Negative Human Papillomavirus Status and Excessive Alcohol Consumption are Significant Risk Factors for Second Primary Malignancies in Japanese Patients with Oropharyngeal Carcinoma†

Yuki Saito1,*, Yasuhiro Ebihara1, Tetsuo Ushiku2, Go Omura1, Kenya Kobayashi1, Mizuo Ando1, Takashi Sakamoto1, Masashi Fukayama2, Tatsuya Yamasoba1 and Takahiro Asakage1

1Department of Otolaryngology, Head and Neck Surgery, University of Tokyo and 2Department of Pathology, University of Tokyo, Tokyo, Japan

*For reprints and all correspondence: Yuki Saito, Department of Otolaryngology, Head and Neck Surgery, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: saitou-tky@umin.ac.jp

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Objective: To determine the clinical significance of human papillomavirus subclinical infection in patients with oropharyngeal squamous cell carcinoma in Japan.

Methods: Over a 9-year period, a retrospective case comparison study of the pathology database was conducted at the University of Tokyo to identify samples of oropharyngeal squamous cell carcinoma. We performed in situ hybridization for human papillomavirus-DNA to identify subclinical human papillomavirus infections among patients with oropharyngeal squamous cell carcinoma. Second primary malignancies were classified as synchronous, if identified within 6 months of the diagnosis of the first tumor, or metachronous, if identified after this 6-month period. Univariate and multivariate analyses using logistic stepwise regression models were performed to identify factors associated with synchronous and metachronous second primary malignancy.

Results: Of the 150 patients with oropharyngeal squamous cell carcinoma, 14% (21/150) and 20.7% (31/150) developed synchronous and metachronous second primary malignancies, respectively. Esophageal carcinoma was the most frequent second primary malignancy (10/21 for synchronous and 10/31 for metachronous second primary malignancies). The prevalence of oropharyngeal squamous cell carcinoma positive for human papillomavirus was 31% (47/150). Multivariate analysis identified alcohol consumption as a significant unfavorable risk factor for the occurrence of synchronous second primary malignancy, and either a human papillomavirus-negative status or N0 classification was a significant unfavorable risk factor for the occurrence of metachronous second primary malignancy.

Conclusions: Evaluation of the human papillomavirus status may help identify patients at risk for metachronous second primary malignancy. Upper gastrointestinal endoscopy is very important in the diagnosis of oropharyngeal squamous cell carcinoma among heavy drinkers in Japan.

Key words: oropharyngeal cancer – HPV – Japan – second primary malignancies

INTRODUCTION

Recently, the identification of human papillomavirus (HPV) infection in oropharyngeal squamous cell carcinoma (OPSCC) has been reported to be of considerable clinical importance and an independent prognostic biomarker (1–6). Most studies on HPV-associated SCC were undertaken in the USA or Europe. Despite the fact that populations in eastern Asia, including Japan, are known to have a relatively high
incidence of several oncogenic viral infections, including Epstein–Barr and hepatitis B virus, the clinical features of HPV-related OPSCC have only rarely been evaluated for patient populations in this region.

In the USA and Europe, patients diagnosed with OPSCC were found to have a lower risk of developing synchronous second primary malignancies (SPM) (7,8). We have previously reported that the incidence of HPV-positive OPSCC in Japan is increasing and that p16 expression and alcohol consumption are significantly associated with the survival of Japanese OPSCC patients (9). Accordingly, we hypothesized that HPV infection and alcohol consumption were also risk factors for SPM in Japan. To test this hypothesis, we analyzed the risk factors for synchronous SPM (SSPM) and metachronous SPM (MSPM) in Japanese OPSCC patients.

PATIENTS AND METHODS

CLINICAL INFORMATION

Between January 2004 and December 2012, 187 patients with OPSCC were treated in the Department of Otolaryngology and Head and Neck Surgery, Tokyo University, Tokyo, Japan. Of these patients, 20 received only palliative treatment because of a poor performance status. Of the 167 patients treated with curative intent, 17 were excluded because of the poor quality of the available histological sample. The clinical charts of the remaining 150 patients (130 men; 20 women; age, 21–90 years; median age, 64 years) were reviewed, retrospectively. Some of these patients (102 patients) were included in our previous study on the prognostic value of p16 expression and alcohol consumption (9). The anatomic locations from which the tissue samples were removed included the tonsil (n = 93), the base of the tongue (n = 41), the soft palate (n = 11) and the posterior pharyngeal wall (n = 5). The TNM staging system was used to classify the tumors, in accordance with the American Joint Committee on Cancer classification. Patient distribution according to TNM stage is shown in Table 1. There were no patients with distant metastasis initially. Paraffin-embedded specimens and tissue sections were retrieved from the Department of Pathology files. The Institutional Review Board (#2487 and #2904) approved the protocol for this study.

Table 1. Distribution of 150 patients according to TNM stage

| T stage | N0 | N1 | N2 | N3 | Total |
|---------|----|----|----|----|-------|
| T1      | 10 | 4  | 12 | 0  | 26 (17.3%) |
| T2      | 20 | 8  | 32 | 5  | 65 (43.3%) |
| T3      | 14 | 3  | 17 | 3  | 37 (24.7%) |
| T4a     | 6  | 7  | 6  | 3  | 22 (14.7%) |
| Total   | 50 (33.3%) | 22 (14.6%) | 67 (44.7%) | 11 (7.3%) | 150 |

DETECTION OF HPV VIRAL DNA USING IN SITU HYBRIDIZATION (ISH-HPV)

HPV-DNA was detected in formalin-fixed paraffin-embedded blocks using a catalyzed signal amplification ISH method (GenPoint signal amplification system for biotinylated probes; Dako Japan Inc., Kyoto, Japan). After 4 μm sections were processed by deparaffinization, heat-induced target retrieval (S1700) in citrate buffer, and digestion using Proteinase K (Dako Japan Inc.,), slides were hybridized using a biotinylated GenPointTM HPV (Types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 specific) probe (DAKO). After initial binding of the streptavidin–horseradish peroxidase complex to the probe, signal amplification was performed using biotinyl tyramide. Positive hybridization signals were visualized by adding the chromogenic substrate dianinobenzidine. A known HPV-positive OPSCC sample served as a positive control. Slides were scored as positive for HPV if a punctate signal pattern was observed in almost all tumor nuclei.

CONSUMPTION OF ALCOHOL AND TOBACCO

All patients were interviewed about their smoking and drinking habits during the first medical examination at our institution before then. In order to identify SPM, all patients were evaluated using upper gastrointestinal endoscopy and chest computed tomography before undergoing treatment with curative intent. Lesions were considered to be SPMs if they were distinct, solid cancers and histologically proven to be inconsistent with recurrence or metastatic disease. An SPM was classified as SSPM, if identified within 6 months of OPSCC diagnosis, or MSPM if identified beyond this 6-month period.

TREATMENT

All cases were reviewed by a multidisciplinary tumor board to determine whether the primary treatment course should be surgery or definitive radiotherapy/cisplatin-based chemoradiotherapy. Of 63 patients undergoing primary surgical treatment, 10 were treated with either postoperative radiotherapy alone or adjuvant chemoradiotherapy on the basis of pathologic risk factors. Recently, cisplatin-based induction chemotherapy has been indicated for some cases of Stages III and IV disease, and it was decided that good responders should be treated with radiation therapy instead of surgery. Initial treatment according to T classification is summarized in Table 2. Treatment decisions did not take into account p16 or HPV status until 2011, because these markers were not routinely examined prior to treatment in our institution before then. In order to identify SPM, all patients were evaluated using upper gastrointestinal endoscopy and chest computed tomography before undergoing treatment with curative intent. Lesions were considered to be SPMs if they were distinct, solid cancers and histologically proven to be inconsistent with recurrence or metastatic disease. An SPM was classified as SSPM, if identified within 6 months of OPSCC diagnosis, or MSPM if identified beyond this 6-month period.

Table 2. Initial treatment according to T classification

| Initial treatment | T1 | T2 | T3 | T4a | Total |
|------------------|----|----|----|-----|-------|
| Radiotherapy (%) | 10 (38) | 40 (62) | 27 (73) | 10 (45) | 87 (58) |
| Surgery (%)      | 16 (62) | 25 (38) | 10 (27) | 12 (55) | 63 (52) |
| Total            | 26 | 65 | 37 | 22 | 150 |
and 95% confidence interval (CI) were calculated to determine the effect of each variable on the outcome with an OR of < 1.0. A P value of < 0.05 was considered statistically significant. Receiver operating characteristic (ROC) analysis (for SPM data) and recursive partitioning analysis (for MSPM data) were performed using the ‘Epi’ and ‘party’ software packages in R (R Foundation for Statistical Computing, Vienna Austria; available at: http://www.R-project.org [28 March 2013, date last accessed]). All analyses were performed using Microsoft Excel version 2010 (Microsoft, Redmond, WA, USA), StatFlex version 6.0 (Artech Co., Ltd.), and R (version 2.15.2).

RESULTS

SSPM AND MSPM IN OPSCC PATIENTS

Table 3 summarizes the occurrence of SSPM and MSPM together with relevant clinical data for the 150 OPSCC patients.

|                      | Synchronous SPM | Metachronous SPM |
|----------------------|------------------|-------------------|
|                      | Positive (%)     | Negative (%)      | Positive (%) | Negative (%) | P value | Positive (%) | Negative (%) | P value |
| Gender               |                  |                   |               |               |         |               |               |         |
| Male                 | 17 (81)          | 113 (88)          | 27 (87)       | 103 (87)      | 0.89    |               |               |         |
| Female               | 4 (19)           | 16 (12)           | 4 (13)        | 16 (13)       |         |               |               |         |
| Age, range (median)  | 57–81 (63)       | 21–90 (64)        | 45–80 (63)    | 21–90 (65)    | 0.84    |               |               |         |
| Tobacco smoking      | 0–129 (40)       | 0–170 (36.7)      | 0–129 (40)    | 0–170 (30)    | 0.10    |               |               |         |
| Alcohol consumption  | 0–3000 (900)     | 0–4000 (400)      | 0–2400 (800)  | 0–4000 (400)  | 0.28    |               |               |         |
| Clinical T classification |                |                   |               |               |         |               |               |         |
| T1–2                 | 13 (62)          | 78 (60)           | 19 (61)       | 72 (61)       | 0.93    |               |               |         |
| T3–4                 | 8 (38)           | 51 (40)           | 12 (39)       | 47 (39)       |         |               |               |         |
| Clinical N classification |              |                   |               |               |         |               |               |         |
| N0                   | 6 (29)           | 44 (34)           | 17 (55)       | 33 (28)       | 0.04    |               |               |         |
| N1–3                 | 15 (71)          | 85 (66)           | 14 (45)       | 86 (72)       |         |               |               |         |
| Clinical stage       |                  |                   |               |               |         |               |               |         |
| Stage I–II           | 4 (19)           | 25 (19)           | 10 (32)       | 19 (16)       | 0.04    |               |               |         |
| Stage III–IV         | 17 (81)          | 104 (81)          | 21 (68)       | 100 (84)      |         |               |               |         |
| ISH-HPV              |                  |                   |               |               |         |               |               |         |
| Positive             | 6 (29)           | 41 (32)           | 3 (10)        | 44 (37)       | 0.0035  |               |               |         |
| Negative             | 15 (71)          | 88 (68)           | 28 (90)       | 75 (63)       |         |               |               |         |
| SPM location<sup>a</sup> |            |                   |               |               |         |               |               |         |
| Head and neck        | 3                | 9                 |               |               |         |               |               |         |
| Esophagus            | 10               | 10                |               |               |         |               |               |         |
| Lung                 | 3                | 2                 |               |               |         |               |               |         |
| Intestinal           | 4                | 10                |               |               |         |               |               |         |
| Urogenital           | 1                | 5                 |               |               |         |               |               |         |

HPV, human papillomavirus; SPM, second primary malignancies.

<sup>a</sup>Some patients had more than one second primary carcinoma.
Of these, 21 patients developed SSPM (14%) and 31 patients developed MSPM (20.7%). Univariate analysis identified a positive association between SSPM and alcohol consumption ($P = 0.0027$) and between MSPM and clinical stage, clinical N classification, and ISH-HPV negativity ($P = 0.04, 0.04$ and $0.0035$, respectively).

**LOGISTIC REGRESSION ANALYSIS**

Age, pack-years, clinical stage, clinical T and N classifications, sex, p16 positivity and ISH-HPV positivity were not found to be statistically significant variables for SSPM using a stepwise selection method, although an independent risk factor for SSPM was alcohol consumption (OR 1.001, 95% CI; 1.0004–1.0017, $P = 0.0022$). In contrast, age, pack-years, sex, p16 positivity, alcohol consumption, clinical stage and clinical T classification were not statistically significant variables for MSPM, but both ISH-HPV negativity (OR 0.288, 95% CI; 0.102–0.810) and a lower N classification (OR 0.64, 95% CI; 0.422–0.978) were found to be independent risk factors (Table 4).

ROC analysis was also performed to determine the cutoff value for alcohol consumption that would optimize its specificity and specificity for predicting SSPM. This was 800 drinks/week-years, with a sensitivity of 71.4% and a specificity of 68.5%. When cases were stratified according to this cutoff, 93.7% of patients below the threshold were negative for SSPM.

Recursive partitioning analysis identified ISH-HPV positivity as the major determinant of MSPM, followed by clinical N1–3. We classified the risk of MSPM among Japanese patients with OPSCC (Fig. 1) into categories of low risk (8.5% MSPM), intermediate risk (16.9% MSPM) and high risk (44.7% MSPM). Patients who had ISH-HPV-positive tumors were in the low-risk group, patients who had ISH-HPV-negative tumors with clinical N1–3 were in the intermediate-risk group and patients who had ISH-HPV-negative tumors with clinical N0 were in the high-risk group.

**DISCUSSION**

SPMs have been one of the leading causes of mortality in patients with head and neck squamous cell carcinoma (HNSCC). Jain et al. (7) recently reported that the risk of SPM has markedly changed for patients with OPSCC. Whereas OPSCC was once associated with the highest risk of SPM, it is now associated with the lowest risk of SPM among HNSCC subsites. This change was attributed to the increased prevalence of HPV-positive OPSCC, which is lower in East Asia than in the USA and Europe. Furthermore, polymorphisms in the aldehyde dehydrogenase-2 ($ALDH2$) gene, which encodes a key enzyme responsible for the elimination of acetaldehyde, vary with different ethnic backgrounds and are associated with esophageal and head and neck cancers (10). Correspondingly, our study, albeit retrospective, showed that alcohol consumption was a significant risk factor for SSPM and that negative ISH-HPV and a low clinical N classification were significant risk factors for MSPM.

An $ALDH2$ gene polymorphism results in the mutant $ALDH2*2$ allele (Glu487Lys), which encodes an inactive subunit. Individuals with the $ALDH2*1/2*2$ genotype would be predicted to have only 6.25% of the normal $ALDH2*1$ protein level, explaining the dominant effect of the $ALDH2*2$ mutation. Approximately 40% of Japanese individuals have inactive forms of the $ALDH2$ gene due to the presence of the $ALDH2*2$ allele (11). The incidence of this allele varies with ethnicity; it is prevalent among East Asians but has not been found in Caucasians or Africans. An inactive form of $ALDH2$ is a risk factor for synchronous or metachronous multiple cancers associated with esophageal and head and neck cancer in Japanese men (11).

The patterns with which SPMs in head and neck cancer patients occur differ between Japan and Europe (12). Lubin et al. (13) reported on these patterns in patients exposed to various levels of smoking and found that they were similar to those observed in other smoking-related malignancies such as lung, esophageal and bladder cancer. However, the most frequent SPM in Japan is upper GI tract carcinoma (12). Tanabe et al. (14) reported that high alcohol consumption ($>75$ g per day) increased the risk of esophageal SPM, whereas smoking was not related to SPM. The current study showed that heavy drinkers ($>800$ drinks/week-year) were at a significantly increased risk of SSM. This result does not conflict with that of Tanabe et al. (14) as 800 drinks/week-years is approximately equal to 80 g/day for 20 years. Therefore, our results further emphasize the importance of upper gastrointestinal endoscopy for heavy drinkers with OPSCC in Japan, like other eastern

**Table 4.** Multivariate logistic regression models for synchronous and metachronous SPM

| Variable                        | Odds ratio | 95% Confidence interval | $P$   |
|---------------------------------|------------|-------------------------|-------|
| **Synchronous SPM**             |            |                         |       |
| Alcohol (drinks/week-years)     | 1.001      | 1.0004–1.0017            | 0.0022|
| Sex (male vs female)            | 2.97       | 0.81–10.95               | 0.102 |
| **Metachronous SPM**            |            |                         |       |
| HPV (positive vs negative)      | 0.206      | 0.06–0.727               | 0.014 |
| Clinical N classification (0–3) | 0.616      | 0.40–0.948               | 0.028 |

**Figure 1.** Classification of the 150 oropharyngeal squamous cell carcinoma patients according to the risk of metachronous second primary malignancies using recursive partitioning analysis.
Asian countries (15). Furthermore, treatment of superficial esophageal SCC by endoscopic mucosal resection has markedly improved the prognosis of these patients (16), whilst the prognosis of patients with advanced esophageal SCC remains dismal. Therefore, for heavy drinkers, it is vital to screen the upper aerodigestive tract for neoplasms, regardless of any symptoms. This will also help to reduce the associated medical costs.

The effect of continued tobacco use and alcohol consumption on the development of MSPM after treatment for a HNSCC continues to be debated in western countries (17). As the cases in our study were not fully evaluated in terms of whether they stopped smoking or drinking alcohol after treatment for OPSCC, the influence of continuing these habits on the development of MSPM could not be analyzed.

HPV-positive OPSCC patients had the lowest risk (8.5%) of MSPM in our cohort. Ang et al. (1) reported that the incidences of MSPM amongst HPV-positive and HPV-negative OPSCC patients were 5.9% and 14.6%, respectively, \((P < 0.05)\) and that the site of SPM did not differ between HPV-positive and HPV-negative OPSCC. In a case–control study of oral cancer (18), the risk of SPM in the aerodigestive tract (the oral cavity, pharynx, larynx, esophagus and lung) increased with both tobacco smoking and alcohol consumption. HPV-positive OPSCC patients tended to smoke and drink less than HPV-negative patients, and the relatively low risk of MSPM in HPV-positive OPSCC patients is consistent with this. Reports of HPV-related SPM are rare (19). The reason for this is unclear, but we speculate that HPV viral DNA integration is confined to the neoplastic tissue (20), and the field cancerization effect, first suggested by Slaughter et al. (21), would thus not be observed in HPV-related OPSCC.

The risk of MSPM is relatively high for lower clinical N and ISH-HPV-negative tumors, although the reasons for this remain unclear. One possibility is that the patients with ISH-HPV-negative, superficial tumors in our cohort were at risk of ‘field cancerization,’ and therefore, the risk of MSPM was actually higher than that in the other two cohorts. In one study, in at least 35% of the oral and oropharyngeal tumors analyzed, the lesion was surrounded by mucosal epithelium carrying genetic changes (22). SPM has been shown to carry CND1 and EMS1 amplifications and gain of BCL2L1 mutations (23). Furthermore, genetic variation within the PI3K/PTEN/AKT/mTOR pathway was sufficient to identify HNSCC patients at a high risk of SPM or recurrence (24). However, the genetic alterations carried by HNSCC tumors with associated lymph node metastasis have not been fully evaluated. Therefore, we speculate that the different genetic alterations between N0 and N1–3 tumors might have been related to the risk of MSPM in the ISH-HPV-negative OPSCC cases in our study.

The other possibility is that the methods for detecting HPV differ between studies. In our report, we used ISH-HPV to test for HPV. However, ISH-HPV is known to be less sensitive and more specific than the polymerase chain reaction-based method for detecting HPV (25), and thus, some HPV-positive OPSCCs might be just below the threshold of ISH-HPV detection. As HPV-positive OPSCCs more commonly involve regional neck disease (26), a low nodal stage with a higher SPM rate might be a surrogate marker for HPV-negative disease, and as noted above, HPV-positive OPSCC is associated with a lower rate of SPM.

The difference in the prevalence of HPV-associated OPSCC between different ethnic groups has been reported (2). Although the clinical features of HPV-positive Asian OPSCC patients were generally similar to those of their USA and European counterparts, the incidence of HPV-positive tumors among OPSCC cases was only 30–50% in Japan (9,27). As ISH-HPV-positive OPSCC patients have only a low risk of SPM, which concurs with recent studies conducted in the USA (8), testing for ISH-HPV in archived specimens would be particularly beneficial for ‘minor’ HPV-related OPSCC patients who have already been treated for this malignancy in Japan.

CONCLUSIONS
The current incidence of HPV-related OPSCC in Japan is ≏30%. Heavy drinkers (>800 drinks/week-years) were at a significantly increased risk of SPM, and the evaluation of HPV status may help identify patients at risk for MSPM.

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Conflict of interest statement
None declared.

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