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Epstein–Barr virus prevalence in oral squamous cell cancer and in potentially malignant oral disorders in an eastern Hungarian population

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We tested 65, 44, and 116 patients with oral squamous cell cancer (OSCC), oral leukoplakia (OL), and oral lichen planus (OLP) against 68 age-matched controls for the presence of Epstein–Barr virus (EBV). Apparently healthy mucosa was simultaneously sampled and examined in all patients. Paraffin-embedded tissue sections of all EBV-positive patients with OSCC were examined for latent membrane protein-1 (LMP-1) expression (demonstrable in most EBV-associated malignancies) using immunohistochemistry. The prevalence of EBV in the controls and in OSCC, OL, and OLP lesions was 19.1%, 73.8%, 29.5%, and 46.6%, respectively, and 66.2%, 22.7%, and 31.9% in the healthy mucosa of patients, respectively. The prevalence of EBV in OSCC patients was significantly higher than in controls or in respective samples of the other two patient groups both in the lesion and in the healthy mucosa. Comparisons including only patients with EBV-negative lesions yielded similar results. Lesions of patients with OLP, but not of patients with OL, differed significantly from controls in EBV prevalence. In OSCC, LMP-1 expression was not detected, and EBV carriage was not significantly associated with any risk factors and did not influence the outcome. Although a high prevalence of EBV was found in OSCC, comparable carriage rates on healthy mucosa of patients indicated that an aetiological role of EBV is unlikely.

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Epstein–Barr virus (EBV) is a double-stranded DNA virus belonging to the *Gammaherpesvirinae* subfamily. Epstein–Barr virus has a lifelong persistence in the human host, where it is common and is distributed worldwide in the normal population (1). The virus is ubiquitous, with a seroprevalence of between 50 and 95% (2). After primary infection, EBV establishes a latent infection in a small proportion of B lymphocytes as well as in oronasopharyngeal and salivary gland epithelial cells, periodically replicates in the oropharynx or in salivary gland epithelium, and is then shed in the saliva (3).

It is well known that EBV is associated with a variety of malignant disorders such as nasopharyngeal carcinoma (4), Burkitt's lymphoma, Hodgkin's disease, and B-cell lymphoma (5, 6). It is frequently associated with malignant and benign diseases of the immunocompromised (e.g. different B-cell lymphomas and oral hairy leukoplakia) (2, 5). Besides these established disease associations, a role of EBV has been presumed in many different malignant diseases, such as leiomyosarcoma, gastric adenocarcinoma, certain non-B-cell lymphomas (1), and oral squamous cell cancer (OSCC) (7), as well as in some benign and potentially malignant oral lesions and diseases, including oral lichen planus (OLP) (8), gingivitis, and periodontitis (2).

Several viral proteins have been found be involved in the transforming activity of, and in the carcinogenesis induced by, EBV; these include the latency-associated proteins latent membrane proteins 1 and 2 (LMP-1 and LMP-2) and Epstein–Barr virus nuclear antigens (EBNA1–6) (3, 5). Of these, LMP-1, EBNA-2, and EBNA-3 are indispensable for the immortalization of B cells; LMP-1 was also shown to be involved in the transformation of murine fibroblasts (3, 5). Some of these proteins were demonstrated, by immunohistochemical analyses, to be present in EBV-associated malignant cells (5).

The prevalence of EBV in oral samples varies widely in different studies. Most south-east Asian studies found a high prevalence of EBV and concluded an aetiological role of EBV in OSCC (9, 10); and in an Egyptian population this was also supported by immunohistochemical detection of LMP-1, the EBV antigen associated with transforming activity (11, 12). By contrast, North American studies, as well as West and North European

studies, regularly report lower prevalences of EBV and conclude that the aetiological role of EBV is doubtful in OSCC (13–15).

The aim of the present study was to collect data on the prevalence of EBV DNA in patients with OSSC, OLP, and oral leukoplakia (OL) in an eastern Hungarian population with a relatively high incidence of OSCC. To track the source of EBV in these patients we also took a sample of the apparently healthy mucosa of the patients simultaneously when taking a sample of the lesion.

Material and methods

Study groups, specimens, and DNA extraction

All patients enrolled in the study attended the Department of Oral and Maxillofacial Surgery and the Department of Periodontology, Faculty of Dentistry, University of Debrecen, Hungary, during 2003–2007. Histopathological results based on targeted biopsy were available at the time of sample collection in each patient. We included OSCC patients if (i) they were newly diagnosed patients and (ii) they did not undergo neoadjuvant chemotherapy or radiotherapy before the surgical intervention and specimen collection. Similarly, we included patients with potentially malignant oral lesions if (i) they were newly diagnosed patients and (ii) they did not receive any therapy for their lesion before sampling. All individuals fulfilling the inclusion criteria and agreeing to participate were enrolled. Sixty-five patients with OSCC (51 men, 14 women; mean age 54.4 yr; age-range 25–80 yr), 44 patients with OL (14 men, 30 women; mean age 56.3 yr; age-range 29–91 yr), and 116 patients with OLP (29 men, 87 women; mean age 55.0 yr; age-range 23–79) were enrolled in the study. The age-matched control group consisted of 68 individuals without a history of oral disease or malignancy, with a healthy oral mucosa (16 men, 52 women; age-range 22–77 yr; mean age 52.5 yr). Control individuals were from the same geographical area as the patients (eastern Hungary) and had been referred to the Faculty of Dentistry for regular oral screening. Written informed consent was collected from each patient enrolled. The study was approved by the local ethics committee (approval number: 2273–2004).

Data were also collected on exposure to known risk factors of OSCC relevant in the region (smoking, alcohol consumption) and pathological characteristics [localization, tumour node metastasis (TNM) stage according to the Tumour-Node-Metastasis staging of carcinomas and histological grade of the tumour]. To assess complication-free survival (survival without recurrence, growth of new tumour or metastasis), patients were followed-up after surgical intervention; the mean follow-up time was 31 months (range: 1.5–60 months).

As certain clinical variants of OLP (erosive and atrophic forms; EA-OLP) are more prone to malignant transformation than others (plaque-like and reticular forms; non-EA-OLP), and, similarly, there are OLs with higher (non-homogeneous OL; erythroleukoplakia and verrucous forms) and with lower (homogeneous OL) risks of malignization, potentially malignant oral lesions (OL and OLP) were divided into two respective groups according to clinical appearance associated with higher risk of malignant transformation, to subgroups EA-OLP (59 individuals: 13 men, 46 women; mean age 57.5 yr; age-range 23–79 yr) and non-EA-OLP (57 individuals: 16 men, 41 women; mean age 52.4 yr; age-range 24–75 yr), as well as to non-homogeneous OL (14 individuals: 5 men, 9 women; mean age 51.5 yr; range 29–71 yr) and homogeneous OL (30 individuals: 9 men, 21 women; mean age 58.5 yr; age-range 35–91 yr).

Excised tissue samples of patients with OSCC were obtained, during surgical intervention, from the centre of the tumour. Another part of the tissue sample was submitted for histopathological and immunohistochemical analyses (see below). In OL and OLP groups, exfoliated cells were collected from the surface of oral lesions. In all groups of patients, before specimen collection from the lesions, exfoliated cells were harvested from the apparently healthy mucosa at the farthest possible site from the lesion. Control specimens consisted of cytobrush-harvested exfoliated buccal epithelial cells collected from healthy individuals. To minimize contamination of the exfoliated cell samples with saliva, sampling was preceded by two thorough mouth rinses with physiological saline.

DNA was isolated using TRI Reagent (Sigma, St Louis, MO, USA), according to the manufacturer's recommendations for tumour tissue samples and exfoliated cells, from OSCC patients. Extraction of DNA from samples of exfoliated cells from patients with OL and OLP, and from control individuals, was performed with proteinase K digestion followed by treatment with 5 M NaCl, and DNA was precipitated using 96% ethanol.

EBV detection by polymerase chain reaction

We used a nested polymerase chain reaction (PCR), amplifying a 97-bp region of the internal repeat of the *Bam*H1-W fragment of the EBV genome, constructed from two previously described overlapping PCR assays (16). Briefly, primers EBV-F (5′-GAGACCGAAGTGAAG GCCCT-3′) and EBV-R (5′-ACAGCTCCTAAGAAGG CACC-3′) were used to amplify a 171-bp product, and then primers EBV B-F (5′-GCCAGAGGTAAGTGGACTTT-3′) and EBV B-R (5′-GAGGGGACCCTGAGACGGGT-3′) were used to amplify a 97-bp fragment within the amplimer yielded by the first round of PCR. Both PCR assays were performed in a final volume of 25 μl containing 250 μM of each dNTP, 25 pmol of each primer, and 0.5 U of GoTaq DNA polymerase in 1 × PCR buffer containing $MgCl_2$ (supplied by the manufacturer; Promega, Madison, WI, USA). Thermal profiles were: 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min in both rounds. DNA from the EBV-positive B95-8 cell line was used as a positive control.

Detection of LMP-1 using immunohistochemistry

Immunohistochemistry was performed with the Dako-Cytomation LSAB+ System AP (Dako Denmark, Glostrup, Denmark), using monoclonal mouse antibodies against LMP-1, according to the manufacturer's recommendations. Two paraffin-embedded tumour-tissue sections were tested in all 48 patients with EBV-positive OSCC tumour tissue. A paraffin-embedded lymph node preparation from a patient with EBV-positive Hodgkin's disease was used as a positive control, while lymph node sections from EBV-negative Hodgkin's disease patients served as negative controls.

Statistical analysis

Statistical comparison of prevalence data was performed using chi-square and Fisher's exact tests. Logistic regression was used to analyze the association between EBV carriage and patient characteristics (gender and age) as well as the clinical appearance of OLP. Because of the small number of patients with non-homogeneous OL, the clinical appearance of OL was not analyzed statistically. The association of tumour characteristics (localization, histological grade, and TN stage) with exposure to risk factors (smoking and alcohol consumption) in patients with OSCC was also analyzed using logistic regression. Tumour-free survival of OSCC patients was analyzed using the Kaplan–Meier test. All tests were carried out with a confidence interval (CI) of 95% using SPSS 15.0 for Windows software.

Results

EBV prevalence data

The prevalence of EBV DNA was 19.1% (13/68) in the control group. In patients with OSCC, OL, and OLP, the carriage rates in the lesion were 73.8% (48/65), 29.5% (13/44), and 46.6% (54/116), respectively, and 66.2% (43/65), 22.7% (10/44), and 31.9% (37/116) in the apparently healthy mucosa, respectively. If patients were divided according to the presence or absence of EBV DNA in their lesions, prevalence rates in the apparently healthy mucosa of patients with EBV-positive vs. EBV-negative lesions were 34/48 (70.8%) vs. 9/17 (52.9%), 5/13 (38.5%) vs. 5/31 (16.1%), and 29/54 (53.7%) vs. 8/62 (12.9%) for OSCC, OL, and OLP patients, respectively. When patients with OLP were divided, the two subgroups showed comparable EBV prevalences, both in the lesion (45.6%, 26/57 in non-EA-OLP; and 47.5%, 28/59 in EA-OLP) and on the healthy mucosa (33.3%, 19/57 in non-EA-OLP; and 30.5%, 18/59 in EA-OLP).

Statistical analysis of prevalence data

The prevalence of EBV in the lesion, as well as on the apparently healthy mucosa, of patients with OSCC, was significantly higher than the prevalence in the controls or in respective samples of the other two groups of patients (Table 1). Regarding only patients with EBV-negative lesions, similar results were found (Table 2). However, when regarding only patients carrying EBV DNA in the lesions, mucosal carriage rates of patients with OSCC and patients with OLP were not significantly different, but were significantly higher than the prevalences of EBV in mucosal controls or in patients with OL (Table 2).

No significant differences were detected in the prevalence of EBV between patients with OL and controls (Table 1). By contrast, patients with OLP carried EBV DNA more frequently than controls in the lesion but not on the healthy mucosa. However, the prevalence of EBV on the apparently healthy mucosa was found to be significantly higher in patients with EBV-positive lesions compared with controls, but not in patients with EBV-negative OLP lesions compared with controls (Table 2).

Comparing patients with the two potentially malignant oral disorders, positivity in the lesion was significantly higher for OLP than for OL. Regarding EBV carriage on the healthy mucosa, OLP and OL patients never differed significantly; neither when comparing the total patient populations, nor when examining patients with EBV-positive or EBV-negative lesions separately (Table 1 and Table 2). The prevalence of EBV in

Table 1

Statistical comparison of Epstein–Barr virus (EBV) prevalence data in different study groups

	Lesion			Apparently healthy mucosa		
	OLP ($n = 116$)	OL ($n = 44$)	OSCC ($n = 65$)	OLP ($n = 116$)	OL ($n = 44$)	OSCC ($n = 65$)
Controls ($n = 68$)	$P < 0.001$	NS	$P < 0.001$	NS	NS	$P < 0.001$
OLP ($n = 116$)		$P = 0.049$	$P < 0.001$		NS	$P < 0.001$
OL ($n = 44$)			$P < 0.001$			$P < 0.001$

NS, not significant; OL, oral leukoplakia; OLP, oral lichen planus; OSCC, oral squamous cell cancer.

Table 2

Statistical comparison of Epstein–Barr virus (EBV) prevalences in apparently healthy mucosa

Apparently healthy mucosa of EBV-positive patients				Apparently healthy mucosa of EBV-negative patients			
	OLP ($n = 54$)	OL ($n = 13$)	OSCC ($n = 48$)		OLP ($n = 62$)	OL ($n = 31$)	OSCC ($n = 17$)
Controls ($n = 68$)	$P < 0.001$	NS	$P < 0.001$	Controls ($n = 68$)	NS	NS	$P = 0.010$
OLP ($n = 54$)		NS	NS	OLP ($n = 62$)		NS	$P = 0.001$
OL ($n = 13$)			$P = 0.050$	OL ($n = 31$)			$P = 0.018$

NS, not significant; OL, oral leukoplakia; OLP, oral lichen planus; OSCC, oral squamous cell cancer.

EA-OLP and non-EA-OLP patients did not differ significantly either in the lesion or on the healthy mucosa; therefore, the two subgroups were not examined in further comparisons.

Immunohistochemistry

The lymph node sections of the Hodgkin's disease patients (positive control) were consistently strongly positive for EBV, but none of the 48 samples tested, or the lymph nodes from EBV-negative Hodgkin's disease patients, was found to be LMP-1 positive.

Analysis of the association of EBV with clinicopathological data

Epstein–Barr virus-positive and EBV-negative OSCC patients did not statistically differ in patient characteristics (age, gender) and exposure to risk factors (smoking and alcohol consumption) or clinical data (localization, TN stage, and histological grade of the tumour). The presence of EBV in the lesion or in the apparently healthy mucosa was not found to influence survival, and did not increase the risk of poor outcome.

In the case of patients with OL, younger age (under 55 yr), but not gender, was a risk factor associated with EBV carriage in the lesion [odds ratio (OR) = 4.09; CI = 1.02–16.40; $P = 0.047$]. In patients with OLP, EBV infection appeared more frequently in men than in women (OR = 2.82; CI = 1.17–6.79; $P = 0.02$), but age was not significantly associated with EBV carriage. Epstein–Barr virus carriage did not influence the risk of unfavourable clinical appearance (EA-OLP).

Discussion

Data on the aetiological role of EBV in OSCC is controversial. Generally, studies finding a low prevalence of EBV concluded no, or a negligible, aetiological role (14, 17, 18); by contrast, studies reporting a relatively high prevalence of EBV frequently supported an aetiological role (7, 19). In some cases, immunohistochemistry has been used to confirm (11) or to reject (14) the possibility of the role of EBV in OSCC. Curiously, most studies concluding an aetiological role of EBV in OSCC were conducted on south-eastern Asian or North African populations with a high prevalence of EBV-associated nasopharyngeal carcinoma (6, 9, 10).

The prevalence of EBV in our study was high compared with European studies (13) and was comparable to data from studies supporting an aetiological role (9–12), suggesting that EBV may have a role in OSCC in the study population. However, we believe that the high proportion of EBV carriers in our OSCC patients is more likely to be a result of other factors (e.g. tumour-induced B-lymphocyte activation and/or infiltration of the tumour tissue by B cells).

Tumour tissue and apparently healthy mucosa showed almost the same EBV prevalence rate in OSCC patients, which is, to our knowledge, an issue as yet unexamined. Moreover, even patients with EBV-negative tumours had a notably high carriage rate of EBV on the healthy mucosa (significantly higher than in the oral controls, but comparable to that of patients with EBV-positive tumours). This means that although EBV DNA is present in the majority of the OSCCs, it is prevalent to a comparable degree also on the normal mucosa of OSCC patients. This situation is completely different from that of human papillomaviruses (viruses suspected to occur more strongly in the background of OSCC), in which the carriage of human papillomaviruses on the apparently healthy mucosa of patients with virus-free tumours was almost the same as that in the controls (Szarka *et al*., in press). In brief, including the healthy mucosa of the patients in the analysis revealed that EBV is not associated with the lesion itself, but rather with OSCC as a disease.

The aetiological role of EBV in OSCC cannot entirely be ruled out, as it is possible that it contributes to carcinogenesis, but only in a minority of the tumours, an effect that is not demonstrable as a result of being obscured by the other more important effects in the relatively small populations used in most studies, including the present study. However, the uniform lack of LMP-1 expression in the OSCC tumour cells, found in this study as well as in previous studies in European patients (7), represents another strong argument against the aetiological role.

The high prevalence of EBV DNA in the tumour tissue may be a result of infiltrating B lymphocytes and macrophages infected with EBV and/or to higher susceptibility of the tumour keratinocytes to EBV infection caused by an altered immunological environment arising from the presence of the tumour (14). These factors may, similarly, also increase the prevalence of EBV in the healthy mucosa.

This assumption is supported by the prevalence data in the potentially malignant lesions. In our data set, patients with OLP and OSCC, but not with OL, carried significantly more EBV DNA than controls. Similar data have been reported by Sand *et al*. (8). The difference between OLP and OL can conveniently be explained by the distinct pathomechanisms of the two diseases: OLP is a disease with a primarily autoimmune pathomechanism (20–22); and OL develops mostly on the background of chronic mechanical or chemical irritation (23, 24). It is tempting to speculate that the autoimmune processes lead to an oral environment more favourable for EBV re-activation and shedding, probably provoked and maintained by the altered cytokine profile (25). As immunological changes are present both in OLP and in OSCC, but are not characteristic in OL, this similarity may be the basis of the higher prevalence in both OSCC and OLP.

Data provided in the literature suggest that the role of EBV in OSCC may vary according to geographical regions, for example, in some populations (e.g. Japanese, Chinese, North Africans), EBV may play a role in OSCC (9, 10, 26); by contrast, in North American and North European populations the role is less probable (14, 15). Our data suggest that the Hungarian population belongs to the latter group.

Notably, those populations in which the aetiological role of EBV in OSCC is more probable are generally the same, where EBV-associated nasopharyngeal carcinoma is prevalent (6, 27). This suggests that the genetic and/or habitual factors aiding EBV in the pathogenesis of nasopharyngeal carcinoma may similarly provide an opportunity for EBV to contribute to the development of OSCC in these populations.

Our data draw attention to the fact that high prevalences of EBV in oral lesions compared with controls do not always indicate a true association, and inclusion of the apparently healthy mucosa/tissues into similar studies aid in the correct interpretation of the data.

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Insert double quotation marks	(As above)	Y or Y and/or Y or Y
Insert hyphen	(As above)	⊢
Start new paragraph	⌐	⌐
No new paragraph	⌐	⌐
Transpose	⌐	⌐
Close up	linking ⁀ characters	⁀
Insert or substitute space between characters or words	/ through character or ⌐ where required	Y
Reduce space between characters or words	| between characters or words affected	⊤