Clinical characteristics and treatment outcome of oropharyngeal squamous cell carcinoma in an endemic betel quid region

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The clinical characteristics of oropharyngeal squamous cell carcinoma (OPSCC) may be different between endemic and non-endemic regions of betel nut chewing. The impact of combined alcohol drinking/betel quid chewing/cigarette smoking (ABC) exposure on the survival of OPSCC remains unclear. We reviewed the medical records of OPSCC patients between 1999 and 2013. Immunohistochemical staining of p16 and HPV genotype detection by DNA Polymerase chain reaction were both performed for each tumor. A total of 300 eligible patients including 74 HPV+ OPSCC patients and 226 HPV− OPSCC patients were enrolled. The 5-year disease-free survival rates for the HPV−, HPV+ OPSCC with and without ABC patients were 49.8%, 58.4% and 94%, respectively. The 5-year overall survival rates for the patients with HPV−, HPV+ OPSCC with and without ABC patients were 46%, 57.4% and 86%, respectively. Advanced locoregionally disease (T3/T4, N2/N3), HPV− OPSCC, combined 2 or all ABC exposure were the independent adverse prognostic factors for disease-free and overall survival. Therefore, our data suggest that in an endemic region of betel quid chewing, HPV− OPSCC comprises the majority of OPSCC and has a worse survival. Combined 2 or all ABC exposure had a significant negative impact on disease-free and overall survival.

Human papillomavirus (HPV) has been shown to be one of the causes of oropharyngeal squamous cell carcinoma (OPSCC), especially in patients without traditional risk factor exposure. The virus contains two oncogenes, E6 and E7, which inactivates p53 and retinoblastoma, respectively. Both pathways are involved in the carcinogenesis of HPV+ OPSCC. Whole exome sequencing data have demonstrated that HPV+ OPSCC has different genetic alterations from HPV− OPSCC, which is mostly caused by cigarette smoking. In comparison with HPV− OPSCC, HPV+ OPSCC has better treatment response to chemoradiotherapy (CRT) and has a better survival. The 8th American Joint Committee on Cancer (AJCC) staging system downgrades HPV+ OPSCC staging and clinical trials on de-intensified treatment in HPV+ OPSCC are ongoing right now.

In developed Western countries, HPV+ OPSCC is more common than HPV− OPSCC nowadays. In the United States, HPV+ OPSCC accounts for more than 70% of all OPSCC. In Taiwan, HPV− OPSCC is still more common than HPV+ OPSCC. The reason for a higher frequency of HPV− OPSCC in Taiwan is due to betel quid chewing and cigarette smoking still being common. The prevalence of current betel quid chewer and cigarette smoker among men in Taiwan are about 10% and 30%, respectively. Moreover, most of the patients with HPV− OPSCC and many patients with HPV+ OPSCC in Taiwan also have two or all exposures of alcohol drinking, betel quid chewing and cigarette smoking (ABC), which all are the risk factors strongly associated with OPSCC. Therefore, clinical characteristics and treatment outcome of OPSCC in Taiwan may be different from...
those in other populations with only single or two exposures of alcohol drinking and cigarette smoking, without betel quid chewing. The aims of this study are to show the clinical characteristics of OPSCC in an endemic region of betel quid chewing and to analyse the impacts of ABC exposure on the survival of the OPSCC patients.

Results

Patient demographics. A total of 300 eligible patients diagnosed with OPSCC, including 74 (25%) patients with HPV+ OPSCC and 226 (75%) patients with HPV− OPSCC, were enrolled in this study. The mean age of all OPSCC patients included in our series was 54 ± 10 years (range, 29–83 years). The mean age of the HPV− OPSCC patients, HPV+ OPSCC without and with ABC exposure patients were 53 ± 10 years (range, 29–80 years), 56 ± 12 years (range, 29–82 years) and 57 ± 11 years (range, 37–83 years), respectively (p = 0.15). The prevalence of HPV+ OPSCC after 2004 was higher than that before 2004. Among 226 patients with HPV− OPSCC, 191 (85%) patients had ABC exposure. Among 74 patients with HPV+ OPSCC, 38 (51%) patients had and the other 36 (49%) patients did not have ABC exposure. For all OPSCC patients, it was highly associated between alcohol drinking and betel quid chewing (r = 0.64), between betel quid chewing and cigarette smoking (r = 0.55) and between alcohol drinking and cigarette smoking (r = 0.69).

The clinicopathological characteristics are listed in Table 1. Female patients were significantly more common in the group of HPV+ OPSCC without ABC exposure than the groups of HPV− OPSCC and HPV+ OPSCC with ABC exposure (p = 0.001). HPV+ OPSCC had advanced nodal disease significantly more common than HPV− OPSCC (p = 0.02). Two hundred (89%) of 226 HPV− OPSCC were p16 negative and 206 (91%) of HPV− OPSCC were HPV DNA PCR negative. Sixty (81%) of 74 HPV+ OPSCC contained HPV subtype 16 and the other 14 (19%) tumor contained other types or multiple infections of high-risk HPV (subtypes 33, 35, 56, 58, 68).

Survival outcome of OPSCC patients based on different HPV and ABC status. The follow-up period was from 1 to 213 months, with a mean of 63 ± 50 months. The mean follow-up periods of the patients with HPV+ and HPV− OPSCC were 78 ± 42 and 58 ± 51 months, respectively. The time to disease progression was from 1 to 210 months, with a mean of 50 ± 50 months. The 5-year disease-free and overall survival rates of all patients were 48.3% and 52.3%, respectively. The 5-year disease-free survival rates of the patients with HPV− OPSCC, HPV+ OPSCC with and without ABC exposure were 49.8%, 58.4% and 94%, respectively (Fig. 1a). After adjusting with major competing risk factor (death) by cumulative incidence competing risk (CICR) method, the adjusted 5-year disease-free survival rates of the patients with HPV− OPSCC, HPV+ OPSCC with and without ABC exposure were 52.7%, 60.5% and 94.4%, respectively. The 5-year overall survival rates of the patients with HPV− OPSCC, HPV+ OPSCC with and without ABC exposure were 46%, 57.4% and 86%, respectively (Fig. 1b). The disease-free and overall survival between HPV− OPSCC and HPV+ OPSCC with ABC exposure were not significantly different (p = 0.2, p = 0.13, respectively), but there were significant differences in both disease-free and overall survival between HPV− OPSCC with and without ABC exposure (p = 0.001, p = 0.002, respectively). The 5-year local recurrence rates of the patients with HPV− OPSCC, HPV+ OPSCC with and without ABC exposure were 28.6%, 15.4% and 3.2%, respectively (Fig. 1c). After adjusting with major competing risk factor (death) by CICR method, the adjusted 5-year local recurrence rates of the patients with HPV− OPSCC, HPV+ OPSCC with and without ABC exposure were 26.2%, 13.2% and 2.87%, respectively. The 5-year regional recurrence rates of the patients with HPV− OPSCC, HPV+ OPSCC with and without ABC exposure were 16.8%, 11.3% and 0%, respectively (Fig. 1d). After adjusting with major competing risk factor (death) by CICR method, the adjusted 5-year regional recurrence rates of the patients with HPV− OPSCC, HPV+ OPSCC with and without ABC exposure were 15.5%, 10.5% and 0%, respectively. The 5-year distant-metastasis rates of the patients with HPV− OPSCC, HPV+ OPSCC with and without ABC exposure were 20.5%, 22.3% and 2.9%, respectively (Fig. 1e). After adjusting with major competing risk factor (death) by CICR method, the adjusted 5-year distant-metastasis rates of the patients with HPV− OPSCC, HPV+ OPSCC with and without ABC exposure were 19.7%, 2.9% and 6.0%, respectively, but the 10-year cumulative rates of the metachronous second primary malignancy of those groups were 38.3%, 29.4% and 10.9%, respectively (Fig. 1f). After adjusting with major competing risk factor (death) by CICR method, the adjusted 5-year cumulative rates of the metachronous second primary malignancy of the patients with HPV− OPSCC, HPV+ OPSCC with and without ABC exposure were 12.6%, 2.63% and 5.64%, respectively.

Risk stratification for survival outcomes of the OPSCC patients. The univariate analyses of all possible factors on survival are shown in Table 2. Male gender (p = 0.001), locally advanced T3/T4 tumor (p < 0.001), negative p16 staining (p < 0.001), negative HPV DNA PCR (p < 0.001), alcohol drinking (p < 0.001), betel quid chewing (p < 0.001) and cigarette smoking (p < 0.001) were the adverse prognostic factors for disease-free survival. Male gender (p = 0.001), locally advanced T3/T4 tumor (p < 0.001), negative p16 staining (p < 0.001), negative HPV DNA PCR (p < 0.001), alcohol drinking (p < 0.001), betel quid chewing (p < 0.001) and cigarette smoking (p < 0.001) were the adverse prognostic factors for overall survival. The multivariant analyses by Cox Proportional Hazard Model for the disease-free and overall survivals are shown in Fig. 2a,b. After adjusting with major competing risk factor (death) by Subdistribution Hazard Model, the significant risk factors for disease-free survival are shown in Fig. 2c. Locoregionally advanced tumor (T3/T4 and N2/N3), combined 2 ABC exposure, all ABC exposure and HPV− OPSCC were the independent adverse factors for both disease-free and overall survival. The impacts of locally advanced T3/T4 tumor on disease control and survival stratified by HPV are shown in Fig. 3. In HPV+ OPSCC, T3/T4 tumor had similar survival and locoregional control to T1/T2 tumor. In HPV− OPSCC, T3/T4 tumor had significantly worse disease-free survival.
survival ($p < 0.001$, Fig. 3a), overall survival ($p < 0.001$, Fig. 3b), local control ($p = 0.001$, Fig. 3c) and distant failure ($p < 0.001$, Fig. 3e), than T1/T2 tumor.

The ABC exposure was also an independent adverse factor for disease-free survival. Multiple ABC exposure was the independent adverse factor for disease-free and overall survival. The patients with two or all ABC exposure had the worst disease-free and overall survival than those with single risk factor exposure, in both

| Characteristics | HPV− OPSCC (n = 226) | HPV+ OPSCC with ABC exposure (n = 38) | HPV+ OPSCC without ABC exposure (n = 36) | P value |
|-----------------|------------------------|----------------------------------------|------------------------------------------|---------|
| Age (years)     |                        |                                        |                                          |         |
| Mean ± Standard deviation (range) | 53 ± 10 (29–80) | 57 ± 11 (37–83) | 56 ± 12 (29–82) | 0.15*   |
| ≥50             | 138 (61.06%)           | 26 (68.42%)                            | 24 (66.67%)                             | 0.61    |
| ≤50             | 88 (38.94%)            | 12 (31.58%)                            | 12 (33.33%)                             |         |
| Gender          |                        |                                        |                                          |         |
| Male            | 210 (92.92%)           | 37 (97.37%)                            | 26 (72.22%)                             | 0.001** |
| Female          | 16 (7.08%)             | 1 (2.63%)                              | 10 (27.78%)                             |         |
| Primary Tumor   |                        |                                        |                                          | 0.07*   |
| Tonsil          | 131 (57.96%)           | 28 (73.68%)                            | 27 (75%)                                |         |
| Tongue base     | 74 (32.74%)            | 7 (18.42%)                             | 6 (16.67%)                              |         |
| Soft palate     | 18 (7.96%)             | 3 (7.89%)                              | 1 (2.78%)                               |         |
| Multifocal      | 3 (1.33%)              | 0                                      | 2 (5.56%)                               |         |
| T classification|                        |                                        |                                          |         |
| T3, T4          | 102 (45.13%)           | 18 (47.37%)                            | 10 (27.78%)                             | 0.13    |
| T1, T2          | 124 (54.87%)           | 20 (52.63%)                            | 26 (72.22%)                             |         |
| N classification|                        |                                        |                                          | 0.02    |
| N2, N3          | 136 (60.18%)           | 27 (71.05%)                            | 30 (83.33%)                             |         |
| N0, N1          | 90 (39.82%)            | 11 (28.95%)                            | 6 (16.67%)                              |         |
| Carcinogen exposure |              |                                        |                                          |         |
| Alcohol         | 169 (74.79%)           | 24 (63.16%)                            | 0                                        |         |
| Cigarette       | 185 (81.86%)           | 38 (100%)                              | 0                                        |         |
| Betel quid      | 133 (58.85%)           | 31 (81.58%)                            | 0                                        |         |
| Treatment       |                        |                                        |                                          | 0.007** |
| Single modality (RT or OP) | 15 (6.64%) | 5 (13.16%) | 0 |         |
| Two-modality    | 141 (62.39%)           | 28 (73.68%)                            | 19 (52.78%)                             |         |
| Three-modality  | 70 (30.97%)            | 5 (13.16%)                             | 17 (47.22%)                             |         |
| Tumor p16 status|                        |                                        |                                          | <0.001  |
| ≥70%            | 26 (11.50%)            | 38 (100%)                              | 36 (100%)                               |         |
| <70%            | 200 (88.50%)           | 0                                      | 0                                        |         |
| Tumor HPV Genotype |                    |                                        |                                          | <0.001  |
| Type 16         | 15 (6.64%)             | 31 (81.58%)                            | 29 (80.56%)                             |         |
| Others (Type 33,35,56,58,68) | 4 (1.77%) | 6 (15.79%) | 5 (13.89%) |         |
| Multiple (≥2, Type 16+) | 1 (0.44%) | 1 (2.63%) | 2 (5.56%) |         |
| Negative        | 206 (91.15%)           | 0                                      | 0                                        |         |
| Disease failure pattern |                |                                        |                                          | 0.05    |
| Local recurrence| 61 (26.99%)            | 5 (13.16%)                             | 2 (5.56%)                               |         |
| Regional recurrence | 36 (15.93%) | 4 (10.53%) | 0 | 0.013** |
| Distant metastasis | 38 (16.81%) | 8 (21.05%) | 1 (2.78%) | 0.07    |
| Second primary malignancy | 46 (20.35%) | 3 (7.89%) | 3 (8.33%) |         |
| Calendar year at diagnosis |              |                                        |                                          | 0.003   |
| 1999–2003       | 64 (28.32%)            | 3 (7.89%)                              | 3 (8.33%)                               |         |
| 2004–2008       | 86 (38.05%)            | 14 (36.4%)                             | 19 (52.78%)                             |         |
| 2009–2013       | 76 (33.63%)            | 21 (55.26%)                            | 14 (38.89%)                             |         |

Table 1. The clinicopathological characteristics of oropharyngeal squamous cell carcinoma. Abbreviation: HPV, human papillomavirus; HPV−, HPV-negative; HPV+, HPV-positive; OPSCC, oropharyngeal squamous cell carcinoma; RT, radiotherapy; OP, curative operation alone; *using one-way ANOVA test; **using Fisher's exact test.
Among the patients with HPV− OPSCC, the 5-year disease-free survival rates of the patients with 2 or more ABC exposure, single ABC exposure and no exposure were 40.5%, 66.2% and 82.9%, respectively (p < 0.001), and the 5-year overall survival rates of the patients with 2 or more ABC exposure, single ABC exposure and no exposure were 37.5%, 55.6% and 82.8%, respectively (p < 0.001). Among the patients with HPV+ OPSCC, the 5-year disease-free survival rates of the patients with 2 or more ABC exposure, single ABC exposure and no exposure were 55.7%, 71.4% and 94%, respectively (p = 0.001), and the 5-year overall survival rates of the patients with 2 or more ABC exposure, single ABC exposure and no exposure were 50.9%, 85.7% and 86%, respectively (p < 0.001), which were all better than those with HPV− OPSCC, respectively (Table 2).

Discussion

The HPV+ OPSCC is a subtype of OPSCC, which is associated with a better response to treatment and a better outcome. In western countries, where is betel quid non-endemic region, the HPV+ OPSCC patients tend to be younger, have a higher socioeconomic status and are less likely to smoke6,8,9. However, it has been demonstrated that each of ABC exposures is strongly associated with the risk for OPSCC13,14. Among them, the betel quid may be the most potent carcinogen associated with non-nasopharyngeal carcinoma head and neck cancer (HNC), including OPSCC13,14. Because most patients with OPSCC in Taiwan have two or all exposures of ABC, especially the betel quid, which is different from that in other populations with only alcohol or cigarette exposure, the clinical characteristics and survival of OPSCC in Taiwan may not be the same as those in non-endemic region of betel quid chewing.

Firstly, the incidence and prevalence trends of HPV− and HPV+ OPSCC were different between the non-endemic and endemic regions of betel quid chewing. The incidences of HPV− OPSCC in the non-endemic betel quid regions are decreasing15,16. On the contrary, the incidence of OPSCC is increasing because of the rapidly rising incidence of HPV+ OPSCC, which accounts for more than 70–90% of all OPSCC8,9,16. In an endemic region of betel quid chewing, HPV− OPSCC still accounts for the majority of OPSCC17. Our previous population-based studies showed that the incidences of all HNC associated with ABC including OPSCC in Taiwan are still increasing to date and only about one-third of OPSCCs were HPV+ OPSCC10,18. The incidence of HPV+ OPSCC is really rising in the recent years in Taiwan, similar to other developed countries because of the changes of sexual behaviour among younger age cohorts, but HPV− OPSCC is still more common than HPV+ OPSCC and the incidence of HPV− OPSCC is also increasing with a similar rate18.

Secondly, it has been shown that HPV+ OPSCC has significantly less locoregional recurrence and distant failure so that the survival of HPV+ OPSCC is much better than that of HPV− OPSCC6,19,20. This study showed the same finding that HPV+ OPSCC, especially without ABC exposure, also has a significantly better treatment response, less recurrence and a better survival in an endemic region of betel quid chewing. However, because the majority of the OPSCC tumor was HPV−, the overall survival in an endemic region of betel quid chewing was poorer than that in non-endemic region of betel quid chewing. The overall survival of OPSCC in this study (5-year overall survival: 52.3%) was still lower than that of the SEER database in USA (5-year overall survival: 65.8%)21 although the treatment outcome of other HNCs in Taiwan, for example, oral cavity cancer, is comparative to that in the developed countries22,23.

Furthermore, it has been shown that cigarette smoking worsens the survival of the patients with HPV+ OPSCC in Western countries and the patients with HPV+ OPSCC and cigarette smoking is classified as an intermediate risk group in terms of treatment outcome24. In an endemic region of betel quid chewing, the majority
| Factors                          | 5-year Disease-Free Survival | P value | 5-year Overall Survival | P value |
|---------------------------------|-------------------------------|---------|-------------------------|---------|
| Age (years)                     |                               |         |                         |         |
| >50                             | 58.4%                         | 0.41    | 51.8%                   | 0.46    |
| ≤50                             | 53.4%                         |         | 53.2%                   |         |
| Gender                          |                               |         |                         |         |
| Male                            | 52.9%                         | 0.001   | 49.4%                   | 0.001   |
| Female                          | 88.9%                         |         | 81.3%                   |         |
| Primary Tumor                   |                               |         |                         |         |
| Tonsil                          | 58.6%                         | 0.22    | 52.4%                   | 0.68    |
| Tongue Base                     | 50.1%                         |         | 51.6%                   |         |
| Soft Palate                     | 53.1%                         |         | 55.0%                   |         |
| Multifocal                      | 0%                            |         | 40.0%                   |         |
| T classification                |                               |         |                         |         |
| T3/T4                           | 41.8%                         | <0.001  | 37.4%                   | <0.001  |
| T1/T2                           | 67.4%                         |         | 63.6%                   |         |
| N classification                |                               |         |                         |         |
| N2/N3                           | 53.0%                         | 0.09    | 50.1%                   | 0.14    |
| N0/N1                           | 62.5%                         |         | 56.0%                   |         |
| Tumor p16 status                |                               |         |                         |         |
| Negative                        | 47.5%                         | <0.001  | 44.0%                   | <0.001  |
| Positive                        | 73.7%                         |         | 68.7%                   |         |
| HPV DNA PCR status              |                               |         |                         |         |
| Negative                        | 47.2%                         | <0.001  | 43.7%                   | <0.001  |
| Positive                        | 75.6%                         |         | 71.0%                   |         |
| Treatment                       |                               |         |                         |         |
| Single modality (RT or OP)      | 50.3%                         | 0.26    | 57.0%                   | 0.54    |
| Two-modality                    | 54.1%                         |         | 48.0%                   |         |
| Three-modality                  | 62.5%                         |         | 59.7%                   |         |
| Alcohol drinking                |                               |         |                         |         |
| Positive                        | 43.1%                         | <0.001  | 39.8%                   | <0.001  |
| Negative                        | 78.1%                         |         | 74.6%                   |         |
| Betel quid chewing              |                               |         |                         |         |
| Positive                        | 39.2%                         | <0.001  | 35.7%                   | <0.001  |
| Negative                        | 75.6%                         |         | 71.9%                   |         |
| Cigarette smoking               |                               |         |                         |         |
| Positive                        | 46.2%                         | <0.001  | 42.5%                   | <0.001  |
| Negative                        | 83.9%                         |         | 80.3%                   |         |
| HPV and ABC exposure history    |                               |         |                         |         |
| HPV − OPSCC                     | 49.8%                         | <0.001  | 46.0%                   | <0.001  |
| HPV + OPSCC with ABC            | 58.4%                         |         | 57.4%                   | <0.001  |
| HPV + OPSCC without ABC         | 94%                           |         | 86.0%                   |         |
| HPV − OPSCC and ABC exposure    |                               |         |                         |         |
| Combined 2 or all exposure      | 40.5%                         | <0.001  | 37.5%                   | <0.001  |
| Single exposure                 | 66.2%                         |         | 55.6%                   |         |
| No exposure                     | 82.9%                         |         | 82.8%                   |         |
| HPV + OPSCC and ABC exposure    |                               |         |                         |         |
| Combined 2 or all exposure      | 55.7%                         | 0.001   | 50.9%                   | <0.001  |
| Single exposure                 | 71.4%                         |         | 85.7%                   |         |
| No exposure                     | 94%                           |         | 86.0%                   |         |

Table 2. Univariate analysis of the prognostic factors for survival. Abbreviation: HPV, human papillomavirus; HPV −, HPV-negative; HPV +, HPV-positive; PCR, Polymerase chain reaction; IHC, immunohistochemistry; ABC, Alcohol drinking/Betel quid chewing/Cigarette smoking; OPSCC, oropharyngeal squamous cell carcinoma; RT, radiotherapy; OP, curative operation alone.
of patients with HPV−OPSCC and about half of the patients with HPV+OPSCC chewed betel quid in addition to alcohol drinking and cigarette smoking. This study showed that ABC exposure had a significantly worse disease-free and overall survival for all OPSCC patients, either the patients with HPV−OPSCC or the patients with HPV+OPSCC. However, it is difficult to clarify the impact of each risk factor exposure, especially the betel quid, on treatment outcome because the majority of the patients had two or all ABC exposures, especially betel quid chewers. Therefore, we stratified the patients by single or combined two or all ABC exposures in this study, which is easier and more clinically relevant. Combined two or all ABC exposure had the independent worst effect on disease-free and overall survival of OPSCC patients. The patients with HPV+OPSCC and combined two or all ABC exposures had a significantly poorer prognosis than those with single exposure, and so did the patients with HPV−OPSCC. The 5-year disease-free and overall survival rates of the patients with HPV−OPSCC and 2 or more ABC exposures were 55.7% and 50.9%, which were as poor as those with all HPV−OPSCCs (49.8%, 46%, respectively). Therefore, the negative impact of traditional carcinogen exposure, especially combined exposure, on the survival of the patient should be considered in terms of prognosis although this study still agrees with down-staging of HPV+OPSCC in the American Joint Committee on Cancer 8th Edition Cancer Staging system because of no differences of local control and survival between T3/4 and T1/2 HPV+OPSCC. In an endemic region of betel quid chewing, not only betel quid but also betel quid and alcohol should be considered as the adverse prognostic factors because the patients who had two or all ABC exposures had the worst prognosis, even with HPV+OPSCC. For the clinical trials on the de-intensified treatment for HPV+OPSCC, it may be necessary to take combined ABC exposure, not only cigarette smoking, into account in allocating the patients. For choice

Figure 2. The multivariable analyses of all prognostic factors for survival using Cox proportional hazards model with a forward selection procedure (age work as continuous variable; all other factors work as categorical variable): (a) disease-free survival; (b) overall survival; (c) The adjusted multivariant analyses with competing risk factor, death, by Subdistribution hazards model for disease-free survival (Abbreviation: HR: hazard ratio; ABC, Alcohol drinking/Betel quid chewing/Cigarette smoking; OPSCC, oropharyngeal squamous cell carcinoma; HPV, human papillomavirus; HPV−, HPV-negative).
of treatment in clinical practice, the patients with HPV+ OPSCC and two or more ABC exposures may be not a good candidate for the de-intensified treatment approach.

Second primary malignancy also causes patients’ death25. HPV− OPSCC has a significantly higher risk for metachronous second primary malignancy, which further lowers the survival, than HPV+ OPSCC, because the patients with HPV− OPSCC usually have carcinogen exposure, which causes field cancerization on the upper aerodigestive tract26, but HPV infection doesn’t cause obvious field cancerization27. Especially in Taiwan, most patients have multiple ABC exposures so that the prevalence and the cumulative incidence of second primary malignancy may be higher than those in other countries where are not endemic regions of ABC25,28,29. In this study, about 20% of patients with HPV− OPSCC had metachronous second primary malignancy, much higher than 8% of patients with HPV+ OPSCC. Therefore, the follow-up strategy for HPV− OPSCC should be different and more aggressive than that of HPV+ OPSCC in order to improve the survival by early diagnosis of the second primary malignancy in addition to recurrent diseases. Regular screening of synchronous/metachronous second primary malignancy should be considered in patients with OPSCC in an endemic region of betel quid chewing, especially for esophageal cancer, which is not uncommon and is the most lethal and difficult to diagnose at early stage without endoscopy screening28. Nowadays, endoscopic screening for second primary esophageal cancer is suggested for patients with pharyngeal cancers at diagnosis or during the follow-up after treatment in most hospitals in Taiwan, trying to improve the survival28,30.

There are some limitations in our series. First, with regard to the dose of ABC consumption, it is a little difficult to quantify the dose of alcohol/betel quid/cigarette exposure for every patient is this retrospective study. Therefore, it is really difficult to calculate the dose-relationship between the risk and the alcohol/betel quid/cigarette exposure from this cohort. Second, although the method we used to define HPV+ OPSCC, positive HPV− Polymerase chain reaction (PCR) sequencing and p16 immunohistochemistry (IHC) staining >70%, has been reported to have adequate sensitivity and specificity in detecting HPV-associated OPSCC31, there may be some discrepancies in the interpretation of our results in relation to real HPV-associated OPSCC.

In conclusion, in an endemic region of betel quid chewing, the majority of OPSCC patients still is HPV− OPSCC although the incidence of HPV+ OPSCC is also increasing. The treatment response and prognosis of HPV+ OPSCC without ABC exposure are good, which are similar to those in the non-endemic region of betel quid chewing. The locally advanced (T3/T4) tumor, HPV negative status and combined two or all ABC exposure were the independent adverse prognostic factors for disease-free and overall survival. HPV− OPSCC has a significantly higher risk for second primary malignancy.

Methods

Patient population. We retrospectively reviewed the medical records of the patients who were diagnosed with OPSCC and received curative-intended treatment at National Taiwan University Hospital between January 1999 and August 2013. Ethical approval for this study was obtained from institutional board at National Taiwan University Hospital (Approval Number: 201011033RC). Informed consent was obtained from all participants. All methods used in this study were performed by the relevant guidelines and regulations. The inclusion criteria were the OPSCC patients with corresponding Formalin-Fixed, Paraffin-Embedded tissue for IHC staining and HPV PCR analyses. The exclusion criteria included the patient with previously treated OPSCC, the patient with malignancies other than OPSCC, the patient with a simultaneous second primary cancer, and the patient with a...
previous history of radiotherapy involving the head or neck region due to other diseases. The TNM status of each tumor was classified according to the 2010 criteria of the AJCC32.

**Primary OPSCC p16 IHC staining.** Primary tumor sections of 4-μm thickness were deparaffinized and pre-treated for antigen retrieval by autoclave heating (121 °C) in 10 mM sodium citrate buffer (pH 6.0) for 10 min. These sections were blocked for endogenous peroxidase activity with 3% H2O2 in methanol for 10 min and then washed in phosphate-buffered saline (PBS). Thereafter, the sections were immersed in UltraVision Protein Block (Thermo Fisher Scientific, Fremont, USA) for 10 min, covered with a primary rabbit monoclonal antibody specific for p16 (clone: EP1215Y, Epitomics, Abcam Company, Burlingame, CA, USA) and incubated for one hour at room temperature. Immunoreactions were performed using UltraVision Quanto Detection System HRP DAB (Thermo Fisher Scientific, Fremont, USA). Immunohistochemical evaluation of p16 in OPSCC specimens was based on the intensity and extent of nuclear and cytoplasmatic reactivity. Positive p16 expression was defined as strong and diffuse nuclear and cytoplasmatic staining in 70% or more of the tumor cells4. Two independent pathologists (Y-L.C. and C-T.W.) were involved in the assessment of tumor p16 expression.

**Primary OPSCC HPV PCR analysis.** We used a commercial EasyChip® HPV blot kit (King Car, Taiwan) to carry out the HPV genotyping of the polymerase chain reaction (PCR) products of HPV and GAPDH. The quality of the HPV blot meets the requirement for class III GMP certification. The kit allows specific detection of 39 HPV genotypes (HPV types 6, 11, 16, 18, 26, 31, 32, 33, 35, 37, 39, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 72, 74, 82, CP8061, CP8304, L1AE5, MM4, MM7 and MM8, as well as three intrinsic factors.

**Statistical analysis.** In this study, the tumor with both positive p16 staining (≥70%) and positive HPV DNA PCR test (detectable HPV genotype) was regarded as HPV+ OPSCC24.35. All statistical analyses were performed using the SPSS software package, version 16.0 (SPSS Inc., Chicago, IL). The one-way analysis of variance (ANOVA) was used to determine difference in age among the patients with HPV+ tumor, HPV+ without tumour and with and ABC exposure. Fisher’s exact tests and chi-square tests were used to determine differences in other clinical characteristics. The data of ABC exposure were obtained from medical and/or cancer registry record of our hospital. All patients with the history of daily exposure more than 6 months would be regarded as positive13. The correlation among ABC exposure was tested using the Phi coefficient. The starting point of the follow-up period was defined as the completion of the comprehensive treatment for each patient. The end point of the follow-up period was defined as the time when the patient expired, lost to follow-up, or June 2017. The primary outcomes were disease-free and overall survival. The secondary outcomes were local recurrence, regional recurrence and distant failure. The rates of disease-free survival, overall survival, local recurrence, regional recurrence and distant failure were calculated using the Kaplan-Meier product limit method. All sites of persistent, residual or recurrent tumor were recorded as treatment failures in terms of the disease-free survival; and all deaths were recorded against the overall survival parameter. Significance levels among the curves were determined using the log-rank test. The CICR method was used to adjust the major competing factor, death, for cumulative rates of disease-free survival, local recurrence, regional recurrence and distant failure. A multiplicative Cox proportional hazards model with a forward selection procedure was used to estimate the effects of all possible covariates (Age work as continuous variable; all other factors work as categorical variable) on disease-free and overall survival endpoints. Finally, the Subdistribution hazard model was used to adjust the major competing risk factor, death, for disease-free survival. A p-value < 0.05 was considered statistically significant.

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**References**

1. Gillison, M. L. et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J. Natl. Cancer Inst.* 92, 709–720 (2000).
2. Fakhry, C. & Gillison, M. L. Clinical implications of human papillomavirus in head and neck cancers. *J. Clin. Oncol.* 24, 2606–2611, https://doi.org/10.1200/jco.2006.06.1291 (2006).
3. Leemans, C. R., Braakhuis, B. J. & Brakenhoff, R. H. The molecular biology of head and neck cancer. *Nat. Rev. Cancer* 11, 9–22, https://doi.org/10.1038/nrc2982 (2011).
4. Stransky, N. et al. The mutational landscape of head and neck squamous cell carcinoma. *Science* 333, 1157–1160, https://doi.org/10.1126/science.1208130 (2011).
5. Agrawal, N. et al. Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science* 333, 1154–1157, https://doi.org/10.1126/science.1206923 (2011).
6. Ang, K. K. et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N. Engl. J. Med.* 363, 24–35, https://doi.org/10.1056/NEJMoat0912217 (2010).
7. Lydiatt, W. M. et al. Head and Neck cancers-major changes in the American Joint Committee on cancer eighth edition cancer staging manual. *CA Cancer J. Clin.* 67, 122–137, https://doi.org/10.3322/caac.21389 (2017).
8. Stein, A. P. et al. Prevalence of Human Papillomavirus in Oropharyngeal Cancer: A Systematic Review. *Cancer J.* 21, 138–146, https://doi.org/10.1097/pco.0000000000001115 (2015).
9. Chatrvedi, A. K. et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J. Clin. Oncol.* 29, 4294–4301, https://doi.org/10.1200/jco.2011.36.4596 (2011).
10. Hwang, T. Z., Hsiao, J. R., Tsai, C. R. & Chang, J. S. Incidence trends of human papillomavirus-related head and neck cancer in Taiwan, 1995–2009. *Int. J. Cancer* 137, 395–408, https://doi.org/10.1002/ijc.29330 (2015).
11. Ko, Y. C., Chang, T. A., Chang, S. J. & Hsieh, S. F. Prevalence of betel quid chewing habit in Taiwan and related sociodemographic factors. *J. Oral. Pathol. Med.* 21, 261–264 (1992).
12. Tham, J., Sem, G., Sit, E. & Tai, M. C. The ethics of betel nut consumption in Taiwan. *J. Med. Ethics* 43, 739–740, https://doi.org/10.1136/medethics-2016-103990 (2017).
13. Hsu, W. L. et al. Lifetime risk of distinct upper aerodigestive tract cancers and consumption of alcohol, betel and cigarette. Int. J. Cancer 135, 1480–1486, https://doi.org/10.1002/ijc.28791 (2014).

14. Lee, K. W. et al. Different impact from betel quid, alcohol and cigarette: risk factors for pharyngeal and laryngeal cancer. Int. J. Cancer 117, 831–836, https://doi.org/10.1002/ijc.21237 (2005).

15. Chaturvedi, A. K., Engels, E. A., Anderson, W. F. & Gillison, M. L. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. J. Clin. Oncol. 26, 612–619, https://doi.org/10.1200/JCO.2007.14.1713 (2008).

16. Chaturvedi, A. K. et al. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. J. Clin. Oncol. 31, 4550–4559, https://doi.org/10.1200/JCO.2013.50.3870 (2013).

17. Bandhary, S. K. et al. Detection of Human Papilloma Virus and Risk Factors among Patients with Head and Neck Squamous Cell Carcinoma Attending a Tertiary Referral Centre in South India. Asian Pac. J. Cancer Prev. 19, 1325–1330, https://doi.org/10.22034/apjcp.2018.19.5.1325 (2018).

18. Hwang, T. Z., Hsiao, J. R., Tsai, C. R. & Chang, J. S. Incidence trends of human papillomavirus-related head and neck cancer in Taiwan, 1995–2009. Int. J. Cancer 137, 395–408, https://doi.org/10.1002/ijc.29330 (2015).

19. Fung, N., Faraji, F., Kang, H. & Fakhry, C. The role of human papillomavirus on the prognosis and treatment of oropharyngeal carcinoma. Cancer Metastasis Rev. 36, 449–461, https://doi.org/10.1007/s10555-017-9686-9 (2017).

20. Marur, S., D’Souza, G., Westra, W. H. & Forastiere, A. A. HPV-associated head and neck cancer: a virus-related cancer epidemic. Lancet Oncol. 11, 781–789, https://doi.org/10.1016/S1470-2045(10)70017-6 (2010).

21. Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Mortality - All COD, Aggregated With State, Total U.S. (1969–2015) <Katrina/Rita Population Adjustment>, National Cancer Institute, DCCPS, Surveillance Research Program, released December 2017. Underlying mortality data provided by NCHS (www.cdc.gov/nchs).

22. Liao, C. T. et al. Comparative clinical outcomes of Taiwanese patients with resected buccal and tongue squamous cell carcinomas. Oral. Oncol. 67, 95–102, https://doi.org/10.1016/j.oraloncology.2017.02.011 (2017).

23. Taiwan Cancer Registry Annual Report, 2015, Health Promotion Administration of Ministry of Health and Welfare [online], https://www.hpa.gov.tw/Pages/Detail.aspx?nodeId=269&pageId=8084 (Accessed December 31, 2018).

24. Mirghani, H. et al. Smoking impact on HPV driven head and neck cancer’s oncological outcomes? Oral. Oncol. 82, 131–137, https://doi.org/10.1016/j.oraloncology.2018.05.007 (2018).

25. Liao, I. L., Chou, H. W., Wang, C. T., Chung, C. S. & Lai, M. S. The impact of second primary malignancies on head and neck cancer survivors: a nationwide cohort study. PLoS One 8, e62116, https://doi.org/10.1371/journal.pone.062116 (2013).

26. Saito, Y. et al. Negative human papillomavirus status and excessive alcohol consumption are significant risk factors for second primary malignancies in Japanese patients with oropharyngeal carcinoma. Jpn. J. Clin. Oncol. 44, 564–569, https://doi.org/10.1093/jjco/hyy042 (2014).

27. Rietbergen, M. M. et al. No evidence for active human papillomavirus (HPV) in fields surrounding HPV-positive oropharyngeal tumors. J. Oral. Pathol. Med. 43, 137–142, https://doi.org/10.1111/op.12123 (2014).

28. Huang, Y. C. et al. Regular screening of esophageal cancer for 248 newly diagnosed hypopharyngeal squamous cell carcinoma by unsedated transnasal esophagogastroduodenoscopy. Oral. Oncol. 55, 55–60, https://doi.org/10.1016/j.oraloncology.2016.01.008 (2016).

29. McCarey, P. O. J. et al. Rigid Esophagoscopy for Head and Neck Cancer Staging and the Incidence of Synchronous Esophageal Malignant Neoplasms. JAMA Otolaryngol. Head. Neck Surg. 142, 40–45, https://doi.org/10.1001/jamaoto.2015.28215 (2016).

30. Wang, W. L. et al. The benefit of pretreatment esophageal screening with image-enhanced endoscopy on the survival of patients with hypopharyngeal cancer. Oral. Oncol. 49, 808–813, https://doi.org/10.1016/j.oraloncology.2013.04.009 (2013).

31. Prigge, E. S., Arbyn, M., von Knebel Doeberitz, M. & Reuschenbach, M. Diagnostic accuracy of p16(INK4a) immunohistochemistry in oropharyngeal squamous cell carcinomas: A systematic review and meta-analysis. Int. J. Cancer 140, 1186–1198, https://doi.org/10.1002/ijc.30516 (2017).

32. Edge, S. B. & Compton, C. C. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann. Surg. Oncol. 17, 1471–1474, https://doi.org/10.1245/s10434-010-0988-5 (2010).

33. Huang, H. J. et al. Human papillomavirus genotyping by a polymerase chain reaction-based genechip method in cervical carcinoma treated with neoadjuvant chemotherapy plus radical surgery. Int. J. Gynecol. Cancer 14, 639–649, https://doi.org/10.1111/j.1043-8547.2004.1418.x (2004).

34. Mena, M. et al. Double positivity for HPV-DNA/p16(INK4a) is the biomarker with strongest diagnostic accuracy and prognostic value for human papillomavirus related oropharyngeal cancer patients. Oral. Oncol. 78, 137–144, https://doi.org/10.1016/j.oraloncology.2018.01.010 (2018).

35. Fakhry, C. et al. Human Papillomavirus Testing in Head and Neck Carcinomas: ASCO Clinical Practice Guideline Endorsement of the College of American Pathologists Guideline. J. Clin. Oncol. 36, 3152–3161, https://doi.org/10.1200/jco.18.00684 (2018).

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Competing interests
The authors declare no competing interests.

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