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Epstein-Barr Virus Latent Membrane Protein-1 (LMP-1) Expression in Oral Squamous Cell Carcinoma

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Objectives: **Epstein-Barr virus (EBV) is frequently associated with malignant cell transformation through the action of the oncoprotein latent membrane protein-1 (LMP-1). The present study aimed to determine the presence of EBV in oral squamous cell carcinomas (OSCCs) and the expression of LMP-1 in neoplastic cells of EBV-positive OSCCs.** ***Study Design/Methods:*** **In a retrospective study of 78 OSCCs, we investigated the presence of the DNA of EBV by polymerase chain reaction, the expression of the oncoprotein LMP-1 by immunohistochemistry, and the presence of EBV-encoded RNA (EBER) by in situ hybridization.** ***Results:*** **EBV DNA was detected in 19.2% of the cases. Expression of LMP-1 in neoplastic cells was found in 85.7% of the EBV-positive OSCCs. EBV presence was significantly more frequent (*P* <.05) in OSCCs localized on the lateral tongue. EBV-positive OSCCs more frequently presented (*P* <.05) greater nuclear atypia.** ***Conclusion:*** **EBV can appear in latent form in OSCC and express its main oncoprotein, LMP-1.** ***Key Words:*** **Epstein-Barr virus, latent membrane protein-1, oral squamous cell carcinoma.**

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INTRODUCTION

Epstein-Barr virus (EBV) is frequently associated with malignant cell transformation, above all through the action of the oncoprotein latent membrane protein-1 (LMP-1),[1–4] present during viral persistence. LMP-1 expression in the immunodepressed host can induce the oncogenic transformation of B lymphocytes and the appearance of lymphoproliferative processes.[5–8] However, the detection of EBV DNA in other tumors of immunocompetent patients, such as nasopharyngeal carcinoma (NPC),[9–12] Burkitt's lymphoma (BL),[13,14] Hodgkin's lymphoma,[15] non-Hodgkin's lymphoma,[16,17] thymic lymphoepithelioma, and carcinoma of the salivary glands,[18] suggests the implication of EBV in the pathogenesis of these tumors.[19]

In recent years, some researchers have pointed out the presence of EBV in tissue samples from OSCCs.[20–23] The aim of the present study was to analyze the presence of EBV in OSCCs and to determine whether the virus in OSCC-positive patients is latent and expresses LMP-1, its main oncoprotein.

MATERIALS AND METHODS

Patients

We studied 87 consecutive patients with OSCC referred to University Hospital, Granada, Spain, from 1988 to 1991. Nine patients were excluded for inadequate data (n = 4) or poor condition of paraffin block (n = 5). The final study sample comprised 78 patients with complete medical records and adequate paraffin block of the primary tumor (68 males [87%], 10 females [13%]; ages, 62.7 ± 12.2 y [mean ± standard deviation]). The following data were gathered from the medical records: tumor localization, tumor size, invasion of cervical nodules, distant metastasis, and Tumor, Nodes, and Metastases (TNM) stage at the diagnosis. Survival was analyzed until May 1996.

Control Subjects

Fifty healthy age- and sex-matched subjects were enrolled as control subjects from among individuals attending the School of Dentistry of the University of Granada for dental extraction. After written consent was obtained, a biopsy was done on the gingival mucosa adjacent to the extraction zone for use in the present study.

Histopathologic Study

A 5-μm section of each sample of tumor tissue was fixed in formol and embedded in paraffin. Staining with hematoxylin–eosin was performed. The histopathologic analysis was always performed by the same specialist pathologist and included the following parameters, evaluated as noted in parentheses: degree of tumor differentiation (well-differentiated, moderately differen-

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tiated, poorly differentiated); keratin production (absent: 0 horn pearls/40× field; minimum: 1 horn pearl/40× field; moderate: 2 horn pearls/40× field; and intense ≥3 horn pearls/40× field); cytologic grade (grade I: ≥75% mature nuclei; grade II: 25%–75% mature nuclei; and grade III: <25% mature nuclei); number of mitosis/8 fields at 40× (mild: 0–1 mitosis; moderate 2–5 mitosis; and intense >5 mitosis); intensity of peritumoral inflammatory infiltrate (mild, moderate, or severe, according to the pathologist's assessment); and type of tumoral growth (solid: neoplastic cells are infiltrated forming solid masses; cord: the neoplastic cells infiltrate forming thick cords; small groups of tumor cells and individual cells).[24]

Microbiologic Study

Polymerase chain reaction (PCR). A commercially available PCR (Herplex; Pharmagen, Madrid, Spain) was used. This assay was selected for its high level of sensitivity.[25] 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, inactivated by heating at 96°C for 10 minutes, followed by 10 minutes ultracentrifugation. Five μL were then used for the PCR. A cell suspension of EBV containing p3HR1 cell line was used as a 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[25] and included a set for the viral DNA polymerase region. The following primers were used: 5'CGAGTCATCTACGGGGGACACGGA3' and 5'AGCACCCCCACATATCTCTTCTT3'.

PCR[25] and hybridization[26] of EBV were performed as previously described (40 cycles of denaturalization at 96°C for 30 sec; annealing at 52°C for 1 min; extension at 72°C 30 sec; and final extension at 72°C for 10 min). The amplicon was detected with an enzyme linked immunosorbent assay with peroxidase that has the specific probe for EBV. A positive result was recorded when the absorbance of the sample was double the absorbance of the negative control. The assay had an internal positive control to detect the presence of inhibition. To ensure the accuracy of the results, positive samples were retested with the assay to detect the presence of EBV DNA and the mean value of the absorbances was used, provided the deviation did not exceed 10%. If it exceeded 10%, the test was repeated again. To further improve the clinical sensitivity of the assay, when the result was negative, the DNA was purified (DNA Purification Kit, Pharmagen) and concentrated by ultrafiltration from 200-μL aliquots of the samples, and the work was repeated.

In situ hybridization (ISH). EBV-coded RNA (EBER) hybridization was performed with a commercially available kit (In situ Hybridization, Biogenex, San Ramon, CA). Briefly, 5-$\propto$m sections were dewaxed in xylol–alcohol and the endogenous peroxidase was blocked with 0.5% hydrogen peroxide in methanol. The sections were digested with 200 μL proteinase K in Tris-buffered saline (pH 7.6) for 30 minutes at 37°C. Then 25 μL of fluorescein-labeled NCL-VEB probe (Biogenex) were added and incubated for 16 hours at 37°C. A 1:40 solution of anti-FITC rabbit antibody (Dako Corp., Carpinteria, CA) was added for 30 minutes at 37°C followed by mouse anti-rabbit IgG peroxidase conjugate for 30 minutes. Finally, diamino-benzidine substrate was added. The presence of brown nuclear staining was evaluated.

Immunohistochemistry (IMH). LMP-1 expression was studied with a five-step alkaline phosphatase antialkaline phosphatase (APAAP) immunohistochemical technique, as previously reported.[27] Sections of 5-μm thickness were dewaxed in xylene–alcohol and initially incubated with primary antibody anti-LMP-1 (Dako Corp.) for 2 hours. This reagent (IgG1 κ isotype) consists of a cocktail of four clones (CS 1–4), each of which detects a distinct epitope of a 60 Kdal latent membrane protein of EBV. The slides were then incubated for 40 minutes with rabbit antimouse immunoglobulin antibodies (1:40 dilution; Dako Corp.) followed by APAAP immune complexes (1:50 dilution). Incubations with the latter reagents were repeated for 20 minutes. Antibody localization was achieved with an alkaline phosphatase reaction using naphtol AS-MX phosphate (Sigma Chemical Co., St. Louis, MO) as substrate and Fast Red TR (Sigma Chemical Co.) as chromogen. Levamisole was included in the final reaction mixture to inhibit endogenous alkaline phosphatase. All incubations were performed at room temperature. Paraffin sections from a case of LMP-positive Hodgkin's disease were used as positive controls. Tris buffer and isotype-specific mouse immunoglobulin were substituted for the primary antibody as negative controls.

Statistical Analysis

The χ^2 test and Spearman's non-parametric correlation coefficient were used to measure the association between the variables under study. Survival was analyzed by actuarial method.[28] The influence of individual variables on survival was determined with the Lee-Desu test and the possibility of confusion or interaction with Cox's proportional risks model.[29] All data were processed with the SPSS program for Windows, version 6.01 (SPSS Inc., Chicago, IL).[30]

RESULTS

After the application of PCR, the DNA of EBV was detected in 15 cases (19.2%). The DNA of EBV was detected in none of the control samples. In no case was EBER RNA detected with in situ hybridization. The immunohistochemistry technique to detect the expression of LMP-1 protein could only be used in 14 of the 15 patients with EBV-positive OSCC, because there was an inadequate tissue sample for one of these patients. Twelve of the 14 EBV-positive OSCCs (85.7%) expressed LMP-1 in neoplastic cells (Table I; Fig. 1) and 11 of them also ex-

TABLE I.
Results for LMP-1 Expression of the EBV-Positive OSCCs.

Case No.	LMP-1 in Neoplastic Cells	LMP-1 in Infiltrating Lymphocytes
1	+	–
2	+	+
3	–	–
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	+	+
11	+	+
12	+	+
13	–	–
14	+	+
15	ND	ND

+ = positive; EBV = Epstein-Barr virus; OSCCs = oral squamous cell carcinomas; LMP-1 = latent membrane protein-1; ND = not determined.

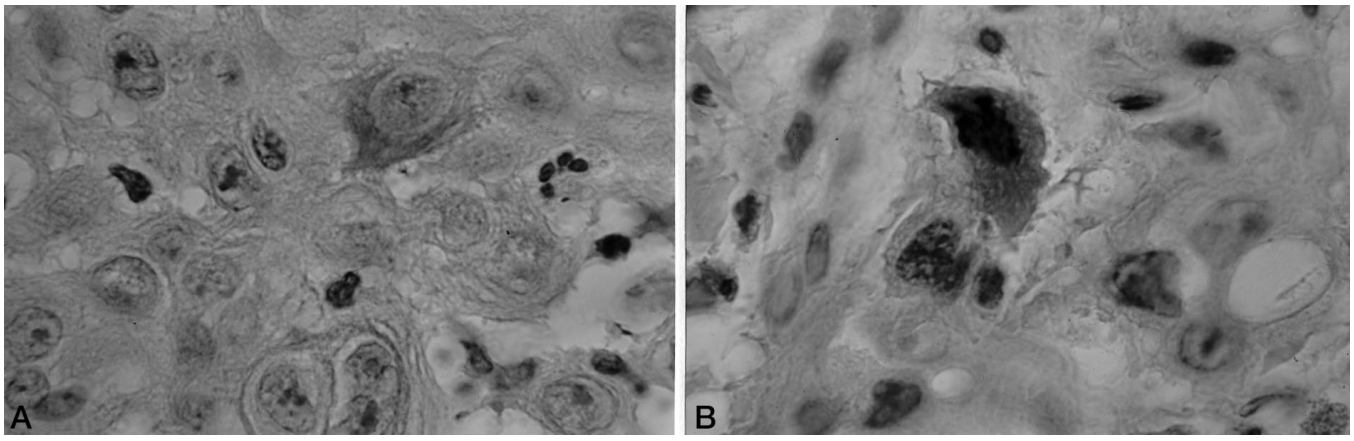

Fig. 1. Expression of LMP-1 in neoplastic cells. (**A**) Membrane localization. (**B**) Cytoplasmic localization.

pressed LMP-1 in the infiltrating lymphocytes of the tumor. All the OSCCs that expressed LMP-1 in infiltrating lymphocytes of the tumor also showed LMP-1 expression in neoplastic cells. No oncoprotein expression was found in non-neoplastic epithelium adjacent to tumors that expressed LMP-1. Table II lists the localization of all the tumors and the EBV-positive tumors. The statistical analysis showed a significant association ($P < .05$) between the presence of EBV DNA and tumor localization on the lateral edge of the tongue. A greater nuclear atypia was observed in EBV-positive than in EBV-negative OSCCs ($P < .05$). Tables III and IV show the survival findings and the variables that affected patients with OSCC in the univariate analysis of survival. The application of Cox's regression analysis (multivariate analysis of survival) revealed that only the localization (lower lip vs. other locations) significantly affected survival. After the exclusion of cancers of the lower lip, the mean survival of patients with EBV-positive OSCC was 21 months, whereas that of patients with EBV-negative OSCC was over 120 months ($P = .21$).

TABLE II.
Localization of the OSCC in All Cases and in the EBV-Positive OSCCs.

Location	Tumors (%)	Tumors with EBV DNA (%)
Lower lip	35 (45.5)	4 (11.4)
Lateral of tongue	11 (14.3)	6 (54.5)
Dorsum of tongue	8 (10.4)	1 (12.5)
Floor of mouth	6 (7.8)	0
Gingiva	6 (7.8)	0
Retromolar trigonum	4 (5.2)	3 (75)
Upper lip	3 (3.9)	0
Buccal mucosa	2 (2.6)	0
Hard palate	1 (1.3)	0
Base of tongue and pharynx	1 (1.3)	0
Unknown localization	1	1 (100)
Total	78 (100)	15 (19.2)

OSCC = oral squamous cell carcinoma; EBV = Epstein-Barr virus.

DISCUSSION

In the present study, 19.2% of the patients with OSCC were EBV-positive. Results reported in the literature vary widely between 0% and 100% positive cases.[20–23,31–33]

This wide variability may have two main causes. First, PCR does not discriminate for the origin of the amplified viral DNA (neoplastic cells, lymphocytes, saliva); and second, some authors[21–23] obtained the samples by oral smears, which could increase the possibilities of amplifying the DNA of the virus present in the saliva. In the present study, we overcame these drawbacks by using the immunohistochemical detection of LMP-1, which identifies the tissue localization of the EBV in EBV-positive tumors. No EBV DNA was detected in any of our control subjects. Scully et al.[34] reported 20% positive findings in healthy oral mucosa using PCR on samples obtained with exfoliative cytology, which, in our view, may also amplify the DNA present in saliva or lymphocytes. D'Costa et al.[23] found that 4% of his control group were EBV-positive. His control samples comprised apparently healthy oral mucosa contralateral to the site of an oral cancer in patients who smoked tobacco. This control procedure is questionable, because the effect of the tobacco may give rise to disorders (epithelial dysplasia) that in some cases can only be detected by histopathologic study.[35]

In human neoplasias strongly associated with EBV, the virus is frequently found to be latent, and it is of interest to know whether the virus is also latent in EBV-positive OSCC cases. We studied two markers of viral latency, EBER RNA (ISH) and LMP1 (IMH). No EBER RNA was found in any of our study samples, consistent with reports by Kobayhashi et al.,[33] who detected no EBER-1 using in situ hybridization, and by Cruz et al.,[36] who detected no EBERs expression in a series of 36 OSCCs that showed EBV DNA by PCR.

Of the 14 EBV-positive tumors in the study, 85.7% (12 tumors) expressed LMP-1 in neoplastic cells. Reports on the percentage of cases expressing LMP-1 in EBV-positive tumors at other locations vary between 19%[37] and 65%.[38] Only one other study[33] has demonstrated expression of LMP-1, the principal oncoprotein of EBV, in OSCC.

TABLE III.
Actuarial Life Table in Patients With OSCC (n = 78).

Interval (mo)	Entering	Withdrawn	Exposed to Risk	Dead	Cumulative Proportion Surviving (at end) $P \pm$ SE
0–12	78	0	78.0	6	0.923 ± 0.030
12–24	72	1	71.5	13	0.755 ± 0.049
24–36	58	0	58.0	5	0.690 ± 0.053
36–48	53	0	53.0	2	0.664 ± 0.054
48–60	51	0	51.0	1	0.651 ± 0.054
60–72	50	11	44.5	0	0.651 ± 0.054
72–84	39	3	37.5	0	0.651 ± 0.054
84–96	36	2	35.0	0	0.651 ± 0.054
96–108	34	7	30.5	0	0.651 ± 0.054
108–120	27	25	14.5	0	0.651 ± 0.054
120+	2	2	1.0	0	0.651 ± 0.054
Total		51		27	

Half-life = 120+ months.
OSCC = oral squamous cell carcinoma; SE = standard error.

Horiuchi et al.[20] reported a lack of LMP-1 expression in oral cancer, contradicting our findings. LMP-1 reduces the response of cells to normal differentiation signals, increases their invasiveness in the collagen matrix,[15] and can transform human fibroblasts and keratinocytes.[39] LMP-1 also induces resistance to apoptosis in B cells through the activation of transcription factors NFκB and AP1.[1] LMP-1 is a highly immunogenic protein that pro-

TABLE IV.
Parameters That Influence Survival in the Univariate Analysis.

Variable/Categories	Accumulated Survival ± SD		Comparison* z,p
	At 36 mo	60 mo	
Primary localization			
Other	0.51 ± 0.08	0.44 ± 0.08	z = 20.376
Lower lip	0.94 ± 0.03	0.94 ± 0.04	$P < 0.001$
TNM stage			
I/II	0.93 ± 0.04	0.91 ± 0.04	z = 27.265
III/IV	0.40 ± 0.08	0.34 ± 0.08	$P < 0.0001$
Tumor size			
T1	0.89 ± 0.04	0.85 ± 0.05	z = 19.952
T2/T3/T4	0.41 ± 0.09	0.38 ± 0.09	$P < 0.0001$
Distant metastasis			
No	0.80 ± 0.04	0.77 ± 0.05	z = 15.434
Yes	0.28 ± 0.12	0.21 ± 0.11	$P < 0.0001$
Degree of differentiation			
WDEC	0.86 ± 0.06	0.83 ± 0.07	z = 5.648
MDEC/PDEC	0.56 ± 0.07	0.54 ± 0.07	$P < 0.01$
Nuclear grade			
Grade I (low)	0.85 ± 0.06	0.85 ± 0.06	z = 6.481
Grade II (moderate)/III (marked)	0.59 ± 0.07	0.5262 ± 0.07	$P < 0.01$
Epstein-Barr Virus			
Negative	0.78 ± 0.05	0.77 ± 0.05	z = 10.566
Positive	0.40 ± 0.01	0.27 ± 0.11	$P < 0.001$

*Lee-Desu test, with one degree of freedom.
z = contrast statistic; p = statistical significance. The survival curves of the two categories considered (accumulated survival at 36 and 60 mo) are compared for each variable; WDEC = well-differentiated epidermoid carcinoma; MDEC = moderately differentiated epidermoid carcinoma; PDEC = poorly differentiated epidermoid carcinoma. The parameters with no influence on survival were: degree of production of keratin, no. of mitosis, intensity of peritumoral inflammatory infiltrate, and type of tumoral growth.

motes a specific response in cytotoxic T lymphocytes toward LMP-1-expressing cells.[40] This could condition a clonal selection by which only the LMP-1-negative cells survive,[41] which would account for the presence in our series of two tumors that were EBV-positive and LMP-1-negative. Nevertheless, it is also possible that the virus was not latent in these two tumors or even that the IMH technique produced false-negative results. Interestingly, while the localization of this protein is transmembranous, cytoplasmic expression was also observed in the present study. Murray et al.[42] accounted for this phenomenon by the phosphorylization of the protein and its binding to the cellular cytoskeleton.

Some authors have studied the timing of EBV infection in the neoplastic evolution process. The demonstration of latent infection in preinvasive lesions adjacent to NPCs[43] and the clonal expansion of the EBV in this type of lesion suggest that the viral infection precedes the development of the tumor.[20,22,23,44,45] We cannot confirm this observation in OSCC, because we found no case of LMP-1-positive cells in non-neoplastic epithelium adjacent to the cancer.

A major finding of our study is the significant frequency with which EBV-positive OSCCs appear on the lateral edge of the tongue. Mao et al.[21] obtained similar results. It is difficult to account for this observation, which has not been elucidated by analyses of the topographic distribution of the EBV receptor in epithelial cells of the oral mucosa.[46–49] Wide studies would be of interest to determine why the lateral edge of the tongue is the localization of choice for both the productive infection by EBV (oral hairy leukoplakia) and the latent infection.

Another important finding is the significant association between the presence of EBV in the OSCCs and the degree of nuclear atypia. A marked association between the presence of EBV and the degree of differentiation of the neoplasia has been demonstrated in NPCs. A study by Raab-Traub et al.[50] indicated that well-differentiated NPCs contain fewer copies of the EBV genome than do undifferentiated NPCs. Our findings show that in at least one of the parameters used to determine the degree of tumor differentiation, i.e., nuclear atypia, there is a certain similarity between EBV-positive OSCCs and NPCs. Finally, although the presence of the DNA of EBV does not significantly influence the survival of patients with OSCC, our survival curve for patients with intraoral tumors, excluding cancer of the lower lip, shows a tendency to significance. Wider studies are required to elucidate this issue.

To summarize, we conclude that Epstein-Barr virus can infect in latent form the oral mucosa outside the nasopharyngeal area. The presence of LMP-1, the principal oncoprotein of the virus, in many EBV-positive OSCCs indicates that this latent infection could in some way influence the oncogenic transformation of the infected oral epithelium.

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