RESEARCH ARTICLE

Prevalence of Human Papillomavirus Infection in Oral Squamous Cell Carcinoma: a Case-control Study in Wuhan, China

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Abstract

   High risk forms of the human papilloma virus (HPV) are generally accepted as necessary causative agents for cervical cancer. Recently, a possible relation between HPV and oral squamous cell carcinoma (OSCC) has also been noticed. The present study was conducted to investigate the prevalence of HPV infection in OSCCs in Wuhan city. DNA samples were collected from fresh tissues in 200 patients with OSCC and 68 normal controls. The polymerase chain reaction and direct sequencing were used to identify the HPV types in the samples. The prevalence of HPV of all types in the OSCC group was higher than in the control group (55/200 vs 2/68, OR=11.5, 95% CI=2.6-50.2). HPV16 and HPV18 were the main types detected, with HPV6 was the only low-risk type identified. High-risk HPV types HPV16 and HPV18 are prevalent in OSCC patients and may participate in the development of OSCC with traditional risk factors, tobacco and alcohol, possibly exerting synergistic effects. The results of multinomial logistic regression showed that those who smoked, consumed alcohol and with HPV infection have the highest risk of developing oral cancer (OR=13.3, 95% CI=3.1-56.8). Adjusted for age, smoking and alcohol use, HPV infection was independently associated with oral squamous cell carcinoma.

Keywords: HPV - oral SCC - PCR - epidemiology - risk factor - interaction - smoking - alcohol

Introduction

   Oral cancer, the sixth most common cancer worldwide, includes a group of neoplasms affecting any region of the oral cavity, oropharyngeal regions and salivary glands, which accounts for the most majority of head and neck cancer. The term OSCC (oral squamous cell carcinoma) tends to be used to describe squamous cell carcinoma of oral cancer (Markopoulos, 2012). OSCC is the most common histologic type (Argiris et al., 2008; Marur et al., 2010), which constitutes about 90% of all oral malignancies. In 2013, there will be an estimated 36000 new cases and 6850 deaths from oral cancer in America (Mitka, 2013).

   Tobacco, alcohol, poor oral hygiene, chronic inflammation and genetics are important risk factors for OSCC. Syrjänen et al. first proposed in 1983 that HPV might be involved in the development of some OSCCs based on morphological and immunohistochemical features indicative of HPV infection (Syrjanen et al., 1983). In recent years, HPV has been recognized as an independent risk factor for a subset of OSCC (Choi et al., 2008; Marur et al., 2010). More than 200 different human papillomavirus (HPVs) types have been characterized (Psyrri et al., 2008), and at least 40 types can infect human genital tract (Munoz et al., 2003), of which HPV16 and HPV18 as main high-risk types are more closely

linked with malignant tumors. Recent study has shown that HPV genomes integrated in head and neck cancer express chimeric virus-cell mRNAs, which is similar to those found in cervical cancers (Lace et al., 2011). HPV-associated OSCCs tend to be poorly differentiated with basaloid features in histology, frequently presenting at an advanced stage. Patients with HPV-positive OSCC often have an improved survival compared with HPV-negative OSCC patients (Fakhry et al., 2008; Psyrri et al., 2008; Pai et al., 2009; Gillison et al., 2012).

   Since HPV-16 DNA was first detected in head and neck squamous cell carcinoma (HNSCC) in 1985 (Loning et al., 1985), HPV sequences have been repeatedly detected in a variable proportion of OSCC, from as few as 3.9% to 43.5% (Herrero et al., 2003; Lee et al., 2012; Mondal et al., 2013). It was reported that the incidence of OSCC that potentially HPV-related increased in the USA from 1973-2004 partly due to the changes in sexual behavior, especially in young adults (Chaturvedi et al., 2008; Jemal et al., 2011). A meta-analysis (1988-2007) showed that the prevalence of HPV DNA in OSCC was 38.1% (Termine et al., 2008). HPV-16 genomic DNA can be detected in 16.5% to 19% of oral cancers in the USA (Schwartz et al., 1998; Smith et al., 2004). While the meta-analysis of Chinese population indicated that the overall rate of HPV-positive OSCC was 58.0% and HPV-16 infection accounted for 47.47% of OSCC (Zhu et al., 2012). Our

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previous study with 44 patients showed that the incidence of HPV infection in OSCC in Wuhan was 40.4% (Zhao et al., 2009). This discrepancy might attribute to the different anatomic locations of tumors and techniques used to detect HPV-DNA (Termine et al., 2008). But the epidemiology studies including our previous study in Chinese population were almost limited by small samples, which cannot precisely reflect the real situation of HPV infection in China. Thus, in order to help understand the epidemiology of HPV infection in OSCC in Wuhan city of China, we performed a case-control study that enrolled 200 samples and 68 controls.

Materials and Methods

Patients

This case-control study was conducted in patients with newly diagnosed squamous cell carcinomas of the oral cavity seen at School & Hospital of Stomatology, Wuhan University and the Central Hospital of Wuhan between 2009 and 2013. 200 qualified case patients have a confirmed diagnosis of oral squamous cell carcinomas without tumors in other parts of the body. The control group consisted of 68 patients without a history of cancer who were seen for 3rd molar removal between 2009 and 2013 at those two hospitals. All patients enrolled were informed and consent to this study. Sample collection was permitted only when agreements were reached.

Specimen and Data Collection

Specimens were collected from both tumor tissues of case patients and normal tissues of control patients. All tissue samples were collected and stored at -80°C immediately until use.

All the information about patient was obtained from direct patient interview and medical records, which was written down by collectors. The obtained information included patient's demographic characteristics, medical history, family history of cancer, lifetime history of tobacco and alcohol use and pathological diagnosis.

DNA extraction and PCR

DNA extraction of samples was accomplished with QIAamp Extraction Kit (Qiagen, USA) within 48h after specimens were obtained. Human β-globin gene was amplified by polymerase chain reaction (PCR) with PC03/PC04 primers to confirm the integrity of DNA. Samples were analyzed by PCR performed with GP5+/GP6+ primers that targeting conserved sequences within the capsid gene L1. Positive control (containing HPV18 DNA extracted from Hela cells) and negative control (containing DNA purified from HPV negative cell line 293) were used for each reaction. Each reaction contained 50ul mixture that is consistent of 50mM KCI, 10mM Tris-HCI, 200μM of each dNTP, 1 U DNA polymerase (Fermentas, Canada), 100pmol of each primer of GP5+/GP6+ and 1 μg DNA template. A 3 min denaturation step at 94°C was followed by 35 cycles of amplification with a PCR Thermo Cycler (AB Applied Biosystems, USA). Each cycle included a 30s denaturation step at 94°C, an annealing step to 45°C for 30s and a chain elongation step to 72°C for 50s. The

last cycle was extended by a 10 min elongation at 72°C. The reaction temperature was adjusted according to the instruction of the DNA polymerase. PCR products were run on a 1.5% agarose gel containing 0.1ul/ml Dured and visualized under an ultraviolet transilluminator. The positive PCR products were then sequenced by Sangon Biotech (Sangon, China) for the HPV genotyping.

Statistical analysis

Analyses were conducted with SPSS20.0 for Mac (SPSS Inc, USA). Standard method was used for the statistical analysis of case-control study. Multinomial logistic regression models were used to estimate odds ratios (OR) for the association between oral cancer risk and factors in this study. The associated 95% confidence intervals (CIs) were calculated using the standard errors from the corresponding logistic regression models and the normal approximation. A *p*-value of less than 0.05 for associations was considered significant.

Results

In this study, there were 200 case patients and 68 control patients were eligible, of which the tumor samples were squamous cell origin. Male patients account for 71.5% in OSCC group.

The overall prevalence rate of HPV infection was higher in case patients than in control subjects (27.5% versus 2.9%, respectively, Table 1). Compared to HPV-negative patients, HPV- positive patients had an increased risk of OSCC (OR=11.5, 95% CI=2.6-50.2) when odd ratios were adjusted for age, tobacco use and alcohol consumption. The presence of HPV DNA in OSCC was not associated with tumor's anatomic location and clinical stage.

HPV16 was detected in 39 of 200 OSCC patients (19.5%), which accounted for 71% of HPV positive tumor samples. HPV 18 was detected in 15 of 200 tumor samples (7.5%). No HPV 16 was found in control specimens, only HPV 18 was detected in control samples (2.9%, 2/68). There was only one patient harboring coinfection of HPV6 and HPV 18 in OSCC group. No other HPV types were found in this study.

Patient demographic risk factors were examined and odds ratios were calculated, adjusting for age, tobacco use, alcohol consumption and HPV status (Table 2). Logistic regression analysis suggested that risk of OSCC increased with age (≤55 years versus >55 years, OR=3.4, 95% CI =1.8-6.7). OSCC is apt to be a disease that

Table 1. Human Papillomavirus (HPV) Prevalence and Risk of Head and Neck Cancer

Result	No. of case patients (%) (N=200)	No. of control subjects (%) (N=68)	OR (95% CI)
HPV negative	145 (72.5)	66 (97.1)	1.0 (referent)
HPV positive	55 (27.5)	2 (2.9)	11.5 (2.6to50.2)
HPV16	39 (19.5)	0	
HPV18	15 (7.5)	2 (2.9)	
HPV coinfection	1 (0.5)	0	

OR, odds ratio; CI, confidence interval

Prevalence of Human Papillomavirus Infection in Oral Squamous Cell Carcinoma in Wuhan, China

Table 2. Demographic Characteristics and Risk Factors for OSCC

	OSCC (%) (N=200)	Control (%) (N=68)	P-value*	OR (95%CI)
Age				
18-55	81 (40.5)	51 (75.0)		1.0 (referent)
≥56	119 (59.5)	17 (25.0)	0.000	3.4 (1.8-6.7)
Sex				
male	143 (71.5)	27 (39.7)	0.019	2.4 (1.2-5.0)
female	57 (28.5)	41 (60.3)		1.0 (referent)
Tobacco use				
yes	100 (50.0)	13 (19.1)	0.015	2.7 (1.2-6.0)
no	100 (50.0)	55 (80.9)		1.0 (referent)
Alcohol use				
yes	71 (35.5)	6 (8.8)	0.063	2.7 (0.9-7.7)
no	129 (64.5)	62 (91.2)		1.0 (referent)
Anatomic site				
tongue	74 (37.0)	0		
gingiva	38 (19.0)	68 (100.0)		
buccal mucosa	31 (15.5)	0		
floor of mouse	16 (8.0)	0		
oropharynx	9 (4.5)	0		
soft palate	8 (4.0)	0		
hard palate	6 (3.0)	0		
lip	7 (3.5)	0		
alveolar bone	11 (5.5)	0		
Tumor stage				
I-II	181 (90.5)			
III-IV	19 (9.5)			

OR, odds ratio; CI, confidence interval; *The values were ajusted for age (18-55 and ≥56), tobacco use, alcohol consumption and HPV status

Table 3. Multivarible Logistic Regression Model of Risk Factors for OSCC

Variables	No. of patients (N=268)	*P* value	OR (95% CI)
none*	117		1.0
HPV only	28	0.001	12.5 (3.0-52.9)
Smoking only	35	0.000	4.2 (2.2-8.2)
Alcohol consumption only	7	0.000	5.7 (2.3-13.8)
HPV + Smoking	11	0.000	13.3 (3.1-56.7)
HPV + Alcohol	3	0.001	13.1 (3.1-56.1)
Smoking + Alcohol	52	0.001	3.2 (1.2-8.6)
HPV+Smoking+Alcohol	15	0.000	13.3 (3.1-56.8)

OR, odds ratio; CI, confidence interval; *Reference group

occurs mainly in the elderly. It seemed that men were more inclined to have cancers than women (OR=2.4, 95% CI=1.2-5.0). Compared with never-users of tobacco and alcohol, the tobacco users (OR=2.7, 95% CI=1.2-6.0) and alcohol users (OR=2.7, 95% CI=0.9-7.7) have statistically significantly increased risk of OSCC. Tobacco use and HPV infection were two independent risks in our case-control study. However, the alcohol use is not a significant risk factor according to our result (*p*>0.05). However, when odds ratios were just adjusted for tobacco and alcohol, the alcohol use is statistically significant risk factor for OSCC (*p*<0.05).

The multinomial logistic regression model for exploring the relevant risk factors for developing OSCC also showed the synergistic effect between the risk factors. People who smoke, with or without alcohol consumption, and with HPV infection had the highest risk of developing OSCC when compared with non-smoker, non-drinker, and without HPV infection (OR=13.3, 95% CI=3.1-56.8). Among people with HPV infection, smokers (OR=13.3, 95% CI=3.1-56.7) had a higher risk of developing OSCC

than non-smokers (OR=12.5, 95% CI=3.0-52.9). Alcohol consumption also had the same synergistic effect as smoking. Drinkers with HPV infection (OR=13.1, 95% CI=3.1-56.1) increased the risk of OSCC compared with non-drinkers (OR=12.5, 95% CI=3.0-52.9). The details were shown in Table 3.

Generally, The result of multinomial logistic regression found that those who smoked, consumed alcohol and with HPV infection have the highest risk to develop cancer (OR=13.3, 95%CI=3.1-56.8). Oral HPV infection is strongly associated with OSCC among patients with or without the established risk factors of tobacco and alcohol use. The incidence rate for OSCC was higher among persons aged over 55 years than younger individuals.

Discussion

Squamous cell carcinoma is the most common histologic type in oral cancer, accounting 90% of oral neoplasms (Hennessey et al., 2009). Recent reports suggest an increasing incidence in OSCC in many regions of world. Tobacco and alcohol consumption are implicated in 75% of OSCC (Mitka, 2013), and smoking accounts for 42% of deaths from cancers of oral cavity and heavy alcohol consumption for 16% of the deaths (Jemal et al., 2011). The rest 25% of OSCC are mainly attributed to HPV infection. Although at least 15 HPV types are thought to have oncogenic potential, the most prevalent type caused HPV-associated oral squamous cell cancers is HPV16, the same type that leads to HPV-associated anogenital cancers (Marur et al., 2010). Previous study confirmed that oral HPV infection increased the risk of tumorigenesis of oral cancer independent of tobacco and alcohols and suggested HPV-positive oral cancer comprise a distinct molecular, clinical and biological disease that has a markedly improved therapeutic responses and prognosis (Gillison et al., 2000; Fakhry et al., 2008).

As the best of our knowledge, so far, it is the first time to explore relationship of HPV infection and oral cancer in Wuhan city in the south of China using case control study, which has much more cases involved than other studies in China including our previous study (Zhao et al., 2009). In this study risk factors for oral cancer besides tobacco and alcohols were also examined. In our study, presence of OSCC increased with age (≤55 years versus >55 years, OR=3.4, 95% CI=1.8-6.7), which is consistent with other study showing the increases of incidence of oropharynx cancer mainly among persons aged 55 to 64 years old (Jemal et al., 2013).

Tobacco use is a very important risk factor in the development of OSCC. The epidemiological study of oral cancer in Asia indicated that some common factors like use of tobacco, alcohol and quid chewing are the main reasons for the increasing incidence of oral cancer in Asian countries (Krishna Rao et al., 2013). The prevalence of smoking in OSCC group in this study was 50%, which is higher than that in other studies (Lin et al., 2011). However, selection bias is unavoidable in case-control studies. The smoking men account for 67.8% (97/143) of all male patients, while the smoking women only account for 5.2% (3/57) of the female population. In the

control group, the proportions were 33.3% (9/27) and 9.8% (4/41), respectively. Tobacco use is proved to be an independent risk factor in this study (*p*=0.015). People consumed tobacco have increased risk in developing OSCC compared with non-smokers (OR=2.7, 95% CI=1.2-6.0), which is lower than other study (Tachezy et al., 2009). Alcohol consumption is also as an important risk factor for the prevalence of OSCC. Ever use of alcohol was associated with a 1.67 fold increased risk of oral cancer (Anantharaman et al., 2014). However, it is not proved that alcohol use is the risk of OSCC with adjusted *p*-value 0.063 in our study, which is different from other studies in other countries (Zygogianni et al., 2011; Markopoulos, 2012).

It was shown in this study tobacco use and alcohol consumption had a synergistic effect on the development of OSCC in HPV-positive patients. The data of multinomial logistic regression indicated that in the HPV-positive population, smokers or drinkers had a higher incidence of OSCC than non-smokers or non-drinkers, respectively.. the HPV-positive patients who both smoked tobacco and drank alcohol had the highest risk to develop cancer. Further study need to be done to explore the effects and mechanism of tobacco and alcohol on the development of OSCC in Chinese population.

The discrepancy of different HPV prevalence found in OSCC in different studies might attribute to different anatomic locations of tumors, different sample origins (such as formalin-fixed, fresh biopsies, or oral exfoliated cells), different techniques used to detect HPV existence (such as PCR, P16 immunohistochemistry and in situ hybridization) (Termine et al., 2008) and different population involved in epidemiology studies. For example, the epidemiology in USA is about 38% infection in OSCC, whereas the meta-analysis of Chinese population indicated the prevalence in China is 47.47% (Termine et al., 2008; Zhu et al., 2012). Furthermore, the case numbers enrolled in epidemiology study in China were much smaller than those in other countries. It's necessary to proceed large sample study to reveal the HPV infection situation in OSCC in Chinese population.

In our study, HPV prevalence was 27.5% in OSCC patients and only 2.9% in controls. HPV16 was the most prevalent common type detected. Only one case had coinfection with HPV6 and HPV18 in case patient. In the control group, both two HPV positive samples were caused by HPV18. A Malaysia research suggested HPV18 may be not a crucial factor in Malaysian OSCC according to the result of low percentage (1/30) of HPV18 (Goot-Heah et al., 2012). The similar result obtained in the control group in our study indicated other HPV types should be paid more attention. Other study reported that men with an oral HPV6 infection had 2.9 times the risk for oral cancer of noninfected men, and men with HPV16 infection had 6.2 times the risk for oral cancer of noninfected men (Maden et al., 1992). The overall HPV prevalence in our study was lower than the case-control study in Mexico, which reported that 43.5% of 62 OSCC were HPV positive (Anaya-Saavedra et al., 2008); and higher than the case-control study in Canada, which reported 19% of 72 cases were HPV positive (Pintos et al., 2008). In these studies,

the identification of viral DNA sequences was performed with different primers located in HPV L1 region with PCR method.

HPV6 was the only low-risk HPV type found in our study. Although HPV6, together with HPV11, were considered low-risk HPV types and uncommonly found in malignant lesions. Syrjanen speculated that low-risk HPV6 was not entirely benign in the head and neck region since it can be found in a minority of head and neck squamous cell cancer (Syrjanen, 2010). However, it remained unclear in what proportion of HPV6 and HPV18 were allocated in our case, and whether such concomitant co-infection increased or decreased the risk of the development of OSCC. As far as we know, HPV6 is the major etiological agent of anogenital warts and recurrent respiratory papillomatosis, and has been included in the quadrivalent prophylactic HPV vaccine (Lacey et al., 2006). The impact of HPV6 involved in OSCC should be further studied.

According to the current data, the most common anatomic site of HPV-positive OSCC is oral tongue. But there is some other controversial definitions need to be clarified. In many documents, scientists tend to classified base of tongue into oropharyngeal and focus on the oropharyngeal squamous cell cancer (Herrero et al., 2003; D'Souza et al., 2007), whereas in other countries, authors tend to classified all oral anatomic site into the oral cancer which included oral cavity and oropharyngeal (Pintos et al., 2008; Markopoulos, 2012). Here, we prefer to the latter classification. Unlike some other studies that analyze for oral cancer other than oropharyngeal site showed low prevalence and non significant association between HPV and oral cancer (Smith et al., 2004), our data indicated that HPV is highly-associated with oral cancer (including oral cavity and oropharyngeal site) and act as an independent risk factor combined with other risk factors such as tobacco and alcohol.

In summary, the results of our survey have indicated that HPV was strongly associated with oral cancer in a group of Chinese patients in south of China, suggesting that HPV, as an independent risk factor, plays an important role in oral carcinogenesis. Also, tobacco use and age increase as traditional risk factors were confirmed in our study. Furthermore, it has been demonstrated in this study that tobacco use and alcohol consumption have the synergistic effect with HPV infection to the development of OSCC.

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