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HPV-mediated OPSCC in minority nationalities region along the border of South China: a multi-center study

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Abstract

Background In recent years, the incidence of HPV-mediated oropharyngeal squamous cell carcinoma (OPSCC) has shown an increasing trend. Guangxi Zhuang Autonomous Region is a border region with ethnic minorities, and current research on HPV-mediated OPSCC still remains a gap. Therefore, HPV-mediated OPSCC cases and their HPV infection sub-types were detected in this retrospective and multi-center study, aiming to explore the incidence situation of HPV-mediated OPSCC in minority nationalities region along the border of South China.

Methods A total of 361 cases of OPSCC specimens collected from nine hospitals in Guangxi Zhuang Autonomous Region from 2005-2023 were analyzed using a combined detection method incorporating p16 immunohistochemistry (IHC), RNAscope HPV HR18 in situ hybridization (ISH) and gene chip technology to identify HPV-mediated OPSCC cases and their HPV sub-types. Differences in characteristics between HPV-mediated and non-HPV-mediated OPSCC were assessed using the chi-square test or Fisher’s exact test, and survival analyses were performed using Kaplan-Meier analysis with the log-rank test. Independent prognostic factors were evaluated by Cox regression to derive hazard ratios (HRs), with patients alive at last follow-up or

lost to follow-up censored in the overall survival (OS) analysis.

Results HPV-mediated OPSCC accounted for 12.7% (46/361) of all the OPSCC cases. Among the 46 HPV-mediated OPSCC cases, HPV-16, 18, 33, 52 and 58 were detected in 42 cases. HPV-16 infection rate was the highest among the 5 sub-types, accounting for 92.8% (39/42). Single HPV sub-type infection was observed in 97.6% (41/42) of cases. The proportion of Zhuang and other minority ethnic patients with HPV- mediated OPSCC was higher than that of patients with non- HPV-mediated OPSCC , but the difference was not significant (P=0.06). T-stage, recurrence status and surgical intervention were factors influencing overall survival (OS) in patients with HPV-mediated OPSCC.

Conclusion HPV-mediated OPSCC cases in minority nationalities region along the border of South China account for approximately 13%. HPV-16 is the predominant sub-type and single sub-type infection is the main infection pattern of HPV-mediated OPSCC in this area.

Keywords HPV, OPSCC, multi-center

Oropharyngeal carcinoma (OPC) is a common malignant tumor in the head and neck region, with over 90% of cases being oropharyngeal squamous cell carcinoma (OPSCC). OPSCC refers to carcinoma originating from the oropharyngeal mucosal epithelium with squamous differentiation including tonsil, palate tonsils, base of tongue (posterior 1/3 of the border groove), soft palate, lateral pharyngeal wall and posterior pharyngeal wall. Since the 1970s,

with the decline in smoking rates, the incidence of head and neck squamous cell carcinoma (HNSCC) have gradually decreased in Europe and North America. However, the incidence of OPSCC has shown an increasing trend in recent years. Among these, the increasing incidence of HPV-mediated OPSCC exhibits a more pronounced trend, currently accounting for 20-25% of all HNSCC cases[1], indicating that HPV infection has become a new risk factor. HPV infection can occur in multiple anatomical sites of the head and neck, but primarily induces carcinogenesis in the oropharynx[2]. HPV-16 is the most common high-risk HPV (HR-HPV) sub-type in OPSCC. Over 70-90% of HPV-mediated OPSCC cases resulted from a single infection with HPV-16, while approximately 5% were caused by HPV-18[3]. Patients with HPV-mediated OPSCC predominantly affected young Caucasian people and males, with a male-to-female ratio of 4:1[4]. The risk factors of HPV-mediated OPSCC were primarily associated with sexual behaviors such as oral or vaginal intercourse with multiple sexual partners, while the relationship with alcohol and/or tobacco abuse was not significantly correlated[5]. Therefore, the rising incidence of HPV-mediated OPSCC might be associated with modern lifestyle changes and has gradually become an independent disease entity.

Globally, HPV-positive OPSCC accounted for 20.6% of all OPSCC cases before 2015[6], while this figure rose to 33% by 2021[7]. Data from the National Cancer Data Base indicated that HPV-mediated OPSCC accounted for 68% of all OPSCC cases, and this proportion continues to show an upward trend[8].

According to estimates by the Centers for Disease Control (CDC), there were over 16,000 HPV-positive OPSCC cases annually in the United States, accounting for 60%-80% of all OPSCC cases in the country. The incidence rate and associated healthcare burden have surpassed those of cervical cancer[9, 10]. It was reported that the overall HPV positivity rate among OPSCC cases was 16% in Europe, 36% in Central and South America, and only 17% in Asia[11-14]. Another survey indicated that the highest detection rates of HPV positive OPSCC were observed in North America (56%) and Japan (52%) and relatively lower rates of 17% in Southern Europe and 13% in other regions of the world, with 45% in Australia, 39% in Northern and Western Europe, 38% in Eastern Europe[15]. This demonstrates that the incidence of HPV-mediated OPSCC varies across different countries and regions globally.

China is a unified multi-ethnic country with vast territory and a total of 56 nationalities (the Han nationality and 55 minority nationalities). The Han nationality account for approximately 91.1% of the national total. Besides, the Zhuang nationality is the largest minority nationalities with a population of nearly 17,000,000, primarily distributed in the Guangxi Zhuang Autonomous Region. Guangxi Zhuang Autonomous Region is one of the five minority nationality autonomous regions in China, located on the border of South China with its southwestern part adjacent to Vietnam[16]. There are many minority nationalities living here, such as the Zhuang, Yao and Miao nationality. The overall economy of Guangxi Zhuang Autonomous Region ranks relatively low

nationwide with poor living conditions and scarce medical resources[17]. The residents exhibit low educational attainment and generally weak health awareness, with dietary and lifestyle habits potentially differing from those in other regions of China and even other countries. The studies on HPV-mediated OPSCC still remains a gap in this minority nationalities region along the border of South China.

Therefore, supported by multiple hospitals in Guangxi Zhuang Autonomous Region, this multi-center and retrospective study aims to detect HPV-mediated OPSCC from OPSCC cases and their HPV sub-types, in order to explore the incidence situation and HPV infection pattern of HPV-mediated OPSCC in minority nationalities regions along the border of South China.

Methods

1 Sample In accordance with the multi-center research principles, patients diagnosed with squamous cell carcinoma (SCC) of the tonsils, base of tongue, soft palate and other oropharyngeal sub-regions (including multiple affected oropharyngeal sub-regions) through pathological examination, who received treatment at Guangxi Medical University Hospital of Stomatology, The First Affiliated Hospital of Guangxi Medical University, People' s Hospital of Guangxi Zhuang Autonomous Region, The Second Affiliated Hospital of Guangxi University of Chinese Medicine, Liuzhou People' s Hospital, Affiliated Hospital of Guilin Medical University, Beihai People' s Hospital, Affiliated Hospital of Youjiang Medical University for Nationalities, Hezhou People' s

Hospital and Guigang City People' s Hospital from January 2005 to December 2023 were selected as the study subjects. Detailed clinical data and paraffin-embedded specimens of the patients with OPSCC were collected. The TNM clinical stage of the patients with OPSCC was classified according to the American Joint Committee on Cancer (AJCC), the eighth edition in 2017[18]. The pathological diagnosis and grading of all patients were confirmed by two consultant pathologists. Paraffin-embedded specimens were sectioned into 5μm serial sections for Hematoxylin- Eosin (HE) staining, immunohistochemistry (IHC) staining and in situ hybridization (ISH).

The inclusion criteria for this study were as follows: (1) All patients' paraffin sections were stained with HE staining and diagnosed as SCC. (2) Detailed and complete medical records were available. (3) Complete paraffin-embedded specimens were obtained for serial section.

2 Staining Method

2.1 HE staining HE staining was performed on paraffin sections of all OPSCC cases. The paraffin sections were dewaxed with xylene and rehydrated with a gradient alcohol solution. The nuclei and cytoplasm were stained with hematoxylin and eosin, respectively. Subsequently, the sections were dehydrated with a gradient alcohol solution, cleared with xylene and mounted with neutral gum.

2.2 p16 IHC p16 IHC was performed on paraffin sections of all OPSCC cases to preliminarily screen HPV-mediated (p16+) OPSCC cases. After

deparaffinization and rehydration, antigen retrieval was performed using Tris-EDTA (ZLI-9068, ZhongshanJinQiao, Beijing, China). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. After washing in PBS, sections were incubated in primary antibodies (ZM-0205-6, ZhongshanJinQiao, Beijing, China) overnight at 4℃ and in secondary antibody (PV-6000-6, ZhongshanJinQiao, Beijing, China) for 20 min at 37 ℃. Color was developed with diaminobenzidine (ZLI-9019, ZhongshanJinQiao, Beijing, China), and the sections were counterstained with hematoxylin. PBS, as a substitute for the primary antibody, was used as a negative control.

According to Guideline From the College of American Pathologists [19]and AJCC Cancer staging manual[18], HPV-mediated (p16+) SCC is defined as non-keratinized SCC with p16 IHC staining showing more than 70% moderately-to-strongly positive nuclei and cytoplasm in tumor cells without HPV DNA/RNA testing. Furthermore, studies have shown the proportion of keratinizing tumors detected in specimens from patients with HPV-positive OPSCC in Asia was higher than that in other regions[20, 21]. Therefore, keratinized/non-keratinized SCC with p16 IHC staining showing more than 70% moderately-to-strongly positive in both tumor nuclei and cytoplasm without HPV DNA/RNA detection was classified as HPV-mediated (p16+) SCC in this study.

2.3 RNAscope HPV HR18 ISH RNAscope HPV HR18 ISH was performed complementally on OPSCC samples which were p16-positive by IHC

staining but had not been met the diagnostic criteria (p16-positive tumor cells less than 70% and/or p16-positive weakly in tumor cells), aim at detecting HR-HPV mRNA expression in paraffin-embedded sections to further identify HPV-mediated OPSCC cases. HR-HPV E6/E7 mRNA ISH was performed manually using the RNAscope HPV-HR18 probe (RS-8002, ZhongshanJinQiao, Beijing, China). After heat treatment and protease pretreatment, 5μm paraffin sections were hybridized with single mixed probe targeting 18 HR-HPV sub-types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82). The sections were sequentially hybridized with a pre-amplification probe, amplification probe and horseradish peroxidase-labeled probe, followed by color development using diaminobenzidine. All steps were performed manually under appropriate controls. The presence of punctate brown signals in the cytoplasm and/or nucleus is considered RNAscope-positive.

2.4 Gene Chip HPV-mediated OPSCC samples confirmed by p16 IHC and RNAscope HPV HR18 ISH were subjected by using gene chip technology to detect HPV sub-types, aiming to analyse the infection patterns of HPV sub-types in HPV-mediated OPSCC. The HPV sub-types identified in the sequenced cases included 18 HR-HPV sub-types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) and 5 low-risk sub-types (6, 11, 42, 43 and 81).

The paraffin sections were placed in a 1.5 ml centrifuge tube for dewaxing. The centrifuge tube was added 150-200μl lysis buffer to suspend the precipitate and centrifugated at 13,000 rpm at room temperature for 5-10 min. After

discarding the supernatant, the PN-HPV nucleic acid extraction kit (PN-16E, Yaneng BIO, Shenzhen, China) was used for DNA extraction. Take 5 μL of each DNA sample as template for PCR. Hybridization was performed by an automated nucleic acid molecular hybridization instrument. The specific hybridization operational steps were carried out in accordance with the instructions provided in the PCR-Reverse Point Hybridization HPV Genotyping Detection Kit (20233400811, YanengBIO, Shenzhen, China). HPV sub-type was determined basing on the chromogenic site. The positive quality control exhibits chromogenic signals at the corresponding genotype loci and quality control loci on the hybrid membrane strip, while no coloration is observed at other loci. The negative quality control only produces a chromogenic signal at the control site on the hybrid membrane strip.

3 Statistical Analysis

The the differences among various characteristics between HPV-mediated and non-HPV-mediated OPSCC was assessed using the chi-square test or Fisher's exact test. Survival analyses were evaluated using Kaplan-Meier analysis combined with the log-rank test. The independent prognostic factors were analyzed by Cox regression model to derive the hazard ratio (HR). In the overall survival (OS) analysis, patients who were still alive at the last follow-up or had discontinued subsequent follow-ups were censored. The survival curves between groups were compared using the Log-rank test, and the curve plots were generated with GraphPad Prism 9 (GraphPad, San Diego, U.S.A). All

statistical tests were two-tailed, with $P<0.05$ considered statistically significant. All data were analyzed by SPSS 25.0 (IBM, New York, U.S.A).

Results

Subject recruitment According to the case data collected from multiple centers, 374 cases of OPSCC patients were recorded during the study period (2005-2023). Among these OPSCC, 13 patients lacked paraffin-embedded specimens and eventually 361 fulfilled our validation criteria.

HPV status According to the aforementioned diagnostic criteria, 39 HPV-mediated (p16+) OPSCC cases were identified preliminary in 361 OPSCC cases. Further RNAscope HPV HR18 ISH was performed on 25 p16+ OPSCC cases that did not meet the diagnostic criteria, yielding positive results for HPV RNAscope-positive in 7 cases. Therefore, carcinogenesis of 46 OPSCC cases (12.7%) was associated with HPV in this study. The staining of p16 IHC and RNAscope HPV HR18 ISH were detailed in Figures 1-3.

Among the 46 cases of HPV-mediated OPSCC, 42 cases was detected expression of HPV genotype, achieving a concordance rate of 91.3%. A total of 5 HR-HPV genotypes—16, 18, 33, 52 and 58—were detected, while no low-risk types were identified. HPV-16 exhibited the highest infection rate, accounting for 92.8% (39/42) of all HPV-positive cases, and the next genotype was HPV-33(4.7%).

Among the 5 HPV genotypes identified in this study, HPV-16 was detected in both single and co-infection cases. HPV-33 and 58 were exclusively present in

single infection, whereas HPV-18 and 52 were observed only in the sole case of co-infection (HPV16, 18 and 52). There were a total of 41 cases (97.6%) of single infection, of which 38 were due to HPV-16 and 2 were due to HPV-33.

3 Clinical Characteristics Compared with non-HPV-mediated OPSCC, HPV-mediated OPSCC were more prevalent among female patients (30.4% vs. 12.7%, *P*=0.002). Patients with HPV-mediated were younger than patients with non-HPV-associated OPSCC (mean age: 55.1 years vs. 57.3 years), although the difference was not statistically significant (*P*=0.223). The vast majority of HPV-mediated OPSCC patients were married (96.1%) and conceived (98.3%).

Regarding lifestyle habits, the smoking prevalence (annual smoking index ≥20 packs/year) was significantly lower in HPV-mediated OPSCC patients compared to non-HPV-mediated OPSCC patients (30.2% vs. 49.5%, *P*=0.017). The majority of HPV-mediated OPSCC patients had no history of alcohol consumption (63.0%) and even no history of betel nut chewing. Regarding medical history, 39.1% of patients with HPV-mediated OPSCC had systemic diseases, while only a minority had previously suffered from head and neck cancer or other malignancies. Only a few HPV-mediated OPSCC patients suffered from head and neck radiotherapy or had family history of cancer.

HPV-mediated OPSCC most commonly occurred in the tonsils (26.7%) and soft palate (21.7%), whereas its incidence in the base of tongue (32.6% vs. 44.1%) and other oropharyngeal walls (19.6% vs. 28.6%) was significantly lower than that of non-HPV-mediated OPSCC (*P*=0.042). Lymph node

metastasis occurred in 54.3% of cases. No distant metastases were observed in any of the HPV-mediated OPSCC cases. Only 4.3% of the cases were associated with tumors in other sites. The treatment primarily consisted of either surgery or comprehensive sequential treatment primarily based on surgery.

4 Histopathological Characteristics The histological characteristics of patients with HPV-mediated and non-HPV-mediated OPSCC were detailed in Table 2. The pathological classification of HPV-mediated OPSCC predominantly consists of moderately or poorly differentiated SCC, which was significantly higher than that of non-HPV-mediated OPSCC (P=0.001). HPV-mediated OPSCC exhibited a significantly higher proportion of non-keratotic cases (P=0.002). The tumor of HPV-mediated OPSCC were often accompanied by extensive necrosis (50.0%), high nuclear/cytoplasmic ratio (73.9%), significant cellular heterogeneity (69.6%) and showed a higher propensity for Basaloid differentiation (P=0.016).

5 Survival Analysis The follow-up deadline for all patients was 31th December, 2024. After thorough review of case records and follow-up data, detailed information was obtained for 339 patients with OPSCC (45 cases of HPV-mediated OPSCC and 294 cases of non-HPV-mediated OPSCC), achieving a follow-up rate of 94%.

5.1 Comparison of Prognosis Between Patients with HPV-mediated and Non-HPV-mediated OPSCC

The follow-up period for patients with HPV-mediated OPSCC ranged from 2

to 199 months, with a median survival time of 145.3 months. The follow-up period for patients with non-HPV-mediated OPSCC ranged from 2 to 221 months, with a median survival time of 123.6 months. Since the proportion of patients with HPV-mediated/non-HPV-mediated OPSCC exceeded 50% at all time points, the median survival period could not be estimated. Patients with HPV-mediated OPSCC exhibited higher 1-year, 3-year and 5-year OS compared to patients with non-HPV-mediated OPSCC (Figure 5&Table 3), though the difference was not statistically significant (*P*=0.077).

5.2 Multivariate analysis of prognosis in patients with HPV-mediated/non- HPV-mediated OPSCC

For patients with HPV-mediated OPSCC, each additional T-stage increased the mortality risk by 3.82-fold (95%CI: 1.127-12.968; *P*=0.031). Patients with postoperative recurrence had a 100.97-fold increased risk of mortality compared to those without recurrence (95%CI: 5.613-1816.348; *P*=0.002). Surgical intervention reduced the mortality risk by 98.8% compared to non-surgical treatment (HR=0.012, 95% CI: 0.001-0.228, *P*=0.003) (Table 4).

For patients with non-HPV-mediated OPSCC, the mortality risk was reduced by 43.8% in non-drinkers compared to drinkers (HR=0.562, 95%CI: 0.333-0.946, *P*=0.030). Patients who experienced postoperative recurrence had a 2.17-fold increased mortality risk compared to those without recurrence (95% CI: 1.452-3.254, P <0.001). The mortality risk in patients who received surgical treatment was reduced by 86.2% compared to those who did not (HR = 0.138,

95% CI: 0.090-0.212, P <0.001) (Table 5).

Discussion

Detection of HPV are widely employed in cervical cancer screening and vaccine efficacy evaluation. In recent years, with the increasing number of studies linking HPV infection to OPSCC, the detection has been progressively applied in the detection of HPV-mediated OPSCC. Compared to HPV-mediated cervical cancer, HPV-mediated OPSCC typically exhibits lower viral loads and expression levels. Its carcinogenic mechanisms may involve multiple pathways including DNA, RNA and protein levels. Detection of HPV in OPSCC is often performed using tissue samples, with a variety of testing methods available[22]. However, there is no unified and widely recognized method for HPV detection. Common approaches include PCR[23, 24], HPV DNA/mRNA ISH[25-28], gene chip technology[29], as well as p16 IHC[19, 28, 31-32] and Western blot[28]. Emerging detection techniques include droplet digital PCR (ddPCR)[33-35], loop-mediated isothermal amplification (LAMP)[36, 37] and next-generation sequencing (NGS)[38]. Different detection methods are suitable for various research purposes, and the type of specimen must be considered. Diagnosis of HPV infection requires testing the expression of HPV DNA, mRNA or oncoprotein in the specimen.

Among various detection methods for HPV infection, p16 IHC is currently the most cost-effective approach and has been widely adopted clinically for early screening of patients with HPV-mediated OPSCC. However, p16 IHC may

also yield false-positive results and should be combined with other detection methods for definitive diagnosis. HPV E6/E7 mRNA ISH is the “gold standard” for identifying HPV-mediated OPSCC[39-41].This method exhibits high detection performance but requires stringent sample quality and preservation conditions (fresh or frozen tissues). Due to the high cost and technical complexity of HPV mRNA ISH, its routine clinical application has not been widely adopted. HPV-DNA ISH can confirm whether OPSCC was caused by HPV- induced carcinogenesis, but it detects only a limited number of HPV genotypes and may yield false-negative results, leading to a high rate of missed diagnosis. Therefore, HPV-DNA ISH is also rarely employed in clinical practice[22].

Given the significant variation in the reported prevalence of HPV-mediated OPSCC worldwide (0%–86%)[42-44], it is essential to tailor prevention strategies specifically for the epidemiological context of this disease in the minority nationalities region along the border of South China. Therefore, this study employed a combined approach of p16 IHC and RNAscope HPV HR18 ISH to conduct HPV testing on OPSCC cases from multiple centers in Guangxi Zhuang Autonomous Region, revealing that HPV-mediated OPSCC accounted for 12.7% of all OPSCC cases in the minority nationalities region along the border of South China. Globally, the proportion of HPV-positive OPSCC cases among all OPSCC cases increased from 20.6% reported before 2015[6] to 33% in 2021[7]. The results of this study were lower than the global overall level. Compared with foreign countries, the result of this study was closer to the levels observed

in Thailand (11.5%-14.5%)[45, 46] and Southern Europe (10%-18%)[47-49] but significantly lower than those in North America (60%-68%)[8, 50]. Compared with various regions of China, the overall rate was closer to that in East China (11.7%-18.3%)[51, 52], yet lower than that in South China (20.8%-29.4%)[53, 54].

As this study included multi-center OPSCC cases from Guangxi Zhuang Autonomous Region, the case coverage was extensive and comprehensive. Furthermore, by incorporating the latest diagnostic criteria[18, 19] and the histological morphology of HPV-mediated OPSCC in Asia[20, 21], the obtained composition ratio of HPV-mediated OPSCC was sufficiently representative to the actual incidence of HPV-mediated OPSCC in the minority nationalities region along the border of South China. This study represented a preliminary attempt in Guangxi Zhuang Autonomous Region to detect HPV-mediated OPSCC in OPSCC cases by an approach of p16 IHC screening combined with HPV-mRNA ISH supplementary validation. By leveraging the advantages of both p16 IHC (cost-effective, mature technology and well-defined diagnostic criteria) and HPV-mRNA ISH (high detection sensitivity), this approach reduced unnecessary resource and cost waste as well as minimizing missed diagnoses of HPV-mediated OPSCC.

This study identified HPV-16 as the most prevalent infection genotype of HPV-mediated OPSCC in the minority nationalities region along the border of South China, accounting for 92.8% of all positive cases, which is consistent with numerous reports[7, 55-58]. The four most prevalent HPV genotypes, following

HPV-16, were HPV-33, 18, 52 and 58 in sequence. This indicated that HPV-16 and HPV-33 may be the two most common infection HPV genotypes in Guangxi Zhuang Autonomous Region, with similar pattern also observed in Denmark[59] and Italy[58]. A study conducted among the population in East China revealed that HPV-16 was the most prevalent HPV genotype in HPV-mediated OPSCC cases (89.36%), followed by HPV-18, 33 and HPV-35[51]. Interestingly, all patients with HPV-mediated OPSCC were infected with HPV-16 in another cohort study in East China[52].

Regarding the relationship between single or multiple HPV infections and HPV-mediated OPSCC, current reports are inconsistent. A multi- center study conducted in the southeastern coastal regions of China revealed that the predominant HPV-mediated HNSCC infections were HPV-16, 18, 11 and 82 in sequence. Additionally, dual infections involving HPV-11/16, HPV-16/18 and HPV-6/16, as well as multiple infections including HPV-16/18/33 and HPV-11/16/18/33, were identified in individual cases[57]. Another study conducted in northeastern of Italy found that all patients with HPV-mediated OPC in the region from 2000 to 2018 exhibited single infections with HR-HPVs, including HPV-16, 33, 58 and 18[58].

In this study, all HPV-mediated OPSCC were caused by HR-HPV genotypes (primarily HPV-16), which is consistent with existing research findings. However, the distribution and infection patterns of HPV genotypes differed from those reported in international and domestic studies across various regions. The

reasons for these differences can be attributable. On one hand, the detection methods of HPV genotype were various. On the other hand, these differences might be attribute to ethnicity, economy and culture in various country and region. Therefore, this study concludes that the infection genotypes of HPV-mediated OPSCC in the minority nationalities region along the border of South China are predominantly characterized by single HPV-16 infections, with a minority cases involving co-infections of multiple HR-HPV genotypes. This finding further confirms that HPV-16 remains the primary oncogenic genotype closely associated with carcinogenesis induced by HPV in the oropharynx. Furthermore, HPV-33 is the second most prevalent HPV genotype after HPV-16 in HPV-mediated OPSCC, which may account for the distinct pattern of HPV infection in the minority nationalities region along the border of South China compared to other areas domestically or internationally.

The characteristics of patients with HPV-mediated OPSCC were largely consistent with reported findings: the patients were predominantly younger, less likely to be smokers or alcohol consumers and exhibit earlier T stage[53, 61-63], further demonstrating that HPV-mediated OPSCC represents a distinct clinical entity. Notably, this study for the first time found that the proportion of Zhuang patients with HPV-mediated OPSCC (43.5%) was close to that of Han Chinese patients (50.0%) and higher than that of Zhuang patients with non-HPV-mediated OPSCC (28.9%). These results indicate potential differences in HPV-mediated/non- HPV-mediated OPSCC among ethnic minority groups in

China, warranting further in-depth research.

Smoking prolongs the duration of persistent HPV infection in the oral cavity, indicating that smoking may play a promoting role in the carcinogenic process of HPV in the oropharynx[64, 65]. However, up to 72% of OPSCC patients lack traditional risk factors such as tobacco use and alcohol consumption, but instead carcinogenesis induced by HPV infection[66, 67]. Only a minority of patients with HPV-mediated OPSCC were heavy smokers compared to patients with non-HPV-mediated OPSCC[43, 68-71]. The data from this study do not yet provide sufficient evidence to establish an association between the development of HPV-mediated OPSCC and tobacco/alcohol abuse, suggesting that HPV-mediated OPSCC is primarily a disease caused by viral infection.

The OS of patients with HPV-mediated OPSCC was higher than those of patients with non-HPV-mediated OPSCC in this study, which is largely consistent with the conclusion that the prognosis of HPV-mediated OPSCC generally surpasses that of non-HPV-mediated OPSCC[50, 69, 70]. However, this result did not conclusively demonstrate a difference in prognosis between HPV-mediated and non-HPV-mediated OPSCC ethnic minority regions along the southern border of China (P=0.077). Both recurrence and surgical treatment were significant factors influencing OS of patients with HPV-mediated and non-HPV-mediated OPSCC in this study. Furthermore, T-stage was a factor influencing OS in patients with HPV-mediated OPSCC, indicating that larger volume of tumor primary site, postoperative recurrence and absence of surgical

intervention may lead to a poorer prognosis in these patients. The prognosis of patients with HPV-mediated and non-HPV-mediated OPSCC can be assessed based on these various factors in clinical practice.

The data from this multi-center study are representative of the Guangxi Zhuang Autonomous Region and accurately reflect the true incidence of HPV-mediated OPSCC in the ethnic minority regions along the southern border of China, thereby aiding in the identification of high-risk populations for HPV-mediated OPSCC in these areas. However, this study also had certain limitations. Due to some objective constraints, gene chip or HPV-mRNA ISH was unable to perform for all OPSCC cases in this study. Future research may utilize various methods to perform HPV-related testing on all OPSCC cases and compare the sensitivity and specificity of different detection approaches.

Conclusion

This multi-center study has provided insights into the incidence and characteristics of HPV-mediated OPSCC in the ethnic minority regions along the southern border of China, which is crucial for monitoring the change of future trends. If our population follows global trends, we should anticipate and prepare for an increase in HPV-mediated OPSCC. It is imperative to conduct further research to explore the use of economic, rapid and efficient HPV detection methods for screening for HPV-mediated OPSCC.

List of abbreviations

AJCC: American Joint Committee on Cancer

CDC: Centers for Disease Control

HE: Hematoxylin- Eosin

HR: hazard ratio

HR-HPV: high-risk HPV

HNSCC: head and neck squamous cell carcinoma

IHC: immunohistochemistry

ISH: in situ hybridization

OPC: Oropharyngeal carcinoma

OPSCC: oropharyngeal squamous cell carcinoma

OS: overall survival

SCC: squamous cell carcinoma

Declarations

Ethics approval and consent to participate

The following information was supplied relating to ethical approvals (i.e., approving bodyand any reference numbers):

Permission was obtained from the Ethics Committee of Guangxi Medical University College & Hospital of Stomatology [No. 2022-KY-E- (149)] and The First Affiliated Hospital of Guangxi Medical University (No. 2022021).

Consent for publication

The ethical review board approved the consent procedure and execution of this project. This study was granted an exemption from obtaining consent.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

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Table 1 Association between HPV status and clinical features of OPSCC

Variable Quantity	Sum n(%)	HPV-mediated OPSCC n(%)	Non-HPV-mediated OPSCC n(%)	*P*
Gender				
Male	307（85.0%）	32（69.6%）	275（87.3%）	**0.002**
Female	54（15.0%）	14（30.4%）	40（12.7%）	
Age	57.00±11.10	55.13±9.61	57.27±11.29	0.223
Nationality				
Han	236（65.4%）	23（50.0%）	213（67.6%）	0.060
Chuang	111（30.7%）	20（43.5%）	91（28.9%）	
other	14（3.9%）	3（6.5%）	11（3.5%）	
Marital status				
unmarried	3（0.8%）	0（0.0%）	3（1.0%）	0.189
married	347（96.1%）	43（93.5%）	304（96.5%）	
divorced	5（1.4%）	2（4.3%）	3（1.0%）	
widowed	6（1.7%）	1（2.2%）	5（1.6%）	
Childbearing history				
Childless	6（1.7%）	1（2.2%）	5（1.6%）	0.561
Fertilized	355（98.3%）	45（97.8%）	310（98.4%）	
Annual smoking index				
<20 package·year	192（53.2%）	33（69.8%）	159（50.5%）	**0.017**
≥20 package·year	169（46.8%）	13（30.2%）	156（49.5%）	
Drinking				
No	188（52.1%）	29（63.0%）	159（50.5%）	0.111
Yes	173（47.9%）	17（37.0%）	156（49.5%）	
Betel nut chewing				
No	357（98.9%）	46（100.0%）	311（98.7%）	1.000
Yes	4（1.1%）	0（0.0%）	4（1.3%）	
History of systemic diseases				
No	205（56.8%）	28（60.9%）	177（56.2%）	0.550
Yes	156（43.2%）	18（39.1%）	138（43.8%）	
History of other cancer				
No	291（80.6%）	39（84.8%）	264（83.8%）	0.867
Yes	60（19.4%）	7（15.2%）	51（16.2%）	
Family history of cancer				
No	351（97.2%）	44（95.7%）	307（97.5%）	0.485
Yes	10（2.8%）	2（4.3%）	8（2.5%）	

Primary site				
Palatine tonsil	56（15.5%）	12（26.1%）	44（14.0%）	**0.042**
Base of tongue	154（42.7%）	15（32.6%）	139（44.1%）	
Soft palate	52（14.4%）	10（21.7%）	42（13.3%）	
Other oropharyngeal walls	99（27.4%）	9（19.6%）	90（28.6%）	
Nodal status				
No	159（44.0%）	25（54.3%）	134（42.5%）	0.132
Yes	202（56.0%）	21（45.7%）	181（57.5%）	
T-stage				
T1	50（13.5%）	10（21.7%）	40（12.3%）	0.058
T2	152（41.0%）	19（41.3%）	133（40.9%）	
T3	77（20.8%）	12（26.1%）	65（20.0%）	
T4	92（24.8%）	5（10.9%）	87（26.8%）	
N-stage				
N0	159（44.0%）	25（54.3%）	134（42.5%）	**0.015**
N1	58（16.1%）	12（26.1%）	46（14.6%）	
N2	135（37.4%）	9（16.9%）	126（40.0%）	
N3	9（2.5%）	0（0.0%）	9（2.9%）	
M-stage				
M0	352（97.5%）	46（100.0%）	306（97.1%）	0.610
M1	9（2.5%）	0（0.0%）	9（2.9%）	
Group staging				
I	62（17.2%）	26（56.5%）	36（11.4%）	**<0.001**
II	73（20.2%）	13（28.3%）	60（19.0%）	
III	58（16.1%）	7（15.2%）	51（16.2%）	
IV	168（46.5%）	0（0.0%）	168（53.3%）	
Synchronous tumors				
No	347（96.1%）	44（95.7%）	303（96.2%）	0.860
Yes	14（3.9%）	2（4.3%）	12（3.8%）	
Recrudescence				
No	284（78.7%）	41（89.1%）	243（77.1%）	0.064
Yes	77（21.3%）	5（10.9%）	72（22.9%）	
Treatment modalities				
Primary surgery	107（29.6%）	17（37.0%）	90（28.6%）	
Primary radiotherapy	7（1.9%）	1（2.2%）	6（1.9%）	
Primary chemotherapy	4（1.1%）	0（0.0%）	4（1.3%）	
Primary surgery +	82（22.7%）	12（26.1%）	70（22.2%）	

radiotherapy/chemotherapy				
Chemo-irradiation	17（4.7%）	2（4.3%）	15（4.8%）	
Primary surgery + concurrent chemo-irradiation	87（24.1%）	9（19.6%）	78（24.8%）	0.925
Primary surgery ± chemotherapy±radiotherapy +immunotherapy	6（1.7%）	1（2.2%）	5（1.6%）	
Primary surgery ± chemotherapy±radiotherapy +target therapy	3（0.8%）	0（0.0%）	3（1.0%）	
Palliative treatments	48（13.3%）	4（8.7%）	44（14.0%）	
Primary curative treatment				
Surgery-based	281（77.8%）	38（82.6%）	243（77.1%）	0.808
RT-based	24（6.6%）	3（6.5%）	21（6.7%）	
Chemotherapy-based	8（2.2%）	1（2.2%）	7（2.2%）	
Palliative treatments	48（13.3%）	4（8.7%）	44（14.0%）	

Table 2 Differences in the histologic characteristics of HPV-meidated and non-HPV-mediated OPSCC

Variable Quantity	Sum n(%)	HPV-mediated OPSCC n(%)	Non-HPV-mediated OPSCC n(%)	*P*
Pathologic diagnosis				
Squamous cell carcinoma	350（97.0%）	44（95.7%）	306（97.1%）	0.092
Papillary squamous cell carcinoma	3（0.8%）	2（4.3%）	1（0.3%）	
Basoloid squamous cell carcinoma	6（1.7%）	0（0.0%）	6（1.9%）	
Spindle cell squamous cell carcinoma	2（0.6%）	0（0.0%）	2（0.6%）	
Grade of differentiation				
Well	157（43.5%）	13（28.3%）	144（45.7%）	**0.001**
Well to moderate	46（12.7%）	1（2.2%）	45（14.3%）	
Moderate	108（29.9%）	22（47.8%）	86（27.3%）	
Moderate to poorly	16（4.4%）	3（6.5%）	13（4.1%）	
Poorly	34（9.4%）	7（15.2%）	27（8.6%）	
Basaloid differentiation				
Yes	58（16.1%）	13（28.3%）	45（14.3%）	**0.016**
No	303（83.9%）	33（71.7%）	270（85.7%）	
Keratinization				
Yes	250（69.3%）	23（50.0%）	227（72.1%）	**0.002**
No	111（30.7%）	23（50.0%）	88（27.9%）	
Degree of necrotic region				
High	180（49.9%）	23（50.0%）	157（49.8%）	0.422
Moderate	87（24.1%）	14（30.4%）	73（23.2%）	
Low	94（26.0%）	9（16.9%）	85（27.0%）	
Nuclear/cytoplasm ratio				
High	214（59.3%）	34（73.9%）	180（57.1%）	0.086
Moderate	125（34.6%）	11（23.9%）	114（36.2%）	
Low	22（6.1%）	1（2.2%）	21（6.7%）	
Degree of nuclear pleomorphism				
Severe	217（60.1%）	32（69.6%）	185（58.7%）	0.332
Moderate	92（25.5%）	8（17.4%）	84（26.7%）	
Mild	52（14.4%）	6（13.0%）	46（14.6%）	

Table 3 Comparison of overall survival in HPV-mediated / non-HPV-mediated OPSCC patients

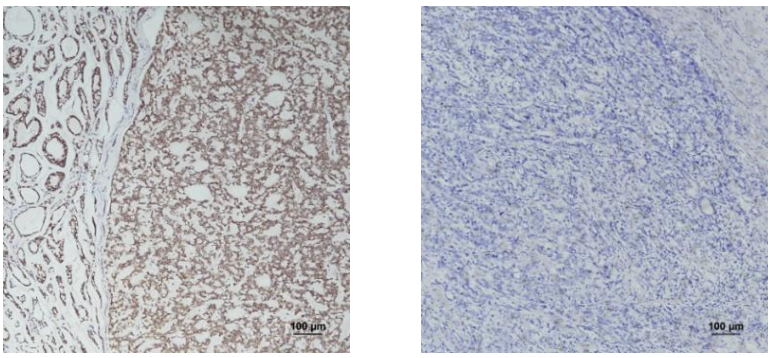
OS	HPV-mediated OPSCC	Non-HPV-mediated OPSCC	χ^2	*P*
1 year	84%	75%	3.122	0.077
3 years	73%	69%		
5 years	69%	54%		

Table 4 Cox multivariate regression analysis results of OS in HPV-mediated OPSCC

Factor	B	SE	Wald	*P*	HR (95%CI)
Age	0.227	0.808	0.079	0.779	1.255 (0.258-6.113)
Sex	-1.001	1.492	0.451	0.502	0.367 (0.02-6.836)
Annual smoking index	1.154	1.458	0.626	0.429	3.17 (0.182-55.238)
Drinking	-1.006	1.639	0.377	0.539	0.366 (0.150-9.076)
Grade of differentiation	-1.559	1.044	2.23	0.135	0.21 (0.027-1.628)
T-stage	1.341	0.623	4.633	**0.031**	3.824 (1.127-12.968)
N-stage	1.395	1.483	0.884	0.347	4.034 (0.220-73.863)
Group staging	-0.302	0.927	0.106	0.745	0.74 (0.120-4.551)
Recrudescence	4.615	1.474	9.797	**0.002**	100.972 (5.613-1816.348)
Primary curative treatment	-4.437	1.509	8.648	**0.003**	0.012 (0.001-0.228)

Table 5 Cox multivariate regression analysis results of OS in non-HPV-mediated OPSCC

Factor	B	SE	Wald	*P*	HR (95%CI)
Age	0.166	0.215	0.6	0.439	1.181 (0.775-1.798)
Sex	0.368	0.356	1.066	0.302	1.444 (0.719-2.903)
Annual smoking index	0.252	0.268	0.88	0.348	1.286 (0.760-2.175)
Drinking	-0.577	0.266	4.698	**0.030**	0.562 (0.333-0.946)
Grade of differentiation	-0.118	0.198	0.354	0.552	0.889 (0.603-1.311)
T-stage	0.039	0.127	0.092	0.762	1.039 (0.810-1.334)
N-stage	0.084	0.154	0.3	0.584	1.088 (0.805-1.470)
Group staging	0.157	0.188	0.7	0.403	1.17 (0.810-1.691)
Recrudescence	0.776	0.206	14.223	**<0.001**	2.174 (1.452-3.254)
Primary curative treatment	-1.982	0.22	81.138	**<0.001**	0.138 (0.090-0.212)



A B

Figure 1 RNAscope HPV HR18 in situ hybridization staining

A. HPV-mediated OPSCC. B. Non-HPV-mediated OPSCC

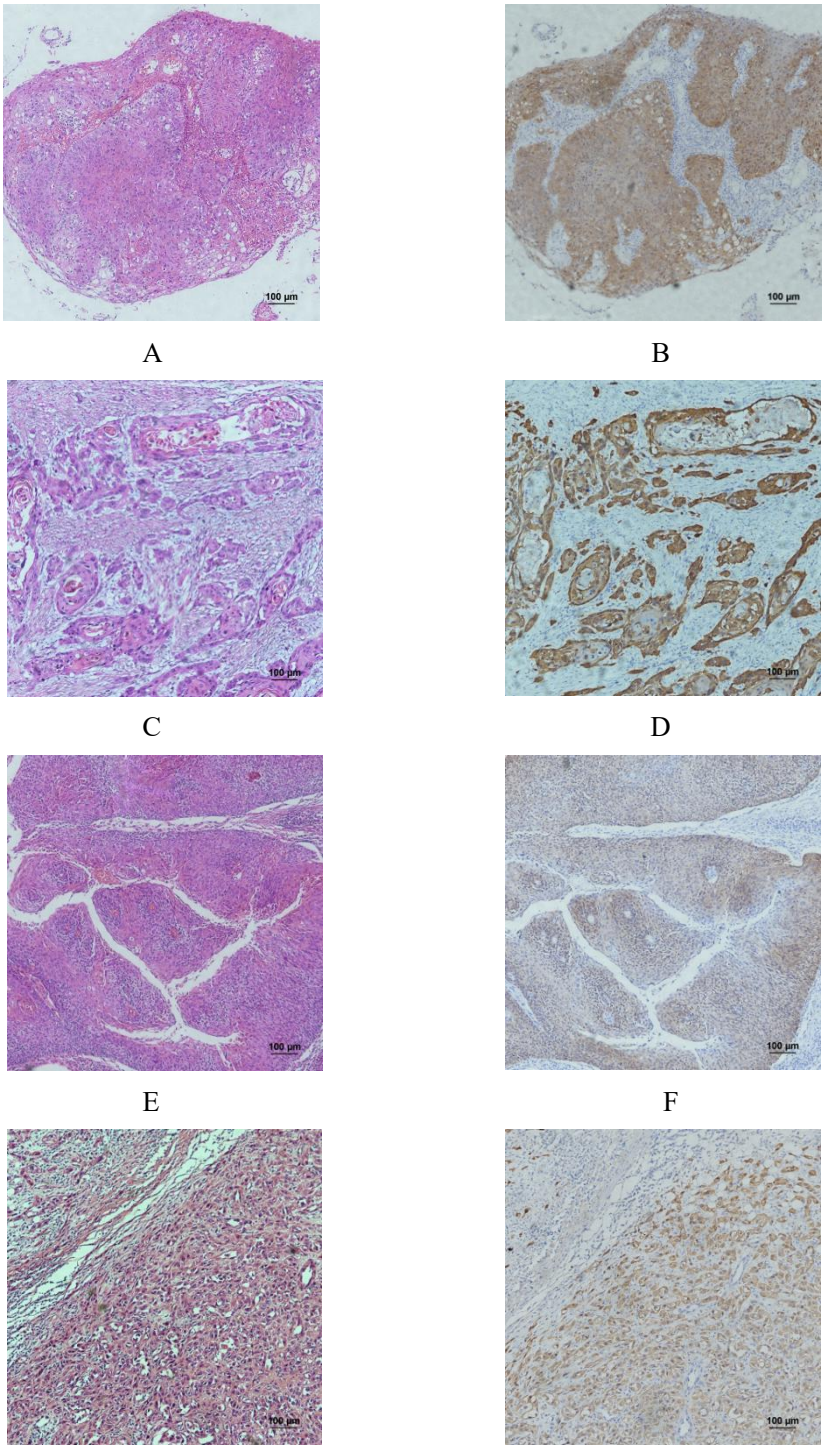


Figure 2 HE and p16 IHC staining of HPV-mediated OPSCC（100×）

A. HE of HPV-mediated tonsillar SCC. B. p16 staining of HPV-mediated tonsillar SCC. C. HE of HPV-mediated base of tongue SCC. D. p16 staining of HPV-mediated base of tongue SCC. E. HE of HPV-mediated soft palate SCC. F. p16 staining of HPV-mediated soft palate SCC. G. HE of HPV-mediated OPSCC (other oropharyngeal sites). H. p16 staining of HPV-mediated OPSCC (other oropharyngeal sites).

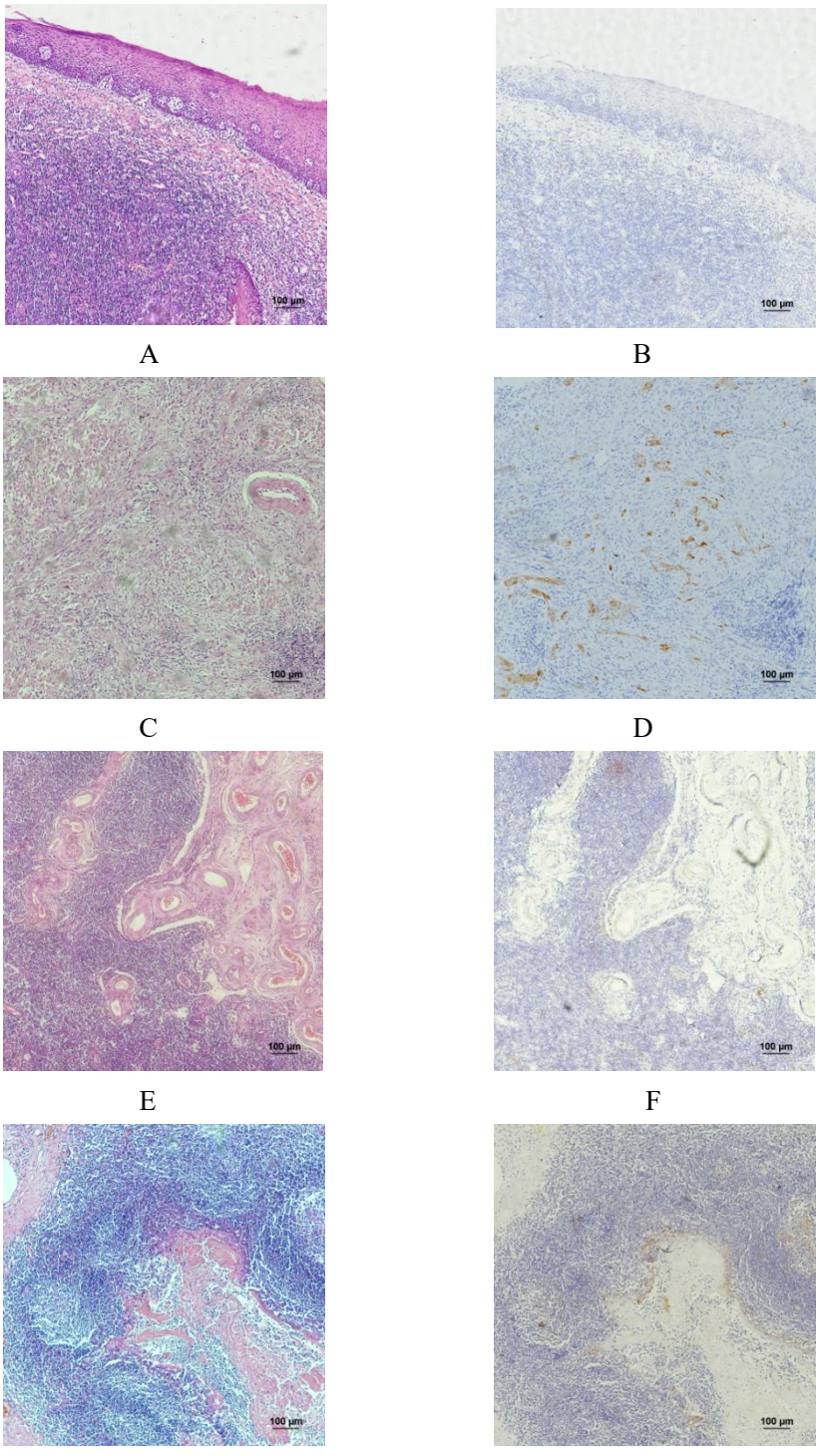
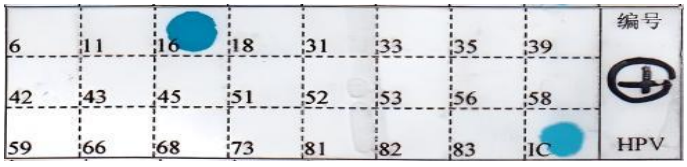
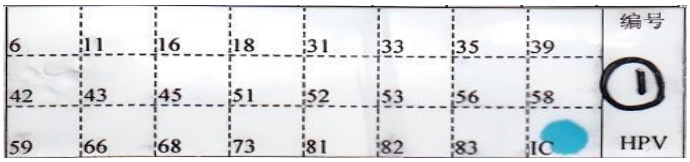


Figure 3 HE and p16 IHC staining of non-HPV-mediated OPSCC（100×）

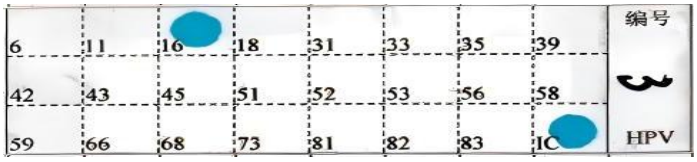
A. HE of non-HPV-mediated tonsillar SCC. B. p16 staining of non-HPV-mediated tonsillar SCC (p16 negative). C. HE of non-HPV-mediated base of tongue SCC. D. p16 staining of non-HPV-mediated base of tongue SCC (p16 scattered positivity). E. HE of non-HPV-mediated soft palate SCC. F. p16 staining of non-HPV-mediated soft palate SCC (p16 negative). G. HE of non-HPV-mediated OPSCC (other oropharyngeal sites). H. p16 staining of non-HPV-mediated OPSCC[other oropharyngeal sites(p16 staining was absent in tumor cells)] .



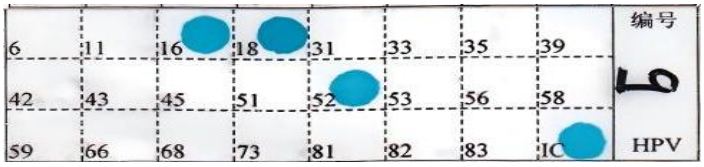
A



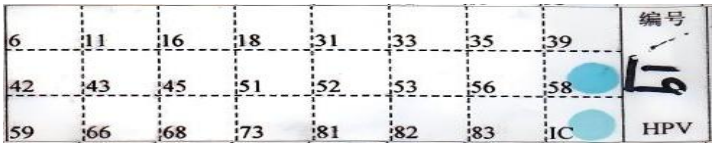
B



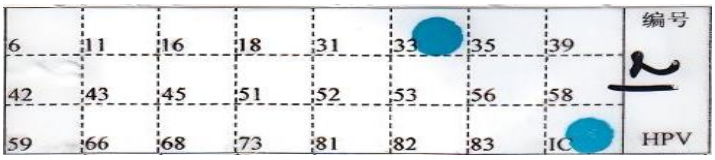
C



D



E



F



G

Figure 4 Gene Chip results

A. The positive control was HPV16 infection. B. The negative control showed no color development at any site except for the IC site, which exhibited blue spots. C. Sample No.3 was an HPV16 infection. D. Sample No.9 was a multiplex co-infection with HPV16, HPV18 and HPV52. E. Sample No.19 was an HPV58 infection. F. Sample No.21 was an HPV33 infection. G. Sample No.22 was HPV-negative.

Note: The HPV gene chip typing detection technology can simultaneously identify 23 HPV sub-types, including 17 high-risk types (HPV 16,18,31,33,35,39,45,51,52,53,56,58,59,66,68,73 and 82) and 6 low-risk types (HPV 6, 11,42,43,81 and 83). It can not only identify single but also composite HPV sub-type infections. When the IC site on the sample membrane strip appears blue, it indicates that the detection result is valid.