

Importance of human papillomavirus infection in squamous cell carcinomas of the tongue in Guangdong Province, China

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Abstract

Objective: Tongue squamous cell carcinoma is one of the most common oral tumors. Human papillomavirus (HPV) infection has been proposed as a risk factor for head and neck squamous cell carcinoma, particularly oropharyngeal squamous carcinoma.

Methods: In this study, we retrospectively analyzed HPV infection in 121 Chinese patients with tongue squamous cell carcinoma in Guangdong Province. Polymerase chain reaction of HPV DNA and immunohistochemistry staining of p16 protein were used to identify the presence of HPV in formalin-fixed paraffin-embedded samples.

Results: HPV DNA was detected in 15.7% (n = 19) of tongue squamous cell carcinoma patients, with HPV16 being the most common type (n = 8, 42.1%). p16 staining did not correlate with detection of HPV DNA. Male sex was associated with HPV-positive tongue squamous cell carcinoma, whereas there were no significant differences in alcohol consumption, smoking, or age when tumors were stratified by HPV.

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Conclusion: Our study showed that HPV infection contributed to tongue squamous cell carcinoma in a small cohort of patients in Guangdong Province, China. Further investigation is needed to confirm whether HPV is a causal factor for tongue squamous cell carcinoma.

Keywords

Tongue squamous cell carcinoma, human papillomavirus, China, HPV16, oral cancer, p16

Date received: 21 June 2019; accepted: 3 December 2019

Introduction

Oral squamous cell carcinoma (OSCC) is the eighth most common cancer worldwide, and fewer than 60% of patients survive beyond 5 years.¹ OSCC includes cancers of the lips, teeth, gums, the anterior section of the tongue, buccal mucosa, hard palate, and other anatomical sites.² Of these, the most common is tongue cancer, which is one of the worst OSCC in terms of prognosis.^{3,4} According to the International Classification of Diseases (ICD-10), oropharyngeal cancer includes primary cancer that originates in the pharynx wall, tonsil, tongue root, and soft palate. Therefore, in tongue cancer, the anterior section of the tongue is considered an oral cancer, whereas the base of the tongue is a component of oropharyngeal cancer. Despite advances in surgery, radiotherapy, and chemotherapy, the mortality rate for this disease has not improved in the past 40 years.⁵⁻⁷ To better individualize treatment of tongue squamous cell carcinoma (TSC), recent studies have focused on finding additional risk factors and biomarkers for this rapidly growing patient category.⁸

Smoking and alcohol consumption are the main causes of oral cancer.⁹ A reduction in the number of tobacco-related tumors (including cancers of the floor of the mouth, cheek, gums, and other sites) has been observed because of anti-tobacco campaigns throughout the developed world.¹⁰

However, a global study of over 80,000 patients in 22 countries shows that the incidence of TSC is increasing worldwide.¹¹ This phenomenon suggests that TSC may have new risk factors affecting its incidence. Recent studies have shown that head and neck squamous cell carcinoma (HNSCC) is associated with human papillomavirus type 16 (HPV16) infection, especially in oropharyngeal squamous carcinoma (OPSS).¹² In the United States, HPV-driven OPSS is as high as 75%.¹³

HPV is a double-stranded DNA virus that only infects epithelial cells. Persistent HPV infection, especially infection with HPV16 and HPV18, is associated with the development of cervical, anorectal, and a subset of oropharyngeal malignancies. The early HPV protein E6/E7 affects the p53 and retinoblastoma genes respectively, which contribute to cell transformation.¹⁴ HPV-related HNSCC responds well to radio- and chemotherapy and has a better prognosis than HPV-negative HNSCC.^{13,15} HPV-associated HNSCC can be diagnosed by detecting the p16 protein in the tumor and by identifying integrated E7 DNA. p16 is a product of the *CDKN2A* gene, which disappears in HPV-negative HNSCC tumors but is overexpressed in HPV-related HNSCC. HPV E7 can be detected by DNA in situ hybridization or PCR. In situ hybridization has high sensitivity and can detect the integrated HPV DNA.

Diagnosis of HPV-related HNSCC can most reliably be achieved by immunohistochemical (IHC) detection of p16 followed by in situ hybridization of E7 HPV DNA or by E6/E7 real-time PCR.¹⁶

There does not appear to be enough epidemiological data to link OSCC and HPV infection.¹⁷ Published studies vary in the reported prevalence of HPV in tongue cancer. For example, the prevalence of HPV infection in oral cavity cancers varies from 0%¹⁸ to 100%.¹⁹ In addition, a worldwide study found an increase in the incidence of tongue cancer but an association between HPV and tongue cancer could not be determined.¹¹ Research on OSCC related to HPV is rare in China. Data sources are scattered and the results are controversial.^{20–23}

China is undergoing rapid social and economic changes, and people's living habits, including their sexual behaviors, are changing rapidly. Therefore, it is necessary to study the role of HPV in OSCC in depth. In this study, we explored the correlation between TSC and HPV in a cohort of 121 patients with TSC in Guangdong Province, China.

Materials and methods

Samples and patient cohort information

We retrospectively enrolled patients with a pathologically confirmed TSC, who were treated in the relevant departments of Foshan First People's Hospital and the First Affiliated Hospital of Guangdong Pharmaceutical University, Guangdong Province between 2011 and 2018 (Guangzhou), and 2012 and 2017 (Foshan). A whole cohort of patients from the two hospitals was analyzed. Criteria for the inclusion of subjects were as follows: (1) initial tumor sites included squamous cell carcinoma of tongue including root, body, and tip of the tongue; (2) all newly treated

patients had complete clinical and pathological data; (3) no other primary cancers were present; and (4) good quality and ample tissue samples were available for PCR and IHC analysis. Exclusion criteria were as follows: (1) primary tumor location was unknown or metastasis occurred from primary nasopharynx to the adjacent tongue root and other related sites; (2) patients had incomplete medical records; (3) death due to non-TSC causes; or (4) patients with concurrent nasopharyngeal cancer related to Epstein–Barr virus (EBV) or infection with human immunodeficiency virus (HIV).

Patients who met the inclusion criteria were chosen from the participating hospitals. In total, 131 patients met the preliminary screening criteria, 10 of whom were excluded by exclusion criteria. Finally, 121 patients were selected. TSC was classified as tongue root and non-tongue-root (including tongue body and tip) cancers.

Ethics statement

The study was approved by the Human Ethics Committee of the First Affiliated Hospital of Guangdong Pharmaceutical University and the First People's Hospital of Foshan, China. The ethical approval codes were GYFY201703 and FHPH20161215, respectively. Because this was a retrospective clinical study using paraffin-embedded pathological tissue, written informed consent from patients was not required.

HPV DNA extraction and PCR analysis

DNA was extracted from three pieces of 5- μ m-thick formalin-fixed paraffin-embedded (FFPE) tumor tissue sections using TaKaRa MiniBEST FFPE DNA Extraction Kit (TaKaRa Bio Group, Shiga, Japan) according to the manufacturer's protocol. This commercial kit is a genomic DNA purification kit for FFPE

tissue samples, which uses a deparaffinization method without xylene. Paraffin was eliminated during a single step of incubation in mineral oil at 80°C for 1 minute. Then, the water bath was maintained at 56°C for 1 hour after 20 µL of proteinase K (20 mg/mL) and 10 µL of RNase A (10 mg/mL) were added. After repeated elution, samples were eluted in 50 µL of elution buffer in 65°C for immediate use or stored at -20°C.

PCR was performed using a GoTaq Green Master Mix (Promega, Madison, WI, USA) PCR kit that contained 12.5 µL of PCR mixed buffer, 2 µL of primer, 2 µL of DNA template, and 8.5 µL of nuclease-free water. Forty cycles of amplification were performed on a Bio LifeEco PCR machine (ABI Veriti96 PCR, Thermo Fisher Scientific, Waltham, MA, USA) after an initial denaturation step of 5 minutes at 94°C. For HPV16 E7, the primers were (forward) 5'-CCCAGCTGTAATCATGCATGGAGA-3' and (reverse) 5'-GTGTGCCCCATTAACAGGTCTTCC A-3'. For non-HPV16, the primers were (MY09) 5'-CGTCCMARRGGAWACTGATC-3' and (MY11) 5'-GCMCAGGGWCATAAYAATGG-3'. For β -globin (*HBB*), the primers were (forward) 5'-AGGAGAA GTCTGCCGTTACTG-3' and (reverse) 5'-CCGAGCACTTTCTTGCCATGA-3'. In each batch of tests, HPV16-positive cervical cancer samples were used as a positive control and nuclease-free water was used as a negative control. PCR conditions for HPV16 and β -globin were 94°C for 2 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 1 minute for a total of 35 cycles. For HPV16-negative samples, the PCR conditions were the same as those of HPV16-positive samples except that the annealing was at 55°C for 1 minute. PCR amplicons were analyzed by 2% agarose gel

containing ethidium bromide and identified under UV light.

The concentration of the extracted DNA was determined by NanoDrop (NanoDrop 2000/2000c; Thermo Scientific, Waltham, MA, USA). Ninety percent of the DNA samples had a concentration of 30 ng/L, 10% of the DNA had a concentration <30 ng/L, and a small proportion had a concentration <5 ng/L. However, as measured by optical density value at 260 nm, the purity of all DNA samples was between 1.8 and 2.0, indicating a low level of protein and RNA contamination in the samples. The β -globin gene (*HBB*) was amplified from each sample as the housekeeping gene: all patient specimens included in the analysis had the band indicating the presence of *HBB*.

HPV type determination

HPV genetic material and genotypes were identified using the PCR-RDB HPV genotyping assay (Yaneng Biotech, Guangzhou, China). This test combines PCR amplification of target DNA and reverse line-blot hybridization for the identification of 23 anogenital HPV DNA genotypes, including 17 high-risk (HR) HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) and 6 low-risk (LR) HPV types (6, 11, 42, 43, 81, and 83). It contains an internal control (a gene probe corresponding to *HBB*) that confirms the presence of sufficient cellular material in the biological samples. The blue spots on the strip are considered positive by direct observation when the bands of the internal *HBB* control are positive. Hybridization and HPV genotyping were performed as described by the manufacturer in a recent study.²⁴ HPV types were identified by comparing the hybridization signals with the linear array HPV reference guide. Both positive and negative controls were

used and adequately typed to validate the genotyping test.

Immunohistochemistry

The p16 monoclonal antibody (E6H4), immunohistochemistry kits, and DAB (3,3-diaminobenzidine) chromogenic reagents were all purchased from Roche Biotechnology (Greenfield, IN, USA). Paraffin sections were routinely deparaffinized and subjected to immunohistochemical staining using a fully automated immunohistochemical stainer (Benchmark XT from Roche Biotechnology). p16 immunostaining was evaluated by two pathologists and defined as positive if a diffuse strong cytoplasmic and nuclear reaction was identified in 70% or more of the tumor area.²⁵ Positive reactions within individual cells or small cell clusters were considered negative.

Statistical analysis

The survival rates for patients with various TSC were compared by log-rank analysis. Risk factors between HPV-positive and HPV-negative TSC and concordance of HPV and p16 staining were analyzed by Fisher's exact test. *P*-values < 0.05 were considered significant. All statistical analyses were conducted using GraphPad Prism (version 6, GraphPad Software Inc., San Diego, CA, USA).

Results

Demographic and clinical characteristics of the study patients

This study was a retrospective analysis of 131 patients with a diagnosis of TSC, treated in two major Chinese hospitals over a period of up to 10 years, 121 of whom had sufficient clinical data and pathological material for inclusion in the current study. The clinical and demographic

findings of the study patients are summarized in Table 1. Cancers of the tongue body or anterior tip (non-tongue-root; 73.55%) accounted for most of the included subjects, although the spectrum of cancer sites was significantly different between the two participating hospitals (Table 2).

HPV-positivity in tongue cancers and concordance with p16

To determine the presence of HPV infection in TSC, paraffin-embedded tissue was tested for the presence of the HPV E7 gene DNA. HPV was first tested for by non-genotype-specific PCR, and then by PCR reverse dot blot hybridization for HPV subtypes. Among 121 patients, HPV DNA was detected in 15.7% (*n* = 19) patients, with HR HPV in 52.6% (*n* = 10) and LR HPV in 1 patient (5.3%). Both HR and LR types were observed in 15.8% (*n* = 3) patients and unknown subtypes were observed in 26.3% (*n* = 5). The most common type was HPV16 (*n* = 8, 42.1%). More than one HPV infection was identified in 15.8% of tongue cancers. HPV16 was frequently associated with other HPV types. Five subtypes (16, 18, 82, 6, and 11) were detected in total. Interestingly, five patients had subtypes that could not be detected by the current subtyping method, suggesting that more rare subtypes were present in tongue cancers (Figure 1).

Overall, 19 (15.7%) patients tested positive for HPV DNA by PCR and only 6 (4.9%) were positive for p16. Moreover, p16 status was a relatively poor predictor of HPV status as detected by PCR: only 1/121 (0.8%) patients was positive for both PCR and p16 (Figures 2 and 3, Table 2).

HPV-positive tongue cancer classified by different tumor locations and risk factor analysis

Of the 121 patients, 32 had tongue root cancers (26.45%) and 5 of these were

Table 1. Demographics and clinical characteristics of patients with tongue carcinoma in two hospital cancer cohorts (2011–2018) (n = 121).

Parameter	Values (Foshan)	Values (Guangzhou)	Values (total)
Age at diagnosis in years (SD)	59 (11.1)	51 (14.8)	54 (14.3)
Sex (n [%])			
Male	23 (79.31)	60 (65.22)	83 (68.59)
Female	6 (20.68)	32 (34.78)	38 (31.40)
Nationality (n [%])			
Han	29 (100.0)	92 (100.0)	121 (100.0)
Minority	0	0	0
Smoking use (n [%])			
Yes	19 (65.52)	32 (34.78)	51 (42.15)
No	7 (24.14)	60 (65.21)	67 (55.37)
Not recorded	3 (10.34)	0	3 (2.47)
Alcohol use (n [%])			
Yes	12 (41.37)	17 (18.48)	29 (23.96)
No	15 (51.72)	75 (81.52)	90 (74.38)
Not recorded	2 (6.89)	0	2 (1.65)
Initial tumor stage (n [%])			
Tumor category			
Tis	0	0	0
T1	3 (10.34)	18 (19.56)	21 (17.35)
T2	14 (48.28)	23 (25.00)	37 (30.57)
T3	4 (13.79)	36 (39.13)	40 (33.06)
T4	5 (17.24)	15 (16.30)	20 (16.53)
Tx	3 (10.34)	0	3 (2.48)
Nodal category (n [%])			
N0	9 (31.03)	58 (63.04)	67 (55.37)
N1	8 (27.59)	23 (25.00)	31 (25.62)
N2	5 (17.24)	9 (8.78)	14 (11.57)
N3	1 (3.45)	2 (2.17)	3 (2.48)
Nx	6 (20.69)	0	6 (4.96)
Metastasis category (n [%])			
M0	23 (79.31)	92 (100.00)	115 (95.04)
M1	3 (10.34)	0	3 (2.47)
Mx	3 (10.34)	0	3 (2.47)
Primary treatment (n [%])			
Surgery	29 (100.0)	92 (100.0)	121 (100.0)
Chemoradiation	0	0	0
Not recorded	0	0	0

n, number; SD, standard deviation.

HPV-positive; 26 patients were men (81.25%) and 6 were women (18.75%). Eighty-nine patients—57 men (65%) and 32 women (35%)—had tongue body or tip cancers (73.55%) and 14 of these were HPV-positive. Regarding tumor localization

(Figure 2), no significant difference was detected between HPV-positive and HPV-negative patients (Fisher Exact test). In addition, we analyzed the relationship between smoking, alcohol consumption, age, and sex in HPV-related TSC (Figure 4).

Table 2. Tumor HPV and p16 status.

Tumor location	Tumor subtype	PCR+ p16+	PCR+ p16-	PCR- p16+	PCR- p16-	Total
Foshan	Tongue root	1	4	2	22	29
	Tongue tip or body	0	0	0	0	0
	Total	1	4	2	22	29
Guangzhou	Tongue root	0	0	1	2	3
	Tongue tip or body	0	14	2	73	89
	Total	0	14	3	75	92
All tumor total		1	18	5	97	121

HPV, human papillomavirus; p16, p16 protein.

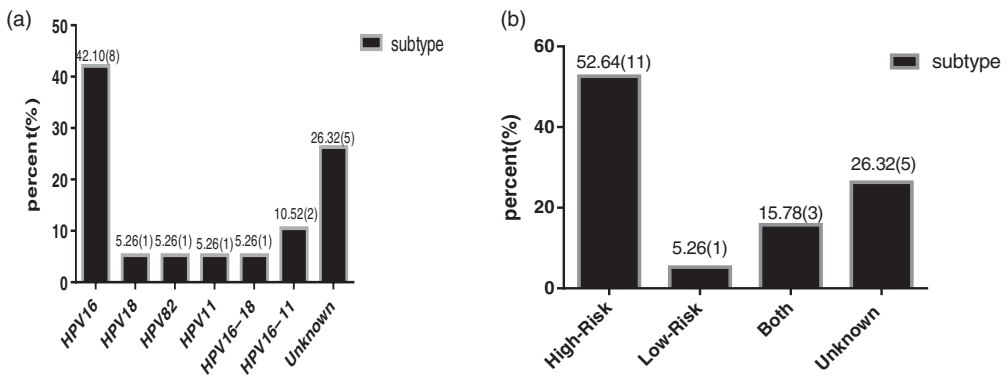


Figure 1. HPV subtypes detected among patients with tongue squamous cell carcinoma. a) HPV subtypes detected in tongue cancers. b) HR and LR subtypes detected in tongue cancers. Among 121 patients, HPV DNA was detected in 19 patients (15.7%), with HR HPV in 52.64% (n = 10), LR HPV in 5.26% (n = 1), HR and LR in 15.79% (n = 3), and unknown subtypes in 26.32% (n = 5). The most common subtype was the HR HPV16 (42.10%). More than one HPV type was identified in 15.78% of HPV-positive tongue cancers. HPV, human papillomavirus; HR, high risk; LR, low risk.

Male sex was associated with having HPV-positive TSC ($P=0.034$), whereas no significant differences were found with respect to age, smoking, or alcohol consumption habits, although HPV-positive TSC patients were younger than HPV-negative TSC patients.

Survival time between HPV-positive and HPV-negative tongue cancers

The difference in overall survival time between HPV-positive and HPV-negative TSC was analyzed for 103 patients with complete survival data, 17 of whom were

HPV-positive. The overall survival of HPV-positive TSC patients was not significantly different from that of HPV-negative patients. Patients were then divided by their initial tumor stages (T1 to T4). No significant difference between HPV-positive and HPV-negative patients could be detected for T1, T2, T3, T4, or T total (Figure 5).

Discussion

Among 121 TSC patients, HPV DNA was detected in 15.7% (n = 19), with HR-HPV in 68.5% (n = 13), LR-HPV in 21.1% (n = 4), and unknown subtypes in 33.3%

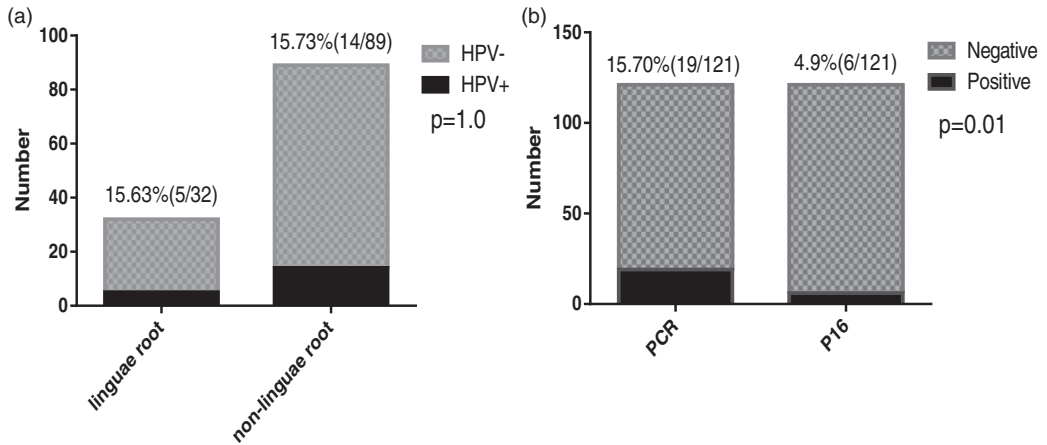


Figure 2. Tumor localization and HPV DNA and p16 positive rate in tongue carcinoma. a) Tongue cancers were divided into tongue root, tongue body, or tongue tip cancers (non-lingual root). HPV-positivity was not significantly different between tongue root and non-tongue-root cancers. b) Nineteen (15.7%) out of 88 patients were positive for HPV DNA by PCR and 6 (4.9%) were positive for p16 protein by IHC. HPV, human papillomavirus; IHC, immunohistochemistry.

(n = 5) patients. The most common type was HPV16 (n = 8, 42.1%). Compared with a study in Taiwan, where the HR-HPV infection rate was 100% in OSCC,¹⁹ the presence of HR-HPV was lower in our study. However, it did appear to be consistent with another recent study that reported an overall prevalence of HPV infection in OSCC of 21.2% and an increase in LR-HPV subtypes in squamous cell carcinoma of the oral cavity in a cohort of Taiwanese patients.²⁶ A recent study by our group found that HPV16 was the dominant HPV subtype among HNSCC patients in the same cohort of patients as in the current study,²⁷ accounting for over 90% of all detected HPV subtypes. Thus, it seems that the tongue may be a suitable site for other HPV subtypes to establish infection. However, the biological significance of this is unclear. Whether HPV is causal for TSC needs to be investigated.

The prevalence of HPV infection in oral cavity cancers varied from 5.9%²⁰ to 100%²³ in previous studies in China. In one study, the prevalence of oral cavity

cancers ranged from 5.9% to 25%,¹⁹ with TSC accounting for approximately 20% of those cases (n = 10). Similarly, HPV DNA was detected in 15.7% (n = 19) of patients with TSC in our study. Studies with a positivity rate exceeding 90% usually have few patients included.^{22,23}

In the current study, HPV infection was based on HPV PCR results, not on p16 positivity. Recent studies have shown that overexpression of p16 in TSC is not associated with HPV infection.²⁸ Our study showed that p16 positivity was not correlated with HPV DNA PCR (Figure 3, Table 2), consistent with other recent findings.²⁶ Detection of HPV via PCR directly in tumor tissue seems to be the standard method²⁹ to identify HPV-driven TSC. p16 cannot be used as a surrogate marker for HPV infection but rather is an indicator of worse prognosis in TSC.²⁸ Our study found that p16-positive patients had a better prognosis than p16-negative patients, although the difference was not significant, which may be due to the small sample size (Figure 6).

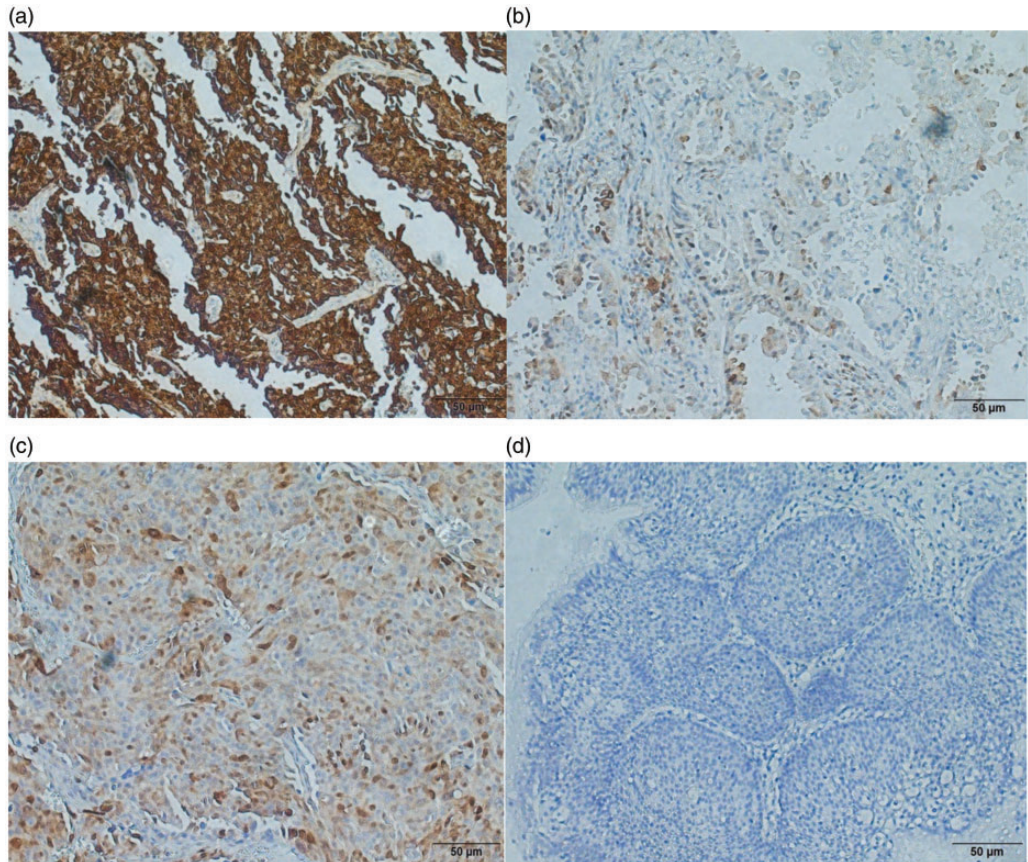


Figure 3. p16 immunostaining. a) p16-positive quality control; a high proportion of cytoplasm and nucleus is visible. b) p16-negative quality control; blue and yellow staining is mainly confined to the cytoplasm or nucleus. c) p16-positive specimen; the cytoplasm and nucleus are mainly stained yellow and brown. d) p16-negative specimen; no yellow and brown staining evident for cytoplasm and nucleus.

Guangdong Province does not have comprehensive data on cancer statistics, which is a problem when comparing results from China and Western countries. However, Guangdong Province accrued nearly 1,400 cases of oral cancer from 2004 to 2013, of which tongue cancer accounted for 54.4% (about 761 cases).³⁰ TSC is therefore not very common in Guangdong Province.

We found a significant difference in sex distribution between the HPV-positive and HPV-negative TSC patients in the current study, where men were disproportionately

affected. Similarly, a greater increase in the age-standardized incidence rate of TSC has been observed in men compared with women.¹¹ A higher prevalence of HPV in younger patients than in older patients was also noted (Figure 4). This difference was not significant, perhaps because of the small sample size in the current study.

The overall survival time of patients with HPV-positive TSC was longer than that of HPV-negative TSC patients, although this was not significant. This trend may be reflected by the younger age

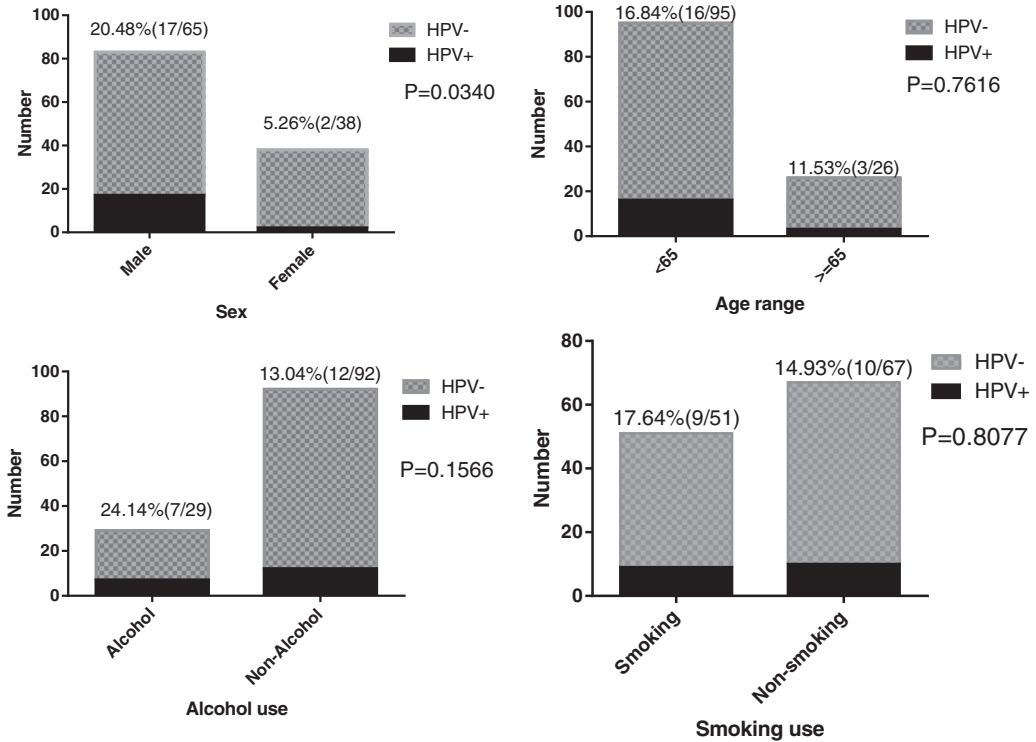


Figure 4. Risk factor analysis between HPV-positive and HPV-negative TC. HPV-positive TC was more common in male than in female patients ($P = 0.034$), whereas no significant differences were detected for age ($P = 0.7616$), smoking ($P = 0.8077$), or drinking habits ($P = 0.1577$). HPV, human papillomavirus; TC, tongue cancer.

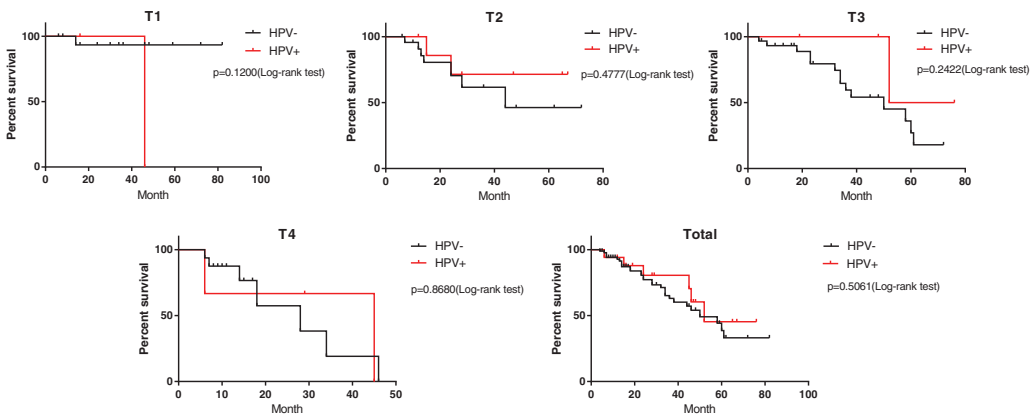


Figure 5. Survival time of patients with HPV-positive (HPV+) and HPV-negative (HPV-) tongue cancers. The overall survival time was longer for HPV+ TC patients than for HPV- TC patients, although not significant ($P = 0.1701$, log-rank test). Patients were then divided by their initial tumor stages of T1–T4 and no significant difference was found between HPV+ and HPV- patients: T1 ($P = 0.1200$), T2 ($P = 0.4777$), T3 ($P = 0.2422$), and T4 ($P = 0.8680$).

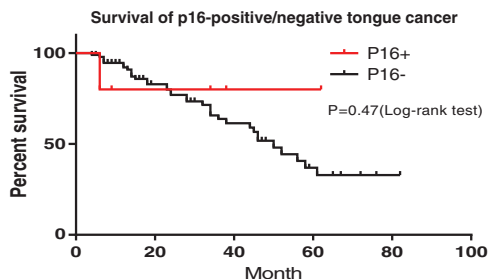


Figure 6. Survival of patients with p16-positive and p16-negative tongue cancer. p16-positive tongue cancer patients had a better prognosis than p16-negative patients, although this was not statistically significant ($P=0.47$), perhaps owing to the small sample size.

of HPV-positive TSC patients. HPV-driven HNSCC are well established as separate diseases from non-HPV-related HNSCC, especially oropharyngeal cancers, which respond better to chemo- and radiotherapy and where survival is typically longer.³¹ HR-HPV is related to the development of cervical cancer and other anogenital cancers. A prophylactic HPV vaccine was introduced to the market over a decade ago and is effective against HPV infection. HPV infection has been drastically reduced in those countries where the HPV vaccine is widely used, as have cervical abnormalities.³² The HPV vaccine in China is currently aimed at preventing HPV infection in women and has not yet been introduced in men. Based on our results, we believe that men should be included in the vaccination program to better prevent HPV infection because they are disproportionately affected by OSCC driven by HPV.

Tongue squamous cell carcinoma has the worst prognosis of all OSCC types, and the incidence of TSC is on the rise.¹¹ Therefore, it is necessary to strengthen research on the treatment and prevention of TSC, especially that related to HPV infection. The disease could be largely prevented by vaccination against HPV infection.

Conclusion

In our current study with a small cohort of patients with TSC, HPV-related TSC accounted for a proportion of total TSC in Guangdong Province, China. Both low- and high-risk HPV subtypes were observed in TSC. Further investigation is needed to confirm whether HPV is a causal factor for TSC. Preventive procedures against HPV should be considered for this subset of TSC patients, particularly in men,³³ to reduce HPV infection in the oral cavity.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This project was partly supported by the Foshan City Council Research Platform Fund (no. 2015AG1003); National Natural Science Foundation of China (no. 81472451); Science and Technology Research Program of Guangdong Province (no. 2016A020213001) and Science and Technology Research Program of Guangzhou City (no. 20180310064).

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