Human Papillomavirus as an Independent Predictor in Oral Squamous Cell Cancer

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Abstract

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Aim There is an increasing evidence for the role of high risk human papillomavirus (HPV) in the pathogenesis of oral squamous cell carcinoma (OSCC). The purpose of this study is to evaluate the relevance of HPV infection to the survival and prognosis of OSCC.

Methodology Fifty-two patients with OSCC were followed from 4 to 88 months with a median of 50.7 months. HPV DNA was identified in formalin-fixed, paraffin-embedded tumor specimens by nested PCR with MY09/MY11 and GP5⁺/GP6⁺ primer pairs and the HPV genotype was determined by direct DNA sequencing. Association between the HPV status and risk factors for cancer as well as tumor-host characteristics were analyzed.

Survival curves were calculated by the Kaplan-Meier method and analyzed using the log-rank test.

Results HPV was found in 40.4% of the tumors with HPV16 accounting for 63.5%, HPV18 for 30.8%, HPV6 for 3.9% and HPV11 for 1.8%. No infection with more than one HPV genotype was detected. HPV infection was significantly associated with poor histological grade, TNM stage I - II, alcohol usage and no smoking status. Multi-variate analysis showed that HPV had an independent prognostic effect on the overall survival after adjusting other confounding factors such as histological grade, TNM stage and tobacco usage. The presence of HPV was significantly correlated with a better survival in patients with OSCC.

Conclusion HPV infection can act as an independent predictor for the survival and prognosis of OSCC.

Keywords human papillomavirus (HPV), oral squamous cell carcinoma (OSCC), survival analysis

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Introduction

Oral cancer is the sixth most prevalent cancer worldwide, ranking third in developing countries and eighth in developed countries, and China (incl. Hong Kong) is involved in one of the six high-risk areas and countries worldwide (Syrjänen, 2005). In China, the incidence of oral cancer has jumped from 81/1,000,000 to 202/1,000,000 in the past few years, and estimates of mortality in China approach 10,000 annually. Since oral squamous cell carcinomas (OSCCs) are prone to be detected at its late stage, the overall survival rate of such

kind of diseases can only reach about 40%–50%.

Alcohol use and cigarette smoking are well-known risk factors for the development of oral cancer (Decker *et al.*, 1982). In 1983, human papillomavirus (HPV) was first described as a factor involved in the development of OSCC (Syrjänen *et al.*, 1983). From then on, the presence of different types of HPV has been detected in oral cancers, which are mainly HPV16 and HPV18 with noticeable detection rates from 8% to 64% (Kansky, *et al.*, 2003; Lo Muzio *et al.*, 2004; Hansson *et al.*, 2005).

Figure 1 Products of MY-PCR (450 bp) and MY/GP-PCR (142 bp)

Lane 1: DL2000 DNA marker; lane 2: blank control; Lane 3: Wi38 cells (negative control); Lane 4 and 8: HeLa cells (positive control); Lane 5, 6 and 9: positive cases; Lane 7, 10 and 11: negative cases.

The presence of HPV DNA in a significant fraction of oral cancer, combined with the evidence that HPV status may affect the clinical outcomes in cervical or anogenital cancer patients (Lombard *et al.*, 1998), led to a number of studies pointing a possible use of HPV diagnosis as an additional aid for survival prognosis in oral cancers (Schwartz *et al.*, 2001; Ritchie *et al.*, 2003), while other groups found no differences in survival by HPV status (Brandwein *et al.*, 1994). However, many of these studies did not use detection methods with high sensitivity, screen for all of the HPV types, nor distinguish between the HPV types, and/or did not control for other confounding factors suspected to influence the survival in cancer patients such as tumor histology, histological grade, TNM stage, alcohol use and cigarette smoking.

OSCC is the most common type of oral cancers which accounts for 80% of the cases. Because some previous studies have found no change in survival rates by HPV status, while other studies show a more pronounced association, the question remains whether HPV exerts any influence on prognosis and survival in OSCC patients. Thus, the purpose of this study is to investigate the relationship between HPV status and the survival of OSCC patients to remedy deficiencies of previous studies.

Materials and methods

Subjects

Fifty-two OSCC patients were obtained from School & Hospital of Stomatology, Wuhan University from 1999 to 2001. Tumor sites included tongue ($n=21$), cheek ($n=11$), gingival ($n=11$),

floor of mouth ($n=2$), palate ($n=5$), and lip ($n=2$). Clinical staging according to the 1997 criteria of the International Union Against Cancer (Sobin *et al.*, 1997) was evaluated by reviewing the medical charts and pathologic records. Risk factors such as tobacco and alcohol exposure were investigated by interview. All patients had undergone surgery immediately after their first diagnosis.

Tissue specimen preparation and PCR-based HPV typing

The 10% formalin-fixed and paraffin-embedded tumor specimens were serially sectioned (9 sections) on a microtome. The first and last sections were stained with hematoxylin-eosin to confirm the OSCC diagnosis and to determine the histological grade according to the World Health Organization criteria. The intermediate seven sections of 4 μ m each were used for DNA extraction with GENTRA DNA Tissue Kit (Gentra Systems Inc., USA). β -globin PCR was used as an internal control for the equal loading of DNA (Saiki *et al.*, 1985).

When β -globin was not detected in one sample, additional sections were taken from the same sample and reanalyzed. For HPV DNA detection, nested PCR (using two pairs of primers: MY09/MY11 and GP5⁺/GP6⁺) was performed as described elsewhere (Remmerbach *et al.*, 2004) with smart changes. Three controls were used for each sample described as blank control (containing all reagents except the template DNA), negative control (containing DNA purified cultures of HPV-negative cell line Wi38) and positive control (containing DNA purified cultures of HeLa cells infected with HPV18). All the experiments were duplicated (Figure 1). The PCR assays were performed using

Gene Amp PCR System 9700 (PE Applied Biosystems, USA), and the PCR products were analyzed on 2% agarose gels.

The decision of HPV genotyping was based on the DNA sequencing of MY⁻ or MY/GP⁺ PCR fragments. Amplification products were purified by QIAGEN PCR purification and directly sequenced by fluorescent dye-labeled dideoxynucleotides and cycle sequencing methods using the Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, USA). Sequence analysis was performed on the ABI PRISM 310 Genetic Analyser (PE Applied Biosystems, USA). Alignments were obtained from the GenBank on-line BLAST server, and downloaded HPV sequences from the HPV database (<http://hpv-web.lanl.gov>). All the working surfaces were treated with sodium hypochlorite before and after operation to prevent contamination.

Statistical Analysis

Analyses were conducted with Windows SPSS ver. 12 (SPSS Inc., USA). Association between the HPV status, OSCC risk factors, as well as other characteristics of patients were analyzed in cross

tables by Pearson's χ^2 test and with Fisher's exact test for small groups. Survival was measured by the time from the date of diagnosis to the death of patient. The deaths caused by OSCC were considered as outcomes; while the deaths caused by other factors were censored and the missing values were replaced by series mean method. Univariate analysis with Cox's proportional hazards model was deployed to determine every identified prognostic factor; multivariate analysis with Cox's proportional hazards model was used to explore combined effects. A difference of $P < 0.05$ was considered statistically significant.

Results

HPV infection status analysis

HPV was detected in 40.4% (21 of 52) of the tumors by nested MY/GP PCR, with HPV16 accounting for 63.5%, HPV18 for 30.8%, HPV6 for 3.9% and HPV11 for 1.8%. No other HPV types or coinfection were detected in our study. Table 1 shows the correlation between several clinicopathologic factors and HPV infection. It

Table 1 Correlation between HPV infection and clinicopathologic characteristics for 52 OSCC patients

	n (%)	HPV infection			OR (95% CI)
		Positive	Negative	P-value	
Age					
≤50	17 (32.7)	8	9	0.494	1.0
>50	35 (67.3)	13	22		0.7 (0.2–2.1)
Gender					
Male	35 (67.3)	15	10	0.602	2.8 (0.8–9.9)
Female	17 (32.7)	6	11		1.0
Histological grade					
Poor	25 (48.1)	20	5	0.000*	104.0 (11.2–962.1)
Well+Moderate	27 (51.9)	1	26		1.0
TNM stage					
I + II	22 (42.3)	17	5	0.000*	22.1 (5.2–94.2)
III+IV	30 (57.7)	4	26		1.0
Drinker					
No	31 (59.6)	7	5	0.001*	1.0
Yes	21 (40.4)	14	26		0.4 (0.1–1.4)
Smoker					
No	21 (40.4)	18	3	0.000*	56.0 (10.2–308.5)
Yes	31 (59.6)	3	28		1.0

* $P < 0.05$ by χ^2 test.

suggested that HPV infection was significantly correlated with poor histological grade, TNM stage I - II, alcohol usage and no smoking status. However, no statistically significant correlation was found between patients' age or gender and the HPV infection.

Survival analysis

The follow-up period ranged from 4 to 88 months with a median of 50.7 months. Among the 52 patients, 48 patients (92.3%) lived for at least 5 years. Twenty-five patients (48.1%) died of OSCC, 1 patient (1.9%) died of other reasons than OSCC, 24 patients (46.2%) were known to be alive and 2 patients (3.8%) were lost at the end of the follow-up period. Survival curves were calculated by the Kaplan-Meier method and analyzed using the log-rank test for a total of 52 cases (Figure 2). HPV-positive patients showed better overall survival outcomes than did HPV-negative patients. Besides HPV status, histological grade, TNM stage and exposure to tobacco were found to be significantly associated with a worse prognosis by univariate analysis (Cox's proportional hazards model) (Tables 2, 3). This multivariate analysis was used to control for the four prognostic variables mentioned above. All variables had an independent prognostic effect on the OSCC survival

except for the exposure to tobacco.

Discussion

In our study, HPV was found to have an independent prognostic effect on the overall survival of OSCC after adjusting other confounding factors such as histological grade, TNM stage and tobacco usage. The presence of HPV was significantly correlated with a better survival in patients with OSCC excluding other small histological types of oral cancer to reduce confusion. Unlike previous

Figure 2 Kaplan-Meier survival curve according to HPV infection ($P=0.0022$) for 52 OSCC patients

Table 2 Prognostic factors by univariate analysis (Cox's proportional hazards model)

Variables	Hazard ratio*	P-value
Age ($\leq 50 / > 50$)	2.027 (0.760–5.407)	0.158
Gender (Male/Female)	1.299 (0.542–3.113)	0.557
Histological grade (W+M/P)	2.360 (1.041–5.349)	0.040
TNM stage (I + II / III+IV)	25.630 (3.490–188.195)	0.001
Drinker (No/Yes)	0.541 (0.233–1.254)	0.152
Smoker (No/Yes)	2.907 (1.159–7.292)	0.023
HPV (Negative/Positive)	0.243 (0.091–0.651)	0.005

Abbreviations: W, well; M, moderate; P, Poor. *95% confidence interval.

Table 3 Prognostic factors by multiunivariate analysis (Cox's proportional hazards model)

Variables	Hazard ratio*	P-value
Histological grade (W+M/P)	0.036 (0.004–0.300)	0.002
TNM stage (I + II / III+IV)	26.383 (3.988–174.562)	0.001
Smoker (No/Yes)	1.618 (0.369–7.097)	0.542
HPV (Negative/Positive)	0.134 (0.018–0.978)	0.048

*95% confidence interval.

studies, nested PCR was employed to obtain more sensitive HPV detection and more accurate HPV infection rates, while the HPV genotype was determined by direct DNA sequencing. More than 90% of the patients had a follow-up until death for more than 5 years. When investigating the relationship between OSCC HPV status and survival, some other confounding factors were adjusted.

This study found that the HPV-positive tumor rate is 40.4%, higher than other reported studies for oral cancer (Yang *et al.*, 2004). One possible explanation could be the sensitive PCR assay that was adopted in our study to overcome this specific problem (Remmerbach *et al.*, 2004). As previously reported, this study confirmed that HPV16 and HPV18 were the two most prevalent HPV types (Syrjänen, 2005). Interestingly, the low-risk HPV types 6 and 11 were also found in our studied cases, although in very small percentages compared to HPV16 and HPV18. This may be caused by the use of MY and GP consensus primers, which allowed for a broad number of HPV genotypes to be detected.

Three risk factors that were highly associated with HPV detection have also been discussed elsewhere (Ritchie *et al.*, 2003; Dahlstrom *et al.*, 2003; Sugiyama *et al.*, 2003; Tachezy *et al.*, 2005; Campisi *et al.*, 2006). Many studies performed by multivariate analysis on HNSCC (Ritchie *et al.*, 2003; Dahlstrom *et al.*, 2003) and one study (Sugiyama *et al.*, 2003) on OSCC have found a correlation between HPV infection and poor histological grade. The same result was obtained in the present study (OR=104.0, 95% CI: 11.2–962.1). With respect to TNM clinical stage, no link with HPV status was found in most studies on OSCC. These findings were consistent with those reported by Campisi *et al.*, who found TNM stage II and its constituent T2 significantly associated to HPV infection by multivariate analysis and a fuzzy logic (FL) technique (membership functions as input, the ANFIS methodology, and the Sugeno's model of first order) (Campisi *et al.*, 2006). We also located the relationship between the TNM stage I–II and HPV infection (OR=22.1, 95% CI: 5.2–94.2). Tumors with HPV infection tended to be associated with a decreased exposure to tobacco (OR=56.0, 95% CI: 10.2–308.5). Our result is in consensus with some other investigations

which considered smoking as a protective effect on HPV status in the oral cavity (Ritchie *et al.*, 2003; Tachezy *et al.*, 2005). It has been suggested (Ritchie *et al.*, 2003) that smoking could increase the keratinization of oral mucosal which may make the mucosa more resistant to minor trauma, a proposed mechanism for the necessary infection of the basal layer cells by HPVs.

Interestingly, the presence of HPV was significantly correlated with a better survival of OSCC in the present study, and its prognostic effect on the overall survival was independent after adjusting other confounding factors such as histological grade, TNM stage and tobacco usage. Schwartz *et al.* also demonstrated that the presence of HPV16 DNA is independently related to a favorable prognosis in patients with OSCC in a population study of 254 cases (Schwartz *et al.*, 2001). Another large, well-controlled study found that oral cancers containing oncogenic HPV types had 74% less risk of disease-specific mortality (Gillison *et al.*, 2000). This study provides evidence that the presence of HPV predicted a better survival.

Nevertheless, in a mixed group of head and neck tumors, Hoffmann *et al.* failed to detect the difference in the overall survival rate between HPV-positive and HPV-negative cancer patients; however, a better survival of HPV-positive patients was demonstrated compared to HPV-negative patients when the same initial nodal status was given (Hoffmann *et al.*, 2005). By investigating 101 HNSCC patients, Pinto *et al.* also found no significant difference for survival according to the HPV status from HNSCC tumors (Pinto *et al.*, 1999). This study demonstrated evidence, apparently in contrast but not easily comparable since these studies were mainly carried out with HNSCC. Brandwein *et al.* examined the presence of HPV DNA in the archival specimens of oral carcinomas from 64 patients and found no difference in survival according to HPV status in patients with stage I–II disease; but they observed poorer survival for patients with HPV-positive stage III–IV disease compared with patients who had HPV-negative stage III–IV disease (Brandwein *et al.*, 1994). However, the population was also characterized by a high degree of occupational exposure to chemical and metallurgic carcinogens. These patients were in addition more likely to be

HPV-positive (57%), which could in part explained the apparent difference in survival.

One explanation of the association between the presence of HPV and the better survival may be the increased sensitivity of HPV-positive tumors to radiotherapy. The strong inverse association between HPV and survival among those who received radiotherapy compared with those who received no radiotherapy has been found (Schwartz *et al.*, 2001). Previous studies have noticed that patients with HPV-positive tumors are less likely to have p53 mutations. The absence of p53 mutations in the tumor cell may allow an enhanced response to radiotherapy (Tachezy *et al.*, 2005; Hoffmann *et al.*, 2005). Hafkamp *et al.* suggested that radiotherapy may decrease the ability of HPV-E6 and E7 to interact with p53 and pRb which supported the hypothesis above (Hafkamp *et al.*, 2004). However, Ritchie *et al.* found no statistically significant interaction between HPV status and radiotherapy on survival in oral and oropharyngeal cancers by considering separate effects for surgery and radiotherapy (Ritchie *et al.*, 2003). Moreover, better survival was still found in HPV-positive tumors after adjusting for the presence of a p53 mutation (Campisi *et al.*, 2006). Unfortunately, the present study lacks the information of radiotherapy after operation.

Another explanation has been introduced by the indirect effect of HPV infection on field cancerization which may influence the survival of some HNSCC tumors. It was found that HNSCCs expressing HPV-16 E6 and E7 show substantially lower rates of loss of heterozygosity in chromosomal regions 3p, 9p and 17p compared to HPV-negative tumors (Braakhuis *et al.*, 2004). One could therefore speculate that HPV-induced HNSCCs may be more univocal, whereas those arising from tobacco originate in several areas due to field cancerization. Statistically speaking, having multiple foci may eventually lead to one focus growing faster and thus becoming the dominating lesion. HPV foci, on the other hand, are fewer and would have a lower chance of producing a “champion” lesion that would quickly take over the epithelium (Schlecht *et al.*, 2005). Gillison *et al.* proposed that HPV-positive tumors may show less exposure to alcohol and tobacco (Gillison *et al.*, 2000). The loss of the two well-known risk factors may

decrease the chance of field cancerization and therefore would increase the survival. As shown in our study, patients with HPV-positive tumors indeed had less exposure to tobacco, but more likely to be alcohol consumers. Furthermore, HPV was shown to have an independent prognostic effect even after adjusting the risk factor of smoking. Similar findings had been made in two studies (Schwartz *et al.*, 2001; Ritchie *et al.*, 2003). In the past decades significant progress has been achieved in the field of HPV vaccine development to reduce the incidence of cervical cancer, which has drawn people’s attention to the role of HPV in oral cancer for the biological similarities between the epithelium of the cervix and the oral cavity. Although the effect of HPV on oral cancer remains contentious and the mechanism has yet been unknown, our study did find that HPV was an independent prognostic factor that predicted better survival in OSCC, the dominant subset of oral cancer. This finding raises the prognostic value of HPV and furthermore provides an option for improving the prognosis and quality of life for patients with HPV-associated OSCC by HPV vaccine.

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