

Role of high-risk human papillomavirus in the etiology of oral and oropharyngeal cancers in Thailand: A case–control study

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Abstract

Background: Among developing countries, Thailand shows no increase in the incidence of human papillomavirus–driven oropharyngeal cancer. The causal role of human papillomavirus infection in this pathology has not been researched thoroughly.

Methods: A hospital-based, case–control study was performed which included 104 patients with newly diagnosed oral and oropharyngeal squamous cell carcinomas and 104 individuals without cancer. The Cervista high-risk human papillomavirus and 16/18 assays were used to detect human papillomavirus. Odds ratios were used to assess the association between high-risk genotypes of human papillomavirus and the cancers.

Results: High-risk human papillomavirus was detected in 4 of 52 (7.7%) oral cancer cases, 6 of 52 (11.5%) oropharyngeal cancer cases, and 1 of 104 (0.96%) control subjects. Of 104 cancer patients in the study, 83 were smokers. High-risk human papillomavirus was significantly associated with oropharyngeal cancer (odds ratio = 13.44, 95% confidence interval = 1.6–114.8) but was nonsignificantly associated with oral cancer (odds ratio = 8.58, 95% confidence interval = 0.9–78.9). However, after adjustment for smoking, high-risk human papillomavirus was determined to be nonsignificantly associated with oropharyngeal cancer (adjusted odds ratio = 5.83, 95% confidence interval = 0.8–43.5).

Conclusion: Although low human papillomavirus prevalence was observed, the rate of high-risk human papillomavirus infection in the cancer group was still higher than that in the control group. Smoking may have an influence on the etiology of human papillomavirus–related cancers. However, the study is underpowered to clarify the role of human papillomavirus as the independent risk factor for oral and oropharyngeal cancers in the Thai population.

Keywords

Human papillomavirus, human papillomavirus, smoking, oral cancer, oropharyngeal cancer, prevalence, etiology, Thailand

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Introduction

Head and neck cancer is one of the most frequently diagnosed cancers worldwide. The cause of head and neck cancer has been attributed to several factors, including smoking, concurrent smoking and alcohol consumption, betel quid chewing, and virus infections, such as Epstein–Barr virus and human papillomavirus (HPV).^{1–3} There are more than 100 types of HPV, of which at least 14 are cancer causing (also known as high-risk type). HPV type 16 is the predominant type found in HPV-related head and neck squamous cell cancer.

HPV-related head and neck squamous cell cancer has attracted much attention in recent years. Cumulative evidence shows that HPV infection is one of the significant risk factors for oropharyngeal squamous cell carcinoma;

however, the relation between HPV and other head and neck squamous cell cancers remains elusive.⁴

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A downward trend in smoking and an increased HPV exposure cause an increasing incidence of oropharyngeal squamous cell cancer and a decline of oral squamous cell cancer incidence in many developed countries.⁵

Despite aggressive anti-smoking campaigns, the incidence rates of oral and oropharyngeal squamous cell cancers in Thailand remained stable for the last decade.⁶ The factors that contribute to these trends, especially HPV infection, have not been researched thoroughly. Thus, we conducted a study to investigate the role of high-risk HPV (HR-HPV) in oral and oropharyngeal squamous cell cancers in order to understand the actual situation of head and neck squamous cell cancer etiology in Thailand.

Materials and methods

Study design and data collection

This case–control study was conducted at Chonburi and Lopburi cancer hospitals. These hospitals have been assigned as referral centers for cancer patients from 20 provinces in Eastern and Central Thailand. Patients were nonrandomly enrolled. The following inclusion criteria were used: a new diagnosis of oral or oropharyngeal cancer, Thai nationality, and histological features of squamous cell carcinoma. Patients with lip cancers and cancers without clearly demarcated tumor epicenter were excluded. Ethical approval for the study was granted by the respective hospital ethics committees. All study participants provided informed consent prior to their enrollment in this study.

Sample size calculation

The sample requirement for this case–control study was calculated using Epi Infotm version 7.2.1.0 (Division of Health Informatics and Surveillance, Center for Surveillance, Epidemiology and Laboratory Services, Atlanta, GA). The software requires as inputs the estimated HPV infection rates of cases and control subjects. Because no HPV study in Thailand with comparable HPV testing methods was conducted, the assumed HPV infection rates were based from data in other countries. We estimated the presence of HPV infection to be at least 20% in patient cases and less than 2% in control subjects.^{7,8} The study was an unmatched design. The control-to-case ratio for each cancer site was 2:1. The desired power to detect a difference was 85% with a 95% confidence interval (CI). Minimum sample size was estimated to be 44 patients and 88 control subjects. Therefore, we aimed to recruit at least 50 oral cancer cases, 50 oropharyngeal cancer cases, and 100 control subjects.

Selection of the control group

The control subjects were recruited from healthy persons and noncancer patients who visited the clinic for a checkup

examination. After the enrollment of cancer cases, subjects who matched the case patients by age (± 3 years) and sex were invited to participate in the study.

Specimen collection and HPV testing

Epithelial cell samples were collected by primary tumor brushing in the cancer group and normal mouth and throat mucosa brushing in the control group. The brush device was suspended in PreservCyt[®] transport medium (Hologic, Inc., Marlborough, MA). All specimens were submitted to Chonburi cancer hospital laboratory, and HPV tests were performed within 1 month after collection. The Cervista[®] HPV assays (Hologic, Inc., Madison, WI) were selected for this study mainly because they were used at that time for gynecologic Papanicolaou tests.

The Cervista assays use the Invader[®] technology (Hologic, Inc., Madison, WI), a signal amplification method for detection of specific nucleic acid sequences. The Cervista HR-HPV assay detects the presence of 14 HR-HPV types, namely, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. The Cervista HR-HPV assay does not differentiate HPV types; thus, Cervista HPV 16/18 assay was used in conjunction with Cervista HR-HPV assay to assess the presence or absence of HPV types 16 and 18.^{8,9} Tests were conducted according to the manufacturer's guidelines. Inadequate specimens were excluded from the study.

Smoking, alcohol consumption, and betel quid use criteria

Subjects who smoked more than 100 cigarettes in their lives were categorized in the smoking group. Alcohol drinkers were defined as individuals who had consumed at least one drink per week for at least 1 year. Subjects who mentioned the daily frequency of betel quid chewing were categorized in the betel quid use group, regardless of duration.

Statistical analysis

Pearson chi-square or Fisher exact test was used for comparing categorical variables between studied groups. Fisher exact test was used if any expected count in the 2×2 table was less than 5. Odds ratios (ORs) were used to assess the association between the cancers and risk factors. Adjusted ORs were calculated by the Cochran–Mantel–Haenszel method, using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL). A p -value < 0.05 was considered statistically significant.

Results

In total, 104 patients and 104 control subjects were nonrandomly enrolled between January and December 2016. Characteristics of patients with cancer are shown in Table 1.

Table 1. Characteristics and HPV status of oral and oropharyngeal cancers.

| Patients and tumor characteristics | Total (n = 104) | OC (n = 52) | OP (n = 52) | p-value |
|------------------------------------|-----------------|--------------|--------------|---------|
| Sex | | | | |
| Male | 77 (74.0%) | 29 (55.8%) | 48 (92.3%) | <0.001 |
| Female | 27 (26.0%) | 23 (44.2%) | 4 (7.7%) | |
| Age (years) | | | | |
| At diagnosis | 60.2 (31–90) | 60.1 (31–90) | 60.4 (35–86) | 0.429 |
| ≤44 | 11 (10.6%) | 7 (13.5%) | 4 (7.7%) | |
| 45–65 | 60 (57.7%) | 27 (51.9%) | 33 (63.5%) | |
| ≥66 | 33 (31.7%) | 18 (34.6%) | 15 (28.8%) | |
| Stage | | | | |
| I, II | 19 (18.3%) | 13 (25%) | 6 (11.5%) | 0.076 |
| III, IV | 85 (81.7%) | 39 (75%) | 46 (88.5%) | |
| Histologic subtypes | | | | |
| WD-SCC | 55 (52.9%) | 35 (67.3%) | 20 (38.5%) | |
| MD-SCC | 44 (42.3%) | 16 (30.8%) | 28 (53.8%) | |
| PD-SCC | 4 (3.9%) | 0 (0%) | 4 (7.7%) | |
| Bas-SCC | 0 (0%) | 0 (0%) | 0 (0%) | |
| SP-SCC | 1 (0.96%) | 1 (1.9%) | 0 (0%) | |
| Smoking | 83 (79.8%) | 34 (65.4%) | 49 (94.2%) | |
| Alcohol consumption | 75 (72.1%) | 31 (59.6%) | 44 (84.6%) | 0.004 |
| Betel quid chewing | 13 (12.5%) | 12 (23.1%) | 1 (1.9%) | 0.002 |
| HR-HPV positive | 10 (9.6%) | 4 (7.7%) | 6 (11.5%) | 0.741 |
| HPV 16 | 6 (5.8%) | 2 (3.8%) | 4 (7.7%) | 0.678 |
| HPV 18 | 0 (0%) | 0 (0%) | 0 (0%) | – |

OC: oral cancer; OP: oropharyngeal cancer; WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated; Bas: basaloid; SP spindle cell; SCC: squamous cell carcinoma; HPV: human papillomavirus; HR-HPV: high-risk human papillomavirus.

Smoking was more frequently observed in men. The percentages of smoking were 94.8% in male patients and 37.0% in female patients. All betel quid users in this study were elderly women aged 55–87 years. A total of six (5.8%) patients did not have identifiable risk factors.

The oral squamous cell cancer group comprised 52 patients; 4 (7.7%) patients had positive HR-HPV results revealed by Cervista assay. Subsequent test with Cervista HPV 16/18 assay showed positive results of HPV type 16 in two of these four patients.

The oropharyngeal squamous cell cancer group had 52 patients; 6 (11.5%) patients had positive HR-HPV results revealed by Cervista assay. HPV type 16 was detected in four of these six patients. Only one patient with no history of smoking had a positive result for HPV type 16. Table 2 shows the comparison between HPV-positive and HPV-negative cancers.

The most common histologic subtypes in the oral and oropharyngeal squamous cell cancer groups were well and moderately differentiated types, respectively. A total of four patients with oropharyngeal squamous cell cancer had a poorly differentiated morphology. Of the four patients with a poorly differentiated morphology, one patient had a positive result for HPV type 16. No basaloid squamous cell cancer was found in this study.

Comparison between the cases and control subjects is shown in Table 3. One control subject was positive for

non-16/18 HR-HPV. The case and control subject groups had similar age and sex characteristics.

Table 4 shows the statistical analysis of the association between cancers and risk factors. Smoking was significantly associated with both oral and oropharyngeal squamous cell cancers. Betel quid chewing was significantly associated with oral cancer. HR-HPV was significantly associated with oropharyngeal squamous cell cancer (OR=13.44, 95% CI=1.6–114.8) but was nonsignificantly associated with oral squamous cell cancer (OR=8.58, 95% CI=0.9–78.9). However, HR-HPV alone, excluding the confounding effect of smoking, was nonsignificantly associated with oropharyngeal squamous cell cancer (adjusted OR=5.83, 95% CI=0.8–43.5). Because no control subject was positive for HPV type 16, the calculations of ORs for HPV type 16 were corrected by adding 0.5 to zero values in the contingency tables. HPV type 16 was significantly associated with oropharyngeal squamous cell cancer (OR=19.39, 95% CI=1.02–367.4).

Discussion

Background of Thailand

Thailand is located in Southeast Asia and has a population of 65 million. The five most common types of cancer diagnosed

Table 3. Characteristics of case and control subjects.

| Variables | Cancers | Controls | p-value |
|---------------------|--------------|--------------|---------|
| Age (years) | | | |
| At diagnosis | 60.2 (31–90) | 59.3 (31–90) | – |
| ≤44 | 11 | 13 | 0.834 |
| 45–65 | 60 | 56 | |
| ≥66 | 33 | 35 | |
| Sex | | | |
| Male | 77 | 77 | 1.000 |
| Female | 27 | 27 | |
| Smoking | 83 (79.8%) | 36 (34.6%) | <0.001 |
| Alcohol consumption | 75 (72.1%) | 34 (32.7%) | <0.001 |
| Betel quid chewing | 13 (12.5%) | 6 (5.8%) | 0.092 |
| HR-HPV positive | 10 (9.6%) | 1 (0.96%) | 0.010 |
| HPV 16 | 6 (5.8%) | 0 (0%) | 0.029 |
| HPV 18 | 0 (0%) | 0 (0%) | – |

HPV: human papillomavirus; HR-HPV: high-risk human papillomavirus.

within Thai people are liver and bile duct, lung, breast, head and neck, and cervical cancer.¹⁰

The most common type of head and neck cancer in Thailand is oral cancer. Oropharyngeal cancer is the fourth most common type after oral cancer, nasopharyngeal cancer, and laryngeal cancer. The highest incidence of oral cancer is found in Eastern Thailand. About 4000 Thais are diagnosed with oral and oropharyngeal cancers each year.^{10,11}

Smoking, by far, is the biggest cause of head and neck squamous cell cancer among Thais. Of the five patients, three with oral cancer mentioned tobacco use.¹¹ Since the popularity of betel quid chewing habit has declined, the percentage of oral cancer patients with a history of betel quid chewing decreased from 50.2% to 13.0% in a decade.^{10,11}

The data and statistics on HPV infection and related diseases have been publicized by many researchers around the world. However, little information is available about the correlation between HPV and head and neck squamous cell cancer in Thailand. To the best of our knowledge, this is the first

Table 2. Comparisons between HPV-positive and HPV-negative cancers.

| Patients and tumor characteristics | HR-HPV positive | HR-HPV negative | p-value |
|---|-----------------|-----------------|---------|
| Number of patients | 10 | 94 | |
| Sex | | | |
| Male | 10 (100%) | 67 (71.3%) | 0.060 |
| Female | 0 (0%) | 27 (28.7%) | |
| Age (years) | | | |
| At diagnosis | 56.3 (36–77) | 60.7 (31–90) | |
| ≤44 | 2 (20%) | 9 (9.6%) | 0.492 |
| 45–65 | 6 (60%) | 54 (57.4%) | |
| ≥66 | 2 (20%) | 31 (33.0%) | |
| Oral cavity | | | |
| All | 4 | 48 | 0.522 |
| Oral tongue | 2 | 28 | |
| Floor of mouth | 1 | 3 | |
| Palate | 1 | 3 | |
| Retromolar trigone | 0 | 1 | |
| Buccal and gum | 0 | 13 | |
| Oropharynx | | | |
| All | 6 | 46 | |
| Tonsil | 2 | 21 | |
| Base of tongue | 1 | 9 | |
| Other and overlapping sites in oropharynx | 3 | 16 | |
| Histologic subtypes | | | |
| WD-SCC | 2 (20%) | 53 (56.4%) | |
| MD-SCC | 7 (70%) | 37 (39.4%) | |
| PD-SCC | 1 (10%) | 3 (3.2%) | |
| Bas-SCC | 0 (0%) | 0 (0%) | |
| SP-SCC | 0 (0%) | 1 (1.1%) | |
| Smoking | 9 (90%) | 74 (78.7%) | 0.683 |
| Alcohol consumption | 8 (80%) | 67 (71.3%) | 0.722 |
| Betel quid chewing | 0 (0%) | 13 (13.8%) | 0.356 |

HPV: human papillomavirus; HR-HPV: high-risk human papillomavirus; WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated; Bas: basaloid; SP spindle cell; SCC: squamous cell carcinoma.

Table 4. Statistical analysis of the association between cancers and risk factors.

| Factors | Oral cancer group | | | | Oropharyngeal cancer group | | | |
|---------------------|-------------------|---------|------------------------------|---------|----------------------------|---------|--------------------------------|---------|
| | OR (95% CI) | p-value | Adjusted OR (95% CI) | p-value | OR (95% CI) | p-value | Adjusted OR (95% CI) | p-value |
| HR-HPV | 8.58 (0.9–78.9) | 0.057 | 4.67 ^a (0.5–44.1) | 0.179 | 13.44 (1.6–114.8) | 0.018 | 5.83 ^a (0.8–43.5) | 0.086 |
| HPV 16 ^b | 10.35 (0.5–219.6) | 0.134 | – | – | 19.39 (1.02–367.4) | 0.048 | – | – |
| Smoking | 3.57 (1.8–7.2) | <0.001 | 4.21 ^c (1.9–9.2) | <0.001 | 30.85 (9.0–105.9) | <0.001 | 32.83 ^c (8.8–122.6) | <0.001 |
| Alcohol consumption | 3.04 (1.5–6.1) | 0.002 | 1.90 ^a (0.8–4.3) | 0.126 | 11.32 (4.8–26.7) | <0.001 | 4.02 ^a (1.5–11.0) | 0.007 |
| Betel quid chewing | 4.90 (1.7–14.0) | 0.003 | 6.25 ^a (2.1–18.6) | 0.001 | 0.32 (0.04–2.7) | 0.298 | 0.31 ^a (0.03–3.5) | 0.342 |

OR: odds ratio; CI: confidence interval; HPV: human papillomavirus; HR-HPV: high-risk human papillomavirus.

^aORs were adjusted for smoking.

^bZero values in contingency tables were corrected by adding 0.5.

^cORs were adjusted for HR-HPV and betel quid chewing.

case-control study of HPV-related oral and oropharyngeal squamous cell cancers in the Thai population.

Prevalence of HR-HPV infection in the patients and control subjects

Previous studies showed various prevalence rates of HPV infection in head and neck squamous cell cancer, depending on the type of assay, the tumor site, and the location of study. There are various approaches to HPV testing of clinical samples from the head and neck. The p16 immunohistochemistry and DNA polymerase chain reaction (PCR)-based techniques are among the frequently used HPV tests.^{12–16} The prevalence of HPV infection in oropharyngeal squamous cell cancer was considerably higher than that in other squamous cell cancers of the head and neck.¹² In particular, HPV type 16 accounts for more than 85% of all HPV-related head and neck squamous cell cancers.^{12,13} The reported prevalence rates of HPV in related cancers were high in North America and many developed countries. A report from the United States found that 71.0% of the patients with oropharyngeal squamous cell cancer had related HPV infection.^{6,13} However, many countries reported low or zero prevalence of HPV-related oral and oropharyngeal squamous cell cancers.^{14–16} Genetic and host susceptibility to HPV infection, environmental factors, and culture may cause the differences in HPV infection and HPV-related cancer prevalence between countries.¹⁷

A total of four patients (7.7%) in the oral squamous cell cancer group and six patients (11.5%) in the oropharyngeal squamous cell cancer group had positive HPV tests. These prevalence rates were relatively low when compared with the rates found in developed countries. HR-HPV infection prevalence in the oropharyngeal squamous cell cancer group was higher than that in the oral squamous cell cancer group, but the difference was not statistically significant ($p=0.741$).

In the normal oral mucosa, previous studies have reported a great variation in HPV rates detected, from 0% all the way to 81.1%. This seems to depend on the population studied,

the type of HPV testing, and collection techniques (e.g. brush, swab, rinse, spit, and biopsy). High HPV-positive rates were usually reported by studies with PCR-based techniques.¹⁸ HR-HPV infection rate (0.96%) in the control subjects from this study was comparable to the rate (1.2%) found in the normal mucosa study using similar test and specimen collection techniques.⁷

Sexual behaviors, such as oral sex or oral-anal sex, are strong risk factors for HPV infection in the mouth and throat.¹⁹ The low prevalence rate in this study may reflect cultural characteristics in Thailand that are not conducive to oral HPV transmission. Many Thais conceive that nongenital sexual acts are associated with impurity and immorality.²⁰ This belief may reduce HPV transmission to the mouth and throat. Furthermore, HPV infection prevalence in the healthy population affects the occurrence of HPV-related diseases. Thailand is one of the countries with a low HPV infection rate. For example, the studies on cervical cancer screening programs of Thai women reported 6.3%–8.2% HR-HPV infection rates.^{21–23} However, the US studies reported 21.1%–23.0% infection rates, which were three times higher than the rates in Thailand.^{24,25} A recent study has reported that the Asian population exhibits a lower probability of acquiring new HPV infections.²⁶ The mechanism of how race influences HPV infection is unknown.

Low presence of HPV in the general population and the cultural beliefs in Thailand could contribute to the low prevalence of HPV-related oral and oropharyngeal squamous cell cancers.

Role of HR-HPV as an etiologic factor

Smoking was the most common risk factor in this study. The percentage of smoking in the oropharyngeal squamous cell cancer group was significantly higher than that in the oral squamous cell cancer group ($p<0.001$). Smoking was found to be an independent risk factor for oral and oropharyngeal squamous cell cancers. In contrast to previous studies which reported that HPV-related cancers occur usually in younger

and nonsmoking male patients, in this study, HR-HPV infection was more common among male smokers.^{3,27} This can be caused by a high proportion of smoking patients. In addition, no significant difference in age was observed between HPV-positive and HPV-negative cancers ($p=0.492$).

Studies performed in various countries have suggested that HR-HPV is an independent risk factor for oral and oropharyngeal squamous cell cancers.^{27,28} Similarly, our study found a significant association between HR-HPV and oropharyngeal squamous cell cancer, but it was underpowered to reach a conclusion about the relationship between HR-HPV and oral squamous cell cancer. Moreover, the ORs for HR-HPV associated with both cancers, after adjustment for smoking, were considerably lower than the crude ORs. This suggested that smoking was a strong confounder of the association between HR-HPV and oral and oropharyngeal squamous cell cancers. However, unadjusted and adjusted ORs for HR-HPV associated with the cancers need to be interpreted with caution. The precision of these results was limited by a wide CI. The stratified analysis was not accurate due to many subgroups in the stratification contained few or no subjects enrolled. Thus, further larger studies are necessary in order that the role of HPV as the independent risk factor for oral and oropharyngeal squamous cell cancers in the Thai population can be clarified.

Strengths and limitations of the study

This study used the Food and Drug Administration (FDA) approved, commercially available HPV test, unlike most prior HPV studies which used in-house assays. The Cervista assays have proven intra- and inter-laboratory reproducibility.²⁹ The presence of an internal control in the Cervista assays reduces the possibility of false-negative results due to insufficient DNA present. Specimens were newly collected to avoid errors from contamination and deterioration.

The main limitation in this study is insufficient sample size, especially in the control group, making an underpowered statistic. In fact, the number of samples in this study was fairly comparable with those in other studies,^{30–32} and sample size requirement was calculated with a valid method. Much lower than expected HPV prevalence found in this study was likely to be the cause of this limitation. As mentioned in the “Materials and methods” section, due to lack of suitable data relating to HPV prevalence in the study population, the sample size requirement was calculated based on available data in other countries. Based on the HPV infection rate found in this study, the sample size needs to be doubled in order to reach an acceptable level of statistical power. Moreover, in order to avoid having no case or no control in a stratum, we may need to increase the sample size to three or four times of its original size so that the stratified analysis can yield a reliable result. These required numbers for sample size are too high to reasonably achieve within the study setting for this investigation.

There were other factors that might affect the credibility of the low HPV infection results found in this study. Initially, the study did not use the traditional HPV testing modalities, such as p16 immunohistochemistry screening followed by *in situ* hybridization or DNA PCR-based techniques. Although the Cervista assays have been used by others for head and neck tumor material testing,^{8,9} the assays still do not have regulatory approval for use outside the setting of the detection of cervical HPV DNA. In addition, nonrandom sampling method used in this study might create selection bias.

Conclusion

The study investigated the role of HR-HPV in the etiology of oral and oropharyngeal squamous cell cancers in a developing country with no increase in the incidence of HPV-driven oropharyngeal squamous cell cancer. The prevalence rates of HR-HPV infection in oral and oropharyngeal squamous cell cancers were relatively low when compared with the rates in other developed countries. Smoking may have an influence on the etiology of HPV-related cancers. So far, the study can merely point out that HR-HPV plays a lesser role than other well-established risk factors, such as smoking and betel chewing. Although the study is underpowered to clarify the role of HPV as the independent risk factor for oral and oropharyngeal squamous cell cancers in the Thai population, it adds to the very few studies done in Thailand. Results from this study provided an approximate sample size needed for future studies in Thailand and other countries with a similar epidemiologic trend. Due to low prevalence of HPV, the potential benefit of HPV vaccine on the occurrence of head and neck squamous cell cancer in Thais is still in question. Smoking prevention remains the most important strategy to control these cancers.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

Ethical approval for this study was obtained from the hospital ethics committee (approval number: 13/2015).

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Informed consent

Written informed consent was obtained from all subjects before the study.

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