RESEARCH ARTICLE

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HPV-Related Oropharyngeal Cancer in Southern Thailand: Proportion Trend and Survival Outcome

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Abstract

Background: Persistent high-risk human papillomavirus (HPV) infection is one of the major etiologies of oropharyngeal squamous cell carcinoma (OPSCC). This study aimed to determine the proportion, temporal trend, and prognostic significance of HPV-related OPSCC in Thai patients. **Methods:** The study included patients with OPSCC who were treated at Songklanagarind Hospital (Songkhla, Southern Thailand) from 2009 to 2020. HPV status was screened by *p16* expression using immunohistochemistry and confirmed by real-time polymerase chain reaction. Cox regression was used to determine prognostic significance. **Results:** The overall proportion of HPV+ OPSCC was 15.3% (95% confidence interval [CI]: 12.1–18.5) with a slightly increased proportion from 10.6% in 2009–2010 to 16.5% (2019–2020) (P for trend = 0.166). Among the HPV+ cases, HPV16 was detected in 65.3%, HPV18 in 34.7%, and other high-risk HPV types in 24%. Patients with P16+ or HPV+ OPSCC had significantly better overall survival (hazard ratio [HR]: 0.63, 95% CI: 0.45–0.90 and HR: 0.63, 95% CI: 0.45–0.88, respectively). **Conclusion:** Thai patients in the southern region have a low proportion of HPV-related OPSCC with an increasing trend. Both P16 expression and HPV DNA status are strong independent prognostic factors of OPSCC.

Keywords: Human papillomavirus- genotype- oropharyngeal squamous cell carcinoma- prognosis

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Introduction

Oropharyngeal cancer is an important cancer worldwide with a global age-standardized incidence rate (ASR) of 1.8 per 100,000 men [1]. In Thailand, the nationwide ASR is 1.7 per 100,000 and it is 2.0 per 100,000 in Songkhla, Southern Thailand [2]. Squamous cell carcinoma is the main histologic type of oropharyngeal cancer (OPSCC). A global rising incidence of OPSCC, which is found to be strongly associated with persistent infection of high-risk (HR) human papillomavirus (HPV) in oropharyngeal mucosa, has been increasing over the past few decades [3]. The proportion and the increasing rate of HPV-related OPSCC vary in different regions or countries due to various factors, especially sexual practice. Currently, HPV-related OPSCC accounts for 50–70% of all OPSCC in Northern Europe and the United States while it accounts for 28%–38% in East Asian countries [4- 9]. Clinicopathological features of HPV-related OPSCC are unique and are found in younger and non-smokers and are more likely to be non-keratinizing SCC [10]. Importantly, it has a much better survival outcome compared to non-HPV-related tumors [11]. Therefore, HPV status is incorporated in the current edition (8th) of Tumor, Node, and Metastasis staging of oropharyngeal cancers [12]. Additionally, data on HPV-related OPSCC in certain countries or different regions of the country should be studied for proper patient care and treatment management.

HPV is a double-stranded circular DNA virus. More than 200 HPV types infect human cells [13]. They are classified into high-risk (HR) and low-risk HPV types [14]. At least 14 HR HPV types are identified, including HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. HPV16 is the main HPV type found in cervical cancers and other mucosal sites, including OPSCC. HPV-related carcinogenesis is driven by the two oncogenic proteins, E6 and E7. E6 binds and inactivates P53, thereby inhibiting apoptosis. The viral E7 binds to pRb and separates E2F from pRb, leading to cell cycle progression [15]. This triggers *p16* to exert its function by inhibiting CDK4-mediated phosphorylation of pRb. In routine practice, *p16* overexpression by immunohistochemistry (IHC) is accepted as a surrogate marker of HPV infection [16, 17]. However, additional tests with higher sensitivity

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or specificity, including DNA- or RNA-based methods are recommended in the case of an equivocal *p16* expression [18].

A vast majority of studies regarding the prevalence of HPV-related OPSCC and its temporal trend are from Western countries [4, 7] and only a few studies are from East Asian countries [19, 6, 8, 20]. Recently, studies from central and northeastern regions of Thailand reported an overall proportion of HPV-related OPSCC of 11.5%–17.7% [21- 23]. The current study presented the overall proportion of HPV-related OPSCC and its temporal trend over 12 years in a cohort of patients treated in a tertiary university hospital in Southern Thailand. Additionally, clinicopathological characteristics and the prognostic significance of HPV-related OPSCC were evaluated. *p16* IHC was used as a screening marker for HPV infection and a real-time polymerase chain reaction (PCR) for HPV DNA detection as a confirmation method.

Materials and Methods

Patients and clinical data

The study included patients with primary OPSCC, treated at Songklanagarind Hospital from January 2009 to December 2020. This 1000-bedded tertiary university hospital in Songkhla province provides comprehensive care serving a population in the southern region. The majority (more than 80%) of cancer patients in the region who require radiation or chemotherapy or need complex surgeries are referred to this hospital. The oropharyngeal site was defined following the International Classification of Diseases version 10 (ICD-10), as a base of the tongue, tonsil, soft palate, uvula, pharyngeal wall, and overlapped area of the oropharynx. Only patients with available paraffin-embedded tissue blocks in the Department of Pathology were included, and tumor samples with limited tumor cells for IHC staining were excluded.

Demographic and clinical data, including age, sex, history of smoking, alcohol drinking, betel nut chewing status, tumor site, clinical stage, date of last follow-up, and status of last follow-up, were retrieved from electronic medical records. Pathological information was obtained from pathological reports. Clinical staging was based on the American Joint Committee on Cancer 7th (2009–2017) and the 8th edition cancer staging (starting in 2018). The date and cause of death were obtained from the database of the hospital cancer registry, which was updated through the National Civil Database bi-annually. The study was approved by the Human Research Ethics Committee of the Faculty of Medicine, Prince of Songkhla University (REC.63-241-5-1).

Immunohistochemistry (IHC) for p16 expression and evaluation

The 3-μm-thick sections were deparaffinized with xylene and rehydrated in graded alcohol. An automated immunostainer (Leica BOND-MAX, Melbourne, Australia) was used for IHC for *p16* expression. Antigens were retrieved in the Tris–EDTA buffer (Bond Epitope Retrieval Solution 2, Leica Biosystem, Newcastle Upon Tyne, UK), pH 9, in a pressure cooker at 95°C for 4 min. Sections were first incubated with bond peroxidase-blocking reagent (Bond Polymer Refine Detection, Leica Biosystem, Newcastle Upon Tyne, UK) and then with primary antibodies against *p16* at a dilution of 1:5 (clone E6H4, CINtec® *p16* Histology; Roche, Tuscon AZ, USA). A bond polymer refine detection kit (Leica) was used to detect the antigen-antibody reaction, followed by color development using 3,3'-diaminobenzidine as a chromogen and Meyer's hematoxylin as a counterstain.

Immunostaining for the *p16* was evaluated by the percentage of positively stained tumor cells. The intensity of staining was scored as strong (3+), moderate (2+), weak (1+), or negative (0). Moderate to strong intensities and diffuse nuclear and cytoplasmic staining in ≥70% of the tumor cells were considered positive for *p16* expression. All sections were independently examined by a senior pathologist and a third-year resident. Discrepancies were resolved by a discussion on a multi-head microscope.

HPV DNA detection

HPV DNA detection was done for the purpose of this study. All *p16*+ tumors and 50 random *p16*− tumors were confirmed for the presence of HPV. DNA by real-time PCR. The QIAamp® DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany) was used to extract DNA from 5 to 10 5-micron (depending on tissue size) tissue cut from paraffin-embedded tissue blocks and stored at −20°C until used. The 14 HR HPV with 16/18 Genotyping Real-time PCR Kit (HBRT-H14; Hybribio, Chaozhou, China) was used for real-time PCR. The kit has been designed to detect 14 HR HPV types, including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, with specific detection of HPV16 and 18 genotypes. Cellular internal control was included for each sample to monitor the whole testing process, starting from DNA extraction to signal detection. Bio-Rad CFX Manager (C1000 Touch Thermal Cycler, Bio-Rad, Germany) was used to assess results following the manufacturer's instructions. Positive controls were valid when the threshold cycle (Ct) was ≤36, while negative controls were valid when undetected. Samples were re-run if either control was deemed invalid. The PCR results were interpreted as HPV type 16, HPV type 18, other HR types, or negative for detection.

Statistical analysis

Descriptive analysis of clinicopathological data was presented in percent, mean (standard deviation), and median (interquartile range [IQR]) as appropriate. The chi-square test or Fisher's exact test was used to test the comparison of clinicopathological variables of HPV+ versus HPV− OPSCC as appropriate. The proportion of *p16*+ OPSCC and HPV+ OPSCC by 2-year intervals were calculated along with a 95% confidence interval (CI). The chi-square test was used to test the significance of the trend of proportion. The Kaplan–Meier method was used to estimate the overall survival (OS) and the logrank test was used to test for differences between survival curves. Cox regression analysis was used to obtain independent associations of *p16* and HPV status with OS. All variables were tested for proportional hazard assumption, and a stratified Cox regression model was applied if the variable

did not meet the assumption criteria. A p value of < 0.05 was considered statistically significant. The R Program version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for all analyses.

Results

Patient characteristics

There was a total of 560 patients with OPSCC treated at our institute for a period of 12 years. Tissue blocks of 65 patients were not available and 5 blocks had inadequate tissue for IHC staining, leaving 490 cases for the final analysis. Table 1 shows the clinicopathological characteristics of the study cohort. The median age was 65 years. Most patients were male (93.9%) and had a history of smoking (88.5%) and alcoholic drinking (79.8%). The majority of patients were at stage III or IV at diagnosis (80.5%).

Frequency and trend of p16-positive and HPV-positive OPSCC

Positive *p16* expression was found in 73 of 490 cases (14.9%). HPV PCR was performed in 67 *p16*+ samples because six cases of *p16*+ samples had inadequate DNA. HPV DNA was detected in 75 cases, of these, 62/67 of *p16*+ tumors and 13/50 *p16*− tumors. The concordance rate between *p16* IHC and HPV PCR was 84.6% (75/117). The estimated overall proportion of HPV+ OPSCC was 15.3% (95% CI: 12.1–18.5) (75/490).

Among HPV+ samples, HPV16 was detected in 65.3% (49/75), HPV18 in 34.7% (26/75), and other HR types in 24% (18/75). Table 2 shows the mutually exclusive distribution of HPV type. Mono-infection (only one HPV type detected) was found in 78.7% and multiple infections in 21.3%. The result regarding the temporal trend of proportion revealed a trend toward an increasing proportion of HPV+ tumors from 10.6% (2009–2010) to 16.5% (2019–2020) (p for trend = 0.166, Figure 1).

Association of clinicopathological characteristics with HPV status

Table 3 shows the clinicopathological characteristics among patients with HPV+ and HPV− OPSCC. Patients with HPV+ tumors were more likely to be younger, female patients, non-smokers, and non-betel chewers. HPV+ tumors were more likely to be moderately or poorly differentiated SCC (76%) compared to HPV tumors (59.4%). The distribution of tumor size and clinical stage were not different between the two groups.

Association of p16 expression and HPV status with OS

The median follow-up time was 13.3 months (IQR: 5.6–31.1 months). The median survival time of the entire cohort was 14.7 months (95% CI: 12.9–16.9 months). Kaplan–Meier analysis revealed significantly better OS in patients with *p16*+ and HPV+ tumors than those with *p16*− and HPV− tumors (p < 0.001) (Figure 2). The median survival time of patients with *p16*+ or HPV+ was 33.3 months compared to 13.6 months in patients with *p16*− or HPV− tumors.

Cox regression analysis for OS

Univariate Cox regression revealed a significant association in age, clinical stage, treatment, *p16* expression, and HPV status with the increased risk of death (Table 4). Multivariable analysis revealed that the treatment did not meet the proportional hazard assumption, thus we used stratified Cox regression by fitting the model according to the strata of treatment. *p16* expression and HPV status were separately evaluated in a multivariate model as they are highly correlated. Table 5 shows the final multivariate model. *p16*+ (HR: 0.63, 95% CI: 0.45–0.90) and HPV+ tumors (HR: 0.63, 95% CI: 0.45–0.875) were strongly associated with favorable survival outcomes.

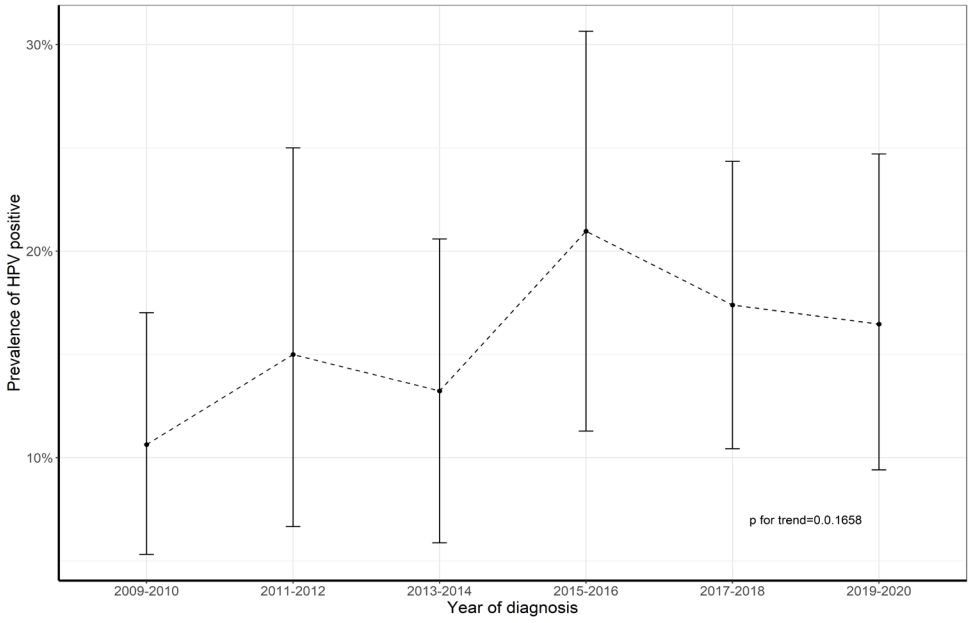

Figure 1. The Proportion of HPV+ OPSCC (%) with a 95% Confidence Interval from 2009 to 2020 by a 2-Year Interval.

Table 1. Clinicopathological Characteristics of Patients (n = 490).

Variables	Number (%)
Age, median (interquartile range)	65 (56–74)
Sex	
Male	460 (93.9)
Female	30 (6.1)
Smoking	
No	50 (10.4)
Yes	433 (89.6)
Alcohol drinking	
No	92 (19)
Yes	391 (81)
Betel nut chewing	
No	321 (68.2)
Yes	150 (31.8)
Tumor site	
Base of tongue	189 (38.6)
Tonsil	182 (37.1)
Soft palate	87 (17.8)
Posterior pharyngeal wall	11 (2.2)
Overlapped area	21 (4.3)
Clinical stage	
Stage I	35 (7.3)
Stage II	59 (12.3)
Stage III	74 (15.4)
Stage IV	313 (65.1)
Treatment	
Surgery only	22 (4.5)
Radiation only	70 (14.3)
Surgery with radiation/chemotherapy	58 (11.8)
Radiochemotherapy or chemotherapy	171 (34.9)
Supportive treatment	169 (34.5)
Tumor differentiation	
Well	185 (37.8)
Moderate	203 (41.4)
Poor	102 (20.8)
Lymphovascular invasion	
Yes	10 (2)
No	480 (98)

Discussion

This study revealed a low proportion of HPV+ OPSCC (15.4%); however, an increasing trend of this proportion is evident. The proportion of HPV+ OPSCC increased from 13% to 16.5% in 12 years. Consistent with most previous studies, our study supported the evidence of favorable survival outcomes in patients with HPV-related OPSCC.

A high prevalence of HPV-related OPSCC has been reported in various countries, especially those in North America and Europe [5, 9]. In our study, we revealed a low prevalence of HPV+ tumors among OPSCC (15.4%).

Table 2. Mutually Exclusive Distribution of HPV Type among the HPV-Positive Cases.

HPV type	Number	Percent
HPV 16 alone	38	50.7
HPV 18 alone	11	14.7
Other HR types	10	13.3
HPV 16 and 18	8	10.7
HPV 18 and other HR types	5	6.7
HPV 16, 18, and other HR types	2	2.7
HPV 16 and other HR types	1	1.3
Total	75	100

HPV, human papillomavirus; HR, high-risk

As OPSCC patients need complex treatments, they are majorly referred to our institute, therefore, our results, more or less, represent the figure of HPV-related OPSCC in the southern Thai population. Our study showed almost

Table 3. Clinicopathological Characteristics of Patients Classified by HPV DNA Status.

	HPV-positive (N = 75)	HPV-negative (N = 409)	
Variables	Number (%)		p value
Age, median (interquartile range)	60 (50–68.5)	67 (58–75)	<0.001
Sex			0.03
Male	66 (88)	389 (95.1)	
Female	9 (12)	20 (4.9)	
Smoking			<0.001
Yes	56 (76.7)	372 (92.1)	
No	17 (23.3)	32 (7.9)	
Alcohol drinking			0.767
Yes	58 (79.5)	327 (80.9)	
No	15 (20.5)	77 (19.1)	
Betel use			0.029
Yes	15 (20.8)	133 (33.8)	
No	57 (79.2)	260 (66.2)	
Tumor site			0.104
Base of tongue	19 (25.3)	168 (41.1)	
Tonsil	35 (46.7)	144 (35.2)	
Soft palate	15 (20)	72 (17.6)	
Posterior pharynx	3 (4)	8 (2)	
Overlapped area	3 (4)	17 (4.2)	
Clinical stage			0.116
Stage I	9 (12.3)	25 (6.2)	
Stage II	10 (13.7)	47 (11.7)	
Stage III	14 (19.2)	58 (14.4)	
Stage IV	40 (54.8)	272 (67.7)	
Tumor differentiation			0.002
Well	18 (24)	166 (40.6)	
Moderate	32 (42.7)	170 (41.6)	
Poor	25 (33.3)	73 (17.8)	
Lymphovascular invasion			0.658
Yes	73 (97.3)	401 (98)	
No	2 (2.7)	8 (2)	

Table 4. Univariate Cox Regression Analysis for OverAll Survival of Patients with Oropharyngeal Cancer.

Variable	HR (95% CI)	p value
Age	1.02 (1.01–1.03)	<0.001
Sex (male vs female)	1.25 (0.79–1.98	0.348
Smoking (yes vs no)	1.48 (1.01–2.16)	0.044
Alcohol drinking (yes vs no)	1.11 (0.84–1.45)	0.465
Betel use (yes vs no)	1.22 (0.97–1.52)	0.085
Tumor site (ref = base of tongue)		
Tonsil	0.79 (0.62–1.01)	0.056
Soft palate	0.9 (0.68–1.2)	0.486
Posterior pharyngeal wall	0.9 (0.44–1.84)	0.78
Overlapped area	1.52 (0.94–2.46)	0.086
Clinical stage (ref = stage I)		
Stage II	1.68 (0.96–2.94)	0.067
Stage III	2.23 (1.31–3.81)	0.003
Stage IV	2.72 (1.68–4.4)	<0.001
Tumor differentiation (ref = well)		
Moderate	0.96 (0.76–1.2)	0.701
Poor	0.72 (0.54–0.96)	0.027
lymphovascular invasion (yes vs. no)	0.66 (0.31–1.39)	0.276
Treatment (ref = supportive)		
Surgery	0.21 (0.11–0.38)	<0.001
Radiation	0.45 (0.33–0.62)	<0.001
Surgery with radiation or CMT	0.22 (0.15–0.32)	<0.001
Radiochemotherapy or CMT	0.34 (0.26–0.43)	<0.001
p16+ vs *p16*-	0.53 (0.38–0.73)	<0.001
HPV+ vs HPV-	0.56 (0.41–0.77)	<0.001

CI, confidence interval; CMT, combined-modality treatment; HPV, human papillomavirus; HR, high-risk; ref: reference

Table 5. Multivariate Cox Regression Analysis for Overall Survival of Patients with Oropharyngeal Cancer.

Variables	HR (95% CI)†	p value
Clinical stage (ref = stage I)		
Stage II	1.49 (0.848–2.633)	0.165
Stage III	2.12 (1.216–3.698)	0.008
Stage IV	2.32 (1.393–3.874)	0.001
p16+ vs *p16*−‡	0.63 (0.448–0.897)	0.01
HPV+ vs HPV−‡	0.63 (0.454–0.875)	0.006

†, adjusted by strata of treatment; ‡, HPV status and *p16* expression were separately tested; Abbreviations: CI: confidence interval; HPV: human papillomavirus; ref: reference

exactly similar to the previous three studies from other regions of Thailand and other studies from Southeast Asia. These included two reports from the central region of Thailand [22, 23] and one report from the northeastern region [21] which revealed the proportion of HPV-related OPSCC of 14.5%, 15.6%, and 17.7% from a total sample size of 110, 64 and 96, respectively. These three studies used PCR-based methods for HPV detection, thus the results are comparable. A study from Malaysia also revealed a low proportion of HPV-related OPSCC (16.7%) [24]. All of this evidence may indicate a low proportion of HPV-related OPSCC in Southeast Asian populations. However, a higher prevalence of HPV-related OPSCC (28%–38%) was reported in other Asian countries, including Japan, China, and Taiwan [19, 6, 8]. Reports from Middle East Asia also documented a high prevalence (up to 80%) of HPV+ OPSCC [25, 26]. This is probably due to the more Westernized lifestyle of certain population groups as well as other factors in these countries compared to the Southeast Asian populations.

We found an increasing trend of HPV+ OPSCC proportion from 10.56% in (2009–2010) to 16.5% (2019–2020), but with no statistical significance, probably due to the small number of cases in each time interval. Nevertheless, our results, more or less, represent the figure of HPV-related OPSCC in the southern Thai population. The increasing trend of HPV+ OPSCC has also been reported by the study from the northeastern region [21]. This study reported a significant increase in HPV prevalence by 2% annually from 16% in 2012 to 26% in 2017. The study used the same HPV PCR detection kits as our study, but their result may be more solid as they performed HPV DNA analysis in all tumors while our study confirmed the presence of HPV DNA only in *p16*+

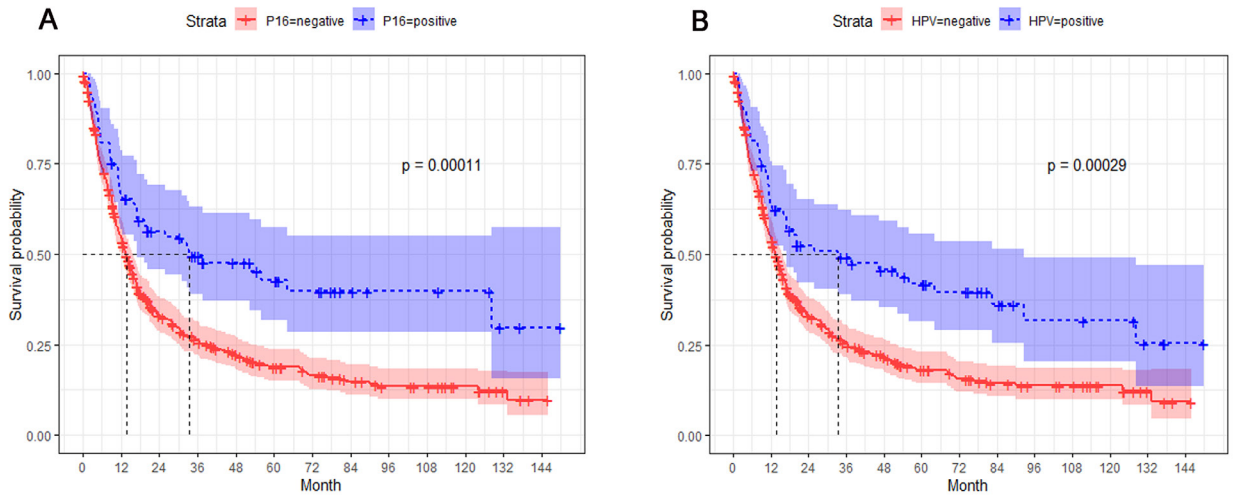

Figure 2. Kaplan–Meier Survival Curves According to *p16* Expression (A) and HPV DNA Status (B).

tumors and a random set of *p16*− cases. Other East Asian countries, including Taiwan and Korea, also reported an increasing trend of HPV+ OPSCC. A large study from Taiwan revealed an increasing trend for 18 years, but with no statistical significance [19]. Another study from South Korea demonstrated a significantly increased HPV+ OPSCC proportion from 33.3% in 2008–2009 to 83.3% in 2020 [27]. However, this study used *p16* expression to define HPV status which might lead to overestimated HPV+ rate. All this evidence indicates an increasing trend of HPV-related OPSCC in the Asian population which is similar to Western countries, although the rate of increase is controversial.

HPV16 is consistently reported as the major genotype (>85%) in HPV-related OPSCC in the Western population [28-30]. This information in the Asian population is scarce. One large study from Taiwan [19] and from Thailand [21] reported a high frequency of HPV16 up to 83% and 82%, respectively. However, the representative HPV type in the latter study may be limited due to the small number of HPV+ cases (n = 17). Interestingly, our study revealed a different result of a considerably lower proportion of HPV16 (62.67%). Additionally, our cohort had a higher proportion of multiple infections (21.3%) compared to <10% in the aforementioned studies [28, 19]. The difference in the frequency of specific HPV genotypes might have a clinical impact. A recent systematic review [31] revealed a significant impact on survival in three of six studies, of which two studies revealed a better survival among HPV16 cases compared to other HR HPV genotypes while the other one revealed the reverse results. Therefore, the determination of specific HPV genotypes in HPV-related OPSCC may be important for patient management; however, further meta-analysis or future trials are needed.

The association of clinicopathological characteristics with HPV status in OPSCC appears to be similar to previous studies in Western countries and Thailand [32, 21, 23]. Patients with HPV+OPSCC are younger, non-smokers. The tumor occurs more frequently at the tonsil and has poorly differentiated histology. The results regarding prognostic HPV status are also consistent with previous studies [32, 28]. Patients with HPV+ tumors (HR: 0.63, 95% CI: 0.45–0.88) had better OS. The prognostic value of *p16* expression was exactly equivalent to that of HPV DNA status (HR: 0.63, 95% CI: 0.45–0.89). This supports the clinical utility of *p16* expression evaluated by IHC in clinical practice.

Our study has some limitations. We could not evaluate a portion of patients treated in our hospital (about 12%) due to unavailable tissue blocks. In addition, not all tumor samples were tested for HPV DNA analysis. We performed DNA analysis in *p16*+ samples and selected *p16*− samples; therefore, the HPV prevalence in this study may be underestimated. Additionally, this is a hospital-based study, thus the results may not represent the incidence and trend of HPV-related OPSCC in the population. However, this study is a large series of its kind and is the largest study regarding HPV-related OPSCC in Thailand.

In conclusion, the present study reports a potentially increasing proportion of HPV-related OPSCC in the Southern Thailand population, although the overall proportion is low. HPV-related OPSCC, evaluated by either *p16* IHC or HPV PCR analysis, was confirmed to be associated with favorable survival outcomes.

Author Contribution Statement

P.S. collected the data, performed statistical analysis and drafted the manuscript. K.J. collected the clinical data and drafted the manuscript. A.D. designed the study and drafted the manuscript. J. J. performed laboratory work. P. T. designed the study, performed statistical analysis, and reviewed & edited the manuscript.

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Conflict of interest

The authors declare no conflicts of interest.

Ethical issue

The study was approved by the Human Research Ethics Committee of the Faculty of Medicine, Prince of Songkhla University (REC.63-241-5-1). Individual informed consent was waived due to the nature of the study.

Data Availability Statement

The data presented in this study are available upon request from the corresponding author.

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