Int J Clin Exp Pathol 2016;9(2):1839-1848
www.ijcep.com /ISSN:1936-2625/IJCEP0019654

Original Article

Human papillomavirus-related squamous cell carcinomas of the oropharynx and sinonasal tract in 156 Chinese patients

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Received November 12, 2015; Accepted January 12, 2016; Epub February 1, 2016; Published February 15, 2016

Abstract: Aims: High-risk variants of human papilloma virus (HPV) may cause oropharyngeal squamous cell carcinomas (SCCs). Recent studies have also reported HPV infection in the sinonasal region. However, the relationship between HPV infection and oropharyngeal/sinonasal SCC in Chinese patients has seldom been reported. In this study, we determined the prevalence of HPV infection in 156 Chinese patients with primary oropharyngeal/sinonasal SCC and investigated the relationship between HPV infection and prognosis. Methods and results: HPV infection was conducted by in situ hybridization. Among 156 patients with oropharynx/sinonasal SCC, thirteen cases (eight from the oropharynx and five from the sinonasal tract) were positive for HPV, 7 and 2 of which were positive for HPV type 16/18 and type 31/33, respectively. The expression of p16 and p53 was examined by immunohistochemistry; p16 expression significantly positively correlated with HPV status (69% vs. 13% for HPV positive vs. negative samples, $P < 0.001$). Univariate survival analysis showed that HPV infection was significantly associated with better survival. However, this association was no longer observed when the data were corrected for age, tumor stage, nodal stage, and p16 expression. Conclusions: The prevalence of HPV infection is relatively low among these Chinese patients with oropharyngeal/sinonasal SCC. HPV infection was not associated with patients' survival, whereas age, tumor stage, nodal stage and p16 expression showed significant association in this study.

Keywords: Human papillomavirus, oropharyngeal, sinonasal, squamous cell carcinoma, p16, survival analysis

Introduction

Human papillomavirus (HPV) is one of the most common sexually transmitted infectious viruses in the world. HPV can be categorized into low-risk (including HPV types 6 and 11) or high-risk types (including HPV types 16 and 18) according to its epidemiological association with cervical cancer [1]. The high-risk types of HPV are detected in 99% of cervical cancers, with HPV types 16 and 18 present in approximately 70% of cervical cancers worldwide [2]. In addition to the obvious association with cervical cancer, HPV infection has recently also been reported in head and neck squamous cell carcinomas (HNSCC) [3]. According to the CDC, up to 60% of oropharyngeal carcinomas that were diagnosed between 1998 and 2003 were related to HPV. An increasing number of reports have also suggested that HPV type 16 may be a causative agent in many oropharyngeal carcinomas, particularly in the tonsils and at the base of the tongue [4-6]. In addition to oropharyngeal carcinomas, carcinomas in the sinonasal tract are also associated with HPV infection, although the reported prevalence rate of HPV infection varies among different studies [7-19].

Because HPV is strongly associated with HNSCC, researchers have now classified HNSCC into HPV+ HNSCC and HPV- HNSCC [6, 20]. The features of these two types of HNSCC, such as the risk factors, genetics, histological expression, degree of differentiation, clinical characteristics and prognosis, are distinctive. In general, HPV+ HNSCC is more closely correlated with HPV infection than with tobacco and alcohol consumption. The expression of p16, an established biomarker for the function of the HPV E7 oncoprotein, positively correlates with

tumour HPV status [21], whereas the expression of p53 negatively correlates with tumour HPV status. HPV+ HNSCCs tend to be low-differentiated nonkeratinizing carcinomas [22-27]. Interestingly, the response rates to chemotherapy and chemoradiation are higher in patients with HPV+ HNSCC than in patients with HPV- HNSCC, resulting in more favourable prognoses for the HPV+ HNSCC patient group [22].

Although the relationships between HPV infection and HNSCC have been extensively investigated, most studies have been based on data collected from people in Western countries. The prevalence of HPV infection in Western counties can be attributed to its transmission via sexual behaviours, such as oral sex and multiple sex partners, particularly among young adults < 45 years of age [26-32]. However, these sexual behaviours remain strictly censored in China, and the effect of this censorship on the prevalence of HPV infection in HNSCC is unknown. Thus, this study aimed to determine the prevalence of HPV infection among 156 Chinese patients with oropharyngeal and sinonosal carcinoma and to then investigate the impact of HPV status on the prognosis of HNSCC.

Methods

Patients and specimens

A total of 156 carcinomas of the oropharynx (tonsil or base of tongue) and sinonasal tract (nasal cavity and paranasal sinuses) were obtained from the surgical pathology archives of the department of pathology at Beijing Tong Ren Hospital. The primary carcinomas were diagnosed from 2000 to 2013 and included 66 carcinomas of the oropharynx (33 of the tonsil, 33 of the base of the tongue) and 90 carcinomas of the sinonasal tract. The patients' clinical information was obtained from the medical records. Additional information was derived from the follow-up visits. All slides were reviewed, and the tumours were categorized according to histological criteria.

Tissue microarrays

Tissue microarrays (TMA) were constructed from the archived paraffin tissue blocks of 121 oropharyngeal and sinonasal carcinomas. Two to three cores of each case were included; each core tissue measured 1 mm in diameter. The microarray also included control cores from HPV-positive cervical carcinomas as a positive control and normal epithelium (oropharynx) as a negative control. Haematoxylin and eosin staining of the first and last sections of each TMA served to confirm the quality of the cores. Thirty-five cases were too small to be included in the tissue microarrays and were therefore tested on whole slides [7].

Immunohistochemistry

Immunohistochemical staining for p16 and p53 was performed on 4-μm sections using a Biocare Intellipath FLX immunohistochemical Automated Staining Instrument (Biocare Medical, LLC, Concord, CA, USA) following the manufacturer's instructions. p16 was detected with a rabbit anti-human monoclonal antibody (clone, 16P04/JC2; Invitrogen Corp., Carlsbad, CA, USA) at a 1:100 dilution; antigens were retrieved using a pressure cooker and 10 mM ethylenediaminetetraacetic acid at pH 9.0 for 2 minutes. p53 was detected with a mouse anti-human monoclonal antibody (clone, EP9; Invitrogen Corp., Carlsbad, CA, USA) at a 1:1500 dilution; antigens were retrieved using a pressure cooker and 10 mM citrate buffer at pH 6.0 for 2 minutes. If strong and diffuse nuclear and cytoplasmic staining was detected in ≥ 70% of the tumour, p16 expression was scored as positive [7]. p53 staining was semiquantitatively scored according to the percentage of positively stained cells (+, < 25%; ++, 25-50%; +++, 50-75%; ++++, > 75%) [8].

In situ hybridization for HPV

HPV-DNA was detected by in situ hybridization (ISH) on 4-μm tissue sections from formalin-fixed, paraffin-embedded tumour blocks. Two different detection methods were used. Type-specific assays for HPV 16/18, and 31/33 were performed using ISH for biotin labelled DNA probes (PanPath B.V., Budel, Netherlands) according to the manufacturer's instructions. The signals were amplified by the consecutive application of streptavidin-HRP complex and biotinyl tyramide. Positive hybridization signals were visualized by incubation with the chromogenic substrate diaminobenzidine, which stained nuclei a punctate brown.

Table 1. HPV Status of oropharyngeal and sinonasal carcinomas by tumour type

	Sinonasal carcinoma			Oropharyngeal Carcinoma			Total HPV+
	Keratinizing	Nonkeratinizing	Total	Keratinizing	Nonkeratinizing	Total	
HPV cocktail	2	3	5	3	5	8	13
Type 16/18	1	1	2	1	4	5	7
Type 31/33	0	1	1	0	1	1	2
HPV negative	41	44	85	45	13	58	143
Total HNSCCs		90			66		156

Table 2. Immunohistochemical expression of p16, p53 and HPV detection

Immunohistochemistry	HPV+ (n=13)	HPV- (n=143)	P
p16	9	19	0.000
p53	3	48	0.5

The Wide Spectrum HPV Biotinylated DNA probe detects HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51 and 52 (DAKO, Glostrup, Denmark) and was used according to the manufacturer's manual. The ISH Detection System K0601 (DAKO, Glostrup, Denmark) utilizes alkaline phosphatase (AP)-conjugated streptavidin to localize biotinylated probes. The site of hybridization was visualized using the colorimetric reaction of the enzyme conjugate with its substrate, BCIP (5-bromo-4-chloro-3-indolyl phosphate), and the concomitant reduction of NBT (nitroblue tetrazolium). This reaction deposits an insoluble blue-purple product at the site of hybridization; neutral red was used as a counterstain.

For both detection assays, ISH for HPV DNA was scored as HPV positive when the reaction product was seen in the tumour cell nuclei, either in homogeneous or punctate patterns. HPV-positive controls were HPV-16/18- and HPV-31/33-positive cervical cancers.

Statistical analysis

The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Fisher's exact test was used to test for the qualitative variables. Student's t test and an analysis of variance (ANOVA) were applied for quantitative variables. The difference in the survival rate between the HPV-positive and HPV-negative patients was analyzed with the Kaplan-Meier method, and the significance of the differences between the two groups was compared with a log-rank test. Univariate and multivariable Cox regression models were applied to evaluate the association between HPV status and survival using the R packages "survival" and "survcomp". All tests were two-sided, and the significance level was set at $P < 0.05$.

Results

The prevalence of HPV infection and the immunohistochemical characteristics of the patients with oropharynx/sinonasal SCCs

ISH showed that 13 (8%) cases were HPV positive, of which 5 (6%) and 8 (12%) were sinonasal and oropharynx carcinomas, respectively (**Table 1**). Of the 13 HPV-positive samples, seven (54%) were infected with the high-risk HPV types 16/18, and five of these samples were oropharynx carcinomas.

In general, SCCs can be classified into two histological subtypes: keratinizing (K-SCCs) and nonkeratinizing (NK-SCCs). Of the 13 HPV-positive SCCs, 28% were K-SCCs and 72% were NK-SCCs (**Table 1**). NK-SCCs were slightly more prevalent in HPV-positive SCCs than in HPV-negative SCCs, but this difference was not significant (8 of 13 (62%) vs. 57 of 143 (40%), P=0.15). Because we did not find a significant difference in the HPV infection rate between sinonasal and oropharyngeal carcinomas, we hereafter combined the two carcinomas for analysis.

We further examined the expression of p16 and p53 in SCCs using immunohistochemical staining. The expression of p16 in SCCs was significantly associated with HPV infection: p16 was detected in 9 of 13 HPV-positive SCCs but only in 19 of 143 HPV-negative SCCs (69% vs. 13%, $P \leq 0.001$). The expression of p53 in SCCs was not associated with HPV infection. Although 10

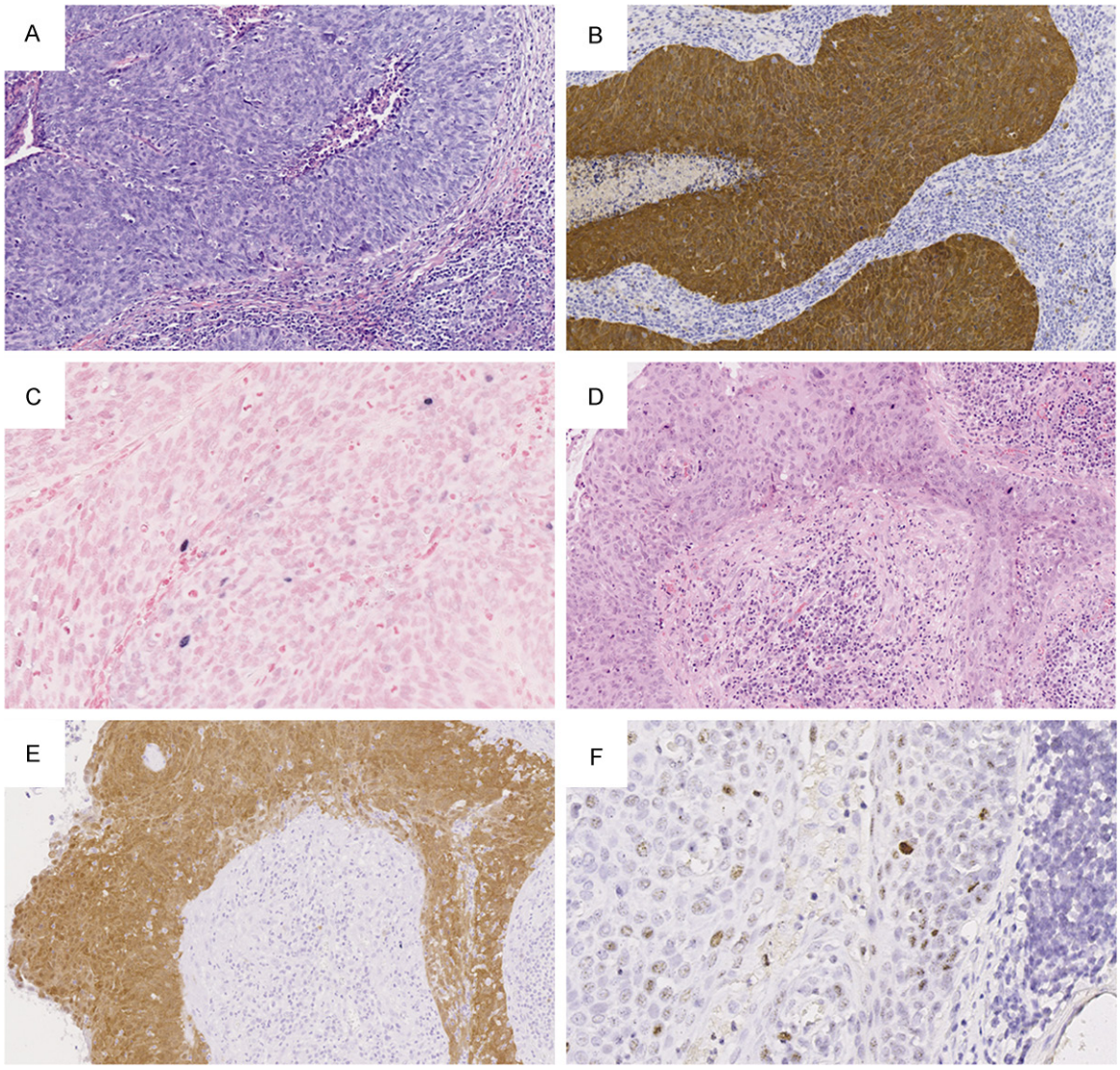

Figure 1. HPV- and p16-positive oropharyngeal and sinonosal carcinomas are shown (A-C and D-F of the same case). (A) Haematoxylin and eosin morphology showing nonkeratinizing carcinoma with basal cell features and comedo-type necrosis (B) p16-positive tumour by immunohistochemistry showing strong and diffuse nuclear and cytoplasmic staining; (C) Wide Spectrum HPV positivity by ISH with blue dots staining the tumour nuclei; (D) Keratinizing carcinoma by haematoxylin and eosin staining; (E) p16 expression with strong and diffuse nuclear and cytoplasmic staining (F) HPV 16/18-positive tumour by ISH with punctate brown staining in the nuclei.

of 13 HPV-positive samples did not or weakly expressed p53, most HPV-negative samples also expressed low levels of p53 (**Table 2**).

The histological characteristics and expression of p16 protein in HPV-positive tumours classified by HPV type (16/18 or wide spectrum HPV) are shown in **Figure 1**.

Clinical characteristics of the patients with SCCs and survival analysis

The clinical characteristics of the 156 patients are summarized in **Table 3**. The following clinical characteristics did not significantly differ between HPV-positive and HPV-negative SCCs: age, male: female ratio, smoking, carcinoma sites, histological types and tumor stage of tumors. However, we found that patients diagnosed with HPV-positive SCCs tended to be younger than patients with HPV-negative SCCs (the median ages of patients with HPV-positive and HPV-negative SCCs were 48 and 57, respectively). Most of HPV-positive SCCs were in the stage (III + IV) (11 out of 13), while only 2 HPV-positive SCCs were in the early stage (P=0.075).

Table 3. Demographic, clinical and HPV status by group

Group No.	HPV-Positive (n=13)	HPV-Negative (n=143)	P
Age			0.244
Median age (range)	48 (20-72)	57 (25-87)	
Sex (%)			0.736
Male	11 (85)	107 (75)	
Female	2 (15)	36 (25)	
Smoking history (%)			0.398
Never/unknown	4 (44)	63 (31)	
Yes	9 (56)	80 (69)	
Smoking, pack years (%)			0.614
< 20	2 (22)	11 (14)	
≥ 20	7 (78)	69 (86)	
Site (%)			0.156
Sinonasal tract	5 (38)	85 (59)	
Oropharynx	8 (62)	58 (41)	
Stage (%)			0.075
I + II	2 (15)	62 (43)	
III + IV	11 (85)	81 (57)	
Tumor Stage (%)			0.414
T1	2 (15.4)	19 (13.3)	
T2	4 (30.8)	53 (37.1)	
T3	6 (46.2)	39 (27.3)	
T4	1 (7.7)	32 (22.4)	
Nodal Stage (%)			0.529
N0	6 (46)	79 (55)	
N1-3	7 (54)	64 (45)	
Histological type (%)			0.263
Keratinizing	5 (38)	79 (55)	
Nonkeratinizing	8 (62)	64 (45	

Of the 156 SCC patients, median follow-up time was 23 months (range, 2-146 months). Twenty-seven patients could not be contacted to obtain follow-up data; consequently, the follow-up information of only 129 patients was examined. Seventy-three patients received primary surgery and radiotherapy, 27 and 56 tumors received surgery or radiotherapy alone, respectively. Chemotherapy was administered to 17 patients.

A Kaplan-Meier survival analysis of the patients revealed that the overall survival of HPV-positive patients was significantly better than that of HPV-negative patients (P=0.045, by the log-rank test) (**Figure 2A**). The estimated 1-, 3-, and 5-year overall survival rates of HPV-positive patients were 100.0% (95% CI, 1), 90.0% (95% CI, 73.2% to 1) and 68.6% (95% CI, 44.5% to 1), respectively, whereas the 1-, 3-, and 5-year overall survival rates of HPV-negative patients were 81.3% (95% CI, 74.4% to 88.8%), 52.3% (95% CI, 43.3% to 63.2%), and 46.7% (95% CI, 37.2% to 58.7%), respectively. The progression-free survival of patients with HPV-positive tumours was also better compared than that of patients with HPV-negative tumours (P=0.03, by the log-rank test) (**Figure 2B**). The estimated of 1-, and 3-, and 5-year progression-free survival rates for HPV-positive patients were 100.0% (95% CI, 1), 74.1% (95% CI, 52.6% to 1), and 74.1% (95% CI, 52.6% to 1), whereas they were 66.0% (95% CI, 57.7% to 75.4%), 49.1% (95% CI, 39.8% to 60.5%), and 46.4% (95% CI, 36.5% to 58.8%) for HPV-negative patients.

Although HPV infection seemed to be significantly associated with better survival outcome, age, smoking, stage, tumor stage, nodal stage and p16 expression were also significantly associated with both overall survival and progression-free survival based on a Cox univariate regression analysis (**Table 4**). We performed a multivariate survival analysis to identify the primary factor among these variables. Interestingly, p16 expression, age, tumor stage, nodal stage still remained significantly associated with both overall survival and progression-free survival (**Table 4**). HPV infection was no longer significant predictors for survival outcomes in the multivariate analysis (**Table 4**). Thus, these data suggest that HPV infection might be a confounding factor for survival outcomes. However, given that the sample size is relatively small, this conclusion may change upon the addition of more samples.

Discussion

HNSCCs are known to be associated with smoking and alcohol abuse. Recently, the high-

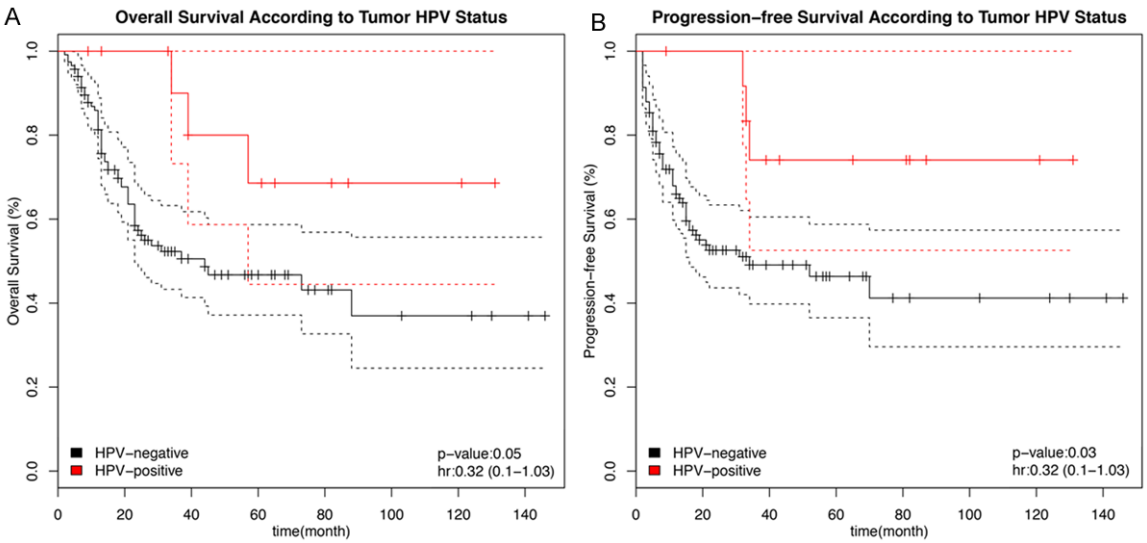

Figure 2. Kaplan-Meier Estimates of survival among SCC patients categorized by HPV tumour status. Overall survival (A) and progression-free survival (B) are shown according to stratification based on tumour HPV status. The Kaplan-Meier curves are indicated by solid lines, and the associated 95% confidence intervals are shown in dashed lines. Red and black denote HPV-positive and HPV-negative samples, respectively. The overall survival and progression-free survival of patients with HPV-positive tumours was significantly better than that of patients with HPV-negative tumours ($P < 0.05$ for both comparisons by the two-sided log-rank test).

Table 4. Overall survival and progression survival by univariate and multivariate analysis

Characteristic	Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P
Overall survival						
Age (> 60 vs. ≤ 60)	5.256	2.966-9.312	0.000	5.569	3.049-10.169	0.000
Sex (female vs. male)	0.549	0.277-1.089	0.086			
Smoke (Yes vs. No)	1.939	1.113-3.378	0.019	1.108	0.598-2.054	0.744
HPV (+ vs. -)	0.309	0.096-0.993	0.049	0.519	0.132-2.036	0.347
p16 (+ vs. -)	0.275	0.123-0.616	0.002	0.237	0.104-0.538	0.001
Type (NK-SCC vs. K-SCC)	0.620	0.359-1.071	0.087			
Stage (III + IV vs I + II)	3.838	1.988-7.413	0.000	0.787	0.184-3.373	0.747
T stage (T3+T4 vs. T1+T2)	2.242	1.635-3.075	0.000	1.428	1.000-2.041	0.050
N stage (N1-3 vs. N0)	5.594	3.050-10.258	0.000	4.266	2.031-8.963	0.000
Progression-free survival						
Age (> 60 vs.≤ 60)	5.454	3.077-9.670	0.000	5.664	3.121-10.281	0.000
Sex (women vs. man)	0.536	0.270-1.062	0.074			
Smoke (Yes vs. No)	1.829	1.051-3.184	0.033	1.126	0.614-2.063	0.701
HPV (+ vs. -)	0.304	0.095-0.979	0.046	0.570	0.148-2.202	0.415
p16 (+ vs. -)	0.292	0.130-0.655	0.003	0.244	0.107-0.557	0.001
Type (NK-SCC vs. K-SCC)	0.598	0.346-1.033	0.065			
Stage (III + IV vs I + II)	3.326	1.737-6.370	0.000	0.519	0.122-2.212	0.375
T stage (T3+T4 vs. T1+T2)	2.140	1.564-2.927	0.000	1.475	1.027-2.120	0.035
N stage (N1-3 vs. N0)	5.063	2.772-9.247	0.000	3.606	1.763-7.375	0.000

HR indicates hazard ratio; NK-SCC nonkeratinizing squamous cell carcinomas; K-SCC keratinizing squamous cell carcinomas.

risk variants of HPV were also reported to be associated with HNSCCs and have been suggested to be the etiologic agent for some HNSCCs, most notably in the tonsils and at the

base of the tongue [4-6]. A recent meta-analysis shown an overall HPV infection rate in North American and Europe patients with oropharyngeal cancer increased significantly over time: from 40.5% (95% CI, 35.1-46.1%) to 72.2% (95% CI, 52.9-85.7%), between 2000 and 2009, Which was higher than that for non-oropharyngeal cancer (21.8%; 95% CI, 18.9-25.1%) [33]. In this study, the prevalence of HPV infection was approximately 6% and 12% among these patients with sinonasal and oropharyngeal SCCs, respectively. These rates are lower than those reports in the above. Multiple factors contribute to this disparity in the prevalence of HPV. One of the possible explanations for the low proportion may lie in a lower exposure to HPV, either due to a lesser presence of HPV in the general population in china or to different sexual behavior. Such as oral sex and multiple sex partners. According to an analysis of the Swedish cancer registry data (1958-1996), husbands of women with cervical cancer had a significantly increased risk of developing tonsillar carcinoma [31]. In contrast, the estimated incidence of cervical cancer remains much lower in China, with an age-standardized rate of 6.8 per 100,000 in the year 2000 [34]. The lower incidence of cervical cancer in China suggests that sexual behaviours remain under stringent cultural censoring, which partly explains the lower prevalence of HPV infection among Chinese patients with HNSCC.

In the analysis, p16 expression remained significantly associated with better both overall survival and progression-free survival. When we studying the relationship between p16 expression and HPV infection, we observed intense and diffuse p16 expression in 9 (69%) HPV-positive carcinoma samples. This findings is reasonable, because the overexpression of p16 is accepted as a marker of a subset of HPV-related carcinomas, such as those of the vulva, [35] cervix, [36] penis, [37] and tonsil [6, 23, 28]. Conversely, p16 was overexpressed in 19 out of 143 HPV-negative tumours. Some previous reports found as many as 30% of p16 positive tumors but were HPV negative [38]. A recent study conducted by Klingenberg et al. also reported that p16 overexpression is frequently detected in tumour-free tonsil tissue and not associated with HPV [39]. So the question is “What is the mechanism for the p16 expression without detectable HPV?” First, this finding may be attributed to other viruses that contribute to p16 overexpression and inactivate Rb gene in the same manner as the HPV oncoprotein E7 [40, 41] or the unidentified HPV genotypes that cannot be detected by current HPV-specific test, which contain the consensus HPV DNA sequences. Second, Lewis Jr et al speculated that HPV is also involved in tumor carcinogenic process. But some tumors would have favorable progress genetically and HPV might have been shed. Such tumors are lack of active viral E7 expression and Rb gene is deleted or suppressed. Alternatively, another speculation is that the tumors develop completely independently from HPV, however as a tumor suppressor gene, p16 may play an important role in their good treatment respronsiveness [42]. However, this possibility needs to be verified with more clinical data.

The inverse correlation between p53 and HPV-positive carcinomas of the head and neck has been documented in previous studies [43-45]. As many as 10 out of 13 HPV- positive samples did not or weakly expressed p53 in our study, which showed the opposite trend compared to HPV-negative carcinomas. The HPV viral E6 protein can cause a p53 gene mutation that inactivates p53 protein and interferes with p53 function by targeting it for ubiquitination and degradation [46, 47]. Some authors have identified a subgroup of HNSCCs that is associated with HPV and the overexpression of p53 protein because of the mutation of p53 [43, 44]. Our results are also similar to those of Hafkamp et al., who detected p53 overexpression in 3 of 13 HPV-positive cases (23%) via immunohistochemistry [45].

In this study, we found that HPV infection was not significantly associated with survival outcome if other risk factors, such as age, smoking, tumor stage and p16 expression, were taken into consideration. This finding is different from that of D’Souza et al.’s study, in which they used multivariate logistic-regression models to show that HPV infection favourably impacts the survival of SCC patients [5]. This difference may be due to the following reasons: First, the prevalence of HPV infection may be population specific; thus, the relationship between HPV infection and survival outcomes is expected to differ by population. Second, the number of HPV-positive samples investigated

in this study was small, and our conclusions may change with the inclusion of more HPV samples. Third, the expression of p16, smoking, tumor stage and age were significantly associated with patient survival as well as HPV infection in this study; however, these factors were not controlled in D'Souza et al.'s study, which likely resulted in bias in their data. Nevertheless, the lack of association between HPV infection and survival in Chinese SCC patients suggests that large-scale studies are needed to elucidate the effect of HPV infection on the survival of SCC patients.

In summary, our study identified a relatively low prevalence of HPV among these Chinese patients with oropharyngeal/sinonasal SCC. An association between HPV infection and survival was not observed among oropharyngeal/sinonasal SCC patients when age, smoking, and other factors were taken into consideration.

Disclosure of conflict of interest

None.

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