTION OF CORTACTIN IN CELL MIGRATION REGULATION IN ORAL CARCINOGENESIS

Cortactin overexpression may specifically contribute to a gain in cell migration and invasiveness in oral carcinogenesis. This has been extensively studied in HNSCC/OSCC in vitro and in vivo (Clark et al., 2009; van Damme et al., 1997; Patel, Incognito, Schechter, Wasilenko, & Somers, 1996; Rothschild et al., 2006; Schuuring, Verhoeven, Litvinov, & Michalides, 1993; Yamada, Yanamoto, Kawasaki, Mizuno, & Nemoto, 2010), explaining the association between *CTTN*/cortactin and HNSCC/OSCC amplification and overexpression and M+ and N+ status (Chen et al., 2004; van Kempen et al., 2015; Liu et al., 2009; Sugahara et al., 2011; Xia, Chen, Li, & Zeng,

2007; Yamada et al., 2010). It has been reported that low cortactin expression levels in oral carcinogenesis involve the cytoplasmic localization of this protein (Schuuring et al., 1993). As a result of *CTTN* gene amplification, a frequent event in OSCC (Clark et al., 2009; van Damme et al., 1997; Patel et al., 1996; Ramos-García, Ruiz-Ávila, et al., 2017; Rothschild et al., 2006; Schuuring et al., 1993; Yamada et al., 2010), and the overexpression and/or post-translational modifications of this gene, cortactin appears to abandon the cytoplasm and accumulate in the cytoskeleton periphery, where it binds to the Arp2/3 complex to regulate cortical actin polymerization and branched actin network formation, and also in the membrane at cell–extracellular matrix contact sites (van Damme et al., 1997; Patel et al., 1996; Rothschild et al., 2006; Schuuring et al., 1993). Both events are essential for the dynamic remodeling of the plasmatic membrane and the formation of protrusive structures and are necessary for the onset of cell motility.

Tumor cells with increased motility and metastatic potential exhibit disorders in the actin cytoskeleton, whose strictly ordered and rigid structure becomes more disordered and irregular, with fewer actin polymers, reducing its structural resistance and increasing its elasticity (Guck et al., 2005). These changes have also been observed in malignant keratinocyte cell lines (Katsantonis et al., 1994), especially in HSC3 cells derived from the lymph node metastasis of a lingual squamous cell carcinoma (Kinoshita et al., 2012), finding that cancer cells with migratory phenotype were longer than those with inhibited cell migration. This morphologic change was accompanied by cytoskeleton reorganization and modifications in F-actin structural layout. Katsantonis et al. (Katsantonis et al., 1994) evaluated these changes by studying the differential distribution of actin filaments in normal cells and malignant keratinocytes, with the latter showing markedly reduced polymerized actin levels and less stable actin networks, with lower structural resistance. The loss of cortactin-mediated cytoskeleton rigidity also appears essential for acquisition of the migratory phenotype (Yilmaz & Christofori, 2009).

The influence of cortactin post-translational modifications on increased cell migration was also evaluated in malignant oral keratinocytes. In a study of EGF-stimulated HNSCC cell lines, Van Damme et al. (van Damme et al., 1997) observed a change in the conformational structure of cortactin, which largely contained phosphoserine and phosphothreonine residues, associated with increased motility. Another study of HNSCC cell lines attributed cortactin phosphorylation to its interaction with MEK and Erk (Campbell et al., 1999) in response to the downstream activation of the MAPK signaling pathway (Hayes et al., 2013). Thus, post-translational cortactin modifications may regulate its cell sublocalization and accelerate cell migration and invasiveness in OSCC (van Damme et al., 1997). There is some evidence that cortactin also interacts with the nontyrosine kinase receptor Src during oral carcinogenesis. Huang et al. (Hwang, Park, & Chung, 2013) observed reduced Src and cortactin phosphorylation in OSCC cell lines after treatment with the inhibitor epigallocatechin-3 gallate, a polyphenol component of green tea, as well as lower cell migration and invasiveness. Siqueira et al. (Siqueira et al., 2016) observed in lingual SCC line CAL27 that peptide CL16 of laminin 111, a membrane protein associated with cell migration, enhanced invadopodial formation and activity by regulating Src and cortactin phosphorylation. In HNSCC cell lines (HN31, UMSCC1, and 1483), Ammer et al. (Ammer et al., 2009) reported reduced Src activation and downstream inhibition of cortactin phosphorylation in Tyr-421 with the use of saracatinib, an inhibitor of Src tyrosin kinase activity. Other investigations in HNSCC cell lines by the same group (Hayes et al., 2013; Kelley, Ammer, et al., 2010) also observed cortactin phosphorylation in tyr-421 (Hayes et al., 2013; Kelley, Ammer, et al., 2010; Rothschild et al., 2006), -466 and -482 (Rothschild et al., 2006), which appears to result from upstream activation of the EGFR-Src-cortactin cascade (Hayes et al., 2013; Rothschild et al., 2006). With regard to this cascade, Hofman et al. (Hofman et al., 2008) are the only authors to evaluate the relationship between cortactin and EGFR, observing overexpression of both proteins in most HNSCC cases in their series and its association with a more advanced TNM stage. Hwang et al. (Hwang, Park, Cha, Kim, & Chung, 2012) observed high EGFR expression in invadopodia that was significantly correlated with cortactin overexpression in vivo in OSCC animal models (athymic nude mice); they also reported a significant inhibition of invadopodial formation and of extracellular matrix degradation after treatment with specific inhibitors of EGFR AG1478 and PD153035. It has been observed that Abl-kinase, an upstream component of EGFR signaling, negatively regulates invadopodial formation and cell migration in HNSCC lines, apparently by inhibiting the EGFR-Src-cortactin cascade through inhibition of heparin-binding EGF-like growth factor (HB-EGF) (Hayes et al., 2013). However, although most studies have indicated a close EGFR–cortactin connection, some authors have reported contradictory results (Fantozzi et al., 2008).

Cortactin also appears to play a role in oral cancer cell migration through its relationships with the Arp2/3 complex. In a study of HNSCC cell lines, Rothschild et al. (Rothschild et al., 2006) observed binding between cortactin and the Arp2/3 complex, whose activation increased the migration and invasiveness of the cells, finding that this effect disappeared after gene silencing using CTTN siRNA. Cortactin overexpression was largely observed in the peripheral cytoplasm and front cell edge membrane and presumably contributed to cortical actin polymerization, branched actin formation, and the development of actin-based projections, which are all related to increased invasiveness. Similar results were also reported for the overexpression of Arp2/3, Arp2 (Abraham et al., 2001), and ARPC5 (Kinoshita et al., 2012) subunits.

The proinvasive effect of cortactin appears to result from its regulation of MT1-MMP, MMP-2, and MMP-3 in invadopodia, and cortactin downregulation was associated with fewer invadopodia and reduced extracellular matrix degradation capacity in HNSCC cell lines (Clark et al., 2007). In another study, the cortactin expression rate was higher in OSCC 43B cells, which have a fibroblast morphology, than in noninvasive OSCC 43A cell line, characterized by cortactin downregulation and an epithelioid morphology (Takkunen, Hukkanen, Liljeström, Grenman, & Virtanen, 2010).

4.1 | Invasive functions of cortactin not related to invadopodial formation

It has recently been reported that cortactin affects cell migration *via* interactions with proteins without tyrosine kinase receptor functions, including focal adhesion kinase (FAK), a cytoplasmic protein encoded by *PTK2*, characterized as a substrate of Src, whose gene overexpression and amplification appear to play a major in the migration and invasiveness of human cancers (Sulzmaier, Jean, & Schlaepfer, 2014). De Vicente et al. (de Vicente et al., 2012) observed a significant increase in cortactin and FAK expression with a higher degree of dysplasia in dysplasias that became OSCCs. The synergic effect of these two proteins has been documented in vitro and in vivo in HNSCC models (Hwang et al., 2013; Lin, Sun, & Wu, 2014).

You et al. (You et al., 2012) observed a significant association between cortactin overexpression and E-cadherin downregulation in HNSCC. This suggests that the invasiveness gain related to cortactin functions may be at least in part due to the invasiveness associated with loss of adhesion molecule expression and with the onset of epithelial–mesenchymal transition, for which E-cadherin loss is essential (González-Moles, Ruiz-Ávila, Gil-Montoya, Plaza-Campillo, & Scully, 2014; González-Moles et al., 2004, 2013).

5 | OTHER EMERGING FUNCTIONS OF CORTACTIN IN OSCC

Oncogenic actions other than invasiveness gain have also been reported for cortactin, including increased angiogenesis, cell proliferation, exosome secretion, and effects on the tumor microenvironment. Clark et al. (Clark et al., 2009) reported significantly increased cortactin expression in vascularized areas of HNSCC vs control samples, possibly due to activation of the GEP100-Arf6-AMAP1-cortactin pathway by vascular endothelial growth factor 2 (VEGFR2) (Hashimoto et al., 2011). They also described a significant reduction in the proliferation rate after cortactin knockdown (Clark et al., 2009), likely attributable to cell cycle promotion via the cortactin-RhoA-Skp2 pathway, which has been observed in HNSCC cell lines with 11q13 amplification and cortactin overexpression (Croucher, Rickwood, Tactacan, Musgrove, & Daly, 2010). Skp2 is a component of the SCF ubiquitin ligase complex that marks $p27^{kip1}$ and $p57^{kip2}$ for degradation, with the consequent downregulation of different members of the Cip/Kip family and the promotion of cell cycle S-phase entry (Croucher et al., 2010). Exosomes are vesicles loaded with DNA, RNA, proteins, lipids, and metabolites, which are released in the extracellular space in different cell types and regulate physiological and pathological processes, including cancer (Kalra, Drummen, & Mathivanan, 2016). In a recent study of HNSCC cells, cortactin was found to regulate exosome secretion by binding to the Arp2/3 complex and branched actin, controlling the traffic of vesicles and establishing sites for the docking of multivesicular late endosomes (MVEs) to the plasmatic membrane (Sinha et al., 2016). This process appears to be negatively regulated by coronin 1b and positively regulated by Rab27a, which can inhibit the repression exerted by coronin 1b and restore the cortactin-regulated exosome secretion process downstream (Sinha et al., 2016). The tumor microenvironment comprises noncancerous cells in the tumor (immune cells, fibroblasts, and blood vessel cells), whose dynamic interactions with cancer cells exert oncogenic functions at all stages of carcinogenesis (Balkwill, Capasso, & Hagemann, 2012). There has been little published research on the interactions of cortactin with the tumor microenvironment in OSCC. A recent study of hypopharyngeal carcinoma cells associated the phosphorylation of cortactin by infiltrating neutrophils in the tumor stroma with increased cell migration (Dumitru et al., 2013). Some authors have reported that macrophages in the tumor microenvironment regulate the formation of invadopodia in cancer cells during intravasation, a key event in the metastatic cascade (Fidler, 2003), allowing tumor cells to degrade and pass through barriers during transendothelial migration (Pignatelli et al., 2016; Roh-Johnson et al., 2014). Given the important role of cortactin in invadopodia regulation, further research is warranted on the interaction between the tumor microenvironment and cortactin and its involvement in tumor cell intravasation.

6 | MECHANISMS OF CORTACTIN OVEREXPRESSION IN OSCC

Gene amplification. The main cortactin overexpression mechanism is related to amplification of the *CTTN* gene (*aka EMS1*), which is located in chromosome band 11q13 and encodes cortactin. Amplification of 11q13 and oncogenes in this region is a frequent event in OSCC and essential for OSCC onset and progression (Ramos-García, Ruiz-Ávila, et al., 2017). High *CTTN* mRNA expression and protein overexpression are consistently observed in tumors with gene amplification (Freier et al., 2006, 2010; Huang, Godfrey, Gooding, McCarty, & Gollin, 2006; Järvinen et al., 2008; Liu et al., 2009), indicating that the action of this gene in oral oncogenesis is highly dependent on its amplification rather than other overexpression mechanisms. *CTTN* is amplified in 10 - 57% of OSCC cases (Ambatipudi et al., 2011; Freier et al., 2006, 2010; Huang et al., 2006; van Kempen et al., 2015; Liu et al., 2009; Uchida et al., 2011; Xia et al., 2007) (Table 2) and in 20% of oral potentially malignant disorders (OPMDs) (de Vicente et al., 2012; Xia et al., 2007). However, *CTTN* appears to participate later in the multistep process of oral carcinogenesis in comparison to 11q13 amplicon genes (Ramos-García, Ruiz-Ávila, et al., 2017), with maximum amplification/expression levels in OSCCs that are close to metastasis. *CTTN* has persistently been found co-amplified alongside a series of neighboring genes in the 11q13.2-q13.4 amplicon core, including *CPT1A*, *MRPL21*, *IGHMBP2*, *MRGPRF (aka MRGF)*, *TPCN2*, *MYEOV (aka OCIM)*, *CCND1*, *ORAOV1 (aka TAOS1)*, *FGF19/4/3*, *ANO1 (aka TMEM16A*, *ORAOV2*, *TAOS2* o *DOG1)*, *FADD*, *PPFIA1*, *CTTN*, *SHANK2*, *and NUMA1* (Ramos-García, Ruiz-Ávila, et al., 2017). *CTTN* and *SHANK2* (Freier et al., 2006) may have a cooperative effect on tumor cell motility and invasiveness in OSCC, given that both genes

TABLE 2 Cortactin deregulation in head and neck squamous cell carcinoma

Mechanism of deregulation	Study year	Study population	Sample size	Methods	Clinicopathological associations	Frequency (%)
CTTN amplification						
	Takes et al. (1997)	HNSCC	31	Southern blot (probe U21C8)	N+ status	23.80
	Rodrigo et al. (2000)	HNSCC	104	Differential PCR	High T status N+ status Advanced clinical stage High histological grade Recurrent disease Decreased disease-specific survival	20.19
	Chen et al. (2004)	OSCC	13	Comparative genomic hybridization	N+ status	69.0
	Huang et al. (2006)	OSCC	31	Quantitative microsatellite analysis	N/A	32.25
	Freier et al. (2006)	OSCC	40	Comparative genomic hybridization	N/A	35.0
	Rothschild et al. (2006)	HNSCC	39	Fluorescence in situ hybridization	N+ status	28.20
	Xia et al. (2007)	OSCC	33	Differential PCR	High T status N+ status Advanced clinical stage High histological grade	57.57
	Liu et al. (2009)	OSCC	82	Quantitative PCR	Any association	45.12
	Rodrigo et al. (2009)	HNSCC	202	Differential PCR	High T status N+ status Advanced clinical stage High histological grade Increased recurrence Decreased disease-specific survival	37.12
	Uchida et al. (2011)	OSCC	50	Comparative genomic hybridization	Any association	10.0
	Sugahara et al. (2011)	OSCC	10	Comparative genomic hybridization	N+ status	30.0
	Ambatipudi et al. (2011)	OSCC	60	Comparative genomic hybridization	N/A	47.0
	van Kempen et al. (2015)	OSCC	164	Multiplex ligation probe amplification	N+ status Decreased disease-free survival	14.63
Cortactin overexpression						
	Freier et al. (2006)	OSCC	12	Real-time quantitative PCR	N/A	16.66
	Rothschild et al. (2006)	HNSCC	39	IHQ (monoclonal antibody clone 4F11)	N+ status	28.20

(Continues)

TABLE 2 (Continued)

Mechanism of deregulation	Study year	Study population	Sample size	Methods	Clinicopathological associations	Frequency (%)
	Hofman et al. (2008)	HNSCC	176	IHQ (monoclonal antibody clone 4F11)	Advanced clinical stage High histological grade Increased recurrence Decreased overall survival and disease-free survival	43.75
	Gibcus et al. (2008)	HNSCC	106	IHQ (monoclonal antibody clone 30)	Decreased disease-specific survival	48.11
	Liu et al. (2009)	OSCC	49	IHQ (insufficient information)	N+ status	75.51
	Rodrigo et al. (2009)	HNSCC	86	IHQ (monoclonal antibody Clone 30)	N+ status	56.97
	Yamada et al. (2010)	OSCC	70	IHQ (monoclonal antibody Clone 4D10)	High T status N+ status Invasive pattern of invasion	45.71
	de Vicente et al. (2012)	OSCC	17	IHQ (monoclonal antibody Clone 30)	Increased cancer risk	76.47
	You et al. (2012)	HNSCC	97	IHQ (monoclonal antibody Clone 30)	High T status Increased recurrence and/or metastasis High histological grade Decreased overall survival and relapse-free survival	20.61
	Liu et al. (2016)	OSCC	122	IHQ (polyclonal antibody Clone G-20)	High T status N+ status Advanced clinical stage Increased recurrence Decreased disease-specific survival	53.27
Cortactin splice variants						
SV1-cortactin overexpression	van Rossum et al. (2003)	HNSCC cell lines	15	Real-time semiquantitative PCR	N/A	≈33
SV2-cortactin overexpression	van Rossum et al. (2003)	HNSCC cell lines	15	Real-time semiquantitative PCR	N/A	≈2.5
Mutations						
Affecting proteolysis	No studies	HNSCC	–	–	–	–

Notes. HNSCC: head and neck squamous cell carcinoma; IHQ: immunohistochemistry; N/A: not available; OSCC: oral squamous cell carcinoma; PCR: polymerase chain reaction.

encode proteins associated with the cytoskeleton, which may explain the strong association between chromosome band 11q13 and lymph node metastasis (Freier et al., 2006; Ramos-García, Ruiz-Ávila, et al., 2017). With respect to its possible co-amplification with genes located in chromosomal regions other than 11q13, the *Cancer Genome Atlas* (Cancer Genome Atlas Network, 2015) found co-amplification of *CTTN* and 11q22 with genes included in this region, *YAP1* and *BIRC2*, to be a characteristic feature of smoking-related HNSCC/*HPV*(-). There are also reports on the co-amplification of 11q13 with the 7p11 region, an amplicon that harbors the *EGFR* oncogene (Garnis, Campbell, Zhang, Rosin, & Lam, 2004). *CTTN* amplification may have a direct oncogenic function, being identified by numerous research groups as a crucial event in cortactin-mediated cell migration regulation in OSCC. *CTTN* amplification translates into a major increase in cortactin expression, which has been correlated with a rise in cell migration and invasion in numerous in vitro and in vivo studies of oral carcinogenesis (van Damme et al., 1997; Patel et al., 1996; Rothschild et al., 2006; Schuuring et al., 1993).

Alternative splicing. Besides the canonical shape of cortactin (full-length canonical cortactin protein), two additional isoforms

have been identified as a result of alternative splicing in the maturation of *CTTN* mRNA, SV1-cortactin, and SV2-cortactin (van Rossum et al., 2003), whose structural organization is very similar to the canonical form (Ohoka & Takai, 1998; van Rossum et al., 2003). These isoforms can also bind to F-actin, interact with the Arp2/3 complex, and hence promote actin polymerization. Alternative splicing does not appear to affect the subcellular localization of the resulting isoforms, although these variants were associated with significantly reduced cell migration in comparison with cells that overexpressed the canonical form (van Rossum et al., 2003). SV1-cortactin has been found in OSCC cell lines (van Rossum et al., 2003) (Table 2), suggesting that alternative splicing may be a regulating mechanism of cortactin-mediated cell motility. This event may have oncogenic implications, modulating the functional activity of cortactin, as occurs with other relevant oncogenes in cancer.

7 | CLINICOPATHOLOGICAL AND PROGNOSTIC IMPLICATIONS OF *CTTN*/CORTACTIN IN HUMAN CANCER AND IN OSCC

Gene amplification and cortactin overexpression have both been frequently correlated with parameters that imply a worse prognosis in OSCC, including lymph node involvement, larger tumor size, advanced clinical stage, differentiation degree, poor response to treatment, and reduced survival (Rivera, Oliveira, Costa, De Rossi, & Paes Leme, 2017).

Lymph node metastasis development has attracted particular attention in studies of *CTTN*/cortactin (Chen et al., 2004; van Kempen et al., 2015; Liu et al., 2009; Sugahara et al., 2011; Xia et al., 2007; Yamada et al., 2010). Advanced N+ status has been associated with the overexpression of cortactin (Yamada et al., 2010) and its mRNA (Zhou et al., 2006) in OSCC. Furthermore, DNA copy number gain and *CTTN* amplification have been reported to behave as markers of occult metastasis in stage I and II OSCC (van Kempen et al., 2015). The influence of gene amplification on N+ status was even more marked when *CTTN* was co-amplified with other genes such as *TPCN2* or *MYEOV* (Sugahara et al., 2011). These associations have also been described in HNSCC (Hofman et al., 2008; Rodrigo, García, Ramos, Lazo, & Suárez, 2000; Takes et al., 1997; You et al., 2012) and in breast, lung, colon, and prostate cancers, among others (Chuma et al., 2004; Hirakawa et al., 2009; Hsu et al., 2009; Li et al., 2001, 2008; Nakane et al., 2012; Noh et al., 2013; Tsunoda et al., 2011; Wang et al., 2009), and they appear to result from the aforementioned actions of cortactin on cell migration in cancer.

Various studies have also demonstrated a significant association between *CTTN*/cortactin amplification/overexpression and advanced cancer T stage in OSCC (van Kempen et al., 2015; Liu et al., 2016; Xia et al., 2007) and in HNSCC (Gibcus et al., 2008; Hofman et al., 2008; Rodrigo et al., 2000, 2009; You et al., 2012), HNSCC cell lines and in vivo models (Clark et al., 2009). *CTTN* amplification and cortactin overexpression have been significantly associated with poor differentiation degree in OSCC (Xia et al., 2007) and HNSCC (Hofman et al., 2008; Rodrigo et al., 2000; You et al., 2012) and with reduced survival (Gibcus et al., 2008; Hofman et al., 2008; Rodrigo et al., 2000, 2009; You et al., 2012).

8 | IMPLICATIONS OF CTTN/CORTACTIN IN OPMDS

The possible participation of cortactin has been postulated in early stages of OSCC and HNSCC, and a marked increase in cortactin expression has been observed in OPMDs (Liu et al., 2016; de Vicente et al., 2012), oral dysplasias (de Vicente et al., 2012), and laryngeal dysplasias (Rodrigo et al., 2011). *CTTN* amplification has also been reported in early stages of HNSCC at rates ranging between 8 and 20% (Rodrigo et al., 2009, 2011; Xia et al., 2007). With respect to the timing of its participation in head and neck carcinogenesis, *CTTN*/cortactin amplification/overexpression has been described at all pre-invasive stages, from epithelial hyperplasia to severe dysplasia/carcinoma in situ (Hsu et al., 2008; Liu et al., 2016; Rodrigo et al., 2009; Xia et al., 2007). Cortactin expression appears to increase with a higher degree of epithelial dysplasia, suggesting that this protein may be a predictor of cancer risk (Rodrigo et al., 2009, 2011; de Vicente et al., 2012).

9 | CTTN/CORTACTIN AS THERAPEUTIC TARGET IN OSCC

This review confirms the high incidence of *CTTN* gene amplification and aberrant cortactin expression in numerous human cancers, including OSCC, and they appear to play an essential role in cell migration gain and in lymph node involvement (N+ status) and distant metastasis (M+ status). Some authors have demonstrated the sensitivity of cancer cells, mainly those with a migratory phenotype, to *CTTN*/cortactin inhibition, which has prompted research into CTTN/cortactin as therapeutic target.

As depicted in Figure 4, strategies for CTTN/cortactin targeting in OSCC treatment can be classified as: (1) direct cortactin inhibition, (2) action on cortactin expression regulation pathways, (3) action on chromosome band 11q13, and (4) a combination of these actions with other antitumor drugs.

1. Direct cortactin inhibition. No inhibitory molecules have been designed that selectively target CTTN/cortactin, probably attributable to the lack of enzymatic activity and the intracellular localization of this complex. However, in vitro CTTN/cortactin knockdown appears to reduce cell migration. Yamada et al. (Yamada et al., 2010) evaluated the effects of silencing cortactin mRNA in the OSCC SAS cell line, finding a marked reduction of 13.2% to 0.02% in the invasion index of these cells. Rothschild et al. (Rothschild et al., 2006) also reported that the downregulation of cortactin expression levels by siRNA in HNSCC cell

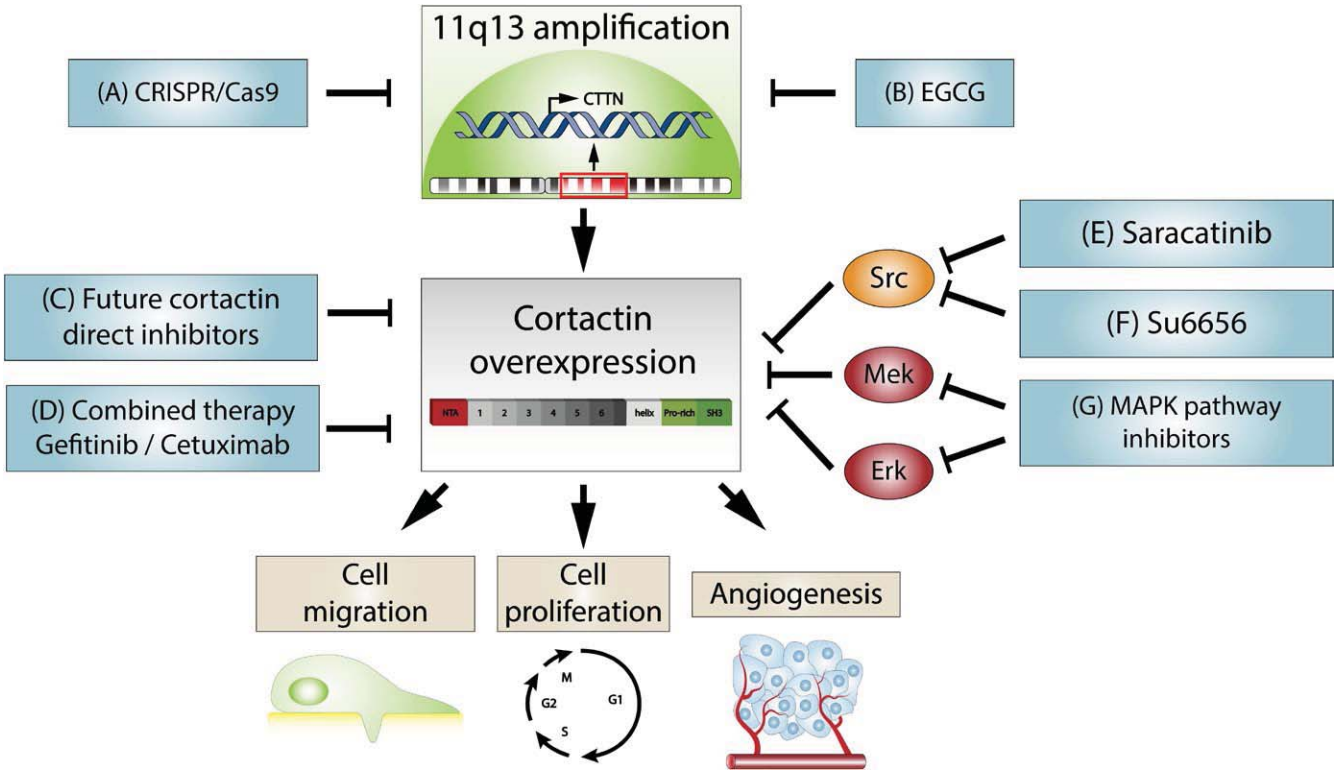

FIGURE 4 Cortactin as therapeutic target in oral cancer. Suggestions for future research lines. Observations in cell lines and animal studies have suggested that cortactin targeting has therapeutic potential. Further research on unexplored aspects of cortactin is warranted, given evidence on the oncogenic functions of this protein. One therapeutic approach would be to target genes amplified in chromosome band 11q13. The following strategies have been proposed: (a) Elimination of amplified CTTN gene by CRISPR/Cas9 genome edition technology (Doudna & Charpentier, 2014; Jubair & McMillan, 2017); (b) the independent or cooperative repression of oncogenes harbored by amplicon 11q13 (CTTN, CCND1, and FADD) using epigallocatechin-3 gallate (Alway et al., 2014; Hwang et al., 2013; Ramos-García, Gil-Montoya, et al., 2017); (c) the development and utilization of selective cortactin inhibitor molecules, given the high incidence of cortactin overexpression in HNSCC and OSCC; (d) the administration of anti-EGFR drugs (e.g., cetuximab or gefitinib), based on reports of EGFR involvement in pathways that oncogenically activate cortactin (EGFR-Src), and of drugs aimed at co-amplification of their respective chromosome bands (7p11 and 11q13) (Garnis et al., 2004), with co-expression of their products (Hofman et al., 2008); in fact, combined therapy with gefitinib and future cortactin inhibitory molecules (b+c) may be relevant, given the known involvement of cortactin in acquired HNSCC treatment resistance (Timpson et al., 2007), while a further option would be to target pathways that regulate oncogenic cortactin functions; (e) the utilization of saracatinib, which selectively inhibits Src activity and appears to reduce Src activation and inhibit that of its substrates downstream, blocking cortactin phosphorylation in Tyr421 (Ammer et al., 2009); saracatinib has been found to act dose-dependently and reduce cortactin expression levels at high doses (0.5–1 μM) (Ammer et al., 2009); (f) the utilization of SU6656, another Src-selective inhibitor, which also suppresses the invadopodial function in HNSCC, likely by blocking the EGFR-Src-cortactin-invadopodia pathway (Hayes et al., 2013); and (g) administration of inhibitors of the MAPK pathway, for example, PD98059, to block members of this pathway that interact with cortactin, for example, MEK and Erk (Campbell et al., 1999; van Damme et al., 1997; Hayes et al., 2013)

lines with amplified CTTN (UMSCC19, UMSCC2, and MSK921) significantly reduced the invasiveness and migration of the cells. In addition, the use of inhibitor molecules that selectively target its different binding companions ablated protein–protein interactions (e.g., saracatinib, Su6656, or PD98059; see below). We reviewed current clinical trials and found no trial evaluating CTTN/cortactin (Source: clinicaltrials.gov) except for a study of its expression in esophageal SCC (NCT01240369), which is limited by its observational cross-sectional design. A search of patents on direct cortactin inhibition (Source: patentscope.wipo.int) revealed a recent filing (CN106916816) on the use of a siRNA molecule that multitargets CTTN/cortactin and appears to stably and adequately inhibit its expression in a lung cancer cell line. In 2005, another patent (JP2007074989) described a polypeptide inhibitor of cortactin–AMAP1 binding in invasive breast cancer, and the authors (Hashimoto et al., 2006) subsequently reported that blockade of this interaction by P4-TAT and UCS15A peptides can inhibit the invasiveness of numerous breast/lung cancer and glioblastoma cell lines.

2. Targeting of cortactin expression regulation pathways. To date, research has centered on the upstream inhibition of cortactin expression regulators *via* the EGFR-Src-cortactin signaling pathway. Although some publications describe cortactin as an upstream substrate of the MAPK pathway (via Ras-Raf-MEK-cortactin and *via* Ras-Raf-MEK-Erk-cortactin), there has been no evaluation of the effects on cortactin of the suppression of this pathway by

inhibitory molecules in oral carcinogenesis. Rothschild et al. (Rothschild et al., 2006) achieved HNSCC cell migration inhibition using gefitinib, a selective EGFR inhibitor molecule, and this inhibition was correlated with phosphorylation downregulation in cortactin tyrosine residues and with blockade of the EGFR-Src-cortactin pathway. They also found that the degree of gefitinib-mediated motility inhibition was inversely proportional to cortactin expression levels. In studies of HNSCC cell lines and in vivo models, Ammer et al. (Ammer et al., 2009) observed that administration of saracatinib, a selective inhibitor of Src activity, dose-dependently reduced Src activation and inhibited activation of its substrates downstream, blocking cortactin phosphorylation in Tyr421; these authors reported a reduction in cortactin expression at high doses (0.5–1 μM). Saracatinib was also reported to inhibit invadopodial formation, accompanied by a reduction in membrane cortactin levels (Ammer et al., 2009). More recently, the same group (Hayes et al., 2013) observed that EGFR-Src pathway blockade by gefitinib, saracatinib, and SU6656, another selective Src inhibitor suppressed the invadopodial function in HNSCC cell lines, probably by blocking the EGFR-Src-cortactin-invadopodia pathway. The clinical trial registry contains a phase II trial (NCT00513435) on treatment with saracatinib (175 mg/day/8 weeks) in a small sample of patients (n = 9) with recurrent or metastatic HNSCC. Although the tolerability of saracatinib was good, it did not prove clinically effective (Fury et al., 2011). Huang et al. (Hwang et al., 2013) successfully used epigallocatechin-3 gallate to inhibit the migration and invasion of OSCC cells in vitro and in vivo (athymic nude mice). They described its action as being in part due to the downregulation of Src and cortactin, which promoted actin cytoskeleton remodeling and the functional repression of invadopodial formation, attributing the suppression of cortactin to an upstream regulatory effect (Src-cortactin pathway). With regard to possible Src-mediated cortactin inhibition, both proteins are known to interact directly through the Src recombinant SH2 domain, and this interaction can be prevented by the presence of ligands that contain the specific sequence of binding with the SH2 domain of Src -pTyr-Glu-Glu-Ile [pYEEI]- (Nam et al., 2004). Further research is warranted on the design of inhibitors that include this tetrapeptide.

3. Targeting of chromosome band 11q13. As already noted, co-amplification of an array of neighboring genes in 11q13 is a frequent finding in OSCC (Ramos-García, Ruiz-Ávila, et al., 2017). The oncogenes in this amplicon (*CCND1*, *CTTN*, *FADD*, *FGF3/4*, *ORAOV1*, *ANO1*, among others) may explain the influence of 11q13 amplification on different clinicopathological characteristics of OSCC, including lymph node involvement, poor tumor differentiation, or poor survival. One possible therapeutic approach could be the utilization of CRISPR/Cas9 genome edition technology to eliminate the *CTTN* gene. CRISPR-associated protein Cas9 is an endonuclease that uses a guide sequence within an RNA duplex (tracrRNA:crRNA) to form base pairs with DNA target sequences, allowing Cas9 to introduce a site-specific DBS into the DNA (Doudna & Charpentier, 2014). The multiple advantages of this system include the simplicity of its programming and the efficacy and precision of its targeting, making it therapeutically plausible to edit, modify, regulate, and label genomic loci in a wide range of cells and organisms (Doudna & Charpentier, 2014). Future studies are warranted on the utilization of this technology against *CTTN* gene amplification in OSCC, given the resulting overexpression of cortactin, a key oncoprotein in the migration of OSCC cells. According to the concept of "oncogenic addiction" (Weinstein & Joe, 2006), cancer cells often acquire an appreciable dependence on certain oncoproteins/oncogenes, allowing them to maintain an oncogenic phenotype and survive. This concept is especially relevant in relation to *CTTN*/cortactin in OSCC, because inactivation of an isolated oncoprotein can suffice to block the proliferation and survival of cancer cells, despite multiple summative oncogenic events. Hence, the utilization of CRISPR/Cas9 technology against certain oncogenes (e.g., *CTTN*) shows considerable promise (Jubair & McMillan, 2017). However, it has not been elucidated whether this influence results from individual actions of these oncogenes or from their cooperative effects. It appears important to evaluate the impact on HNSCC of the simultaneous inhibition of the different members of the 11q13 amplicon, which might be an effective therapeutic strategy, but no drugs have been designed for this purpose to date. The effects of epigallocatechin-3 gallate on cortactin reported above (Hwang et al., 2013) were attributed to the upstream inhibition of Src, but this drug has also proven active against *CCND1*/cyclin D1, the most widely studied complex of 11q13 amplicon to date (Ramos-García, Gil-Montoya, et al., 2017, Ramos-García, Ruiz-Ávila, et al. 2017). Hence, the inhibitory activity of epigallocatechin-3 gallate on cortactin *via* the Src pathway may not be the sole suppressor mechanism (Hwang et al., 2013), which may plausibly involve cooperative repression of the different oncogenes in the 11q13 amplicon. Thus, Alway et al. (Alway, Bennett, Wilson, Edens, & Pereira, 2014) reported a marked downregulation of *FADD* in vivo after treatment with epigallocatechin-3 gallate, and *CCND1*, *CTTN*, and *FADD* have been proposed as candidate amplicon drivers of the amplification of chromosome band 11q13 in OSCC (Ramos-García, Ruiz-Ávila, et al., 2017).

4. Combined therapy. The administration of a combination of drugs that act on cortactin with other antitumor drugs may be justified by the influence of *CTTN*/cortactin amplification on the treatment resistance of tumor cells (Albertson, 2006). Thus, *CTTN* amplification may confer gefitinib resistance, and Timpson et al. (Timpson et al., 2007) found that the aberrant expression of cortactin enhanced resistance to gefitinib-based treatment in HNSCC cell lines. Emerging evidence of resistance to treatment suggests that the combination of gefitinib with therapeutic agents that target cortactin may have synergetic effects. This would require the previous selection of patients with cortactin overexpression/*CTTN* amplification. This therapeutic modality is showing promise in the case of cyclin D1/*CCDN1* in HNSCC and may also be of interest for *CTTN*/cortactin. Finally, combined therapy may also be relevant due to the possible upstream regulation of cortactin *via*

EGFR, and the inhibition of both EGFR and cortactin may have synergic effects. EGFR may be a priority target among upstream cortactin expression regulators, given its known participation in oral oncogenesis through the co-amplification of its respective chromosome bands, 7p11 and 11q13 (Garnis et al., 2004), and the co-expression of its products in OSCC (Hofman et al., 2008). Currently, the EGFR inhibitor cetuximab is the first selective agent approved by the FDA for HNSCC treatment.

10 | REFLECTIONS AND FUTURE RESEARCH LINES

There is a need for further research on key aspects of cortactin biopathology with a likely influence on oral oncogenesis. Thus, an alternative splicing mechanism generated two little-known cortactin isoforms, SV1 and SV2, which have been related to oral oncogenesis in isolated studies (van Rossum et al., 2003). In addition, cortactin degradation processes regulated by calpain 2, which are also poorly understood, appear to be important to attain the intracellular levels of cortactin required to exert its oncogenic actions. Future studies on calpain2/cortactin interactions are therefore warranted, characterizing other negative regulators of cortactin expression and examining the ubiquitination and proteomic degradation of the complex, among other issues. There is also a need to improve characterization of the regulators of cortactin translocation to the cell periphery, which is essential to form the protrusive structures required for cell migration. Further knowledge is also required on the relationship between cortactin and the Arp2/3 complex, which is much less known in comparison to the interactions between cortactin and Src and is likely to promote the formation of these structures through its influence on F-actin. Increased research is warranted on the interactions of cortactin with numerous proteins. Cortactin is a substrate of various proteins with tyrosine kinase (e.g., Abl, Syk, and Fer), serine/threonine kinase (e.g., different members of the MAPK pathway), histone deacetylase (HDAC), and histone acetyltransferase (HAT) activities, and it directly interacts with numerous proteins *via* its SH3 domain (e.g., CortBP1, WASL, WIPF1, dynamin2, MIM1, Fgd1, EC-MLCK, Hip1R). Other molecules with which cortactin can bind include CD44, ZO-1, p120-catenin, B-catenin, E-cadherin, FAK, vinculin, Alix, talin, paxillin, and ASAP-1, among others.

11 | CONCLUSIONS

CTTN gene amplification and the overexpression of its product cortactin are frequent and relevant findings in human carcinogenesis, particularly in oral carcinogenesis. The amplification of chromosome band 11q13 is a key event in tumor onset and progression and represents the cortactin overexpression mechanism *par excellence* in OSCC. The downregulation of cortactin expression can promote cell migration in cancer by different mechanisms, including F-actin network regulation *via* the Arp2/3 complex, post-translational modifications of cortactin regulated by proteins with varied enzymatic activity (e.g., Src), direct interaction with different binding partners *via* its SH3 domain (e.g., dynamin2), and the regulation of actin-based protrusive structures (lamellipodia and invadopodia). However, the best characterized mechanisms in oral carcinogenesis are phosphorylation *via* Src and the regulation of invadopodial formation and persistence, which produce the increased cell migration necessary for acquisition of a metastasizing invasive phenotype. *CTTN*/cortactin amplification/overexpression in OSCC patients has been associated with clinicopathological parameters that imply a poor prognosis, notably locoregional lymph node involvement, which may be related to the regulation of cell migration by cortactin. Although cortactin expression is highest in late stages of oral carcinogenesis, some publications support the possible participation of cortactin in early stages of oral and head and neck carcinogenesis, given its null expression in healthy tissue. Finally, cortactin represents an attractive therapeutic target in human cancer, particularly in OSCC and HNSCC.

CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTIONS

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How to cite this article: Ramos-García P, González-Moles MÁ, González-Ruiz L, et al. An update of knowledge on cortactin as a metastatic driver and potential therapeutic target in oral squamous cell carcinoma. *Oral Dis*. 2018;00:1–23. https://doi.org/10.1111/odi.12913

Oral Oncology 83 (2018) 96–106

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Review

Prognostic and clinicopathological significance of cyclin D1 expression in oral squamous cell carcinoma: A systematic review and meta-analysis

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ARTICLE INFO

Keywords:
Cyclin D1
CCND1
Oral cancer
Prognosis
Meta-analysis

ABSTRACT

Objectives: To evaluate the prognostic significance of cyclin D1 (CD1) overexpression in OSCC.
Material and methods: We searched studies published before August 2017 (Pubmed, Embase, Web of Science, Scopus). We evaluated the quality of the studies included (Quality in Prognosis Studies [QUIPS] tool). The impact of CD1 overexpression on overall survival and disease-free survival, T status, N status, stage, and histological degree was meta-analyzed. We analyzed heterogeneity among studies, conducted sensitivity analyses, analyzed small-study effects, and conducted subgroup analyses.
Results: 31 studies (2942 patients) met inclusion criteria. Qualitative evaluation demonstrated that not all studies were performed with the same rigor, finding the greatest risk of bias in the study confounding domain. Quantitative evaluation showed that CD1 overexpression had a strong statistical association with worse overall survival (HR = 2.00, 95% CI = 1.59–2.51, $p < 0.001$), worse disease-free survival (HR = 1.46, 95% CI = 1.13–1.87, $p = 0.003$), higher T status (OR = 1.51, 95% CI = 1.07–2.13, $p = 0.02$), N+ status (OR = 2.16, 95% CI = 1.60–2.92, $p < 0.001$), advanced stage (OR = 1.44, 95% CI = 1.15–1.81, $p = 0.002$), and high histological grade (OR = 1.60, 95% CI = 1.12–2.29, $p = 0.010$). We observed heterogeneity in all parameters except for disease-free survival and clinical stage. We found effect of small studies on T and N status. The tonguel SCC subgroup showed the strongest association between CD1 overexpression and worse development. In addition, application of a cutoff point ≥10% tumor cells with nuclear CD1 expression maintained most of the significant associations reported.
Conclusions: These findings indicate that immunohistochemical assessment of CD1 overexpression may be useful as a prognostic biomarker for OSCC.

Introduction

Oral cancer has a worldwide incidence of 300,400 cases and is responsible for 145,400 deaths a year (GLOBOCAN, IARC, WHO) [1]. Oral squamous cell carcinoma (OSCC) represents around 90% of malignant oral neoplasms [2] and has a 5-year survival rate of 50–60% [2,3]. Prediction of the prognosis is of major importance and is usually based on the Tumor Node Metastasis (TNM) staging system, with N+ status and the presence of extracapsular spread predicting a worse prognosis [4,5]. The prognostic value of molecular biomarkers has attracted considerable research interest [6,7], and evidence has accumulated on a key role for cyclin D1 (CD1) in oral oncogenesis [8]. CD1 is encoded by the CCND1 gene in chromosomal band 11q13 [9] and promotes G1 cell cycle progression, regulating cell proliferation [10]. CD1 functions that have emerged over the past few years include cell growth regulation, mitochondrial activity modulation, DNA repair, and cell migration control [8,11]. The frequent amplification and overexpression of the CCND1 gene and its CD1 protein [8,9,12] has been strongly implicated in the development of breast, lung, and colon cancers, melanoma, and head and neck squamous cell carcinomas, including OSCC [8,13]. Since its first description [14], numerous publications have explored a possible relationship between CD1 expression and OSCC prognosis [8,9], associating its overexpression with risk factors for a poor prognosis, including N+ and T status, advanced

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https://doi.org/10.1016/j.oraloncology.2018.06.007
Received 4 April 2018; Received in revised form 7 June 2018; Accepted 9 June 2018
Available online 18 June 2018

clinical stage, undifferentiated tumor, and reduced survival [8]). Out of a panel of candidate biomarkers of oral carcinogenesis, one of the best performances was observed for CD1 [8,15], but its prognostic value in OSCC remains controversial [8] and it is not utilized as a standardized marker in the clinical setting. In this systematic review and meta-analysis, we have carried out qualitative and quantitative analyses of scientific evidence on the prognostic significance of CD1 in OSCC with the objective to establish whether its overexpression can predict the progression of this disease. If this association is confirmed, CD1 expression may be useful in routine clinical practice for the prognosis of patients with OSCC and for therapeutic decision-making, with potential benefits for their survival.

Material and methods

This systematic review and meta-analysis complied with PRISMA guidelines [16] and closely followed the criteria of *Cochrane Prognosis Methods Group* [17], *Cochrane Handbook for Systematic Reviews of Interventions* [18], and *Centre for Reviews and Dissemination (CRD)'s guidance for undertaking reviews in healthcare* [19].

Protocol

In order to minimize the risk of bias and improve the transparency, precision, and integrity of this study, we registered a protocol on its methodology *a priori* in the PROSPERO international prospective register of systematic reviews (www.crd.york.ac.uk/PROSPERO, registration number CRD42018081746) [20]. The protocol adheres to PRISMA-P guidelines to ensure a rigorous approach [21].

Search strategy

We searched Pubmed, Embase, Web of Science, and Scopus databases for studies published at any time before the search date (July 2017). Searches were conducted by combining thesaurus terms used by the databases (e.g., MeSH and EMTREE) with free terms. In order to maximize sensitivity, the search strategy in Pubmed combined the following terms: ("cyclin d1"[MeSH Terms] OR ("cyclin"[All Fields] AND "d1"[All Fields]) OR "cyclin d1"[All Fields] OR "cyclind1"[All Fields] OR "ccnd1"[All Fields] OR "ccnd 1"[All Fields]) AND ("mouth"[MeSH Terms] OR "mouth"[All Fields] OR "oral"[All Fields]) AND ("carcinoma, squamous cell"[MeSH Terms] OR ("carcinoma"[All Fields] AND "squamous"[All Fields] AND "cell"[All Fields]) OR "squamous cell carcinoma"[All Fields]). An equivalent search strategy was adapted to the syntax of each database consulted (see protocol).

We also manually screened the reference lists of retrieved studies for additional relevant studies. All references were managed using software Mendeley v.1.17.10 (Elsevier. Amsterdam, The Netherlands), and duplicate references were eliminated.

Eligibility criteria

Study eligibility criteria were applied independently by two authors (PRG and MAGM). Any discrepancies were resolved by consensus.

Inclusion criteria: (1) Original research studies published in English. (2) Evaluation of CD1 expression using immunohistochemistry (IHC) in human tissues from primary OSCCs. (3) Analysis of the association between CD1 overexpression with at least one of the following clinicopathological and/or prognostic variables: T status, N status, histological grade, clinical stage, overall survival (OS), or disease-free survival (DFS). OS was defined as the time elapsed from date of diagnosis/surgery to date of death by any cause. DFS was defined as the time elapsed from surgery to the detection of locoregional or distant recurrence or to death without recurrence. Given the lack of international consensus standards to define survival endpoints, we included studies that used the direct designation of the aforementioned terms (OS/DFS) or other terms that are defined in the original studies as in the present article (e.g., recurrence-free survival) (4). The names and affiliations of authors and the recruitment period and setting were examined to determine whether studies were conducted in the same study population. In such cases, we included the most recent study or that which published more complete data.

Exclusion criteria were: (1) Reviews, meta-analyses, case reports, editorials, letters, abstracts from scientific meetings, personal opinions or comments, book chapters, and any study in a language other than English. (2) Study with no OSCC cases. (3) *In vitro* or animal studies. (4) Studies using techniques other than IHC or analyzing CCND1 gene alterations alone. (5) Studies with no analysis of relationships with clinicopathological and/or survival variables of interest. (6) Studies with insufficient data to estimate odds ratios (ORs) in analyses of clinicopathological variables, and studies of time-to-event variables alone (OS/DFS) that reported inadequate data for survival analysis, e.g., hazards ratio (HR) with 95% confidence interval (CI).

We selected articles in two phases, first screening the titles and abstracts of retrieved articles in an initial selection, and then reading the complete text of the article selected, excluding articles that did not meet the review eligibility criteria.

Data extraction

Two authors (PRG and MAGM) independently extracted data from the articles selected for reading of the complete texts, completing a data collection form in a standardized manner using Excel v.2015 (Microsoft. Redmond, WA). These data were additionally reviewed by two different authors (LGR and IRA), solving discrepancies by consensus. Data were gathered on the first author, year of publication, study country and continent, sample size, tumor localization, recruitment period, treatment modality, follow-up time, anti-CD1 antibody used, intracellular immunostaining (nuclear/cytoplasmic), cutoff point, CD1 overexpression (high/low), N and T status, histological grade, clinical stage, and survival data (OS and DFS).

Evaluation of quality and risk of bias

Two authors (PRG and MAGM) evaluated the quality of studies and the risk of bias using the Quality in Prognosis Studies (QUIPS) tool of the *Cochrane Prognosis Methods Group* [22], which explores six main potential bias domains: (1) Study participation, (2) Study attrition, (3) Prognostic factor measurement, (4) Outcome measurement, (5) Study confounding, and (6) Statistical analysis and reporting [23]. The risk of bias was evaluated as low, moderate, or high for each domain. Discrepancies were resolved by consensus.

Statistical analysis

CD1 expression was considered as "high" (above cutoff) or "low" (below cutoff). ORs with 95% CIs were calculated to determine the correlation between CD1 expression and clinicopathological variables in patients with OSCC. We used HRs with 95% CIs to estimate the impact of CD1 expression on time-to-event variables (OS and DFS). When reported, HRs and 95% CIs were directly extracted from the original articles. When HRs were determined in univariate and multivariate models, we used data from the multivariate model. When HR data were not reported, these were calculated following the methods of Parmar et al [24] and Tierney et al [25] or, in some studies, relative risk (RR) values and adjusted ORs were extracted as an approximation of the same measure [26]. When only a survival curve was given, data were extracted using Engauge Digitizer 4.1 (open-source digitizing software developed by M. Mitchell).

In the meta-analysis, studies were grouped by association measure. Combined associations were analyzed using both fixed-effect models (Mantel-Haenszel methods and inverse variance) and random-effect

models (DerSimonian and Laird method). Forest plots were created to graphically represent the general effect and for its subsequent analysis. Heterogeneity among studies was evaluated using Cochran's Q test (based on the chi-square test) [27]. Given the low statistical power of this test, $p < 0.1$ was considered significant, assuming apparent heterogeneity and consequently using a random-effect model to calculate combined estimations. We also used Higgins I^2 statistic to quantify the percentage heterogeneity, considering values of 25, 50, and 75% to indicate low, moderate, and high heterogeneity, respectively [27,28].

We also conducted stratified analyses (by continent, intraoral tumor site, anti-CD1 antibody, cutoff point, and immunostaining pattern) to identify possible sources of heterogeneity and to analyze the relationship of CD1 overexpression with prognostic and clinicopathological variables in these subgroups.

We conducted sensitivity analyses, evaluating the influence of each individual study on the estimation of the general effect, thereby testing the reliability of combined results [29]. Accordingly, the meta-analysis was repeated sequentially, omitting one study each time and representing the results graphically. Finally, we constructed funnel plots and used Egger's regression test ($p_{Egger} < 0.1$) to evaluate "small-study" effects, including publication bias [30–32]. Stata version 14.1 (Stata Corp, College Station, TX, USA) was employed for all tests in the meta-analysis, using commands written by the user [33]; $p < 0.05$ was considered significant.

Results

Results of the literature search

The flow diagram in Fig. 1 depicts the review selection process and the results obtained. We retrieved a total of 1786 records published before August 2017: 358 from PubMed, 540 from Embase, 476 from the Web of Science, 412 from Scopus, and 2 from the reference list screening. After eliminating duplicates, 784 studies were considered potentially eligible. After screening their titles and abstracts, 71 were selected for reading of the complete text. After excluding studies that did not meet all eligibility criteria, 31 studies were finally included in the review for qualitative evaluation and quantitative meta-analysis [34–64].

Study characteristics

Table 1 summarizes the characteristics of the 31 selected studies, which reported on a total of 2942 patients with OSCC. Sample sizes ranged between 29 and 290 patients. The studies were conducted in all continents except for Africa and comprised 23 Asian and 8 non-Asian studies (3 in Europe, 3 in South America, 1 in North America, and 1 in Australia). CD1 expression was mainly assessed in the nucleus, although five studies evaluated both nuclear and cytoplasmic immunostaining. The cutoff point for CD1 overexpression varied among studies (range of 1–50%), although 10% was the most frequent choice (in 12 studies). Various anti-CD1 antibodies were used, most commonly P2D11F11, DCS-6, SP4, and 5D4 monoclonal antibodies.

This meta-analysis evaluated the prognostic value of CD1 expression for the OS of 1524 patients with OSCC (enrolled in 15 studies), the DFS of 831 patients (enrolled in 5 studies), the T status (T3/4 *vs.* T1/2) of 1493 patients (enrolled in 15 studies), the N status (N+ *vs.* N−) of 2225 patients (enrolled in 21 studies), the clinical stage (III/IV *vs.* I/II) of 1526 patients (enrolled in 15 studies), and the histological grade (II/III *vs.* I) of 1901 patients (enrolled in 20 studies). It was possible to conduct the meta-analysis using all of these variables because they were each represented by a quantitatively adequate sample.

Qualitative evaluation

The qualitative analysis was conducted using the *QUIPS* tool, which evaluates potential sources of bias in six domains (Fig. 2):

Study participation. The risk of this bias was high in 42% of the reviewed studies, moderate in 39%, and low in 19% (Fig. 2). The most frequent biases were the inadequate description of patient characteristics (age, sex, etc.), failure to report the study period or place of recruitment, and the inclusion of patients outside the population of interest.

Study attrition. The risk of this bias was high in 58% of the studies, moderate in 26%, and low in 16% (Fig. 2). The most important bias was the failure to report on patients lost to the follow-up. No study described any attempt to gather information on patients who dropped out or on their characteristics or the reasons for this loss to follow-up, which are essential data to evaluate any differences with the characteristics of the final study sample.

Prognostic factor measurement. The risk of this bias was high in 29% of the studies, moderate in 29%, and low in 42% (Fig. 2). The main potential biases were the use of an inappropriate method to measure CD1 expression and the application of inappropriate cutoff points (e.g., use of optimized cutoff points based on data analysis, which can introduce strong biases in research studies [65]). The most frequent potential biases include inadequate information on the IHC technique and/or measurement system used to measure CD1 expression levels. Occasionally, the immunostaining pattern was not specified (nuclear or nuclear/cytoplasmic) or there were no images of the IHC technique.

Outcome measurement. The risk of this bias was high in 42% of the studies, moderate in 42%, and low in 16% (Fig. 2). The most frequent potential biases were: the non-definition of survival parameters, largely attributable to the lack of international consensus on survival endpoint nomenclature); and the failure to correctly report the classification system used (e.g., the edition of the AJCC/UICC TNM staging system, subject to periodic changes [66]) or the method employed to analyze different clinicopathological parameters.

Study confounding. The risk of this bias was moderate in 26% of the studies and low in 74% (Fig. 2), finding the lowest potential bias in this domain. The most frequent potential biases were the failure to consider confounders in the study design or to measure all potential confounders. No study provided *a priori* clear definitions of the potential confounding factors considered or subsequently discussed these factors or the biological mechanisms by which they might influence the impact of CD1 overexpression on study variables.

Statistical analysis and reporting. The risk of this bias was considered to be high in 35% of the reviewed articles, moderate in 52%, and low in 13% (Fig. 2). The main potential biases detected were inappropriate statistical analysis, erroneous data reporting, and the application of incorrect cutoff points. More frequent, although of lesser importance, were selective reporting of results and the lack of adequate information to determine whether analyses (e.g., Kaplan-Meier curves, CIs, etc.) were properly conducted.

Quantitative evaluation (meta-analysis)

Association between CD1 overexpression and survival in patients with OSCC

Overall survival (OS). A low degree of agreement with Higgins cutoff points was found in the reviewed studies ($p = 0.068$, $I^2 = 37.9\%$; Fig. 3A). The random-effect model indicated a statistically significant relationship between CD1 overexpression and poor OS (HR = 2.00, 95% CI = 1.59–2.51, $p < 0.001$; Fig. 3A).

Disease-free survival (DFS). There was no significant heterogeneity among studies ($p = 0.389$, $I^2 = 3.1\%$; Fig. 3A). The fixed-effect model indicated a statistically significant relationship between CD1 overexpression and reduced DFS (HR = 1.46, 95% CI = 1.13–1.87, $p = 0.003$; Fig. 3A).

Association between CD1 overexpression and clinicopathological variables of patients with OSCC

T status. There was a low degree of heterogeneity among studies

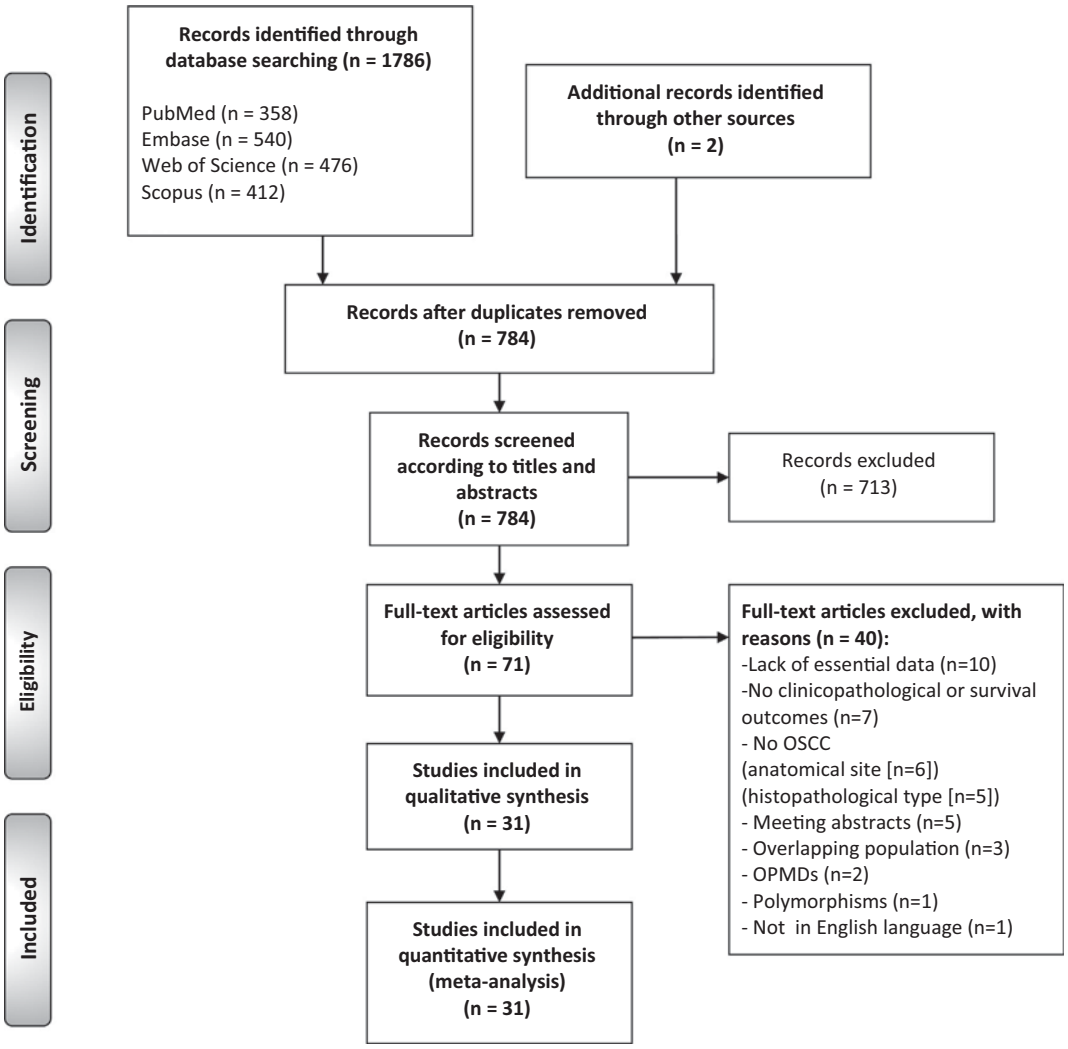

Fig. 1. Flow chart of the process of identification and selection of relevant studies analyzing the prognostic and clinicopathological significance of CD1 in OSCC.

($p = 0.03$, $I^2 = 45\%$; Fig. 3B). The random-effect model indicated a statistically significant relationship between CD1 overexpression and locally advanced carcinomas (T3/4) (OR = 1.51, 95% CI = 1.07–2.13, p = 0.02; Fig. 3B).

N status. There was a low degree of heterogeneity among studies ($p = 0.01$, $I^2 = 46.7\%$; Fig. 3B). The random-effect model indicated a statistically significant relationship between CD1 overexpression and the presence of lymph node metastasis (N+) (OR = 2.16, 95% CI = 1.60–2.92, $p < 0.001$; Fig. 3B).

Clinical stage. There was no significant heterogeneity among the studies ($p = 0.586$, $I^2 = 0.0\%$; Fig. 3C). The fixed-effect model indicated a statistically significant relationship between CD1 overexpression and advanced disease stage (III/IV) (OR = 1.44, 95% CI = 1.15–1.81, p = 0.002; Fig. 3C).

Histological grade. There was moderate heterogeneity among studies ($p = 0.004$, $I^2 = 51.8\%$; Fig. 3C). The random-effect model indicated a statistically significant relationship between CD1 overexpression and more advanced histological grade (II/III) (OR = 1.60, 95% CI = 1.12–2.29, p = 0.010; Fig. 3C).

Quantitative evaluation (secondary analyses)

Sensitivity analysis

The general results did not substantially vary after the sequential repetition of meta-analyses, omitting one study each time. This suggests that the combined estimations reported do not depend on the influence of a particular individual study (Figs. 3A–3C).

Analysis of the effects of small studies

Observation of the asymmetry of the funnel plots constructed (Figs. 3A–3C) and the statistical tests conducted for the same purpose confirm the absence of "small-study" effects on the prognostic (OS [$p_{Egger} = 0.717$], DFS [$p_{Egger} = 0.471$]), clinicopathological (clinical stage [$p_{Egger} = 0.177$], and histological [$p_{Egger} = 0.461$]) variables with the exception of T status ([$p_{Egger} = 0.024$]) and N status [$p_{Egger} = 0.015$], for which bias, e.g., publication bias, could not be ruled out.

Subgroup analysis

In the stratified analysis of *OS* results (Table 2), numerous subgroups maintained a statistically significant association (Asian group [HR = 2.17, 95% CI = 1.69–2.78, $p < 0.001$], Japanese group [HR = 2.01, 95% CI = 1.39–2.92, $p < 0.001$] and non-Japanese group [HR = 2.29, 95% CI = 1.65–3.19, $p < 0.001$], tongue SCC [OR = 2.51, 95% CI = 1.73–3.64, $p < 0.001$], mixed subsites [including intraoral localizations other than the tongue] [HR = 1.86, 95% CI = 1.40–2.48, $p < 0.001$], nuclear IHC staining [HR = 2.03, 95% CI = 1.65–2.50, $p < 0.001$], combined nuclear-cytoplasmic staining [HR = 2.26, 95% CI = 1.10–4.62, p = 0.03], use of monoclonal antibodies P2D11F11 [HR = 2.42, 95% CI = 1.33–4.42, p = 0.004] and

Table 1
Characteristics of the included studies (n = 31).

Study	Year	Country	Sample size	Tumor subsite	Recruitment period	Treatment	Follow-up (months)	Anti-Cyclin D1 antibody	IHC pattern	Cutoff point (%)	Cyclin D1 (+) (%)
Bova *et al.*	1999	Australia	147	Tongue	N/A	Sx, Rt	1–186 (mean 57)	P2D11F11 (monoclonal)	Nuclear	10	68.02
Kuo et al.	1999	Taiwan	88	Tongue, gingiva, floor of mouth, buccal mucosa, lip	1991–1995	Sx, Rt	> 60	N/A	Nuclear	10	44.32
Lam et al.	2000	Hong-Kong	56	Tongue, gingiva, floor of mouth, buccal mucosa, palate, rtm, oropharynx	1988–1996	Rt	> 72	N/A	Nuclear	5	62.50
Mineta et al.	2000	Japan	94	Tongue	1977–1995	Sx	> 60	5D4 (monoclonal)	Nuclear	50	18.08
Smith et al.	2001	USA	56	Oral cavity and oropharynx	1981–1992	Sx, Rt	> 72	A-12 (monoclonal)	Nuclear	5	19.64
Goto et al.	2002	Japan	41	Tongue	1981–1998	N/A	2–133 (mean 36.3)	DCS-6 (monoclonal)	Nuclear	34	34.15
De Vicente et al.	2002	Spain	35	Tongue, gingiva, floor of mouth, buccal mucosa, rtm, palate,	1990–1999	Sx, Rt	6–107 (mean 68)	P2D11F11 DCS-6 (monoclonals)	Nuclear	50	17.14
Vora et al.	2003	India	84	Tongue	1986–1990	Sx, Rt, Ct	30–50	P2D11F11 (monoclonal)	Nuclear Cytoplasmic	1	61.90
Miyamoto et al.	2003	Japan	41	Tongue, gingiva, floor of mouth, buccal mucosa,	1999–2001	Sx	7.7–39.3 (median 25.4)	DCS-6 (monoclonal)	N/A	10	65.85
Liu et al.	2004	Taiwan	55	Buccal mucosa	1985–1996	Sx, Rt, Ct	4–147	P2D11F11 (monoclonal)	Nuclear Cytoplasmic	10	32.73
Shiraki et al.	2005	Japan	140	Tongue, gingiva, floor of mouth, buccal mucosa, lip	1986–1998	Sx	5–134 (median 66)	5D4 (monoclonal)	Nuclear	10	38.57
Soni et al.	2005	India	220	Tongue, gingiva, buccal mucosa, lip	1993–1999	Sx, Rt, Ct	94 (median 21)	R-124 (monoclonal)	Nuclear	10	60.9
Tsuzuki et al.	2005	Japan	58	Tongue, gingiva, floor of mouth, buccal mucosa, palate, oropharynx	1984–1996	Sx, Rt, Ct	60	DCS-6 (monoclonal)	N/A	20	34.48
Wang et al.	2006	Japan	30	Tongue	1999–2000	N/A	9–78 (median 52.1)	N/A	Nuclear	10	60.0
Maahs et al.	2007	Brazil	45	Oral cavity	1991–2001	Sx	> 48	N/A	Nuclear	N/A	33.33
Angadi et al.	2007	India	41	Oral caviy	N/A	N/A	N/A	DCS-6 (monoclonal)	Nuclear	N/A	70.73
Shah et al.	2009	India	135	Tongue, buccal mucosa	2000–2003	Sx, Rt, Ct	24	P2D11F11 (monoclonal)	Nuclear Cytoplasmic	10	31.85
Mishra et al.	2009	India	51	Tongue, gingiva, buccal mucosa, lip	N/A	N/A	N/A	A-12 (monoclonal)	Nuclear	N/A	31.37
Yun et al.	2010	Japan	50	Tongue	2004–2006	Sx	12–60 (median 40)	P2D11F11 (monoclonal)	Nuclear	10	58.0
Das et al.	2011	India	45	Tongue, gingiva, floor of mouth, buccal mucosa, rtm, lip	2001–2006	Sx	N/A	5D4 (monoclonal)	Nuclear	5	66.67
Feng et al.	2011	China	217	Tongue, gingiva, floor of mouth, buccal mucosa, palate, oropharynx, nasal sinus	1999–2005	Sx, Rt, Ct	> 100	EPR2241 (monoclonal)	N/A	N/A	N/A
Perisanidis et al.	2012	Austria	111	Oral cavity, oropharynx	2001–2008	Sx, Ct	> 60 (median 48)	SP4 (monoclonal)	N/A	5	27.03
Saawarn et al.	2012	India	40	Tongue, gingiva, buccal mucosa, lip	N/A	N/A	N/A	N/A	Nuclear Cytoplasmic	1	45.0
Huang et al.	2012	Taiwan	264	Tongue, gingiva, floor of mouth, buccal mucosa, rtm, palate, lip	1999–2005	Sx, Rt, Ct	> 120 (median 46.5)	SP4 (monoclonal)	Nuclear	10	36.74
Jia et al.	2013	China	81	Tongue	2008–2011	Sx	9–48 (median 24)	N/A	N/A	25	46.91
Guimarães et al.	2015	Brazil	29	Tongue	2006–2013	Sx, Rt, Ct	5–81 (mean 24.14)	SP4 (monoclonal)	Nuclear	19 cells per field	51.72
Suresh et al.	2015	India	105	Tongue, gingiva, floor of mouth, buccal mucosa	2006–2011	Sx	N/A	EP-12 (monoclonal)	Nuclear	40	44.76
Zhang et al.	2015	China	109	Tongue	2007–2013	Sx	N/A	Sc-753 (Policlonal)	Nuclear	10	68.81
Gupta et al.	2016	India	290	Tongue, gingiva, buccal mucosa, rtm, palate, lip	2009–2012	Rt, Ct	24 (mean 10.84 median 9)	N/A	Nuclear	10	89.31
Silva et al.	2017	Brazil	32	Tongue, gingiva, floor of mouth, rtm, palate	N/A	Sx	12	N/A	Nuclear Cytoplasmic	30	78.12
Noorlag et al.	2017	Netherlands	152	Tongue, floor of mouth	2004–2010	Sx, Rt, Ct	> 24	SP4 (monoclonal)	Nuclear	15	40.79

IHC, immunohistochemistry; N/A, not available; Sx, surgery; Rt, radiotherapy; CT, chemotherapy; rtm, retromolar trigone.

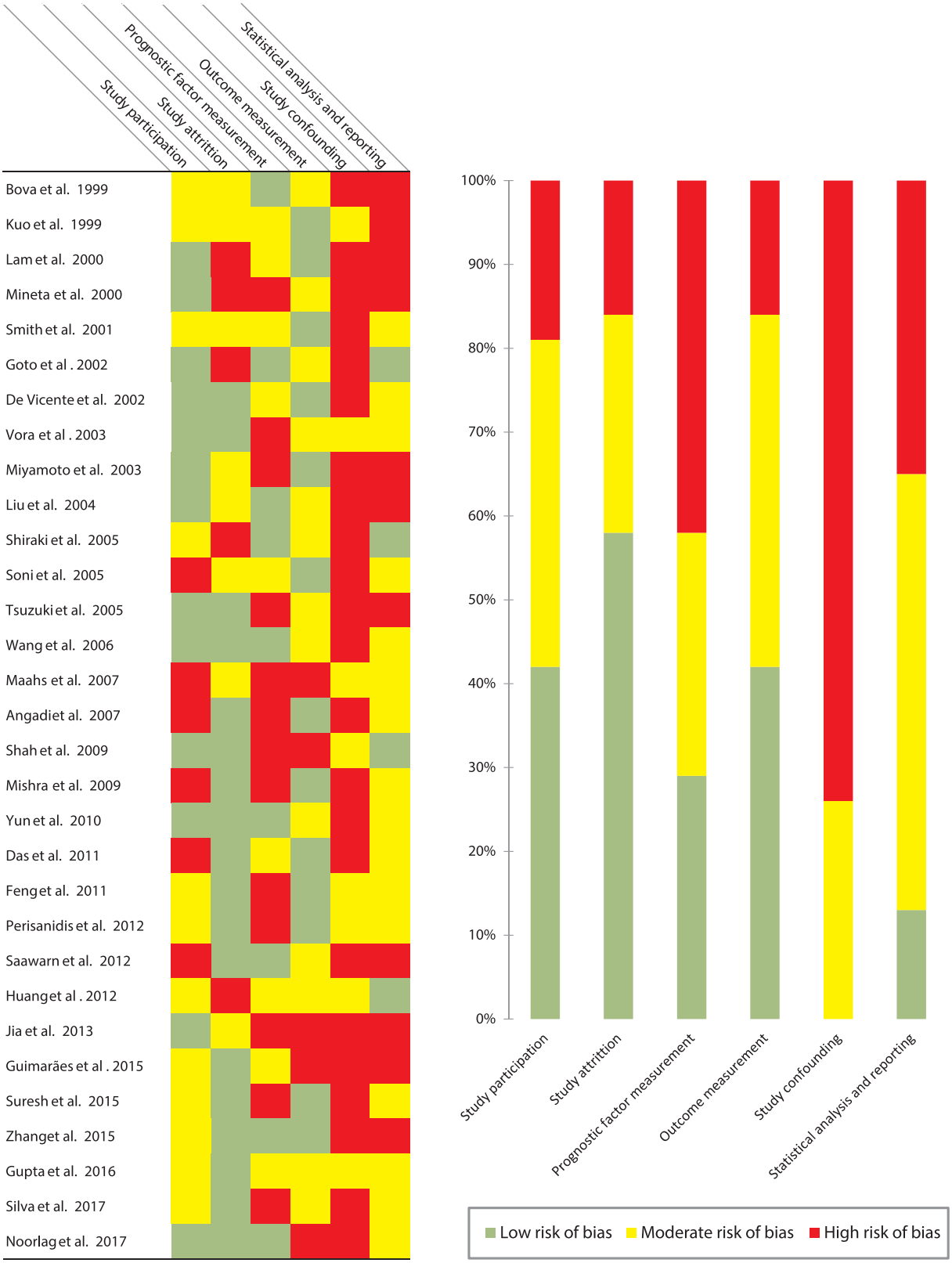

Fig. 2. Evaluation of the risk of bias using the Quality in Prognosis Studies (QUIPS) tool.

5D4 [HR = 2.30, 95% CI = 1.52–3.47, p < 0.001] or other varied antibodies [HR = 2.72, 95% CI = 1.80–4.11, p = 0.01], and cutoff point > 10% [HR = 2.14, 95% CI = 1.41–3.24, p < 0.001] and of 10% [HR = 2.02, 95% CI = 1.62–2.51, p < 0.001]). We found no potential source of severe heterogeneity in subgroup analysis. Some subgroups showed moderate heterogeneity (Asian group [p = 0.08, I^2 = 41.1%], non-Japanese group [p = 0.04, I^2 = 57.4%], mixed subsites [p = 0.03, I^2 = 50.2%], combined nuclear-cytoplasmic IHC staining [p = 0.03, I^2 = 67.0%], and use of monoclonal antibody P2D11F11 [p = 0.008, I^2 = 55.5%]).

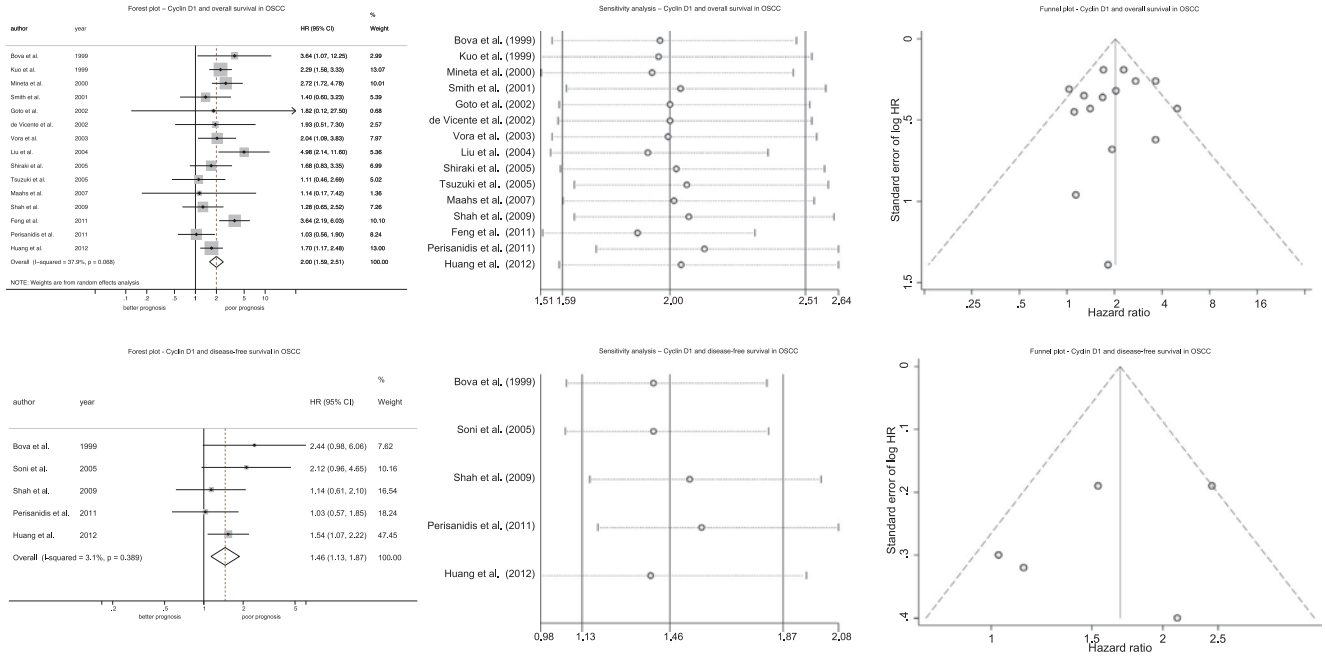

Fig. 3A. Forest plots of the association of cyclin D1 overexpression with overall survival (OS) (random-effect model) and disease-free survival (DFS) (fixed-effect model) parameters in OSCC. Sensitivity analyses graphically represent the results of the meta-analysis of OS and DFS parameters, sequentially omitting one study each time. Funnel plots graphically represent the analysis of "small-study" effects on OS and DFS.

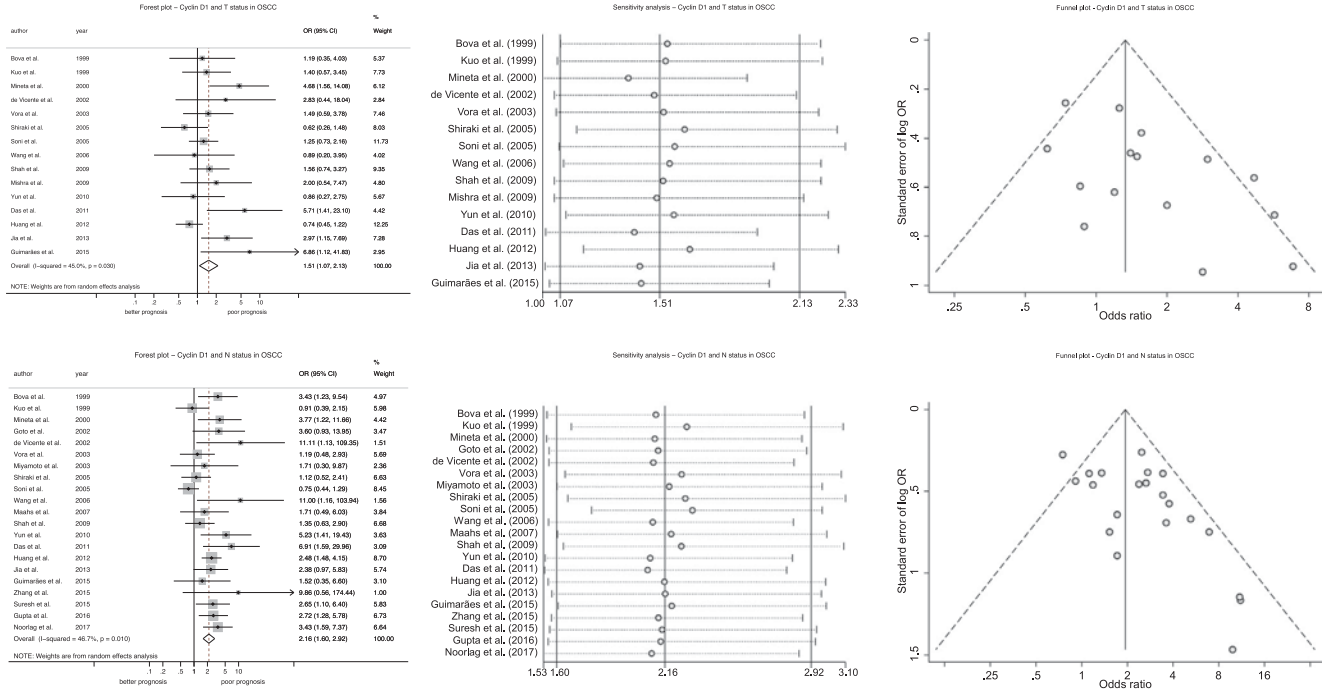

Fig. 3B. Forest plots of the association of cyclin D1 overexpression with T status (random-effect model) and N status (random-effect model) in OSCC. Sensitivity analyses graphically represent the results of the meta-analysis of T and N status parameters, sequentially omitting one study each time. Funnel plots graphically represent the analysis of "small-study" effects on T and N status.

In the stratified analysis of *DFS* results (Table 2), some subgroups maintained statistically significant associations (Asian group [HR = 1.50, 95% CI = 1.12–2.01, p = 0.006], mixed subsites [HR = 1.40, 95% CI = 1.07–1.81, p = 0.01], nuclear IHC staining [HR = 1.71, 95% CI = 1.25–2.33, p = 0.001], and cutoff point of 10% [HR = 1.57, 95% CI = 1.19–2.08, p = 0.001]). We found no potential sources of heterogeneity in the subgroup analysis.

In the stratified analysis of *T status* (Table 2), three subgroups maintained statistically significant associations, non-Japanese group (OR = 1.50, 95% CI = 1.01–2.22, p = 0.04), tongue SCC (OR = 1.93, 95% CI = 1.26–2.97, p = 0.02) and cutoff point > 10% (OR = 3.46, 95% CI = 1.77–6.78, p ≤ 0.001). We observed a significant but moderate degree of heterogeneity in the Japanese subgroup (p = 0.04, I^2 = 65.0%). We also detected important sources of heterogeneity when stratifying by the anti-CD1 antibodies used (SP4 [p = 0.02, I^2 = 81.6%] and 5D4 [p = 0.003, I2 = 82.4%] clones).

In the stratified analysis of *N status* (Table 2), numerous subgroups maintained statistically significant associations (by ethnic group, Asian

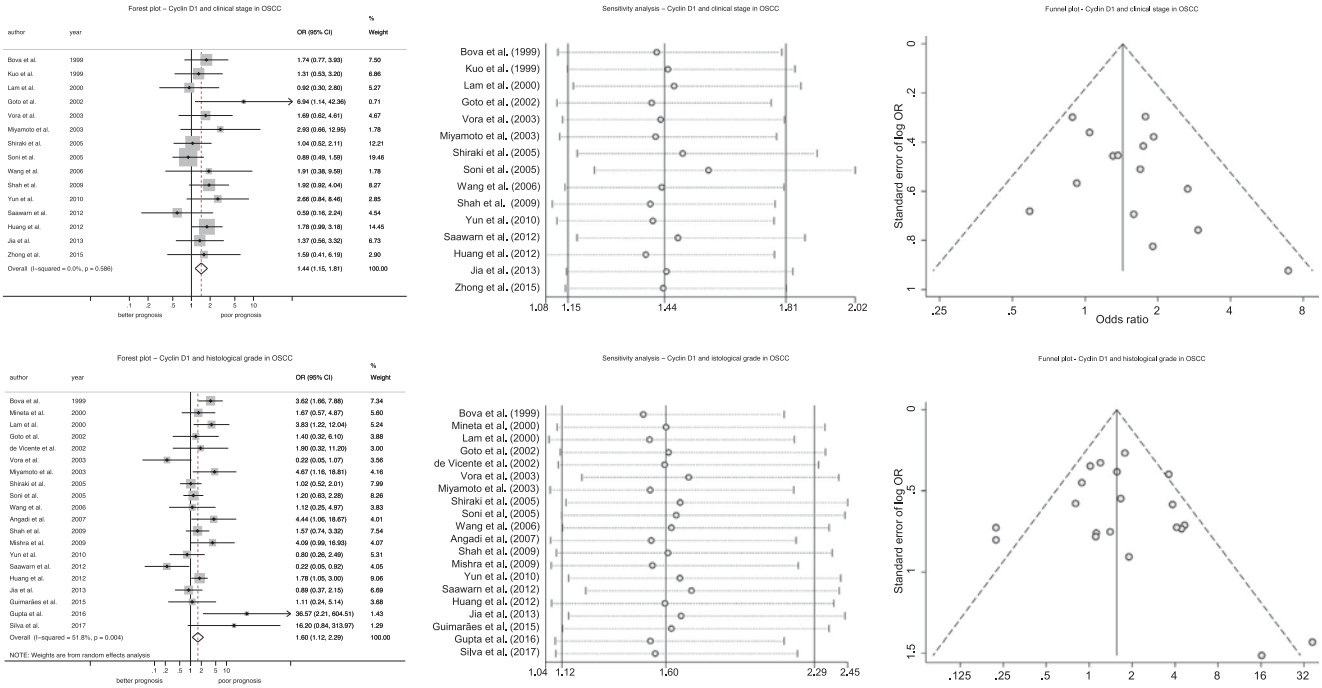

Fig. 3C. Forest plots of the association of cyclin D1 overexpression with clinical stage (fixed effect model) and histological grade (random effect model) in OSCC. Sensitivity analyses graphically represent the results of the meta-analysis of clinical stage and histological grade, sequentially omitting one study each time. Funnel plots graphically represent the analysis of "small-study" effects on clinical stage and histological grade parameters.

[OR = 2.02, 95% CI = 1.43–2.87, p < 0.001] and non-Asian [OR = 3.01, 95% CI = 1.83–4.94, p < 0.001], Japanese group [OR = 2.45, 95% CI = 1.53–3.92, p < 0.001] and non-Japanese group [OR = 1.80, 95% CI = 1.19–2.72, p = 0.005]; by anatomical subsite, tongue [OR = 2.87, 95% CI = 1.94–4.24, p < 0.001], and mixed subsites [OR = 1.88, 95% CI = 1.27–2.77, p = 0.002]; by IHC staining, nuclear pattern [OR = 2.40, 95% CI = 1.66–3.47, p < 0.001]; by antibody, monoclonal antibodies P2D11F11 [OR = 1.98, 95% CI = 1.25–3.12, p = 0.03] and SP4 [OR = 2.62, 95% CI = 1.74–3.94, < 0.001], and by cutoff point, > 10% [OR = 3.20, 95% CI = 2.12–4.84, p < 0.001] and 10% [OR = 1.86, 95% CI = 1.20–2.89, p = 0.006]). We also found a significant but moderate degree of heterogeneity in the following subgroups: non-Japanese group (p = 0.01, I^2 = 58.5%), mixed subsites (p = 0.006, I^2 = 57.8%), nuclear IHC staining (p = 0.004, I^2 = 54.4%), cutoff point < 10 (p = 0.006, I2 = 59.2%), and use of monoclonal antibody 5D4 (p = 0.05, I2 = 67.5%); and a severe degree of heterogeneity in the use of other varied anti-CD1 antibodies (p = 0.008, I^2 = 74.7%) and cutoff point < 10% (p = 0.04, I^2 = 75.2%).

In the stratified analysis of *clinical stage* (Table 2), numerous subgroups maintained statistically significant associations (Asian group [OR = 1.44, 95% CI = 1.15–1.81, p = 0.004], Japanese group [OR = 1.75, 95% CI = 1.07–2.87, p = 0.03] and non-Japanese group [OR = 1.33, 95% CI = 1.01–1.74, p = 0.04], tongue SCC [OR = 1.87, 95% CI = 1.23–2.83, p = 0.003], nuclear IHC staining [OR = 1.43, 95% CI = 1.10–1.86, p = 0.01], antibodies P2D11F11 [OR = 1.91, 95% CI = 1.22–2.98, p = 0.004] and DCS-6 [OR = 4.08, 95% CI = 1.29–12.85, p = 0.01], and cutoff point of 10% [OR = 1.47, 95% CI = 1.13–1.90]). As in the global analysis, we detected no potential sources of heterogeneity in any of the subgroups analyzed.

In the stratified analysis of *histological grade* (Table 2), some subgroups maintained statistically significant associations (non-Asian subgroup [OR = 3.08, 95% CI = 1.67–5.70, p = 0.005], mixed subsites [OR = 1.97, 95% CI = 1.22–3.20, p = 0.006], nuclear IHC staining [OR = 1.91, 95% CI = 1.49–2.45, p = 0.001], antibodies DCS-6 [OR = 3.12, 95% CI = 1.39–7.00, p = 0.007] and SP4 [OR = 1.69, 95% CI = 1.03–2.78, p = 0.04], and cutoff point of 10% [OR = 1.71, 95% CI = 1.12–2.61, p = 0.01]). We found moderate heterogeneity in the anatomical subsite group (tongue SCC [p = 0.07, I^2 = 47.2%] and mixed subsites [p = 0.007, I^2 = 57.5%]), which suggests that this is not a potential source of heterogeneity. We found potential sources of moderate heterogeneity in the Asian group (p = 0.007, I^2 = 52.9%), non-Japanese group (p = 0.001, I^2 = 66.5%), and cutoff point of 10% (p = 0.04, I2 = 50.2%). We also found two sources of important heterogeneity with the use of monoclonal antibody P2D11F11 (p = 0.008, I^2 = 74.7%) and cutoff point < 10% (p = 0.002, I^2 = 84.4%).

Discussion

Our systematic review and meta-analysis of 31 studies with a total population of 2942 patients demonstrate that CD1 overexpression is correlated with worse OS and DFS outcomes and is associated with higher T and N+ status, advanced clinical stage, and high histological grade. Amplification of the CCND1 gene is the main oncogenic mechanism underlying CD1 overexpression in OSCC [9]; however, the alteration of pathways frequently involved in human carcinogenesis (MAPK, Wnt, NF-κβ…) can also transcriptionally activate the CCND1 gene by increasing CD1 overexpression [8,12]. These mechanisms almost inevitably lead to uncontrolled cell proliferation [8,67,68], with the consequent development of larger OSCCs and a higher risk of lymph node involvement. The association between CD1 overexpression and N + status can also be attributed to emerging functions of CD1 related to cell migration regulation [69–71], including its involvement in the development of the actin-based protrusive structures (lamellipodia and invadopodia) necessary for cell migration and metastasis ([72,73]).

Only one meta-analysis has been published to date on the involvement of CD1 in OSCC prognosis (15 studies, 1251 patients) [74], with controversial conclusions, and it only included studies on Asian populations. The present meta-analysis, which used a different analysis technique, includes populations worldwide and contains more studies (n = 31) and more patients (n = 2942), increasing the reliability of the results. Zhao et al. [74] evaluated the heterogeneity of the studies in their meta-analysis using the Q test (p < 0.10) and I^2 test (> 50%), whereas we used the Q test (p < 0.10) to calculate heterogeneity and

the I^2 test to evaluate its magnitude, which is a more sensitive approach to assess studies in a meta-analysis. In addition, the absence of qualitative analysis in the study by Zhao et al. [74] limits the evaluation of biases and final estimator. According to our qualitative evaluation, carried out using the QUIPS tool of the Cochrane Prognosis Methods Group [22], the studies in our meta-analysis had similar experimental designs but were not all conducted with the same rigor. Most potential biases were caused by the failure to consider confounding factors (study confounding domain) and by the application of inappropriate statistical analyses (statistical analysis and reporting domain). The prognostic factor measurement domain also harbored numerous potential biases, mainly due to the use of inappropriate CD1 expression measurement methods and cutoff points. Future studies on the prognostic value of CD1 in OSCC should consider the potential biases reported in this meta-analysis, using items in the QUIPS tool to improve and standardize future research [22].

The heterogeneity of the studies in our meta-analysis was generally poor, especially in relation to OS outcomes and T and N status. There was moderate heterogeneity in histological grade analysis but none in DFS or clinical stage. In our analysis of the impact of CD1 overexpression on subgroups and our evaluation of the potential sources of heterogeneity, the patients with SCC of the tongue showed the strongest association between CD1 overexpression and a worse prognosis for OS (HR = 2.51, 95% CI = 1.73–3.64, $p < 0.001$ *vs.* HR = 1.86, 95% CI = 1.40–2.48, $p < 0.001$) and, especially, for T status (OR = 1.97, 95% CI = 1.26–2.97, $p = 0.02$ *vs.* OR = 1.27, 95% CI = 0.85–1.95, $p = 0.25$), N status (OR = 2.87, 95% CI = 1.94–4.24, $p < 0.001$ *vs.* OR = 1.88, 95% CI = 1.27–2.77, $p = 0.002$), and clinical stage (OR = 1.87, 95% CI = 1.23–2.83, $p = 0.003$ *vs.* OR = 1.28, 95% CI = 0.97–1.69, $p = 0.09$). Prognostic OSCC studies have generally described those in the tongue as carrying the worst prognosis due to its lymphatic richness and the frequently late diagnosis, among other factors. It is also now understood that this tumor has differential molecular characteristics with regard to CD1 overexpression [8,75], which behaves as a highly negative prognostic marker at this site. Our meta-analysis demonstrates that patients with tongue SCC can especially benefit from the evaluation of CD1 overexpression in their prognostic assessment. CD1 overexpression also had a particularly negative impact on the OS and DFS of Asian patients and on the T status of non-Japanese Asian populations, probably attributable to the widespread habit of smoking or chewing tobacco or betel in that continent, which would be the main cause of CCND1/CD1 amplification in OSCC [8,9]. Liu et al [76] reported a high rate of CCND1 amplification in OSCC among Asian patients who chewed betel quid. Our subgroup analysis revealed that most of the statistically significant associations were maintained when the cutoff point was ≥10% CD1-positive tumor cells, with the exception of T status, suggesting that this cutoff should be used in the prognostic assessment of individual patients.

The wide range of CD1 expression (18.08–89.31%) reported by the reviewed studies may have various possible explanations. These include geographic variations in the etiological factors associated with oral cancer, as observed for alterations in other genes. Methodological differences may also play a role, and our meta-analysis points to variability in the selection of monoclonal anti-CD1 antibody and in immunohistochemical procedures as potential sources of heterogeneity.

Our meta-analysis has some limitations. First, the restriction to studies published in English means that information published in other languages would have been missed. Second, we included studies that did not directly report HR values in the survival analysis, although this weakness was countered by calculating HRs from the data provided by these studies, following Parmar et al. [24] and Tierney et al [25]. Third, analysis of "small-study" effects showed a slight right skewness of funnel plots for T and N status at visual ($p_{Egger} = 0.024$) and statistical ($p_{Egger} = 0.015$) level, probably attributable to a "small-study" publication bias frequently encountered in the literature on prognostic biomarkers in cancer, where there is a tendency to publish only positive results [77]. Despite these limitations, the results of our meta-analysis are robust, demonstrating a strong statistical association of CD1 overexpression with clinicopathological and survival variables and including a sensitivity analysis.

In conclusion, the consistent findings analyzed in this systematic review and meta-analysis indicate that IHC assessment of CD1 overexpression may be useful as a prognostic biomarker for OSCC. This is especially important in tongue SCC, the most frequent intraoral site that also carries the worst prognosis. According to our findings, cases should be considered positive when the percentage of tumor cells with nuclear CD1 expression is 10% or above. IHC is a simple and inexpensive technique routinely applied in pathology laboratories in an automatized manner. Its application can improve the prognostic evaluation of patients with OSCC and therefore the planning of their treatment, which may have positive repercussions on their survival.

Conflict of interest

None declared

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.oraloncology.2018.06.007.

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Table 2. Sugroups analysis.

Outcomes	Subgroups	No. Of studies	Stat. Model	Pooled data		Heterogeneity		
				OR (95% CI)	*P*	*Q*	P_{het}	I^2 (%)
T status	All	15	R	1.51 (1.07-2.13)	0.02	25.48	0.03	45.0
	Ethnic group							
	Asian	12	R	1.43 (0.99-2.06)	0.053	21.55	0.03	49.0
	Non-Asian	3	F	2.26 (0.94-5.42)	0.10	2.56	0.28	22.0
	Asian populations							
	Non-Japanese	8	R	1.50 (1.01-2.22)	0.04	12.81	0.08	45.3
	Japanese	4	R	1.21 (0.47-3.16)	0.69	8.57	0.04	65.0
	Subsite							
	Tongue	7	F	1.93 (1.26-2.97)	0.02	8.97	0.17	33.1
	Mixed	8	R	1.27 (0.85-1.91)	0.25	12.48	0.09	43.9
	IHC pattern							
	Nucl.	12	R	1.45 (0.95-2.20)	0.08	22.05	0.02	50.1
	Nucl.+cyt.	2	F	1.53 (0.86-2.73)	0.15	0.01	0.94	0.0
	N/A	1	—	—	—	—	—	—
	Antibody							
	P2D11F11	4	F	1.33 (0.83-2.15)	0.23	0.81	0.85	0.0
	DCS-6	0	—	—	—	—	—	—
	SP4	2	R	1.89 (0.22-16.37)	0.56	5.43	0.02	81.6
	N/A	3	—	—	—	—	—	—
	Cutoff point							
	>10	3	F	3.46 (1.77-6.78)	<0.001	0.43	0.80	0.0
	10	8	F	1.01 (0.77-1.32)	0.95	5.30	0.62	0.0
	<10	2	R	2.63 (0.71-9.65)	0.146	2.46	0.12	59.4
	N/A	2	—	—	—	—	—	—

Table 2. Subgroups analysis (Continuation).

Outcomes	Subgroups	No. of studies	Stat. model	Pooled data		Heterogeneity		
				OR (95% CI)	*P*	*Q*	P_{het}	I^2 (%)
N status	All	21	R	2.16 (1.60-2.92)	<0.001	37.51	0.01	46.7
	Ethnic group							
	Asian	16	R	2.02 (1.43-2.87)	<0.001	31.27	0.008	0.0
	Non-Asian	5	F	3.01 (1.83-4.94)	<0.001	3.02	0.55	52.0
	Asian populations							
	Non-Japanese	10	R	1.80 (1.19-2.72)	0.005	21.69	0.01	58.5
	Japanese	6	F	2.45 (1.53-3.92)	<0.001	8.04	0.15	37.8
	Subsite							
	Tongue	7	F	2.87 (1.94-4.24)	<0.001	7.88	0.45	0.0
	Mixed	8	R	1.88 (1.27-2.77)	0.002	26.05	0.006	57.8
	IHC pattern							
	Nucl.	17	R	2.40 (1.66-3.47)	<0.001	35.12	0.004	54.4
	Nucl.+cyt.	2	F	1.28 (0.72-2.30)	0.402	0.05	0.83	0.0
	N/A	2	—	—	—	—	—	—
	Antibody							
	P2D11F11	4	F	1.98 (1.25-3.12)	0.03	5.40	0.14	44.4
	DCS-6	2	F	2.69 (0.92-7.90)	0.07	0.43	0.51	0.0
	SP4	3	F	2.62 (1.74-3.94)	<0.001	1.04	0.59	0.0
	5D4	3	R	2.72 (0.89-8.24)	0.08	6.16	0.05	67.5
	Other	4	R	2.49 (0.73-8.52)	0.15	11.84	0.008	74.7
	N/A	5	—	—	—	—	—	—
	Cutoff point							
	>10	6	F	3.20 (2.12-4.84)	<0.001	1.87	0.87	0.0
	10	11	R	1.86 (1.20-2.89)	0.006	24.50	0.006	59.2
	<10	2	R	2.60 (0.47-14.46)	0.28	4.02	0.04	75.2
	N/A	2	—	—	—	—	—	—

Table 2. Subgroups analysis (continuation).

Outcomes	Subgroups	No. of studies	Stat. model	Pooled data		Heterogeneity		
				OR (95% CI)	*P*	*Q*	P_{het}	I^2 (%)
Clinical stage	All	15	F	1.44 (1.15-1.81)	0.002	12.25	0.59	0.0
	Ethnic group							
	Asian	14	F	1.42 (1.12-1.80)	0.004	12.01	0.53	0.0
	Non-Asian	1	—	1.74 (0.77-3.93)	0.18	—	—	—
	Asian populations							
	Non-Japanese	9	F	1.33 (1.01-1.74)	0.04	5.90	0.66	0.0
	Japanese	5	F	1.75 (1.07-2.87)	0.03	5.27	0.26	24.1
	Subsite							
	Tongue	7	F	1.87 (1.23-2.83)	0.003	2.98	0.81	0.0
	Mixed	8	F	1.28 (0.97-1.69)	0.09	7.05	0.42	0.8
	IHC pattern							
	Nucl.	11	F	1.43 (1.10-1.86)	0.01	9.82	0.46	0.0
	Nucl.+cyt.	3	F	1.52 (0.88-2.60)	0.20	2.37	0.31	15.6
	N/A	1	—	—	—	—	—	—
	Antibody							
	P2D11F11	4	F	1.91 (1.22-2.98)	0.004	0.42	0.94	0.0
	DCS-6	2	F	4.08 (1.29-12.85)	0.01	0.52	0.47	0.0
	SP4	1	—	1.78 (0.99-3.18)	0.05	—	—	—
	5D4	1	—	1.04 (0.52-2.11)	0.91	—	—	—
	Other	2	F	0.98 (0.57-1.66)	0.91	0.60	0.44	0.0
	N/A	5	—	—	—	—	—	—
	Cutoff point							
	>10	2	R	2.53 (0.54-11.85)	0.24	2.50	0.11	60.0
	10	10	F	1.47 (1.13-1.90)	0.005	6.88	0.65	0.0
	<10	3	F	1.07 (0.56-2.03)	0.83	1.65	0.44	0.0

Table 2. Subgroups analysis (continuation).

Outcomes	Subgroups	No. of studies	Stat. model	Pooled data		Heterogeneity		
				OR (95% CI)	*P*	*Q*	P_{het}	I^2 (%)
Histological grade	All	20	R	1.60 (1.12-2.29)	0.01	39.45	0.004	51.8
	Ethnic group							
	Asian	16	R	1.45 (0.99-2.14)	0.057	31.82	0.007	52.9
	Non-Asian	4	F	3.08 (1.67-5.70)	0.005	3.36	0.34	10.6
	Asian populations							
	Non-Japanese	10	R	1.51 (0.86-2.66)	0.15	26.90	0.001	66.5
	Japanese	6	F	1.29 (0.83-1.98)	0.25	4.67	0.46	0.0
	Subsite							
	Tongue	8	R	1.19 (0.67-2.11)	0.54	13.25	0.07	47.2
	Mixed	12	R	1.97 (1.22-3.20)	0.006	25.88	0.007	57.5
	IHC pattern							
	Nucl.	14	F	1.91 (1.49-2.45)	0.001	19.53	0.11	33.4
	Nucl.+cyt.	4	R	0.80 (0.18-3.56)	0.76	12.53	0.006	76.1
	N/A	2	—	—	—	—	—	—
	Antibody							
	P2D11F11	4	R	1.18 (0.44-3.17)	0.739	11.86	0.008	74.7
	DCS-6	3	F	3.12 (1.39-7.00)	0.007	1.69	0.43	0.0
	SP4	2	F	1.69 (1.03-2.78)	0.04	0.32	0.57	0.0
	5D4	2	F	1.17 (0.66-2.08)	0.58	0.58	0.45	0.0
	Other	3	F	1.55 (0.89-2.67)	0.16	2.46	0.29	18.8
	N/A	6	—	—	—	—	—	—
	Cutoff point							
	>10	5	F	1.51 (0.87-2.62)	0.28	3.96	0.41	0.0
	10	9	R	1.71 (1.12-2.61)	0.01	16.06	0.04	50.2
	<10	3	R	0.60 (0.08-4.50)	0.62	12.88	0.002	84.5
	N/A	3	—	—	—	—	—	—

Table 2. Subgroups analysis (continuation).

Outcomes	Subgroups	No. of studies	Stat. model	Pooled data		Heterogeneity		
				HR (95% CI)	*P*	*Q*	P_{het}	I^2 (%)
Overall survival	All	15	R	2.00 (1.59-2.51)	<0.001	22.55	0.07	37.9
	Ethnic group							
	Asian	10	R	2.17 (1.69-2.78)	<0.001	15.29	0.08	41.1
	Non-Asian	5	F	1.39 (0.91-2.11)	0.13	3.60	0.46	0.0
	Asian populations							
	Non-Japanese	6	R	2.29 (1.65-3.19)	<0.001	11.75	0.04	57.4
	Japanese	4	F	2.01 (1.39-2.92)	<0.001	3.34	0.34	10.2
	Subsite							
	Tongue	4	F	2.51 (1.73-3.64)	<0.001	0.92	0.82	0.0
	Mixed	11	R	1.86 (1.40-2.48)	<0.001	20.10	0.03	50.2
	IHC pattern							
	Nucl.	9	F	2.03 (1.65-2.50)	<0.001	4.80	0.78	0.0
	Nucl.+cyt.	3	R	2.26 (1.10-4.62)	0.03	6.07	0.05	67.0
	N/A	3	—	—	—	—	—	—
	Antibody							
	P2D11F11	4	R	2.42 (1.33-4.42)	0.004	6.75	0.08	55.5
	DCS-6	2	F	1.16 (0.50-2.69)	0.72	0.12	0.73	0.0
	SP4	2	F	1.41 (0.87-2.26)	0.16	1.88	0.17	46.7
	5D4	2	F	2.30 (1.52-3.47)	<0.001	1.19	0.27	16.1
	Other	3	F	2.72 (1.80-4.11)	0.01	3.92	0.14	49.0
	N/A	2	—	—	—	—	—	—
	Cutoff point							
	>10	4	F	2.14 (1.41-3.24)	<0.001	3.01	0.39	0.2
	10	6	F	2.02 (1.62-2.51)	<0.001	8.53	0.13	41.4
	<10	3	F	1.43 (0.97-2.11)	0.10	2.34	0.31	14.5
	N/A	2	—	—	—	—	—	—

Table 2. Subgroups analysis (continuation).

Outcomes	Subgroups	No. of studies	Stat. model	Pooled data		Heterogeneity		
				HR (95% CI)	*P*	*Q*	P_{het}	I^2 (%)
Disease-free survival	All	5	F	1.46 (1.13-1.87)	0.004	4.13	0.39	3.1
	Ethnic group							
	Asian	3	F	1.50 (1.12-2-01)	0.006	1.52	0.47	0,0
	Non-Asian	2	R	1.47 (0.64-3.39)	0.361	2.43	1.12	58.8
	Asian populations							
	Non-Japanese	3	F	1.50 (1.12-2-01)	0.006	1.52	0.47	0,0
	Japanese	0	—	—	—	—	—	—
	Subsite							
	Tongue	1	—	2.44 (0.98-6.07)	0.055	—	—	—
	Mixed	4	F	1.40 (1.07-1.81)	0.01	2.79	0.42	0.0
	IHC pattern							
	Nucl.	3	F	1.71 (1.25-2.33)	0.001	1.19	0.55	0.0
	Nucl.+cyt.	1	—	1.14 (0.61-2.12)	0.68	—	—	—
	N/A	1	—	—	—	—	—	—
	Antibody							
	P2D11F11	2	F	1.45 (0.87-2.42)	0.24	1.84	0.17	45.5
	DCS-6	0	—	—	—	—	—	—
	SP4	2	F	1.38 (1.01-1.88)	0.11	130	0.25	22.8
	5D4	0	—	—	—	—	—	—
	Other	1	—	2.12 (0.96-4.67)	0.06	—	—	—
	N/A	0	—	—	—	—	—	—
	Cutoff point							
	10	4	F	1.57 (1.19-2.08)	0.001	2.50	0.48	0.0
	<10	1	—	1.03 (0.57-1.86)	0.92	—	—	—

Stat., statistical; F, fixed-effects model; R, random-effects model; OR, odds ratio; HR, hazard ratio; CI, confidence intervals; Nucl., nuclear; cyt., cytoplasmic; N/A, not available.

Received: 14 August 2017 | Revised: 21 August 2017 | Accepted: 23 August 2017

DOI: 10.1111/odi.12752

PROF SCULLY MEMORIAL ISSUE

WILEY ORAL DISEASES

Significance of cytoplasmic cyclin D1 expression in oral oncogenesis

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Objectives: To examine cytoplasmic cyclin D1 expression levels in oral carcinogenesis and evaluate their possible oncogenic significance and their clinicopathological and prognostic implications.

Materials and Methods: Immunohistochemical analysis of 69 oral squamous cell carcinomas (OSCCs) was performed, revealing 23 with cytoplasmic cyclin D1 expression. We analyzed the association of the percentage of cyclin D1-positive cells and the intensity of expression with TNM classification, tumor stage, differentiation degree, cell morphology, and Ki-67 expression.

Results: Cytoplasmic cyclin D1 expression was associated with advanced tumor stage, poor differentiation, elevated Ki-67 expression, and the presence of invasive cell morphology, indicators of a poor prognosis. An association was observed between nuclear and cytoplasmic expressions of cyclin D1.

Conclusions: Cytoplasmic expression of cyclin D1 appears to possess functions related to increased cell migration and invasion in OSCC.

KEYWORDS

cancer, CCND1, Cyclin D1, oral, oral carcinogenesis

1 | INTRODUCTION

Cyclin D1 (CD1) is a protein encoded by the *CCND1* gene, located in chromosomal band 11q13, which promotes cell cycle progression from G1 to S phase (Sherr & Roberts, 2004). Its expression is frequently altered in human cancer, especially in oral squamous cell carcinoma (OSCC) (Ramos-García, Gil-Montoya et al., 2017). Gene amplification is the most important overexpression mechanism in OSCC, with an amplification rate more than twofold higher than observed in other cancers (Ramos-García, Ruiz-Ávila et al., 2017). CD1 overexpression is often associated with indicators of a poor prognosis, including high T and N stage, advanced stage, and poor differentiation, and with non-response to treatment and reduced survival, suggesting that CD1 may play a key role in oral carcinogenesis (Ramos-García, Gil-Montoya et al., 2017).

Analysis of the oncogenic activity of CD1 overregulation in OSCC has usually been restricted to its nuclear expression, with little research on its cytoplasmic expression. Reports on the possible clinicopathological implications of cytoplasmic CD1 expression in pancreatic, bladder, liver, prostate, thyroid, colon, and ovarian cancers (Comstock, Revelo, Buncher, & Knudsen, 2007; Dhar et al., 1999; Holland et al., 2001; Lebe et al., 2004; Pickett, Agoff, Widman, & Bronner, 2005; Sato et al., 1999; Tut et al., 2001) prompted the present investigation in OSCC. Accordingly, the objectives of this study were to examine cytoplasmic CD1 expression levels in OSCC and evaluate their possible oncogenic significance and clinicopathological and prognostic implications.

2 | MATERIAL AND METHODS

Immunohistochemical expressions of CD1 and Ki-67 were evaluated in 69 OSCCs from patients treated in the Hospital Complex of Jaen (Spain). The study was conducted with the approval of the Ethics and Research Committee at the Faculty of Dentistry, University of Granada (Granada, Spain). Inclusion criteria were

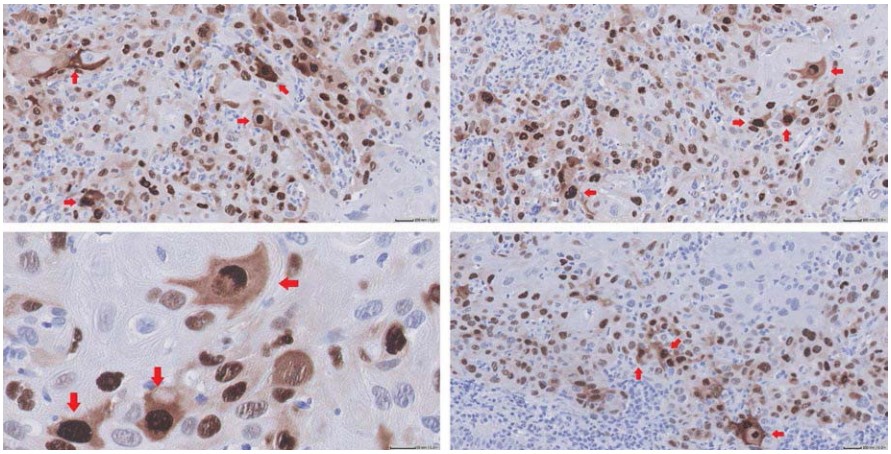

FIGURE 1 Intense nuclear and cytoplasmic CD1 expression in oral squamous cell carcinoma (OSCC). The four panels correspond to the same patient. All of these with an area of 0.191 mm^2 (equivalent to 40× magnification), with the exception of the lower left panel, which has a greater magnification (with an area equivalent to 200×). Red arrows indicate tumor cells with protrusive structures (invasive morphology)

TABLE 1 Description of patients with oral tumors that showed cytoplasmic CD1 expression (*n* = 20)

Variable	*n* (%)
Sex	
Female	4 (20.0)
Male	16 (80.0)
Age (years)	
Range	46-87
mean ± *SD*	63 ± 12
Missing (*n*)	3
Tobacco	
Non-smoker	2 (15.4)
11–20 cig./day	6 (46.2)
>20 cig./day	5 (38.5)
Missing (*n*)	7
Alcohol	
No	2 (16.7)
Yes	10 (83.3)
Missing (*n*)	8
N° tumors	
1	17 (85.0)
3	2 (10.0)
4	1 (5.0)

the availability of data on clinicopathological and outcome variables and an immunohistochemical study of adequate quality. An automated immunohistochemical technique was applied, using cyclin D1 monoclonal rabbit anti-human antibody (Clone EP12) and monoclonal mouse anti-human Ki-67 antigen (Clone MIB-1) (Dako, Carpinteria, CA, USA). The primary antibody was replaced with phosphate-buffered saline for the negative control. Tissue from an OSCC with known CD1 and Ki-67 expressions was used as positive control. The Philips Intelli Site Ultra Fast Scanner system (Philips Digital Pathology Solutions, Best, The Netherlands) was applied to digitalize the slides, and the Philips IMS viewer system (Philips Digital Pathology Solutions) was used to randomly capture four digital images with an area of 0.191 mm^2, equivalent to 40× magnification. Expressions were measured in each area by a semi-automated cell count technique using Adobe Photoshop CC v.2017 software (San Jose, CA, USA). Out of the initial series of 69 OSCCs, those with nuclear and cytoplasmic CD1 expression (*n* = 23: corresponding to 20 patients, three of them had >1 tumor) were elected for this study. Brown labeling was considered positive for both markers. A grid was placed over each area to facilitate an ordered and meticulous count, minimizing possible errors. After obtaining total and positive cell counts, the mean percentage expression was calculated for each tumor tissue area and each case. The intensity of nuclear and cytoplasmic CD1 expression was classified as mild, moderate, or intense. We recorded the presence in each area of cells with a morphology considered to be invasive (Figure 1), defined by fingerlike projections of the cell membrane invaginated among adjacent tumor cells, which corresponded to lamellipodia and invadopodia, actin-based protrusive structures (Chhabra & Higgs, 2007). Evaluations were always conducted by the same observer (P.R-G.).

SPSS-Windows 15.0 (SPSS Inc., Chicago, IL, USA) was used for the descriptive statistics, while SUDAAN 7.0 (RTI, RTP, NC) was employed for the analytical statistics (*P*-value calculations) to account for clustering (multiple oral cancers per patient); specific tests used are reported in Table footnotes.

3 | RESULTS

Tables 1 and 2 exhibit the clinicopathological outcomes of the patients and tumors in the study. Table 3 compares the count of CD1-positive tumor cells with the expression intensity, which was increased in tumors with higher percentage of CD1-positive cells ($p = .03$). Table 4 compares the percentage of cells expressing cytoplasmic CD1 and its intensity with clinicopathological variables. A statistically significant association was found between nuclear and cytoplasmic CD1 expression (data not shown); thus, 45% of tumors with intense nuclear expression showed mild cytoplasmic expression, while 100% of tumors

TABLE 2 Description of oral tumors with cytoplasmic CD1 expression (n = 23[a])

Variable	*n* (%)
Localization	
Mouth floor	6 (26.1)
Tongue	11 (47.8)
Others	6 (26.1)
Clinical presentation of the tumor	
Ulcer	7 (41.2)
Tumor	6 (35.3)
Leukoplakia + Tumor/Ulcer	4 (23.5)
Missing (*n*)	6
Tumor size	
T1	5 (22.7)
T2	11 (50.0)
T3 (*n* = 1)-T4 (*n* = 5)	6 (27.3)
Missing (*n*)	1
Adenopathies	
N0	13 (59.1)
N1	4 (18.2)
N2 (*n* = 4)-N3 (*n* = 1)	5 (22.7)
Missing (n)	1
Metastasis	
M0	14 (63.6)
M1 (*n* = 1)-MX (*n* = 7)	8 (36.4)
Missing (n)	1
Stage	
I	5 (22.7)
II	7 (31.8)
III (*n* = 2)-IV (*n* = 8)	10 (45.5)
Missing (*n*)	1
Degree of tumor differentiation	
Good	4 (20.0)
Moderate	8 (40.0)
Poor	8 (40.0)
Missing (*n*)	3
Morphology	
Non-invasive	13 (56.5)
Invasive	10 (43.5)
Tumor Ki-67 (%)	
0–24	3 (13.0)
25–49	9 (39.1)
50–74	8 (34.8)
75-100	3 (13.0)
mean ± *SD*	51 ± 21

[a]Corresponding to 20 patients.

TABLE 3 Statistically significant associations between the expression intensity and percentage of oral squamous cell carcinoma cells with nuclear and cytoplasmic CD1 expression

Expression intensity	*n* (%)	% positive cells Mean ± *SD*
Nucleus		43 ± 24
Mild	–	–
Moderate	6 (26.1)	25 ± 7
Intense	17 (73.9)	50 ± 24
P-value[a]		<0.001
Cytoplasm		18 ± 23
Mild	11 (47.8)	7 ± 6
Moderate	4 (17.4)	16 ± 16
Intense	8 (34.8)	34 ± 32
P-value[b]		0.031

[a]T test adjusted for multiple cancers per patient using the DESCRIPT procedure in SUDAAN.
[b]ANOVA adjusted for multiple cancers per patient using the REGRESS procedure in SUDAAN.

with moderate or intense cytoplasmic expression showed intense nuclear expression (p = .041). A statistically significant correlation was also found between the percentage of cells with nuclear CD1 expression and the percentage with cytoplasmic expression (r = .64, p < .001).

4 | DISCUSSION

Our results demonstrate that cytoplasmic CD1 expression is a frequent event in OSCC (33.3% of the cases in our series), significantly associated (or with values close to significance) with numerous poor prognosis parameters, including advanced tumor stage, poor differentiation, elevated Ki-67 expression, the presence of cells with invasive morphology, and high T and N classification (Table 4). These findings suggest that cytoplasmic CD1 expression has oncogenic functions and should be considered in studies that address the influence of CD1 on OSCC outcomes.

The mechanisms regulating cytoplasmic CD1 accumulation are poorly understood. In the present series of OSCC patients, a strong association was found between cytoplasmic and nuclear CD1 expression in both positive cell count (p = .041) and expression intensity (p < .001). Hence, cytoplasmic CD1 overexpression in OSCC may be related to the considerable amplification of chromosome band 11q13 genes, which has been described as the most important mechanism for regulating CD1 expression in oral carcinogenesis (Ramos-García, Ruiz-Ávila et al., 2017). This amplification would generate a massive ribosomal production of CD1, which would form cytoplasmic complexes with CDKs4/6. Although a proportion of these complexes translocate

TABLE 4 Association[a] of clinicopathological variables with Ki-67 and cytoplasmic CD1 expression in oral squamous cell carcinomas with this expression (n = 23)

		Intensity		Cell count (%)	
Variable	***n*[b]**	**% Mi-Mo-I[c]**	***P*-value**	**Mean ± *SD***	***P*-value**
Tumor size			0.474		0.082
T1	5	80-20-0		6 ± 4	
T2	11	36-9-55		25 ± 30	
T3-T4 (n = 5)	6	50-17-33		12 ± 13	
Adenopathies			0.082		0.424
N0	13	46-15-39		29 ± 28	
N1	4	50-25-25		17 ± 13	
N2-N3	5	60-0-40		10 ± 14	
Metastasis			0.863		0.396
M0	14	57-7-36		14 ± 21	
M1-MX	8	38-25-37		23 ± 27	
Stage			0.089		0.051
I	5	80-20-0		6 ± 4	
II	7	14-14-71		30 ± 36	
III-IV	10	60-10-30		13 ± 13	
Degree of differentiation			0.591		0.007
Good	4	5-0-25		3 ± 1	
Moderate	8	50-12-38		21 ± 28	
Poor	8	37-25-38		24 ± 24	
Morphology			0.020		0.069
Non-invasive	13	77-23-0		10 ± 10	
Invasive	10	10-10-80		28 ± 31	
Ki-67 (%)			0.113		0.004
0-24	3	100-0-0		4 ± 2	
25-49	9	78-22-0		8 ± 6	
50-74	8	12-25-63		35 ± 32	
75-100	3	0-0-100		17 ± 17	

[a]*P*-values are calculated with SUDAAN 7.0 to account for clustering (multiple cancers within the patient), using CROSSTAB (chi-square test) for "Intensity," DESCRIPT (t test) to compare percentage cell counts with "Metastasis" and "Morphology," and REGRESS (ANOVA) to compare percentage cell counts with the remaining variables in column 1.
[b]Differences with n = 23 correspond to missing values for column 1 variables.
[c]Percentages (without decimals) of Mi(ld)–Mo(derate)–I(ntense) categories.

to the nucleus, where they exert their oncogenic functions, a certain amount of free or complex-bound CD1 would likely remain in the cytoplasm due to nuclear CD1 saturation.

There has been little research on the relationship between cytoplasmic CD1 expression and clinicopathological variables in cancer. However, the correlation between cytoplasmic CD1 expression and clinicopathological indicators of a poor prognosis in the present OSCC series is in line with previous reports in pancreatic, bladder, and liver cancers (Lebe et al., 2004; Sato et al., 1999; Tut et al., 2001). Although there are marked differences among these cancers (including OSCC), they all share a pathogenesis that is highly dependent on tobacco, which appears to be a key mechanism in the amplification of chromosome band 11q13. This may support our hypothesis of an association between cytoplasmic CD1 expression and gene amplification. This is especially important in OSCC, which has been associated with a rate of 11q13 amplification (46% of cases) that is more than twofold higher than observed in other human cancers (Ramos-García, Ruiz-Ávila et al., 2017). Conversely, cancers in which cytoplasmic CD1 expression has been associated with a favorable prognosis (prostate, thyroid, and colon) (Comstock et al., 2007; Holland et al., 2001; Pickett et al., 2005) largely correspond to adenocarcinomas with a less tobacco-dependent pathogenesis and lower 11q13 amplification rates.

Cytoplasmic CD1 expression has been associated with the acquisition of oncogenic functions related to cell migration through interaction with filamin A (Zhong et al., 2010), p27, ROCKII (Li et al.,

2006) and, more recently, with paxillin (Fusté et al., 2016). CD1-CDk4 complexes were found to interact with paxillin in the cytoplasm by direct binding and to phosphorylate this protein at ser-83 and -178, producing the downstream activation of Rac1 and thereby enhancing cell migration (Fusté et al., 2016). Furthermore, phosphorylated paxillin has been observed colocated alongside CD1-CDK4 complexes in specialized cell peripheral structures compatible with actin-based protrusive structures (Fusté et al., 2016). Accordingly, cytoplasmic CD1 may play a role in regulating increased cell motility *via* this (CD1-CDK4/6)-paxillin-Rac1 pathway, which may augment the invasive potential of OSCC cells. Paxillin appears to be linked to the regulation of actin-based protrusive structures in the cell periphery, which are produced in cancer cells by changes in the reorganization of their actin cytoskeleton and are required for the acquisition of migratory and invasive capacities (Brown & Turner, 2004; Chhabra & Higgs, 2007). In particular, paxillin appears to be involved in regulating the two types of actin-based protrusive structure that are most important for cell migration in cancer: lamellipodia, which are essential for cell locomotion, and invadopodia, whose high capacity for extracellular matrix degradation facilitates invasion (Brown & Turner, 2004; Chhabra & Higgs, 2007). Lamellipodia and invadopodia are presumably compatible with our classification of an invasive morphology, which was significantly associated with more intense cytoplasmic CD1 expression and a higher percentage of CD1-positive cells in the present OSCC series. We therefore propose that cytoplasmic CD1 expression may increase the migration and invasiveness of OSCC cells by regulating the formation of actin-based protrusive structures (lamellipodia and invadopodia), which may be generated upstream by paxillin activation.

AUTHOR CONTRIBUTION

Study concepts: Pablo Ramos-García, Manuel Bravo, Lucía González-Ruiz and Miguel Ángel González-Moles. Data acquisition: Pablo Ramos-García, Manuel Bravo, Lucía González-Ruiz and Miguel Ángel González-Moles. Quality control of data and algorithms: Pablo Ramos-García, Manuel Bravo, Lucía González-Ruiz and Miguel Ángel González-Moles. Data analysis and interpretation: Manuel Bravo, Miguel Ángez González-Moles and Pablo Ramos-García. Manuscript preparation: Pablo Ramos-García, Manuel Bravo, Lucía González-Ruiz and Miguel Ángel González-Moles. Manuscript editing: Miguel Ángez González-Moles and Pablo Ramos-García. Manuscript review: Pablo Ramos-García, Manuel Bravo, Lucía González-Ruiz and Miguel Ángel González-Moles.

CONFLICT OF INTERESTS

None to declare.

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How to cite this article: Ramos-García P, Bravo M, González-Ruiz L, González-Moles MÁ. Significance of cytoplasmic cyclin D1 expression in oral oncogenesis. *Oral Dis*. 2018;24:98–102. https://doi.org/10.1111/odi.12752

Archives of Oral Biology 97 (2019) 12–17

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Asymmetrical proliferative pattern loss linked to cyclin D1 overexpression in adjacent non-tumour epithelium in oral squamous cell carcinoma

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ARTICLE INFO

Keywords:
Cyclin D1
CCND1
Oral cancer
Premalignant fields

ABSTRACT

Objective: To evaluate cyclin D1 overexpression in oral squamous cell carcinomas and adjacent non-tumour epithelium as a biomarker of premalignant fields and a risk factor for multiple tumour development.
Design: We studied cyclin D1 expression in 54 patients with 68 oral squamous cell carcinomas plus adjacent non-tumour epithelia characterized as close (n = 58) or distant (n = 41) from the invasion point. Randomized 40x fields were evaluated (4 in tumour tissue and 1 each in close and distant non-tumour epithelium). Expression in non-tumour epithelium was evaluated in basal, parabasal, middle-third and upper-third compartments.
Results: Cyclin D1 overexpression was found in both carcinomas and non-tumour epithelia. Nuclear expression in basal and parabasal layers of distant epithelium was significantly increased in patients with multiple tumours ($p < 0.001$). A significant association between cyclin D1 overexpression in different epithelial layers was found in both close and distant epithelia. A significant association was found between nuclear expressions of cyclin D1 and Ki-67 in the basal layer of distant epithelium ($p = 0.02$).
Conclusions: Cyclin D1 overexpression is an early event in oral carcinogenesis linked to loss of the physiological asymmetrical proliferation pattern. Cyclin D1 overexpression in basal and parabasal layers of epithelia distant from the invasion point may act as a potential marker of premalignant fields and multiple tumour development.

1. Introduction

Among patients who have suffered a first oral squamous cell carcinoma (OSCC), the incidence of a second is estimated to be 17–30%, with an annual risk of 3–10% (Tabor et al., 2001). After tumour excision, development of a new cancer may result from the continued presence of a genetically altered "premalignant" field (Braakhuis, Tabor, Kummer, Leemans, & Brakenhoff, 2003). The so-called *secondary field tumours* that develop in these fields share some of the oncogenic alterations of the primary tumour and its adjacent tissue, including p53 mutations and loss of heterogeneity (LOH) in 3p, 9p and 17p (Mao et al., 1996; Partridge et al., 2000; Rosin et al., 2000). Because of the increased risk of multiple tumour development, the diagnosis of premalignant fields is crucial (Braakhuis et al., 2002) and has major prognostic and therapeutic implications. However, the reliable diagnosis of premalignant fields currently requires molecular techniques (LOH and mutational analysis) that are not in routine use due to their cost and complexity (Braakhuis et al., 2002).

Our group has reported on biomarkers that can be detected by immunohistochemistry (IHC) techniques widely applied in pathology labs and could improve the identification of patients at risk of secondary field tumour development (Garcia et al., 2016; González-Moles et al., 2010; González-Moles, Bravo et al., 2013; González-Moles et al., 2014; Gonzalez-Moles, Scully, & Ruiz-Avila, 2012). In studies of the adjacent non-tumour epithelium (ANTE) of OSCCs linked to premalignant fields, we detected a change from the physiological asymmetric proliferative pattern towards a symmetric proliferative pattern. In this new pattern, division of the cells no longer gives rise to amplifying transitory cells,

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https://doi.org/10.1016/j.archoralbio.2018.10.007
Received 11 June 2018; Received in revised form 20 September 2018; Accepted 6 October 2018

probably due to oncogenic aggressions, but rather generates only the clonal progeny of premalignant basal cells (presumably stem cells), which accumulate in the basal layer (Garcia et al., 2016; González-Moles et al., 2014; González-Moles, Scully, Ruiz-Ávila, & Plaza-Campillo, 2013). We previously associated this change in labial epithelium carcinogenesis with the overexpression of cyclin D1 (Garcia et al., 2016), a key protein in the progression through the G1 to S phases of the cell cycle. Cyclin D1 is encoded by the CCND1 gene, located on chromosome band 11q13 (Ramos-García, Gil-Montoya et al., 2017), and both gene and band are frequently amplified in OSCC (Ramos-García, Gil-Montoya et al., 2017; Ramos-García, Ruiz-Ávila et al., 2017) essentially due to oncogenic aggression from tobacco products. CCND1 gene amplification is currently considered the primary mechanism of cyclin D1 overexpression, considered a prominent step in oral oncogenesis (Ramos-García, Gil-Montoya et al., 2017; Ramos-García, Ruiz-Ávila et al., 2017).

With this background, the objective of this study was to evaluate cyclin D1 overexpression in ANTE of OSCCs as a marker of premalignant fields and of multiple tumour development risk.

2. Materials & methods

This retrospective study included 54 patients (40 males) with 68 OSCCs treated in the Jaen Hospital Complex (Jaen, Spain); their mean (SD) age was 63.0 ± 12.0 years (range, 45–87 years). All patients signed an informed consent form during their hospital stay to allow the conservation and future use of any biological tissues for research purposes. After study approval was granted by the hospital ethical committee, we reviewed the clinical records of the patients, gathering relevant clinical and pathological data. This study focussed on the ANTE of OSCC, which was classified as close (≤1 cm from the invasion point; n = 58) or distant (> 1 cm from the invasion point; n = 41) following previously published criteria (González-Moles et al., 2010).

A secondary tumour was recorded as present if the following criteria were met: arising from the superficial epithelium and not from the deep surgical margin of the tumour (Johnson et al., 2005); complete resection of the primary tumour with histopathologically confirmed negative margins (Braakhuis et al., 2002); at least 2 cm of tumour-free epithelium between tumours, based on clinical, surgical and histopathological findings (Hong et al., 1990); and ≥6 months between tumour onsets (Hashibe et al., 2005).

2.1. Immunohistochemistry

IHC study of tumour and ANTE samples was carried out by the Pathology Department of Granada University Hospital Complex, using 5 sections of 4 μm cut from paraffin-embedded specimens. Peroxidase-antiperoxidase and avidin-biotin techniques were applied, using Autostainer Link equpiment (Dako, Carpinteria, CA, USA) and EnVision FLEX reagents (K8002; Dako) according to the manufacturers' instructions. In this system, deparaffinization and rehydration is followed by recovery of the heat-induced epitope, with all slides being heated equally to ensure the reproducibility of the process. Monoclonal rabbit anti-human cyclin D1 antibody (Clone EP12; Dako) and Mib-1 anti-Ki-67 antibody (Dako) were used, as recommended for the automated system. Counterstaining with EnVision Flex Haematoxylin (K8008; Dako) stains nuclear areas light blue in samples mounted in DPX. As negative controls, primary antibodies were replaced with phosphate buffered saline. Positive controls were OSCC tissues known to express cyclin D1 and Ki-67. There was no control group for cyclin D1 expression, widely reported to be negative in healthy oral tissue (Das, Khare, Singh, & Sharma, 2011; Goto, Kawano, Kobayashi, Sakai, & Yanagisawa, 2002; Kuo, Lin, Hahn, Cheng, & Chiang, 1999; Wang et al., 2006; Wong et al., 2003). All slides were digitalised using a Philips IntelliSite Ultra Fast Scanner (Philips Digital Pathology Solutions, Best, Netherlands). IHC expression was evaluated in tumour and ANTE samples. Philips IMS viewer (Philips Digital Pathology Solutions) was used to capture digital images (0.191 mm^2) of randomized areas, including one image of close ANTE, one of distant ANTE and four images of the tumour field. This system allows high magnification (40X) and resolution of the digital images in a reproducible manner. Cyclin D1 and Ki-67 expressions were measured with a semi-automated counting system using Adobe Photoshop CC v.2017 (San Jose, CA, USA), evaluating both nuclear and cytoplasmic staining for cyclin D1 and nuclear staining alone for Ki-67. Positivity for both markers was defined by a brown staining. Expression in the ANTE was determined in each of four levels: basal, parabasal (formed by approximately 3 rows of cells), middle-third and upper-third. The total number of cells and the number of positive cells were counted to yield the mean percentage marker expression for each layer and for the entire image.

2.2. Statistical methods

SPSS v15.0 (SPSS Inc., Chicago, IL, USA) was used for descriptive statistics and the SUDAAN 7.0 (Research Triangle Institute, Durham, NC, USA) for analytical statistics (calculation of p value) to account for clustering (multiple tumours in the same patients). Tests used are indicated in table footnotes.

3. Results

The study included 54 patients (40 males) with 68 OSCCs; their mean ± standard deviation age was 63.0 ± 12.0 years (range, 45–87 years); 15 of the patients (27.8%) had more than one tumour (Table 1). Table 2 exhibits the clinical-pathological data for the tumours, including Ki-67 expression, revealing that the OSCC diagnosis continues to be late in a large percentage of cases, which is one of the main prognostic factors in this disease, being associated with high T, N, M, and stage.

The mean nuclear expression of cyclin D1 in the tumours was 28.7% ± 21.5% (Fig. 1A,B) and the mean cytoplasmic expression of cyclin D1 for all tumour cells, was 6.4% ± 16.1% (Fig. 1C). Table 3 displays associations between the cyclin D1 expression in ANTE and the number of tumours. The nuclear expression of cyclin D1 in basal and parabasal layers of distant ANTE was significantly higher in patients who developed more than two tumours ($p < 0.001$) (Fig. 1D).

Results in Table 4 reveal a trend between high cyclin D1 expression in the different compartments of close ANTE and high cyclin D1

Table 1
Description of patients with oral tumours (n = 54).

Variable	n (%)
Sex	
Female	14 (25.9)
Male	40 (74.1)
Age (years)	
Range	45-87
Mean ± Standard Deviation	63 ± 12
Unknown	12
Tobacco	
No	8 (28.6)
Yes	20 (71.4)
Unknown	26
Alcohol	
No	8 (33.3)
Yes	16 (66.7)
Unknown	30
Number of tumours	
1	39 (72.2)
2	11 (20.4)
3	2 (3.7)
4	2 (3.7)
Mean ± Standard Deviation	1.39 ± 0.74

Table 2
Description of the tumours with cyclin D1 expression (n = 68[a]).

Variable	n (%)
Location	
Floor of Mouth	11 (17.7)
Tongue	35 (56.5)
Other	16 (25.8)
Missing (n)	6
Clinical presentation of the tumour	
Ulcer	18 (38.3)
Swelling	15 (31.9)
Other[b]	14 (29.8)
Missing (n)	21
Size	
T1	16 (27.6)
T2	22 (37.9)
T3 (n = 4)-T4 (n = 16)	20 (34.5)
Missing (n)	10
Adenopathy	
N0	37 (63.8)
N1	13 (22.4)
N2 (n = 6)-N3 (n = 1)-Nx (n = 1)	8 (13.8)
Missing (n)	10
Metastasis	
M0	44 (75.9)
M1 (n = 1)-MX (n = 13)	14 (24.1)
Missing (n)	10
Stage	
I	13 (22.4)
II	14 (24.1)
III	11 (19.0)
IV	20 (34.5)
Missing (n)	10
Degree of differentiation	
Good	18 (34.6)
Moderate	22 (42.3)
Poor	12 (23.1)
Missing (n)	16
Ki-67 in tumour (%)	
0-24	13 (19.1)
25-49	28 (41.2)
50-74	20 (29.4)
75-100	7 (10.3)
Mean ± Standard Deviation	45 ± 20

[a] Corresponding to 54 patients.
[b] Leukoplakia + swelling (n = 7), Lichen planus + ulcer (n = 1), Erythroleukoplakia (n = 3) y Leukoplakia + ulcer (n = 3).

expression in the corresponding compartments of distant ANTE.

Table 5 exhibits the associations found between cyclin D1 and Ki-67 expressions in ANTE. Significant associations were found between the nuclear expressions of cyclin D1 and Ki-67 in the basal layer of distant ANTE (p = 0.02) and between the cytoplasmic expression of cyclin D1 and nuclear expression of Ki-67 in the basal and parabasal layers of close ANTE and in all layers of distant ANTE (Fig. 1D).

4. Discussion

In this study, overexpression of cyclin D1 was observed in OSCC and ANTE samples, mainly localized in the nuclei of epithelial cells in basal, parabasal and middle-third compartment (Table 3, Fig. 1D). As reported in two systemic reviews, some authors observed that cyclin D1 alterations have a marked effect on the normal maturation and architecture of oral epithelia and may possibly participate in dysplasia development (Nankivell, Weller, McConkey, Paleri, & Mehanna, 2011; Smith, Rattay, McConkey, Helliwell, & Mehanna, 2009). Given that epithelial dysplasia is a recognized risk marker of cancer development in oral epithelia, it is tempting to speculate that OSCC derives from adjacent oral epithelium through the development of dysplasia and that molecular changes in this epithelium probably precede those that appear in the tumour. However, this hypothesis has not been definitively

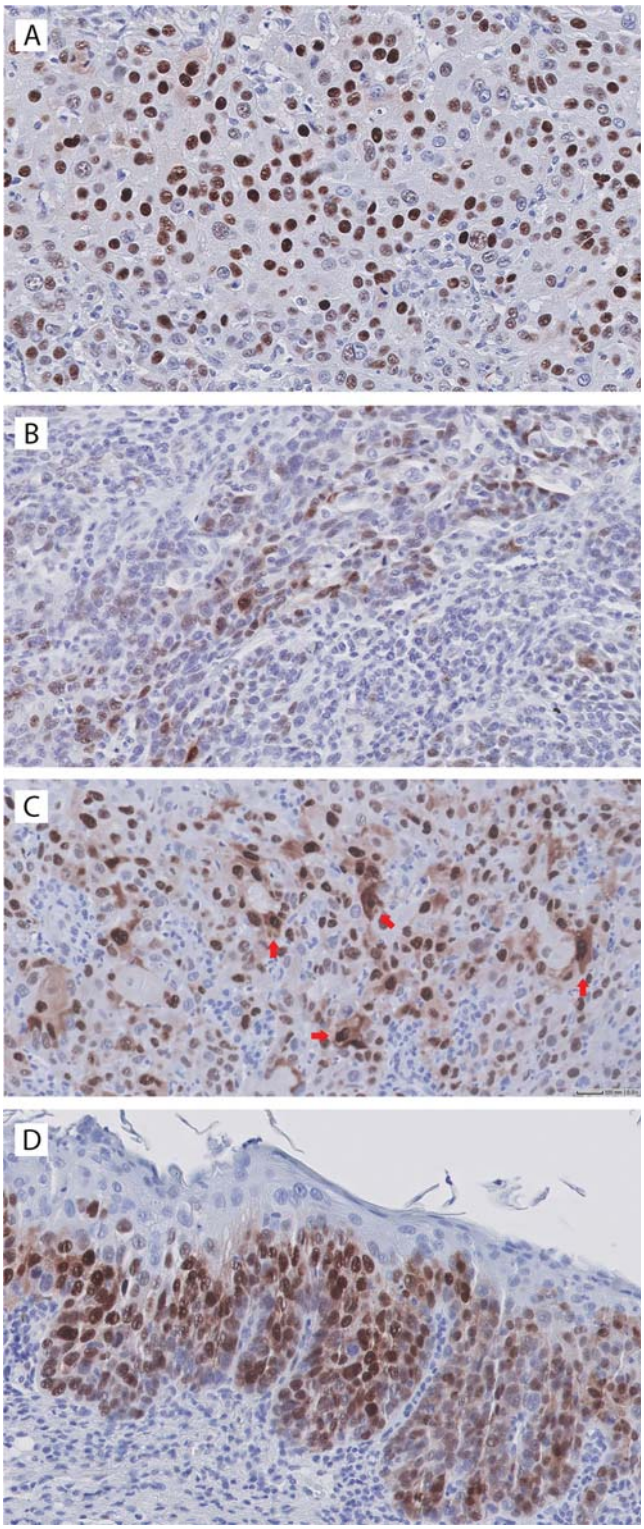

Fig. 1. a) Nuclear expression of cyclin D1 in OSCC (IHC, 40x), depicting a tumour with high cyclin D1 expression, above the mean for tumours in the series (> 28.7%). b) Nuclear cyclin D1 expression in OSCC (IHC 40x), depicting a tumour with an expression close to the mean for tumours in the series (28.7%). c) Nuclear and cytoplasmic expression of cyclin D1 (red arrows) in OSCC (IHC, 40x). d) Nuclear and cytoplasmic expression of cyclin D1 in basal, parabasal and middle-third compartments of distant ANTE (IHC, 40x) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
Cyclin D1 expression in close and distant adjacent non-tumour epithelium (n = 68, from 54 patients) as a function of the number of tumours/patient.

	All		Grouped by nº tumours/patient							
			With 1 tumour [A]		With 2 tumours [B]		With > 2 tumours [C]		Comparison	
Variable	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	Global p[a]	Pairwise[b]
Close Adjacent Non-Tumour Epithelium										
Basal Nucleus expression	58	17.4 ± 19.1	33	21.1 ± 21.4	15	10.5 ± 13.1	10	15.6 ± 16.7	0.148	
Parabasal Nucleus expression	58	38.7 ± 21.5	33	41.9 ± 22.5	15	32.2 ± 22.5	10	38.0 ± 15.5	0.430	
Middle-Third Nucleus expression	58	14.0 ± 16.7	33	13.2 ± 16.5	15	11.2 ± 13.6	10	21.0 ± 20.9	0.561	
Upper-Third Nucleus expression	58	1.7 ± 4.7	33	0.8 ± 3.5	15	1.6 ± 3.8	10	5.0 ± 7.7	0.384	
Basal Cytoplasm expression	58	1.7 ± 13.1	33	3.0 ± 17.4	15	0.0 ± 0.0	10	0.0 ± 0.0	0.317	
Parabasal Cytoplasm expression	58	1.9 ± 13.1	33	3.1 ± 17.4	15	0.0 ± 0.0	10	0.5 ± 1.6	0.284	
Middle Third Cytoplasm expression	58	0.1 ± 0.6	33	0.1 ± 0.5	15	0.0 ± 0.0	10	0.4 ± 1.2	0.222	
Upper Third Cytoplasm expression	58	0.0 ± 0.0	33	0.0 ± 0.0	15	0.0 ± 0.0	10	0.0 ± 0.0	–	
Distant Adjacent Non-Tumour Epithelium										
Basal Nucleus expression	41	9.6 ± 13.3	22	8.9 ± 11.9	11	4.0 ± 5.9	8	19.3 ± 19.4	< 0.001	AB ≠ C
Parabasal Nucleus expression	41	33.4 ± 22.3	22	31.5 ± 23.5	11	26.7 ± 17.4	8	48.1 ± 20.6	0.001	AB ≠ C
Middle Third Nucleus expression	41	15.1 ± 17.2	22	17.0 ± 19.4	11	8.5 ± 8.9	8	19.0 ± 18.7	0.117	
Upper Third Nucleus expression	41	2.5 ± 9.8	22	1.7 ± 4.7	11	0.5 ± 1.2	8	7.5 ± 21.2	0.320	
Basal Cytoplasm expression	41	0.0 ± 0.2	22	0.1 ± 0.3	11	0.0 ± 0.0	8	0.0 ± 0.0	0.313	
Parabasal Cytoplasm expression	41	0.5 ± 2.4	22	0.8 ± 3.2	11	0.0 ± 0.0	8	0.6 ± 1.6	0.225	
Middle Third Cytoplasm expression	41	0.7 ± 4.1	22	1.2 ± 5.6	11	0.0 ± 0.0	8	0.5 ± 1.4	0.267	
Upper Third Cytoplasm expression	41	0.1 ± 0.8	22	0.2 ± 1.1	11	0.0 ± 0.0	8	0.0 ± 0.0	0.313	

[a] REGRESS procedure in SUDAAN.
[b] DESCRIPT procedure in SUDAAN. "≠" = statistically significant ($p < 0.05$).

proven. Cyclin D1 is a key protein in oral oncogenesis, increasing cellular proliferation and migratory capacity and interfering in cellular differentiation and mitochondrial metabolism (Ramos-García, Gil-Montoya et al., 2017), functions considered to be hallmarks of cancer (Hanahan & Weinberg, 2011). Studies have reported that overexpression of cyclin D1, amplification of its gene CCND1 or overexpression of its mRNA are absent in healthy oral epithelium (Das et al., 2011; Goto et al., 2002; Kuo et al., 1999; Wang et al., 2006; Wong et al., 2003). In our view, cyclin D1 overexpression in epithelium adjacent to OSCC suggests that amplification of the gene and consequent overexpression of the protein are an early oncogenic event in oral carcinogenesis (Kövesi & Szende, 2006; Ramakrishna et al., 2013; Rousseau, Lim, Lin, & Jordan, 2001). Cyclin D1 overexpression in the basal layer of ANTE indicates that the basal layer cells, presumably stem cells, are targets for oncogenic aggressions that lead to oral carcinogenesis.

We also observed a significant association between cyclin D1 overexpression and increased cellular proliferation in the basal layer of distant ANTE. As noted in the Introduction, normal basal cells follow a physiological pattern of asymmetric proliferation, characterized by a low basal proliferation that gives rise to a population of highly proliferative amplifying transient cells in the parabasal layer. In our study, the accumulation of proliferative cells in the basal layer was significantly associated with the overexpression of cyclin D1, indicating

Table 5
Association (r = Pearson's correlation) between expressions of Cyclin D1 and Ki-67 in Adjacent Non-Tumour Epithelium (ANTE) (n = 68, from 54 patients).

	Ki-67		
Variable	n	r	p-value
Close ANTE	58	0.07	0.565
Basal Nucleus expression	58	0.08	0.462
Parabasal Nucleus expression	58	0.17	0.248
Middle-Third Nucleus expression	58	-0.07	0.394
Upper-Third Nucleus expression	58	0.14	< 0.001
Basal Cytoplasm expression	58	0.14	< 0.001
Parabasal Cytoplasm expression	58	0.13	0.137
Middle-Third Cytoplasm expression	58	–	–
Upper-Third Cytoplasm expression			
Distant ANTE	41	0.29	0.020
Basal Nucleus expression	41	0.08	0.584
Parabasal Nucleus expression	41	0.06	0.714
Middle-Third Nucleus expression	41	0.18	0.163
Upper-Third Nucleus expression	41	0.18	< 0.001
Basal Cytoplasm expression	41	0.21	< 0.001
Parabasal Cytoplasm expression	41	0.19	< 0.001
Middle-Third Cytoplasm expression	41	0.18	< 0.001
Upper-Third Cytoplasm expression			

a: REGRESS procedure in SUDAAN.

Table 4
Association (r = Pearson's correlation) between cyclin D1 expression in close and distant adjacent non-tumour epithelium (ANTE) (n = 68, from 54 patients).

Close ANTE	Distant ANTE							
	DEBN	DEPN	DEMN	DEUN	DEBC	DEPC	DEMC	DEUC
Basal Nucleus expression	0.34*	0.35*	0.39*	0.10	0.29	0.35	0.31	0.29
Parabasal Nucleus expression	0.46*	0.58*	0.30*	0.02	0.20	0.28	0.23	0.20
Middle-Third Nucleus expression	0.12	0.20	0.31*	0.13	0.30	0.38	0.32	0.30
Upper-Third Nucleus expression	0.33*	0.19	0.19	0.54*	-0.07	-0.02	-0.04	-0.07
Basal Cytoplasm expression	–	–	–	–	–	–	–	–
Parabasal Cytoplasm expression	0.16*	0.36*	0.12	-0.05	-0.03	0.30*	0.10	-0.03
Middle-Third Cytoplasm expression	0.06	0.13	0.12	-0.06	-0.04	0.22	0.09	-0.04
Upper-Third Cytoplasm expression	–	–	–	–	–	–	–	–

a: REGRESS procedure in SUDAAN.
D = Distant; E = Epithelium; B = Basal; P = Parabasal; M = Middle-third; U = Upper-Third; N = Nucleus; C = Cytoplasm.
* $p < 0.05$.

that this oncoprotein may act by inducing a change from an asymmetric proliferative pattern to a symmetrical pattern. In the latter pattern, a premalignant basal cell (premalignant stem cell) gives rise to two premalignant stem cells instead of an amplifying transient cell in the parabasal layer. We previously described the role of cyclin D1 in changing the proliferative pattern in labial oncogenesis (Garcia et al., 2016), reporting that the increased proliferative activity of basal cells can exceed the capacity of the basal layer to house the clonal premalignant stem cell population, consequently resulting in the progressive occupation of more superficial epithelial layers (González-Moles et al., 2014). In our opinion, this reasoning explains the overexpression of cyclin D1 in the parabasal and middle-third compartment of ANTE which correlates with the overexpression in the basal layer.

In the present study, the nuclear cyclin D1 expression in close ANTE was observed in cases with the highest expression in distant ANTE. We found a significant association of basal and parabasal nuclear cyclin D1 expressions between epithelia distant and close to the tumour invasion point. We consider that this reflects the distribution of this molecular alteration throughout the premalignant oral epithelium, mainly in basal and parabasal layers, with the main consequence being increased cell proliferation and genomic instability. Clonal accumulation of cyclin D1-overexpressing cells in premalignant epithelium, especially in basal and parabasal layers, may progressively replace normal cells due to their superior proliferative capacity, creating premalignant fields that extend in the oral mucosa. This suggests that the oncogenic functions linked to cyclin D1 alterations are not only an early feature of oral carcinogenesis but can also extend to wide areas of the oral mucosa, supporting the proposition that nuclear cyclin D1 overexpression is an important oncogenic phenomenon in the genesis and expansion of premalignant fields.

Interestingly, basal and parabasal overexpression of cyclin D1 in distant ANTE was significantly correlated with the risk of multiple tumour development (> 2 tumours). Thus, cyclin D1-overexpressing and actively-proliferating epithelial cells may develop, as commented, a genomic instability that makes them more prone to acquire new oncogenic events, which will ultimately allow subclones to invade In previous studies (Garcia et al., 2016; González-Moles et al., 2014; González-Moles, Bravo et al., 2013), we have described the importance of molecular changes in basal and parabasal epithelial layers for multiple tumour development. Hence, basal and parabasal overexpression of cyclin D1 in distant ANTE may be a useful marker of premalignant fields and multiple tumour risk.

Finally, some areas of ANTE showed an evident if scant expression of cytoplasmic cyclin D1 (Table 3). This localization of the protein has only been reported by a few authors (Das et al., 2011; Maahs, Machado, Jeckel-Neto, & Michaelses, 2007), and there has been no interpretation of its function. In a previous study (P. Ramos-García, Bravo, González-Ruiz, & González-Moles, 2018), we reported the association of cytoplasmic cyclin D1 expression in tumour cells with an invasive morphology, advanced tumour stage, poorer differentiation and increased Ki-67 expression. Cytoplasmic cyclin D1 may play a role in the regulation of increased cellular mobility *via* the cyclin D1-CDK4/6-paxillin-Rac1 pathway. This is because paxillin appears to be involved in the regulation of actin-based protrusive structures at the cell periphery that are necessary for the acquisition of migratory capacities (Chhabra & Higgs, 2007; Fusté et al., 2016). In this way, cytoplasmic cyclin D1 overexpression in premalignant epithelia may contribute to the expansion of premalignant fields.

In conclusion, these findings confirm that cyclin D1 overexpression is an early event in oral carcinogenesis, linked to a change towards an abnormal symmetric proliferative pattern. Accordingly, cyclin D1 overexpression, especially in basal and parabasal layers of distant ANTE, may be a useful marker of premalignant fields and multiple tumour risk. Detection by IHC of cyclin D1 overexpression in ANTE, especially distant ANTE, from patients who have suffered a first tumour, is a routine, automated and inexpensive procedure that could make a substantial contribution to the therapeutic planning and follow-up of patients with OSCC.

Declarations of interest

None.

Author contribution

All authors have read and approved the final article.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee. and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Title: Prognostic relevance of tumor cyclin D1 expression in oral cancer

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Declarations of interest: none

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Abstract word count: 144

Manuscript word count: 3133 (references included)

Number of references: 50

Number of figures: 1

Number of tables: 3

2 Oct 2018

Abstract

Objective. To evaluate the prognostic value of cyclin D1 overexpression in oral squamous cell carcinomas (OSCCs).

Study Design. A retrospective immunohistochemical study was conducted of cyclin D1 and ki-67 expression in 68 OSCCs from 54 patients. Cases were scanned using a digital pathology system. The tumor expression of markers was assessed in four randomly selected fields (40x), and a semi-automatized count was conducted of cyclin D1-positive and -negative cells.

Results. Cyclin D1 overexpression was frequent (28.7 %) in this OSCC sample. It was significantly and positively associated with the following poor prognostic factors: low tumor differentiation degree (p=0.030), invasive morphology (p=0.045), and proliferative phenotype according to tumor cell ki-67 expression(p=0.018).

Conclusion. Cyclin D1 overexpression is a frequent event in oral carcinogenesis and is associated with poor prognostic factors. Measurement of cyclin D1 expression may be useful to predict the prognosis of patients with OSCC.

Keywords: Cyclin D1; CCND1; Oral carcinogenesis; Oral; Cancer.

Introduction

Oral cancer is a growing worldwide health problem with 300,400 new cases and is responsible for 145,400 deaths a year (GLOBOCAN, IARC, WHO) [1]. Oral squamous cell carcinoma (OSCC) represents around 90 % of malignant oral neoplasms [2] and has a 5-year survival rate of 50-60 % [2,3]. Numerous studies have focused on the prognostic

value of emerging molecular biomarkers [4,5], and our group has proposed immunohistochemical biomarkers that may improve prediction of the prognosis of individual patients [6–10]. In particular, cyclin D1 has been widely reported to play a key role in head and neck oncogenesis [11,12]. Our recent meta-analysis demonstrated that cyclin D1 overexpression has a negative impact on the prognosis of patients with OSCC [13], and it has also been associated with labial [14] and oral [15] oncogenesis.

Cyclin D1 protein is encoded by the *CCND1* gene in chromosome band 11q13 and promotes cell cycle progression during the G1-S phase [16]. It has also been attributed with numerous emerging functions, including the regulation of cell migration and mitochondrial metabolism and the inhibition of cell differentiation and DNA repair [17]. Gene amplification is the main mechanism of cyclin D1 overexpression in OSCC, showing a two-fold higher rate than observed in other human cancers [18]. However, numerous molecular alterations have also been implicated in cyclin D1 dysregulation and oncogenic activation, including chromosomal translocations, mutations, polymorphisms, and the activation of pathways involved in human carcinogenesis (MAPK, PI3K, Wnt, NF-κβ) [11,19]. Cyclin D1 overexpression is frequently associated with T and N status, advanced clinical stage, high histological grade, reduced survival, and lack of response to treatment, among other poor prognostic factors [11], and it has been described as one of the markers with greatest potential prognostic value in oral cancer patients [12,13].

With this background, the objective of this study of patients with OSCC was to evaluate the prognostic value of cyclin D1 overexpression as measured by an semi-automatized immunohistochemical technique.

Material and methods

We conducted a retrospective study of 54 patients aged between 45 and 87 yrs (63.0 ± 12.0 yrs) with 68 OSCCs under treatment in the Hospital Complex of Jaen (Spain); 40 patients (74.1 %) were males (table I). All participants had signed informed consent to the preservation and subsequent use of their biological samples for research. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors. After approval of the study by the ethics committee of the hospital, we reviewed the hospital records of the patients and gathered data on the clinicopathological characteristics of their lesions.

Immunohistochemistry

An immunohistochemical study of cyclin D1 and Ki-67 proteins was conducted in the Pathology Department Granada University Hospital Complex (Spain). Peroxidase-antiperoxidase and avidin-biotin techniques were applied in five 4-µm sections from each paraffin block (tumor), utilizing automated Autostainer Link equipment (Dako, Carpinteria, CA, USA) and EnVision™ FLEX reagents (K8002; Dako) according to the manufacturer's instructions. This system permits dewaxing and rehydration followed by heat-induced epitope recovery. The reproducibility of the process was ensured by loading the whole coverslip, guaranteeing the identical heating of all sections in each cycle. We used rabbit monoclonal anti-human cyclin D1 antibody (Clone EP12) (Dako) and primary Mib-1 antibody against ki-67 (Dako), recommended by the manufacturer for this automated system. Counterstaining was done using the EnVision™ Flex

Hematoxylin system (K8008; Dako), which stains in light nuclear blue, followed by permanent mounting of the samples in DPX. For the negative control, the primary antibody was replaced by saline phosphate buffer. Tissue from an OSCC of known cyclin D1 and Ki67 expression served as positive control. No control group was used for cyclin D1 expression, given the well-documented and consistent negativity for cyclin D1 of healthy oral epithelia [20–27]. Slides were digitalized using a Philips IntelliSite Ultra-Fast Scanner (Philips Digital Pathology Solutions, Best, The Netherlands), and the tumor expression of markers was evaluated in four randomly selected tumor fields of 0.191 mm^2 (equivalent to 40x magnification) with the Philips IMS viewer (Philips Digital Pathology Solutions), which offers high magnification, definition, and reproducibility. The expression in each field was measured with a semi-automated cell count technique [15], using Adobe Photoshop CC v.2017 (Adobe, San Jose, CA, USA) and minimizing errors by placing a grid over each area. We evaluated nuclear immunopositivity for cyclin D1 and Ki-67, considering brown labeling as positive for both markers. We counted total cells and positive cells and established a mean percentage of expression in each field. Out of the 68 carcinomas under study, we selected those with both nuclear and cytoplasmic expression of cyclin D1 (n=23), recording the presence of cells with a consistent morphology of spindle-shaped prolongations of the cell membrane invaginated between adjacent tumor cells corresponding to lamellipodia and invadopodia, actin-based protrusive structures [28]. This cell morphology was considered invasive, based on the results of a previous study [15,29]. The aim was to explore the relationship between the percentage of cells with nuclear immunopositivity and the presence of an invasive morphology. All evaluations were conducted by two observers (M.A.G-M and P.R-G).

Statistical methods

SPSS-Windows 15.0 (SPSS Inc., Chicago, IL) was used for descriptive statistics and SUDAAN 7.0 (Research Triangle Institute, Durham, NC, USA) for analytical statistics to account for clustering (multiple OSCCs per patient). Tests applied are indicated in table footnotes.

Results

We analyzed 54 patients with a total of 68 OSCCs (Table I). More than one OSCC was present in 15 (27.8 %) of these patients. Table II exhibits the clinicopathological features of the tumors and their proliferation rate (ki-67 expression); 20 tumors (34.5 %) were T3 or T4, and 21 (36.2%) were N+.

The mean nuclear expression of cyclin D1 in tumor cells was 28.7 % (±21.5 %) in the whole series, 32.7 % (±23.7 %) in patients with one tumor, 18.3 % (±16.0 %) in those with two tumors, and 27.2 % (±16.3 %) in those with more than two tumors (data not shown in tables).

Table III displays the statistical associations found between the expression of nuclear cyclin D1 and the clinicopathological variables under study. Nuclear cyclin D1 expression was significantly associated with high histological grade (p=0.030). In tumors with cyclin D1 expression in both nucleus and cytoplasm (n=23) (Fig. 1), the percentage of immunopositive cells was significantly associated with an invasive morphology (p=0.045). Nuclear expression of cyclin D1 was significantly associated with tumor cell ki-67 expression (p=0.018).

Discussion

Our study demonstrates that cyclin D1 overexpression is frequent in OSCC and is significantly associated with poor prognostic factors, including low degree of tumor differentiation, proliferative subtype, and invasive morphology of tumor cells.

Cyclin D1 is a key protein in oral oncogenesis, increasing cell proliferation [11,16] and exerting numerous emerging functions [17], including the regulation of cell migration and mitochondrial metabolism and the inhibition of cell differentiation and DNA repair, considered hallmarks of cancer [30]. Amplification of the *CCND1* gene is the main oncogenic mechanism of cyclin D1 overexpression in OSCC [18], although its transcriptional activation can also be increased by the alteration of pathways involved in human carcinogenesis such as MAPK, Wnt, and NF-κβ, among others [11,19]. These mechanisms lead to uncontrolled cell proliferation, which would hypothetically lead to the development of larger OSCCs and increase the risk of lymph node involvement [11,30,31]. However, in the present and previous studies [24,25,32–42], nuclear expression of cyclin D1 was not significantly associated with larger tumor size [24,25,32–38] or with the presence of lymph node metastases [24,33–36,39–42]. Our recent meta-analysis [13] on the prognostic value of cyclin D1 reported the influence of its overexpression on T and N status, especially in tongue cancer. Tongue carcinoma, which behaves differently from other intraoral carcinomas (higher T and N status and worse survival) may also be different from a molecular perspective, especially with respect to cyclin D1 overexpression. Hence, discrepancies between the present findings and previous studies may be due to their inclusion of tumors from different sites in which the importance of cyclin D1 may vary. We also observed a statistically significant association between the nuclear expression of cyclin D1 and low tumor differentiation degree, as also reported in our meta-analysis [13]. Molecular mechanisms regulating increased proliferation *via*

cyclin D1 would alter the homeostasis of cells towards a more proliferative phenotype [11], typically associated in cancer with an immature and oncogenically more aggressive cell phenotype [30]. Some studies have suggested a putative role for cyclin D1 in the inhibition of cell differentiation in various cell lineages, either through the formation of complexes with their binding partners CDKs 4 and 6 [43,44] or independently of CDK activity [45,46]. A recent study by our group [15] observed a less differentiated morphology in OSCC cells with cyclin D1 overexpression and a nuclear-cytoplasmic expression pattern and found that tumors with this immunostaining pattern were significantly associated with a low differentiation degree and an invasive morphology. Numerous mechanisms have been proposed to underlie the contribution of cyclin D1 to the acquisition of oncogenic functions related to cell migration, mainly through interaction with filamin A [47], $p27^{kip1}$[48], and/or ROCKII [49] molecules. Cytoplasmic cyclin D1 may also regulate cell migration *via* the (cyclin D1-CDK4/6)-paxillin-Rac1 pathway [29]. Our previous study [15] reported a significant association of cytoplasmic cyclin D1 expression in OSCC cells with this invasive morphology. We have also described a role for cyclin D1 in the regulation of actin-based protrusive structures (lamellipodia and invadopodia) in the cell periphery that are essential for migration [28,50]. It has not been elucidated whether this putative role is exclusively related to the cytoplasmic expression of cyclin D1 or also involves its nuclear expression, as observed in the present study. Finally, we found an association between expression levels of cyclin D1 and ki-67, indicating the importance of cyclin D1 in the regulation of its canonic function, cell proliferation.

In conclusion, these findings confirm the association of cyclin D1 overexpression with poor prognostic factors in OSCC (low differentiation degree, ki-67 overexpression, and invasive morphology), supporting the usefulness of its assessment for predicting the prognosis of these patients.

Acknowledgement

We would like to thank the research group CTS-392 (Plan Andaluz de Investigación, Junta de Andalucía, Spain).

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Figure caption

The image depicts numerous tumor cells with nuclear expression (red arrow) or with both nuclear and cytoplasmic expression (yellow arrow). Some of the cells with nuclear and cytoplasmic expression show cytoplasmic prolongations that characterize an invasive morphology [15](immunohistochemical technique; 40x).

Figure

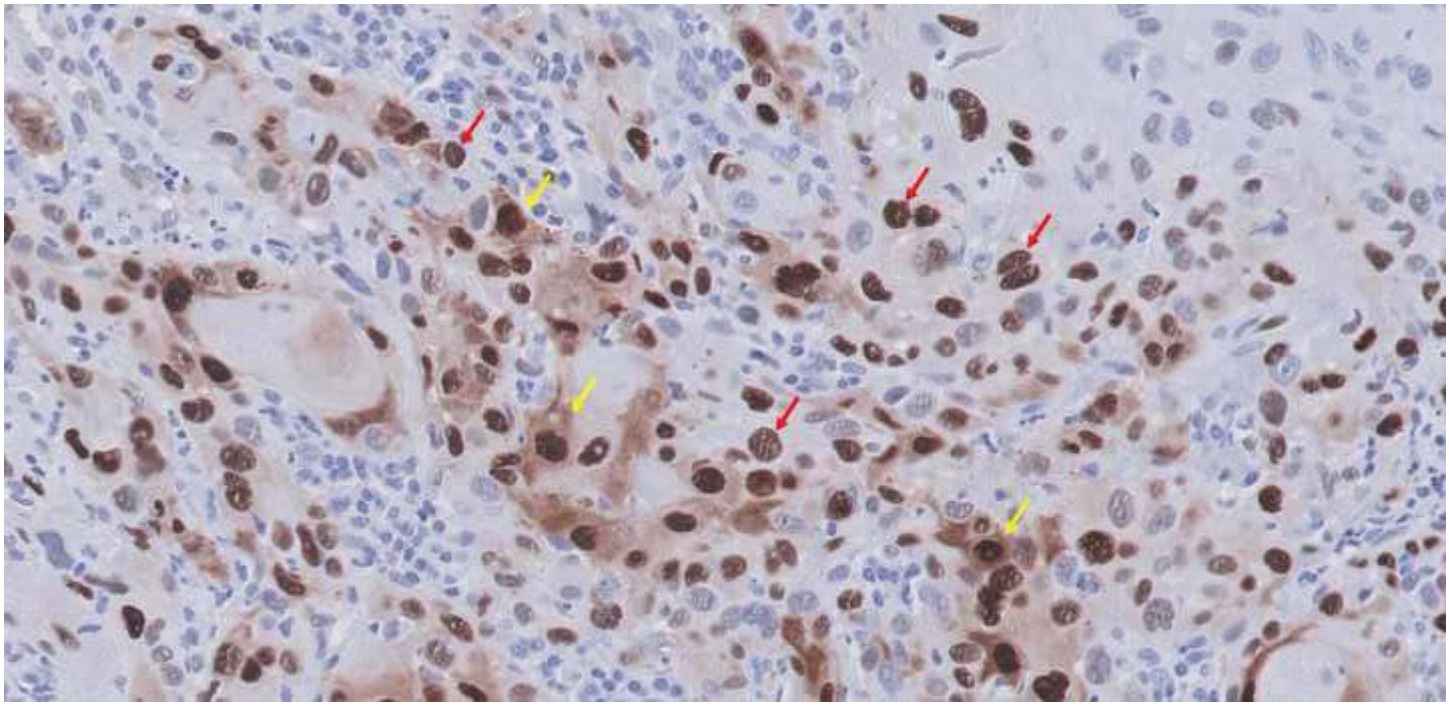

Table I

Table I. Characteristics of patients (n=54) with OSCCs (n=68)

Variable	n (%)
Sex	
Female	14 (25.9)
Male	40 (74.1)
Age (yrs)	
Range	45-87
mean±sd	63±12
Missing	12
Tobacco	
No	8 (28.6)
Yes	20 (71.4)
Missing	26
Alcohol	
Non-drinker	8 (33.3)
Yes	16 (66.7)
Missing	30
Nº tumors	
1	39 (72.2)
2	11 (20.4)
3	2 (3.7)
4	2 (3.7)
mean±sd	1.39±0.74

Table II

Table II. Description of oral tumors with cyclin D1 expression (n=68[a])

Variable	n (%)
Localization	
Mouth floor	11 (17.7)
Tongue	35 (56.5)
Other	16 (25.8)
Missing (n)	6
Clinical presentation	
Ulcer	18 (38.3)
Tumor	15 (31.9)
Other[b]	14 (29.8)
Missing (n)	21
Tumor size	
T1	16 (27.6)
T2	22 (37.9)
T3 (n=4)-T4 (n=16)	20 (34.5)
Missing (n)	10
Adenopathy	
N0	37 (63.8)
N1	13 (22.4)
N2 (n=6)-N3 (n=1)-Nx (n=1)	8 (13.8)
Missing (n)	10
Metastasis	
M0	44 (75.9)
M1 (n=1)-MX (n=13)	14 (24.1)
Missing (n)	10
Stage	
I	13 (22.4)
II	14 (24.1)
III	11 (19.0)
IV	20 (34.5)
Missing (n)	10
Tumor differentiation degree	
High	18 (34.6)
Moderate	22 (42.3)
Low	12 (23.1)
Missing (n)	16
Tumor Ki-67 (%)	
0-24	13 (19.1)
25-49	28 (41.2)
50-74	20 (29.4)
75-100	7 (10.3)
mean±sd	45±20
Morphology	
Non-invasive	13 (56.5)
Invasive	10 (43.5)
Missing (n)	45

a: Corresponding to 54 patients
b: Leukoplakia+tumor (n=7), Lichen planus + ulcer (n=1), Erythroleukoplakia (n=3), and Leukoplakia + ulcer (n=3).

Table III

Table III. Association of clinical-pathological variables and tumor Ki-67 expression with tumor cyclin D1 expression (% cell count) in oral tumors (n=68)

Variable	n[a]	Nucleus (mean±sd)
Tumor size		
T1	16	26±21
T2 (n=21)+T3-T4 (n=5)	40	28±22
p value[b]		0.703
Adenopathy		
N0	36	27±21
N1 (n=12) + N2-N3 (n=8)	20	29±22
p value[b]		0.795
Metastasis		
M0	42	24±17
M1-MX	14	38±30
p value[b]		0.128
Stage		
I	13	30±21
II	13	32±26
III	11	26±16
IV	19	24±22
p value[c]		0.341
Degree of differentiation		
High	18	20±15
Moderate(n=20)+Low(n=12)	32	33±24
p value[b]		0.030
Morphology		
Non-invasive	13	35±22
Invasive	10	55±23
p value[b]		0.045
Ki-67	65	
r[d]		0.28
p value[c]		0.018

a: Differences with n=68 due to missing values.
b: DESCRIPT SUDAAN procedure 7.0 to account for clustering (multiple cancers within patients).
c: REGRESS SUDAAN procedure 7.0 to account for clustering (multiple cancers within patients).
d: r = Pearson correlation.

*Title Page (with author names and affiliations)

Prognostic and clinicopathological significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma: systematic review and meta-analysis

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Word Count – 4961 (main text) + 89 (figure captions)

Conflict of interest statement

None declared

PROGNOSTIC AND CLINICOPATHOLOGICAL SIGNIFICANCE OF *CTTN*/CORTACTIN ALTERATIONS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA: SYSTEMATIC REVIEW AND META-ANALYSIS

ABSTRACT

Objectives: To evaluate the prognostic significance of *CTTN*/cortactin alterations in head and neck squamous cell carcinoma (HNSCC).

Material and methods: We searched PubMed, Embase, Web of Science, and Scopus for studies published before May 2018, evaluating their quality with the Quality in Prognosis Studies (QUIPS) tool. We conducted a meta-analysis to quantify the impact of *CTTN*/cortactin alterations on clinicopathological and survival variables. We examined the heterogeneity among studies, conducted sensitivity analyses, analyzed small-study effects, and carried out subgroup and meta-regression analyses.

Results: Eighteen studies (1,633 patients) met inclusion criteria. Qualitative evaluation demonstrated that not all studies were conducted with the same rigor, and the highest risk of bias was due to the failure to control for confounders. Quantitative evaluation revealed a strong association of *CTTN*/cortactin alterations with N+ status (OR=2.34, 95%CI=1.83-3.00, p<0.001), higher T status (OR=2.65, 95%CI=1.54-4.55, p<0.001), advanced clinical stage (OR=2.23, 95%CI=1.67-2.98, p<0.001), high histological grade (OR=2.09, 95%CI=1.36-3.23, p=0.001), and lower overall survival (OS) (HR=2.39, 95%CI=1.66-3.44, p<0.001). We found heterogeneity in T status, histological grade, and OS and observed small-study effects on N status and OS. In subgroup analyses, a significant association of *CTTN* amplification and cortactin overexpression with the above variables was preserved, and the meta-regression analysis found no significant difference between the prognostic values of these two alterations. The strongest association between *CTTN*/cortactin alterations and a worse outcome was observed in the subgroup of Asian patients and in the subgroup of pharyngolaryngeal SCCs. Finally, important differences were found in the selection of anti-cortactin antibodies for immunohistochemical analysis.

Conclusions: These results indicate that *CTTN*/cortactin alterations should be evaluated to predict the prognosis of patients with HNSCC.

Keywords: *CTTN,* cortactin, head and neck cancer, oral cancer, pharyngeal cancer, laryngeal cancer, prognosis, meta-analysis.

INTRODUCTION

Head and neck cancer is the sixth most frequent type of cancer, with more than 600,000 new cases/year, and it is responsible for around 300,000 deaths/year [1,2]. Head and neck squamous cell carcinoma (HNSCC) represents >90% of these malignant neoplasms and constitutes a heterogeneous group of tumors with anatomical, clinical, histopathological, and molecular differences [1,3]. Prediction of the prognosis for individual patients is highly important, given the 5-year survival rate of around 60% [4,5]. The tumor node metastasis (TNM) system is an important prognostic tool in HNSCC, and N+ status is associated with the worst prognosis [6]. Currently, research is focused on the prognostic value of emerging molecular biomarkers that may serve as a complement in clinical practice [7,8]. Accumulated evidence suggests that *CTTN*/cortactin alterations play a key role in head and neck oncogenesis, particularly in the development of lymph node metastasis [9–11].

Cortactin is a protein encoded by the *CTTN* gene in chromosome band 11q13 [11]. *CTTN* is often amplified due to the frequent amplification of this chromosomal locus, promoting the overexpression of its product, cortactin [11], which is an important event in head and neck carcinogenesis [12]. Cortactin downregulation appears to be associated with characteristically invasive cancers such as melanoma [13] and it has been most widely investigated in the setting of head and neck cancers [11]. Its name, cortactin, derives from its vicinity to cytoskeletal structures of cortical actin [14]. In order to acquire a migratory phenotype, cancer cells must undergo changes in the regulation of their actin cytoskeleton and develop protrusive structures (invadopodia and lamellipodia) on their membrane [15,16]. Cortactin binds to and regulates filamentous actin, enhancing the polymerization and reticular assembly of actin monomers, stabilizing branched actin networks and regulating their structural organization [17,18]. In addition, the interactions of cortactin with numerous proteins, notably Src and the Arp2/3 complex, give it an important role in the regulation of various cell events that are also related to regulation of the aforementioned protrusive structures [17,18]. *CTTN*/cortactin alterations (amplification and overexpression) contribute to a gain in cell migration and invasiveness and have been extensively studied *in vitro* and *in vivo* in HNSCC [11]. Since the first reports [19,20], numerous authors have addressed the relationship of *CTTN*/cortactin alterations with the development of lymph node metastasis and reduced survival in HNSCC [11,21]. Recent studies reported that cortactin may be involved in the regulation of numerous emerging functions in cancer cells, including cell proliferation, angiogenesis, and exosome secretion, thereby playing a role in the tumor microenvironment, which may explain its association with poor prognostic factors for HNSCC besides high N+ status, such as high T status, advanced clinical stage, and poor tumor differentiation [11].

Cortactin is considered as a superior prognostic marker among candidate biomarkers for oral carcinogenesis [9]. However, the prognostic value of *CTTN*/cortactin alterations in HNSCC remains controversial [11] and its usefulness in routine clinical practice has yet to be established. We therefore considered it timely to conduct a systematic review and meta-analysis for the qualitative and quantitative analysis of current scientific evidence on the prognostic significance of the amplification of *CTTN* and the overexpression of its product cortactin in HNSCC. Our objective was to explore whether these alterations can predict the development of the disease, especially the onset of lymph node metastasis, and can therefore

serve as a complementary prognostic tool in routine clinical practice and to assist selection of the optimal therapeutic approach, with the ultimate aim of improving the survival of patients with HNSCC.

MATERIAL AND METHODS

This systematic review and meta-analysis complied with PRISMA guidelines [22] and closely followed the criteria of the *Cochrane Prognosis Methods Group* [23], *Cochrane Handbook for Systematic Reviews of Interventions* [24], and *Center for Reviews and Dissemination (CRD)'s guidance for undertaking reviews in health care* [25].

Protocol

In order to reduce the risk of bias and to improve the transparency, precision, and integrity of this systematic review and meta-analysis, we recorded *a priori* a protocol on its methodology in the PROSPERO international prospective register of systematic reviews (www.crd.york.ac.uk/PROSPERO, registration number CRD42018105228) [26]. The protocol was developed in accordance with PRISMA-P guidelines [27].

Search strategy

We searched PubMed, Embase, Web of Science, and Scopus databases for studies published before May 2018, with no lower date limit. Searches combining database thesaurus terms (MeSH and EMTREE) and free terms. We maximized the sensitivity of the PubMed search was maximized by using the following strategy: ("cortactin"[MeSH Terms] OR ("cortactin"[All Fields] OR "cttn"[All Fields] OR "ems1"[All Fields] OR "ems 1"[All Fields]) AND ("head"[MeSH Terms] OR "head"[All Fields] OR "neck"[MeSH Terms] OR "neck"[All Fields] OR "mouth"[MeSH Terms] OR "mouth"[All Fields] OR "oral"[All Fields] OR "pharynx"[MeSH Terms] OR pharyn*[All Fields] OR oropharyn*[All Fields] OR nasopharyn*[All Fields] OR hypopharyn*[All Fields] OR "larynx"[MeSH Terms] OR laryn*[All Fields] OR "nose"[MeSH Terms] OR "nose"[All Fields] OR "nasal"[All Fields]) AND ("carcinoma, squamous cell"[MeSH Terms] OR ("carcinoma"[All Fields] AND "squamous"[All Fields] AND "cell"[All Fields]) OR "squamous cell carcinoma"[All Fields]). An equivalent search strategy was adapted to the syntax of each consulted database (see protocol).

The reference lists of retrieved records were manually screened for further relevant studies. All references extracted by the search were managed using Mendeley v.1.17.10 software (Elsevier. Amsterdam, The Netherlands); duplicate references were eliminated.

Eligibility criteria

Inclusion criteria: (1) Original research studies published in English; (2) Evaluation of *CTTN* amplification or cortactin overexpression in human tissues from primary HNSCCs; (3) Analysis of the association of *CTTN* and/or cortactin alterations with at least one of the following clinicopathological and/or prognostic variables: N status, T status, clinical stage, histological grade, overall survival (OS), and disease-free survival (DFS). OS was defined as the time interval between the date of diagnosis or surgery to the date of death from HNSCC or any other cause. DFS was defined as the time interval between surgery and the presence of locoregional or distant recurrence or death without recurrence. Given the lack of an international consensus on the definition of survival endpoints, studies using terms other than OS or DFS (e.g., recurrence-free survival) were also included when the definitions in the original studies were the same as used in our meta-analysis.

Exclusion criteria: (1) Reviews, meta-analyses, case reports, editorials, letters, abstracts of scientific meetings, personal opinions or comments, book chapters, and any study in a language other than English; (2) No relation to HNSCC; (3) Reports of *in vitro* or animal experiments; (4) Evaluations of *CTTN* gene alterations other than gene amplification (e.g., polymorphisms) and studies of chromosome band 11q13 that do not specifically discriminate the *CTTN* gene; (5) No analysis of the relationship of alterations with the clinicopathological or survival variables of interest; (6) Insufficient data for the estimation of odds ratios (ORs) for clinicopathological variables and, in studies only reporting time-to-event variables (OS/DFS), the absence of hazards ratios (HRs) with 95% confidence intervals (CIs) and the lack of adequate data for their calculation by survival analysis.

We first screened the titles and abstracts of retrieved articles to select those that appeared to meet the review eligibility criteria. In a second phase, we examined the full texts of the selected articles and removed any that did not fulfil these criteria.

Data extraction

Two authors (PRG and MAGM) independently extracted data from the articles selected for full text study in a standardized fashion using an Excel data collection template (Excel v.2015, Microsoft. Redmond, WA, USA). The extracted data were then reviewed by two different authors (LGR and AA). Discrepancies were solved by consensus. Data were gathered on: first author, year of publication, country and continent of the study, sample size, tumor location, recruitment period, treatment modality, follow-up time, alteration under study (*CTTN* amplification and/or cortactin overexpression), methodology, and the frequency of alterations. In immunohistochemical studies, information was also recorded on the cutoff point, anti-cortactin antibody, and intracellular immunopositivity pattern (membrane/cytoplasm). We also recorded the data required to analyze the parameters of N and T status, histological grade, clinical stage, OS, and DFS.

Quality evaluation

Two authors (PRG and MAGM) evaluated quality using the Quality in Prognosis Studies (QUIPS) tool (*Cochrane Prognosis Methods Group)* [28]. This tool includes six important potential bias areas in the selected studies: [1] study participation, [2] study attrition, [3] prognostic factor measurement, [4] outcome measurement, [5] study confounding, and [6] statistical analysis and reporting [29]. The risk of bias was classified as low, moderate, or high for each domain. Discrepancies were solved by consensus.

Statistical analysis

We considered *CTTN* amplification as "positive" or "negative" in agreement with the methodology assumed by the authors of each study. Cortactin expression was considered "high" or "low" according to the cutoff values provided by the authors of each study. When each individual study analyzed the amplification and the overexpression of *CTTN*/cortactin, both data were gathered and analyzed separately. We used odds ratios (ORs) with their corresponding 95% CIs as association measurement to determine the correlation between *CTTN*/cortactin alterations and clinicopathological variables of the patients. We used HRs with their corresponding 95% CIs as association measurement to estimate the impact of *CTTN*/cortactin alterations on time-to-event variables (OS and DFS). When authors published the HRs with 95% CIs, these were directly extracted from the original articles. When studies published HR in different models (univariate and multivariate), data were extracted from the multivariate model, with its superior fit of potentially confounding variables. If these data were not explicitly provided by authors, they were calculated using the methods of Parmar *et al.* [30] and Tierney *et al.* [31]. When a study only reported survival curves, we extracted the data from Kaplan-Meier curves with Engauge Digitizer 4.1 software (open-source digitizing software developed by M. Mitchell). In some studies with no reported HRs, the adjusted relative risk (RR) or OR was estimated and considered as an approximation of this measurement [32].

In the meta-analysis, we grouped the different association measurements derived from the individual studies. We estimated combined associations using both fixed-effect models (Mantel-Haenszel and inverse variance methods) and a random-effect model (DerSimonian and Laird method). The general effect was graphically represented and subsequently analyzed using forest plots. We evaluated the heterogeneity among studies using the Cochran's Q test based on the chi-square test [33]. Given the low statistical power of this test, the level of significance established was $p<0.1$, assuming apparent heterogeneity and consequently using a random-effect model to calculate combined estimations. We also quantified the proportion of heterogeneity with Higgins' I^2 statistic, in which values of 25, 50, and 75% can be considered as low, moderate, or high heterogeneity, respectively [33,34].

We also conducted sensitivity analyses, evaluating the influence of each individual study on the estimation of the general effect, thereby testing the reliability of combined results [35]. Accordingly, we sequentially repeated the meta-analysis after omitting one study in turn and graphically represented the results. We also constructed funnel plots and used Egger's regression test ($p<0.1$) to evaluate small-study effects, e.g., publication bias [36–38].

We conducted subgroup analyses (*CTTN*/cortactin alteration, ethnicity, tumor location, anti-cortactin antibody, and cutoff point subgroups) to identify possible sources of heterogeneity and to examine the relationships of *CTTN*/cortactin alterations with prognostic and clinicopathological variables in each subgroup. Finally, we used meta-regression techniques to compare the prognostic value of *CTTN* gene amplification and cortactin overexpression.

Software Stata version 14.1 (Stata Corp, College Station, TX, USA) was used for all statistical analyses, using commands written by the user [39]. $P<0.05$ was considered significant in the meta-analysis.

RESULTS

Results of the search of the literature

The flow chart in Figure 1 depicts the search and selection process and its results. We identified a total of 342 records published before May 2018: 73 from PubMed, 105 from Embase, 75 from the Web of Science, 89 from Scopus, and 2 from the reference lists of retrieved studies. After elimination of duplicate records, the title and abstract of 133 potentially eligible studies were screened, leading to the selection of 52 studies for evaluation of their full text. Among these articles, 18 met all eligibility criteria for inclusion in the qualitative study and quantitative meta-analysis [40–57].

Study characteristics

Table 1 summarizes the characteristics of the 18 selected studies, which include 1,633 patients with HNSCC. The sample size of these studies ranges between 13 and 222 patients. *CTTN* amplification was investigated by seven studies, cortactin overexpression by nine, and both alterations by two. The role of *CTTN*/cortactin was studied in oral cavity carcinomas by nine studies, in pharyngolaryngeal SCCs by six, and in mixed HNSCCs (subgroup of studies on combinations of these SCCs and/or carcinomas at other sites) by three. Six studies were conducted in Asian countries and twelve in non-Asian countries (11 in Europe and 1 in North America). In immunohistochemical studies, the cutoff point for cortactin overexpression was 50% in five studies, lower than 50% in four studies, while no cutoff point was used in three studies, which evaluated the overexpression according to color intensity. The studies used numerous different anti-cortactin antibodies, the most frequent being monoclonal clone 30 (5 studies).

This meta-analysis evaluated the prognostic value of *CTTN*/cortactin alterations in the N status (N+ *vs*. N-) of 1,222 patients (recruited in 14 studies; 2 studies [48,49] analyzed both alterations [n=16]), in the T status (T3/4 *vs*. T1/2) of 997 patients (recruited in 10 studies; 1 study [49] analyzed both alterations [n=11]), in the clinical stage (III/IV *vs*. I/II) of 1,083 patients (recruited in 10 studies; 1 study [49] analyzed both alterations [n=11]), in the histological grade (II/III *vs*. I) of 1,064 patients (recruited in 9 studies; 1 study [49] analyzed both alterations [n=10]), and in the OS of 1,118 patients with HNSCC (recruited in 8 studies; 1 study [49]

analyzed both alterations [n=9]). We decided not to meta-analyze the variable DFS, given that only two studies [47,51] investigated this parameter (273 patients).

Qualitative evaluation

Qualitative analysis was conducted using the QUIPS tool, which evaluates potential sources of bias in six domains (Fig. 2):

Study participation. The studies had a high (39%), moderate (39%), or low (22%) risk of bias (Fig. 2), most frequently related to the lack of information on study period and/or place of recruitment, to an inadequate description of patient characteristics (age, hazardous habits, etc.), and to the inclusion of patients who do not represent the population of interest.

Study attrition. The studies evaluated had a high (11%), moderate (11%), or low (78%) risk of bias (Fig. 2), most frequently due to insufficient or no data on the follow-up period. No study described attempts to collect information on patients lost to the follow-up or the reasons for this loss.

Prognostic factor measurement. The studies had a high (44%), moderate (17%), or low (39%) risk of bias (Fig. 2), most frequently due to insufficient information on the immunohistochemical technique or on the system for measuring cortactin expression levels. In some studies, the immunopositivity pattern (cytoplasmic or membrane) was not reported or immunohistochamical images were not depicted. More important limitations such as the use of inappropriate methods for cortactin expression measurement or inappropriate cutoff points were less frequent.

Outcome measurement. The studies had a high (33%), moderate (33%), or low (33%) risk of bias (Fig. 2), most frequently due to failure to define the survival outcomes evaluated (essential due to the lack of international consensus on survival endpoints) and a lack of information on the classification system followed (e.g., the edition of the AJCC/UICC TNM staging system [58]), or on the method used to analyze clinicopathological variables.

Study confounding. The studies had a high (89 %) or moderate (11%) risk of bias (Fig. 2), most frequently due to failures to consider any or all potential confounders. None of the studies provided clear definitions of the confounding factors evaluated or discussed potential candidate confounders or the biological principles by which they might distort the impact of *CTTN*/cortactin alterations on study variables.

Statistical analysis and reporting. The studies had a high (44%), moderate (28%), or low (28%) risk of bias (Fig. 2), most frequently due to an inappropriate statistical analysis, inadequate data to evaluate the analysis is adequate (e.g., Kaplan-Meier curves), erroneous data, and potential bias from the use of inappropriate cutoff points. Selective outcome reporting, a frequent bias in this domain, was not observed in any study.

Quantitative evaluation (meta-analysis)

Association between CTTN/cortactin alterations and the clinicopathological variables of patients with HNSCC

N status. There was no significant heterogeneity among studies (p=0.139, I^2=28.4%; Fig. 3, Table 2). The fixed-effect model revealed a statistically significant relationship between *CTTN*/cortactin alterations and the presence of lymph node metastasis (N+) (OR=2.34, 95% CI=1.83-3.00, p<0.001; Fig. 3, Table 2).

T status. There was a moderate degree of heterogeneity among studies according to the Higgins cutoff point (p=0.004, I^2=61.1%; Table 2). The random-effect model indicated a statistically significant relationship between CTTN/cortactin alterations and locally advanced carcinoma (T3/4) (OR=2.65, 95% CI=1.54-4.55, p<0.001; Table 2).

Clinical stage. There was no significant heterogeneity among studies (p=0.116, I^2=35.3%; Table 2). The fixed-effect model indicated a statistically significant relationship between *CTTN*/cortactin alterations and advanced stage of the disease (III/IV) (OR=2.23, 95% CI= 1.67-2.98, p<0.001; Table 2).

Histological grade. There was a low level of heterogeneity among studies (p=0.058, I^2=45.3%; Table 2). The random-effect model indicated a statistically significant relationship between *CTTN*/cortactin alterations and more advanced histological grade (II/III) (OR=2.09, 95% CI=1.36-3.23, p=0.001; Table 2).

Association between CTTN/cortactin alterations and survival of patients with HNSCC

Overall survival (OS). There was a high degree of heterogeneity among studies, according to Higgins cutoff points (p<0.001, I^2=79.4%; Table 2); therefore, the results for this variable should be interpreted with caution. The random-effect model indicated a statistically significant relationship between *CTTN*/cortactin alterations and poor OS (HR=2.39, 95% CI=1.66-3.44, p<0.001; Table 2).

Disease free-survival (DFS). This variable was not considered in the meta-analysis of this variable, because only two studies including DFS met the meta-analysis eligibility criteria. Both of these studies described a statistically significant association between cortactin overexpression and poor DFS (Hofman *et al.* [47]: HR=3.00, 95% CI=1.30-7.00, p=0.01; You *et al.* [51]: HR=3.27, 95% CI=1.62-6.60, p=0.001).

Quantitative evaluation (secondary analyses)

Sensitivity analysis

In general, the results were not substantively changed after the sequential repetition of meta-analyses, omitting one study in turn, and statistical significance was not lost for any study variable. The study by Bissinger *et al.* [54] exerted the highest influence on the final estimator

in the meta-analyses conducted, mainly in relation to N status (OR=2.82, 95% CI=2.12-3.74 after omission of Bissinger *et al.*) (Fig. 4A), T status (OR=3.14, 95% CI=1.94-5.10 after this omission), and clinical stage (OR=2.91, 95% CI=2.07-4.10 after this omission). These findings suggest that the combined estimations in the main meta-analyses do not depend on the influence of a particular study. However, this study (54) is likely to have had a greater influence in subgroup analyses, and some of the results are probably conservative and underestimated.

Analysis of small-study effects

Visual inspection of the asymmetry of funnel plots and of the statistical tests conducted for this purpose confirmed the absence of small-study effects on T status [p_{Egger}=0.292], clinical stage [p_{Egger}=0.106], or histological grade [p_{Egger}=0.340]) but not on N status ([p_{Egger}=0.019) (Fig. 4B) and OS [p_{Egger}=0.002], for which biases (e.g., publication bias) cannot be ruled out.

Analysis of subgroups

In N-status stratified analyses *(table 2)*, a statistically significant association was maintained for both *CTTN* and cortactin alterations (amplification: OR=2.26, 95% CI=1.52-3.37, p<0.001; overexpression: OR=2.75, 95% CI=1.71-4.44, p<0.001), both ethnic groups (Asian: OR=3.38, 95% CI=1.83-3.00, p<0.001; non-Asian: OR=2.02, 95% CI=1.50-2.71, p<0.001), oral squamous cell carcinoma (OSCC) (OR=2.78, 95% CI=1.68-4.60, p<0.001), and pharyngolaryngeal squamous cell carcinoma (SCC) (OR=2.54, 95% CI=1.67-3.87, p<0.001), 1-50% cutoff point (OR=3.08, 95% CI=1.10-8.59, p=0.032), staining intensity ≥moderate (OR=3.91, 95% CI=2.36-6.45, p<0.001), and the subgroup of other antibodies (antibody other than monoclonal clone 30) (OR=2.89, 95% CI=1.67-4.99, p<0.001). We found a significant (p<0.1) and moderate degree of heterogeneity in the cortactin overexpression (p=0.092, I^2=41.2%) and OSCC (p=0.079, I^2=43.2%) subgroups and a high degree of heterogeneity in the monoclonal clone 30 subgroup (p=0.010, I^2= 78.1%).

In T status-stratified analyses *(table 2)*, a statistically significant association was maintained for both *CTTN* and cortactin alterations (amplification: OR=3.94, 95% CI=2.04-7.63, p<0.001; overexpression: OR=2.65, 95% CI=1.54-4.55, p=0.012) and for the Asian (OR=4.47, 95% CI=2.63-7.59, p<0.001); pharyngolaryngeal SCC (OR=2.38, 95% CI=1.47-3.84, p<0.001); mixed HNSCC (OR=6.34, 95% CI=2.36-16.98, <0.001); staining intensity ≥moderate (OR=2.12, 95% CI=1.16-3.87, p=0.014); and "other antibody" (OR=2.89, 95% CI=1.67-4.99, p<0.001) subgroups. We found a moderate and significant degree of heterogeneity in the cortactin overexpression (p=0.004, I^2=66.8%), non-Asian patient (p=0.041, I^2=54.3%), clone 30 (p=0.038, I^2=69.3%) and other antibody (p=0.067, I^2=54.4%) subgroups, and a high degree of heterogeneity in the OSCC (p=0.002, I^2= 76.0%) and cutoff point >50% (p=0.007, I^2= 80.0%) and 1-50% (p=0.015, I^2=76.1%) subgroups.

In clinical stage-stratified analyses *(table 2)*, a statistically significant association was maintained for both *CTTN* and cortactin alterations (amplification: OR=4.25, 95% CI=2.20-8.23, p<0.001; overexpression: OR=1.94, 95% CI=1.18-3.18, p=0.009), both ethnic (Asian: OR=3.26,

95% CI=1.86-5.73, p<0.001; non-Asian: OR=1.95, 95% CI=1.40-2.74, p<0.001), pharyngolaryngeal SCC (OR=3.41, 95% CI=1.76-6.61, p<0.001), mixed HNSCC (OR=2.14, 95% CI=1.28-3.59, p=0.004), staining intensity ≥moderate (OR=3.85, 95% CI=1.97-7.53, p<0.001), and "other antibody" (OR=2.51, 95% CI=1.57-4.02, p<0.001) subgroups. We found no potential source of high heterogeneity in the subgroup analyses. There was significant low heterogeneity of cortactin overexpression subgroup (p=0.098, I^2=46.1%); and moderate heterogeneity in OSCC subgroup (p=0.014, I^2=71.8%).

In histological grade-stratified analyses *(table 2)*, a statistically significant association was maintained for both *CTTN* and cortactin alteration (amplification : OR=2.70, 95% CI=1.66-4.39, p<0.001; overexpression: OR=1.76, 95% CI=1.01-3.08, p=0.046) subgroups and for the non-Asian (OR=2.34, 95% CI=1.62-3.37, p<0.001), pharyngolaryngeal SCC (OR=2.12, 95% CI=1.23-3.41, p=0.002), mixed HNSCC (OR=3.29, 95% CI=1.89-5.72, p<0.001), and cutoff point >50% (OR=2.65, 95% CI=1.50-4.68, p=0.001) subgroups. No potential source of high heterogeneity was observed in the subgroup analyses. We found significant low heterogeneity in the cortactin overexpression subgroup (p=0.068, I^2=49.0%); and moderate heterogeneity in the Asian patient (p=0.030, I^2=66.4%), OSCCs (p=0.079, I^2=55.9%), cutoff point <50% (p=0.070, I^2=69.6%), and "other antibody" (p=0.015, I^2=71.4%) subgroups.

In OS-stratified analyses *(table 2)*, a statistically significant association was maintained for both *CTTN* and cortactin alteration (amplification: HR=2.10, 95% CI=1.40-3.16, p<0.001; overexpression: HR=2.50, 95% CI=1.60-3.90, p<0.001], both ethnic subgroup (Asian: HR=3.40, 95% CI=2.07-5.59, p<0.001; non-Asian: HR=2.16, 95% CI=1.47-3.18, p<0.001), pharyngolaryngeal SCC (HR=2.76, 95% CI=1.36-5.58, p=0.005), mixed HNSCC (HR=3.17, 95% CI=2.03-4.95, p<0.001), cutoff point >50% (HR=1.76, 95% CI=1.19-2.61, p=0.005), staining intensity ≥moderate (HR=3.45, 95% CI=2.20-5.42, p<0.001), and both antibody (clone 30: (HR=3.58, 95% CI=1.46-8.75, p=0.005; other antibody (HR=1.78, 95% CI=1.06-2.98, p=0.002) subgroups. We found significant moderate heterogeneity in the OSCC (p=0.104, I^2=62.2%) and in "other antibody" (p=0.108, I^2=55.1%) subgroups; and high heterogeneity in the cortactin overexpression (p<0.001, I^2=83.7%), non-Asian patient (p<0.001, I^2=79.3%), pharyngolaryngeal SCC (p<0.001, I^2=87.2%), cutoff >50% (p=0.004, I^2=77.6%), and 30 antibody clone (p<0.001, I^2=90.3%) subgroups.

Meta-regression

Meta-regression analysis results showed no statistically significant difference between *CTTN* gene amplification and the overexpression of its product cortactin in the prognostic value of N status (p=0.756), T status (p=0.783), clinical stage (p=0.124), histological grade (p=0.480) or OS, p=0.812).

DISCUSSION

This systematic review and meta-analysis included 18 studies that analyzed a total of 1,633 patients. *CTTN*/cortactin alterations were found to be correlated with N+ status, high T status,

advanced clinical stage, high histological grade, and worse survival outcomes in patients with HNSCC.

The main mechanism of cortactin overexpression is the amplification of its gene, *CTTN*, located in chromosome band 11q13 [11]. The amplification of 11q13 and the oncogenes in this region is a frequent event in HNSCC and exerts a major influence on its onset and progression [12]. Amplification of *CTTN* is consistently associated with high expression of its mRNA and with cortactin overexpression in tumor tissue, suggesting that actions of this gene in head and neck oncogenesis are predominantly determined by its amplification rather than by other overexpression mechanisms [48,59–62]. These *CTTN*/cortactin alterations play an essential role in the regulation of lamellipodia and invadopodia, whose formation and activation is responsible for an increase in cell migration and invasion [13,15,16] and consequent N+ status, related to a poor prognosis and worse survival [9–11]. The association in HNSCCs of *CTTN*/cortactin alterations with higher T status and high histological grade may be attributable to emerging functions recently attributed to cortactin, notably those related to an increase in cell proliferation [11]. Clark *et al*. [63] observed a significant reduction in proliferation after cortactin knockdown, likely due to cell cycle progression *via* the cortactin-RhoA-Skp2 pathway, as observed in HNSCC cell lines with 11q13 amplification and cortactin overexpression [64]. The relationship of *CTTN*/cortactin alterations with a more advanced clinical stage in HNSCCs would be explained by their aforementioned association with larger tumor size and with lymph node metastasis.

Studies included in the meta-analysis had similar experimental designs, but our qualitative evaluation, using the QUIPS tool of the Cochrane Prognosis Methods Group- [28], revealed that they were not all conducted with the same rigor. Most potential biases detected were related to failures to consider relevant confounding factors (study confounding domain), to perform adequate statistical analysis (statistical analysis and reporting domain), and to use appropriate methods to measure cortactin expression (prognostic factor measurement domain). The most frequent types of bias in this type of meta-analysis are in the study confounding domain [28,29]. Future studies on the prognostic value of *CTTN*/cortactin alterations in HNSCC should avoid the above limitations and comply with all items in the QUIPS tool, improving the validity of findings and facilitating comparisons [28].

The heterogeneity of studies in our meta-analysis was generally low, especially in relation to N status and clinical stage, and histological grade, while it was moderate for T status, and high for OS [33,34]. Our results for OS should therefore be interpreted with care. Analysis of the impact of *CTTN*/cortactin alterations in subgroups and evaluation of potential sources of heterogeneity demonstrated that gene amplification and protein overexpression were both significantly associated with all study variables in a highly similar manner, although the relationship with N status and OS was slightly stronger for overexpression and the relationship with T status, clinical stage, and histological grade was stronger for gene amplification. The meta-regression analysis and the visual examination of confidence intervals confirmed the lack of statistically significant difference between *CTTN* amplification and cortactin overexpression detection in the prognostic evaluation of any study variable. This is a highly relevant finding, given that immunohistochemical studies are routinely conducted in pathology laboratories.

In our meta-analysis, the subgroup of Asian patients showed the strongest association between *CTTN*/cortactin alterations and a worse result all study variables with the exception of histological grade. This may be attributable to the heavy tobacco consumption (inhaled or chewed tobacco, betel nut, etc.) typical of this population, the main cause of amplification of chromosome band 11q13 in HNSCC [11,12,65]. Liu *et al*. [48] reported a high *CTTN* amplification in OSCCs from Asian patients who consumed betel nut.

Among the tumor sites considered, a stronger association was found between *CTTN*/cortactin alterations and worse disease outcomes in the subgroup of patients with pharyngolaryngeal SCCs than in the OSCC subgroup, which only showed a statistically significant association with N status. However, this result may have been influenced by the results of Bissinger *et al*. 2017, the study with the greatest potential bias, producing an underestimation of the final estimator of the OSCC subgroup in the study variables. Furthermore, HNSCCs are known to be a heterogeneous group of cancers at anatomical and molecular level [3]. Future studies should investigate the impact of *CTTN*/cortactin alterations on pharynx and larynx SCCs separately in order to elucidate whether the prognostic association is stronger for one rather than the other, given their anatomical and biological differences [3,66].

The most frequently selected antibody was monoclonal clone 30 (n=5 studies), and we compared its usefulness with that of the other antibodies used (n=6 studies). In the clone 30 subgroup, statistical significance was lost for all study variables with the exception of OS, whose results showed highest degree of heterogeneity observed in this meta-analysis. These findings suggest that future studies should center on investigating these other antibodies (*i.e.:* 4F11, 4D10, H-191, G-20, or EP1922Y), which were associated with less divergent and less heterogeneous results and proved to be more accurate for the prognostic evaluation of HNSCC in comparison to monoclonal clone 30.

Limitations of our meta-analysis include the possible loss of studies published in languages other than English. In addition, we included studies that do not directly report HRs in their survival analyses, although we used the data in these studies to calculate HRs following the methodology of Parmar *et al*. [30] and Tierney *et al*. [31]. Variations in the definition and use of survival endpoints, on which an international consensus has yet to be established, may represent a limitation in the grouping of individual studies for the meta-analysis; thus, differences in survival outcomes considered (e.g., mortality by any cause *vs*. disease-specific) may explain the large between-study differences in OS. Furthermore, our analysis of small-study effects showed a slight skew to the right in the funnel plot for N status and OS both at visual (p_{Egger}=0.019) and statistical (p_{Egger}=0.002) level, likely attributable to small-study publication bias (e.g., a tendency to only publish positive results), as widely observed in the literature on prognostic biomarkers in cancer [67]. Study strengths include the robust results of our meta-analysis, which yielded strong and statistically significant associations of *CTTN/*cortactin alterations with the clinicopathological and survival variables, as depicted in the forest plots, and our performance of a sensitivity analysis.

In conclusion, the consistent results observed in this systematic review and meta-analysis support the importance of evaluating *CTTN/*cortactin alterations for predicting the prognosis of patients with HNSCC. This appears to be especially important for Asian patients with HNSCC.

Future immunohistochemistry studies should use anti-cortactin antibodies that have been found to offer greater precision for the prognosis of HNSCC in comparison to monoclonal clone 30. Given the similar prognostic value for HNSCC observed between gene amplification detection and immunohistochemistry, the latter would appear to be the preferred approach due to its simplicity, low cost, and routine automatized application in pathology laboratories.

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Footnotes

Figure 1. Flow diagram of the identification and selection of relevant studies, analyzing the prognostic and clinicopathological significance of *CTTN*/cortactin alterations in HNSCC.

Figure 2. Evaluation of the risk of bias using the Quality in Prognosis Studies tool (QUIPS).

Figure 3. Forest plot of the association between *CTTN*/cortactin alterations and N status (fixed effect model) in HNSCC.

Figure 4A. Sensitivity analysis graphically representing the results of N status meta-analysis, sequentially omitting one study in turn.

Figure 4B. Funnel plot representing the analysis of small-study effects on N status.

Table 1

Table 1. Characteristics of studies in the meta-analysis (n=18).

Study	Year	Country	Alteration analyzed (sample size)	Tumor subsite	Recruitment period	Treatment	Follow-up (months)	Methods	Anti-cortactin antibody	IHC pattern	IHQ Cutoff point (%)	CTTN cortactin (+) (%)
Takes *et al.*	1997	Netherlands	*CTTN* amplification (21 cases)	Larynx	N/A	Sx, Rt	>24	Southern blot	__	__	__	33.33
Rodrigo *et al.*	2000	Spain	*CTTN* amplification (104 cases)	Oral cavity Oropharynx Hypopharynx Supraglottis Glottis	1992-1994	Sx, Rt	60 Mean 24.4 Median 18	Differential PCR	__	__	__	20.19
Lopes *et al.*	2002	USA	Cortactin overexpression (20 cases)	Oral cavity	N/A	Sx, Rt	8-107	IHC	4F11 (monoclonal)	Membrane Cytoplasm	10	85.00
Chen *et al.*	2004	Taiwan	*CTTN* amplification (13 cases)	Oral cavity	N/A	Sx	N/A	CGH	__	__	__	53.85
Xia *et al.*	2007	China	*CTTN* amplification (33 cases)	Oral cavity	N/A	Sx	N/A	Differential PCR	__	__	__	57.58
Guervos *et al.*	2007	Spain	*CTTN* amplification (56 cases)	Oropharynx Hypopharynx Supraglottis Glottis	1993-1999	Sx, Rt	100	MLPA	__	__	__	33.93
Gibcus *et al.*	2008	Netherlands	Cortactin overexpression (20 cases)	Larynx	N/A	Sx, Rt	>72	IHQ	Clone 30 (monoclonal)	Cytoplasm	13	51.89
Hofman *et al.*	2008	France	Cortactin overexpression (176 cases)	Oral cavity Oropharynx Hypopharynx Larynx	1993-2001	Sx, Rt, Ct	60	IHQ	4F11 (monoclonal)	Membrane Cytoplasm	50	43.75
Liu *et al.*	2009	Taiwan	*CTTN* amplification (82 cases) Cortactin overexpression (49 cases)	Oral cavity	N/A	Sx	N/A	Quantitative PCR and IHQ	Insufficient information	Cytoplasm	50	45.12 (a) 75.51 (o)

Table 1. Characteristics of the included studies (n=18) (continuation).

Rodrigo *et al.*	2009	Spain	*CTTN* amplification (202 cases) Cortactin overexpression (86 cases)	Pharynx Larynx	1992-2002	Sx, Rt	>50	Differential PCR and IHQ	Clone 30 (monoclonal)	Membrane Cytoplasm	Moderate or strong staining	37.13 (a) 43.02 (o)
Yamada *et al.*	2010	Japan	Cortactin overexpression (70 cases)	Oral cavity	N/A	Sx	N/A	IHQ	4D10 (monoclonal)	Membrane Cytoplasm	25	45.71
You *et al.*	2012	South Korea	Cortactin overexpression (97 cases)	Oral cavity Larynx Face Neck Nasal cavity Ear	2000-2008	Sx	8-89 Mean 52.7	IHQ	Clone 30 (monoclonal)	Cytoplasm	50	20.62
Dumitru *et al.*	2013	Germany	Cortactin overexpression (89 cases)	Oropharynx Hypopharynx	1995-2000	Sx, Rt, Ct	60	IHQ	H-191 (polyclonal)	N/A	50	48.31
Ribeiro *et al.*	2014	Portugal	*CTTN* amplification (30 cases)	Oral cavity	2010-2012	Sx, Rt, Ct	N/A	MLPA	__	__	__	66.67
Bissinger *et al.*	2017	Germany	Cortactin overexpression (222 cases)	Oral cavity	2009-2011	Sx, Ct	>200	IHQ	Clone 30 (monoclonal)	N/A	50	55.86
Noorlag *et al.*	2017	Netherlands	Cortactin overexpression (152 cases)	Oral cavity	2004-2010	Sx, Rt, Ct	12	IHQ	Clone 30 (monoclonal)	Cytoplasm	Strong staining	15.79
Liu *et al.*	2017	Taiwan	Cortactin overexpression (122 cases)	Oral cavity	N/A	Sx, Rt, Ct	>40	IHQ	G-20 (polyclonal)	N/A	Moderate staining	53.28
Marioni *et al.*	2018	Italy	Cortactin overexpression (38 cases)	Supraglottis	N/A	Sx	Mean 71.7	IHQ	EP1922Y (monoclonal)	Cytoplasm	1%	89.47

IHC, immunohistochemistry; CGH, comparative genomic hybridization; MLPA, multiplex ligation probe amplification; Sx, surgery; Rt, radiotherapy; CT, chemotherapy; (a), *CTTN* amplification; (o) Cortactin overexpression; N/A, not available.

Figure 1

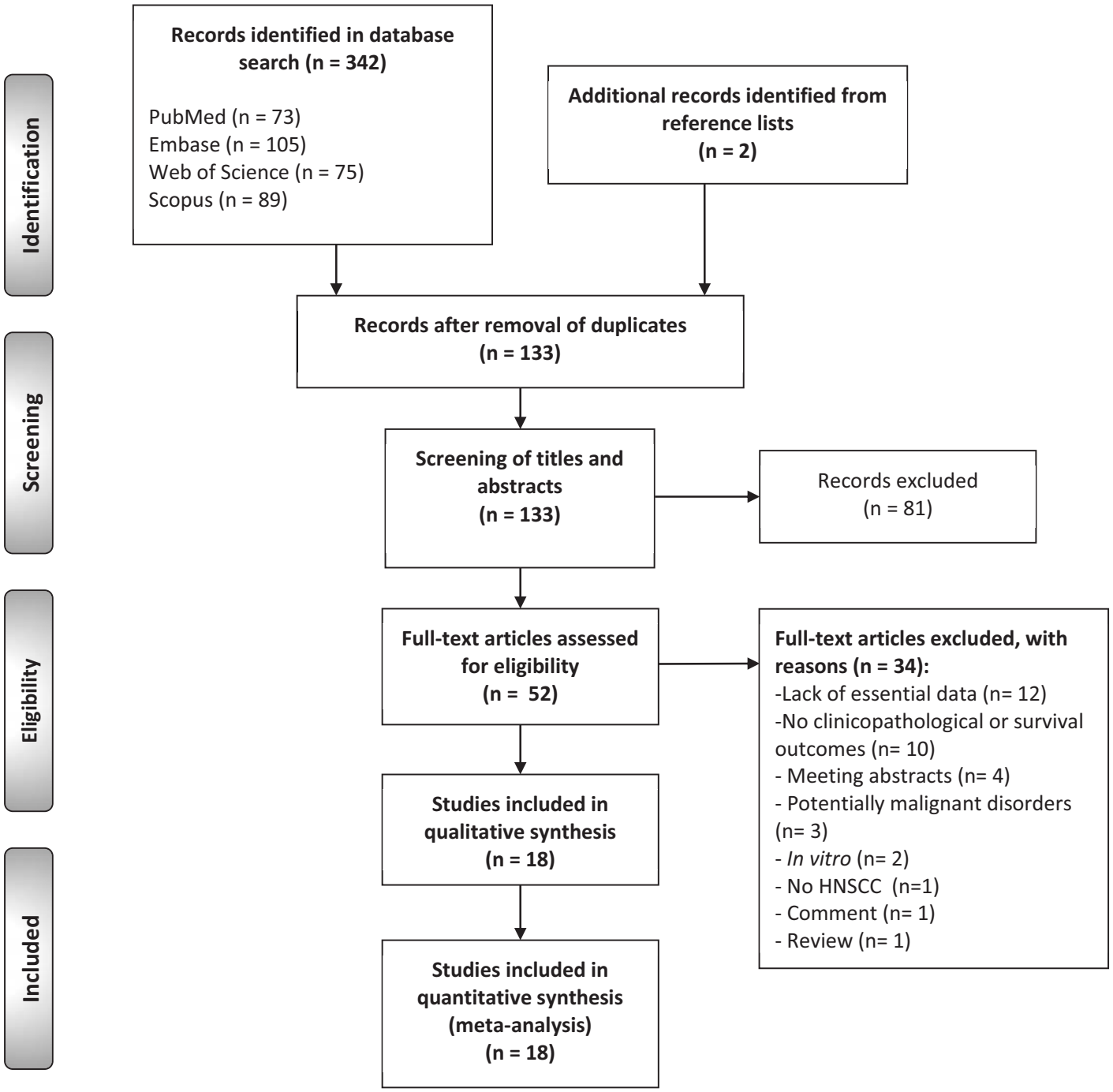

Identification
Screening
Eligibility
Included
Records identified in database search (n = 342)
PubMed (n = 73)
Embase (n = 105)
Web of Science (n = 75)
Scopus (n = 89)
Additional records identified from reference lists (n = 2)
Records after removal of duplicates (n = 133)
Screening of titles and abstracts (n = 133)
Records excluded (n = 81)
Full-text articles assessed for eligibility (n = 52)
Full-text articles excluded, with reasons (n = 34):
-Lack of essential data (n= 12)
-No clinicopathological or survival outcomes (n= 10)
- Meeting abstracts (n= 4)
- Potentially malignant disorders (n= 3)
- In vitro (n= 2)
- No HNSCC (n=1)
- Comment (n= 1)
- Review (n= 1)
Studies included in qualitative synthesis (n = 18)
Studies included in quantitative synthesis (meta-analysis) (n = 18)

Figure 2

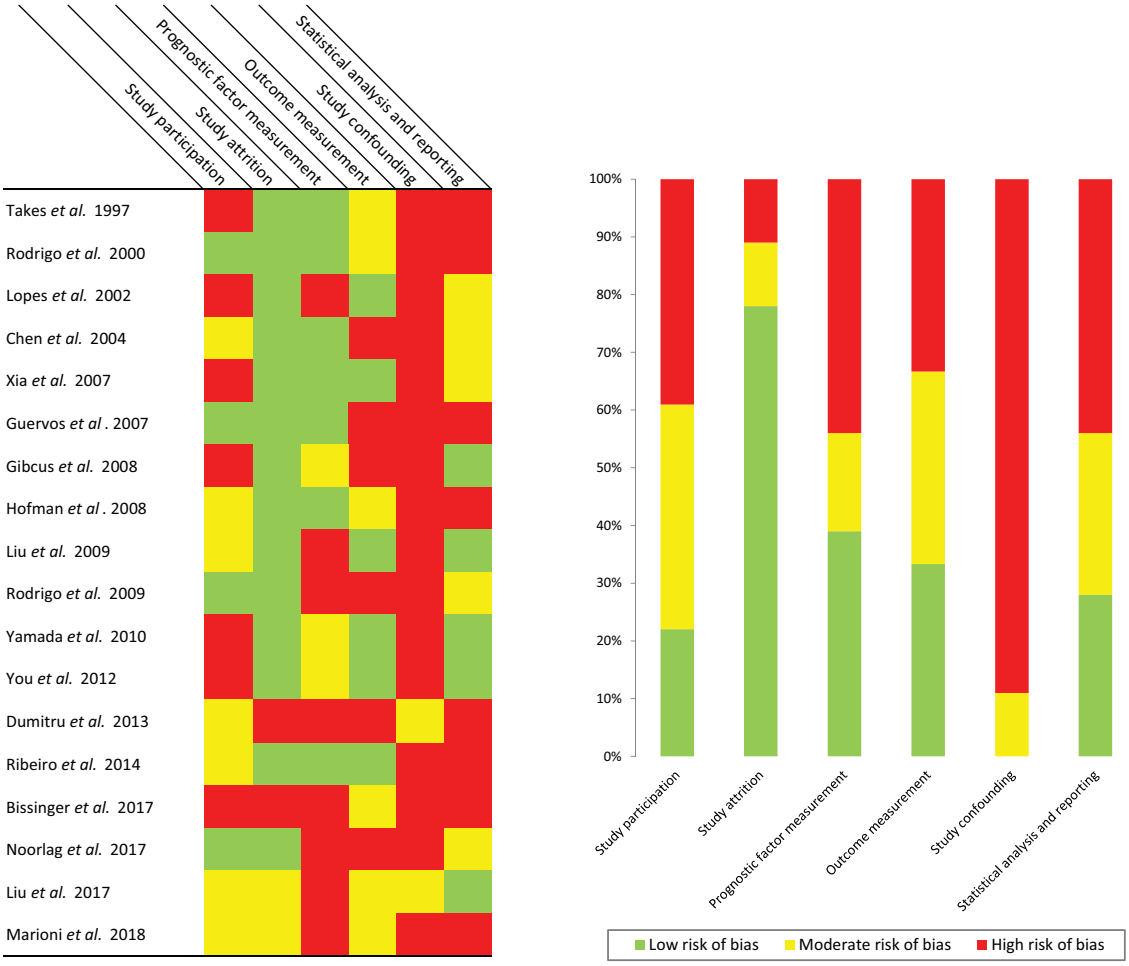

Study participation
Study attrition
Prognostic factor measurement
Outcome measurement
Study confounding
Statistical analysis and reporting
Takes et al. 1997
Rodrigo et al. 2000
Lopes et al. 2002
Chen et al. 2004
Xia et al. 2007
Guervos et al . 2007
Gibcus et al. 2008
Hofman et al . 2008
Liu et al. 2009
Rodrigo et al. 2009
Yamada et al. 2010
You et al. 2012
Dumitru et al. 2013
Ribeiro et al. 2014
Bissinger et al. 2017
Noorlag et al. 2017
Liu et al. 2017
Marioni et al. 2018
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Study participation
Study attrition
Prognostic factor measurement
Outcome measurement
Study confounding
Statistical analysis and reporting
Low risk of bias
Moderate risk of bias
High risk of bias

Figure 3

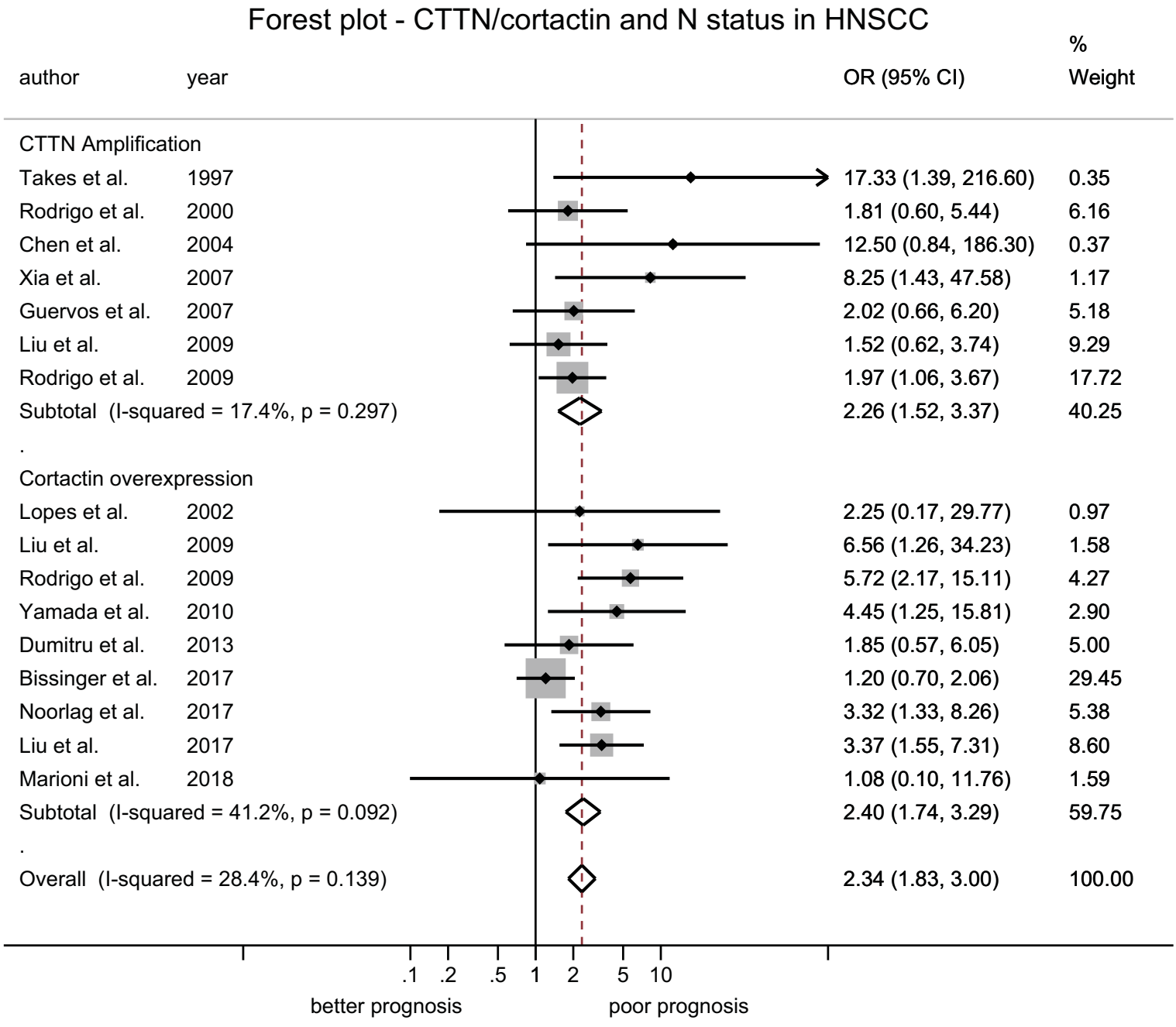
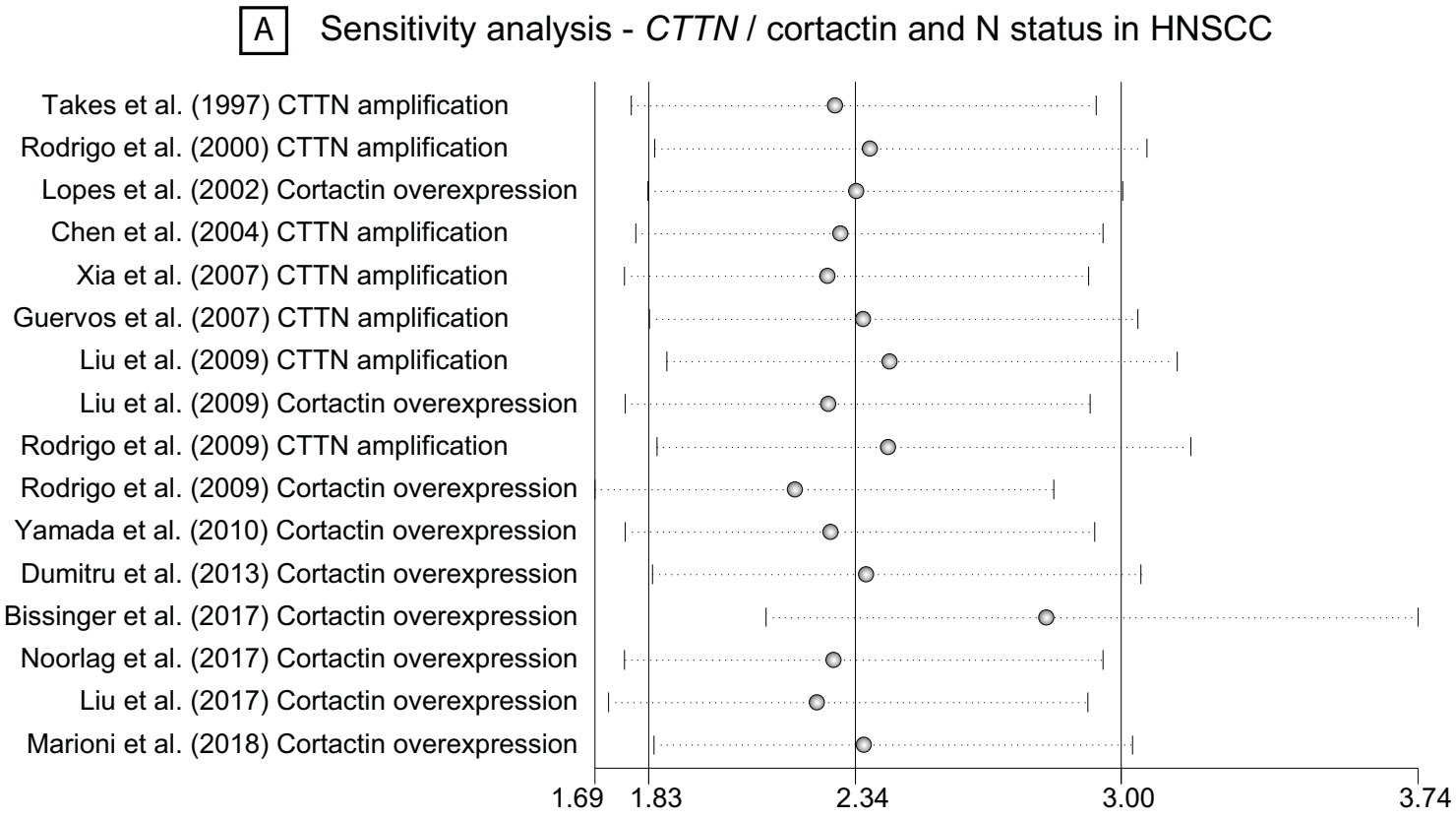

Forest plot - CTTN/cortactin and N status in HNSCC
author
year
OR (95% CI)
% Weight
CTTN Amplification
Takes et al. 1997 17.33 (1.39, 216.60) 0.35
Rodrigo et al. 2000 1.81 (0.60, 5.44) 6.16
Chen et al. 2004 12.50 (0.84, 186.30) 0.37
Xia et al. 2007 8.25 (1.43, 47.58) 1.17
Guervos et al. 2007 2.02 (0.66, 6.20) 5.18
Liu et al. 2009 1.52 (0.62, 3.74) 9.29
Rodrigo et al. 2009 1.97 (1.06, 3.67) 17.72
Subtotal (I-squared = 17.4%, p = 0.297) 2.26 (1.52, 3.37) 40.25
.
Cortactin overexpression
Lopes et al. 2002 2.25 (0.17, 29.77) 0.97
Liu et al. 2009 6.56 (1.26, 34.23) 1.58
Rodrigo et al. 2009 5.72 (2.17, 15.11) 4.27
Yamada et al. 2010 4.45 (1.25, 15.81) 2.90
Dumitru et al. 2013 1.85 (0.57, 6.05) 5.00
Bissinger et al. 2017 1.20 (0.70, 2.06) 29.45
Noorlag et al. 2017 3.32 (1.33, 8.26) 5.38
Liu et al. 2017 3.37 (1.55, 7.31) 8.60
Marioni et al. 2018 1.08 (0.10, 11.76) 1.59
Subtotal (I-squared = 41.2%, p = 0.092) 2.40 (1.74, 3.29) 59.75
.
Overall (I-squared = 28.4%, p = 0.139) 2.34 (1.83, 3.00) 100.00
.1 .2 .5 1 2 5 10
better prognosis
poor prognosis

Figure 4


A
Sensitivity analysis - *CTTN* / cortactin and N status in HNSCC
Takes et al. (1997) CTTN amplification
Rodrigo et al. (2000) CTTN amplification
Lopes et al. (2002) Cortactin overexpression
Chen et al. (2004) CTTN amplification
Xia et al. (2007) CTTN amplification
Guervos et al. (2007) CTTN amplification
Liu et al. (2009) CTTN amplification
Liu et al. (2009) Cortactin overexpression
Rodrigo et al. (2009) CTTN amplification
Rodrigo et al. (2009) Cortactin overexpression
Yamada et al. (2010) Cortactin overexpression
Dumitru et al. (2013) Cortactin overexpression
Bissinger et al. (2017) Cortactin overexpression
Noorlag et al. (2017) Cortactin overexpression
Liu et al. (2017) Cortactin overexpression
Marioni et al. (2018) Cortactin overexpression
1.69
1.83
2.34
3.00
3.74

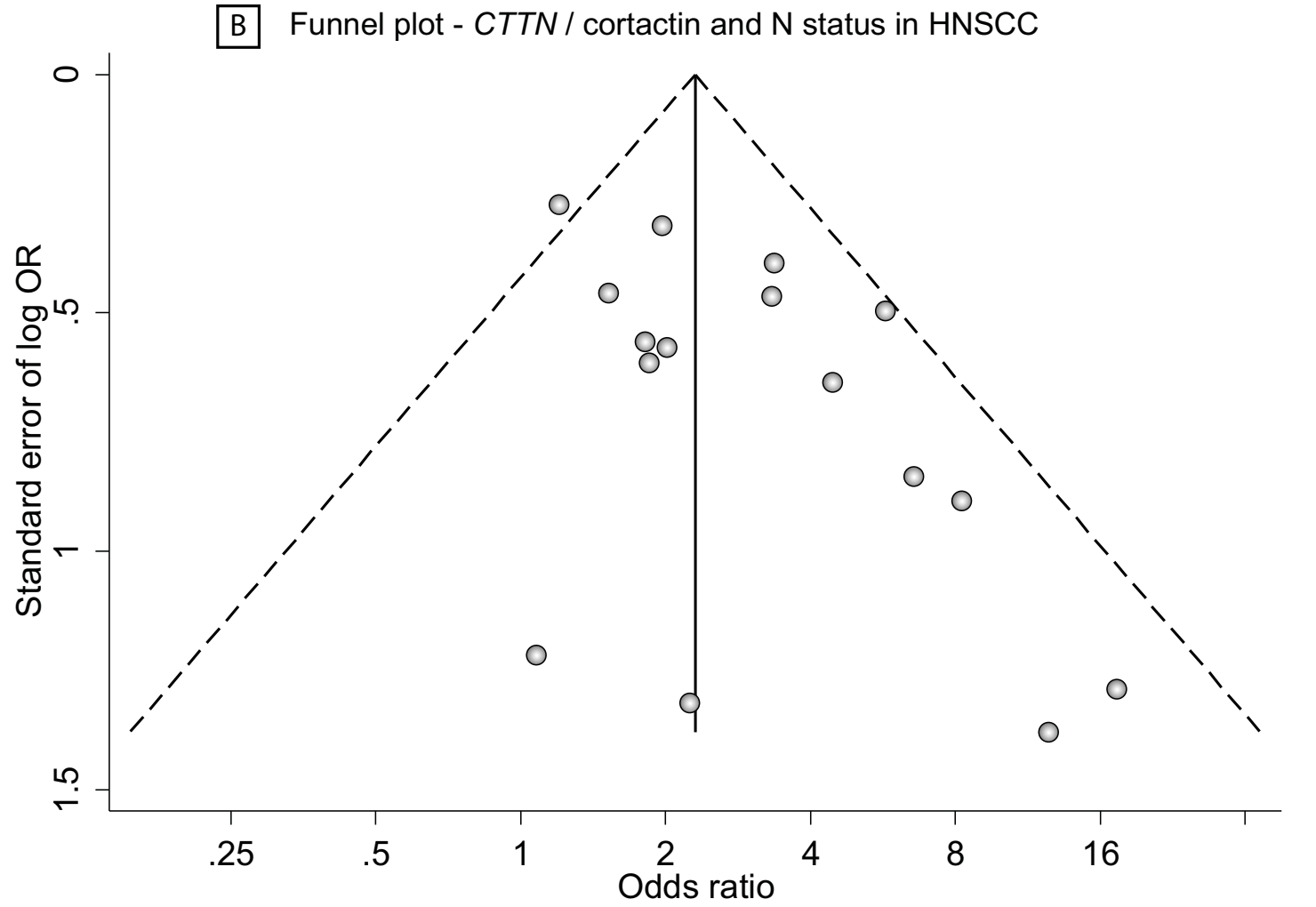

B
Funnel plot - *CTTN* / cortactin and N status in HNSCC
Standard error of log OR
0
.5
1
1.5
.25
.5
1
2
4
8
16
Odds ratio

Table 2. Subgroup analyses

Outcomes	Subgroup	Nº studies	Stat. Model	Pooled data		Heterogeneity		
				OR (95 % CI)	*P*	*Q*	P_{het}	I^2 (%)
N status	All	14+2*	F	2.34 (1.83-3.00)	<0.001	20.95	0.139	28.4
	Alteration							
	Amplification	7	F	2.26 (1.52-3.37)	<0.001	7.26	0.297	17.4
	Overexpression	9	R	2.75 (1.71-4.44)	<0.001	13.61	0.092	41.2
	Ethnic group							
	Asian	6	F	3.38 (1.83-3.00)	<0.001	5.72	0.335	12.6
	Non-Asian	10	F	2.02 (1.50-2.71)	<0.001	12.26	0.199	26.6
	SCC							
	Oral cavity	9	R	2.78 (1.68-4.60)	<0.001	14.09	0.079	43.2
	Pharynx/Larynx	6	F	2.54 (1.67-3.87)	<0.001	6.48	0.262	22.9
	Mixed	1	—	—	—	—	—	—
	Cutpoint**							
	>50	3	F	1.53 (0.97-2.42)	0.069	3.850	0.146	48.0
	1-50%	3	F	3.08 (1.10-8.59)	0.032	1.12	0.571	0.0
	≥mod. Intensity	3	F	3.91 (2.36-6.45)	<0.001	0.86	0.651	0.0
	Antibody**							
	Clone-30	3	R	2.66 (1.00-7.10)	0.051	9.13	0.010	78.1
	Others	5	F	2.89 (1.67-4.99)	<0.001	1.83	0.767	0.0
	N/A	1	—	—	—	—	—	—

*Two studies [48,49] analyzed both alterations. **Overexpression alone

Table 2. Subgroup analyses (Continuation)

Outcomes	Subgroups	Nº studies	Stat. model	Pooled data		Heterogeneity		
				OR (95 % CI)	*P*	*Q*	P_{het}	I^2 (%)
T status	All	10+1*	R	2.65 (1.54-4.55)	<0.001	25.73	0.004	61.1
	Alteration							
	Amplification	3	F	3.94 (2.04-7.63)	<0.001	2.56	0.278	21.8
	Overexpression	8	R	2.65 (1.54-4.55)	0.012	21.07	0.004	66.8
	Ethnic group							
	Asian	4	F	4.47 (2.63-7.59)	<0.001	4.34	0.227	30.9
	Non-Asian	7	R	1.82 (0.98-3.38)	0.056	13.14	0.041	54.3
	SCC							
	Oral cavity	5	R	2.61 (0.91-7.49)	0.074	16.66	0.002	76.0
	Pharynx/Larynx	4	F	2.38 (1.47-3.84)	<0.001	3.97	0.265	24.5
	Mixed	2	F	6.34 (2.36-16.98)	<0.001	0.37	0.542	0.0
	Cutpoint**							
	>50	3	R	2.46 (0.83-7.31)	0.104		0.007	80.0
	1-50%	3	R	1.61 (0.17-15.28)	0.678		0.015	76.1
	≥mod. intensity	2	F	2.12 (1.16-3.87)	0.014		0.347	0.0
	Antibody**							
	Clone-30	3	R	1.76 (0.72-4.34)	0.217	6.52	0.038	69.3
	Others	5	R	2.80 (1.21-6.47)	0.016	8.77	0.067	54.4

*One study [49] analyzed both alterations. **Overexpression alone

Table 2. Subgroup analyses (continuation)

Outcomes	Subgroups	Nº studies	Stat. model	Pooled data		Heterogeneity		
				OR (95 % CI)	*P*	*Q*	P_{het}	I^2 (%)
Clinical stage	All	10+1*	F	2.23 (1.67-2.98)	<0.001	15.46	0.116	35.3
	Alteration							
	Amplification	5	F	4.25 (2.20-8.23)	<0.001	1.62	0.806	0.0
	Overexpression	6	R	1.94 (1.18-3.18)	0.009	9.28	0.098	46.1
	Ethnic group							
	Asian	3	F	3.26 (1.86-5.73)	<0.001	2.90	0.234	31.1
	Non-Asian	8	F	1.95 (1.40-2.74)	<0.001	9.95	0.191	29.7
	SCC							
	Oral cavity	4	R	2.49 (0.95-6.54)	0.065	10.66	0.014	71.8
	Pharynx/Larynx	4	F	3.41 (1.76-6.61)	<0.001	1.48	0.687	0.0
	Mixed	3	F	2.14 (1.28-3.59)	0.004	1.07	0.586	0.0
	Cutpoint**							
	>50	3	F	1.44 (0.97-2.12)	0.068	2.71	0.258	26.2
	1-50%	1	—	—	—	—	—	—
	≥mod. Intensity	2	F	3.85 (1.97-7.53)	<0.001	0.01	0.935	0.0
	Antibody**							
	Clone-30	3	F	1.36 (0.86-2.16)	0.184	3.32	0.190	39.7
	Others	3	F	2.51 (1.57-4.02)	<0.001	2.48	0.289	19.3

*One study [49] analyzed both alterations. **Overexpression alone

Table 2. Subgroup analyses (continuation)

Outcomes	Subgroups	Nº studies	Stat. model	Pooled data		Heterogeneity		
				OR (95 % CI)	*P*	*Q*	P_{het}	I^2 (%)
Histological grade	All	9+1*	R	2.09 (1.36-3.23)	0.001	16.45	0.058	45.3
	Alteration							
	Amplification	3	F	2.70 (1.66-4.39)	<0.001	2.70	0.259	25.9
	Overexpression	7	R	1.76 (1.01-3.08)	0.046	11.76	0.068	49.0
	Ethnic group							
	Asian	4	R	1.81 (0.68-4.82)	0.238	8.94	0.030	66.4
	Non-Asian	6	F	2.34 (1.62-3.37)	<0.001	5.25	0.386	4.8
	SCC							
	Oral cavity	4	R	1.23 (0.74-2.06)	0.467	6.80	0.079	55.9
	Pharynx/Larynx	3	F	2.12 (1.23-3.41)	0.002	2.02	0.365	0.9
	Mixed	3	F	3.29 (1.89-5.72)	<0.001	0.39	0.823	0.0
	Cutpoint**							
	>50	3	F	2.65 (1.50-4.68)	0.001	2.72	0.257	26.5
	1-50%	2	R	2.24 (0.19-26.42)	0.523	3.29	0.070	69.6
	≥mod. Intensity	2	F	1.13 (0.65-1.96)	0.669	0.97	0.325	0.0
	Antibody**							
	Clone-30	3	F	1.77 (1.00-3.14)	0.051	1.25	0.536	0.0
	Others	4	R	1.87 (0.65-5.42)	0.246	10.47	0.015	71.4

*One study [49] analyzed both alterations. **Overexpression alone

Table 2. Subgroup analyses (continuation)

Outcomes	Subgroups	Nº studies	Stat. model	Pooled data		Heterogeneity		
				HR (95 % CI)	*P*	*Q*	P_{het}	I^2 (%)
Overall survival	All	8+1*	R	2.39 (1.66-3.44)	<0.001	38.75	<0.001	79.4
	Alteration							
	Amplification	2	F	2.10 (1.40-3.16)	<0.001	0.21	0.646	0.0
	Overexpression	7	R	2.50 (1.60-3.90)	<0.001	36.88	<0.001	83.7
	Ethnic group							
	Asian	2	F	3.40 (2.07-5.59)	<0.001	0.72	0.397	0.0
	Non-Asian	7	R	2.16 (1.47-3.18)	<0.001	28.97	<0.001	79.3
	SCC							
	Oral cavity	2	R	1.64 (0.87-3.10)	0.129	2.64	0.104	62.2
	Pharynx/Larynx	4	R	2.76 (1.36-5.58)	0.005	23.36	<0.001	87.2
	Mixed	3	F	3.17 (2.03-4.95)	<0.001	1.10	0.576	0.0
	IHQ Cutpoint**							
	>50	4	R	1.76 (1.19-2.61)	0.005	13.41	0.004	77.6
	1-50%	1	—	—	—	—	—	—
	≥mod. Intensity	2	F	3.45 (2.20-5.42)	<0.001	0.72	0.396	0.0
	Antibody**							
	Clone-30	4	R	3.58 (1.46-8.75)	0.005	30.93	<0.001	90.3
	Others	3	R	1.78 (1.06-2.98)	0.029	4.46	0.108	55.1

*One study [49] analyzed both alterations. **Overexpression alone

Abbreviations: Stat., statistical; F, fixed-effects model; R, random-effects model; OR, odds ratio; HR, hazard ratio; CI, confidence intervals; N/A, not available.