BÚSQUEDA DE NUEVOS MARCADORES GENÉTICOS DE SUSCEPTIBILIDAD A LA ENFERMEDAD CELÍACA

TESIS DOCTORAL

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BÚSQUEDA DE NUEVOS MARCADORES GENÉTICOS DE SUSCEPTIBILIDAD A LA ENFERMEDAD CELÍACA

Memoria presentada por la licenciada Blanca Mª Rueda Medina para optar al grado de Doctor en Biología

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1. RESUMEN

La enfermedad celíaca (EC) es una de las patologías autoinmunes complejas más comunes en la actualidad. En su desarrollo es determinante la interacción de factores ambientales y genéticos. El factor ambiental que desencadena la EC es el gluten, una proteína que se encuentra en los productos derivados del trigo, la cebada y el centeno. Su ingesta diaria con la dieta produce una inflamación crónica de la mucosa intestinal en individuos genéticamente predispuestos.

En cuanto a la genética de la EC, la implicación de los genes HLA (human leukocyte antigen)-DQ es la mejor caracterizada por el momento. Un 90% de los pacientes celíacos son portadores de la molécula HLA-DQ2. Además la participación de las moléculas DQ2 es esencial para el desarrollo de la fisiopatología de la enfermedad, puesto que unen con gran afinidad los péptidos derivados del gluten.

El reconocimiento de los complejos DQ2-gluten por los linfocitos T intestinales desencadena una respuesta inflamatoria Th1 caracterizada por la secreción de elevados niveles de interferon gamma (IFNγ). Junto con la activación de la respuesta inmune adquirida, recientemente se ha descrito que ciertos péptidos del gluten pueden estimular la inmunidad innata. En este caso, el principal mediador es la interleuquina 15 (IL-15) que induce la actividad efectora de los linfocitos intraepiteliales (IELs). Todos estos procesos se relacionan con la aparición de una lesión en la mucosa intestinal caracterizada por atrofia de vellosidades intestinales, infiltrado linfocitario e hiperplasia de las criptas.

La interacción entre el gluten y los genes HLA-DQ2 no permite explicar totalmente los mecanismos que llevan al desarrollo de la EC. Se estima que la contribución de los genes HLA al riesgo genético de la EC es sólo de un 40%, sugiriéndose que otros genes podrían influir en la susceptibilidad a esta enfermedad. Al tratarse de una patología genética

compleja espera que haya multitud de genes implicados, cada uno de ellos ejerciendo un efecto modesto.

El grado de conocimiento que existe en la actualidad sobre los genes que intervienen en la EC, a parte de los HLA-DQ es limitado. Por ello se hace necesario realizar una mejor caracterización de la arquitectura genética de esta patología, hecho que contribuiría al mejor entendimiento de los mecanismos moleculares de la EC, al desarrollo de nuevas estrategias de diagnóstico y la identificación de nuevas dianas terapéuticas.

Así el objetivo de este trabajo fue la búsqueda de nuevos factores genéticos asociados con la predisposición a la EC. Para ello se siguió una estrategia basada en la realización de estudios de asociación de genes candidatos, que se seleccionaron en base a su implicación funcional en la patogénesis de la EC.

Como posibles candidatos, en primer lugar se eligieron genes de mediadores proinflamatorios secretados debido a la activación de la respuesta inflamatoria frente al gluten, como el del gen IFNγ (*IFNG*) principal citoquina secretada en la EC, y el de la enzima óxido nítrico sintasa inducible (*NOS2A*) que genera la producción de elevados niveles de óxido nítrico (ON) en los pacientes celíacos. Tanto los altos niveles de IFNγ como el estrés oxidativo creado por las altas concentraciones de NO intervienen en la destrucción de la mucosa intestinal.

También se seleccionaron genes relacionados con la regulación de la respuesta inmune adquirida. En este sentido se propusieron como candidatos interesantes el gen *NFKB1* (factor de transcripción transcripción nuclear kappa B1) uno de los principales factores de transcripción que controlan la expresión de numerosos mediadores proinflamatorios y el gen de la proteína nuclear poli-ADP ribosa polimerasa 1 (*PARP-1*), que también

juega un papel fundamental regulando la cascada de eventos patogénicos que conducen a autoinmunidad.

Asimismo, nos pareció de gran interés explorar la posible implicación en la susceptibilidad a la EC, de genes reguladores de la transmisión de señales a las células T. En este ámbito se escogió el gen *CTLA4* ya que es un importante inhibidor de las señales de activación a través de la vía B7/CD28. También el de la proteín tirosín fosfatasa N22 (PTPN22), una la fosfatasa específica de linfocitos implicada en la señalización a través del receptor de las células T (TCR) y que se ha asociado con numerosas patologías autoinmunes. Igualmente se propuso como gen candidato el del factor de transcrición *FOXP3*, clave para el desarrollo y actividad funcional de las células T reguladoras que median la supresión de linfocitos T autoreactivos.

Por último se seleccionaron como posibles candidatos genes asociados con la respuesta inmune innata, dado reciente papel que se le ha otorgado en las fases iniciales de la respuesta patológica ante el gluten. Se seleccionó el gen *MICA* (moléculas de superficie relacionadas con las moléculas HLA de clase I) por su participación en la estimulación de los IELs, y genes de mediadores típicamente relacionados con la respuesta inmune innata, como la *IL-1, IL-18* y las quimioquinas *MCP-1 y RANTES*.

Estos genes se analizaron mediante la realización de estudios familiares y caso-control considerando diferentes marcadores genéticos asociados a estos genes.

Nuestros resultados sugieren la posible implicación del gen *IFNγ* en la genética de la EC. La presencia del alelo 12 CA del microsatélite (CA)n localizado en el intrón 1 del gen, que se ha asociado con mayores niveles de producción de esta citoquina, confiere un mayor riesgo de padecer la enfermedad. Así mismo, se ha identificado un posible fenómeno de

regulación epigenética asociado a este marcador, que estaría determinado por la herencia de alelos CA de origen paterno. También se observó que los haplotipos del promotor de la *PARP-1* podría ser un nuevo factor de riesgo en la EC, aunque la relevancia de los mismos en la funcionalidad del gen está aún por determinar. Por último, otro hallazgo interesante fue la posible implicación del gen *MICA* en la susceptibilidad a EC. Encontramos que el alelo MICA A5.1 del microsatélite de la región transmembrana de este gen, relacionado con la posible generación de una molécula MICA aberrante, confería un mayor riesgo de EC. La asociación del alelo *MICA A5.1* parece ser independiente de desequilibrio de ligamiento con los alelos HLA-DQ2 y de la forma de presentación clínica de la enfermedad. El análisis del resto de las variantes genéticas analizadas reveló que estos no parecen desempeñar un papel relevante en la predisposición genética a EC.

Los resultados obtenidos en nuestro trabajo junto con los aportados por el resto de la comunidad científica contribuyen al avance en el conocimiento de los factores genéticos que hay detrás del desarrollo de la EC, pero aún queda mucho trabajo por hacer pues nos encontramos muy lejos de la identificación completa de los genes implicados en la EC.

El desarrollo de nuevas estrategias encaminadas a la mejora de los estudios de asociación, como la disponibilidad de poblaciones de estudio con un mayor número de individuos, la identificación de la mayoría de marcadores genéticos distribuidos a lo largo del genoma humano y el desarrollo de plataformas de genotipado a gran escala, abre nuevos caminos que en un futuro próximo podrían llevar al mejor conocimiento de los factores genéticos responsables de las enfermedades autoinmunes complejas como la EC.

2. INTRODUCCIÓN

Los primeros datos escritos que hacen referencia a la enfermedad celíaca (EC) se remontan al siglo II cuando el médico griego Areteo de Capadocia la describió como un síndrome de malabsorción en adultos. Debido a la presencia prioritaria de síntomas gastrointestinales que caracterizaban esta patología la llamó celíaca, palabra griega que significa abdominal. A pesar de las tempranas referencias a la EC, la primera descripción completa de esta patología no se realizó hasta 1888 por Samuel Gee que describió como los síntomas más importantes de la EC la diarrea, el dolor abdominal y el fracaso del crecimiento. El factor causante de la EC permaneció desconocido hasta 1950 cuando el pediatra holandés Willem Dicke determinó que los síntomas de la EC se debían a la ingesta de productos derivados del trigo. Más adelante se pudo establecer que el elemento del trigo responsable del desarrollo de la EC era el gluten (1). Desde entonces se ha producido un gran avance en el conocimiento de las bases fisiopatológicas que llevan al desarrollo de la EC y se han podido describir gran parte de los mecanismos moleculares y celulares que desencadenan esta patología.

2.1 Características clínicas de la EC.

En la actualidad la EC se define como una enteropatía de origen autoinmune que afecta a individuos genéticamente predispuestos tras la ingesta de gluten con la dieta diaria (2).

La ***presentación clínica de la EC*** es muy diversa y pueden aparecer un amplio espectro de síntomas, dependiendo sobre todo de la edad del paciente y de la duración de la enfermedad (3). Otro hecho característico es que durante la evolución de la EC en un individuo existen

periodos de tiempo en los que no aparecen síntomas clínicos obvios. Las formas en las que se puede presentar la enfermedad son las siguientes (4):

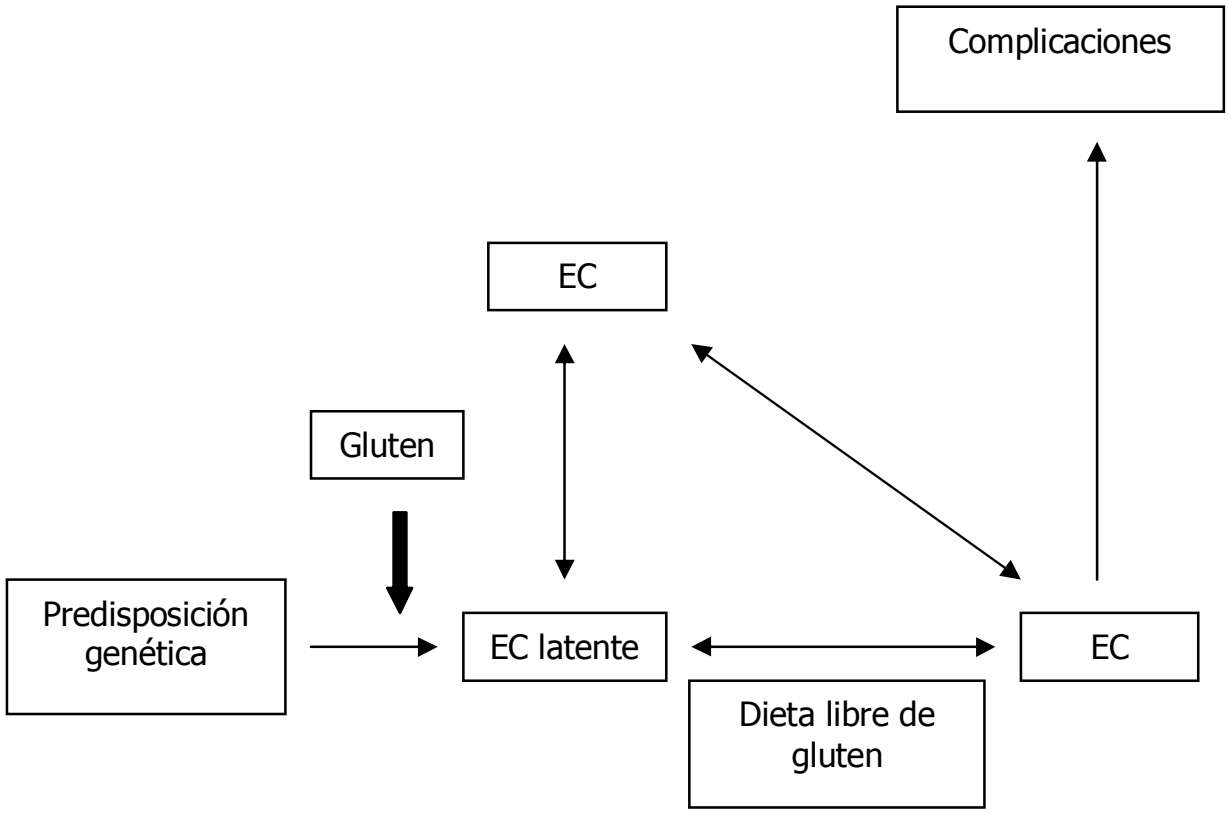
Latente: esta forma es la que precede al diagnóstico de la EC y es la que se presenta cuando los pacientes diagnosticados siguen la dieta libre de gluten. En la biopsia intestinal no aparece atrofia de vellosidades ni hiperplasia, pero persiste el infiltrado linfocitario y se mantiene un bajo grado de inflamación intestinal

Típica o EC activa: se caracteriza por la presencia de síntomas intestinales (diarrea crónica y distensión abdominal) y/o extraintestinales (anemia, fracaso del crecimiento). Aparece atrofia de vellosidades, hiperplasia de las criptas y un elevado número de autoanticuerpos (antiendomisio y antitransglutaminasa) Es la forma más común de presentación en niños pequeños (entre los 6 y 18 meses).

Atípica: se presenta con molestias intestinales leves y una gran variedad de manifestaciones extraintestinales (dermatitis herpetiforme, anemia por deficiencia de hierro, baja estatura, retraso puberal, ataxia cerebral, etc). Se suele diagnosticar en niños de edad más avanzada y adultos.

Silente: se caracteriza por la presencia de una biopsia intestinal positiva para la EC y de autoanticuerpos en un individuo asintomático. Estos individuos son diagnosticados de forma causal mediante rastreos epidemiológicos poblacionales o por acudir al médico debido a otras patologías.

Figura 1. Formas de presentación de la EC.



Parte de estos síntomas son consecuencia de la inflamación intestinal crónica desencadenada por el gluten que produce una lesión de la mucosa intestinal caracterizada por la aparición de infiltrado linfocitario, hiperplasia de las criptas y atrofia de vellosidades intestinales (3). La severidad de la enfermedad se determina por la magnitud de estos cambios histológicos típicos, pudiéndose llegar en los casos mas graves a la atrofia total de las vellosidades intestinales. La confirmación de los cambios histológicos de la mucosa mediante una biopsia intestinal es uno de los requisitos imprescindibles para confirmar el diagnóstico de la EC.

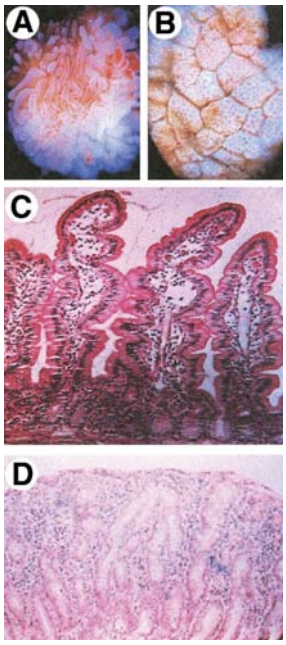


Figura 2. Características histológicas de la lesión celíaca
A: Biopsia intestinal normal con elevado número de vellosidades.
B: Biopsia de un paciente celíaco con atrofia total de vellosidades.
C: Sección de la mucosa intestinal de un individuo sano.
D: Sección de la mucosa intestinal de un paciente celíaco donde se puede apreciar atrofia de vellosidades e hiperplasia de las criptas.

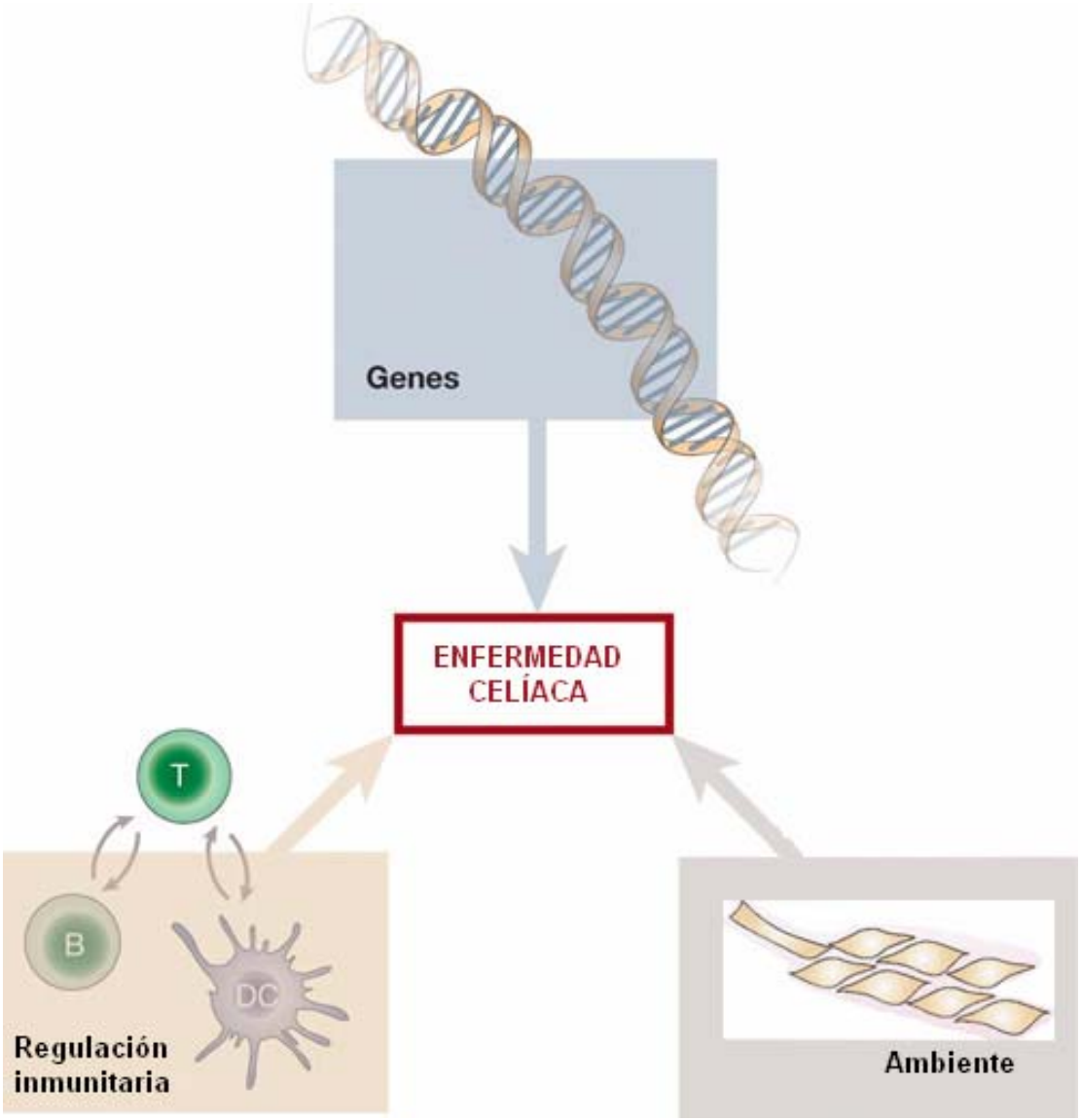
La amplia variedad de formas de presentación de la EC hizo que muchos pacientes permaneciesen sin diagnosticar y por lo tanto se infravaloró ***la prevalencia de la EC*** considerándola como una enfermedad rara. Con la mejora de las técnicas y criterios de diagnóstico hoy día se considera a la EC como una de las patologías autoinmunes mas frecuentes en poblaciones europeas y americanas con una prevalencia estimada del 0.5-2 % y una ratio mujer: hombre 2:1. Por esta razón, se propuso para la prevalencia de la EC el "modelo del iceberg", debido a que por cada caso diagnosticado que emerge, hay aún muchos más que permanecen ocultos (4).

Hay también que destacar que en los pacientes celíacos, tras sufrir largos periodos de la enfermedad, pueden aparecer serias ***complicaciones*** siendo las más comunes: neuropatía periférica, osteopenia, enfermedad tiroidea autoinmune, diabetes mellitus, infertilidad, miocarditis autoinmune, linfomas malignos y adenocarcinomas en el tracto gastrointestinal (3).

Por el momento el único ***tratamiento*** aceptado para la EC es seguir una dieta libre de gluten de por vida, lo cual supone eliminar en la alimentación todos los productos derivados del trigo como el pan y las pastas, así como los derivados de la cebada y el centeno. Con el seguimiento de esta dieta en la mayoría de los pacientes se consigue recuperar hasta un 95 % la normalidad histológica de la mucosa intestinal. Sin embargo, hay pacientes que no mejoran a pesar de seguir una dieta estricta libre de gluten, estos casos se conocen como "sprue celiaco refractario" y los pacientes que lo presentan acaban desarrollando malabsorción severa y atrofia total de vellosidades. Por otra parte, el seguimiento de la dieta libre de gluten es caro y difícil, puesto que gran parte de los productos alimenticios naturales y elaborados contienen gluten. El diseño de nuevas estrategias terapéuticas es por tanto de gran interés para estos pacientes y se está intentando desarrollar nuevas terapias aunque por el momento no han conseguido paliar con éxito los síntomas de los pacientes celíacos (5).

El origen de esta complicada situación se debe a la interacción de tres elementos fundamentales: el ambiente, la alteración del sistema inmunitario y la genética del individuo. La interacción de estos tres factores es esencial para el desarrollo de la EC, hecho que también es imprescindible para la aparición de la mayoría de enfermedades autoinmunes complejas.

Figura 3. Factores que interaccionan para el desarrollo de la EC.



2.2 Los factores ambientales en la EC.

Los cereales suponen la mayor fuente de nutrientes para numerosas comunidades humanas, siendo en el caso de los países occidentales la base de la pirámide alimenticia. Para los pacientes celiacos sin embargo, ciertos cereales son el principal factor ambiental causante de su patología.

Se sabe que las proteínas del trigo, la cebada y el centeno son los desencadenantes esenciales de la EC. Estas proteínas se encuentran en el endospermo del grano de los cereales y su principal función es la de proporcionar una reserva energética para la germinación. Comúnmente se las denomina como ***gluten***, aunque científicamente "gluten" hace referencia al grupo de proteínas contenidas en el grano del trigo. El gluten pertenece a la fracción insoluble de esta reserva proteica y está formado por dos fracciones mayoritarias, las gliadinas y las gluteninas (6). La diversidad biológica de las proteínas del gluten es muy amplia, pudiendo existir en una sola variedad de trigo numerosas variantes alélicas que generan un gran número de proteínas de gluten diferentes.

La principal característica del gluten es su alta riqueza en residuos de prolina (aproximadamente un 20 %) y en glutamina (aproximadamente un 38%). El alto contenido en prolamina lo hace bastante resistente a la digestión por las enzimas digestivas gástricas, pancreáticas e intestinales, resultando en la presencia de péptidos relativamente largos ricos en prolamina y glutamina en el intestino delgado (7).

Figura 4. Ejemplo de la secuencia primaria de un péptido derivado del gluten.

1 VRVPVPQLQPQNPSQQQPQEQVPLVQQQQFLGQQQPFPPQ

41 QPYPQPQPFPSQQPYLQLQPFPQPQLPYSQPQPFRPQQPY

81 PQPQPQYSQPQQPISQQQQQQQQQQQQQQQQQQQILQQILQ

121 QQLIFCMDVVLQQHNIAHGRSQVLQQSTYQLLQELCCQHL

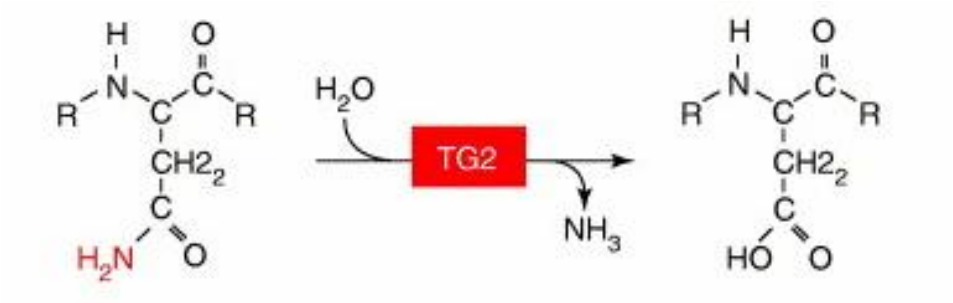
161 WQIPEQSQCQAIHNVVHAIILHQQQKQQQQPSSQVSFQQP

201 LQQYPLGQGSFRPSQQNPQAQGSVQPQQLPQFEEIRNLAL

241 QTLPAMCNVYIAPYCTIAPFGIFGTN

Por otro lado, el alto contenido en residuos de prolina y glutamina hace que estos péptidos sean un substrato ideal para la enzima transglutaminasa de tejidos (TG2). Entre las funciones que leva a cabo la TG2 se encuentra una reacción conocida como deaminación, consistente en la modificación de los residuos de glutamina por ácido glutámico. Se sabe que la TG2 lleva a cabo la deaminación de residuos específicos de glutamina mediante el reconocimiento de motivos QXP, aumentando de esta forma el número de cargas negativas de los péptidos derivados del gluten (8).

Figura 5. Reacción de deaminación.



GLUTAMINA ÁCIDO GLUTÁMICO

Actualmente se conocen un elevado número de péptidos del gluten que se pueden considerar inmunogénicos (7), siendo el prototipo de los mismos el péptido 33-mer, que comprende los residuos 57 al 89 de la gliadina y reune todas las características anteriormente descritas: es altamente estable y presenta una elevada especificidad para la deaminación por la TG2. En su secuencia se pueden encontrar siete epítopos inmunogénicos. (9).

A pesar del prominente papel del gluten como factor ambiental desencadenante de la EC, se cree que otros factores ambientales podrían afectar al desarrollo de esta enfermedad. En este sentido, se ha propuesto que infecciones bacterianas podrían influir en el desarrollo de la EC al aumentar la permeabilidad intestinal (10).

2.3 La respuesta inmune en la EC: mecanismos moleculares y celulares.

El sistema inmune en la mucosa intestinal desempeña dos funciones elementales como son la defensa del organismo frente a infecciones por patógenos y la de reconocer los nutrientes y la flora comensal que son beneficiosos para el individuo y por tanto deben ser tolerados. En el caso de los pacientes celíacos esta última función se altera, y el gluten ingerido con la dieta causa una activación exacerbada de los mecanismos de inmunidad innata y adquirida.

2.3.1 Claves de la activación de la respuesta inmune adquirida frente al gluten.

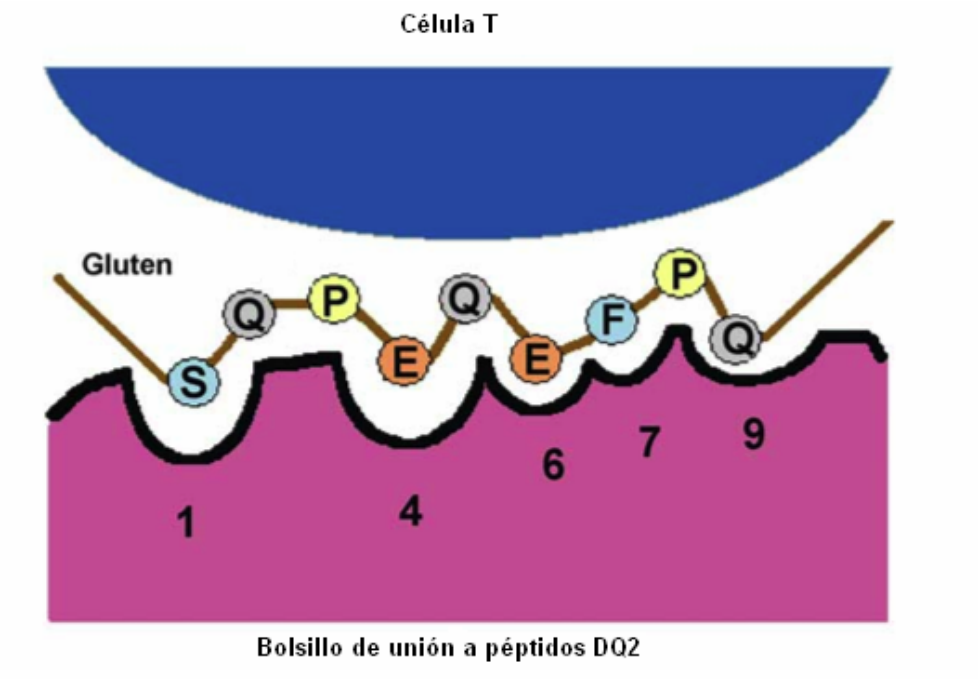
Uno de los eventos centrales en la fisiopatología de la ECC es el desarrollo de una **respuesta T específica ante el gluten.**

Para que esto suceda el paso previo imprescindible es que los péptidos antigénicos del gluten atraviesen la barrera intestinal y lleguen intactos a la lámina propia. Aun no se conoce con exactitud como ocurre este transporte, pero podría ocurrir debido a un aumento de la permeabilidad intestinal en los pacientes celiacos o bien mediante la formación de vesículas en los enterocitos (7).

Una vez alcanzan la lámina propia, los péptidos nativos del gluten y aquellos que han sido deaminados son procesados por las células presentadoras de antígenos (APC) y presentados a los linfocitos T CD4+ gluten-específicos.

La presentación de los péptidos de gluten es altamente específica y se realiza exclusivamente en presencia de las moléculas HLA de clase II DQ2 y DQ8. Pero en los pacientes celíacos se expresan otras muchas moléculas de clase II, lo cual hace preguntarse cual es la característica especial de los heterodímeros DQ2-DQ8 para que presenten con gran afinidad los péptidos antigénicos derivados del gluten. La particularidad de las moléculas DQ2-DQ8 reside en su gruta de unión a péptidos, que en las principales posiciones de anclaje favorece la unión de motivos cargados negativamente (2, 11). Así, las moléculas DQ2-DQ8 están especialmente diseñadas para unir péptidos antigénicos ricos en cargas negativas como es el caso de los derivados del gluten.

Figura 6. Motivo de unión a péptidos de las moléculas DQ2 y DQ8.



Una vez formados, los complejos DQ-gluten capaces de activar eficientemente las células T CD4+ gluten-específicas de la mucosa intestinal, inician el desarrollo de una respuesta inflamatoria tipo Th1, con la consecuente secreción de gran cantidad de mediadores proinflamatorios.

A pesar de que el principal inductor de la polarización hacia una respuesta Th1 es la interleuquina (IL) 12, en la EC la respuesta Th1 parece estar mediada por el interferon alpha (IFN-α) y la IL-18 (12). En la mucosa de los pacientes celíacos con episodios activos de la enfermedad se han detectado altos niveles de expresión de IL-18 y de IFN-α, citoquinas que se han relacionado con la activación y diferenciación de un elevado número de células Th1 (13-15).

La principal citoquina secretada por las células T gluten-específicas activadas es el interferon gamma (IFNγ). Sus niveles de expresión son mucho mayores en los pacientes celíacos con respecto a controles sanos (15-17). El IFNγ ocupa una posición central del proceso fisiopatológico de la EC ya que está implicado en la regulación de los procesos que llevan al

mantenimiento de la respuesta inflamatoria intestinal y en los que generan la lesión de la mucosa. Además del IFNγ, en los pacientes celíacos se han detectado también niveles elevados de otros mediadores proinflamatorios como el factor de necrosis tumoral (TNF-α) o la IL-6 (18, 19).

Junto con la respuesta T gluten-específica, otro evento fundamental que es consecuencia de la estimulación de la respuesta inmune adquirida por el gluten es la **producción de auto-anticuerpos.** En los pacientes celíacos se detectan principalmente auto-anticuerpos dirigidos a la TG2 (antitransglutaminasa, TTGA) y en menores niveles anticuerpos anti-endomisio (EMA) y antireticulina (ARA) (20, 21). El isotipo de los anticuerpos anti-trasnglutaminasa puede ser IgA o IgG , pero los IgA son los que se producen en mayores cantidades en la mucosa intestinal y los que muestran una mayor especificidad utilizándose sus niveles en suero como una de las herramientas de diagnóstico de la EC (22). No se sabe con certeza si estos auto-anticuerpos tienen alguna relevancia en la fisiopatología de la enfermedad, pero se ha sugerido que los anticuerpos antitransglutaminasa podrían influir en la atrofia de vellosidades interrumpiendo la migración de fibroblastos y células epiteliales desde las criptas a las vellosidades intestinales (23). También podrían ser responsables de la aparición de manifestaciones extraintestinales neurológicas, hepáticas o cutáneas (24-26).

El mecanismo por el cual se genera la formación de autoanticuerpos aún no se ha descrito exactamente, pero se cree que en este podrían ser fundamentales los complejos gluten-TG2 que se forman durante la deaminación. Estos complejos serían procesados por las células B y unidos a moléculas HLA-DQ2 de las mismas. Las células T gluten específicas reconocerían estos complejos y podrían proporcionar la señal estimuladora a las células B para la secreción de auto-anticuerpos (27).

2.3.2 La inmunidad innata: papel en la lesión de la mucosa intestinal.

En los pacientes celíacos junto con los linfocitos T CD4+ gluten-específicos, la población de linfocitos intraepiteliales (IELs) también se encuentra dramáticamente expandida. En concreto, las poblaciones mas aumentadas son la de IELs TCR$\alpha\beta$+ CD8+ y TCR$\gamma\delta$+ (28). Estas poblaciones de IELs se caracterizan por sobre-expresar en su superficie receptores activadores de la familia de las células asesinas naturales (NKG2D), a través de los cuales reconocen moléculas HLA no clásicas como las moléculas MICA (moléculas relacionadas con el complejo mayor de histocompatibilidad de clase I) (29). La expresión de moléculas MICA también está inducida en los pacientes celíacos como consecuencia de las condiciones de estrés existentes en la mucosa intestinal debidas al proceso inflamatorio (29, 30). De esta forma los IELs reconocería a los enterocitos estresados y mediarían su muerte, contribuyendo a la atrofia de vellosidades (29, 31).

Todo este proceso parece estar dirigido por un aumento en los niveles de IL-15. En las fases activas de la EC se observan elevados niveles de secreción de esta citoquina en la lamina propia y en el epitelio (32, 33). El aumento de los niveles de IL-15 se correlaciona con la inducción en los IELs de la expresión de los receptores NKG2D, así como con la expresión de moléculas MICA en los enterocitos (29, 31, 33). Otro efecto de la IL-15 sobre los IELs es la inducción en estas células de la secreción de mediadores inflamatorios como el IFNγ y el TNF-α (34). Así, la IL-15 se ha revelado como un importante mediador del daño epitelial en la EC.

El mecanismo por el cual se produce el aumento en los niveles de IL-15 en los enterocitos se conocía pobremente hasta que se identificó un

péptido del gluten conocido como p31-43 que no estimulaba las células T CD4+, pero que era capaz de causar daño en la mucosa intestinal (35, 36). Este péptido produce la activación en los enterocitos de la vía de las proteínas quinasas activadas por mitógenos (MAP quinasas) desencadenando el aumento de la expresión de la IL-15 (37). Aunque el mecanismo exacto por el cual ciertos péptidos del gluten son capaces de estimular la secreción de IL-15 no se conoce aún, se cree que podría ser mediante su unión a un receptor de superficie tipo PPR (receptor específico de reconocimiento de patrón) aún sin identificar o a través de la formación de vesículas. Este péptido constituiría la señal de daño que activaría el sistema inmune innato en los pacientes celíacos (38).

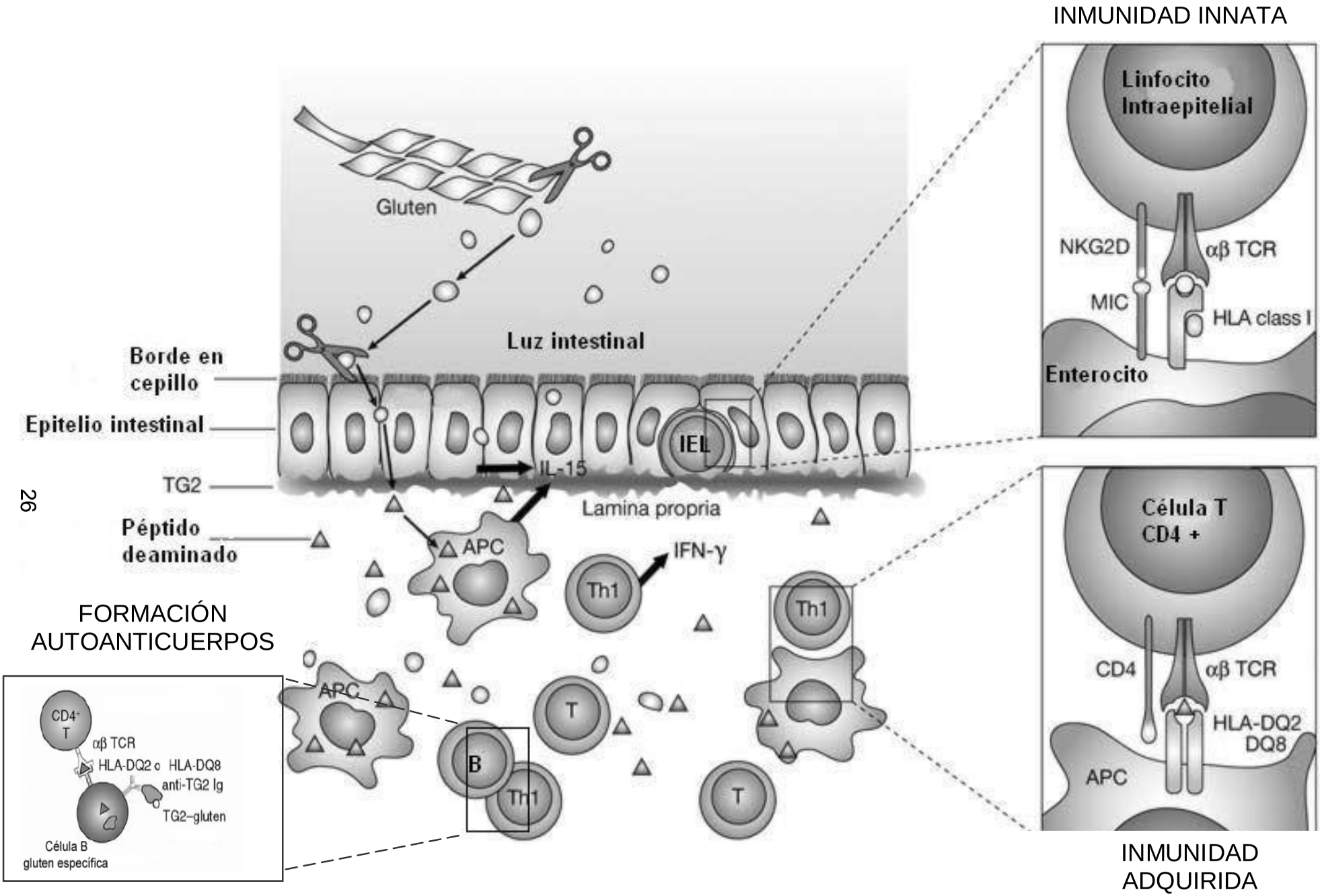


Figura 7. Mecanismos inmunopatogénicos implicados en la EC.

2.4 Bases genéticas de la EC.

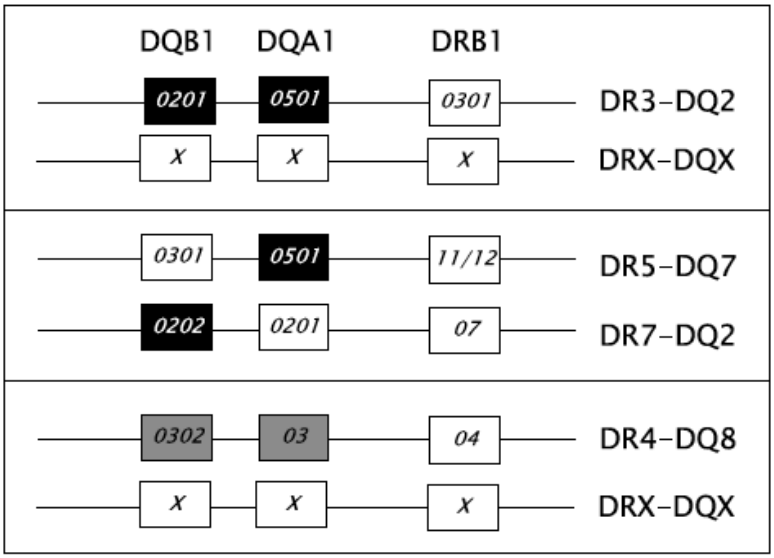
La importancia de los factores genéticos en la predisposición a EC puede deducirse de su alto grado de agregación familiar y por estudios poblacionales. Estudios familiares llevados a cabo en gemelos, han estimado un elevado grado de concordancia para la EC, siendo entre gemelos monocigóticos de un 75%. Además el riesgo relativo entre hermanos (λs) para la EC, definido como el riesgo que el hermano de un paciente tiene de enfermar comparado con el riesgo de la población general, es de 20 a 60 (39).

2.4.1 Papel de los genes HLA en la EC.

La contribución genética mas importante y mejor descrita hasta el momento en la susceptibilidad a la EC, es la de los genes HLA (human leukocyte antigen), concretamente la de los genes HLA-DQ. La asociación genética más fuerte es la de los alelos DQA1*05 y DQB1*02 que codifican respectivamente para las cadenas alpha y betha de la molécula DQ2 (2). Hay que señalar que un 90 % de los pacientes celíacos son portadores del heterodímero DQ2, que puede estar codificado en *cis* por los alelos DQ del haplotipo DR3-DQ2 o en *trans* por los haplotipos DR5-DQ7 y DR7-DQ2 (2). El resto de pacientes son portadores de parte de la molécula DQ2 o del heterodímero DQ8 codificada por los alelos DQA1*03-DQB1*0302 (40).

El riesgo conferido por la presencia de la molécula DQ2 se aumenta enormemente cuando el individuo es homocigoto para el haplotipo DR3-DQ2 o heterocigoto portador de los haplotipos DR3-DQ2/DR7-DQ2. Se ha descrito recientemente que este efecto de dosis parece deberse a un aumento en la expresión de moléculas DQ2 que presentan con gran afinidad péptidos del gluten muy inmunogénicos (41).

Figura 8. Codificación de las moléculas DQ2 y DQ8.



En el contexto de las enfermedades autoinmunes la asociación de la EC con los genes HLA de clase II es de las más fuertes que se han descrito por el momento. Además, la importancia de esta asociación genética reside en que se ha podido establecer cual es la implicación funcional exacta de las moléculas DQ2-DQ8 en la patogénesis de la EC. Como ya se ha comentado anteriormente, las moléculas DQ2-DQ8 son esenciales en la presentación de los péptidos derivados del gluten a los linfocitos T gluten-específicos.

No obstante, se considera que los genes HLA, aunque necesarios para el desarrollo de la EC, no son suficientes. Las moléculas HLA DQ2-DQ8 son bastante frecuentes en la población general (20-30%) que no desarrolla EC (2, 42). Por otra parte, se ha estimado que solo un 40% del riesgo relativo entre hermanos para la EC es atribuible a los genes HLA (43). Esto sugiere que otros genes además de los HLA también están influyendo en la predisposición genética a la EC.

2.4.2 Genética de la EC fuera del sistema HLA.

El papel que los genes localizados fuera de la región HLA desempeñan en la predisposición a EC se conoce de forma limitada, por ello en los últimos años se ha hecho un gran esfuerzo en la identificación de marcadores genéticos que podrían contribuir en la susceptibilidad a EC.

Al tratarse de una enfermedad genéticamente compleja, se espera que haya multitud de genes implicados cada uno de ellos contribuyendo en una pequeña parte al riesgo genético global de padecer la EC. En esta situación para identificar qué genes podrían estar causalmente relacionados con la enfermedad, habitualmente se siguen dos estrategias diferentes: estudios de ligamiento o estudios de asociación de genes candidatos (44).

2.4.2.1 Rastreos sistemáticos del genoma en la EC.

Los estudios de ligamiento pretenden identificar regiones cromosómicas asociadas a enfermedad mediante el análisis de la distribución de determinados marcadores genéticos entre individuos emparentados (parejas de hermanos o familias). Se basan en que las regiones cromosómicas que contienen los genes asociados a la enfermedad deben ser compartidas entre individuos enfermos o trasmitidas a los hijos enfermos. Cuando engloban el análisis de todo el genoma humano se denominan "rastreos sistemáticos del genoma".

Los marcadores genéticos de elección en estos estudios son los microsatélites, consistentes en repeticiones de una secuencia corta de ADN (generalmente entre 2 y 8 pares de bases). Se han localizado un elevado número de microsatélites distribuidos a lo largo de todo el genoma humano afectando principalmente a regiones no codificantes. La principal característica de los microsatélites es su alto grado de variabilidad

presentando generalmente un elevado número de alelos. En consecuencia existen un elevado número de individuos heterocigotos para un determinado microsatélite lo que permite determinar fácilmente su patrón de herencia (45). Actualmente también se están empezando a realizar estudios de este tipo utilizando mutaciones puntuales (SNPs) (45).

En el caso de la EC se han realizado varios rastreos sistemáticos del genoma llevados a cabo en poblaciones de diferente origen étnico (Tabla 1) así como diferentes meta-análisis englobando los resultados de varias de estas poblaciones (46, 47).

El dato más concluyente y reproducible que se ha obtenido en estos estudios es la asociación con la región cromosómica 6p21 conocida como CELIAC 1 en la que se encuentran los genes HLA. También se han encontrado otras regiones cromosómicas posiblemente asociadas a EC como la región 5q31-33 (CELIAC 2), la región 2q33 (CELIAC 3), y muy recientemente la región 19p13 (CELIAC 4), aunque con respecto a ellas existe un menor consenso y las asociaciones encontradas no son reproducible en todas las poblaciones analizadas (42, 48).

Por otra parte, queda pendiente la identificación de los genes causales responsables de la asociación con estas regiones cromosómicas. Por el momento sólo se ha podido caracterizar el gen causal responsable de la asociación encontrada con la región 19p13. Un estudio llevado a cabo en una población holandesa ha revelado que ciertos haplotipos del gen *MYO9B* localizado en esta región confieren un elevado riesgo de padecer EC (49). El gen *MYO9B* codifica para una proteína del citoesqueleto y se cree que podría intervenir en los mecanismos fisiopatológicos de la EC aumentando la permeabilidad intestinal (49).

Tabla 1. Rastreos sistemáticos del genoma llevados a cabo en EC.

Población	Diseño del estudio	Sugerencia de ligamiento [A] ($P<7x10^{-4}$)	Ligamiento significativo [A] ($P<7x10^{-6}$)	Referencia
Irlandesa	Hermanos	6p23, 11p11	-	(50)
Italiana	Hermanos	5q (CELIAC2)	-	(51, 52)
Inglesa	Familias	-	-	(53)
Sueca, Noruega	Hermanos	-	-	(54)
Finlandesa	Hermanos	4p15	-	(55)
Finlandesa	Población aislada	-	15q12	(56)
Norte europea	Familias	-	-	(57)
Norte americana	Familias	3p26, 5p14, 18q23	-	(58)
Holandesa	Hermanos	6q21	19p113.1 (CELIAC 4)	(59)
Holandesa	Familia	9p21-p23	-	(60)
Finlandesa	Hermanos	10p	2q23-q32 (CELIAC 3)	(61)

[A] Criterios propuestos por Lander y Kruglyak (62).

Algunas de estas regiones se han asociado también con susceptibilidad a otras enfermedades autoinmunes como la artritis reumatoide (AR), la diabetes tipo 1 (DT1) o la enfermedad inflamatoria intestinal (EII) apoyando la hipótesis de que parecen existir ciertos factores genéticos comunes de predisposición a diferentes enfermedades autoinmunes, y que por tanto sería factores predisponentes de autoinmunidad (42, 63, 64).

2.4.2.2 Estudios de asociación de genes candidatos.

La segunda estrategia más comúnmente utilizada en la búsqueda de posibles genes asociados con las enfermedades complejas es la realización de estudios de asociación de genes candidatos. Mediante esta aproximación se han estudiado numerosos genes funcionales relacionados con los mecanismos patogénicos e inmunológicos de la EC. Entre los genes estudiados se encuentran el *TNF-α, IL-12, la TG2,* (antígeno 4 de linfocitos T citotóxicos) *CTLA4* o la prolil endopeptidasa (*PREP*) (65-72). Sin embargo, por el momento no se han obtenido datos concluyentes a cerca del papel exacto que estos genes desempeñan en el riesgo genético de EC.

2.4.2.3 Búsqueda de nuevos factores genéticos de susceptibilidad a EC basada en estudios de asociación.

Con la revisión de todos los datos existentes a cerca de la genética de la EC, se puede llegar a la conclusión de que queda aún mucho por hacer, puesto que apenas se ha identificado una pequeñísima proporción de los numerosos genes que se espera estén detrás de la predisposición genética de esta patología.

Como ya se ha comentado, los estudios de asociación son una de las herramientas más utilizadas para la identificación de genes asociados con enfermedades complejas ya que este tipo de análisis es relativamente fácil y asequible para los investigadores.

Los estudios de asociación se pueden llevar a cabo en poblaciones caso-control (constituidas por un grupo bien caracterizado de pacientes y de controles sanos no relacionados) o en grupos familiares (formados por un individuo enfermo y sus progenitores). En el caso de las cohortes caso-control se analizan posibles diferencias en la distribución de determinados marcadores genéticos entre el grupo de los enfermos y los individuos sanos. Cuando se consideran grupos de familias, se determina el grado de desviación en la herencia de los alelos del marcador genético en cuestión con respecto al 50 % esperado por azar (73).

Los estudios de asociación presentan la ventaja de poder detectar asociaciones pequeñas o débiles. Sin embargo, hay que considerar que con los estudios caso-control pueden aparecer resultados no debidos a la presencia falsos positivos. Para evitar estos errores hay que seleccionar un tamaño muestral adecuado y garantizar la homogeneidad de la población. Es imprescindible que el grupo de los controles sea semejante al de los pacientes en términos de origen étnico, exposición a factores ambientales,

edad y sexo. En este aspecto los estudios familiares suponen una ventaja ya que al englobar familias no se corre el riesgo de estratificar la población.

Otro paso fundamental en los estudios de asociación es la elección de los genes candidatos a estudiar. Básicamente se pueden seguir dos estrategias:

Funcional: consistente en seleccionar genes con una implicación clara en los mecanismos patológicos que llevan al desarrollo de la enfermedad.

Posicional: basada en la selección de genes que se localizan en regiones cromosómicas que se han asociado previamente con susceptibilidad a enfermedad en estudios de ligamiento.

También es importante en los estudios de asociación la elección del marcador genético a estudiar. Los marcadores de elección suelen ser las denominadas "variantes causales", es decir aquellas que pueden afectar la funcionalidad del gen, como las localizadas en regiones reguladoras del gen (sitios de unión de factores de transcripción o de procesamiento del ARN) pudiendo alterar la expresión del gen o las que alteran la estructura de la proteína.

Este tipo de estudios ha contribuido en gran medida a la caracterización de la contribución de los genes HLA en enfermedades autoinmunes, y más recientemente han contribuido a la identificación del gen *PTPN22* (proteín-tirosín fosfatasa N22) como uno de los genes más importantes en la susceptibilidad a la AR y otras enfermedades autoinmunes (74).

Siguiendo estos criterios, en la EC se podrían considerar como genes candidatos funcionales interesantes (i) los que codifican para mediadores inflamatorios secretados como consecuencia de la activación de la inmunidad innata frente al gluten, (ii) los relacionados con la regulación de la respuesta inmunitaria o (iii) aquellos que intervienen en la respuesta inmune innata frente al gluten. Dentro de estas categorías, en este trabajo se seleccionaron los siguientes genes candidatos con el objeto de analizar su posible contribución a la EC:

(i) El gen del IFNγ principal mediador proinflamatorio secretado en la EC, y el gen de la óxido nítrico sintasa inducible (*NOS2A*) cuyos niveles se encuentran aumentados en pacientes celíacos y que se sabe que es estimulado por el IFNγ.

(ii) Los genes *NFKB1* y *PARP-1* implicados en la regulación transcripcional de mediadores proinflamatorios. También los genes implicados en el control de la fisiología y la respuesta de las células T como los genes *CTLA4, PTPN22* (proteín-tirosín fosfatasa N22) y el factor de transcripción *FOXP3*.

(iii) El gen *MICA* esencial para la estimulación de la actividad efectora de los linfocitos intraepiteliales y los genes de la *IL-1*α, *IL-1*β, el agonista del receptor de la interleuquina 1 (*IL-1RN*), *IL-18*, *RANTES* (regulated upon activation, normal T-cells expressed and secreted) y el factor quimioatrayente 1 de monocitos (*MCP-1*), mediadores típicamente relacionados con la respuesta inmune innata.

3. JUSTIFICACIÓN Y OBJETIVOS

A pesar del gran conocimiento a nivel molecular de los mecanismos que conducen al desarrollo de la EC, aún quedan numerosas cuestiones por dilucidar, entre ellas porqué solo una pequeña proporción de los individuos de riesgo desarrollan la enfermedad, cual es la conexión exacta entre la respuesta inflamatoria y el daño a la mucosa intestinal o qué mecanismos permiten el paso de los péptidos del gluten a través de la barrera intestinal. Otro de los grandes retos con respecto a la EC son la obtención de mejores herramientas de diagnóstico que permitan detectar las formas silentes de la enfermedad, así como la identificación de nuevas estrategias terapéuticas, ya que el único tratamiento disponible para estos pacientes en seguir una dieta libre de gluten de por vida.

En todos estos ámbitos, la identificación de nuevos marcadores genéticos de susceptibilidad a EC juega un papel esencial ya que podría servir de ayuda para la implantación de mejores herramientas de diagnóstico, llevaría a un mejor conocimiento de las vías fisiopatológicas de la enfermedad y podría ayudar en el desarrollo de nuevas dianas terapéuticas. Así pues el estudio de nuevos genes candidatos implicados en la predisposición genética a EC, se presenta como un campo de investigación muy interesante a la par que necesario para un mejor conocimiento de los complejos mecanismos que intervienen en esta patología.

En base a estos hechos, el objetivo general del presente trabajo fue la identificación de nuevos genes relacionados con la susceptibilidad a EC. Para ello se siguió una estrategia basada en estudios de asociación de genes candidatos funcionales que se llevó a cabo en grupos familiares y grupos caso-control.

Los objetivos concretos plantados fueron los siguientes:

1. Estudiar si el polimorfismo funcional (microsatélite CA) localizado en la región promotora del gen *IFNG* se relaciona con la susceptibilidad a la EC.
2. Determinar la posible asociación del polimorfismo (microsatélite (CCTTT)n) de la región promotora del gen *NOS2A* en la susceptibilidad a la EC.
3. Investigar la influencia del gen del factor de transcripción *NFKB1* en la predisposición genética a la EC mediante el análisis de dos variantes genéticas: microsatélite (CA)n e inserción/delección –94 ATTG.
4. Analizar si los polimorfismos del promotor del gen de *PARP-1* ejercen un papel relevante en la susceptibilidad a la EC.
5. Comprobar en nuestra población la relevancia de variante genética CT60 (A/G) del gen *CTLA4* en la susceptibilidad a la EC.
6. Estudiar el papel en la genética de la EC del polimorfismo funcional –1858 C/T del gen *PTPN22,* que se ha asociado con predisposición a autoinmunidad.
7. Determinar la posible influencia del microsatélite (GT)n del gen *FOXP3* en predisposición genética a la EC.
8. Investigar la implicación del polimorfismo de la región transmembrana (microsatélite (GCT)n) del gen *MICA* en la EC, considerando su posible desequilibrio de ligamiento con los genes HLA-DQ2.
9. Establecer si los genes *IL-1α, IL-1β, IL-1RN, IL-18, RANTES* y MCP-*1*, implicados en la respuesta inmune innata, juegan un papel relevante en la susceptibilidad a la EC.

4. DISCUSIÓN

Puesto que los genes HLA-DQ son el factor genético más fuertemente asociado con la predisposición genética a la EC, previamente a la realización de cualquier estudio que implique el análisis de factores genéticos asociados con esta enfermedad, es de gran relevancia determinar las características de la población de estudio en cuanto a la presencia de los alelos de riesgo HLA-DQ.

Así, previamente al análisis de los genes candidatos seleccionados en este trabajo, se tiparon los pacientes incluidos en el estudio para los alelos DRB1 y DQB1. De esta forma se caracterizó la población para la presencia de la molécula DQ2. El 93% de los pacientes celíacos fueron portadores del heterodímero DQ2 y un 4% portadores de la molécula DQ8. Estos resultados muestran una distribución de los alelos HLA-DQ de riesgo en nuestra población acorde con lo esperado, según los datos reportados de epidemiología genética de la enfermedad celíaca (2).

Tabla 2. Distribución de los haplotipos de riesgo para EC en nuestra población.

HAPLOTIPO HLA clase II	**PACIENTES CELÍACOS 2N=202 (%)**	**CONTROLES 2N=538 (%)**	**P**	**OR**
DR3-DQ2	79 (40)	62 (11.5)	<0.000001	4.9 (3.2-7.4)
DR7-DQ2	51 (25.2)	70 (13)	0.00006	2.3 (1.5-3.5)
DR4-DQ8	11 (5.4)	56 (10.4)	0.04	0.5 (1.5-3.5)
OTROS	61 (20)	350 (53)	<0.000001	0.2 (0.2-0.3)
Presencia de molécula DQ2	**N=101 (%)**	**N=274 (%)**	**P**	**OR**
DQ2+	94 (93)	110 (40)	<0.000001	20 (8.5-49)
DQ2- (DQ8+)	5 (5)	28 (10.2)	0.11	0.4 (0.15-2)
DQ2-	2 (2)	136 (50)	<0.000001	0.02 (0-0.9)

4.1 Genes relacionados con la respuesta inflamatoria en la EC.

La participación de la inmunidad adquirida mediante una respuesta Th1 es uno de los hechos cruciales en la patogénesis de la EC. La respuesta inflamatoria específica ante el gluten está marcada por la activación de los linfocitos T CD4+ gluten-específicos que conlleva a la secreción de mediadores pro-inflamatorios.

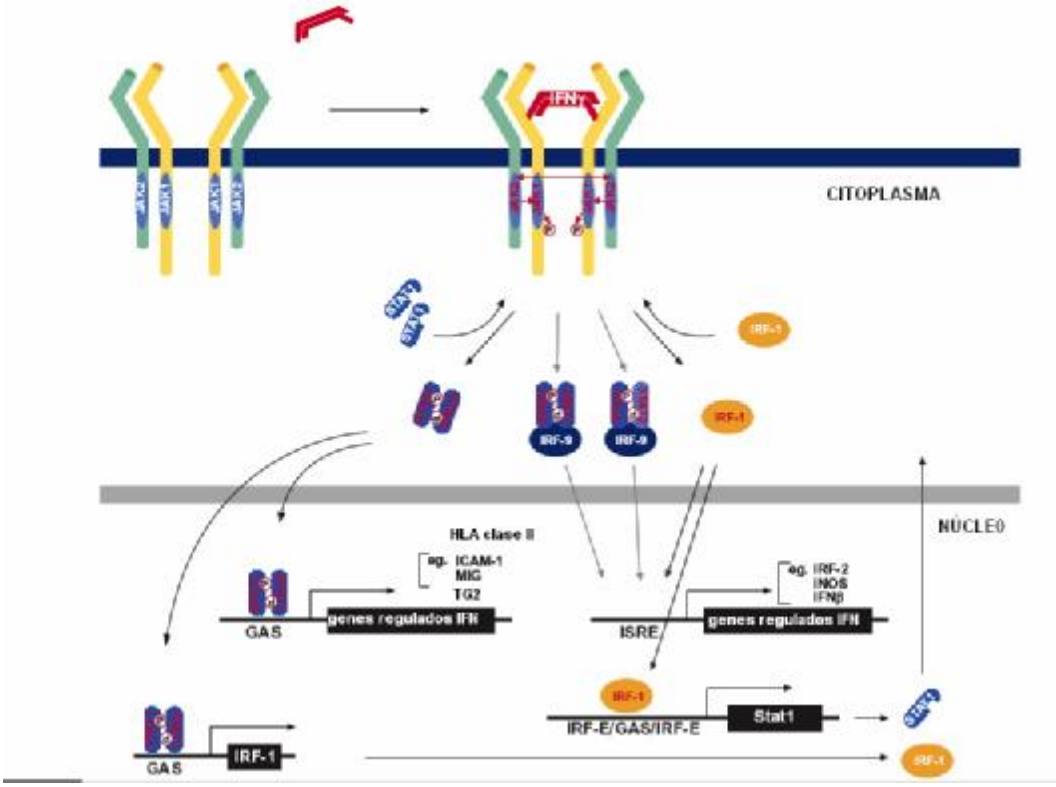
El **IFNγ** es el mediador predominante en esta respuesta. Sus niveles de expresión tanto a nivel intestinal como en sangre periférica en los pacientes celíacos son muy elevados, y se ha observado que se correlacionan con el grado de lesión tisular (12, 17, 75). La función que se atribuye al IFNγ en la EC es la de actuar como un regulador central de numerosos mecanismos efectores implicados en las vías patogénicas de la enfermedad.

Los altos niveles de IFNγ se han relacionado con dos de los mecanismos implicados en la atrofia de las vellosidades intestinales, como son la apoptosis de enterocitos y el aumento de la secreción de enzimas destructoras de la matriz intestinal (metaloproteinasas) (7, 76, 77). Además, el IFNγ también podría contribuir al mantenimiento y la expansión de la respuesta inflamatoria intestinal. La unión del IFNγ a su receptor activa la vía de señalización Jack-STAT1 (factor de transcripción transductor de señales y activador de la transcripción) que estimula la expresión de factores de transcripción como T-bet y el propio STAT-1 implicados en la polarización de las células T hacia un fenotipo Th1 y en la secreción de mediadores proinflamatorios (Figura 9) (78-80). De esta forma se crearía un mecanismo de retroalimentación que fomentaría la cronicidad del proceso inflamatorio en la mucosa intestinal. Por otra parte, el IFNγ facilitaría de forma indirecta la presentación de péptidos

inmunogénicos ya que entre los genes susceptibles de ser inducidos por el IFNγ se encuentran los de moléculas HLA de clase II y el de la *TG2* (81).

Dado su amplio ámbito de actuación en la EC, la regulación del gen del IFNγ, adquiere gran relevancia y cualquier factor capaz de modular sus niveles de expresión podría tener una gran repercusión en la fisiopatología de esta enfermedad. De hecho, el uso de anticuerpos anti-IFNγ se está planteando como posible herramienta terapéutica para los pacientes celíacos (5). En este sentido, se ha documentado la existencia de un microsatélite (CA)n en el intron 1 del gen *IFNG* que puede afectar a la actividad funcional del gen. Estudios *in vitro* e *in vivo* han mostrado que ciertos alelos de este marcador genético se relacionan con mayores niveles de expresión de IFNγ (82).

Figura 9. Vías de acción del IFNγ.



Todos estos hechos nos llevaron a proponer el gen *IFNG* como un candidato muy interesante en la susceptibilidad a la EC. Tras analizar la distribución del microsatélite (CA)n en un amplio grupo de familias celíacas observamos que los dos alelos más frecuentes en la población (12 CA y 13 CA), presentaban un patrón de transmisión alterado y opuesto, caracterizado por el aumento significativo de la transmisión del alelo 12 CA y la menor transmisión del alelo 13 CA a los hijos enfermos (83). Es de destacar que el alelo 12 CA es el que se ha relacionado con mayores niveles de expresión de IFNγ y por lo tanto cabría especular que los altos niveles de IFNγ en los pacientes celíacos podrían estar influenciados por la presencia de este alelo (82).

Debido a la gran relevancia de estos hallazgos, se intentó confirmar esta asociación mediante el estudio de un grupo caso-control de pacientes celíacos e individuos no relacionados. Sorprendentemente, la fuerte asociación encontrada en el estudio familiar no se pudo replicar en el grupo caso-control, en el que no se observó que la frecuencia del alelo 12CA estuviese significativamente aumentada en los pacientes celíacos comparados con los controles (83).

La falta de concordancia entre los resultados de estudios familiares y estudios caso-control puede deberse a la existencia de los llamados "efectos de origen parental" que se diluyen cuando se considera una población caso-control. Un ejemplo de esta situación es la predisposición a obesidad en niños, determinada por la transmisión paterna de ciertos alelos del polimorfismo VNTR del gen de la insulina (84). Para evaluar esta posibilidad en el caso del microsatélite (CA)n del *IFNG*, se realizó un análisis más detallado del estudio familiar en el que se analizó la transmisión de los alelos del microsatélite (CA)n del *IFNG* por separado en los padres y en las madres. Al igual que en el estudio de Le Stunff y col.,

observamos que el patrón de transmisión de los alelos 12CA y 13CA estaba significativamente alterado sólo en el caso de que fuesen heredados de los padres (83). Este hecho podría ser indicativo de la existencia de mecanismos de regulación epigenética, por ejemplo diferentes patrones de metilación en los alelos procedentes de los padres y de las madres (85, 86). Es interesante destacar que se ha sugerido que los patrones de metilación están relacionados con la regulación de la expresión del IFNγ en células T y que estos patrones se heredan de forma estable (87-89). Podría ocurrir entonces que diferentes alelos (CA)n estuvieran asociados con diferentes patrones de metilación del gen *IFNG* y al ser heredados de los padres conferir un mayor riesgo o protección a la EC.

De forma paralela a la obtención de estos resultados, se publicó un estudio en una población holandesa que consideraba también la posible implicación del gen *IFNG* como marcador genético de susceptibilidad a EC, mediante la realización de un estudio familiar y caso-control (17). De acuerdo con nuestros resultados estos autores no observan asociación del microsatélite (CA)n con susceptibilidad a EC en el estudio caso-control. Sin embargo, en este trabajo no se confirma el patrón de transmisión alterado de los alelos CA descrito en nuestra población. Esta falta de concordancia podría atribuirse a diferencias genéticas entre ambas poblaciones. Sin embargo, un hecho fundamental a tener en cuenta es que el numero muestral utilizado en el estudio familiar de Warpenaar y col. es sensiblemente menor al empleado en nuestro trabajo (67 familias holandesas frente a 220 familias españolas) y por lo tanto podría tener un menor poder estadístico para detectar la asociación.

Teniendo en cuenta todas estas evidencias, el microsatélite (CA)n del *IFNG* parece ser uno de los marcadores genéticos que contribuyen en la susceptibilidad a la EC, al menos e nuestra población. Sin embargo, sería

necesario confirmar el grado global de implicación del gen *IFNG* en el riesgo genético a la EC, ampliando los estudios de asociación a otras poblaciones de áreas geográficas diferentes considerando además los posibles efectos de origen parental que parecen existir asociados con el microsatélite CA.

Uno de los genes que se regulan bajo el control del IFNγ es el gen ***NOS2A*****.** La NOS2 es la forma inducible de las enzimas responsables de la síntesis del óxido nítrico (ON). El ON se produce de forma constitutiva por las sintasas endoteliales (eNOS/NOS1) o neuronales y (nNOS/NOS3) y juega un papel relevante en la regulación de los sistemas nervioso, cardiovascular e inmunológico. Sin embargo, la NOS2 produce elevados niveles de ON en respuesta a situaciones de estrés como los procesos infecciosos o autoinmunes (90).

En los pacientes celíacos se ha documentado tanto la presencia de un aumento de la expresión de la NOS2, como la de altos niveles de NO a nivel plasmático e intestinal (91-93). Se cree que la inducción de la NOS2 ocurre en los enterocitos y macrófagos de la lámina propia que liberan al medio altas concentraciones de NO, creándose así una situación de estrés oxidativo que podría contribuir en la lesión de la mucosa intestinal (93, 94). Los mecanismos moleculares responsables de esta fuerte inducción de la NOS2 en los pacientes celíacos no se han identificado claramente, pero se sabe que en la activación de la expresión de esta enzima es fundamental la unión de factores de transcripción como IRF-1, STAT-1 y NF-kB1 en su promotor.

Por lo tanto, diferencias genéticas entre individuos a nivel del promotor del gen *NOS2A* podrían alterar la unión de estos factores de transcripción y afectar la actividad transcripcional del gen. De hecho, la

región promotora del gen *NOS2A* es altamente polimórfica y contiene un elevado número de polimorfismos. De entre todos los marcadores genéticos identificados ha adquirido gran relevancia un microsatélite (CCTTT)n debido a su potencial para afectar a la actividad transcripcional de este gen (95).

Se planteó entonces la posibilidad de que este microsatélite podría ser un marcador genético de predisposición a la EC. A pesar de los datos preliminares, tras analizar la distribución del microsatélite (CCTTT)n en un grupo de familias celíacas no se observó que ningún alelo se asociase con mayor riesgo de padecer EC en nuestra población (96).

El microsatélite (CCTTT)n se ha analizado en relación con la susceptibilidad a otras enfermedades autoinmunes como la DT1, el LES, la (esclerosis múltiple) EM o la EII en las que tampoco se ha detectado un papel relevante de este marcador genético (97-99). Sin embargo, el microsatélite (CCTTT)n parece estar implicado en la genética de la AR confiriendo ciertos alelos protección frente a determinadas complicaciones clínicas y otros susceptibilidad a la enfermedad (100, 101). Por tanto la implicación precisa del microsatélite (CCTTT)n en autoinmunidad no está claramente definida y las diferencias establecidas entre diversas enfermedades podrían ser un reflejo de la distinta relevancia de la NOS2 en los mecanismos patológicos que llevan a cada una de estas enfermedades. Así, mientras que en la AR se ha establecido un claro papel de la NOS2 en los procesos de destrucción articular, en la EC a pesar de encontrarse en elevadas cantidades no se ha podido implicar directamente en ninguno de los mecanismos efectores asociados a la lesión de la mucosa (53).

Nuestro trabajo es el único que por el momento ha analizado el polimorfismo (CCTTT)n del gen *NOS2A* en relación a la EC y según nuestros datos se podría descartar esta variante genética como marcador

genético de predisposición a la EC (96). Posiblemente el aumento de la expresión de la NOS2 en la EC sea una mera consecuencia de la activación de las células productoras de NO en por la acción de otros mediadores inflamatorios más potentes secretados por los linfocitos T CD4+. De hecho está documentado que, previamente al aumento de los niveles de NO, se produce una activación de los granulocitos y macrófagos a nivel intestinal en presencia de IFNγ (102-104).

4.2 Genes implicados en la regulación la repuesta inmune en la EC.

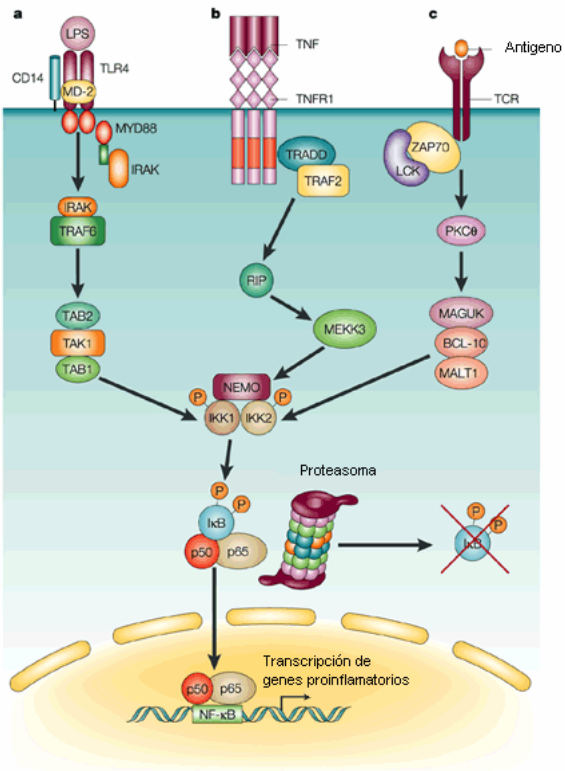
Ya se ha destacado ampliamente a lo largo de este trabajo la gran relevancia que el desarrollo de la respuesta Th1 tiene en la fisiopatología de la EC. No obstante, los mecanismos últimos que conducen a la proliferación de los linfocitos T gluten-específicos y a los elevados niveles de secreción de mediadores proinflamatorios que estos producen, se conocen pobremente. Se sabe que el mantenimiento de la homeostasis de estos procesos inmunológicos es elevadamente compleja y puede llevarse a cabo mediante diferentes niveles de actuación como el control transcripcional de genes implicados en la respuesta inflamatoria o la regulación de la activación de las células T mediante diferentes vías de señalización.

4.2.1 Control transcripcional de la respuesta inmune.

Uno de los factores de transcripción más destacados por su papel central en la regulación de la respuesta inmune innata y adquirida es el ***factor de transcripción nuclear kappa B (NF-kB)***. Las moléculas NF-kB están constituidas por heterodímeros codificados por los genes RELA (p65), NFKB1 (p50;p105), NFKB2 (p52;p100), c-REL y REL., los cuales componen la familia NF-Kb/Rel (105). La forma activada de NF-kB está

constituida por un heterodímeto de p65 asociado con p50-52 y que se localiza a nivel citoplasmático asociado con las proteínas que lo inhiben (IkBs). La activación de NF-kB ocurre rápidamente en respuesta a muy diversos estímulos como la unión de patógenos a receptores de superficie (vía receptores tipo toll), la inducción por citoquinas (TNF-α e IL-1) o tras la activación del TCR (receptor de células T). En todos estos casos se produce la fosforilación, ubiquitinación y degradación de las proteínas IkB que permite la translocación de NF-kB al núcleo. Una vez en el núcleo NF-kB inicia la transcripción de numerosos genes implicados en procesos inflamatorios como citoquinas (IL-1, IL-8, TNF-α), chemoquinas, moléculas de adhesión, metaloproteinasas o el gen *NOS2A* (106). Son numerosas las enfermedades autoinmunes en las que NF-kB se encuentra activado, como la AR, EII o la EM en los lugares en los que se produce la respuesta inflamatoria (106).

Figura 10. Mecanismos de acción de NF-kB.



En la EC también se ha identificado una activación persistente de NF-kB con elevados niveles de las subunidades que lo constituyen (p50/p65) y una alta actividad de unión al DNA (107). También se sabe que NF-kB está implicado en la regulación de la expresión de numerosos mediadores secretados en la respuesta inflamatoria ante el gluten (102, 107, 108). Por lo tanto, este factor de transcripción podría ser un importante modulador de la respuesta inflamatoria en la EC, tanto a nivel molecular como genético.

Dada la relevancia de NF-kB en la regulación de la autoinmunidad e inflamación, la búsqueda de marcadores genéticos capaces de afectar su actividad ha despertado gran interés. En el gen *NFKB1* se describió un microsatélite (CA)n altamente polimórfico asociado con predisposición a la DT1 (109). También en el gen *NFKB1*, muy recientemente se ha observado en su región promotora una inserción/delección (–94ins/delATTG) situada entre dos elementos reguladores (sitio de unión AP-1 y sitio de unión kB) y asociada con susceptibilidad a otra enfermedad intestinal como es la colitis ulcerosa (110). La presencia de la delección de 4 pares de bases (4bp) parece causar la pérdida de capacidad de unión a proteínas nucleares y en consecuencia daría lugar a una menor actividad del promotor (110). Por el momento estos son los dos únicos marcadores genéticos de *NFKB1* con cierta relevancia funcional y/o asociados a autoinmunidad, por lo que nos propusimos analizarlos para determinar su posible contribución genética en la EC.

Mediante un análisis familiar del microsatélite CA y un estudio caso-control bastante extenso del polimorfismo –94ins/delATTG en nuestra población pudimos observar que ningún alelo de estas variantes genéticas se asociaba significativamente con susceptibilidad a la EC (111, 112). Con respecto al microsatélite (CA)n salvo la asociación documentada con la

DT1, en posteriores análisis llevados a cabo en otras enfermedades autoinmunes y en otras poblaciones no han podido confirmar un papel relevante de las repeticiones CA en autoinmunidad. Los avances realizados en la secuenciación del genoma humano con posterioridad a la identificación de este marcador lo han localizado en las proximidades de otro gen aguas abajo de NFkB1. Así pues, este polimorfismo parece no ser un buen marcador genético a considerar en estudios de asociación.

En conformidad con los resultados obtenidos en nuestro análisis, el polimorfismo –94ins/delATTG no se asocia tampoco con otras enfermedades autoinmunes como la AR, el LES o la EII en nuestra población (113, 114). Por el momento el polimorfismo –94ins/delATTG sólo parece jugar un papel relevante en relación a la susceptibilidad a colitis ulcerosa. Aunque se podría descartar que este sea un marcador genético de susceptibilidad a EC en particular, y de atuoinmunidad en general en nuestra población, para esclarecer su relevancia en otras poblaciones y enfermedades autoinmunes sería necesario realizar más estudios de asociación y corroborar la posible relevancia funcional del polimorfismo –94ins/delATTG. Dado el papel central que NF-kB desempeña en el control de los procesos de autoinmunidad y la complejidad de los mecanismos que regulan la activación de este factor de transcripción, parece muy interesante seguir buscando genes candidatos de susceptibilidad a EC en esta vía.

Como continuación de la investigación en este ámbito se planteó como un posible gen candidato el gen de la ***PARP-1.*** La PARP-1 es una proteína nuclear que cataliza la adición de polímeros de ADP-ribosa en diferentes moléculas usando como substrato el NAD+ (115). Su principal función fue descrita como la de intervenir en la reparación del daño al DNA, pero más adelante se ha descubierto que la PARP-1 juega un papel

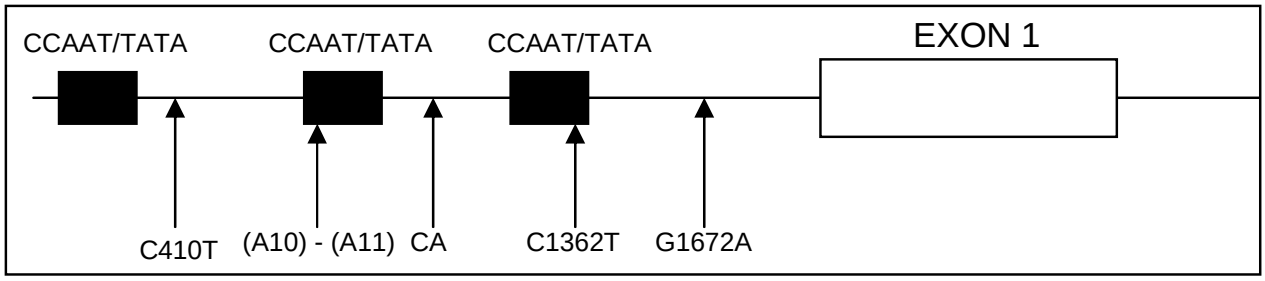
relevante en la regulación transcripcional (115, 116). La PARP-1 es capaz de modificar la estructura de la cromatina favoreciendo así el inicio de los procesos transcripcionales, y además puede alterar de forma directa la actividad de promotores y complejos activadores de la transcripción (115). Se ha demostrado que la PARP-1 interacciona directamente con numerosos factores de transcripción estimulando su actividad, entre los que se encuentra NF-kB (117).

Además de activarse cuando se producen roturas en el ADN, en situaciones de inflamación aguda y crónica se ha observado la activación persistente de la PARP-1 (118). El bloqueo de la actividad de PARP-1 resulta beneficioso en modelos animales de enfermedades autoinmunes como la AR y enfermedad inflamatoria intestinal, ya que produce una reducción de la inflamación y de los mecanismos efectores que causan daño tisular (119-121). Parece pues que la PARP-1 podría estar implicada en la cascada de eventos patogénicos que desencadenan la autoinmunidad y en consecuencia adquiere gran relevancia la fina regulación de su actividad.

Existen numerosos elementos reguladores en el promotor de PARP-1 como sitios de unión para el factor de transcripción ying-yang 1 (YY1) y tres grupos de cajas TATA (122). A lo largo de toda esta región reguladora se han detectado distintas variantes genéticas: tres mutaciones puntuales (SNPs): C410T, C1362T y G1672A, una fragmento de un número variable de repeticiones de A (poly A n) y un microsatélite dinucleotídico CA (123). En un estudio previo de nuestro grupo, se observó que todas estas variantes constituyen dos haplotipos extendidos únicos, definidos en función del número de repeticiones CA: haplotipo A (410T-[A10]-repeticiones cortas CA [83–87 bp]-1362C) y haplotipo B (410TC- [A11]-repeticiones CA largas [89–101 bp]-1362T) (124). Así mismo se observó

que estos haplotipos del promotor de la PARP-1 se asociaron con predisposición genética a la AR y al LES (124).

Figura 11. Localización de las variantes genéticas del promotor de la *PARP-1*.



Todos estos datos nos llevaron a analizar la contribución de los haplotipos localizados en la región promotora del gen de la *PARP-1* en la predisposición a EC. A través de un estudio caso-control se detectó que el haplotipo A del promotor de la PARP-1 era significativamente más frecuente en los pacientes de EC que en los controles, aumentando el riesgo de enfermedad cuando los individuos eran homocigotos para este haplotipo (125). Esta asociación podría tener su explicación funcional en la posible alteración estructural del promotor debido a la variación en el tamaño de las repeticiones CA. El haplotipo A podría causar una deficiente unión de los elementos activadores de la transcripción del gen *PARP-1* y en consecuencia una menor producción de proteína. Al existir una reducción en los niveles de PARP-1 se reduciría su interacción con NF-kB que resultaría en una menor actividad de este factor de transcripción. Aunque a priori esto podría redundar en una disminución de la secreción de mediadores inflamatorios, se ha observado que la inhibición de NF-kB puede resultar en un aumento de la apoptosis de enterocitos y en consecuencia en la aparición de la atrofia de vellosidades (126-128).

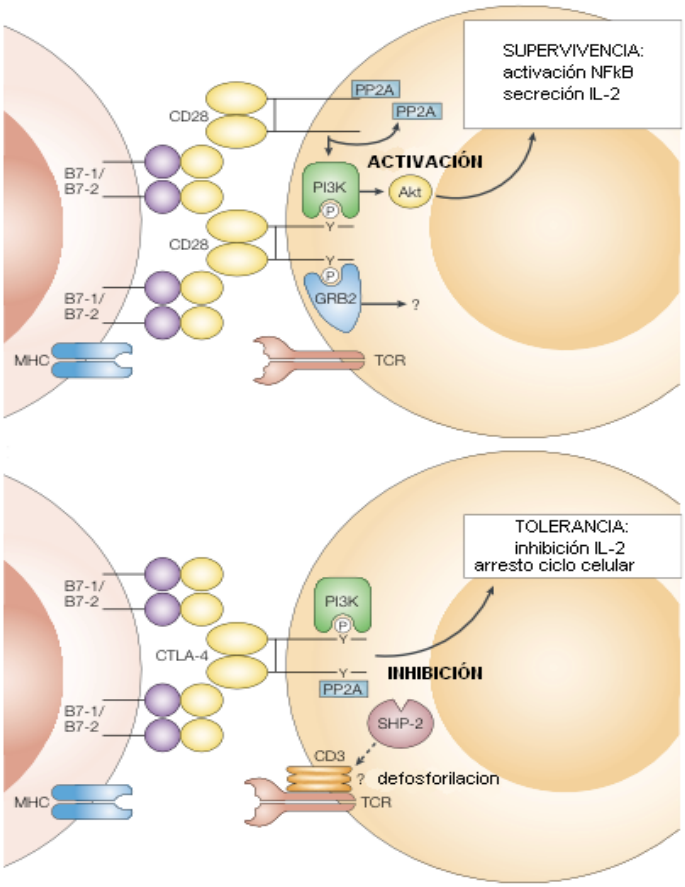
Estos datos dejan abierto un nuevo campo de investigación muy interesante. Quedaría pendiente la confirmación a nivel funcional de los efectos de los haplotipos del promotor de la *PARP-1* en su expresión. Por otra parte, sería de gran interés el estudio del uso de los inhibidores de la PARP-1 como posibles agentes terapéuticos para la EC, aunque una gran dificultad en este sentido es la carencia de modelos animales de EC.

4.2.2 Mecanismos implicados en la regulación de la actividad de los linfocitos T.

La modulación de la proliferación de las células T requiere un delicado equilibrio en la interacción entre moléculas de superficie y receptores activadores e inhibidores. Tras la activación del TCR las células T expresan en su superficie la molécula CTLA-4, que forma parte de la vía B7/CD28-CTLA-4. Mientras que la unión de CD28 a la molécula B7 provoca una señal coestimuladora esencial en el inicio y progresión de la respuesta T, la interacción de CTLA-4 con B7 genera una señal inhibidora de la activación pudiendo de esta forma contribuir a la tolerancia oral (Figura 12) (129).

Debido a su función reguladora y a su implicación en la susceptibilidad a diferentes enfermedades autoinmunes, el gen *CTLA4* se ha propuesto como un posible marcador genético de autoinmunidad (129). Con respecto a la EC este gen también ha despertado gran interés puesto que además ser un excelente candidato funcional, también lo es desde el punto de vista posicional al localizarse en la región cromosómica 2q33 (CELIAC 3), asociada con susceptibilidad a la EC en rastreos sistemáticos del genoma (57).

Figura 12. Esquema de la via coestimuladora B7/CD28-CTLA-4.



De entre las diferentes variantes genéticas descritas para el gen *CTLA4* destaca el polimorfismo CT60 A/G, localizado en la región 3´ UTR del gen, por haberse visto asociado con predisposición a numerosas enfermedades autoinmunes y por su potencial relevancia en la funcionalidad del gen (73). El alelo CT60G se ha relacionado con menores niveles de producción de la forma soluble de la molécula CTLA4 (sCTLA4), quedando libre la molécula B7 lo cual facilitaría la unión de CD28 y en consecuencia el incremento de señales estimuladoras (73). Aunque todos los datos apuntaban a que este marcador podría estar implicado en la susceptibilidad a la EC, el análisis de este polimorfismo en nuestras cohortes caso-control y de familias celíacas no reveló diferencias estadísticamente significativas (130).

Este fue un dato más añadido a la controversia existente con respecto al papel de *CTLA-4* en la EC. Numerosos trabajos en poblaciones de distinto origen étnico han estudiado diferentes polimorfismos del gen CTLA4 (-318C/T, -49A/G o un microsatélite ATn) con resultados muy dispares (54, 72, 131). En cuanto al polimorfismo CT60 A/G los estudios realizados en otras poblaciones, bien no encuentran asociación con la EC o esta es muy débil (54, 70, 132). En conjunto estos datos apuntarían a que la asociación encontrada con la región cromosómica 2q33 podría deberse probablemente a otro gen localizado en la región 3´ de CTLA-4. En este sentido, el gen *ICOS* , que codifica para una molécula estimuladora de las células T secundaria y que se sitúa aguas debajo de CTLA-4, se ha asociado con EC en una población finlandesa (132).

Uno de los mecanismos que regulan finamente la transducción de señales es la fosforilación de tirosinas. Esta es una reacción reversible en la que intervienen dos tipos de enzimas, las proteín-tirosín-kinasas (PTKs) que fosforilan los residuos de tirosina, y las protein-tirosin-fosfatasas (PTPs) que catalizan la desfosforilación. Por medio de este mecanismo se regulan numerosos procesos fisiológicos como diferenciación celular, morfología y movilidad celular, diferenciación y muerte celular, etc (133).

Todas las células del sistema inmune expresan altos niveles de fosforilación de tirosinas y los cambios agudos en este sistema pueden afectar a distintos procesos linfocitarios, como la activación mediada por la interacción antígeno-receptor y la diferenciación o la respuesta ante distintos estímulos (134). En las células T se expresan un amplio rango de PTPs. Recientemente ha despertado gran interés la fosfatasa específica de linfocitos **PTPN22** ó Lyp. Lyp es una proteína intracelular implicada en la señalización a través del TCR ya que se une físicamente mediante un

motivo rico en prolina (P1) al dominio SH3 de la kinasa Csk, e inhibe la transducción de señales atenuando la activación de las células T (135).
Se ha descrito una mutación no sinónima que afecta al motivo P1 de la PTPN22, consistente en un cambio de arginina por triptófano (1858C/T;R620W). Esta variante genética se ha confirmado como un importante determinante de susceptibilidad a enfermedades autoinmunes. Así, el polimorfismo 1858C/T del gen *PTPN22* se ha asociado con riesgo genético de padecer DT1, AR, LES y enfermedad de Graves (GD) (64, 74, 136-140). La extensa replicación de estos hallazgos en numerosas cohortes de origen caucásico, ha convertido la asociación del gen *PTPN22* con estas enfermedades en la mejor establecida después de la de los genes HLA. Por otra parte, su implicación en enfermedades autoinmunes tanto sistémicas como órgano-específicas ha hecho que se proponga el polimorfismo 1858C/T del gen *PTPN22* como un marcador genético común de autoinmunidad (141).

El alelo de riesgo 620W parece estar implicado funcionalmente en la actividad de PTPN22 de dos formas diferentes. En principio se postuló que al alterar la capacidad de unión de Lyp con la CSK podría generar una hiperactivación de las respuestas T patogénicas dando lugar a procesos autoinmunes (136). Datos muy recientes han aportado una nueva visión de la funcionalidad de esta mutación, al demostrarse que puede causar un aumento de la actividad fosfatasa inhibiendo potentemente la señalización mediante CSK (142). Todavía no está claro cómo esta actividad podría redundar en la aparición de enfermedades autoinmunes, pero se baraja la posibilidad de que pueda afectar a la selección tímica y a la actividad de las células T reguladoras (Figura 13) (143).

Todos estos datos sugerían al gen *PTPN22* como un candidato muy atractivo para ser estudiado con respecto a la EC. Nuestro trabajo fue el

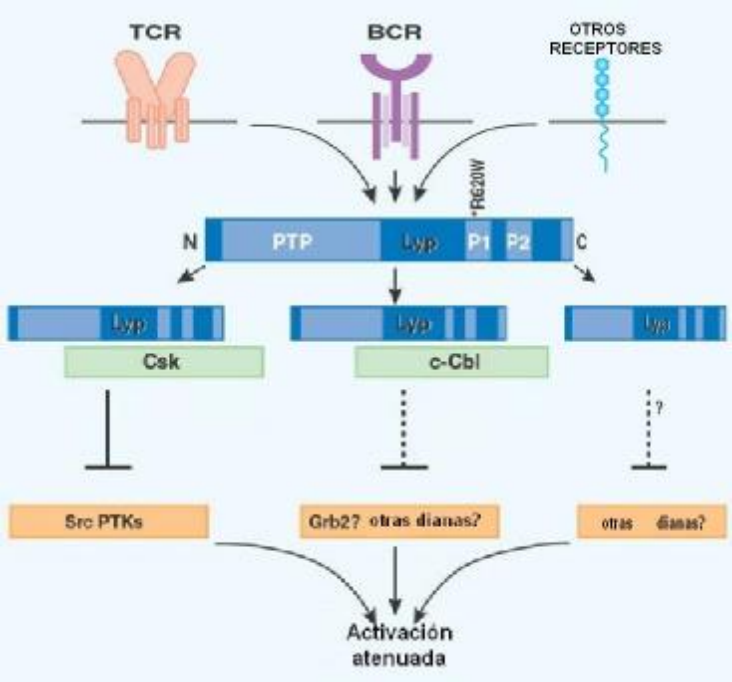
primero en analizar mediante un amplio panel de familias y un estudio caso-control el polimorfismo 1858C/T del gen *PTPN22* como posible marcador genético de predisposición a la EC (144). Los resultados obtenidos no mostraron asociación estadísticamente significativa del polimorfismo 1858C/T con susceptibilidad a la EC (144). La tendencia observada en nuestra población se ha confirmado en dos estudios realizados en cohortes de pacientes celíacos de diferente origen étnico (145, 146). Al igual que para la EC, la variante genética 1858C/T del gen *PTPN22* no se ha podido confirmar como marcador de susceptibilidad en otras enfermedades autoinmunes como la EM, la EII y la espondilitis anquilosante (EA) (139, 147, 148).

Estos hallazgos han planteado una reevaluación del papel de PTPN22 en autoinmunidad. Se propuso la hipótesis de una posible relación de este gen sólo con las enfermedades en las que se produce un elevado número de autoanticuerpos (149). Sin embargo, en la EC también se producen altos niveles de autoanticuerpos que incluso se relacionan con la aparición de manifestaciones extra-intestinales (27).

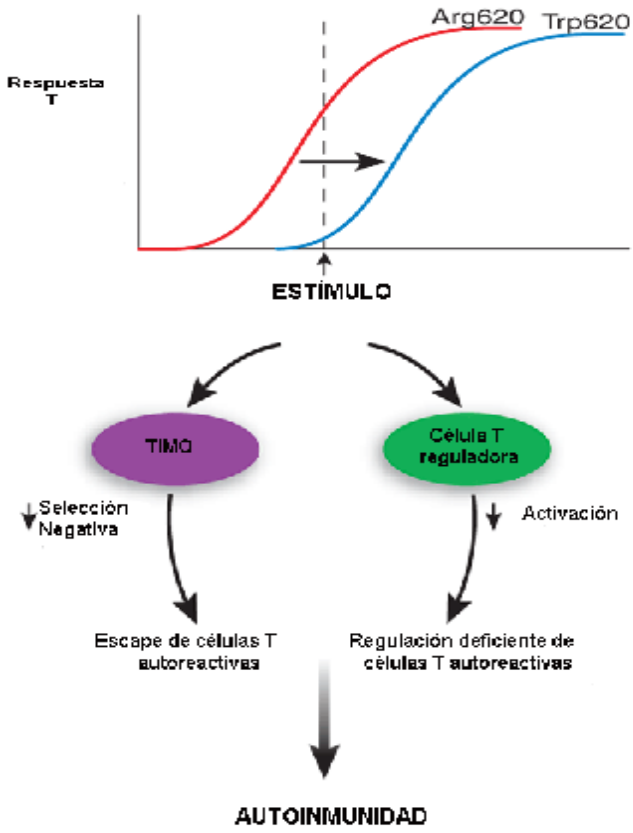
Cabría plantear la posibilidad de que la implicación de PTPN22 en la regulación de la activación de las células T sea distinta según el tipo de enfermedad autoinmune. Las enfermedades en las que la PTPN22 no parece desempeñar un papel relevante afectan al sistema inmune intestinal y cerebral, que presentan características anatómicas y funcionales peculiares. Puede que en estos sistemas otras PTPs jueguen un papel más relevante que la PTPN22. En efecto, una variante genética de CD45 (PTP receptor tipo C) se ha asociado con susceptibilidad a EM. La existencia de un amplio número de miembros de la familia PTP en el sistema inmune, deja abierto un gran campo en el que sería muy interesante la selección de posibles marcadores genéticos para la EC.

Figura 13. Modelos propuestos sobre la implicación funcional del polimorfismo 1858C/T del gen *PTPN22.*

13 A. Pérdida de función.

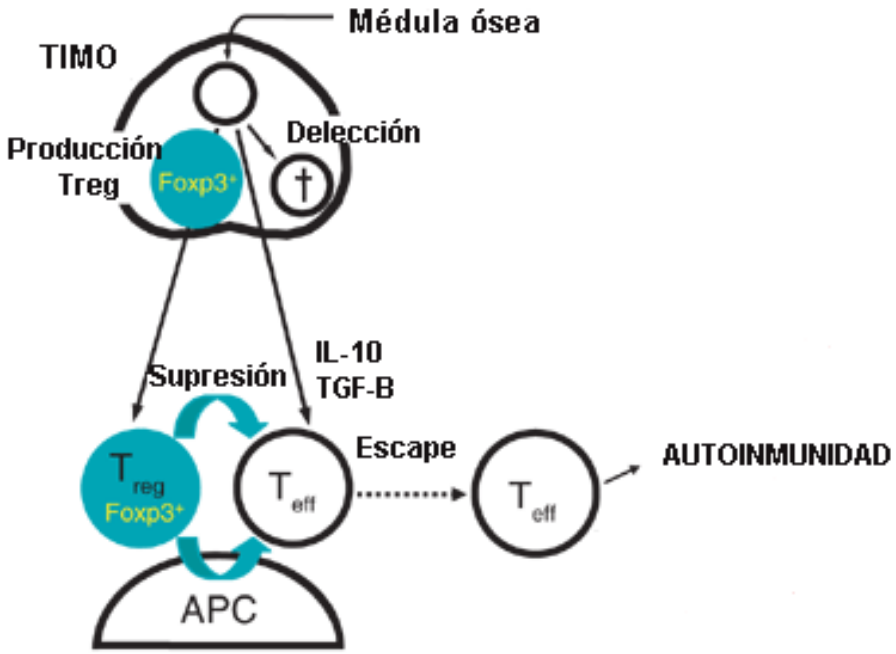


13 B. Aumento de capacidad de fosforilación.



Un capacidad esencial del sistema inmune es la de discriminar entre lo "propio" y lo "no-propio". Aunque para prevenir las infecciones por agentes patógenos se requiere una fuerte inducción del sistema inmune, el mismo tipo de respuesta generada frente a bacterias comensales o a proteínas de la dieta, pueden llevar al desarrollo de enfermedades como en el caso de la EC. La aparición de este tipo de respuestas se previene normalmente mediante un complejo sistema de mecanismos reguladores, entre los que recientemente se ha destacado el papel de los linfocitos T reguladores (Treg) (150). Las células Treg son una subpoblación de linfocitos T CD4+ producidos por el sistema inmune en condiciones normales altamente especializados en la supresión de células T auto-reactivas. Se caracterizan por la expresión constitutiva de la molécula de superficie CD25 y por la expresión específica del factor de transcrición *FOXP3* que es clave para su desarrollo y actividad funcional (150). Está bien documentado que la disminución en el número o en la actividad supresora de las células Treg puede provocar la aparición de enfermedades autoinmunes y también la alteración de otros procesos inmunológicos como la inmunidad tumoral, la alergia o el rechazo de órganos (150-152). Todos estos datos han llevado a considerar a las células Treg como elementos esenciales en el mantenimiento de la homeostasis del sistema inmune. En el ámbito del sistema inmune intestinal aunque no se conoce con exactitud el papel de las células Treg, existen datos de que pueden controlar la respuesta inflamatoria inducida tanto por la respuesta inmune innata como por la respuesta inmune adquirida, mediante mecanismos dependientes de secreción de interleuquina 10 (IL-10) y de factor de crecimiento tisular β (TGF-ß) (153).

Figura 14. Efecto regulador de las células Treg.



Ya se ha comentado que la expresión específica del factor de transcripción FOXP3 es esencial para las células Treg. El gen *FOXP3* localizado en el cromosoma Xp11.23 pertenece a la familia de factores de "transcripción forkhead/winged-helix". Su activación induce la expresión de moléculas asociadas al fenotipo y la actividad de las células Treg, como CD25, CTLA-4 y el receptor GITR (154). Por lo tanto es un regulador crítico de la actividad de las células Treg y por ende de la homeostasis del sistema inmune, de tal forma que cualquier alteración de los niveles de expresión de *FOXP3* podría desencadenar procesos autoinmunes. De hecho, la alteración genética de este importante regulador se ha asociado con la presencia de patologías muy graves, como el síndrome IPEX ("síndrome de inmunodeficiencia ligado al X"), causado por mutaciones recesivas en *FOXP3* y que se asocia con la aparición de DT1 y EII (155).

También se ha descrito otro polimorfismo (microsatélite (GT)n) en el promotor del gen *FOXP3* que parece afectar a los niveles de expresión del gen y que se asoció con susceptibilidad a la DT1 en una población japonesa (156). En vista de estos datos, decidimos estudiar la posible influencia del gen *FOXP3* en la susceptibilidad a EC, utilizando como marcador genético el microsatélite (GT)n.

Nuestros resultados no revelaron asociación de ninguno de los alelos del microsatélite (GT)n del gen *FOXP3* con predisposición genética a EC (157). A la par que el análisis de este marcador genético en EC, se estudió su relevancia en la susceptibilidad a AR, LES y EII confirmándose la falta de asociación de microsatélite (GT)n con estas patologías (157). De acuerdo con lo observado en nuestra población, en un estudio llevado a cabo en una población caucásica (italiana) tampoco se pudo replicar la asociación del microsatélite (GT)n con susceptibilidad a la DT1 (158). La discordancia entre los resultados obtenidos en poblaciones caucásicas y la asiática, podrían deberse a las diferencias étnicas entre ambas poblaciones. De hecho pudimos observar que la distribución de los alelos del microsatélite (GT)n en nuestra población era significativamente distinta con respecto a la japonesa (157). Con respecto a la actividad funcional reportada para el microsatélite (GT)n, hay que ser cautos y tener en cuenta la posible existencia de otros marcadores genéticos en desequilibrio de ligamiento con el microsatélite (GT)n que podrían ser los verdaderos causantes de la alteración de la expresión del gen *FOXP3*.

Por tanto, nuestros datos junto con los obtenidos por Zavattari y col., descartarían la implicación del microsatélite (GT)n del gen *FOXP3* como marcador genético de predisposición a enfermedades autoinmunes al menos en poblaciones de origen caucásico. Sin embargo, dada la obvia implicación de FOXP3 en la regulación de los procesos autoinmunes, no

descartamos la posibilidad de que otros marcadores genéticos puedan afectar la expresión de este gen y sería muy interesante seguir profundizando en el estudio de este gen como candidato de susceptibilidad a la EC y a otras enfermedades autoinmunes.

4.3 Genes de la respuesta inmune innata en EC.

Uno de los hallazgos más relevantes que recientemente se han hecho sobre la patogénesis de la EC, es la existencia de ciertos péptidos del gluten capaces de activar el sistema de inmunidad innata. Entre los eventos clave de esta respuesta está el reconocimiento de los enterocitos por los IELs a través de la interacción MICA-NKG2D.

Las moléculas ***MICA*** junto con las MICB forman la familia de moléculas HLA de clase I no clásicas (MIC), ligandos de los receptores NKG2D expresados en la superficie de células T CD8+ ($\alpha\beta$, $\gamma\delta$ y NK) (159). En condiciones normales la expresión de las moléculas MIC en la superficie celular está prácticamente limitada al epitelio gastrointestinal y tímico (160). Bajo condiciones de estrés se induce la sobre-expresión de moléculas MICA en enterocitos y su reconocimiento por los receptores NKG2D de los linfocitos T $\gamma\delta$+ que están ampliamente expandidos en la EC (28, 161). Estas evidencias sugerían que el gen *MICA* podría ser un candidato funcional muy interesante en la susceptibilidad a la EC.

Localizado a 46 Kb de HLA-B dentro del MHC, el gen *MICA* codifica para una proteína con tres dominios extracelulares, una región transmembrana y una región intracitoplasmática (162). Al igual que los genes clásicos MHC I, MICA se caracteriza por su alto grado de polimorfismo con más de 50 alelos descritos, cuyas variaciones se localizan principalmente en los dominios extracelulares (exones 2, 3 y 4). Además en el exón 5 que codifica para la región transmembrana de la molécula MICA,

se describió un microsatélite polimórfico consistente en una serie de repeticiones (GCT)n cuyos alelos determinan la presencia de un número variable de residuos de alanina. Una variante alélica del microsatélite GCT consiste en la inserción nucleotídica de una G (GCT→GGCT), el alelo MICA A5.1, que causa la alteración del marco de lectura generando un codon de parada prematuro (163). Se cree que este alelo podría dar lugar a una forma soluble de la molécula MICA o alterar su localización en el enterocito de la zona basolateral a la apical (163, 164).

Utilizando como marcador genético el microsatélite (GCT)n mediante un estudio familiar analizamos la contribución del gen *MICA* en la susceptibilidad a la EC en nuestra población (165). Durante el proceso de genotipado, pudimos identificar en la madre de uno de los pacientes la existencia de un nuevo alelo del microsatélite que no se había descrito previamente, consistente en la presencia de siete repeticiones GTC por lo que se denominó alelo MICA A7 (166). El clonaje del gen MICA de esta persona mostró que era portadora del alelo MICA*002, normalmente asociado con el alelo MICA A9. Cabe la posibilidad de que este nuevo alelo se originase debido a la delección de dos repeticiones GTC de un alelo MICA A9 ancestral al ser la madre en la que se detectó el nuevo alelo MICA A7 portadora del alelo MICA A9.

Con respecto al patrón de transmisión de los alelos MICA observamos un aumento significativo de la transmisión del alelo MICA A5.1 a los hijos enfermos (165). Al estar el gen *MICA* localizado dentro de la región MHC, en la proximidad de los alelos HLA-DQ que representan el mayor factor de riesgo genético de la EC, y dado el fuerte desequilibrio de ligamiento existente en la región, era obligatorio determinar si la asociación que habíamos observado se debía a un desequilibrio de ligamiento con los genes HLA-DQ. El estudio de dependencia mostró que el efecto del alelo

MICA A5.1 parecía independiente del desequilibrio de ligamiento con los haplotipos extendidos HLA-DQ2 en nuestra población (165). Es interesante el hecho de que la asociación encontrada sea con el alelo MICA A5.1, el único que por el momento se ha descrito que podría alterar funcionalmente la molécula MICA.

Varias hipótesis podrían explicar la implicación de este alelo en la patogénesis de la EC. Como ya hemos comentado el gen *MICA* es altamente polimórfico y existen numerosos alelos que dan lugar a moléculas MICA con afinidades diferentes por los receptores NKG2D (167). El alelo MICA*008 que está en total desequilibrio de ligamiento con el alelo MICA A5.1 de la región transmembrana, da lugar a moléculas MICA de baja afinidad por el receptor NKG2D (167). Uno de los mecanismos en los que la interacción MICA-NKG2D parece intervenir es la selección tímica del repertorio de linfocitos T CD8+ (168). De esta forma se podría especular que la funcionalidad del alelo MICA A5.1 en la patogénesis de la EC resultaría de su implicación de forma indirecta en el proceso de selección tímica, al asociarse a un alelo MICA de baja afinidad por los receptores NKG2D. También se podría especular que la posible localización del alelo MICA A5.1 en la región apical del enterocito, daría lugar a una disminución en las interacciones MICA–NKG2D desapareciendo una posible señal reguladora para los linfocitos T $\gamma\delta$+. Sin embargo, los recientes descubrimientos hechos a este respecto, apuntan a que la interacción MICA-NKG2D genera una señal de daño que conlleva a la destrucción de la mucosa intestinal (29). Por lo tanto el alelo MICA A5.1 debería conferir protección ya que al presentarse en otra localización dificultaría las interacciones con NKG2D y atenuaría la señal de daño. Además se ha demostrado en biopsias de pacientes celíacos que la presencia del alelo MICA A5.1 no se relaciona con una localización apical de la molécula MICA

(29). Por último, cabe la posibilidad de que el alelo MICA A5.1 dé lugar a una forma soluble de la molécula. Datos muy recientes han demostrado que en los pacientes celíacos se producen elevados niveles séricos de la forma soluble de MICA (MICAs) (29). Sin embargo en este trabajo no se pudo establecer una correlación entre la presencia del alelo MICA A5.1 y los niveles de MICAs.

Nuestro trabajo constituyó el primer estudio familiar de la implicación de los genes MICA en la EC, evitando posibles sesgos debidos a estratificación de la población. Anteriormente, otros estudios caso-control realizados en varias poblaciones españolas, de acuerdo con nuestros datos han determinado la implicación del ge MICA en la EC (169-171). No obstante, los resultados son controvertidos con respecto al alelo MICA GCT asociado, a la independencia con respecto a los genes HLA-DQ2 y en cuanto a la estratificación según las formas clínicas de la enfermedad. Este hecho es común para otras enfermedades autoinmunes, como la DT1, AR o el LES en las que existen resultados controvertidos a cerca del papel del microsatélite (GCT)n en la genética de estas patologías(160). En vista de todos estos datos, hay que considerar la posibilidad de que el microsatélite (GTC)n del gen MICA constituya un marcador posicional y que esté en desequilibrio de ligamiento con otra variante genética, verdadera causante de la asociación con la EC.

Posiblemente el gen *MICA* sea uno de los candidatos más idóneos en la susceptibilidad a la EC, dada su implicación tanto en la genética de la EC como en la inducción de la activación de la inmunidad innata frente al gluten. No obstante, quedan aún por determinar los mecanismos moleculares últimos que hay detrás de la implicación del gen *MICA* en la EC. Entre los grandes retos pendientes estaría la determinar la verdadera independencia de este gen con haplotipos HLA-DQ2 extendidos, la

búsqueda de nuevos marcadores genéticos en regiones reguladoras del gen que podrían determinar su sobre-expresión en los pacientes celíacos y la confirmación de la funcionalidad de los marcadores genéticos identificados o por identificar.

Tras el inicio de la señal de daño que activa la inmunidad innata en las fases iniciales de la EC, una de las consecuencias más inmediatas es el aumento de la expresión de mediadores proinflamatorios típicamente relacionados con la respuesta inmune innata. La IL-15 es el principal de estos mediadores secretados en la EC, pero junto con ella en los pacientes celíacos también se observan elevados niveles de IL-1, IL-18 y chemoquinas (13, 104, 172-175).

La IL-1 es un mediador clave en enfermedades autoinmunes e inflamatorias. EL exceso en los niveles de IL-1 se ha asociado con la aparición de autoinmunidad en numerosos órganos como articulaciones, el sistema nervioso o el tracto gastrointestinal (176). Los genes que codifican los diferentes miembros de la familia de la IL-1 se agrupan en un "cluster" localizado en la región cromosómica 2q12-22 en el que se localizan los genes de la lL-1α, la IL-1β y el agonista del receptor de la IL-1 (IL-1RN) (176). Mientras que la lL-1α y la IL-1β presentan una fuerte capacidad de inducir inflamación, el IL-1RN es un agonista muy efectivo capaz de unirse al receptor de la IL-1 impidiendo así la activación de la respuesta inflamatoria en la célula diana (176). Así el balance entre las dos formas de IL-1 y el receptor IL-1RN es esencial para el mantenimiento de una respuesta IL-1 fisiológica. Todos estos genes son altamente polimórficos y presentan distintos SNPs en sus regiones reguladoras como son el polimorfismo -889 C/T (rs1800587) de la IL-1α y el–511 C/T (rs1143627) de la IL-1β (este último se ha relacionado con diferencias en los niveles de secreción de IL-1β) (177-179). El gen *IL-1RN* se caracteriza por presentar

en su segundo intron un número variable de repeticiones en tandem (VNTR) de 86 bp. El alelo 2 de esta variante genética se ha asociado con susceptibilidad a distintas enfermedades autoinmunes e inflamatorias (180).

Otro miembro muy importante de la familia de la IL-1 es la IL-18. Al igual que la IL-1, el aumento de los niveles de la IL-18 es característico de numerosas patologías autoinmunes, entre ellas la EC (181). La IL-18 podría intervenir en la inducción de la respuesta inflamatoria anti-gluten ya que, en sinergismo con la IL-12, es capaz de inducir la expresión de IFNγ (181). El gen de la IL-18 presenta varios polimorfismos en su región promotora, entre los que destacan el -607 A/C (rs1946518) y el –137 G/C (rs187238), debido a su potencial capacidad de alterar la función del promotor de esta citoquina (182).

Entre las quimioquinas que se presentan en elevados niveles en los pacientes celíacos se encuentran RANTES y MCP-1 (factor quimioatrayente 1 de monocitos). Ambas pertenecen al grupo de quimioquinas inducibles que se producen ante situaciones de estrés y que median el reclutamiento de los linfocitos a los lugares de inflamación (183). Como en los casos anteriores, los genes que codifican RANTES y MCP-1 presentan polimorfismos en sus regiones reguladoras. En el caso de RANTES han cobrado gran relevancia dos variantes genéticas –403 G/A (rs2107538) y –28 G/C (rs2280788) y en el de MCP-1 una –2518 G/A (rs1024611) (184-186) ya que parecen afectar a los niveles de producción de estas quimioquinas.

A pesar de su potencial de modular la magnitud de la respuesta inmune innata, papel que los hace ser interesantes candidatos funcionales para la EC, la posible contribución de estos genes en la predisposición a la EC no se había estudiado previamente. Así, nos pareció muy interesante

analizar los polimorfismos de los genes *IL-1α, IL-1β, IL-1RN, IL-18, RANTES* y *MCP-1* en nuestra cohorte de familias celíacas. A excepción de los polimorfismos de RANTES que mostraron una leve tendencia de asociación con la EC, el resto de variantes alélicas analizadas no manifestó un papel significativo de los genes *IL-1α, IL-1β, IL-1RN, IL-18,* y *MCP-1* en la susceptibilidad a EC (187). Los polimorfismos -403G/A y -28 G/C del promotor de RANTES se han asociado con predisposición a enfermedades autoinmunes como EM, polimialgia reumática (PMR) o con complicaciones clínicas como retinopatía diabética, edad temprana de desarrollo de EM o complicaciones del sistema nervioso en el LES (188-191). Aunque nuestros datos han sugerido un posible papel de *RANTES* en la EC, es necesario clarificar su implicación exacta en la genética de la EC mediante la replicación de estos resultados en otras poblaciones.

El resto de polimorfismos estudiados en este grupo de genes no parecen ser determinantes en la genética de la EC (187). Es posible que los altos niveles de estas citoquinas observados en la EC, se deban más a la acción de otros mediadores más potentes sobre las células que los producen, que al efecto que los polimorfismos podrían ejercer sobre la actividad transcripcional de estos genes.

La revelación del papel de la respuesta inmune innata en la fisiopatología de la EC, supone un ámbito nuevo y todavía poco explorado en la selección de genes funcionales candidatos de susceptibilidad a la EC. En nuestra opinión sería muy interesante continuar estudiando la posible influencia de otros mediadores sobre-expresados en la respuesta inicial frente al gluten.

5. CONCLUSIONES

1. El microsatélite (CA)n del promotor del gen *IFNG* parece desempeñar un papel relevante en la predisposición genética a la EC. La presencia del alelo 12CA, asociado con mayores niveles de producción de esta citoquina, confiere un mayor riesgo de padecer la enfermedad. Así mismo, se ha identificado un posible fenómeno de regulación epigenética asociado a este marcador, que estaría determinado por la herencia de alelos CA de origen paterno.
2. Nuestro trabajo representa el primer análisis de los genes *NOS2A* y *NFKB1* como candidatos funcionales en la susceptibilidad a la EC, y los resultados obtenidos sugieren que el polimorfismo CCTTTn del gen *NOS2A* y las variantes genéticas CA y -94 ins/del del gen *NFKB1* no juegan un papel fundamental en la predisposición genética a la EC.
3. El análisis de los haplotipos conservados del promotor de la *PARP-1* ha revelado su posible papel como nuevos marcadores genéticos de susceptibilidad a EC. El aumento significativo de la frecuencia del haplotipo A en los pacientes celíacos podría asociarse con una menor producción de PARP-1, reduciéndose su unión a NF-kB que no podría ejercer su efecto regulador sobre la apoptosis de enterocitos.
4. Utilizando como marcador genético el polimorfismo CT60, el análisis del gen *CTLA4* en nuestra población apunta a que este gen no es relevante en la susceptibilidad a EC. Nuestros resultados, junto con los aportados por otros grupos de investigación parecen descartar que el gen *CTLA4* sea el responsable de la asociación de la EC con la región cromosómica CELIAC 3 (2q33).

5. Aunque la variante genética 1858C/T del gen *PTPN22* se ha propuesto como un marcador genético común de susceptibilidad a autoinmunidad, nuestros resultados concluyen que este polimorfismo no jugaría un papel relevante en la genética de la EC, hecho que podría reflejar la presencia de diferentes mecanismos reguladores entre diferentes grupos de enfermedades autoinmunes.
6. Según nuestro estudio, el microsatélite (GT)n del gen *FOXP3* no se podría considerar como uno factor de riesgo genético para la EC.
7. En nuestra población, el microsatélite de la región transmembrana del gen *MICA* se presenta como un posible marcador genético de susceptibilidad a la EC. La asociación del alelo MICA A5.1 parece ser independiente de desequilibrio de ligamiento con los alelos HLA-DQ2 y de la forma clínica de presentación de la EC.
8. Los polimorfismos localizados en las regiones reguladoras de los genes *IL-1α, IL-1β, IL-1RN, IL-18, RANTES y MCP-1* no desempeñan un papel relevante como marcadores de susceptibilidad en nuestra población, aunque se observa una tendencia de asociación para el gen *RANTES* que podría sugerir la posible implicación de este gen en la EC y que debería ser confirmada.

6. PERSPECTIVAS

Con este trabajo hemos pretendido colaborar en la caracterización de nuevos factores genéticos determinantes de la predisposición a la EC. Nuestra modesta contribución ha aportado resultados positivos que son llamativos por el hecho de haber permitido relacionar con la genética de la EC a moléculas con un claro impacto en la fisiopatología de la enfermedad. También hay que prestar atención al resto de hallazgos realizados, que no por el hecho de ser negativos son menos importantes. Estos pueden servir de ayuda al resto de la comunidad científica en la selección de los genes candidatos a estudiar, evitando el gasto de recursos y de tiempo en el estudio de marcadores cuya participación en la genética de la EC parece no ser relevante.

En el estudio de la genética de la EC es importante tener presente que al tratarse de una enfermedad compleja se espera que el riesgo genético total esté determinado por muy pocos genes de efecto considerable (como los HLA) y por un elevado número de genes con un efecto modesto. En base al conocimiento actual obtenido acerca de los factores genéticos implicados en la EC, podemos afirmar que nos encontramos muy lejos de esta situación y son muy pocos los genes que se han podido sugerir como posibles factores genéticos de la EC. Es necesario por tanto, el empleo de estrategias más robustas para hacer posible la identificación de los genes causantes de esta enfermedad.

Factores como la heterogeneidad genética entre poblaciones, el uso de tamaños muestrales con bajo poder estadístico, interacciones entre diferentes genes o la presencia de fenotipos complejos, hacen difícil tanto la identificación primaria de genes causativos de la enfermedad como la replicación de los estudios de asociación (192). Para mejorar esta situación se está insistiendo en la necesidad de realizar estudios basados en

poblaciones mucho mayores en las que se incluyan miles de individuos, así como en la replicación de los resultados en poblaciones independientes.

Como ejemplo ilustrativo de esta situación sería necesario contar con una cohorte de 2900 individuos para identificar un marcador genético de susceptibilidad con un impacto moderado en la enfermedad (OR 1.3) y una frecuencia alélica de un 20 %. Pero conseguir un tamaño muestral de tal magnitud no es tarea fácil para un grupo de investigación aislado, por eso se esta tendiendo al establecimiento de colaboraciones internacionales y al desarrollo de los denominados "Bancos de DNA" que intentan recopilar gran cantidad de material biológico e información epidemiológica con el fin de poder realizar estudios genéticos a gran escala.

Por otra parte, dado el alto número de genes que se espera estén implicados, también sería de gran ayuda poder realizar estudios de asociación que incluyesen un gran número de marcadores genéticos que englobasen todo el genoma humano. Los avances realizados en la secuenciación del genoma humano han permitido identificar a lo largo del mismo millones de SNPs disponibles en numerosas bases de datos (http://www.ncbi.nlm.nih.gov/ http://www.ensembl.org). Conjuntamente se ha determinado la existencia de bloques de haplotipos que engloban SNPs con un estrecho grado de desequilibrio de ligamiento, de tal forma que llevando a cabo el tipaje de unos pocos marcadores genéticos (TagSNPs) se puede cubrir una amplia región genómica, hecho que hace mucho más alcanzable el reto de realizar estudios que incluyan un elevado número de marcadores genéticos (http://www.hapmap.org). Estas herramientas junto con el desarrollo de plataformas de genotipado a gran escala (Biochips, Illumina o Maldi-tof) y de programas estadísticos capaces de analizar gran cantidad de información, abren grandes posibilidades para el progreso en el estudio de enfermedades complejas como la EC.

Con la integración de todas estas estrategias se espera que en un futuro próximo se determine la arquitectura genética de la EC, hecho que sería de gran ayuda para el mejor conocimiento de las bases moleculares de esta enfermedad, el establecimiento de estrategias moleculares de diagnóstico y la identificación de nuevas dianas terapéuticas.

7. ANEXO DE PUBLICACIONES

7.1. GENES RELACIONADOS CON LA RESPUESTA INFLAMATORIA EN LA EC

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BRIEF COMMUNICATION

A functional variant of IFNγ gene is associated with coeliac disease

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In coeliac disease (CD) a profile of proinflammatory cytokines are secreted interferon gamma (IFNγ) being one of the most important. A dinucleotide polymorphism consisting of a variable number of CA repeats related with IFNγ production levels, has been reported on the first intron of the IFNγ gene. The aim of this study was to analyse the influence of the functional IFNγ CA repeats in CD predisposition through familial and case–control studies. The familial analysis showed that the 124 bp allele was significantly more transmitted to the affected offspring (P= 0.02), while the 126 bp allele showed a statistically significant nontransmission pattern (P= 0.01). Nevertheless, in the case–control analysis, we could not find a direct association of CA repeats with CD. This fact might be due to parent-of-origin effect in the IFNγ CA polymorphism. Our data suggest a possible role of IFNγ CA polymorphism in CD susceptibility.
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Keywords: *coeliac disease; interferon gamma (IFNγ); microsatellite polymorphism; autoimmunity*

Coeliac disease (CD) affects genetically predisposed individuals after dietary gluten ingestion. Most coeliac patients (90%) are carriers of the DQ2 molecule encoded by DQA1*05/DQB1*02 alleles and a minority of patients bear the DQ8 molecule (DQA1*0301/DQB1*0302). Nevertheless, the contribution of HLA genes for CD genetic predisposition is no more than 40%, suggesting a role for non-HLA genes in CD susceptibility.[1]

The mucosal changes observed in coeliac patients have been related to increased local production of several proinflammatory mediators. Studies in intestinal biopsy specimens from patients with CD have shown that the cytokine expression pattern in response to gluten is strongly dominated by IFNγ and that coeliac patients present an increased expression of IFNγ and a high number of T CD4+ IFNγ-producing cells.[2,3]

A dinucleotide polymorphism consisting of a variable number of CA repeats has been reported on the first intron of the IFNγ gene, which has been related to IFNγ production levels being certain CA repeats associated with higher IFNγ secretion.[4] On this basis, we considered it interesting to analyse the role of the IFNγ gene CAn microsatellite polymorphism with respect to CD susceptibility.

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Accordingly to previous results, the 124 and 126 bp alleles were the most frequently observed in coeliac families[5–7] (Table 1). Interestingly, a distorted transmission pattern was detected for these two common alleles. The 124 bp allele was preferentially transmitted to the affected offspring. In contrast, the 126 bp allele showed a statistically significant nontransmission pattern (Table 1).

To investigate this finding further, we genotyped an additional case–control cohort. As in the family cohort, the most frequent alleles were those with 124 and 126 bp. (Table 2) The overall comparison of CA repeats distribution between CD patients and controls did not give a statistically significant deviation. Similarly, none of the observed alleles independently showed a statistically significant association with susceptibility or protection against the disease.

The discordant lack of association found in case–control led us to perform a more detailed familial analysis considering the transmission of IFNγ CA repeats from mothers and fathers separately to search for a possible parent-of-origin effect. Both the 124 and the 126 bp alleles showed a statistically significant distorted transmission pattern when restricted to fathers whereas when restricted to mothers it was not (TDT_{MvsF} for the 126 bp allele; $P=0.05$, OR 0.54, 95% CI 0.28–1.05) (Table 3). Furthermore, we used the TAT as an alternative method, including only trios with a single heterozygous parent and additionally, we observed evidence for the preferential nontransmission pattern of the 126 bp allele among fathers (TAT_{MvsF} for the 126 bp allele; $P=0.02$, OR = 0.35, 95% CI 0.12–0.97) (Table 3).

Table 1 Transmission pattern of different IFNγ CAn alleles in coeliac families

Allele	*Transmitted*	*Nontransmitted*	*P*	*OR 95% CI*
124 (12 CA)	108 (45.6)	79 (33.3)	0.006	1.67 (1.13–2.47)
126 (13 CA)	84 (35.4)	113 (47.6)	0.006	0.6 (0.41–0.89)
128 (14 CA)	21 (8.8)	23 (9.7)	n.s	
130 (15 CA)	24 (10.1)	22 (9.3)	n.s	

In all, 220 coeliac families of Spanish-Caucasian origin and characterized by the presence of an affected child with CD were collected independently in two Spanish hospitals: 91 families recruited in Granada ('Hospital Materno-Infantil' and 'Hospital Clinico Universitario') and 129 families collected in Madrid ('Hospital Clínico San Carlos'). CAn microsatellite genotyping was performed using primers and conditions previously described.[7] Samples were run on an ABI Prism™ 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) and sized using a GeneScan 672 software. Accuracy of genotypes was determined by sequencing samples covering all CA alleles using homozygous sequenced individuals as internal standard controls. Transmission disequilibrium test (TDT) (which considers only heterozygous parents and assesses the evidence for preferential transmission of one allele over the other) was performed using UNPHASED software.[8]

Table 2 IFNγ CAn microsatellite distribution in CD patients and controls

Allele	*Patients % n=330*	*Controls % n=499*
122 (11 CA)	1 (0.2)	1 (0.1)
124 (12 CA)	314 (47.5)	464 (46.5)
126 (13 CA)	264 (40)	426 (42.7)
128 (14 CA)	40 (6.0)	57 (5.7)
130 (15 CA)	35 (5.3)	44 (4.4)
132 (16 CA)	2 (0.3)	1 (0.1)
134 (17 CA)	4 (0.6)	5 (0.5)

The patients group included one CD patient per family from the Granada and Madrid populations (91 and 129 individuals, respectively) and additionally 110 unrelated CD patients from Madrid. All patients were diagnosed following the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) criteria for CD.[9] Their clinical features were: age at study 7.1±3.9 years; mean age for disease diagnosis 2.7±2.72; gender 60% women : 40% men; anthropometry at diagnosis (weight and height) P 3-100 percentile; mean age of gluten introduction 6.4±1.5 months; typical symptoms 72.2% of patients; atypical symptoms 27.8%. For the control group unrelated healthy individuals from blood banks of Granada (191 samples) and Madrid (308 samples) were collected. All participants were of Spanish-Caucasian origin. Significance was calculated by 2×2 contingency table and Fisher's exact test, to obtain *P*-values, odds ratios (OR) and 95% confidence intervals (CI).

Table 3 Paternal and maternal IFNγ CAn microsatellite transmission pattern within coeliac families

Allele	*TDT*		*TAT*	
	Maternal	*Paternal*	*Maternal*	*Paternal*
124	45 T : 5 NT	51 T : 32 NT[a]	15 T : 16 NT	22 T : 15 NT
126	42 T : 44 NT	29 T : 56 NT[b]	21 T : 14 NT	14 T : 27 NT[c]
128	7 T : 16 NT	14 T : 7 NT	2 T : 7 NT	4 T : 0 NT
130	11 T : 10 NT	12 T : 11 NT	4 T : 5 NT	5 T : 23 NT

T = transmitted; NT = not transmitted.
[a]$P = 0.008$, OR = 2.14, 95% CI = 1.18–3.92.
[b]$P = 0.0002$, OR = 0.34, 95% CI = 0.18–0.62.
[c]$P = 0.005$, OR = 0.3, 95% CI = 0.11–0.78.
We assessed parent of origin effects using two different approximations: first the TDT_{MvsF} (mother *vs* father) that stratify the transmission/nontransmission counts according to whether the mother or the father is the source, and second the transmission asymmetry test (TAT) which omits data from parents when both are heterozygous to ensure statistical independence of parental transmission.[10]

In the present work, we analyse for the first time the IFNγ CA microsatellite in relation to CD. Our results show a possible role for IFNγ CA microsatellite in CD susceptibility. The 124 bp allele was transmitted more significantly to affected offspring. It is worth noting that this allele has been associated with higher levels of IFNγ expression in mitogen-stimulated mononuclear cells *in vitro*.[4] Therefore, it is plausible that the high levels of IFNγ detected in the intestinal mucosa of coeliac patients might be influenced by the presence of the 124 bp allele. While the 126 bp allele, low IFNγ producer, seems to play an opposite protective role, because it has been shown to be significantly nontransmitted to affected sibs.

A paradoxical result was the lack of association in the case–control study, claimed to be more powerful than family-based tests. Similar to our findings in a study of VNRT polymorphism of INS gene in childhood obesity found an association using a familiar approximation but not in a case–control analysis.[11] They concluded that the case–control approach failed to detect an association due to the dilution of paternal effects by noncontributory maternal meioses. Similarly, we detected a possible parent-of-origin effect related to IFNγ CA polymorphism, since we have observed excess in paternal nontransmission of the 126 bp allele to the affected offspring. This finding would provide an explanation for the seemingly discordant results obtained from case–control and familial analysis.

The paternal effect of the 126 bp allele when not transmitted could be related with polymorphic imprinting phenomena. Of note, it has been shown that the differential IFNγ methylation is closely related with IFNγ expression in T cells and that the IFNγ promoter methylation patterns exhibit long-term faithful inheritance.[12,13] On this basis, another possibility is that the role of IFNγ CA allelic variations might be related to genomic imprinting, a mechanism that has been seen to regulate different sets of paternally and maternally expressed genes.[14]

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ELSEVIER

Polymorphism of the Inducible Nitric Oxide Synthase Gene in Celiac Disease

Blanca Rueda, Miguel Angel López-Nevot, Maria Pascual, Eduardo Ortega, Jose Maldonado, Maria L. López, Bobby P. C. Koeleman, and Javier Martín

ABSTRACT: The aim of this study was to investigate the possible association between the inducible nitric oxide synthase (NOS2) gene promoter polymorphism, CCTTTn microsatellite, with celiac disease susceptibility. We carried out a familial study in which 53 Spanish families were genotyped by a polymerase chain reaction (PCR)-based method combined with fluorescent technology. A transmission disequilibrium test was performed to investigate the transmission pattern of the different CCTTTn alleles from parents to affected offspring. The test did not reach any statistically significant difference because none of the CCTTTn repeats was shown to be significantly transmitted to the affected siblings. Our data suggest that the CCTTTn pentanucleotide microsatellite in the NOS2 gene promoter does not play a major role in celiac disease development. *Human Immunology 63, 1062–1065 (2002).* © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: celiac disease; inducible nitric oxide synthase; microsatellite; polymorphism

ABBREVIATIONS

CDC	CD celiac disease
HLA	human leukocyte antigen
IFN-γ	interferon gamma
IDDM	insulin-dependent diabetes mellitus
MHC	major histocompatibility complex
NO	nitric oxide
NOS2	inducible nitric oxide synthase
SNP	single nucleotide polymorphism
TDT	transmission disequilibrium test

INTRODUCTION

Celiac disease (CD) is a chronic inflammatory disorder resulting from dietary gluten ingestion in genetically predisposed individuals. CD susceptibility has a strong genetic component, in which human leukocyte antigen (HLA) genes play an important role [1]. In this sense, DQA1*0501 and DQB1*0201 (DQ2 molecule), located in *cis* in DR3 individuals or in *trans* in DR7/DR5 individuals, are present in 90% of CD patients [1]. Despite the strength of the association between CD and the HLA-DQ2 heterodimer, haplotype sharing studies suggest that HLA genes contribute no more than 40% to the CD genetic predisposition and that genes outside the HLA region might be involved in CD susceptibility [1, 2].

Clinical manifestations of CD are variable, but in all celiac patients there is a mucosal injury characterized by villous atrophy and inflammation, which is known to be associated with enhanced nitric oxide (NO) production, although the exact role of NO in the inflammatory process remains elusive. Studies of intestinal levels of NO have shown that celiac patients have an increased production of NO compared with normal control subjects [3, 4]. In addition, the expression of NOS2 has been demonstrated in the intestine of children with active celiac disease [5–7]). NO is produced constitutively by

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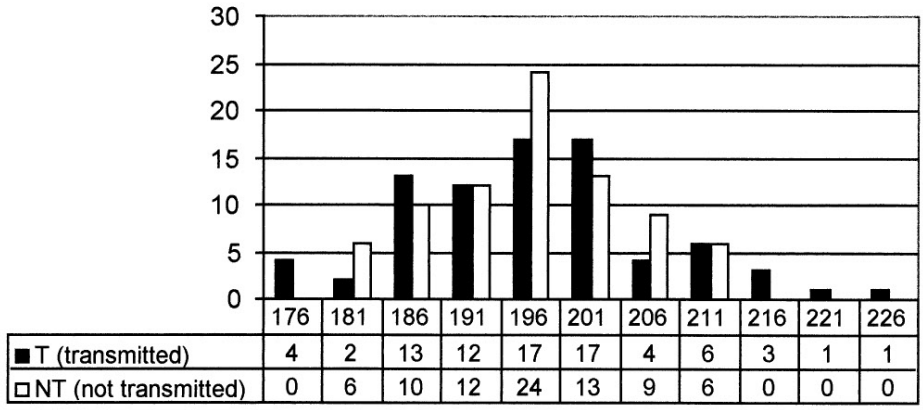

	176	181	186	191	196	201	206	211	216	221	226
■T (transmitted)	4	2	13	12	17	17	4	6	3	1	1
□NT (not transmitted)	0	6	10	12	24	13	9	6	0	0	0

FIGURE 1 Distribution of CCTTT repeats transmission pattern in celiac families.

endothelial or neuronal synthases, or in higher concentrations by inducible NO synthase (iNOS or NOS2) after stimulation of a variety of pro-inflammatory cytokines [8]. The presence of polymorphism in the regulatory region of NOS2 gene may provide an explanation of differential NO production levels observed in CD patients. The NOS2 gene is located at chromosome 17q11.2-12, and three different polymorphisms have been identified in the human NOS2 promoter region; a single nucleotide polymorphism (SNP) G/C at position -954, G-954C [9], and two microsatellite repeats; a biallelic tetranucleotide repeat sequence (TAAA)n [10], and a high polymorphic (nine alleles) CCTTTn repeat [11]. The purpose of the present study was to address the possible contribution of the (CCTTT)n microsatellite polymorphism in CD development.

PATIENTS AND METHODS

Patients

In this study 53 Spanish families consisting of a CD patient and his or her parents were genotyped for the CCTTTn microsatellite. The affected siblings were diagnosed following the European Society for Paediatric Gastroenterology and Nutrition (EPSGAN) criteria for CD [12].

CCTTTn Genotyping

The polymerase chain reaction (PCR)-based genotyping for CCTTTn was performed as described previously [13]. Forward and reverse primers were 5′ACCCCTGGAAGCCTACAACT3′ and 5′GCCACTGCAACCCTAGCCTGTCTCA 3′, respectively. The forward primer was 5′ labeled with the fluorescent dye FAM. PCR aliquots of 0.5 μl were added to 3 μl of formamide and 0.5 μl of internal size standard. Samples were analyzed in denaturing gels (6% acrylamide/7 M urea) and sized using a Genescan TM 672 software (Applied Biosystem, Nieuwekerk/Ijssel, The Netherlands).

Statistical Analysis

Transmission disequilibrium test (TDT) and frequency of transmitted and nontransmitted parental alleles to the patients were determined using the TDT-phase program as described [14]. The test considers only heterozygous parents and assesses the evidence for preferential transmission of one allele over the other. Only one trio per family was studied.

RESULTS AND DISSCUSION

In this study we have selected the multiallelic CCTTTn repeat within the NOS2 promoter region because it is a highly polymorphic marker (14 alleles have been described with heterozygosity of 0.80), and because of its suggested potential to affect NOS2 transcription [13]. We did not consider analyzing the other genetic variations within this region, the G-945C SNP and the TAAAn repeats, because of their previously reported low degree of polymorphism and heterozygosity in our population [15].

Analysis of the multiallelic CCTTTn repeat within the 5′ upstream promoter region of the NOS2 gene in 53 Spanish celiac families showed 11 different alleles comprising repeat and pb ranges 7-17 and 171-121, respectively, according with the Caucasian pattern. Figure 1 shows the distribution of transmitted and nontransmitted NOS2 alleles in the population. We did not find any distorted transmission pattern. The most frequent transmitted repeats were 186 and 201, whereas 196 allele was preferentially nontransmitted to the affected offspring. After applying a TDT it was shown that the overall transmission of CCTTTn alleles to affected siblings reached no statistical deviation.

Different CCTTTn repeat alleles appear to have diverse effects on the ability of the 5′ promoter region to act as an effective transcription regulatory element [15]; nonetheless, caution should be exercised in extrapolating the results of *in vitro* experiments to the individual patient because other factors within the disease environment may affect the NO production and the biologic activity. In addition, further detailed molecular promoter studies using promoter construct and gel retardation assays are needed to define the overall functional importance of NOS2 polymorphism, bearing in mind that other polymorphisms in linkage disequilibrium might be influencing the promoter activity. It is clear that NO plays an important role in autoimmunity and inflammation, but the pathologic processes involved are complex and studies reported so far have been unable to assess the relative importance of NO in relation to other cytokine mediators.

The CCTTTn promoter microsatellite has been analyzed for linkage to other autoimmune conditions in which NOS2-mediated NO production has been implicated as a pathogenic agent. Thus certain CCTTTn alleles have been suggested as being protective in insulin-dependent diabetes mellitus patients with related pathologies such as retinopathy and nephropathy [13, 16], although it has been recently reported as not associated with rheumatoid arthritis or type 1 diabetes in family studies [15, 17]. In the present work, we did not obtain any evidence to suggest that the CCTTTn alleles are associated with predisposition to CD. According to our data, the increased levels of NO in the digestive tract of celiac individuals do not seem to be due to an effect of polymorphism in promoter region of NOS2 gene that mediates an increased expression of NOS2. These high levels of NO may be caused by the action of proinflammatory mediators that are secreted in the gluten-mediated immune response, generating an inflammatory environment in which the stimuli for the induction of NOS2 are present [18, 19].

In summary, this study represents the first work that analyzes the distribution of the CCTTTn pentanucleotide repeat in the NOS2 promoter region with respect to CD, and our data suggest that this polymorphism does not have a major effect on CD predisposition. Genetic susceptibility to CD is complex and heterogenous and possibly involves a large number of polymorphic genes, perhaps each contributing to a small incremental effect. Little is known about the non-HLA susceptibility genes; therefore, it could be of interest to analyze other genes and polymorphism both in the MHC region and outside it to address their potential involvement in the genetic predisposition to CD. Attractive candidates for additional CD genetic risk factors would be cytokine genes that up-regulate NOS2 expression at transcriptional level, especially IFN-γ, which is a powerful inducer of NOS2 expressed at high levels in CD patients [20].

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7.2. GENES IMPLICADOS EN LA REGULACIÓN DE LA RESPUESTA INMUNE EN LA EC

CA microsatellite polymorphism of the nuclear factor kappa B1 gene in celiac disease

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Summary

In the present work, we investigate the possible effect of a CAn microsatellite polymorphism in the nuclear factor kappa B1 (NFKB1) gene on predisposition to celiac disease (CD). Seventy-eight Spanish families with CD were genotyped using a polymerase chain reaction (PCR)-fluorescent method, and the transmission patterns of different CAn alleles were analysed. Furthermore, in order to type the CAn polymorphism more accurately, samples between 126 and 144 bp were cloned and sequenced. A trend of association with the 132-bp allele was found ($P = 0.02$). This allele was more frequently transmitted to affected sibs, although the results of statistical tests were not significant after correction for multiple comparisons. After sequencing, we found that the 132-, 138- and 142-bp alleles had two As at the end of the CA microsatellite, with the other alleles presenting the described pattern (NCB1 nucleotide U60337) for the microsatellite repeats. These results suggest that the NFKB1 CAn microsatellite does not play a major role in CD susceptibility. In addition, a more detailed molecular characterization of the CA microsatellite is described.

Introduction

In patients with celiac disease (CD), there is chronic inflammation of the proximal intestinal mucosa due to an exacerbated immune response after dietary gluten ingestion. A genetic predisposition to the disease has been described, in which human leukocyte antigen (HLA) genes have been strongly implicated, and 90% of CD patients are carriers of the DQ2 heterodimer (DQA1*0501/DQB1*0201) (Sollid, 2002). However, the contribution of HLA genes to disease risk is no more than 40%, suggesting a role for other non-HLA genes in CD susceptibility (Sollid, 2000).

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The physiopathological mechanisms that mediate celiac lesions are complex, implicating T-cell activation, autoantibody secretion, proinflammatory cytokine activity, etc. (Sollid, 2000). This disrupted immune response implies the expression of a great number of genes that must be regulated by transcription factors like nuclear factor kappa B1 (NFkB1), which belongs to NFkB/Related, family of transcription factors and mediates the immune and inflammatory responses of intestinal mucosa, as well as cell growth and apoptosis (Jobin & Sartor, 2000). Increased levels of NFkB1 activation and expression have been described in patients with intestinal autoimmune diseases such Crohn's disease and ulcerative colitis (Schreiber *et al*., 1998), and a predominant role of NFkB p65 in the pathogenesis of chronic intestinal inflammation in animal models has been demonstrated (Neurath & Pettersson, 1997).

The gene coding for NFKB1 is located on chromosome 4q23-q24 and is composed of 24 exons (Héron *et al*., 1995). A polymorphic di-nucleotide repeat consisting of a variable number of CA repeats has been identified in close proximity to the coding region of the human NFKB1 gene (Ota *et al*., 1999), with 13 described alleles. This polymorphism has recently been suggested to have a regulatory influence on the NFKB1 gene and to play a role in the susceptibility to type 1 diabetes mellitus (T1Dm) (Hegazy *et al*., 2001). We thus considered it interesting to investigate the NFKB1 microsatellite polymorphism in relation to CD susceptibility.

Materials and methods

Subjects

A group of 78 Spanish families (including 311 individuals) with a child affected by CD were genotyped for the CAn microsatellite. All participants were of Spanish Caucasian origin and recruited at two hospitals, Materno-Infantil and Hospital Clinico Universitario, in Granada, Spain. The patients were diagnosed following the European Society for Paediatric Gastroenterology and Nutrition (EPSGAN) criteria for CD (Walker-Smith *et al*., 1990). All family members were genotyped, but heterozygous parents were found only in 62 families (186 individuals) out of 78, and therefore only these 62 families were included in the analysis.

CAn microsatellite typing

The NFKB1 CAn microsatellite polymorphism (NCB1 nucleotide U60337 in the Human Genome Browser) was amplified by a polymerase chain reaction (PCR) combined with fluorescent technology. PCR was performed using a 5′ 6-carboxyfluorescein amino hexy labelled forward primer (Applied Biosystems, Warrington, UK) NFKB1 F, 5′-CTT CAG TAT CTA AGA GTA TCC T-3′ and a reverse primer, NFKB1 R 5′-CAA GTA AGA CTC TAC GGA GTC-3′. Fifty ng of genomic DNA was used for amplification in a volume of 25 µl with 1.5 mM Cl_2Mg, 200 mM dNTPs, 1% gelatin, 62.5 pmol of primers and 0.2 U of Biotherm Taq polymerase with the Biotherm buffer (Genecraft, Germany). The PCR conditions were: 5 min at 94 °C, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and finally 72 °C for 5 min. PCR aliquots of 0.5 µl were added to 3 µl of formamide and 0.5 µl of internal size standard. Samples were analysed in denaturing gels (6% acrylamide/7 M urea) and the molecular weight was determined by fluorescence using GeneScan 2.1 and Genotyper 2.5 (Abiprism, Foster City, CA). Individuals homozygous for the 126- and 138-bp alleles were sequenced to confirm the number of CA repeats and used as internal standard controls.

CAn microsatellite cloning and sequencing

PCR products of samples between 126 and 144 bp were cloned using the TOPO-XL PCR cloning kit (Invitrogen, Groningen, the Netherlands) according to the manufacturer's instructions. The samples were then sequenced using the ABI dye terminator cycle sequencing reaction kit (PE Biosystem, Warrington, UK) with the M13 F and M13 R primers supplied by the manufacturer and the exact number of CA repeats of each allele was determined in the Abiprism 3100 Genetic Analyser (Applied Biosystems).

Statistical analysis

We considered an allele as transmitted when it was present in a heterozygous parent as well as in the affected child. The frequencies of transmitted and non-transmitted alleles were compared by the χ^2 test. The *P*-values were corrected with a Bonferroni factor of 10 (the number of alleles determined) and were considered significant at a level of less than 0.05.

Table 1. Allelic frequencies of the NFKB1 CA microsatellite in CD families

Allele	Number of CA repeats	Frequency (%)
126 bp	18	19.9
128 bp	19	9.7
130 bp	20	1.5
132 bp	21	6.8
134 bp	22	9.7
136 bp	23	12.1
138 bp	24	28.6
140 bp	25	5.8
142 bp	26	4.4
144 bp	27	1.5

Results

Table 1 shows the allelic frequencies of CA repeats in the CD families; 10 out of the 13 CAn alleles initially described (Ota *et al.*, 1999) were found, ranging between 126 and 144 bp. Consistent with previous studies (Curran *et al.*, 2002; Miterski *et al.*, 2002), the most frequent alleles in our population were 126 and 138 bp, with total frequencies of 19.9 and 28.6%, respectively. Heterozygous parents were found only in 62 out of 78 CD families and thus only these 62 families were included in the transmission disequilibrium test (TDT) analysis. Figure 1 shows the transmission patterns observed; regarding the most frequent alleles in the population, the 138-bp allele showed a homogeneous transmission pattern with similar transmission/non-transmission ratios, while the 126-bp allele was preferentially not transmitted to the affected children (29.1% transmission vs. 60.9% no transmission), although the difference did not achieve statistical significance. The most frequent transmitted allele was the 132-bp allele (78.5% transmission vs. 21.5% no transmission), showing a trend of association ($P = 0.02$), although after applying the Bonferroni correction the difference was not statistically significant, which may be due to the low frequency of this allele.

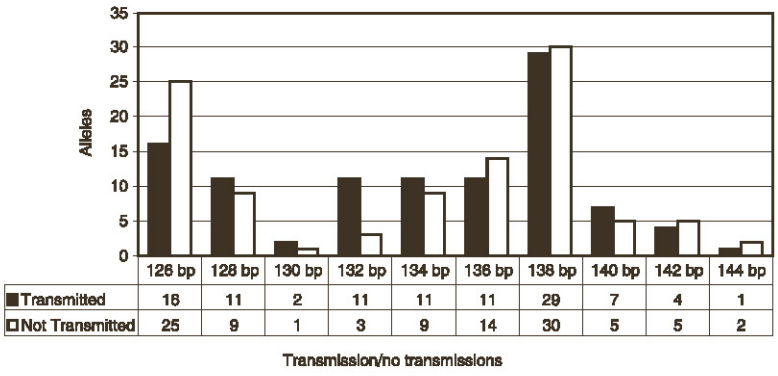

Figure 1. Transmission pattern observed for the CA repeats in CD families. Ten CAn alleles were found in the CD families, with the most frequent alleles being 126 and 138 bp. The 126-bp allele was preferentially not transmitted to the affected children (16 transmitted vs. 25 not transmitted). The 132-bp allele was preferentially transmitted to the affected children (11 transmitted vs. 3 not transmitted).

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Figure 2. Sequencing results of NFKB microsatellite alleles between 126 and 144 bp.

In order to more accurately type the CAn polymorphism, we had cloned and sequenced samples between 126 and 144 bp whose number of CAn repeats was previously assessed on GeneScan. At least three clones and sequences per allele were obtained. As can be seen in Fig. 2, we found that the 132-, 138- and 142-bp alleles had two As at the end of the microsatellite, showing a different pattern from that expected. Instead of 22 CA, 24 CA and 26 CA, respectively, they had 21 CA + AA, 23 CA + AA and 25 CA + AA. It is worth noting that the sequence deposited on the database for the NFKB1 microsatellite corresponds to allele 142 and also shows two As at the 3′ end. The other alleles presented the described pattern (NCB1 nucleotide U60337) for the microsatellite repeats.

Discussion

It is known that the NFKB1 gene plays a critical, evolutionarily conserved role in the coordination of the immune system through its ability of regulate the transcription of a wide variety of genes implicated in the immune response, such as proinflammatory cytokines [i.e. interleukin (IL)-2, IL-6 and tumour necrosis factor-α], adhesion molecules (i.e. vascular adhesion molecule (VCAM) and intercellular adhesion module (ICAM)) and inducible effector enzymes (i.e. inducible nitric oxide synthase (INOS) and cyclooxigenase 2 (COX-2)). Indeed, polymorphisms near the NFKB1 coding region could affect its expression and thus the immune equilibrium.

This is the first study to investigate a possible association between the NFKB1 CAn microsatellite polymorphism and CD. Previous studies have been performed on the NFKB1 CAn microsatellite polymorphism in relation to several autoimmune diseases. In concordance with our data, no association between this polymorphism and multiple sclerosis (Miterski *et al.*, 2002) or breast cancer (Curran *et al.*, 2002) was found. In contrast, Hegazy *et al.* (2001) found that the 138-bp allele was increased in type 1 diabetes patients compared with controls. In this study, the allelic frequencies of CA repeats were quite different from those found in other studies. Hegazy *et al.* (2001) showed that the most frequent alleles in the UK population were 134 bp (19.8%) and 146 bp (28.4%), while in Spanish and other Caucasian populations (Curran *et al.*, 2002; Miterski *et al.*, 2002) these alleles either had low frequencies or were absent. We suggest that the large differences in allelic distribution in the control population found by Hegazy *et al.* (2001) might be due to technical variation. Their method was based on autoradiography, while in other studies (Curran *et al.*, 2002; Gylvin *et al.*, 2002; Miterski *et al.*, 2002), including this study, a fluorescent technology was used, so there will be substantial differences in allelic assignment. This could be the reason why Hegazy *et al.* (2001) described a significant association with T1DM. Of note, a very recent family study could not confirm the highly significant association of NFKB1 (CA) alleles with T1DM (Gylvin *et al.*, 2002).

We also performed a more detailed molecular characterization of the CA microsatellite which could be of interest in further functional analysis to determine the overall importance of the CAn polymorphism and to elucidate how a particular allele might affect gene expression. Furthermore, it would be very interesting to carry out supplementary studies considering the complex pathway that regulates NFkB1 function, investigating the inhibitor kappa B (IkB)/NFkB1 system to determine whether polymorphisms within those genes play a role in autoimmune diseases.

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ORIGINAL ARTICLE

Functional polymorphism of the *NFKB1* gene promoter is not relevant in predisposition to celiac disease

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Abstract

Objective. The nuclear factor (NF)-κB is one of the pivotal regulators of autoimmunity and inflammation, which has been shown to be activated in the inflamed mucosa of patients with celiac disease (CD). Recently, in the *NFKB1* gene promoter region, a common insertion/deletion (−94ins/delATTG) polymorphism located between two putative key promoter regulatory elements was described. The aim of this study was to investigate the contribution of the −94ins/delATTG *NFKB1* gene promoter functional variant to CD genetic predisposition. ***Material and methods***. A case-control cohort comprising 478 patients with CD and 711 healthy controls as well as a panel of 196 celiac families was genotyped for the 94ins/delATTG *NFKB1* polymorphism, using a polymerase chain reaction (PCR) method combined with fluorescence technology. ***Results***. We found no statistically significant differences between CD patients and controls when the −94ins/delATTG genotype and allele distributions were compared. Accordingly, the familial analysis did not reach statistically significant deviation in the transmission of −94ins/del*ATTG* alleles to the affected offspring. ***Conclusions***. From these results, it could be suggested that the −94ins/delATTG *NFKB1* polymorphism does not play a major role in CD susceptibility.

Key Words: *Celiac disease, NFKB1 gene, polymorphism, transmission disequilibrium test*

Introduction

Celiac disease (CD) is an autoimmune disorder caused by intolerance to gluten, a common protein present in wheat and other cereals. CD patients present a complex clinical pattern, with local symptoms (small intestine) characterized by crypt hyperplasia, villous atrophy and lymphocytic infiltration, and additionally systemic manifestations such us, dermatitis herpetiformis or gluten neuropathy [1,2]. One of the key events in CD pathogenesis is the induction of a T-cell-mediated autoimmune response to gluten, which leads to the secretion of a wide spectrum of pro-inflammatory mediators [3]. In this context, there are several genes whose expression is induced implicating the activation of complex mechanisms of transcription regulation.

The NF-κB/Rel family of transcription factors plays a relevant role in the regulation of inflammatory response. Five members are included in the NF-κB/Rel family: NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), RelB, and c-Rel [4]. NF-κB activity is regulated by a family of IκB inhibitor proteins which, after activation, are phosphorylated, ubiquitinated and subsequently degraded allowing NF-κB to translocate to the nucleus.

Activation of NF-κB in the inflamed mucosa of celiac patients was demonstrated. Patients with untreated CD presented higher levels of p50/p65 and NF-κB binding activity than treated patients and controls [5]. Furthermore, recent findings have shown that activation of macrophages by gliadin leads to an elevated phosphorylation of IκBα and increased NF-κB binding activity [6]. Additionally, several genes that show an increased expression in celiac patients, for instance NOS2 or COX2, contain binding sites for NF-κB in their regulatory regions

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[7 9]. On this basis, NF-κB could be proposed as a molecular and genetic target for the modulation of inflammatory response in CD.

Recently, in the *NFKB1* gene promoter region, a polymorphism was identified which seems to be the first potential functional *NFKB1* genetic variant. It consists of a common insertion/deletion (−94ins/delATTG) located between two putative key promoter regulatory elements [10]. The presence of a 4 bp (base pair) deletion resulted in the loss of binding to nuclear proteins leading to reduced promoter activity [10]. Interestingly, the deletion was associated with an increased risk for an inflammatory intestinal disorder ulcerative colitis (UC) [10].

In the present study, we sought to investigate the contribution of the −94ins/delATTG *NFKB1* gene promoter functional variant to CD genetic predisposition.

Material and methods

Patients

The present study included 478 CD patients recruited independently at "Hospital Materno-Infantil" (Granada, Spain), "Hospital Clínico Universitario" (Granada, Spain) and "Hospital Clínico San Carlos" (Madrid, Spain) and 711 unrelated healthy individuals collected at the blood banks of the corresponding cities. Additionally, we extended our study to a panel of 196 celiac families recruited at the same hospitals and characterized by the presence of a child affected by CD.

The participants from both the familial and case-control analysis were of Spanish Caucasian origin. All patients were diagnosed following the European Society for Pediatric Gastroenterology and Nutrition (ESPGAN) criteria for CD [11]. Their age at study was 7.1±3.9 years and the mean age for disease diagnosis was 2.7±2.72; 60% were female and 40% male, showing an anthropometry at diagnosis (weight and height) of P 3 100 percentile. The mean age of gluten introduction was 6.4±1.5 months. Typical symptoms were observed in 72.2% of patients. CD patients were genotyped for DRB1, DQB1 and DQA1.

−94ins/delATTG genotyping

DNA was isolated from anticoagulant-treated peripheral blood mononuclear cells (PBMCs) using standard methods. We determined the −94ins/delATTG genotypes by a polymerase chain reaction (PCR)-based method as described [10]. Briefly, a 289 bp PCR fragment was amplified from genomic DNA using the forward primer 5′-TTT AAT CTG TGA AGA GAT GTG AAT G-3′ and the reverse primer 5′-CTC TGG CTT CCT AGC AGG G-3′. The forward primer was 5′labelled with the fluorescent dye, 6-FAM. The presence or absence of the 4 bp deletion was determined by the size of the labelled PCR product on an ABI 3100 sequencer, using Genescan 672 software (Applied Biosystems, Foster City, Calif., USA). Selected samples were sequenced on the ABI 3100 sequencer. The sequence results accurately confirmed the molecular weight determined by fluorescence labelling.

Statistical analysis

We used the UNPHASED software created for case-control and familial analysis [12,13]. For association studies in the case-control cohort, significance was calculated by chi-square test and the Fisher exact test when necessary, to obtain p-values. The family data were analyzed using the Transmission Disequilibrium Test (TDT), which considers only heterozygous parents and assesses the evidence for preferential transmission of one allele over the other.

An 80% power to detect an estimated effect of the −94ins/delATTG *NFKB1* polymorphism (frequency 0.37) with RR = 1.35 in disease susceptibility was achieved with our cohorts, as calculated using the Quanto v 0.5 software (Department of Preventive Medicine, University of Southern California, Calif., USA.) [14].

Results

Distributions of genotypic and allelic frequencies of −94ins/del*ATTG NFKB1* promoter genetic variant are presented in Table I. No deviation from the Hardy-Weinberg equilibrium was observed in the control population. Allelic and genotypic frequencies for the −94ins/del*ATTG* polymorphism in the control population were similar to those found by Karban et al. [10]. We did not find any statistically significant difference between CD patients and controls when the −94ins/delATTG genotype and allele distributions were compared.

Table I. Distribution −94ins/delATTG of *NFKB1* promoter polymorphism in patients with celiac disease and in controls.

−94ins/delATTG	CD patients (n=478)%	Controls (n=711)%
Ins/ins	193 (40.4)	282 (39.6)
Ins/del	224 (46.7)	321 (45.1)
Del/del	64 (13.4)	108 (15.2)
Ins	605 (63.3)	885 (62.2)
Del	351 (36.7)	537 (37.8)

Abbreviations: CD = celiac disease; ins = insertion; del = deletion.

Additionally, we performed a familial analysis to further confirm these findings. All family members were genotyped for the −94ins/del*ATTG* polymorphism but only data from heterozygous parents and their affected sibs were considered for the TDT analysis. As would be expected from the degree of heterozygosity for −94ins/del*ATTG* polymorphism in our population (H = 0.45), we obtained a considerable number of informative parent trios (n = 176) from the complete material. Accordingly, with the case-control study, the TDT did not reach statistically significant deviation in the transmission of −94ins/del*ATTG* alleles to the affected offspring (Table II).

Discussion

Genetic factors are known to play a relevant role in the etiology of CD. To date, human lymphocyte antigen (HLA) genes form the strongest genetic locus associated with CD predisposition, being a 95% of celiac patients carriers of the DQ2 molecule (DQA1*0301/DQB1*0302) [15]. However, several lines of evidence suggest a role for other genes located outside the HLA region in CD genetic risk [16,17]. Different groups have focused on the contribution of non-HLA genes to CD predisposition, considering both functional and positional candidate genes [18,19]. Nevertheless, the relevance of several potential candidate genes in CD predisposition remains to be addressed.

NF-κB is one of the pivotal regulators of autoimmunity and inflammation, and different pathogenic effects observed for NF-κB in autoimmunity might be caused by an impaired ability to regulate *NFKB1* activation [20]. In fact, genetic polymorphisms interfering with a normal induction of NF-κB activity, such as CARD15 or SUMO 4 polymorphisms, have been associated with genetic risk for different autoimmune disorders [20,21].

The −94ins/del*ATTG* polymorphism of the *NFKB1* gene is located between two regulatory elements, an AP-1 and a κB binding site and seems to affect *NFKB1* promoter activity. In this regard, a decreased promoter activity and loss of nuclear protein binding for the −94delATTG allele were reported [10]. Therefore, the carriage of the putative functional −94delATTG allele could lead to a decrease in NFKB1 production levels affecting the transcription of inflammation-related genes such as, IL-1, IL-6, IL-8 and TNFα, which are implicated in the altered gut immune response in patients with CD [3,4].

For the first time we assessed the contribution of a functional polymorphism within the *NFKB1* gene promoter in CD genetic risk. However, our results show no evidence of an association between the −94ins/del*ATTG NFKB1* promoter polymorphism and CD susceptibility. It is unlikely that our results could have arisen as a result of a type-II error (false-positive), since both case-control and familial approaches showed considerable power to detect the effect of a polymorphism with a moderate impact on CD predisposition.

Similarly to that found for CD, no association of −94ins/delATTG *NFKB1* promoter polymorphism with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) was observed in our population [22]. These findings are in contrast with the association of −94ins/delATTG *NFKB1* promoter polymorphisms with UC predisposition reported by Karban et al. [10]. Interestingly, the implication of −94ins/delATTG *NFKB1* promoter variant in UC has not been replicated in our population [23]. These findings suggest that although NF-κB is an important mediator in autoimmunity, the −94ins/delATTG *NFKB1* variant does not seem to be a common genetic risk marker for autoimmune diseases.

In addition, although a putative functional role for the −94ins/delATTG *NFKB1* polymorphism has been shown, caution should be exercised in extrapolating the results of *in vitro* experiments to the individual patient, since other factors within the disease environment may affect the NF-κB production and the biological activity. Further detailed molecular promoter studies using cell lines of different origins are needed to define the overall functional importance of −94ins/delATTG *NFKB1* polymorphism, bearing in mind that other polymorphisms in linkage disequilibrium might also have an influence on the promoter activity.

In summary, our results together with previous work in which we observed a lack of association of a dinucleotide microsatellite polymorphism (CA)n located in the proximity of the *NFKB1* gene [24] suggest that the described genetic variants of *NFKB1* do not seem to play a relevant role in predisposition to CD in our population.

Table II. Transmission pattern of −94ins/delATTG in *NFKB1* promoter polymorphism celiac disease.

−94ins /delATTG	Transmitted%	No transmitted%	% Transmission
Ins	152	182	45.5
Del	182	152	54.6

Abbreviations: Ins = insertion; del = deletion.

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Poly (ADP-ribose) polymerase-1 haplotypes are associated with coeliac disease

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Summary

In coeliac disease (CD) there is an inflammatory status of the intestinal mucosa because of a high expression of proinflammatory mediators. The nuclear protein poly (ADP-ribose) polymerase-1 (PARP-1) has been implicated in the initial inflammatory response by modulating transcription of inflammation-related genes. The aim of this work was to investigate the role of PARP-1 gene promoter region haplotypes in relation to coeliac disease susceptibility. We analysed a coeliac population consisting of a case-control panel with 120 CD patients and 311 healthy blood donors. A CA microsatellite, as haplotype-defining variant of the whole PARP-1 promoter, was typed using a polymerase chain reaction (PCR) method combined with fluorescence technology. We considered two promoter haplotypes: A defined by short CA alleles (83–87 bp) and B defined by long CA alleles (89–101 bp). Haplotype A was significantly increased within the coeliac patients group (P = 0.007 OR 1.6 95%CI 1.12–2.35). Additionally, we observed a significant dose effect, showing homozygous individuals for haplotype A higher risk for CD susceptibility (P = 0.007, OR 1.79 95%CI 1.14–2.82). Our results provide the first evidence that PARP-1 haplotypes are related with coeliac disease susceptibility.

Introduction

Coeliac disease (CD) is a complex autoimmune disorder characterized by chronic inflammation of the intestinal mucosa because of intolerance to ingested wheat gluten or related proteins from rye and barley (Fasano & Catassi, 2001). The sibling relative risk (λs 20–60) and the high concordance between monozygotic twins (75%) indicate a strong genetic component to CD in which human leukocyte antigen (HLA) genes have been shown to play an important role (Greco *et al*., 2002). In most of coeliac disease patients, the primary HLA association is with the DQ2 molecule (DQA1*05/DQB1*02) and a minority with the DQ8 molecule (DQA1*0301/DQB1*0302) (Louka & Sollid, 2003). Nevertheless, haplotype sharing studies suggest that HLA genes contribute no more than 40% to the CD genetic predisposition, suggesting an implication of non-HLA genes in CD susceptibility (Sollid, 2002).

The altered immune response to gluten is characterized by an inflammatory status of the intestinal mucosa as a result of a high expression of proinflammatory mediators such IFN-γ, TNF-α and IL-6 (Nilsen *et al*., 1998), triggering a typical mucosal lesion characterized by villous atrophy, crypts hyperplasia and increase in intraepithelial lymphocytes (Fasano & Catassi, 2001). It has been recently shown that the nuclear protein poly (ADP-ribose) polymerase-1 (PARP-1) is implicated in the initial inflammatory response by modulating transcription of inflammation-related genes and in apoptosis, among other cellular processes (Oliver *et al*., 1999a). The human PARP-1 gene is located on chromosome 1 and consists of 23 exons. It codifies for a 114-kDa protein constitutively expressed in many tissues including intestine (Hassa & Hottiger, 2002). A microsatellite polymorphism has been described in the 5′ flanking region of the PARP-1 gene, consisting of a variable number of CA repeats (Fougerousse *et al*., 1992). Additionally, four sequence variations have been recently identified in this region: C410T, poly $(A)_n$, C1362T and G1672A (Kato *et al*., 2000) (Fig. 1). These polymorphisms are part of two unique haplotypes of the PARP-1 promoter, which includes four consecutive PARP-1 polymorphisms: haplotype A (410T-[A_{10}]-short CA repeats [83–87 bp]-1362C) and haplotype B (410TC-[A_{11}]-long CA repeats [89–101 bp]-1362T), the CA microsatellite being the haplotype-defining variant of the whole PARP-1 promoter polymorphism (Pascual *et al*., 2003). The CA microsatellite is very close to the binding site of the transcription factor ying yang 1 (YY1), and therefore it might affect the transcription of the PARP-1 gene (Oei & Shi, 2001). Furthermore, it has been shown

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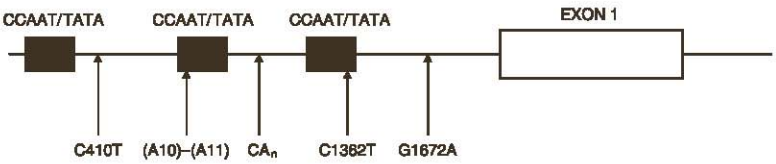

Figure 1. Described polymorphisms within PARP-1 promoter region.

that certain CA microsatellite alleles are related to susceptibility to other autoimmune diseases like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Pascual *et al.*, 2003; Tsao *et al.*, 1999). This evidence, together with the described role of PARP-1 in process related with CD pathophysiology, led us to investigate the possible implication of PARP-1 gene promoter haplotypes in CD susceptibility.

Materials and methods

Patients and controls

In the present work we have analysed a case-control panel consisting of 120 CD patients and 311 healthy blood donors recruited in two Spanish hospitals. An initial group of 77 patients and 252 controls were collected in Hospital Materno-Infantil and Hospital Clinico Universitario (Granada) and a second set of samples (43 CD patients and 59 controls) were obtained from Hospital Universitario La Paz (Madrid).

The participants were of Spanish Caucasian origin. All patients were diagnosed following the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) criteria for CD (Working Group of European Society of Paediatric Gastroenterology & Nutrition, 1990). Their age at study was 7.1 ± 3.9 years and the mean age for disease diagnosis was 2.7 ± 2.72. Sixty per cent were women and 40% men, showing an anthropometry at diagnosis (weight and height) of *P* 3–100 percentile. The mean age of gluten introduction was 6.4 ± 1.5 months. Typical symptoms were observed in 72.2% of patients and 27.8% showed atypical symptoms.

CA microsatellite genotyping

DNA was extracted by standard methods from peripheral blood leukocytes. PARP-1 microsatellite polymorphism was analysed using the method previously described (Fougerousse *et al.*, 1992). Sense and antisense primers were, respectively, 5′-GAT TCC CCA TCT CTT TCT TT-3′ (labelled at the 5′ end with 6-FAM) and 5′-AAA TTG TGG TAA TGA CTG CA-3′. To determine the number of triplet repeats in the TM region, 0.5 μL PCR aliquots were added to 3 μL of formamide and 0.5 μL of internal size standard. Samples were analysed in denaturing gels (6% acrylamide/7 M urea), run on an ABI Prism 377 genetic analyser (Applied Biosystems, Foster City, CA) and sized using a GENESCAN 672 software. Accuracy of genotypes was determined by sequencing selected samples covering all CA alleles; homozygous samples were used as internal standard controls.

Statistical analysis

For association studies in the case-control population, *P* values were calculated by the Chi-square method or the Fisher's exact test when appropriate. Odds ratio (OR) with 95% confidence intervals (95% CI) were calculated according to Woolf's method. All test were made using the UNPHASED software (Dudbridge, 2002; Dudbridge, 2003), and *P* values less than 0.05 were considered significant.

Results

According to our previous findings, we selected the CA microsatellite as a genetic marker to investigate the implication of PARP-1 promoter haplotypes in CD susceptibility. Ten CA microsatellite repeats were found between 83 and 101 bp, being the most frequent CA repeats observed in our population the 85 bp and the 95 bp, which is in concordance with previous data described in Caucasian populations (Pascual *et al.*, 2003; Delrieu *et al.*, 1999; Delrieu *et al.*, 2001). As previously established (Pascual *et al.*, 2003), we grouped CA microsatellite alleles in two haplotypes, CA repeats between 83 and 87 bp were considered as haplotype A, and CA repeats ranging from 89 to 101 bp were grouped as haplotype B. Interestingly it was found that haplotype A was significantly increased within CD patient group with respect to the healthy individuals ($P = 0.007$, OR 1.6 95%, CI 1.12–2.35). Similarly, the overall comparison of genotypes between patients and controls showed a statistically significant difference ($P = 0.02$ by Chi-square test with 3×2 contingency table). Additionally, we observed a significant dose effect, showing homozygous individuals for haplotype A higher risk for CD susceptibility ($P = 0.007$, OR 1.79 95%CI 1.14–2.82) (Table 1).

To investigate a possible interaction between PARP-1 haplotypes and HLA genes, we performed a conditional analysis grouping individuals according to HLA class II typing. The distribution of PARP-1 haplotypes was compared in DQ2 positive or negative CD patients and controls (Table 2). No statistically significant differences were observed between the groups whatever the HLA genotype.

Discussion

Several studies based on animal models with PARP-1 deficient activity have demonstrated the pathophysiological role of this protein in inflammatory disorders (Hassa & Hottiger, 2002). It is worth noting that in a model of inflammatory intestinal disease, such as murine colitis, the PARP-1 blockade results in disease improvement with a

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Table 1. Comparison of PARP-1 haplotype and genotypes frequencies in case-control population

	CD patients	Controls	OR (95%CI)	P (X^2)
Haplotype A, frequency (%)	190 (79.2)	436 (70)	1.60 (1.12–2.35)	0.007
Haplotype B, frequency (%)	50 (20.8)	186 (30)		
Genotype AA, frequency[a] (%)	75 (62.5)	150 (48.2)		
Genotype AB, frequency (%)	40 (33.3)	136 (43.7)		
Genotype BB, frequency (%)	5 (4.2)	25 (8.1)		0.02

[a] For AA vs. other genotypes: $P = 0.007$, odds ratio (OR) 1.79, 95% confidence interval (95% CI) 1.14–2.82.

Table 2. Distribution of PARP-1 haplotypes and HLA typing in case-control population

	CD patients (%)	Controls (%)	P
DQ2+/haplotype A	75.8	66.6	ns
DQ2–/haplotype A	64.3	54.4	ns
DQ2+/haplotype B	24.2	33.4	ns
DQ2–/haplotype B	35.7	45.6	ns

reduction of inflammation and mucosal injury (Zingarelli *et al.*, 1999; Jijon *et al.*, 2000).

Despite those evidences, the precise mechanism underlying the role of PARP-1 during the inflammatory response is not clear. Different reports have shown that PARP-1 acts as coactivator of transcription factors such as NFkB. The strongest indication for a role of PARP-1 in NFkB transcription was the impaired expression of NFkB dependent proinflammatory mediators in PARP-/– mice (Oliver *et al.*, 1999b). In this way it seems that PARP-1 is implicated in the cascade of immune response through regulation of expression of inflammatory mediators, therefore a tight control of PARP-1 gene should be a crucial point to maintain the immune equilibrium. Different regulatory elements have been identified in the PARP-1 promoter region, such as a binding site for YY1 transcription factor, three sets of CCAAAT/TATA boxes and multiple transcription sites (Oei & Shi, 2001), thus genetic variants within this region may affect PARP-1 expression.

Here, we studied a CA microsatellite in the promoter region of PARP-1, which short and long alleles define the two unique promotor haplotypes (Pascual *et al.*, 2003). We detected significant association between this microsatellite and CD.

We hypothesize that the different lengths of CA microsatellite could affect the promoter structure and thus interactions with other molecules, like different NFkB hetero and homodimer complexes. Haplotype A could lead a low expression of PARP-1, reducing its interaction with NFkB molecule. It has been shown that NFkB inhibition leads to an increased enterocite apoptosis (Potoka *et al.*, 2000; Potoka *et al.*, 2002) because of the loss of its regulatory functions in both receptor-mediated and mitochondrial dependent mechanism of apoptosis. An increased enterocite apoptosis has been demonstrated in coeliac patients (Moss *et al.*, 1996) and it is thought to be related with villous atrophy, one of the main characteristics of coeliac lesion. This way, PARP-1 promoter polymorphisms could influence CD susceptibility through its possible role in enterocyte apoptosis regulation.

In summary, we have analysed for the first time the PARP-1 promoter haplotypes in relation to CD and our data strongly suggest an association of PARP-1 with CD predisposition.

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ELSEVIER

CTLA4/CT60 Polymorphism Is Not Relevant in Susceptibility to Autoimmune Inflammatory Intestinal Disorders

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ABSTRACT: The aim of this work was to investigate the possible influence of the recently described CT60 A/G dimorphism of the *CTLA4* (cytotoxic T-lymphocyte antigen 4) gene in the susceptibility to two different autoimmune inflammatory intestinal disorders, inflammatory bowel disease (IBD) and celiac disease. We analyzed a case-control cohort composed of 528 Spanish patients with IBD (284 with Crohn disease and 244 with ulcerative colitis) and 454 unrelated healthy individuals, and additionally a group of 90 celiac disease families. CT60 genotyping was performed with a TaqMan 5′ allelic discrimination assay. After comparing patients with IBD with the control population, we found no significant deviation in the distribution of the alleles or genotypes of CTLA4/CT60 dimorphism. In addition, by means of familial and case-control analysis, no evidence for a statistically significant association was observed between CTLA4/CT60 and celiac disease susceptibility. Therefore, our results suggest that the CTLA4/CT60 polymorphism does not play a major role in inflammatory intestinal disorders. *Human Immunology 66, 321–325 (2005).* © American Society for Histocompatibility and Immunogenetics, 2005. Published by Elsevier Inc.

KEYWORDS: CTLA4; IBD; celiac disease; autoimmunity; polymorphism

ABBREVIATIONS

CTLA4	cytotoxic T-lymphocyte antigen 4
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
OR	odds ratio
SNP	single nucleotide polymorphism
TDT	transmission disequilibrium test

INTRODUCTION

Inflammatory bowel disease (IBD) and celiac disease are chronic autoimmune inflammatory intestinal disorders. The two main forms of IBD, Crohn disease and ulcerative colitis, share some clinical and pathologic features, although disease type can usually be distinguished by anatomic and histologic features [1]. The patients with IBD experienced a chronic relapsing inflammation of the gastrointestinal tract as a result of an inappropriate and exaggerated mucosal immune response to normal constituents of intestinal microflora [1]. In celiac disease, intolerance to dietary gluten leads to inflammation and atrophy of the mucosa [2].

Genetic factors are known to play an important role in determining susceptibility to both IBD and celiac disease [2, 3]. Inflammatory bowel disease epidemiologic and linkage studies have demonstrated that there are many contributing genes in disease susceptibility. Although the nucleotide-binding oligomerization domain 2 has been characterized as the IBD1 locus [4], the identification of other target genes is necessary to understand the mechanisms underlying IBD susceptibility [1, 3]. Regarding celiac disease, the human leukocyte antigen (HLA) genes have been demonstrated to have a relevant role in the genetic predisposition, with 90% of celiac

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patients bearing the DQ2 molecule (DQA1*05/DQB1*02). However, the results of haplotype sharing studies have revealed that the contribution of HLA genes for celiac disease genetic predisposition is no more than 40%, suggesting a role for non-HLA genes in celiac disease susceptibility [2, 5].

T cells play a major role in the mucosal pathogenesis of IBD and celiac disease as well as in the maintenance of oral tolerance [1, 2]. Therefore, molecules that mediate regulation of T-cell activity could influence disease susceptibility. The CTLA4 (cytotoxic T-lymphocyte antigen 4) molecule is a homologue for CD28, and both molecules and their common ligands (B7-1 and B7-2) constitute the B7/CD28-CTLA4 costimulatory pathway for T-cell activation. Whereas the CD28/ligand interaction plays a critical role in increasing and maintaining the T-cell response initiated through T-cell antigen receptor engagement, the CTLA4/ligand interaction has an inhibitory effect on T-cell activation and might contribute to peripheral tolerance [6]. Thus, *CTLA4* is a good candidate gene for susceptibility to both IBD and celiac disease. Furthermore this gene maps within the 2q33 region, which has been demonstrated to be a chromosomal region that confers risk to both IBD and celiac disease [7, 8]. Several polymorphisms have been described in the *CTLA4* gene [9–12]. Among them, a CT60 A/G dimorphism (simple nucleotide polymorphism [SNP] 3087243) has been recently associated with functional relevance and with susceptibility to a variety of autoimmune diseases [13].

The aim of this work was to investigate the possible influence of the recently described CT60 A/G dimorphism of the *CTLA4* gene in the susceptibility to two different autoimmune inflammatory intestinal disorders, IBD and celiac disease.

SUBJECTS AND METHODS

Patients and Controls

We analyzed a case-control cohort composed of 528 Spanish patients with IBD (284 with Crohn disease and 244 with ulcerative colitis) recruited in two hospitals from South Spain (Hospital Virgen de las Nieves, Granada, and Hospital Puerta del Mar, Cádiz). The patients were recruited and diagnosed following standard criteria based on reviews of clinical, endoscopic, radiologic, and histopathologic reports [14]. A total of 454 unrelated healthy individuals were included as controls.

In parallel, a group of 90 celiac families characterized by the presence of a celiac disease–affected sibling was considered for the study. Samples were collected in two hospitals, "Hospital Materno-Infantil" and "Hospital Clínico Universitario" in Granada, Spain. Patients with celiac disease were diagnosed following the European Society for Paediatric Gastroenterology and Nutrition criteria for celiac disease [15]. The same 90 patients with celiac disease were also used for a case-control analysis by comparing them with 202 healthy controls. All study participants—patients with IBD, patients with celiac disease, and controls—were of white Spanish origin.

CTLA4/CT60 Genotyping

DNA from patients and controls was obtained from peripheral blood via standard methods. Genotyping of CT60 was performed by a TaqMan 5′ allelic discrimination assay; the primers, probes, and reaction conditions were similar to those previously described [13]. Briefly the primers sequences were 5′-TGG AAG GTA TCC ATC CTC TTT CCT (forward) and 5′-CAT GCC AAT TGA TTT ATA AAG GAC TGC TA (reverse), and the TaqMan MGB probe sequences were 5′-CTA TTT GGG ATA TAA CAT GGG TT and 5′-ATT TGG GAT ATA ACG TGG GTT; the probe were labeled with the fluorescent dyes VIC and FAM, respectively.

Statistical Analysis

We used the UNPHASED software created for case-control and transmission disequilibrium test (TDT) analysis of haplotypes. For association in the case-control studies, significance was calculated by 2 × 2 contingency table and Fisher exact test, to obtain p values, odds ratios (OR), and 95% confidence intervals. The family data were analyzed by the TDT, which considers only heterozygous parents and assesses the evidence for preferential transmission of one allele over the other [16, 17]. The power of the study to detect an effect of a polymorphism in disease susceptibility was estimated by Quanto 0.5 software (Department of Preventive Medicine, University of Southern California) [18, 19].

RESULTS

Table 1 lists the allelic distribution of CTLA4/CT60 polymorphism in patients with IBD and controls. The allelic distribution in controls was similar to that obtained in other Caucasian populations [20, 21]. Allele frequencies were in Hardy-Weinberg equilibrium in both the patient and control groups. After comparing patients with Crohn disease and patients with ulcerative colitis as two independent groups, with the control population, we found no significant deviation in the distribution of the alleles or genotypes of CT60 dimorphism. Similarly, when both groups were considered as a whole, no statistically significant difference was found between patients with IBD and controls (Table 1).

In addition, in both familial and case-control analyses, no evidence for a statistically significant association was observed between CTLA4/CT60 and celiac disease susceptibility. The TDT analysis revealed no significant

TABLE 1 Genotype and allelic frequencies of CTLA4/CT60 in patients with IBD

Genotype or allele	Patients with IBD			Controls (n = 454), n (%)
	Crohn disease (n = 284), n (%)	Ulcerative colitis (n = 244), n (%)	Total (n = 528), n (%)	
CT60 genotype				
GG	67 (23.6)	64 (26.2)	131 (24.8)	105 (23.1)
AG	143 (50.4)	118 (48.4)	261 (49.4)	229 (50.4)
GG	74 (26)	62 (25.4)	136 (25.8)	120 (26.5)
CT60 allele				
A	277 (48.7)	246 (50.4)	523 (49.5)	439 (48.3)
G	291 (51.2)	242 (49.6)	533 (50.5)	469 (51.7)

deviation of the transmission–no transmission pattern for the CT60 alleles (Table 2). Accordingly, results from case-control analysis demonstrated very similar allelic frequencies among celiac patients and controls, with no statistically significant differences (Table 3).

DISCUSSION

There are two main arguments to choose a good candidate gene in disease-association analyses, based on the positional or the functional implication of the gene in disease susceptibility. Here we have applied these two different strategies to investigate the possible implication of CTLA4/CT60 polymorphism in IBD and celiac disease susceptibility. Genotyping data in multiple SNPs of *CTLA4* obtained in a study concerning other autoimmune diseases (Graves disease, autoimmune hypothyroidism, and type 1 diabetes) suggest that disease susceptibility maps to 6.1-kb 3′ region of the *CTLA4* gene [13], in particular the marker CT60 (SNP3087243), which demonstrated the strongest association. Interestingly, the CT60 G allelic variation was also reported to be correlated with lower mRNA levels of the soluble alternative splice form of *CTLA4* (sCTLA).

The CTLA4/CT60 polymorphism is a good functional candidate marker in IBD. It is known that the mucosal inflammation observed in patients with IBD results from a dysregulation in T-cell balance from an abnormally robust T_H1 (Crohn disease) or T_H2 (ulcerative colitis) response [1]. In this respect, the role of the *CTLA4* gene might be very important as a regulator of T-cell activation and proliferation. Of note, a recent genome scan meta-analysis has shown that the 2q33 region confers susceptibility to IBD [8], demonstrating that the CTLA4/CT60 is a good positional candidate gene to IBD.

TABLE 2 Transmission pattern of CT 60 alleles in patients with celiac disease

Allele	Transmitted, n (%)	Not transmitted, n (%)
G	71 (45.8)	72 (46.4)
A	84 (54.2)	83 (53.6)

In this work, we investigated for the first time the role of CTLA4/CT60 polymorphism in IBD susceptibility, and no evidence of association was found. It is unlikely that our results could have arisen because of a type II error (false-negative results) because, with the sample size used, the IBD combined study had 95% power to detect the effect of a polymorphism, conferring an OR of 1.8 at the 5% significance level (assuming an allele frequency of 50% in the control population). Nevertheless, our study was underpowered to detect smaller effects such as an OR of 1.14 associated with type 1 diabetes [13]. Interestingly, our data are in accordance with those obtained in a study of IBD in a Dutch Caucasian population in which no association between +49A/G and C-318T CTLA4 polymorphisms and IBD was observed [22]. These findings and our results suggest that *CTLA4* polymorphism does not play a major role in IBD susceptibility. However, the contribution of other candidate genes located in close proximity to *CTLA4* in susceptibility to IBD, like the *CD28* and *ICOS* genes, could not be discarded. In fact, very recently, it has been demonstrated that patients with IBD hyperexpress *ICOS* in the gastrointestinal tract [23].

The role of the *CTLA4* gene in celiac disease have been broadly analyzed because it is a very good positional and functional candidate gene to disease susceptibility [7, 24, 25]. Most of the studies have analyzed polymorphisms −318C/T, +49A/G in exon 1 and the AT_n microsatellite, without consistent findings in all populations [20]. The present study found no association between CTLA4/CT60 polymorphism and celiac disease susceptibility. Our analyses in celiac disease case-control and familial cohorts have a 50% power to detect the effect of a polymorphism, conferring an OR of 2.0 (assuming an allele frequency of 50%). In accordance with our findings, recent studies have observed no association

TABLE 3 Genotypic and allelic frequencies of CTLA/CT60 in patients with celiac disease and in controls

Genotype or allele	Patients with celiac disease (n = 90), n (%)	Controls (n = 202), n (%)
CT60 genotype		
GG	16 (17.9)	54 (26.7)
GA	52 (57.3)	101 (50)
AA	22 (24.7)	47 (23.3)
CT60 allele		
G	84 (46.7)	209 (51.7)
A	96 (53.3)	195 (48.3)

[20, 26] or borderline significance [27] between CTLA4/CT60 polymorphisms and celiac disease predisposition.

All these observations suggest that the chromosome 2q33 linked susceptibility in celiac disease might be attributed to another genetic marker. Interestingly, recently, an implication of the *ICOS* gene in celiac disease predisposition has been described [26].

Although the CTLA4/CT60 polymorphism has been proposed as a common genetic marker for autoimmunity [6, 13], studies in autoimmune diseases like rheumatoid arthritis have failed to detect an implication of CTLA4/CT60 polymorphism in disease susceptibility [28, 29]. Our results in IBD and celiac disease are in accordance with these studies, suggesting that the role of CTLA4/CT60 in disease susceptibility might be different depending on the genetic background or environmental factors that influence complex genetic diseases.

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ELSEVIER

C1858T Functional Variant of *PTPN22* Gene Is Not Associated With Celiac Disease Genetic Predisposition

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ABSTRACT: Recent findings have demonstrated that the single nucleotide polymorphism 1858C→T located at the P1 motif of the *PTPN22* (protein tyrosine phosphatase nonreceptor 22) gene has functional relevance and is associated with a variety of autoimmune diseases. The aim of this study was to assess the role of the *PTPN22* 1858C→T polymorphism in the genetic predisposition to celiac disease (CD). We analyzed a case-control cohort composed by 534 patients with CD and 653 healthy controls and additionally a panel of 271 celiac families. The *PTPN22* 1858C→T genotyping was performed by TaqMan 5′ allelic discrimination assay. We did not observed any statistically significant deviation after comparing allele and genotypic frequencies of *PTPN22* 1858C→T between patients with CD and controls. Accordingly, the familial analysis did not reach statistically significant deviation in the transmission of *PTPN22* 1858C→T alleles to the affected offspring. Therefore, our data suggest that the *PTPN22* 1858 single nucleotide polymorphism has no, or only a negligible, effect on CD susceptibility in this Spanish population. *Human Immunology 66, 848–852 (2005).* © American Society for Histocompatibility and Immunogenetics, 2005. Published by Elsevier Inc.

KEYWORDS: celiac disease; protein tyrosine phosphatases; protein tyrosine phosphatase nonreceptor 22 (*PTPN22*) gene; polymorphism; transmission; disequilibrium test

ABBREVIATIONS

CD	celiac disease
Lyp	lymphoid-specific phosphatase
MS	multiple sclerosis
PTP	protein tyrosine phosphatase
PTPN22	protein tyrosine phosphatase nonreceptor 22
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
T1D	type 1 diabetes
TDT	transmission disequilibrium test

INTRODUCTION

Celiac disease (CD) is an autoimmune disorder characterized by a chronic inflammatory status of the intestinal mucosa due to dietary gluten ingestion in genetically predisposed individuals [1]. Human leukocyte antigen (HLA) genes play an important role in CD genetic risk. Most patients with CD (90%) are carriers of the DQ2 molecule encoded by DQA1*05/DQB1*02 alleles, and a minority of patients bear the DQ8 molecule (DQA1*0301/DQB1*0302) [2]. Nevertheless, the results of haplotype sharing studies have described that the contribution of HLA genes for CD genetic predisposition is no more than 40%, suggesting a role for non-HLA genes in CD susceptibility [1].

The development of an antigluten T-cell response is one of the most important events in CD; therefore, the regulation of T-cell responses should be relevant in CD pathogenesis [3]. One important regulatory mechanism in T-cell physiology is tyrosine phosphorylation, which can affect antigen-receptor–mediated lymphocyte activation, cytokine-induced differentiation, and responses to different stimuli [4, 5]. Together with protein tyrosine

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kinases, protein tyrosine phosphatases (PTPs) regulate the reversible phosphorylation of tyrosine residues and are required for a physiological immune response [6]. In this regard, abnormalities in tyrosine phosphorylation have been demonstrated to be involved in the pathogenesis of numerous human diseases from autoimmunity to cancer [7].

A wide range of regulatory PTPs is expressed in T lymphocytes [4]. One of the most recently studied is the PTPN22 (protein tyrosine phosphatase nonreceptor 22), a lymphoid-specific phosphatase (Lyp) [8]. Lyp is an intracellular PTP that is physically bound through one proline-rich motif (referred to as P1) to the SH3 domain of the Csk kinase inhibiting T-cell receptor signaling [9, 10].

A missense single nucleotide polymorphism (SNP) consisting in an arginine to tryptophan change (1858C→T; rs2476601; R620W) located at the P1 motif of *PTPN22* gene has been associated with susceptibility to several autoimmune diseases such as type 1 diabetes (T1D), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Graves disease [11–17]. The *PTPN22* 1858C→T change disrupts the interaction between Lyp and Csk, avoiding the formation of the complex and therefore the suppression of the T-cell activation [11].

Taking into account these findings, the aim of this study was to assess the role of the *PTPN22* 1858C→T polymorphism in the genetic predisposition to CD.

MATERIALS AND METHODS

Patients and Controls

The present study included 534 patients with CD recruited independently at "Hospital Materno-Infantil" (Granada, Spain), "Hospital Clínico Universitario" (Granada, Spain), and "Hospital Clínico San Carlos" (Madrid, Spain) and 653 unrelated healthy individuals collected at the blood banks of the corresponding cities. Cases and controls were matched for age and sex. Additionally, we extended our study to a panel of 271 celiac families recruited at the same hospitals and comprising a child with CD and his or her parents.

The participants from both the familial and case-control analysis were of Spanish white origin. All patients were diagnosed according to the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) criteria for CD [18]. Their age at study was 7.1 ± 3.9 years, and the mean age for disease diagnosis was 2.7 ± 2.72. A total of 60% were women and 40% men, indicating an anthropometry at diagnosis (weight and height) of *P* 3–100 percentile. The mean age of gluten introduction was 6.4 ± 1.5 months. A total of 72.2% of patients exhibited typical symptoms (chronic diarrhea, failure to thrive, abdominal distension), and 28.2% presented atypical symptoms (*i.e.,* anemia, dermatitis herpetiformis, psoriasis, ataxia). Patients with CD were genotyped in DRB1, DQB1, and DQA1.

Genotyping

DNA from all study participants was obtained from peripheral blood by means of standard methods. Samples were genotyped for *PTPN22* 1858C→T variants with a TaqMan 5′ allelic discrimination Assay-by-Design method (Applied Biosystems, Foster City, CA), as previously described [15]. Briefly, the primers sequences were 5′-CCAGCTTCCTCAACCACAATAAATG (forward) and 5′-CAACTGCTCCAAGGATAGATGATGA (reverse), and the TaqMan MGB probes sequences were 5′-CAGGTGTCCATACAGG and 5′-CAGGTGTCCGTACAGG. The probes were labeled with the fluorescent dyes VIC and FAM, respectively.

Statistical Analysis

We used the UNPHASED software created for case-control and familial analysis [19, 20]. For association studies in the case-control cohort, significance was calculated by 2 × 2 contingency table and Fisher's exact test when necessary, to obtain p values. The family data were analyzed by the transmission disequilibrium test (TDT), which considers only heterozygous parents and assesses the evidence for preferential transmission of one allele over the other.

The power of the study to detect the effect of a polymorphism in disease susceptibility was estimated by the Quanto v 0.5 software (Department of Preventive Medicine, University of Southern California) [21].

RESULTS

Table 1 lists the distribution of genotypic and allelic frequencies of *PTPN22* 1858C→T polymorphisms in patients with CD and controls. Data collected from the three hospitals were combined because no statistically significant difference was observed among them. The control population was found to be in Hardy-Weinberg equilibrium. The *PTPN22* 1858C→T allelic frequencies were very similar to those previously described for other white populations, with a low frequency of the T allele (6.2%) [11, 14]. We did not observe any statistically significant deviation after comparing allele and genotypic frequencies of *PTPN22* 1858C→T between patients with CD and controls. In addition, comparison of genotypes carrying T allele (CT + TT *vs* CC) between patients with CD and controls did not reach statistically significant skewing (data not shown).

To further confirm this evidence, we performed a familial analysis. All family members were genotyped for the *PTPN22* 1858C→T polymorphism, but only data from heterozygous parents and their affected sibs were

TABLE 1 Genotypic and allelic frequencies of *PTPN22* 1858C→T polymorphism in patients with celiac disease and controls

PTPN22 1858C→T polymorphism	Patients, *n* (%) (*n* = 534)	Controls, *n* (%) (*n* = 653)	*p* Value	OR (95% CI)
Genotype				
CC	460 (86.2)	576 (88.2)	0.28	0.83 (0.58–1.19)
CT	73 (13.7)	74 (11.3)	0.22	1.24 (0.86–1.78)
TT	1 (0.2)	3 (0.5)	0.42	0.41 (0.02–4.36)
Allele				
C	993 (93.0)	1226 (93.8)	0.37	0.86 (0.62–1.21)
T	75 (7.0)	80 (6.2)	0.37	0.86 (0.62–1.21)

considered for the familial analysis. On the basis of the heterozygosity observed for *PTPN22* 1858C→T in our population ($H = 0.12$), it could be expected that approximately only 60–70 case-parent trios of the complete material ($N = 271$) would be informative. We found a total of 72 families with at least one heterozygous parent, and therefore informative for the TDT test. Accordingly with the case-control analysis, the TDT study for the *PTPN22* 1858C→T genetic variants did not reveal biased transmission of *PTPN22* 1858C→T alleles to the affected offspring (Table 2).

TABLE 2 Transmission pattern of *PTPN22* 1858C→T polymorphism in celiac families

PTPN22 1858C→T allele	Transmitted, *n* (%)	Not transmitted, *n* (%)	*p* Value	OR (95% CI)
C	33 (45.8)	39 (54.2)	0.31	0.72 (0.35–1.45)
T	39 (54.2)	33 (45.8)	0.31	0.72 (0.35–1.45)

DISCUSSION

Recent studies have demonstrated that the functional polymorphism in *PTPN22* is implicated in genetic susceptibility to T1D, RA, SLE, and Graves disease [11–17]. This fact is consistent with a potential role of *PTPN22* 1858C→T as a common susceptibility allele shared among autoimmune diseases. Nevertheless, our results regarding CD point out that CD escapes this role; the *PTPN22* 1858C→T polymorphism does not seem to be a crucial point in susceptibility to CD. It is unlikely that our data could have arisen as a result of a type 2 error (false-negative results) because the power estimation demonstrated that both case-control and familial studies would have considerable power to detect the effect of a polymorphism with moderate impact on CD susceptibility. Assuming a minor allele frequency of 0.07 and RR of 1.8, we would reach 97% power to detect an association in our population with a case-control approach and 80% power by means of a familial analysis.

Interestingly, during the course of this work, a similar study that showed no statistically significant association of the *PTPN22* 1858 C→T polymorphism with CD in a north European white population was published [22]. Similarly, no association of the *PTPN22* 1858 SNP with multiple sclerosis (MS) has been reported [22–24]. The finding that CD and MS are not associated with the *PTPN22* 1858T allele (where other autoimmune diseases, such as T1D, RA, SLE, and Graves disease, are) suggest that the latter diseases share common underlying mechanism that may not play an important role in the predisposition to CD or MS. These findings suggest that the *PTPN22* 1858 genetic variant does not seem to be a common genetic marker for autoimmunity.

It is possible that different populations' genetic backgrounds could condition the effect of the *PTPN22* polymorphism; nevertheless, it seems not to be the reason because the *PTPN22* polymorphism confers susceptibility to other autoimmune diseases such as RA and SLE in populations with the same genetic background [14–16].

The regulation and biology of PTPs in T-cell physiology is complex and implicates a wide spectrum of mediators. Therefore, it might be possible that the implication of *PTPN22* in T-cell activation could not be the same in all autoimmune diseases, and that in certain subgroups of these conditions, other PTP family members may have a more relevant role. In fact, an association of a genetic variant in CD45 (protein tyrosine phosphatase receptor type C) with MS was observed [25].

Similar findings have been reported concerning the influence of *CTLA-4* gene in autoimmunity. Although the *CTLA-4* CT60 marker has been associated with a range of autoimmune diseases, such as T1D, Graves disease, thyroiditis, and SLE [26, 27], no linkage was observed with RA [28, 29] or CD [30]. These findings support the notion that common susceptibility alleles are not shared among all autoimmune diseases, but rather among groups of these conditions.

In summary, our results suggest that the *PTPN22* 1858 SNP has no effect on CD susceptibility in this Spanish population. Although a minor effect of the *PTPN22* SNP cannot be ruled out, this may only be verifiable in an extremely large data set.

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ELSEVIER

Analysis of a GT Microsatellite in the Promoter of the *foxp3/scurfin* Gene in Autoimmune Diseases

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ABSTRACT: The aim of this study was to assess the possible association of the functional $(GT)_n$ microsatellite polymorphism in the *FOXP3* gene with predisposition to several autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), ulcerative colitis (UC), Crohn's disease, and celiac disease. We analyzed a case-control cohort composed of 231 SLE patients, 293 RA patients, 528 inflammatory bowel disease (354 Crohn's disease patients and 260 UC patients) patients, 103 celiac disease patients, and 274 healthy controls ethnically matched. Genotyping of $(GT)_n$ microsatellite was performed by polymerase chain reaction (PCR)-based method combined with fluorescent technology. We found no evidence for association of this polymorphism between controls and these autoimmune disease patients. Additionally, no differences in the genotype and allele distribution were found when patients were stratified according to clinical manifestation. The $(GT)_n$ microsatellite of the *FOXP3* gene may not play a relevant role in the susceptibility to SLE, RA, inflammatory bowel disease, and celiac disease in our population. *Human Immunology 66, 869–873 (2005).* © American Society for Histocompatibility and Immunogenetics, 2005. Published by Elsevier Inc.

KEYWORDS: *FOXP3* gene; systemic lupus erythematosus (SLE); rheumatoid arthritis (RA); ulcerative colitis (UC); Crohn's disease; celiac disease; autoimmune diseases (AID); microsatellite

ABBREVIATIONS

AID	autoimmune diseases
CI	confidence interval
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
T1D	type 1 diabetes
Tregs	regulatory T cells
UC	ulcerative colitis

INTRODUCTION

Autoimmune diseases (AID) affect approximately 5% of the population and are characterized by loss of self-tolerance causing immune-mediated tissue destruction [1]. AID share a number of characteristics that suggest common etiologic pathways or mechanisms, including reactivity to self-antigens, inflammatory manifestation, as well as genetic associations with human leukocyte antigens (HLA) [2]. Although the etiology of AID is

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unknown, it is widely accepted that both environmental and genetic factors are involved in the pathogenesis of these disorders [3]. The genetic background of AID is complex and likely involves multiple genes encoding proteins with significant functions in the regulation of the immune system.

Regulatory T cells (Tregs) are important components of the homeostasis of the immune system, because impaired regulatory T-cell activity can cause AID [4, 5]. There is compelling evidence that the role of Treg is not limited to the prevention of autoimmunity, but is important in controlling virtually all forms of immune response, including inflammation. *FoxP3/Scurfin* gene encodes a protein that is a member of the forkhead/winged-helix family of transcriptional regulators, and is specifically expressed in naturally occurring $CD25^+CD4^+$ regulatory T cells [6, 7]. Furthermore, retroviral gene transfer of Foxp3 converts naive T cells toward a regulatory T-cell phenotype similar to naturally occurring CD4+ regulatory T cells [7]. Thus, *Foxp3* is a master regulatory gene for the development of regulatory T cells.

A rare recessive monogenetic disorder called IPEX (immune dysregulation, polyendocrinopathy, including type 1 diabetes, enteropathy, and X-linked syndrome), is caused by a mutation in the *FOXP3* gene on human chromosome Xp11.23 [8]. Because of the pathologic role in the maintenance of the immune system and the variation in the balance between pro-inflammatory and anti-inflammatory cytokines, dysregulation of *FOXP3/Scurfin* gene expression may lead to the development of AID. Of interest, Bassuny *et al.* reported an association of a functional microsatellite polymorphism $(GT)_n$ of *FOXP3* gene with susceptibility to type 1 diabetes (T1D) in a Japanese population [9].

There is increasing evidence that AID share a common genetic risk factor, which is suggested by the familial aggregation of autoimmunity and also that the chromosomal region linkage to AID tends to overlap. Taking into account these findings, the aim of our study was to examine the possible influence of the functional $(GT)_n$ polymorphism in the *FOXP3* gene on genetic predisposition of a panel of autoimmune diseases, namely rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), and celiac disease.

PATIENTS AND METHODS

Patients

A total of 231 SLE patients, 293 RA patients, 528 IBD patients (354 Crohn's disease patients and 260 UC patients), 103 celiac disease patients, and 274 healthy controls included in this study were recruited from Hospital Virgen de las Nieves (Granada) and Hospital Clínico San Cecilio (Granada), Hospital Puerta del Mar (Cadiz), and Hospital Materno-Infantil (Granada). SLE and RA patients fulfilled the classification criteria of the American College of Rheumatology [10, 11]. Celiac disease patients were diagnosed following the European Society for Pediatric Gastroenterology and Nutrition criteria for celiac disease [12]. IBD patients were diagnosed according to standard clinical, endoscopic, radiologic, and histopathologic criteria [13]. All the subjects, cases and controls, were Caucasian Spanish, living in the same geographic area and were matched for age and sex. All study subjects were included in this study after written informed consent. We obtained approval for the study from all local ethical committees of the corresponding hospitals.

Genotyping

Genomic DNA was isolated from anticoagulant-treated peripheral blood mononuclear cells (PBMCs) using standard methods. Genotyping of the $(GT)_n$ microsatellite marker located between exon −1 and exon 1 of the *FOXP3* gene was performed by a polymerase chain reaction (PCR)-based method as previously described [9], using the following primers: forward, 5′-CAACCATTG-CCCTCATAGAGG-3′, and reverse, 5′-GGCGGTATG-AGATACTCGACCA-3′. The forward primer was 5′ labeled with the fluorescent dye 6-FAM and the lengths of the fragments were analyzed in an ABI PRISM 3100 Genetic Analyzer and using a Genescan 672 software (Applied Biosystems, Foster City, CA). To verify the repeat numbers of each allele we used direct sequencing using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Statistical Analyses

Allelic and genotypic frequencies of *FOXP3* polymorphism were obtained by direct counting. Statistical analysis to compare allelic and genotypic distributions was performed by chi-square test. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated according to Wolf's method. The software used was Statcalc program (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA). p Values were corrected for the number of alleles determined using the Bonferroni test, and p values below 0.05 was considered statistically significant.

RESULTS

The genotype and allele distribution frequencies of the $(GT)_n$ *FOXP3* microsatellite in AID patients and healthy controls is shown in Tables 1 and 2. In both patient and control groups, genotype and allele frequencies did not deviate significantly from those expected from Hardy-Weinberg equilibrium. We found seven different alleles comprising repeat and bp ranges $(GT)_{12}$–$(GT)_{18}$ and 255–267, respectively. The most frequent alleles were $(GT)_{15}$ (54.1%) and $(GT)_{16}$ (36.6%) in the control

TABLE 1 Genotype distribution of *FOXP3/Scurfin* $(GT)_n$ in female and male controls and SLE, RA, CD, UC, and celiac disease patients

Female	SLE (%) (n = 206)	RA (%) (n = 232)	CD (%) (n = 177)	UC (%) (n = 130)	Celiac disease (%) (n = 65)	Controls (%) (n = 147)
$(GT)_{14}/(GT)_{15}$	0	1 (0.4)	8 (4.6)[a]	2 (1.5)	0	1 (0.7)
$(GT)_{14}/(GT)_{16}$	0	0	2 (1.1)	0 (0)	0	0
$(GT)_{15}/(GT)_{15}$	62 (30.1)	72 (31)	45 (25.4)	34 (26.2)	18 (27.7)	44 (30)
$(GT)_{15}/(GT)_{16}$	94 (45.7)	108 (46.6)	74 (41.8)	50 (38.5)	29 (44.6)	62 (42.2)
$(GT)_{15}/(GT)_{17}$	9 (4.3)	8 (3.5)	16 (9)	6 (4.6)	5 (7.7)	8 (5.4)
$(GT)_{16}/(GT)_{16}$	29 (14)	33 (14.2)	14 (7.9)	30 (23.1)[b]	8 (12.3)	18 (12.2)
$(GT)_{16}/(GT)_{17}$	8 (3.9)	8 (3.5)	12 (6.8)	5 (3.8)	2 (3)	5 (3.4)
Others	4 (2)	2 (0.8)	6 (3.4)	3 (2.3)	3 (4.7)	9 (6.1)
Males	**SLE (%) (n = 25)**	**RA (%) (n = 61)**	**CD (%) (n = 107)**	**UC (%) (n = 114)**	**Celiac disease (%) (n = 38)**	**Controls (%) (n = 127)**
$(GT)_{14}$	0	0	2 (1.9)	2 (1.8)	0	0
$(GT)_{15}$	16 (64)	32 (52.5)	59 (55.1)	57 (50)	25 (65.7)	69 (54.3)
$(GT)_{16}$	7 (28)	25 (41)	42 (39.2)	50 (43.8)	11 (29)	47 (37)
$(GT)_{17}$	1 (4)	4 (6.5)	3 (2.8)	5 (4.4)	2 (5.3)	11 (8.7)
Others	1 (4)	0	1 (0.9)	0	0	0

Abbreviations: SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; CD = Crohns disease; UC = ulcerative colitis.

[a] p = 0.03, p corrected = NS.

[b] p = 0.01, p corrected = NS.

group. *FOXP3*genotypes and alleles with a frequency of ≤2% in patients or controls were jointly considered. The allele frequencies observed in our control population were in good agreement with allele frequencies found in other Caucasian South-European population [14]. However, these allele frequencies contrast significantly with those detected in the Japanese population (Table 3).

No statistically significant differences were observed between allele frequencies of SLE, RA, or celiac patients and controls. In addition, we found no association of this polymorphism and genotype frequencies in female and male patients with these AID. With regard to the IBD patients, the $(GT)_{16}/(GT)_{16}$ genotype was slightly increased in UC patients (23.1% versus 12.2% in the control group, p = 0.01, p_c = not significant, OR = 2.15, 95% CI = 1.08–4.29). We observed a statistically significant deviation in the distribution of the $(GT)_{14}$ allele among the female Crohn's patients (5.1% versus

TABLE 2 Distribution of allele frequencies of *FOXP3/Scurfin* $(GT)_n$ in female and male SLE, RA, CD, CU, celiac disease, and healthy controls

Alleles Female	Size (bp)	SLE (%) (n = 412)	RA (%) (n = 462)	CD (%) (n = 354)	UC (%) (n = 260)	Celiac Disease (%) (n = 130)	Controls (%) (n = 294)
$(GT)_{14}$	259	0	1 (0.2)	18 (5.1)[a]	2 (0.8)	0	4 (1.3)
$(GT)_{15}$	261	228 (55.3)	263 (57)	189 (53.4)	127 (48.8)	72 (55.4)	159 (54.1)
$(GT)_{16}$	263	162 (39.3)	182 (39.4)	116 (32.7)	115 (44.2)	47 (36.2)	107 (36.4)
$(GT)_{17}$	265	18 (4.4)	16 (3.4)	30 (8.5)	15 (5.8)	9 (6.9)	19 (6.5)
Others	Others	4 (1)	0	1 (0.3)	1 (0.4)	2 (1.5)	5 (1.7)
Males	**Size (bp)**	**SLE (%) (n = 25)**	**RA (%) (n = 61)**	**CD (%) (n = 107)**	**UC (%) (n = 114)**	**Celiac disease (%) (n = 38)**	**Controls (%) (n = 127)**
$(GT)_{14}$	259	0	0	2 (1.9)	2 (1.8)	0	0
$(GT)_{15}$	261	16 (64)	32 (52.5)	59 (55.1)	57 (50)	25 (65.7)	69 (54.3)
$(GT)_{16}$	263	7 (28)	25 (41)	42 (39.2)	50 (43.8)	11 (29)	47 (37)
$(GT)_{17}$	265	1 (4)	4 (6.5)	3 (2.8)	5 (4.4)	2 (5.3)	11 (8.7)
Others	Others	1 (4)	0	1 (0.9)	0	0	0

Abbreviations: SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; CD = Crohns disease; UC = ulcerative colitis.

[a] p = 0.009, p corrected = 0.05; OR = 3.88 (1.22–13.72).

TABLE 3 Comparative allele frequencies between Japanese and Spanish populations

Alleles	Japanese controls (%) (n = 472)	Spanish controls (%) (n = 421)	p Value	p_c
$(GT)_{12}$	0	3 (0.7)	0.03	ns
$(GT)_{13}$	0	2 (0.5)	ns	ns
$(GT)_{14}$	1 (0.2)	4 (1)	ns	ns
$(GT)_{15}$	154 (32.6)	228 (54.1)	>0.000001	>0.000001
$(GT)_{16}$	296 (62.8)	154 (36.6)	>0.000001	>0.000001
$(GT)_{17}$	11 (2.3)	30 (7.1)	0.002	0.01
$(GT)_{18}$	10 (2.1)	0	0.001	0.006

GT = genotyping; ns = not significant.

1.3% in the control group; p = 0.009, OR 3.88, 95% CI = 1.22–13.72) that turned out to be nonsignificant after applying the Bonferroni test (p_{corr} = 0.05).

DISCUSSION

There is increasing evidence that common genes may underlie autoimmunity [3, 15]. However, evidence that specific risk alleles are associated with multiple AID is relatively sparse. Genes within the MHC complex, and not only the HLA alleles, are associated with multiple autoimmunity diseases [16]. Recently there have also been examples showing that non-HLA complex genes play a role in the development of more than one autoimmune disease. Thus, *CTLA4* gene variants are associated with T1D, Graves' disease, and SLE [17, 18]. In addition, the *PTPN22* R620W polymorphism has been associated with a number of AID [19, 20]. This leads to the hypothesis of common predisposing genes to autoimmunity and consequently when a potential risk factor for an autoimmune disease is discovered, such as the *FOXP3* gene, investigating its involvement in different AID is of interest.

In the current case-control study, the possible association of RA, SLE, IBD, and celiac disease with variants in the *FOXP3* gene was investigated. The data reveal no association between the AID investigated with the *FOXP3* variant that was found to be associated with T1D in the Japanese population.

The possible reason for the lack of association for this polymorphism in our population may result from a *FOXP3/Scurfin* gene located on one of the T1D susceptible loci, Xp11.23, which is not a RA, SLE, IBD, or celiac disease susceptibility loci. However, the absence of linkage evidence in a particular chromosomal region does not mean that genes in that region do not contribute to disease risk. Our negative findings could be due to lack of power to detect a true association in these diseases, specially in Crohn's disease, in which we observed a trend of association for the GT14 allele. Therefore, genotyping in larger number of materials is warranted before drawing any definitive conclusions.

In accordance with our findings, no association was detected between T1D and *FOXP3* polymorphisms in an Italian population [14]. Zavattari *et al.* extensively characterized the *FOXP3* region, searching for genetic variants, and no evidence of association between T1D and the 12 *FOXP3* polymorphisms analyzed was observed. Differences in the ethnicities of the study population may account for the failure to replicate the T1D association with *FOXP3* in a Japanese population [9]. This possibility is consistent with the marked differences in *FOXP3* allele frequencies in the Japanese versus the white population. Alternatively, it is possible that the specific disease-relevant alleles for at least some susceptibility genes vary among different ethnic groups. Thus, our findings together with those of Zavattari *et al.* suggest that the *FOXP3* microsatellite is not a susceptibility allele for most common AID, at least in the Caucasian population. Further studies are warranted to assess the *FOXP3* variants' relevance to AID in the Japanese population.

With regard to the functional relevance of the *FOXP3* gene microsatellite polymorphism, the different microsatellite alleles appear to affect promoter activity of the *FOXP3* gene [9]. Nevertheless, caution should be exercised in extrapolating the results of previous functional assays, because the presence of other polymorphisms within intron zero or other distant genetic variants in strong linkage disequilibrium with *FOXP3* gene microsatellite might be those that are really affecting *FOXP3* gene expression.

In summary, we found no evidence of association between this genetic variant in *FOXP3* gene with AID patients in our Spanish population. Obviously, the participation of FOXP3 protein in autoimmunity is not brought into question by these data, and the existence of other *FOXP3* gene regulatory polymorphisms that might affect *FOXP3* expression and influence AID risk cannot be excluded.

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7.3. GENES DE LA RESPUESTA INMUNE INNATA EN LA EC

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Association of MICA-A5.1 Allele With Susceptibility to Celiac Disease in a Family Study

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OBJECTIVE: The aim of this study was to analyze the role of the major histocompatibility complex class I chain-related gene A (MICA) transmembrane polymorphism in celiac disease (CD) susceptibility.

METHODS: Sixty-one celiac Spanish families were genotyped for MICA transmembrane polymorphism by a polymerase chain reaction method combined with fluorescent technology. A transmission disequilibrium test was performed to investigate the preferential transmission of MICA alleles to the affected offspring.

RESULTS: The MICA A5.1 allele was shown to be significantly transmitted to the affected siblings. This association was independent of the CD-predisposing DQ2 haplotype. Additionally, we classified our celiac families into typical and atypical groups as we found a significant association with MICA A5.1 in typical celiac families. There was also an association tendency with atypical families.

CONCLUSIONS: Our data suggest that the MICA A5.1 allele is associated with CD development independently of DQ2-extended haplotype and clinical forms of CD. (Am J Gastroenterol 2003;98:359–362. © 2003 by Am. Coll. of Gastroenterology)

INTRODUCTION

Celiac disease (CD) is an enteropathic disorder in which genetically susceptible individuals do not tolerate gluten. The mucosal injury mediated by an autoimmune response is characterized by villous atrophy, crypts hyperplasia, and increase in intraepithelial lymphocytes (1). Human leukocyte antigen (HLA) genes play an important role in disease susceptibility. In this sense, DQA1*0501 and DQB1*0201 alleles (DQ2 molecule) are present in 90% of CD patients; however, this haplotype is also present in 20–30% of healthy controls, suggesting that disease susceptibility could be influenced by other genes (1).

The major histocompatibility complex class I chain-related gene A (MICA) could be an attractive candidate for CD susceptibly and for providing some light on the physiopathological mechanisms of CD. This is because MICA is mainly expressed on the GI epithelium and recognized by $\gamma\delta$ T-cells, which are located preferentially in intestinal lymphatic tissue and dramatically expanded in CD (2). The MICA gene is located 46 Kb upstream of HLA-B and encodes a stress-inducible molecule with three extracellular domains (α1, α2, α3), a transmembrane (TM) region, and a cytoplasmatic tail (3). Within the MICA TM region, a microsatellite polymorphism consisting of a trinucleotide repeat (guanine, cytosine, thymine [GCT]) has been described, located in MICA exon 5 and with six reported alleles. Five of them consist of a variable number of GCT repeats, which encode for 4, 5, 6, 9, or 10 alanine residues, respectively (4, 5). Additionally, the MICA A5.1 allele carries a nucleotide insertion (GCT→guanine, guanine, cytosine, thymine [GGCT]), resulting in a frameshift mutation that generates a premature stop codon in the TM region that may not produce a membrane-bound protein but a soluble form (6). Both the possible functional implication of the MICA A5.1 allele and the recently reported association with CD (7, 8) led us to explore the possible contribution of the MICA A5.1 allele to the susceptibility or development of CD in a family-based study.

MATERIALS AND METHODS

Individuals

The present study included 61 Spanish families characterized by the presence of an affected child with CD who were diagnosed following the European Society for Pediatric Gastroenterology and Nutrition criteria for CD (9). All family members were studied, but only data from affected siblings and their parents were used. CD families were recruited from Hospital Materno-Infantil and Hospital Clínico Universitario, Granada, Spain.

MICA Genotyping

DNA was extracted by standard methods from peripheral blood leukocytes. MICA TM microsatellite polymorphism

Table 1. Transmission Disequilibrium Test of the MICA Alleles in Spanish Celiac Families

Allele	Transmitted (%)	Not Transmitted (%)	*p*
179/A4	19 (56)	15 (44)	ns
182/A5	12 (57)	9 (43)	ns
183/A5.1	27 (68)	13 (32)	0.02
185/A6	18 (38)	29 (62)	ns
194/A9	8 (31)	18 (69)	0.05

Extended transmission disequilibrium test as a multi-allelic test for association gives $p = 0.04$.

was analyzed using the method described by Ota *et al.* (6). Polymerase chain reaction primers were MICA 5F 5′-CCT TTT TTT CAG GGA AAG TGC-3′ and MICA 5R 5′-CCT TAC CAT CTC CAG AAA CTG C-3′, which was labeled at the 5' end with 6-FAM. To determine the number of triplet repeats in the TM region, 0.5 μl of polymerase chain reaction aliquots were added to 3 μl of formamide and 0.5 μl of internal size standard. Samples were analyzed in denaturing gels (6% acrylamide/7 mol/L urea) and sized using a Genescan TM 672 software (Applied Biosystem, Nieuwekerk/Ijssel, The Netherlands).

HLA Genotyping

DRB1 and DQB1 typing were carried out by reverse hybridization with sequence-specific oligonucleotides (Inno-Lipa HLA-DQB and DRB, Innogenetics, Zwijndrecht, Belgium). DQA1 typing was deduced from DQB1 and DRB1 typing on the basis of the strong linkage disequilibrium among HLA class II alleles.

Statistical Analysis

The transmission disequilibrium test (TDT) and frequency of transmitted and not transmitted parental haplotypes to the patients were determined using the TDT-phase program as described (10). The test considers only heterozygous parents and assesses the evidence for preferential transmission of one allele over the other (11). As a test for association of MICA independent of HLA class II, we compared the transmission of DQ2 haplotypes carrying the MICA A5.1 allele with the transmission of DQ2 haplotypes not carrying the MICA A5.1 allele. Significance was calculated by a 2×2 contingency table Fisher's exact test. Conditional extended transmission disequilibrium test (CETDT) was used as an overall test for association independent of HLA class II (12).

RESULTS

Table 1 shows the transmission pattern of five different MICA alleles from heterozygous parents to CD patients. The overall transmission of MICA alleles reached statistical deviation ($p = 0.004$), and an increased transmission for the MICA A5.1 allele was observed (transmitted [T]: 61.2%, not transmitted [NT]: 38.7%, $p = 0.02$). Therefore, we investigated whether the MICA A5.1-CD association was independent or attributed to an HLA linkage. It is interesting to note, as depicted in Table 2, that the DQ2-A5.1+ haplotypes are significantly more transmitted than the DQ2-A* (A4, A5, A6, and A9) haplotypes (88% transmission *vs* 52% transmission, respectively, $p = 0.008$). In addition, it is worth noting that the CETDT reached a significant *p* value of 0.005 (data not shown), demonstrating that the influence of MICA A5.1 in CD may be independent of linkage disequilibrium with DQ2-extended haplotypes. Likewise, it is worth noting that within the five families in which one of the progenitors was homozygous for DQ2 and heterozygous for MICA A5.1, four of the five affected siblings inherited the haplotype carrying both the DQ2 and MICA A5.1 (data not shown). With regard to the MICA A9 allele, we observed that it was predominantly not transmitted to the affected siblings (T: 31.8%, NT: 68.2%, $p = 0.05$). Although the data were not statistically significant, a clear tendency could be seen, showing a possible protective role of the MICA A9 allele in CD.

Table 2. MICA A5.1 Allele Transmission Within DQ2 Haplotypes

Haplotype	Transmitted	Not Transmitted	%T
A5.1-DQ2	21	3	88
A*-DQ2	16	15	52

Fisher's exact test: P-heterogeneity = 0.008. T = transmission.

Additionally, we classified the celiac families according to the clinical features of the affected siblings (13) into typical (n = 49; patients with chronic diarrhea, failure to thrive, and abdominal distention) and atypical (n = 12; patients without GI symptoms and diagnosed by other symptoms secondary to malabsortion, such as short stature, anemia, etc, or independent to malabsortion, such as dermatitis herpetiformis, psoriasis, ataxia, etc). Table 3 shows the transmission pattern of MICA TM alleles in typical and atypical families; no significant difference was observed

Table 3. Transmission of the MICA Alleles Classifying Spanish Celiac Families Into Typical and Atypical

Atypical Celiac Families			
Allele	Transmitted (%)	Not Transmitted (%)	*p*
179/A4	4 (80)	1 (20)	ns
182/A5	1 (50)	1 (50)	ns
183/A5.1	6 (85.7)	1 (14.3)	0.167
185/A6	4 (33)	8 (77)	ns
194/A9	2 (25)	6 (75)	ns
Typical Celiac Families			
Allele	Transmitted (%)	Not Transmitted (%)	*p*
179/A4	15 (52)	14 (48)	ns
182/A5	11 (58)	8 (42)	ns
183/A5.1	21 (63.6)	12 (36.4)	<0.05
185/A6	14 (40)	21 (60)	ns
194/A9	6 (33)	12 (77)	ns

between the two groups. The association of MICA A5.1 with typical forms of the disease remains significant ($p < 0.05$) after grouping the families into typical and atypical. The effect of MICA A5.1 within typical forms of CD was again independent of the DQ2 predisposing haplotypes (data not shown). A similar trend was found in the atypical families; nevertheless, this association was not significant (Table 3).

DISCUSSION

Immunopathogenic mechanisms underlying the development of CD remain uncertain, but the implication of $\gamma\delta+$ T-cells has been broadly documented (14). MICA protein is mainly expressed in the GI epithelium and interacts with $\gamma\delta$ T-cells, CD8+ $\alpha\beta$ T-cells, and with natural killer (NK) cells expressing the NKG2D receptor (15). It has been observed that different MICA alleles are associated with weak or strong binding to the NKG2D receptor, MICA*008 being one of the weak binding alleles (16). MICA A5.1 is in linkage disequilibrium with MICA*008 (17). On this basis, it is tempting to hypothesize that the functional role of MICA A5.1 in CD could lie in an altered selection of the T-cell repertory caused by a weak binding of MICA-NKG2D T receptor in the thymus. On the other hand, it is worth noting that MICA A5.1 allele has very recently been localized on the apical side of enterocytes (18), whereas the rest of MICA alleles are found on the basolateral side. Therefore, another hypothesis of MICA A5.1 relevance could be the fact that the lack of signal for $\gamma\delta+$ T-cells will not exert their regulatory function on intestinal epithelium homeostasis.

To date the possible association between CD and MICA genes has only been investigated in case-control studies (7, 8), whereas here we have used a familial-based approach avoiding possible false-positive findings attributed to population stratification. Lopéz-Vázquez *et al.* found an association of the MICA A5.1 allele with atypical forms of CD (8). Nevertheless, if their patients are considered as only one group, the association with the A5.1 allele still remains evident. When we classified our celiac families into typical and atypical, we observed that the association with MICA A5.1 remains significant in typical celiac families, and a trend of association with atypical families was shown, but it was not significant maybe because of the low number of atypical families in our population. Therefore, our results are not altogether in agreement with those of Lopéz-Vázquez *et al.* (8), which did not show an association with typical CD forms. Meanwhile, in our population, MICA A5.1 is clearly associated with the disease within typical families.

The DQ2 heterodimer is the best described marker of susceptibility to CD, with 90% of patients carrying it, and the DQ2 molecule has been indicated to be functionally relevant in the abnormal response to gliadin (19). It is possible that the MICA A5.1 allele could be only an additive allele of susceptibility to disease development, within a conserved major histocompatibility complex haplotype that harbors a combination of genes conferring genetic risk to this autoimmune disease. The current association seen between MICA A5.1 and CD may be attributed to linkage disequilibrium with another gene in this region, which confers genetic risk to disease development.

In summary, our data suggest that the MICA A5.1 allele is associated with developed CD independently of both linkage disequilibrium with HLA-DQ genes and clinical forms of the disease. A larger study with a greater number of atypical families is necessary to better confirm the relationship between MICA A5.1 and CD forms and development.

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Brief communication

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A new allele within the transmembrane region of the human MICA gene with seven GCT repeats

Key words:

HLA; MICA gene; microsatellite; polymorphism

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Abstract: Major histocompatibility complex class I chain-related genes (MIC) belong to a multicopy gene family located within the HLA class I region of chromosome 6. They encode for proteins that have a completely different organization, expression, and products from classical HLA class I gene products. One member of this family is the MICA gene, which is characterized by its high degree of polymorphism, with over 50 MICA alleles described. Moreover, MICA exon 5 presents a microsatellite polymorphism consisting of a variable number of GCT repeats that encode for 4, 5, 6, 9, or 10 alanine residues, and a variant (MICA A5.1) that includes a nucleotide insertion (GCT→GGCT). In this study, we report a novel allele in the transmembrane region of the MICA gene consisting of seven GCT repeats found in a family based study of MICA polymorphism in celiac disease.

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Major histocompatibility complex class I chain-related A (MICA) genes belong to a multicopy gene family located within the HLA class I region in chromosome 6 (1). Seven MIC genes have been described to date; MICC to MICG are pseudogenes, whereas MICA and MICB are functional genes (2). They encode for proteins that have considerable different organization, expression and products from classical HLA class I genes: they do not bind β2 microglobulin or peptide ligands (3), are mainly expressed by epithelial cells following stress (4) and they bind with the NKG2D receptor present in natural killer cells, $\gamma\delta^+$ T cells and $\alpha\beta$ $CD8^+$ T cells (5). The MICA gene lies 40 Kb centromeric to HLA-B and is structured in six exons that encode, respectively, for leader peptide, the extracellular domains α1, α2 and α3, a transmembrane region (TM) and a cytoplasmatic tail (6). As with classical MHC I genes, MICA is characterized by its high degree of polymorphism, with over 50 MICA alleles described, which are mainly localized in extracellular domains (exons 2, 3 and 4) (7). Moreover, MICA exon 5 presents a microsatellite polymorphism consisting in a variable number of GCT repeats that

encode for 4, 5, 6, 9, or 10 alanine residues, and a variant that includes a nucleotide insertion (GCT→GGCT), MICA A5.1 allele, resulting in a frameshift mutation that generates a premature stop codon thought to produce a soluble form of the protein (8, 9). In the present work, we report a novel microsatellite repetition in the MICA gene TM region that codes for seven alanines, and was found in a family based study of MICA polymorphism in celiac disease (10).

A total of 61 Spanish celiac families were analyzed for MICA TM microsatellite polymorphism using the method described by Mizuki and coworkers (11). The PCR primers were MICA 5F 5′-CCT TTT TTT CAG GGA AAG TGC-3′ and MICA 5R 5′-CCT TAC CAT CTC CAG AAA CTG C-3′ which was labeled at the 5′end with 6-FAM. To determine the number of triplet repeats in the TM region, 0.5 μL PCR aliquots were added to 3 μL of formamide and 0.5 μL of internal size standard. Samples were analyzed in denaturing gels (6% acrylamide/7 M urea) and sized using Genescan TM 672 software (Applied Biosystems, Nieuwekerk/Ijssel, the Netherlands). During the process of sizing, we observed a heterozygous mother who presented with one peak corresponding to the MICA A9 allele and another with a novel mobility value of 188 that did not correspond to any of the MICA A alleles described to date (see Fig. 1). We suspected that she might bear a new MICA A allele, and so we proceeded to clone and sequence the MICA genes from this sample.

A PCR was carried out to amplify exons 2–5 of the MICA gene using the primers previously described (12). PCR conditions were 94°C for 2 min; 94°C for 1 min; 63°C for 30 s; 68°C for 5 min and 72°C for 7 min, for 35 cycles. The PCR fragment was then cloned using the TOPO-XL PCR cloning kit (Invitrogen, the Netherlands) according to the manufacturer's instructions. The nucleotide sequence of exons 2–5 was determined on both strands using the M13 F primer, three internal primers MICA Ia (5′CCT CTC CCA AAA CGT GGA-3′; residues 7573–7591), MICA Ib (5′CAG AGA CAG GGA CCT GT-3′), MICA Ic (5′TCA GGG CCA GGG CTG CCC-3′) and M13R primer. A new polymorphism in exon 5, consisting of seven GCT repeats, (Fig. 2) was detected, confirming the results obtained by the Genescan TM 672 software. Following the previously described nomenclature, we named this allele 'A7' (GenBank accession number AY095537). This new MICA 'A7' allele is identical to the MICA*002 allele except for the number of GCT repeats. The MICA*002 allele has been strong associated with the MICA A9 allele (13), however, the recently described MICA A10 allele was also associated with the MICA*002 allele (9). Notably, the new MICA 'A7' reported in this study is also associated with the MICA*002

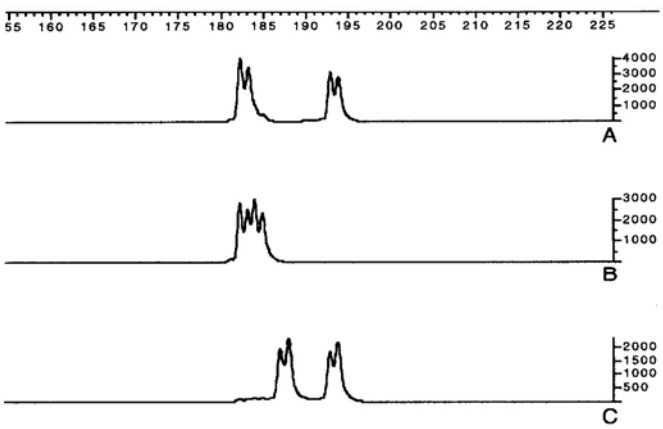

Fig. 1. Novel mobility found by *Genescan* in MICA exon 5. A: sample from affected sib 182/A4-194/A9 (HLA: DR3/DR4; DQB10201/0301). B: father 182/A4-183/A5.1 (HLA: DR1/DR3; DQB10201/0501). C: mother with new peak mobility of 188 corresponding to MICA A7 new allele, and mobility of 194/A9 (HLA: DR4/DR7; DQB10201/0301).

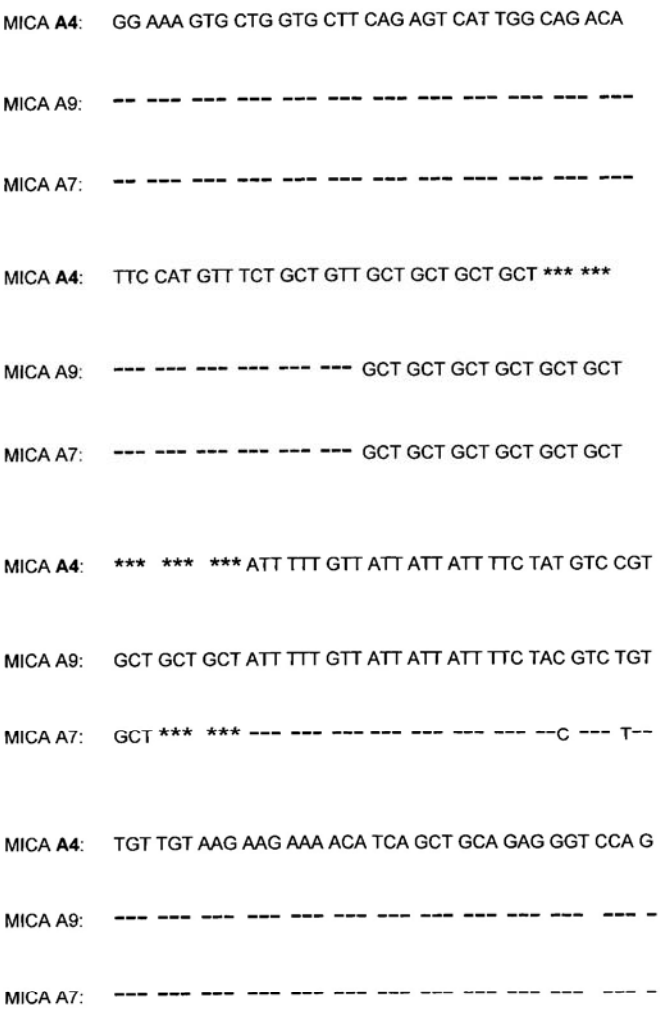

Fig. 2. Sequence of MICA exon 5 with new allele.

allele, and it therefore seems that the MICA*002 alleles always carry a long GCT repeat. It is also possible that the MICA*002-A9 allele was an ancestor of slippage mutations between MICA*002-A10 and the new MICA*002-A7. Further studies will be needed in order to elucidate if this preliminary observation has any functional significance.

MICA genes are mainly expressed in the gastrointestinal epithelium and interact with the γδ T cells, $CD8^+$ αβ T and with natural killer cells (NK) expressing the NKG2D receptor (14). It has been observed that different MICA alleles are associated with weak or strong binding to the NKG2D receptor (15) and that transmembrane MICA alleles are in linkage disequilibrium with those MICA alleles that code for the extracellular domains (13). On this basis, one functional role of the MICA gene in the disease may lie with an altered selection of the T cell repertory caused by weak binding of the MICA-NKG2D T receptor in the thymus.

Very recently, the cellular localization of some of the MICA molecules have been described, for example, those that bear 4, 5, 6, 9 or 10 alanines in the TM region are localized in the basolateral side of the enterocytes, whereas MICA A5.1 is localized in the apical side (16). Another hypothesis of the MICA allele's relevance could be related to changes in signaling for γδ+ T cells that then vary their regulatory function on epithelium homeostasis. Given the current interest for the functional implications of the MICA gene and disease associations, more studies are needed to clarify the role of MICA polymorphism, allele frequencies, and the exact function of the MICA molecules.

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Association study of functional genetic variants of innate immunity related genes in celiac disease

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Abstract

Background: Recent evidence suggest that the innate immune system is implicated in the early events of celiac disease (CD) pathogenesis. In this work for the first time we have assessed the relevance of different proinflammatory mediators typically related to innate immunity in CD predisposition.

Methods: We performed a familial study in which 105 celiac families characterized by the presence of an affected child with CD were genotyped for functional polymorphisms located at regulatory regions of *IL-1α*, *IL-1β*, *IL-1RN*, *IL-18*, *RANTES* and *MCP-1* genes. Familial data was analysed with a transmission disequilibrium test (TDT) that revealed no statistically significant differences in the transmission pattern of the different genetic markers considered.

Results: The TDT analysis for *IL-1α*, *IL-1β*, *IL-1RN*, *IL-18*, and *MCP-1* genes genetic variants did not reveal biased transmission to the affected offspring. Only a borderline association of *RANTES* promoter genetic variants with CD predisposition was observed.

Conclusion: Our results suggest that the analysed polymorphisms of *IL-1α*, *IL-1β*, *IL-1RN*, *IL-18*, *RANTES* and *MCP-1* genes do not seem to play a major role in CD genetic predisposition in our population.

Background

Celiac disease (CD) is an autoimmune disorder of the small intestine in which dietary gluten ingestion leads to a chronic inflammatory status of the mucosa [1]. There is strong evidence for a genetic component for CD, with the HLA genes being the strongest genetic locus associated with CD predisposition known to date. About 95% of CD patients are carriers of the DQ2 molecule, encoded by DQA1*05/DQB1*02 alleles, compared to ~10% of healthy control subjects. Furthermore, the DQ8 molecule (DQA1*0301/DQB1*0302 is also found more frequently in CD patients although to a lesser extent [2]. Finally, a role for genes located outside the HLA region has been suggested since the overall contribution of HLA genes to CD genetic predisposition is no more than 40% [1].

T CD4+ lymphocytes are key elements in the induction and progression of CD pathogenesis. Certain gluten peptides bound to DQ2 or DQ8 molecules cause proliferation and production of proinflammatory cytokines by lamina propria CD4 +T cells [3]. Besides this activation of adaptive immune response, recent evidences suggest that

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there is an implication of the innate immunity in the initial phases of CD [4]. In this regard, some gluten peptides have been demonstrated to drive a danger signal that leads to an activation of the innate immune system [5,6] and additionally it is thought that bacteria may play a role in CD [7]. In fact, CD patients show an up-regulation in the expression of pro-inflammatory cytokines typically related to the innate immune response, such us IL1, IL-18 and chemokines [6,8-10].

The IL1 gene cluster located in the chromosomal region 2q12-22 codifies for three proteins: IL-1α, IL-1β and IL-1 receptor agonist (IL-1RN), of which the two first are strong inducers of inflammation while IL-1RN is an effective antagonist binding to the IL-1 receptor without activating the target cell [11]. These genes are polymorphic bearing well-characterized single nucleotide polymorphisms (SNPs). Polymorphisms in IL-1α at position -889 C/T (rs1800587) and IL-1β at position -511 C/T (rs1143627) were described [12,13]. Furthermore, recent findings showed that the -511 C/T IL-1β genetic variant is related to differences in IL-1β protein secretion [14]. The *IL-1RN* gene contains within its second intron a variable number of an 86-bp tandem repeats (rs380092) [15], showing the allele 2 (IL-1RN*2; two repeats) an increased frequency in a variety of autoimmune and inflammatory disorders [16].

Another important member of the proinflammatory IL-1 family is IL-18, which is thought to be a key regulator of cytokine expression [17]. Furthermore, a role for IL-18 in the induction of an anti-gluten inflammatory response has been suggested [10,18,19]. It is thought that IL-18 gene variation in the promoter region regulates the expression of this cytokine [20]. Interestingly, in the IL-18 promoter region two SNPs -607 A/C (rs1946518) and -137 G/C (rs187238) were described, which are supposed to alter the IL-18 promoter activity [21].

Moreover, raised levels of chemokines such us RANTES (regulated upon activation, normal T-cells expressed and secreted) and monocyte chemoatractant protein-1 (MCP-1) have been observed in the primary immune response to gluten in CD patients [6,8]. Interestingly, genetic variants within regulatory regions that can affect trancription and protein production levels, *RANTES* -403 G/A (rs2107538) and -28 G/C (rs2280788) and *MCP-1* -2518 G/A (rs1024611) SNPs, were described [22-24].

Taking into consideration these findings, in this work we aimed to investigate the possible implication of *IL-1α, IL-1β, IL-1RN, IL-18, RANTES* and *MCP-1* functional polymorphisms in CD susceptibility.

Methods

Patients

In the present work we have analysed a panel of 105 celiac families characterised by the presence of an affected child with CD. The study participants were recruited at "Hospital Materno-Infantil" and "Hospital Clinico Universitario", Granda, (Spain) and were of Spanish Caucasian origin. All patients were diagnosed following the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) criteria for CD [25]. Their age at study was 7.1 ± 3.9 years and the mean age for disease diagnosis was 2.7 ± 2.72. A 60% were women and 40 % men, showing an anthropometry at diagnosis (weight and height) of P 3–100 percentile. The mean age of gluten introduction was 6.4 ± 1.5 months. Typical symptoms were observed in 72.2 % of patients and 27.8 % showed atypical symptoms. All family members were genotyped in DRB1 and DQB1. DQA1 typing was deduced from DQB1 and DRB1 typing on the basis of the strong linkage disequilibrium among HLA class II alleles.

Genotyping

DNA from patients and controls was obtained from peripheral blood using standard methods. For all of the considered SNPs, except *IL-1RN* and *RANTES* -403, samples were genotyped using a Taqman 5' allelic discrimination assay. Table 1 shows the Taqman MGB probes sequences used for each polymorphism provided by the Custom-Taqman-SNP-Genotyping-Assay (Applied Biosystems, Foster City, CA, USA). PCR reaction was carried in a total reaction volume of 5 μl with the following amplification protocol: denaturation at 92°C for 10 min, followed by 50 cycles of denaturation at 92°C for 15 sec and annealing and extension at 58°C for 1 min. Post-PCR, the genotype of each sample was attributed automatically by measuring the allelic specific fluorescence on the ABI PRIM 7900 Sequence Detection Systems using the SDS 2.2.1 software for allelic discrimination (Applied Biosystems, Foster City, CA, USA). *RANTES* -403 genotyping was performed using a TaqMan SNP-Genotyping-Assay (part number: C__15874407_10, Applied Biosystems, Foster City, CA, USA).

The *IL-1RN* polymorphism was genotyped by PCR as previously described [26]. Briefly, we used a froward primer 5'- CTC AGC AAC ACCT CCT AT and reverse primer 5'- TCC TGG TCT GCA GGT AA, two amplify five possible alleles with different PCR fragment size: 410 bp (allele 1: 4 repeats), 240 bp (allele 2: two repeats), 325 bp (allele 3: 3 repeats), 500 bp (allele 4: 5 repeats), and 595 bp (allele 5: 6 repeats).

Statistical analysis

We used the UNPHASED software created for TDT and case-control analysis [27]. We performed a Transmission

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Table 1: Taqman probes used for cytokine genotyping

Polymorphism	Taqman probe sequence
IL-1A -889 C/T (rs1800587)	VIC – CCTTCAATGGTGTTGCC FAM – CCTTCAATGATGTTGCC
IL-1B -551 C/T (rs1143627)	VIC – CTGTTTTTATGGCTTTCA FAM – CTGTTTTTATAGCTTTCA
IL-18 -607 C/A (rs1946518)	VIC – ATCATTAGAATTTTATGTAATAAT FAM – ATCATTAGAATTTTATTTAATAAT
IL-18 -137 G/C (rs187238)	VIC – ACTATTTTCATGAAATCTTTTCT FAM – TTTTCATGAAATGTTTTCT
RANTES -28 G/C (rs2280788)	VIC – CCCCTCAACTGGC FAM – CCCCTGAACTGGC
MCP-1 -2518 G/A (rs1024611)	VIC – CAGACAGCTGTCACTTT FAM – CAGACAGCTATCACTTT

Table 2: Allelic frequencies and percentage of transmission of *IL-1α*, *IL-1β*, *IL-1RN*, *IL-18*, *RANTES* and *MCP-1* genetic variants in CD families.

	Alelle frequency in parents (%)	*T:NT*	% *T*	*P*
IL-1A -551				
C	70.4	111:125	47	0.08
T	29.6	52:38	58	0.08
IL-1B -889				
T	67.6	110:111	49.7	NS
C	32.4	47:46	50.5	NS
***IL-1RN*VNTR**				
1	71.6	107:107	50	NS
2	26.8	43:42	50.6	NS
3	1.6	2:2	50	NS
IL-18 -607				
C	61.7	102:103	49.7	NS
A	38.3	59:58	50.4	NS
IL-18 -137				
G	75.9	124:124	50	NS
C	24.1	47:47	50	NS
RANTES -28				
C	84.1	155:148	51	0.04
G	15.9	3:10	23	0.04
RANTES -403				
G	85.7	141:128	52	0.06
A	14.3	23:36	39	0.06
MCP-1				
A	72.8	117:129	47	NS
G	27.2	48:36	57	NS

***T* = transmitted, *NT* = not transmitted %*T* = percentage transmitted**

Disequilibrium Test (TDT), which assesses allele transmission rates in simplex families and tests for deviation from expected 50% transmission. For the haplotype analysis, pair-wise linkage disequilibrium measures were investigated and haplotypes constructed using the expectation-maximization (EM) algorithm implemented in UNPHASED software. The power of the study to detect an effect of a polymorphism in disease susceptibility was estimated using the Quanto v 0.5 software (Department of

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Table 3: Transmission pattern of haplotypes inferred for IL-18 and RANTES promoter genetic variant in CD families

Gene	Haplotype	Transmitted (%)	No transmitted (%)	*P*
Il-18	-607A/-137G	37 (24)	35 (22.5)	NS
	-607A/-137C	19 (12.2)	20 (13)	NS
	-607C/-137G	99 (63.8)	100 (64.5)	NS
RANTES	-403G/-28A	3 (1.4)	8 (5.2)	NS
	-403A/-28C	19 (12.3)	26 (16.8)	NS
	-403G/-28C	132 (85.7)	119 (81.5)	0.05

Preventive Medicine University of Southern California, California, USA) [28].

Results

IL1 gene cluster

The transmission pattern for *IL-1α* -889, *IL-1β* -511 and *IL-1RN* VNTR polymorphisms is shown in table 2. When transmission of these genetic variants was analysed, none of the alleles showed statistically significant skewing. IL-1α -889 T allele was slightly more transmitted to the affected children (58% transmission for allele T vs 47% for allele C), however the *p* value failed to reach statistically significant level (Table 2). With regard to *IL-1RN* we observed that alleles IL-1RN*1 and IL-1RN*2 were the most frequent in our population (71.6% and 26.8% respectively), accordingly with previously studies in Caucasian populations [26].

IL18 gene

The TDT analysis for -607 A/C and -137 G/C *IL-18* promoter genetic variants did not reveal biased transmission of any of the alleles to the affected offspring (Table 2).

The haplotype estimation for the -607 A/C and -137 *IL-18* promoter variants revealed complete linkage disequilibrium between the two variants (D' = 1). We observed three out of the four possible haplotypic combinations in CD families (Table 3). The transmission pattern of *IL-18* promoter haplotypes did not show any statistically significant skewing (Table 3).

MCP-1 and RANTES

After analyzing the *MCP-1* -2518 G/A alleles transmission we observed that none of the alleles was preferentially transmitted from heterozygous parents to the affected offspring (Table 2). Regarding to the *RANTES* promoter genetic variants the mutant alleles -403 A and -28 G showed an overall allele frequency similar to that expected for Caucasian populations (84.1% and 96.5% respectively in our population) [29,30]. The transmission of both -403G/A and -28 C/G SNPs showed a slightly deviation from the 50% expected transmission pattern (Table 2). Alleles -403 G and -28 C were more transmitted to the affected offspring with borderline significance (P = 0.04 and P = 0.06 respectively) (Table 2). In addition, we estimated haplotypes for both genetic variants. Three out of the four haplotypic combinations were observed, being the -403G/-28C and -403A/-28C haplotypes the most common in CD families. No significant distorted transmission pattern for *RANTES* promoter haplotypes was observed (Table 3).

Discussion

CD is considered a model for autoimmune disorders since many of the components that generate the altered immune response to gluten have been well characterized [1]. However, there are some relevant events of CD pathogenesis that remain unclear, for instance the stimuli that drives the high IFNγ levels in the small intestine of CD patients and why only one out of 20–30 DQ2-positive individuals develops CD [3]. An explanation for these questions might be provided from recent studies that point out a role for the innate immunity in CD [4]. This finding supports a novel focus of research in CD molecular and genetic basis, opening a new field for the functional search of CD candidate genes.

In this work, for the first time we have assessed the relevance of *IL-1α, IL-1β, IL-1RN, IL-18, RANTES* and *MCP-1* genes in CD predisposition. All these genes have been previously associated with susceptibility to several autoimmune disorders [31-40]. However, we failed to detect an association of *IL-1α, IL-1β, IL-1RN, IL-18*, and *MCP-1* genes with CD predisposition using a TDT analysis in our cohort of 105 simplex CD families. Only a borderline significant association of *RANTES* promoter genetic variants with CD predisposition was observed.

Several studies have focused on the role of *RANTES* -403G/A and -28 G/C promoter polymorphisms in susceptibility to different autoimmune disorders. The *RANTES* -403A allele has been associated with susceptibility to multiple sclerosis (MS) and polymialgia rheumatica [41,42]. On the other hand, the *RANTES* -28G allele was observed

to be a genetic risk for clinical complications such us diabetic neprhopathy, early onset of MS, lower levels of C3 in SLE, and higher incidence of central nervous system lupus [37,38,41]. Both *RANTES* -403A and -28G alleles were associated with higher RANTES expression levels [22,23]. However, considering the multiple testing of the 6 different genes of our study, the association observed for *RANTES* promoter variants in our population can not be considered as being significant. Therefore, our results of *RANTES* suggest that further studies should be performed to clarify the role of *RANTES* in CD and autoimmune diseases in general.

Using a familial approach we eliminate the risk of population stratification derived from case-control association studies. In addition, we estimated that our study design would have considerable power to detect the effect of a polymorphism with moderate to high risk for CD. Assuming an additive model, a minor allele frequency of 0.30 (corresponding to a median value of the majority of markers considered) and RR of 1.8 we would reach 81% power to detect an association in our population. Nevertheless, under a dominant model the power drops to 49% and considering a lower disease allele frequency, for instance 0.16 as is the case of *RANTES* -28, our study power would decrease to a 64% for a RR of 1.8, and increases to 82% when we assume a RR of 2.0. For this reason, the low level of significance that our TDT analysis reached for *RANTES* promoter genetic variants might well reflect a true positive, and therefore needs further confirmation using a larger group of CD families.

Taking into account our findings, it is suggested that the analysed genetic polymorphisms of *IL-1α, IL1-β, IL-1RN, IL-18, RANTES* and *MCP-1* genes seem not to play a major role in CD susceptibility in our population. It might be possible that the release of these cytokines and chemokines observed in CD patients could be derived from the activity of other innate immunity related pro-inflammatory mediators with higher influence in disease pathogenesis. In this regard, it is known that in CD the cytokine expression pattern in response to gluten is strongly dominated by IFNγ [43]. Of note, in a recent work we assessed the influence of a functional dinucleotide polymorphism of IFNγ gene in CD predisposition. An association of a higher IFNγ producer allele with CD was observed, supporting a possible explanation for the high levels of INFγ observed in intestinal mucosa of CD patients [44].

Other proinflammatory mediators related with innate immunity such as, TNF-α and IL-12, has been analysed with respect to CD susceptibility. In accordance with our findings no evidence of association was found between IL-12 and CD in two independent studies [45,46]. Regarding TNF-α it has been difficult to dissect the relevance of this genetic marker in CD since it maps within HLA clas III region and it shows linkage disequilibrium with CD disease predisposing DQ2 alleles. In fact controversial results have been obtained, and there is no consensus about an independent or due to linkage disequilibrium role of TNF-α in CD susceptibility [47,48].

Conclusion

Our results suggest that *IL-1α, IL1-β, IL-1RN, IL-18, RANTES* and *MCP-1* genetic variants do not play a major role in CD genetic predisposition, although the suggestive evidence for *RANTES* deserves further investigation. Furthermore, we consider the innate response an intriguing focus of research and it should be of interest to investigate the role of other cytokines up-regulated in the early events of CD.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

B.R., carried out the genotyping and statistical analysis and drafted the manuscript.

A.Z., participated in the genotyping and helped in the use of the ABI PRIM 7900 Sequence Detection Systems and SDS 2.2.1 software.

M.A. L-N., collected the samples and revised the manuscript.

J.M., participated in the manuscript design and coordination and helped to draft the manuscript

B.K., reviewed the statistical analysis and helped to draft the manuscript.

All authors read and approved the final manuscript.

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