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Progressive increase of human papillomavirus carriage rates in potentially malignant and malignant oral disorders with increasing malignant potential

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Introduction: We investigated the potential role of human papillomaviruses (HPVs) in potentially malignant oral disorders, oral leukoplakia (OL) and oral lichen planus (OLP), and in oral squamous cell cancer (OSCC) in an Eastern Hungarian population with a high incidence of OSCC.

Methods: Excised tumor samples (65 OSCC patients) and exfoliated cells from potentially malignant lesions (from 44 and 119 patients with OL and OLP, respectively) as well as from healthy controls (72 individuals) were analysed. OLPs were classified based on clinical appearance, 61 patients had erosive–atrophic lesions (associated with higher malignancy risk, EA-OLP) and 58 had non-erosive non-atrophic lesions (with lower risk of becoming malignant, non-EA-OLP), respectively. Exfoliated cells collected from apparently healthy mucosa accompanied each lesion sample. HPV was detected by MY/GP polymerase chain reaction (PCR) and genotyped by restriction analysis of amplimers. Copy numbers in lesions were determined using real-time PCR. Prevalence rates, copy number distributions, and association with risk factors and diseases were analysed using chi-square test, *t*-test, and logistic regression, respectively.

Results: We detected HPVs significantly more frequently in lesions than in controls ($P \leq 0.001$ in all comparisons). HPV prevalence increased gradually with increasing severity of lesions (32.8, 40.9, and 47.7% in OLP, OL, and OSCC, respectively). Copy number distribution patterns roughly corresponded to prevalence rates, but OLP and OL were comparable. HPV prevalence differed significantly between EA-OLP and non-EA-OLP groups (42.6 vs. 22.4%); EA-OLP group showed a prevalence similar to that found in OL.

Conclusion: HPVs may be involved in the development or progression of not only OSCC but also of potentially malignant oral lesions.

Key words: human papillomavirus; oral carcinogenesis; oral leukoplakia; oral lichen planus; oral squamous cell cancer

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Oral squamous cell cancer (OSCC) causes high mortality in central Eastern Europe including Hungary (5, 26). Manifest malignant disease is preceded by potentially malignant lesions in some cases (17, 23, 28). These potentially malignant disorders include oral lichen planus (OLP) and oral leukoplakia (OL). OLP is associated with various underlying diseases (9, 10, 23, 31) whereas OL is mostly caused by local chemical and mechanical irritants (19, 28). OL is an obligate

precancerous lesion progressing into carcinoma in at least 4–6% of cases according to many authors, with an average yearly rate for progression to malignancy of 1% (27, 28). OLP, in contrast, is a premalignant condition with a 0–5% progression to malignancy, depending on the clinical appearance and the follow-up period (17, 23, 27). OLP may undergo remission upon resolution of the underlying disease. 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) (17, 23, 27). Similarly, there are OLs with higher (non-homogeneous OL; e.g. erythroleukoplakia and verrucous forms) and lower (homogeneous OL) risks of malignant transformation (28).

In the etiology of these potentially malignant disorders and consequent OSCC as well as of other head and neck cancers, the role of physical, chemical, mechanical, and infectious factors has long been suspected. While the importance of smoking, alcohol consumption, and even ultraviolet radiation has been proven, the role of infectious agents has been subject to much debate (11, 13, 19, 20, 23, 28). Hepatitis C virus is thought to play a role in the pathogenesis of OLP (9); Epstein–Barr virus and human papillomavirus (HPV) were both implicated as etiological agents in OSCC, presumably acting through their transforming potential, but the problem is far from resolved (7, 12, 21, 32). Association of HPVs with OSCC was reported by several authors, but causality remains to be proven. Few studies are concerned with the association of HPV with oral potentially malignant disorders, and most of these suffer from low sample size and/or small size of the control population (4, 18, 25, 29). Our aim was to examine the effect of HPV carriage on the risk of development of potentially malignant lesions and OSCC in the same Eastern Hungarian region.

Materials and methods

Study groups, samples, and DNA extraction

All specimens originated from the Departments of Maxillofacial and Oral Surgery and of Periodontology, at the University of Debrecen, Hungary, and were collected between 2003 and 2007. Histopathological results were available at the time of sample collection in each case. Inclusion criteria for OSCC patients were (i) being a newly diagnosed case and (ii) not undergoing neoadjuvant chemo- or radiotherapy before the surgical intervention and sampling. Similarly, inclusion criteria for patients with potentially malignant oral lesions were (i) being a newly diagnosed case; and (ii) not receiving 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 male, 14 female; mean age 54.4 years, range 25–80 years), 44 patients with OL (14 male, 30 female; mean age 56.3 years, range 29–91 years) and 119 patients with OLP (31 male, 88 female; mean age 55.0 years, range 23–79 years) were enrolled. Fifty-eight of the 119 OLP patients (16 male, 42 female; mean age 52.5 years; range 23–75) carried non-EA-OLP lesions, whereas the other 61 OLP patients (15 male, 46 female; mean age 57.4 years, age 23–79 years) had EA-OLP lesions. OLP lesions with mixed clinical presentation were classified as non-EA-OLP only if plaque and reticular areas were seen together; if atrophic or erosive areas were observed, the lesion was regarded as EA-OLP. The most frequent diseases underlying OLP were sideropenic anemia, diabetes mellitus, and chronic viral hepatitis. The small size of the OL population made similar subgrouping inapplicable for OL.

The age-matched control group consisted of 72 individuals without a history of oral disease or malignancy with healthy oral mucosa (19 male, 53 female; mean age 52 years, range 22–77 years). Control individuals were from Eastern Hungary (the same geographic area as the patients) and were referred to the Faculty of Dentistry for regular oral examination. Written informed consent was collected from each enrolled subject.

Excised tissue specimens from OSCC patients were collected during surgical intervention from the center of the tumor. In OL and OLP, exfoliated cells were collected from the surfaces of the lesions. In all patient groups, before sampling of the lesion, exfoliated cells were harvested from apparently normal mucosa at the site farthest away from the lesion. To avoid contamination of samples with saliva, sampling was preceded by two mouth rinses with physiological saline. Exfoliated buccal epithelial cells were collected from controls. As it was shown that samples of excised tissue tend to underestimate HPV prevalence in OSCC compared with samples of cells exfoliated from the tumor surface (16) the differences in the sample collection are expected to lead to underestimation of HPV prevalence in patients with OSCC, but not in other patient groups or in the controls.

DNA was isolated by TRI Reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's recommendations for tumor tissues. Exfoliated cells of OSCC, OL, and OLP patients and controls were digested with proteinase K and treated with 5 M NaCl after digestion; DNA was then precipitated using 96% ethanol.

Polymerase chain reaction assays

For detection and typing of HPVs, we used MY09/MY11-GP5$^+$/GP6$^+$ consensus nested polymerase chain reactions (PCR). HPV genotyping was performed using restriction fragment length polymorphism of the MY or GP amplimers as described previously (2, 24). PCR assays were performed twice using DNA from two independent DNA extraction processes. Identity of HPV types 6, 11, 16, 18, 31, and 33 was confirmed by HPV E7 open reading frame-specific PCR assays (14).

Viral copy numbers were determined for lesion samples carrying types 6, 11, 16, 18, and 33 by means of SYBR Green real-time PCR assays with the type-specific primers (14) using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) (For other virus genotypes the copy number could not be reliably determined because of the insufficient sensitivity of the MY step of the nested PCR assay). Assays were run in ABI Prism 7500 equipment (Applied Biosystems, Foster City, CA). Virus copy numbers were calculated using a standard plasmid dilution series containing DNA (10^{10}–10^0 copies per μl) of the respective HPV types. The detection limit was 10 copies per reaction (100 ng total DNA). All experiments were run in duplicate and repeated twice. Final copy numbers were calculated as the averages of the duplicate experiments and expressed as copy number in 1 μg total DNA. Samples with undetectable HPV in the real-time PCR (containing fewer than 10 copies) were considered to contain 10 copies for statistical purposes. Repeated experiments were used to confirm reproducibility. Melt curve analysis was used to confirm the identity of the PCR product.

Statistical analysis

Prevalence data were analysed with chi-square and Fisher's exact tests. We calculated and compared virus copy number averages found in different patient

Table 1. Human papillomavirus positivity of the different study groups in the lesion and in the apparently normal mucosa

		HPV positivity of the apparently normal mucosa		
	HPV positivity of the lesions	Total	In patients with HPV-positive lesion	In patients with HPV-negative lesion
Control (n = 72)	3/72 (4.2%)			
OLP (n = 119)	39/119 (32.8%)	17/119 (14.3%)	14/39 (35.9%)	3/80 (3.8%)
Non-EA-OLP (n = 58)	13/58 (22.4%)	3/58 (5.2%)	2/13 (15.4%)	1/45 (2.2%)
EA-OLP (n = 61)	26/61 (42.6%)	14/61 (23.0%)	12/26 (46.2%)	2/35 (5.7%)
OL (n = 44)	18/44 (40.9%)	9/44 (20.5%)	8/18 (44.4%)	1/26 (3.8%)
OSCC (n = 65)	31/65 (47.7%)	15/65 (23.1%)	14/31 (45.2%)	1/34 (2.9%)

HPV, human papillomavirus; OLP, oral lichen planus; non-EA-OLP, non-erosive/non-atrophic oral lichen planus; EA-OLP, erosive and/or atrophic oral lichen planus; OL, oral leukoplakia; OSCC, oral squamous cell cancer.

groups using an independent samples t-test. Association between patient data, HPV carriage, and clinical appearance of diseases was evaluated by logistic regression. All statistical tests were carried out with a confidence interval of 95% using SPSS 15.0 for Windows (SPSS, Chicago, IL).

Results

Table 1 summarizes HPV prevalence data in different study populations. Four OLP patients were seropositive for hepatitis C virus, one OL patient was hepatitis B virus positive; because of the low prevalence data, statistical analysis could not be performed for these viruses.

HPV prevalence in controls

Two individuals were HPV16 positive, one individual carried HPV11.

HPV prevalence in lesions

The prevalence of HPV in lesions increased gradually from OLP, through OL, to OSCC. All lesions carried HPV significantly more frequently than healthy controls ($P < 0.001$ in all comparisons). Comparing different patient groups, HPV prevalence differed significantly only between OLP and OSCC patients ($P = 0.047$).

Presence of HPV showed a strongly significant correlation with the presence of all lesions compared with controls: odds ratio (OR) 6.64 [95% confidence interval (CI) 1.79–24.63], 17.09 (4.84–60.38), 15.92 (4.33–59.59), and 20.97 (5.98–73.05) in non-EA-OLP, EA-OLP, OL, and OSCC groups, respectively; ($P \leq 0.005$ in all comparisons).

HPVs were mainly high-risk genotypes (HPV16 and HPV18); the genotype distributions are shown in Table 2.

Dividing the OLP group into subgroups with non-atrophic/non-erosive lesion (non-EA-OLP) and erosive or atrophic lesions (EA-OLP), we found a significant difference between their HPV prevalences ($P = 0.026$). Moreover, while HPV prevalence was significantly higher in both groups compared with controls ($P = 0.001$), non-EA-OLP, but not EA-OLP patients, harbored HPV significantly less frequently than OL and OSCC patients ($P = 0.045$ and $P = 0.003$, respectively). Carriage rates in EA-OLP and OL lesions were essentially the same (Table 1).

HPV prevalence in healthy mucosa of patients

As seen in the lesions, HPV carriage rates also increased gradually. Regarding only patients with HPV-positive lesions, HPV carriage rates on healthy mucosa were markedly higher in all patient groups compared with the controls. Prevalence rates on the apparently healthy mucosa differed significantly from that found in healthy controls ($P < 0.01$ in all comparisons), but not between groups ($P > 0.05$). Prevalence in healthy mucosa of non-EA-OLP and EA-OLP patients did not differ.

HPV prevalence on healthy mucosa of patients with HPV-free lesions was comparable to the prevalence of controls. Genotypes were always low-risk types in patients with HPV-negative lesions (one HPV6 in OSCC, one HPV11 in OL, and three HPV11 in OLP patients). HPV genotypes from the mucosa of patients with HPV-positive lesions were the same as found in the lesion, excepting one HPV16-positive OSCC patient carrying HPV11 in the normal mucosa.

Virus copy numbers in the lesions

Averages of virus copy numbers were 5.2×10^2 (range: 10–840), 6.8×10^3 (range 10–51,000), 7.2×10^3 (range 10–27,000), and 2.4×10^5 (range 90–130,000) per 1 μg total DNA in oral controls and in OLP, OL, and OSCC patients, respectively. Copy number averages were significantly higher in all patient groups than in the controls ($P < 0.005$ in all comparisons). There was no significant difference between OLP and OL patients, but both groups had copy numbers significantly lower than that found in OSCC ($P = 0.016$ and $P = 0.019$, respectively). When dividing OLP group into subgroups as above, copy numbers in non-EA-OLP and EA-OLP subgroups were comparable (7.4×10^3, range 10–51,000 and 6.5×10^3, range 10–24,000, respectively;

Table 2. Distribution of human papillomavirus genotypes in different study groups

	Low-risk HPV genotypes						High-risk HPV genotypes							
	HPV6	HPV11	HPV32	HPV55	HPV57	Total	HPV16	HPV18	HPV31	HPV33	HPV39	HPV51	Total	HPV total
Control (n = 72)		1				1	2						2	3
OLP (n = 119)	2	4	1	1		8	23	5		2	1		31	39
Non-EA-OLP (n = 58)	1	1				2	7	3		1			11	13
EA-OLP (n = 61)	1	3	1	1		6	16	2		1	1		20	26
OL (n = 44)	1		1		1	3	12	2		1			15	18
OSCC (n = 65)		4				4	18	4	1	2	1	1	27	31
Total	3	9	2	1	1	16	55	11	1	5	2	1	75	91

HPV, human papillomavirus; OLP, oral lichen planus; non-EA-OLP, non-erosive/non-atrophic oral lichen planus; EA-OLP, erosive and/or atrophic oral lichen planus; OL, oral leukoplakia; OSCC, oral squamous cell cancer.

$P > 0.05$). Interestingly, copy numbers in the non-EA-OLP subgroup proved comparable to the copy numbers in the control population ($P > 0.05$), but behaved similarly to undivided OLP in other comparisons. Comparisons of EA-OLP yielded results similar to comparisons of undivided OLP.

Risk factors associated with presence of high-risk potentially malignant lesions

EA-OLP is associated with a higher frequency of HPV carriage compared with non-EA-OLP (66.7 vs. 43.8%; OR = 2.57, CI = 1.16–5.72; $P = 0.021$). Age and gender distributions of patients with high-risk and low-risk OLP were similar. HPV status and gender did not differ between OL patients with high-risk and low-risk lesions, but in patients younger than 50 years higher risk OL occurred more frequently than among older patients (50.0 vs. 21.4%; OR = 3.67, CI = 0.97–13.90); however, this correlation was not significant ($P = 0.056$).

Discussion

After much debate, it is now accepted that HPVs have a role in head and neck cancer (21, 25, 29). The importance of this role is still debated, some reports conclude a direct (co)carcinogenic role (18, 21, 25), while other results are interpreted as an association of unknown significance (21, 25). A similarly important, frequently addressed but largely unanswered question is the prognostic significance of HPVs in upper airway malignancies. Furthermore, the relationship between potentially malignant oral lesions and HPVs is even less clear (7, 11, 12, 18, 21, 25, 29).

Relatively few studies have been conducted in populations of patients with potentially malignant oral disorders that are sufficiently large to draw firm conclusions; only two works, those of Campisi et al. (7) and Ostwald et al. (29) were based on histological characterization. The prevalence rates they reported were comparable to but lower than those found in our Eastern Hungarian population. Similar to our data, Campisi et al. (7) found significantly higher HPV carriage rates in patients with oral lesions compared with controls. Ostwald et al. (29) did not include a control population so a similar comparison with our data was not possible.

In our study, the prevalence pattern in different lesions was peculiar. In OLP lesions we found HPVs more frequently in EA-OLP than in non-EA-OLP lesions; erosive and/or atrophic lesions carried HPVs with a similar frequency to that found in OL (Table 1). Consequently, four prevalence rates were observed, (i) control population, (ii) non-EA-OLP, (iii) EA-OLP and OL, and (iv) OSCC. This is also reflected in the prevalence on apparently healthy mucosa. This pattern is unlikely to be biased by differences in samples used, as in OSCC the sampling method is expected to underestimate HPV prevalence compared with using specimens of cells exfoliated from the tumor surface (16).

Though there is the possibility of false negative and false positive results, these are unlikely to bias the results presented. The PCR method used has excellent sensitivity (for 10 copies) (15), therefore this method is the best choice to avoid false negative results (22). Though the occurrence of false positives or cross-contamination can never entirely be ruled out, our data set is unlikely to be biased by these, because (i) all MY/GP nested assays were performed twice using two independent DNA extractions, and (ii) the presence of HPV was confirmed by type-specific PCR (as well as real-time PCR) assays for the most frequent mucosal genotypes. As HPVs unconfirmed by type-specific assays represent a very small proportion of the positives (none, three, two, and two in controls, OLP, OL, and OSCC patients, respectively), these could not alter the results profoundly even if some of them were false positives.

The only study that surveyed different potentially malignant and malignant oral lesions together in a population found a similar stepwise increase in prevalence (29). In contrast, our findings differ from those reported by Campisi et al. (7), reporting no significant difference in HPV prevalence between different clinical variants of OLP or OL. As sample sizes were comparable, this discrepancy may be explained by the profound differences in exposure to risk factors (smoking, alcohol consumption, and dietary habits) (6, 30).

The pattern found in HPV prevalence is strikingly paralleled by the viral copy number data; a stepwise increase in copy number averages was found. However, OLP and OL did not differ in this regard; patient groups with potentially malignant lesions fell between controls and OSCC patients. A further concordance with prevalence data was found for the non-EA-OLP group; the copy number average was closer to that of controls than to EA-OLP or any other patient group.

The HPV prevalence and copy number patterns found correspond strikingly to increasing malignant potential. The risk of progression to malignancy for non-EA-OLPs (approximately 0.5%) was shown to be substantially lower than in EA-OLPs (at least 3.5–4.0%) during similar follow-up periods, the latter risk was comparable to the average risk in OL (3–6%). However, some forms of OL may show substantially higher rates (17, 23, 28). In our study HPV prevalence in EA-OLP and in OL were comparable and well above the prevalence found in non-EA-OLP, which may suggest a connection between differences in HPV prevalence and malignant potential. Testing of these findings in follow-up investigations could shed light on the possibility of a potential prognostic application of HPV detection in oral potentially malignant disorders. If follow-up studies confirm that HPV carriage is associated with a higher risk of malignant transformation, then HPV detection may be of assistance in the assessment of risk of malignant progression for the early and non-invasive warning of potential OSCC development, and in defining a patient population that needs closer supervision.

The progressive increase in HPV prevalence from lesions with low malignant potential to manifest cancer together with the high OR may be explained in two ways. First, HPV plays a supplemental role in inducing or the progression of potentially malignant lesions, or second, lesions provide an environment that is increasingly favorable for HPVs as their malignant potential increases. In both cases the presence of HPV may have prognostic significance (see above). The first option is supported by finding high-risk HPVs consistently and by the low prevalence of low-risk HPV in lesions both in the present and in earlier studies (7, 11, 12, 18, 21, 25, 29).

Another argument for the real etiological role is that patients that are HPV positive in the lesion carried HPVs in the apparently healthy mucosa more frequently than controls or patients with HPV-negative lesions (Table 1). Patients with HPV-negative lesions showed a HPV carriage rate in their healthy mucosa similar to that of controls. HPVs found in patients with HPV-negative lesions were always low-risk types, whereas lesions harbored mostly high-risk HPVs. Consequently, the presence of HPVs on healthy mucosa seems to be a consequence of a HPV-positive lesion.

Several types of cytogenetic and epigenetic alterations were found in the background of malignant transformation of OLP and OL, including loss of p53

function, enhancement of antiapoptotic processes, and increased telomerase activity (27). These processes may be mediated by HPV E6 or E7 oncoproteins (1, 3, 8, 27). Considering the HPV prevalence pattern found, it is tempting to hypothesize that HPVs may play some etiological role in the malignant progression of potentially malignant oral disorders.

While it is obvious that HPVs alone are not capable of mediating malignant transformation of oral lesions, it is conceivable that they can enhance the effect of other carcinogens or agents provoking dysplasia. Our results suggest that the debate on the etiological role of HPVs is probably worth extending to include oral potentially malignant disorders, such as OLP and OL.

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