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Original Article

Human papillomavirus genotypes and p16 expression in oral leukoplakia and squamous cell carcinoma

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Abstract: Several studies have shown a broad variation in the prevalence of human papillomavirus (HPV) in oral leukoplakia (OLK) and oral squamous cell carcinoma (OSCC), whereas the relationship is less well-defined and specific HPV genotypes lack examination in OLK. In the present study, the role of HPV and surrogate p16 expression was investigated to explore the correlation and pathogenesis in OLK and OSCC. Polymerase chain reaction (PCR) and flow-through hybridization technology were utilized to detect HPV genotypes in oral exfoliated cells from 30 healthy volunteers, 103 OLK and 30 OSCC patients. Expression of p16 was assessed by immunohistochemistry (IHC) in biopsies from these OLK and OSCC, in addition to 15 normal oral mucosal tissues as the control group. The healthy controls showed 3.3% (1/30) HPV presence; In OLK and OSCC, the detection rate was 4.9% (5/103), 3.3% (1/30), respectively. No significant relationship between HPV and OLK or OSCC was observed when compared with the control group (P>0.05). All 6 HPV-positive OLK and OSCC cases had p16 overexpression. But the sensitivity of p16 IHC was poor, because 88.4% (38/43) of p16 over-expressed OLK were HPV negative. There was no statistical significance between HPV and the sex, age, site, alcohol consumption, or smoking. These findings suggested HPV had a low prevalence in OLK and OSCC. This suggests the detection of HPV genotypes by PCR in exfoliated cells combined with p16 IHC may be more accurate to represent HPV infection.

Keywords: Oral leukoplakia, human papillomavirus, oral squamous cell carcinoma, p16

Introduction

Oral squamous cell carcinoma (OSCC) currently accounts for more than 90% of oral malignancies in China [1]. Oral leukoplakia (OLK) is the most common pre-malignant lesion of the oral cavity, and 1.36% to 17.9% of OLK can develop into OSCC [2, 3]. The role of human papillomavirus (HPV) in OSCC and OLK is still unclear.

HPV mainly attacks squamous cells of human skin and mucous membranes. HPV infection is etiologically involved in cervical, vaginal, anal, and head-neck tumors, particularly oropharyngeal squamous cell carcinoma (OPSCC). Currently more than 150 types of HPV have been identified based on genomic sequence variations [4]. According to the epidemiological distribution and carcinogenic power in uterine cervical cancers, HPV is classified as high-risk and low-risk types.

The HPV detection rates in OSCC and OLK vary from 0 to 100% [5], or 0-58.3% [6, 7], respectively, mainly related to different specimen sources, detection methods, or targets. [8]. Specimens are mostly taken from fresh frozen or paraffin-embedded tissues. HPV detection methods consist of in situ hybridization, Hybrid Capture, PCR and p16 IHC [9]. p16 is integral to retinoblastoma protein (Rb) that mediates regulation of G1-S phase of the cell cycle. When the HPV viral genome integrates into the cellular DNA, oncoprotein E7 is expressed by host cells

and promotes the phosphorylation of Rb, thereby causing accumulation of p16 protein due to a feedback mechanism [10]. Hence, p16 is considered to be a surrogate marker of HPV positivity [11]. However, a literature search revealed meager data exclusively correlating HPV and p16 in OLK and OSCC.

The aim of this study was to identify the prevalence and types of HPV by PCR in exfoliated cells of a group of Chinese patients with OLK and OSCC. On this basis, to establish an appropriate method for monitoring HPV infection, IHC was used to detect p16 protein in OLK and OSCC tissues.

Materials and methods

Subjects

Between December 2016 and October 2017, oral lesion swabs were collected from 103 OLK and 30 OSCC patients who referred to the Department of Oral Mucosal Diseases and Maxillofacial Surgery, Ninth People's Hospital, Shanghai Jiaotong University School of Medicine for HPV detection. The swabs were then kept in a sterile tube and stored at -20°C immediately. 30 healthy controls were enrolled. In this cross-sectional survey, participants were recruited who met the following criteria: (1) not pregnant when swabs were collected; (2) no history of immunodeficiency disease; (3) no history of radiation or chemotherapy. The exclusion criteria included having a history of other malignant tumors or oral mucosa diseases. All the participants were investigated with a demographic characteristics-related questionnaire. The OLK and OSCC patients further underwent histopathological examination [12, 13]. The study was approved by the Ethics Committee of Shanghai Ninth People's Hospital (2016-79-T36). Informed written consent was obtained from all participants.

Detection and genotypes of HPV DNA by PCR

1 μl supernatant of HPV DNA was extracted from the swabs and then amplified with the L1 consensus HPV MY09/11 primer set. The sequences of the forward and reverse primers used were 5'-CGTCCMARRGGAWACTGATG-3' (MY09) and 5'-GCMCAGGWCATAAYAATGC-3' (MY11). The protocol was carried out in a GeneAmp PCR System 9700 (Chaozhou Hybribio Ltd, Guangdong, China) thermal cycler at 95°C for 9 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. All samples were further genotyped by flow-though hybridization and gene chips. This genotype kit (Chaozhou Hybribio Ltd, Guangdong, China) is able to identify 37 different HPV genotypes, including 17 HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) and 20 LR-HPV genotypes (6, 11, 26, 34, 40, 42, 43, 44, 54, 55, 57, 61, 67, 69, 70, 71, 72, 81, 83, 84).

Immunohistochemical staining for p16

Formalin-fixed paraffin-embedded tissues from all OLK and OSCC patients were sliced into 4 μm thick sections. 15 cases of the control group were excised from the impacted teeth extraction or dental implants surgery. For antigen retrieval, sections were boiled in 0.01 sodium citrate buffer (pH 6.0) for 20 minutes. Samples were exposed to the mouse anti-human monoclonal antibody of p16 (1:100 dilution; Boster, Wuhan, China) for 30 minutes and incubated overnight at 4°C. After rinsing with PBS, pH 7.6, the sections were treated with secondary antibody according to the manufacturer's manual. Subsequently, all sections were visualized by 3,3-diaminobenzidine. The number of positive cells was counted in 5 microscopic fields under light microscopy (×200 magnification) and brown staining of nuclei and/or cytoplasm was interpreted as positive. p16 immunoreactivity was classified as negative (-), moderate (+), and over-expression (++) by multiplying the percentage of positive cells (0-5%, 6-70%, 71-100%) [14, 15].

Statistical analysis

SPSS23.0 (IBM, Armonk, NY) software was used for the statistical analysis. The chi-square test was used to analyze data between different groups of categorical variables. A *P*-value of <0.05 was considered statistically significant.

Results

Prevalence of HPV

The characteristics of the 163 cases and controls included in the analysis are described in **Table 1**. The prevalence of HPV DNA in exfoliated cell samples was higher among OLK cases

Table 1. Characteristics of the control, OLK and OSCC groups

		Control	OLK	OSCC
Mean age (range)		50 (21-74)	55 (26-84)	58 (36-84)
Gender	Male	14	48	14
	Female	16	55	16
Tobacco	No	22	62	18
	Yes	8	41	12
Alcohol	No	21	77	20
	Yes	9	26	10
Lesion site	Tongue		57	13
	Buccal		33	7
	Gingiva		10	8
	Palate		2	0
	Floor of mouth		1	2

OLK, oral leukoplakia; OSCC, oral squamous cell carcinoma.

Table 2. Analysis of HPV of the control, OLK, and OSCC groups

	Group	Age	Sex	Site	Tobacco	Alcohol	HPV type
1	OLK	71	Female	Tongue	No	No	HPV35, 51
2	OLK	57	Female	Tongue	No	No	HPV40
3	OLK	65	Female	Tongue	No	No	HPV82
4	OLK	30	Female	Buccal	No	No	HPV18
5	OLK	66	Male	Palate	Yes	Yes	HPV39
6	OSCC	60	Female	Gingiva	No	No	HPV67
7	Control	30	Male	--	No	No	HPV68

HPV, human papillomavirus; OLK, oral leukoplakia; OSCC, oral squamous cell carcinoma.

(4.9%, 5/103) than among OSCC cases (3.3%, 1/30) and controls (3.3%, 1/30). No statistically significant difference was seen in HPV expression among the groups (χ^2=2.8, *P*=0.9). HPV genotypes were detected as shown in **Table 2**. HPV-18, 35, 39, 40, 51 and 82 were identified in OLK cases. HPV-35, 51 were recognized in one woman.

Relationship between HPV and patient characteristics

Clinicopathologic characteristics of OLK patients according to HPV status are presented in **Table 3**. Although the mean age of the cases was around 55 years, we found a higher prevalence of HPV among older cases. The proportion of HPV-positive OLK cases was higher in females (80%) than males (20%), although this was not statistically significant. HPV-positive cases were common in mild and moderate dysplastic lesions, while none were detected among severe dysplasia. By statistical analysis, no significant relationship was exhibited with HPV DNA detection in patient's tobacco use and alcohol drinking.

The HPV-positive OSCC case was found in a 60-year-old woman. No significant differences were found between HPV-positive OSCC and HPV-negative samples according to gender, tobacco use, alcohol drinking, tumor localization and grade.

Expression patterns of p16 and correlation with HPV status

Further to validate our results, 103 OLK and 30 OSCC samples were evaluated using IHC for p16 expression. The results are shown in **Figure 1**. The rate of p16 over-expression was 66.7% (10/15) in normal control. Among 103 OLK specimens, 43 cases (41.8%) were p16 over-expressed. Over-expression p16 of the entire tumor area was observed in 3.3% (1/30) of OSCC, which the case was HPV-positive. 50% (15/30) of the OSCCs displayed moderate p16 expression, while 46.7% (14/30) did not express p16.

p16 over-expression in OLK did not correlate with clinical variables such as age, sex, lesion site, tobacco and alcohol use (**Table 3**). However, there was a negative correlation between the p16 expression and dysplasia level in OLK. p16 over-expression correlated with HPV positivity in OLK patients (*P*<0.05).

Discussion

There is increasing evidence that HPV is the third causative factor of head and neck tumors after smoking and drinking [16], with HPV16 and 18 as the major types. The HPV contribution in oral cavity malignancies has been an issue of debate. In this case-control study we examined swabs from the lesion surface by

Table 3. Clinicopathologic variables of the oral leukoplakia patients in relation to HPV status and p16 expression

		HPV-PCR		P	p16 expression		P
		Positive	Negative		-/+	++	
Gender	Male	4	51	0.45	32	23	0.99
	Female	1	47		28	20	
Age groups	<60	2	59	0.67	34	27	0.53
	≥60	3	39		26	16	
Tobacco	No	4	58	0.65	33	29	0.20
	Yes	1	40		27	14	
Alcohol	No	4	73	1.00	43	34	0.39
	Yes	1	25		17	9	
Lesion site	Tongue	3	54	0.78	32	25	0.11
	Buccal	1	32		17	16	
	Others	1	12		11	2	
Clinical appearance	Homogeneous	4	56	0.59	33	27	0.43
	Non-homogeneous	1	42		27	16	
Epithelial dysplasia	Mild	3	53	0.24	27	29	0.02*
	Moderate	2	22		14	10	
	Severe	0	23		19	4	

*P<0.05. HPV, human papillomavirus; PCR, polymerase chain reaction.

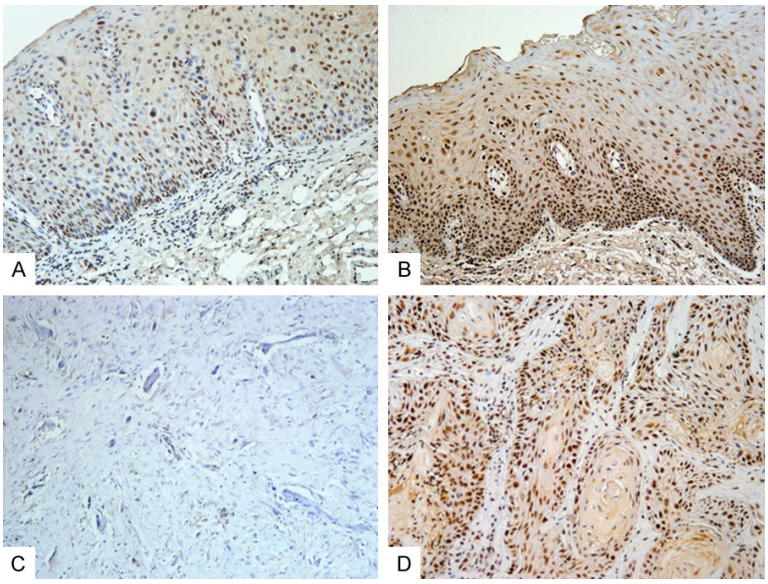

Figure 1. p16 detection by immunohistochemistry with comments on HPV status, assessed by PCR in OLK and OSCC. A. p16-positive OLK showing moderate expression (score, +); HPV DNA (-). B. p16-positive OLK showing overexpression (score, ++); HPV82 (+). C. p16-negative OSCC showing no immunoreactivity (score, -); HPV DNA (-). D. p16-positive OSCC showing overexpression (score, ++); HPV67 (+). Original magnification, ×200.

PCR based detection of HPV viral genome. Compared with traditional methods, the brush test utilized was non-invasive, and ethically accepted by patients. Some scholars have found that the sensitivity of HPV detection in exfoliated cells was higher than that in tissues [17]. This genotyping procedure can detect 37 types of HPV at one time, basically covers the common types detected in the oral cavity [18], and co-infection may be present.

Numerous authors have reported the presence of different HPV subtypes in OLK including HPV6, 11, 16, 18, 31, 33, 52, 53, 68, 81 [19-21]. Yang et al. [21] found that 4 OLK patients simultaneously expressed HPV16, 18; Luo et al. [22] detected 1 OLK with HPV53, 68 co-positive. Our data showed HPV18, 39, 40, and 82 respectively in 4 samples; in addition there was a case with HPV35 and 51 positive. The potential role of different types of HPV in OLK is unknown; the follow-up observation is needed.

We observed HPV positivity in 4.9% of OLK and 3.3% of OSCC. This was in concordance with

previous studies from America and Japan, exhibiting a low prevalence of HPV [5, 23]. It is also observed that HPV infection is more prominent in other countries [7, 20]. Considering sample size limitation, different detection methods, ethnic and geographical factors, HPV frequency in the oral cavity is variable.

It should be noted that most of the HPV-positive specimens in this study belonged to females and were more likely to occur in patients without environmental risk factors (non-smokers or non-drinkers). Our study suggests that HPV is probably linked with a specific subset of OLK and OSCC, which may represent a distinct pathogenic mechanism.

Our data showed that HPV tends to be higher in OLK than in normal controls and OSCC; HPV-positive OLK had a tendency to correlate with mild and moderate dysplasia, although the sample size was limited. Previous studies have also reported the association of HPV with OLK [24]. Yang et al. [22] collected 12 paired cases of OLK and OSCC, 4 cases were infected with HPV16/18 in the non-cancerous stage, and were detected HPV-negative in their cancerous stage. A "hit and run" theory has been proposed to explain that HPV is not required to maintain the malignant state. This notion confirms our speculation that HPV may have an effect in the early stage of OLK carcinogenesis.

HPV DNA detection by PCR cannot distinguish whether HPV is involved in a transient or active infection [25]. Expression of E6/E7 mRNA is considered the gold standard for HPV infection and the result shows a high consistency with p16 IHC [26]. It is well documented that p16 over-expression can sensitively represent HPV infection in OPSCC [27]. Nevertheless, the rate of HPV positivity in OSCC was considerably lower compared to previous reports on OPSCC [28]. Our results showed all the 6 HPV-positive cases of OLK and OSCC exhibited p16 over-expression. However, 88.4% (38/43) of p16 over-expressed OLK cases were HPV negative. Recent data indicated that the concordance between p16 over-expression and HPV positivity in OSCC was weak [29]. For instance, Dediol et al. [30] showed that the sensitivity of p16 for HPV in OSCC was 17% with 22 cases of p16 over-expression being HPV negative. Furthermore, some other mechanisms (e.g., stress, inflammation, aging, senescence, etc.) than E7 oncoprotein may lead to p16 over-expression [31-33]. Hence, p16 expression lacks specificity for the association of HPV in OLK and should not be independently used as a marker for the detection of HPV status. IHC detection of p16 may not only be a morphological indicator for early prevention of OLK, but also could get higher accuracy in assigning diagnosis to HPV infection when combines with HPV genotyping test by PCR.

Regarding the distribution of high-risk and low-risk HPV in OLK and OSCC, some believe that the detection rate of HR-HPV is higher [34, 35]. It has been observed that HR-HPV infection participates in over-expression of p16 protein [36]. However, low-risk subtypes could also be found in the collective presented with p16 over-expression. LR-HPV infection may have a pathogenic effect in oral mucosa. The oncogenic potential of different genotypes may differ from the distribution of cervical and oropharyngeal cancer.

In conclusion, the present study has demonstrated, besides the HPV DNA detection in OLK and OSCC, that further p16 expression would be beneficial for categorizing oncogenic HPV DNA, and the specificity is indefinite. A low prevalence of HPV was detected in OLK and OSCC though different types of HPV can be detected. Considerably less common than that in OPSCC, HPV infection may play a minor role for the histopathologic progression and carcinogenesis of OLK and OSCC.

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Disclosure of conflict of interest

None.

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