Role of the human papillomavirus in malignant transformation of oral leukoplakia distinct from oropharyngeal squamous cell carcinoma: A study of 76 patients with internal-control specimens

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Objective. This study sought to investigate the role of human papillomavirus (HPV) in the carcinogenesis of oral leukoplakia (OLK), with the oral cavity as the site of interest.

Study Design. A total of 76 patients (152 specimens) were enrolled in the study. The patients were divided into 2 groups: the malignant transformation of OLK (OLK-MT) group and the non−malignant transformation of OLK (OLK-non-MT) group. HPV reverse dot blot analysis, HPV DNA polymerase chain reaction (PCR), and p16INK4A immunohistochemistry (IHC) were used to determine HPV infection status.

Results. Transformation of OLK commonly occurred in the lateral/ventral tongue, buccal mucosa, and gingiva. On the basis of the initial analysis of specimens, only 5.3% (4 of 76) of patients were found to be HPV-16 positive, and these patients' final specimens yielded negative results. Overexpression of p16INK4A in the dysplastic stage was associated with the transformation of OLK ($P = .013$; odds ratio = 3.544).

Conclusions. Transformation of OLK was common in patients who are elderly, in females, and in nonsmokers/nondrinkers; lesions were located in the lateral/ventral tongue, with dysplasia and overexpressed p16INK4A seen during the initial stage. HPV may be an opportunistic infection in the oral cavity and may not be a cause of malignant transformation of OLK. p16INK4A expression, which initially increases and then diminishes or disappears, may be an early predictor of malignant transformation. (Oral Surg Oral Med Oral Pathol Oral Radiol 2019;128:273−279)

Oral leukoplakia (OLK), which is the most common lesion among oral potentially malignant disorders (OPMDs), is a precancerous lesion. The global incidence rate of OLK ranges from 0.5% to 3.46%, and the rate of malignant transformation of OLK ranges from 0.7% to 2.9%.[1] Patients with OLK who smoke or chew betel comprise approximately 70% to 90% of patients in the United States.[2] However, other risk factors, such as human papillomavirus (HPV) infection, excessive alcohol consumption, chronic inflammation, and local stimulation, have also been identified. Lesions commonly occur in the buccal mucosa, tongue, floor of the mouth, and palate as either isolated or multiple lesions. Several factors have been associated with the malignant potential of OLK, such as nonhomogeneous leukoplakia; being a non-smoker; large lesions (>5 mm); lesions located in the floor of the mouth, lateral/ventral tongue, or the soft palate; and degree of dysplasia.[3-6]

However, in recent years, researchers have discovered that OPMDs are associated with HPV infections.[7,8] HPV is commonly transmitted through sexual intercourse and is strictly epitheliotropic, infecting the epithelium of the genitourinary tract, respiratory mucosa, and oral mucosa.[9,10] Studies have reported that younger age at first intercourse, multiple sexual partners, oral sexual behavior, human immunodeficiency virus infection, herpes simplex virus infection, and smoking are associated with HPV-related oral lesions.[11-13] High-risk HPV (HR-HPV) has a stronger carcinogenic effect compared with low-risk HPV (LR-HPV). HR-HPV E6 and E7 proteins play key roles in carcinogenesis. HPV-16 and HPV-18 are the most commonly identified genotypes in cervical cancer, and their E6 and E7 proteins have been studied extensively. The HR-HPV E6 protein can degrade the tumor suppressor protein p53 via the ubiquitin pathway. The HR-HPV E7 protein mediates interactions with

Statement of Clinical Significance

Transformation of oral leukoplakia is more likely to occur in the elderly, females, and nonsmokers/drinkers, and it tends to be located in the lateral/ventral tongue, with dysplasia and overexpressed p16INK4A during the initial stage. Human papillomavirus in the oral cavity is uncommon and thus may not be the causative factor in the carcinogenesis of oral leukoplakia.

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product retinoblastoma (pRb), a tumor suppressor gene, leading to the degradation of pRb via proteasomal pathways.[14-16] However, the identification of HPV acting as the etiologic factor in OPMDs remains controversial. In various studies, among patients with OPMDs, the range of HPV prevalence has been reported as 0% to 85%.[17] In one study that included 118 patients with oral squamous cell carcinoma (OSCC), 72 with OLK, and 65 with oral lichen planus, the HPV rates were 43%, 22%, and 15%, respectively.[18] The incidence of HPV-associated oropharyngeal squamous cell carcinoma varied from 36.5% to 90%,[19-21] and HPV-16 was the main subtype (72%−96.1%).[22,23] HPV-16 and HPV-18 were also the predominant HPV subtypes associated with OLK and OSCC.[24] Many procedures with different sensitivities have been proposed for the detection of HPV, such as nucleic acid hybridization assays, signal amplification assays, and nucleic acid amplification methods.[25] The detection of HPV E6/E7 messenger RNA by reverse transcription polymerase chain reaction has been deemed the gold standard, although HPV DNA PCR combined with immunohistochemistry (IHC) testing for the overexpression of p16INK4A is nearly as specific and sensitive.[26]

The relationship between the epidemiology of HPV infection and OLK has been studied. However, the role of HPV in malignant transformation of OLK remains unclear. Therefore, we determined the HPV infection status in sequential specimens from patients with OLK. We also analyzed the association between the clinicopathologic characteristics and the outcome predictors in a Chinese population.

MATERIALS AND METHODS

Sample collection and patient information

Seventy-six patients diagnosed at the Department of Oral Pathology of the Shanghai 9th People's Hospital affiliated with the Shanghai Jiao Tong University School of Medicine between January 2000 and December 2015 who had internal-control specimens were enrolled in the present study. Sufficient follow-up information was available at least every 6 months from telephone follow-up surveys and electronic medical records. The follow-up data included gender, age, lesion location, lesion type, smoking status, alcohol consumption, treatment received, and the time of recurrence. In total, 92.1% (70 of 76) of the study patients were from eastern China, and 7.9% (6 of 76) were from other regions of China. The patients were divided into 2 groups, namely, the malignant transformation of OLK group (OLK-MT group) and the non−malignant transformation of OLK group (OLK-non-MT group), which were composed of 41 patients and 35 patients, respectively. The inclusion criteria were as follows: (1) Samples were selected for examination from the initial and the final specimens; (2) the time interval between the initial and final specimens was greater than 6 months; (3) the OLK-MT group comprised patients who had received a pathologic diagnosis of OLK at the initial examination and were found to have malignant transformation of OLK at the final examination; and (4) the OLK-non-MT group comprised patients who had received a pathologic diagnosis of OLK at the initial examination and were found to have OLK with severe dysplasia at the final examination. The exclusion criteria were as follows: (1) a pathologic diagnosis of oral lichen planus, oral submucous fibrosis, leukoedema, or leukokeratosis; and (2) lesions located in the oropharynx.

DNA extraction

Eight to fifteen 5- to 10-μm thick formalin-fixed paraffin-embedded (FFPE) sections were cut from each sample. If the sample surface had been exposed to the air, the first 2 to 3 sections were discarded. If the HPV DNA PCR results showed that there was positive expression in successive samples, a blank FFPE section was inserted in between successive samples before PCR was resumed to prevent false-positive results caused by cross-contamination. DNA was extracted from 152 specimens by using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and stored at −20°C.

HPV genotyping

The HPV reverse dot blot genotyping kit (Yaneng Bio Technology, Shenzhen, China), which can identify 23 HPV subtypes, including 18 HR-HPV types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -68, -73, -82, and -83) and 5 LR-HPV types (HPV-6, -11, -42, -43, and -44), was used. If a "blue spot" appeared in a certain position on the membrane, it suggested that the specimen had the corresponding HPV subtype infection.

PCR detection

Samples were verified via PCR for HPV DNA, with a HPV-16−positive or HPV-18−positive cervical cancer sample as the positive control, normal muscle tissue as the negative control, and deionized water as the blank control. PCR was performed by using the Takara Biotechnology system (Takara Biotechnology, Dalian, China). The primer sequences and reaction conditions were provided in an earlier study.[27]

IHC and scoring

IHC staining was used to detect the expression of p16INK4A in 152 samples. Four-μm -thick sections were cut from the FFPE samples, deparaffinized, and

rehydrated. Antigen retrieval was performed in 0.01 M citric acid buffer (pH 6.0) in a boiling water bath for 20 minutes. The sections were incubated in 3% hydrogen peroxide at room temperature for 20 minutes to block endogenous peroxidases. Subsequently, the sections were incubated with the primary p16INK4A monoclonal antibody (diluted 1:150; BD Biosciences, Pharmingen, CA;) overnight at 4˚C. Then, the sections were incubated with the secondary antibody at room temperature for 30 minutes, and the Envision system (Dako, Carpinteria, CA) was used to detect the signals. In the IHC detection of p16INK4A, a known HPV-associated cervical cancer section was used as the positive control, and a normal mucosal section was used as the negative control. Phosphate-buffered saline was substituted for the primary antibody in the blank control.

The p16INK4A expression appeared as tan particles in the cytoplasm and nucleus, and a positive result was defined as positive staining of greater than 70% of the squamous epithelial cells.[28]

Statistical Analysis

All statistical analyses were conducted with SPSS version 20.0 (SPSS Inc., Chicago, IL). The frequency data were analyzed by using the χ^2 test or Fisher's exact test. The paired data from 76 patients were analyzed by using the χ^2 test. Predictors of malignant transformation of OLK were assessed by univariate and multivariate logistic regression. P values were two-tailed, and $P \leq .05$ was considered statically significant.

RESULTS

Clinicopathologic characteristics

From the 76 study patients, a total of 152 specimens were obtained, and the initial and final specimens were used. The average follow-up time was 65.8 months in the OLK-MT group and 78.7 months in the OLK-non-MT group. In the OLK-MT group, 65.9% (27 of 41) were females, and 34.1% (14 of 41) were males, whereas in the OLK-non-MT group, 68.6% (24 of 35) were females, and 31.4% (11 of 35) were males. In addition, 23.7% (18 of 76) and 13.2% (10 of 76) patients with OLK had a history of smoking and drinking, respectively; all patients who reported smoking or drinking were males (Table I). Average patient age was 58.1 years at the time of malignant transformation of OLK, and 61.9 years at the time of OLK progressing to OLK with severe dysplasia. The average duration of transformation of OLK was 26.7 months; however, the average duration of progression of OLK to OLK with severe dysplasia was 38 months. Malignant transformation of OLK commonly occurred in the lateral/ventral tongue, buccal mucosa, and gingiva, accounting for 73.2% (30 of 41), 21.9% (9 of 41), and 4.9% (2 of 41), respectively. Malignant transformation of OLK from mild dysplasia, moderate dysplasia, and severe dysplasia occurred in 36.6% (15 of 41), 41.5% (17 of 41), and 21.9% (9 of 41), respectively (see Table I).

Table I. Distribution of clinicopathologic features in 76 patients

Characteristic	*OLK-MT group*	*OLK-non-MT group*	*Statistical significance*
Age (years)			
<60	22	21	NS
≥60	19	14	
Gender			
Male	14	11	NS
Female	27	24	
Tumor Site			
Ventral tongue	30	29	NS
Buccal mucosa	9	5	
Gingiva	2	1	
Smoking			
Nonsmoker	31	27	NS
Smoker	10	8	
Alcohol			
Nondrinker	33	33	NS
Drinker	8	2	
Epithelia Dysplasia			
Mild	15	11	NS
Moderate	17	22	
Severe	9	2	
Time to Progression (months)			
≤12	18	9	NS
≤24	10	9	
≤48	5	8	
≤72	3	2	
>72	5	7	
HPV-16			
Positive	2	2	NS
Negative	39	33	

HPV, human papillomavirus; *NS,* not significant; *OLK,* oral leukoplakia; *MT,* malignant transformation.

HPV status and p16INK4A in the carcinogenesis of OLK

The p16INK4A IHC results showed that 51.2% (21 of 41) of the initial samples and 24.4% (10 of 41) of the final samples were positive in the OLK-MT group ($P = .013$); however, 22.9% (8 of 35) of the initial samples and 20% (7 of 35) of the final samples were positive in the OLK-non-MT group ($P = 1.000$) (Table II; Figure 1A). The results of 23 HPV genotyping tests showed that only HPV-16 infection was detected and that no other HPV subtypes were present (Figure 2). HPV-16 infection was verified via HPV DNA PCR, and the results were consistent with the HPV genotyping method because only 4 (5.3%; 4 of 76) of the samples were positive, with 2 samples in each group (see Table I;

Table II. Comparison of p16INK4A positivity in different groups *via* χ^2 tests

Group	*p16INK4A positive*		*P value*	*Group*	*p16INK4A positive*		*P value*
	Initial	*Final*			*Initial*	*Final*	
OLK-MT group	21	10	.013*	OLK-non-MT group	8	7	1.000

*The difference in p16INK4A overexpression between the initial and final specimens was statistically significant in the OLK-MT group.*OLK,* oral leukoplakia; *MT,* malignant transformation.

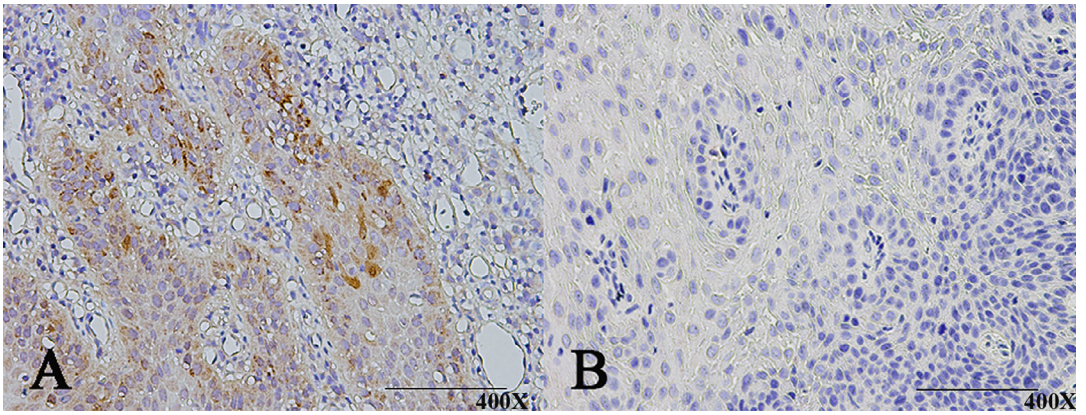

Fig. 1. Immunohistochemical images of p16INK4A.(**A),** Lesions with strong p16INK4A expression in the cytoplasm and nucleus in ≥70% of squamous epithelial cells (× 400 ×). (**B),** Lesions with negative staining for p16INK4A (× 400).

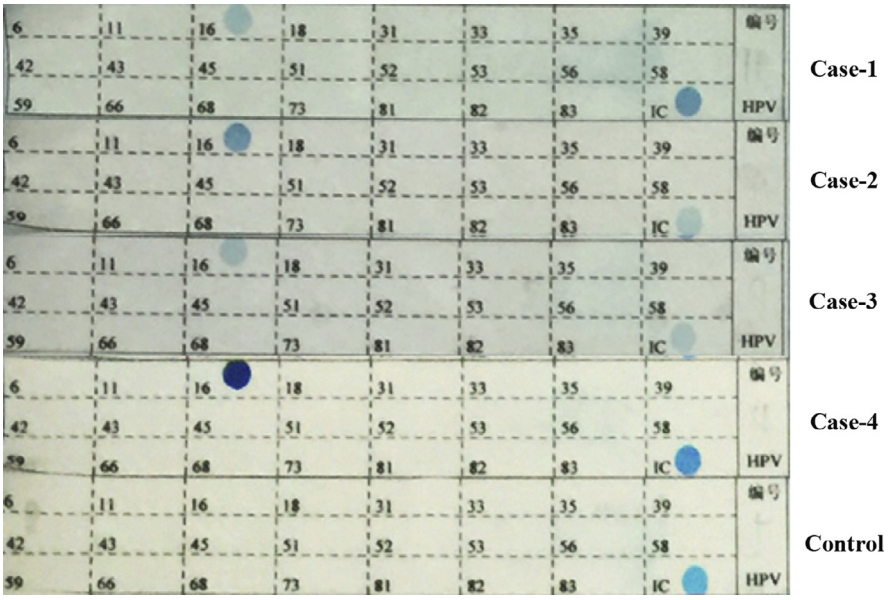

Fig. 2. Human papillomavirus (HPV)-Reverse Dot Blot: HPV genotyping that can identify 23 HPV subtypes; each Arabic numeral represents an HPV subtype. Four cases were identified as positive for HPV-16, and no positive results were obtained for the other tested subtypes of HPV. The different intensities of the positive "blue spot" were caused by different concentrations of DNA. A "blue spot" appeared in the internal control (IC) to indicate the high-quality DNA concentration of the specimen.

Figure 3). In addition, HPV-16 was detected only in the initial stages of OLK, with lesions located in the ventral tongue (3 patients) and in the buccal mucosa (1 patient), all of which were p16INK4A positive; these 4 patients were females who were non-smokers/nondrinkers (Table III).

Subsequently, we integrated the data from the 2 groups and determined that p16INK4A was overexpressed significantly in the initial specimens from the

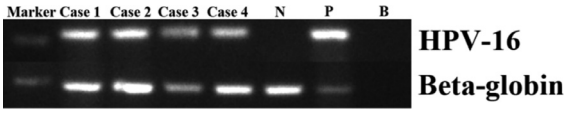

Fig. 3. Human papillomavirus (HPV)-16 DNA polymerase chain reaction (PCR). Agarose gel electrophoresis (AGE) results for HPV-16 E6: *B,* blank. *N,* negative; *P*, positive. Case 1, case 2, case 3, and case 4 were HPV-16 positive. Beta-globin was the internal reference.

Table III. Distribution of clinicopathologic features in the 4 human papillomavirus-16 (HPV-16)–positive cases

Characteristic	*HPV-16 positive*
Gender	Female
Average age	49.8 years
Tumor site	Ventral tongue (3 patients), buccal mucosa (1 patient)
Smoking/Drinking	Never
Epithelia dysplasia	Mild dysplasia (2 patients), moderate dysplasia (2 patients)
p16INK4A	Positive (≥70%)
Periodic detection	Initial stage of oral leukoplakia (OLK)

OLK-MT group than in the initial specimens from the OLK-non-MT group ($P = .011$) (Table IV). Logistic regression analysis indicated that there was an increased risk of malignant transformation of OLK when p16INK4A was overexpressed during the initial stage of OLK, followed by a decrease or disappearance during the final stage ($P = .013$; odds ratio = 3.544; 95% confidence interval [CI] 1.305–9.621) (see Table IV). The area under the receiver operating characteristic curve was 0.675 (95% CI 0.552–0.797, $P = .009$) (Figure 4).

DISCUSSION

This investigation was a study of OLK, with use of internal-control specimens and a large sample size. The strength of our study was that it included 76 patients with internal-control specimens to investigate the risk factors in the carcinogenesis of OLK. In contrast to most other studies on this topic, which focused on a particular stage of OLK or OSCC, this study provided a scientific basis for the potential prevention of malignant transformation of OLK.[29-31] However, there were some limitations to this study, such as lack of specimens with location of lesions in the floor of mouth and in the palate. No lesions at either site exhibited carcinogenesis, as indicated by the internal-control specimens, whereas these 2 sites have been shown to have an increased risk of oral cancer in U.S. studies and other investigations.[4,32,33]

Many studies have reported that 17% to 25% of patients with OLK are diagnosed with epithelial dysplasia and that the rate of malignant transformation of OLK ranges from 0.13% to 17.5%.[5,6,34-36] In addition, the 5-year cumulative rate of malignant transformation of OLK ranges from 1.6% to 14.5%.[6,35] Our results showed that the mean time to malignant transformation commonly was less than 2 years after the initial diagnosis. In particular, an increased risk of malignant transformation of OLK was found in patients who were elderly, female, and nonsmokers/nondrinkers and those who had lesions on the lateral/ventral tongue.

HPV-16 is the most frequently detected HPV subtype in oral cancers, but the role of HPV in malignant transformation is extremely controversial. In this study, the combination of HPV genotyping, HPV DNA PCR, and p16INK4 IHC had a synergistic effect and improved the accuracy of our results. Unlike other studies in which HPV was detected in only one stage of OLK or OSCC separately and in which the role of HPV in the development of OLK could not be explained, our results showed that HPV-16 had a low detection rate (5.3%; 4 of 76). HPV-16 was the only HPV subtype detected and was identified only in females. Interestingly, however, HPV-16 was detected only during the initial stage of OLK, with results for HPV-16 becoming negative in the final stage of this condition; this finding indicated that HPV-16 might be an opportunistic infection during the initial stage of OLK, rather than a cause of the carcinogenesis of OLK.

In our study, we observed that p16INK4A was highly expressed during the initial stage of OLK but exhibited significantly reduced expression during malignant transformation of OLK to OSCC (51.2% vs 24.4%; $P = .013$); however, p16INK4A expression was similar between the initial and final specimens in the OLK-non-MT group (22.9% vs 20.0%; $P = 1.000$). It is worth noting that the p16INK4A expression pattern first increased but then

Table IV. Chi-square and logistic regression analyses of p16INK4A in 76 patients

Variable	χ^2	*Logistic regression*		
	P value	*P value*	*Odd ratio*	*95% confidence interval*
p16INK4A	.011*	.013†	3.544‡	1.305–9.621

*p16INK4A overexpression between OLK-MT group and OLK-non-MT group was statistically significant.
†After adjusting age, sex, smoking, drinking, dysplasia and site in multivariate analysis.
‡p16INK4A overexpression in the initial stage had 3.544 times the risk of cancer than p16INK4A lower or absence expression.

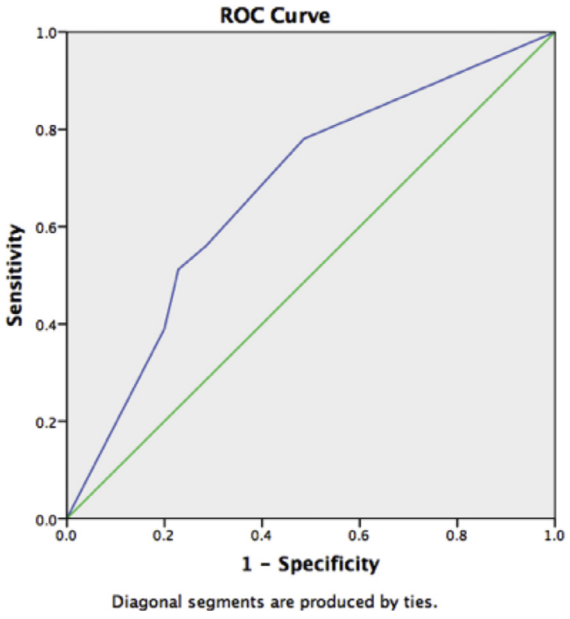

Fig. 4. Receiver operating characteristics (ROC) curve of p16INK4A expression. The area under the curve (AUC) is 0.675 (95% confidence interval [CI] 0.552−0.797; $P = .009$), which represents p16INK4A overexpression in the initial stage has diagnostic value in the carcinogenesis of oral leukoplakia (OLK).

decreased or disappeared in patients who experienced malignant transformation of OLK. The overexpression of p16INK4A exerts a protective effect in the initial phase of a disease, after which it decreases or disappears as the disease progresses.[37-44] In addition, after adjustment for other clinicopathologic features, the high expression of p16INK4A in the early stage of OLK indicated that lesions might have an increased tendency toward malignancy ($P = .013$; odds ratio = 3.544). Therefore, we suggest that p16INK4A expression may serve as a molecular driver of malignancy and thus could be used as a dynamic marker in the detection of malignant transformation of OLK.

CONCLUSIONS

The rate of HPV infection in the oral cavity is very low, and the role of HPV in malignant transformation of OLK has perhaps been overemphasized. However, p16INK4A expression may be a predictor of carcinogenesis of OLK.

DISCLOSURE

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