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Epstein-Barr virus and oral squamous cell carcinoma in patients without HIV infection: viral detection by polymerase chain reaction

Miguel González-Moles[1], José Gutiérrez[2]* Isabel Ruiz[3], Juan A. Fernández[1], M. Rodriguez[2] and José Aneiros[3]

[1]Department of Stomatology, [2]Department of Microbiology and [3]Department of Pathology, University Hospital San Cecilio, University of Granada, Granada, Spain (*Reprint address)

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Abstract

In order to test the hypothesis that Epstein-Barr virus (EBV) may be a cofactor for oral squamous cell carcinoma (OSCC) the authors evaluated tumour cells from OSCC of 108 patients without HIV infection, for the presence of EBV DNA by polymerase chain reaction. The sequences of oligonucleotides used in the amplification and hybridization included a set for the DNA polymerase region. The amplification was detected using an ELISA assay with peroxidase. EBV DNA was detected in 17.59% of the tumours. Inhibition studies showed that the ability to detect EBV DNA was not affected by the pathological material, suggesting that the negative PCR results in these samples were not caused by PCR inhibitors in the biopsy. Results revealed that 63.1% of the tumours (12 cases) were DNA positive affecting the lateral margin of the tongue, and were statistically significant ($p < 0.001$; χ^2). In the pool of tumours with EBV DNA only 26.3% (5 of 19 cases) were well differentiated OSCCs whereas the remaining 73.7% (14 of 19 cases) were moderately and poorly differentiated OSCCs, with a statistical significance of $p = 0.08$; χ^2. This study suggests a relationship between OSCC and EBV.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous herpesvirus which persistently infects most people by adulthood. Most primary infections are asymptomatic, particularly if they occur in early childhood, and the virus then becomes latent in B lymphocytes and epithelial cells of the oropharynx, salivary glands, and cervix. Although EBV is intermittently reactivated, chronic infection is not usually clinically apparent. Exceptionally, EBV causes several neoplastic and non-neoplastic disease syndromes. These include African Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), gastric carcinoma, infectious mononucleosis, lymphocytic interstitial pneumonia in human immunodeficiency virus (HIV) infected children, and oral hairy leukoplakia (OHL) in immunodeficient patients, particularly those infected with HIV (Baskin *et al.*, 1995).

There is a high incidence of undifferentiated nasopharyngeal carcinoma in certain populations which appear to have a causal association with EBV. Moreover, the EBV infection leads in most cases to a chronic carrier state in the epithelial cells (Hording *et al.*,

1994). It is possible that EBV may have a promotive effect during the early stage just after the malignant conversion of epithelial cells and so increases the proliferative rate of carcinoma cells (Zong, 1993). Thus, EBV could be considered a possible candidate for participation in the process of carcinogenesis for two reasons: (1) oral squamous epithelium can potentially support EBV persistence, as exemplified by OHL; and (2) NPC-like squamous cell carcinoma, harbours EBV and viral transcript.

The purpose of the present work was to investigate the presence of EBV by polymerase chain reaction (PCR) in formalin-fixed, paraffin-embedded specimens from oral squamous cell carcinoma (OSCC).

Materials and methods

Patients

HIV-free patients (108 *in toto*) with OSCC were included in this study. The patients were 92 males and 16 females, whose ages ranged from 40 to 85 years (mean 62.4 years). The oral locations of the tumours are shown in Table 1. The patients were selected and identified from the records of the Pathology Department at the University Medical Centre St Cecil (Granada, Spain). All the patients were diagnosed before 1992 and revised in September 1996. Neoplasms were selected so that most categories of neoplasia were included, but the selection was not intended to represent the actual frequency of each category. The clinical study included age, sex, tumoral location, tumoral size, invasion of cervical nodes, distant metastases, TNM stage (pathological system of classification of tumours, nodes and metastases) at the moment of diagnosis and the present state of the patients (dead or alive).

All tumours were evaluated for the following pathological parameters using haematoxylin and eosin staining of formalin-fixed, paraffin-embedded tissue sections: tumoral differentiation (well differentiated, moderately well differentiated and poorly differentiated squamous cell carcinoma), keratin production (minimum, moderate, maximum), cytological grade (grade 1 defined as minimal nuclear abnormalities; grade 2 defined as moderate nuclear abnormalities and grade 3 as maximal nuclear abnormalities), mitotic count (minimum; 0–1 mitosis/8/fields/40x; moderate, 2–5 mitoses/8/fields/40x; and maximum, >5 mitoses/8/fields/40x), inflammatory infiltrate intensity (minimum, moderate, maximum) and type of tumoral growth (solid masses, cords, small groups and dissociated tumoral cells). The histopathological findings are summarized in Table 3.

Epstein-Barr virus PCR

A commercially available PCR (Herplex^R, Pharmagen) was used, and 108 samples were tested. The assay was chosen because of its high level of sensitivity (Tenorio *et al.*, 1993). Formalin-fixed paraffin-embedded sections were studied using standard methods for PCR. Genomic DNA was extracted from tissue blocks by digestion with proteinase K (3 h at 56°C) and Tween 20, which was inactivated by heating at 96°C for 10 min, and 10 min ultracentrifugation (Maniatis *et al.*, 1982). Five µl were then used for PCR. A cell suspension of the EBV-containing P3HR1 cell line was used as positive control. All the oligonucleotides used for PCR amplification in this study were synthesized by the solid-phase triester method. The sequences of oligonucleotides used in the amplification and hybridization of EBV have been previously reported (Tenorio *et al.*, 1993) and included a set for the DNA polymerase region.

Table 1 Tumoral location

Site	Number of tumours (%)	Number of tumours with EBV DNA
Base of the tongue	10 (9.25)	2 (10.52)
Lateral tongue	13 (12.03)	5 (26.32)
Floor of mouth	4 (3.70)	0
Buccal mucosa	4 (3.70)	0
Upper lip	3 (2.77)	0
Lower lip	44 (40.74)	4 (21.05)
Gingiva	7 (6.48)	0
Fauces	5 (4.63)	1 (5.26)
Hard palate	2 (1.85)	1 (5.26)
Angle of the mouth	3 (2.77)	0
Floor of the mouth and lateral tongue	5 (4.62)	3 (5.26)
Floor of the mouth, lateral tongue and fauces	7 (6.48)	3 (5.25)
Alveolar mucosa and hard palate	1 (0.92)	0
Total	108 (100)	19 (100)

The PCR (Tenorio *et al.*, 1993) and hybridization (Navarrete *et al.*, 1994) of EBV were performed as previously described (40 cycles of denaturalization at 96°C for 30 s; hybridization at 52°C for 1 min; extension at 72°C for 30 s, and a final extension at 72°C for 10 min). The amplicon was detected with an ELISA assay with peroxidase which has the specific probe for EBV. A positive result was considered when the absorbance of the sample was double the absorbance of the negative control. The assay had an internal positive control of amplification to detect the presence of inhibition. To avoid false-positive amplifications, procedures recommended for preventing contaminations were strictly observed (Kwok and Higuchi, 1989). In addition, all tubes, pipette tips, and reagents, except for primers and Taq polymerase, were exposed to 254 nm of UV light in a nucleic acid linker oven (Stratalinker UV Crosslinker, Stratagene) before use.

The positive samples were tested again with the assay to detect the presence of EBV DNA and the mean value of absorbance was used, provided the deviation did not exceed 10%, to ensure the accuracy of the results. If it exceeded 10% the test was repeated. In order to further improve the clinical sensitivity of the assay, in the negative results DNA was purified (DNA Purification kit, Pharmagen), and concentrated by ultrafiltration from 200 µl aliquots of the samples and the work was repeated. Statistical analysis of data was carried out using the χ^2 test.

Results

EBV DNA was detected in 17.59% of the tumours examined (19 out of 108). Inhibition studies showed that the ability to detect EBV DNA was not affected by the pathological material, suggesting that the negative PCR results in these samples were not caused by PCR inhibitors in the biopsy. No increase in the clinical sensitivity of the PCR was found after purification and concentration of the samples.

Results of clinical and histopathological studies are shown in Tables 2 and 3, respectively. We found 63.1% of the *tumours (12* cases) with EBV DNA affecting the lateral *margin of the tongue, and* this result *was statistically significant for the* *located in the*

tumours with EBV DNA
were well-differentiated OSCCs
% (14 of 19 cases) were moderately and
dated OSCCs, with a statistical significance of $p = 0.08$.

Table 2 Clinical finding in EBV positive tumours

Case	Age (yr)	Sex	Location	Size (cm)	N	M	Clinical TNM stage	Date of diagnosis	Actual situation Sept 1996
1	78	♂	Lower lip	1.5	No	No	T1 N0 M0	Jun 1988	D
2	78	♂	Lower lip	1.0	No	No	T1 N0 M0	Feb 1987	A
3	58	♂	Fauces	3.5	Yes	No	T3 N1 M0	Aug 1991	A
4	64	♂	Fauces	4.0	No	No	T4 N0 M0	Mar 1991	D
5	81	♀	Lateral tongue	2.5	Yes	No	T2 N1 M0	Aug 1989	D
6	48	♂	Floor of mouth and lateral tongue	4.0	No	No	T4 N0 M0	Feb 1991	D
7	59	♂	Floor of mouth, lateral tongue and fauces	5.0	Yes	No	T4 N1 M0	Jan 1991	D
8	64	♀	Lateral tongue	1.5	No	No	T1 N0 M0	May 1991	A
9	70	♀	Hard palate	4.0	No	No	T3 N0 M0	Apr 1991	A
10	85	♀	Lower lip	1.5	Yes	No	T1 N0 M0	Jan 1990	D
11	45	♂	Floor of mouth, lateral tongue and fauces	4.5	No	No	T4 N0 M0	Jul 1988	D
12	76	♂	Lateral tongue	1.5	No	No	T1 N0 M0	Sep 1987	D
13	65	♂	Lateral tongue	3.0	Yes	No	T2 N1 M0	Sep 1989	D
14	63	♂	Lateral tongue	3.0	Yes	No	T4 N2 M0	Mar 1991	A
15	64	♂	Floor of mouth, lateral tongue and fauces	5.0	No	No	T4 N0 M0	Sep 1991	A
16	60	♂	Floor of mouth, lateral tongue and fauces	4.0	No	No	T4 N0 M0	May 1990	D
17	46	♂	Floor of mouth, lateral tongue	4.5	No	No	T4 N0 M0	May 1990	D
18	73	♂	Lateral tongue	2.5	No	No	T2 N0 M0	Apr 1991	A
19	59	♂	Lower lip	1.5	No	No	T1 N0 M0	Apr 1987	A

N, ganglionar affectation; M, distant metastases; D, death related with tumour; A, alive.
♂, male; ♀, female. TNM, tumours, nodes amd metastases.

Discussion

Various neoplasms were related to EBV infection (Khanna et al., 1995). EBV infection has been detected in epithelial malignancies, including carcinoma of the palatine tonsil, the supraglottic larynx, and the salivary gland. It has also been shown that EBV replicates in oropharyngeal epithelial cells and in the ductal epithelium of the salivary gland, with detection of the virus in the saliva after infectious mononucleosis (Raab-Traub *et al.*, 1991).

Previously, Jiang and Yao (1994) found EBV DNA in squamous cell carcinomas derived from the palate (75%) and tonsils (22%)

Table 3 Histopathological finding in EBV positive tumours

Case	Level of absorbance	Tumoral histopatho-logical diagnosis	Keratin production	Cytological abnormality	Mitotic count	Inflammatory infiltrate intensity	Tumoral growth
1	2.0	PD	Min	2	Mod	Mod	DTC
2	1.2	MWD	Mod	1	Min	Min	SMTC
3	1.3	MWD	Mod	2	Max	Max	SMTC
4	1.4	MWD	Max	2	Max	Mod	SMTC
5	1.0	WD	Max	2	Min	Mod	SMTC
6	1.3	MWD	Mod	2	Max	Max	LGTC
7	1.5	MWD	Mod	2	Max	Min	LGTC
8	1.0	PD	Min	2	Min	Mod	DTC
9	1.0	WD	Max	1	Min	Max	SMTC
10	1.2	MWD	Mod	2	Min	Max	CTC
11	1.6	WD	Max	2	Min	Mod	SMTC
12	1.8	MWD	Mod	2	Mod	Max	SMTC
13	1.7	MWD	Mod	2	Mod	Mod	LGTC
14	1.3	PD	Min	2	Mod	Max	DTC
15	1.2	WD	Max	2	Min	Mod	SMTC
16	1.4	MWD	Mod	3	Min	Mod	SMTC
17	1.3	MWD	Mod	3	Max	Min	CTC
18	2.6	WD	Max	1	Mod	Max	SMTC
19	1.0	MWD	Max	1	Max	Max	SMTC

WD, well differentiated; MWD, moderately well differentiated; PD, poorly differentiated; SMTC, solid masses of tumoral cells; LGTC, little groups of tumoral cells; CTC, cords of tumoral cells; DTC, dissociated tumoral cells; Min, minimum; Mod, moderate; and Max, maximum, results.

in nearly all malignant cell nuclei of positive sections. Moreover, Horiuchi *et al.* (1995) using PCR found EBV genomes in 52.8% of OSCC, and Mao and Smith (1993) detected EBV DNA in 35% of OSCC. Our results (17.59%) differ from those of previous workers. The ability of our assay to detect ten EBV genome equivalents was superior to those reported in previous studies and an alternative explanation could be the differences in the extent of the disease in the patients studied. Jiang and Yao (1994) did not record the extent of the disease in their series, and therefore a direct comparison was not possible.

Conversely, Talacko *et al.* (1991) did not find EBV genomes in twenty OSCC and Lam *et al.* (1995) were unable to detect EBV in oesophageal carcinomas. It is also possible to argue geographical

differences in the distribution of the EBV infections to explain the various percentages of EBV positive tumours previously found (Shijders *et al.*, 1990; Sixbey *et al.*, 1989).

An important aspect of the clinical studies was related to the location of EBV positive tumours. We located 63.1% of the tumours (12 cases) with EBV DNA which affected the lateral margin of the tongue. By comparing different sites of EBV DNA infections, Mao and Smith (1993) discovered that the lateral border of the tongue had the highest infection rate (45%). Indeed, the lateral margin of the tongue was the preferred site of OHL and this location could be the most favoured site for EBV, not only for immunodeficient patients (*i.e.* HIV-infected) but also for cancer patients.

Regarding patient age, a high percentage (68.4%) of cases with EBV DNA were over 60 years. In the Mao and Smith (1993) investigation this age group was also the most affected in both the control group and in patients with OSCC. Based on this evidence two observations can be made. First, that immunological differences may appear in the older patient which facilitate the proliferation of EBV; and second, the high incidence of positive cases around 60 years may simply agree with the normal age range of oral cancer.

An important aspect of the histopathological study of OSCC-EBV positives was the degree of tumoral differentiation. In NPC the association with EBV has proved to be consistent regardless of the degree of differentiation of the neoplasm and the extent of lymphocytic infiltrate. The results of Raab-Traub *et al.* (1987) indicated that, although all histological subtypes of NPC contained EBV DNA, a consistent picture emerged that well-differentiated NPC contained a much lower number of EBV genome equivalents. It is possible that the abundance of EBV DNA was related to the state of differentiation, and EBV genomes were amplified in the undifferentiated cell. Similarly, Pearson *et al.* (1983) found EBV serological profiles similar to those of control populations in patients with well-differentiated NPCs, and thus did not consider them to have special association with EBV infection. In the present study we found only 26.3% of well-differentiated OSCCs with EBV DNA (5 of 19 cases) whereas the remaining 73.7% (14 of 19 cases) with EBV DNA were moderately and poorly differentiated OSCCs. These results may be comparable with those of Raab-Traub *et al.* (1987) in NPC.

Although the results of the present work suggest that EBV DNA detection in OSCC might explain the relation between virus and tumour, this will have to be established with specific pathogenic mechanisms. Therefore, it is important to determine whether the detection of EBV DNA in a particular tissue represents transient

reactivated viral replication, a latent infection which might result in EBV-induced cellular proliferation, or whether it is due, like Hodgkin's lymphoma, to EBV-positive cells. Alternatively, it could be present due to superinfection of transformed tumour cells. Proving causality between EBV and tumorogenesis has been difficult despite the demonstration of EBV positivity in many tumours. Epithelial cells of the oropharynx, salivary glands, and cervix are primary sites of productive infection with EBV. While virions and early viral capsid antigens occur in the more superficial cell layers, reflecting productive infection, the EBV receptor was expressed only on immature cells. The EBV nuclear antigens were expressed solely in undifferentiated basal cells. This suggests that the stem cells of the basal layer remained constantly infected, although virus was produced only by terminally differentiating cells in more superficial epithelial layers (Baskin *et al.*, 1995).

Other investigators were unable to demonstrate EBV genome or gene products in the basal layers and suggested that these lesions were caused by repeated direct infection of superficial epithelial cells with virus from salivary glands (Sandvej *et al.*, 1992). Several lines of evidence from the work of Baskin *et al.* (1995) suggest that the epithelium may become infected by the haematogenous route, rather than from the surface, and that local or systemic immune defects allow the excessive EBV replication associated with the development of tumours. Squamous epithelial cells may become infected by circulating EBV-carrying lymphocytes, as suggested for gastric epithelial cells (Fukayama *et al.*, 1994).

It is well known that EBV can immortalize B cells *in vitro*, but EBV alone may not be sufficient for tumour development, as is exemplified by the complementation of the activated c-*myc* oncogene and EBV in Burkitt's lymphoma (Lombardi *et al.*, 1987). Thus, it is possible that the EBV acts in conjunction with other factors to induce full transformation. Genetic complementation can occur *in vitro* with dual viral infections (Flamand *et al.*, 1993), and the EBV may interact with one another to induce neoplastic transformation.

In summary, we have identified oral squamous cell carcinoma associated with EBV DNA sequences in 17.59% of tumours examined.

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