

# Human papillomavirus and *p53* mutations in head and neck squamous cell carcinoma among Japanese population

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## Key words

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We aimed to reveal the prevalence and pattern of human papillomavirus (HPV) infection and *p53* mutations among Japanese head and neck squamous cell carcinoma (HNSCC) patients in relation to clinicopathological parameters. Human papillomavirus DNA and *p53* mutations were examined in 493 HNSCCs and its subset of 283 HNSCCs. Oropharyngeal carcinoma was more frequently HPV-positive than non-oropharyngeal carcinoma (34.4% vs 3.6%,  $P < 0.001$ ), and HPV16 accounted for 91.1% of HPV-positive tumors. In oropharyngeal carcinoma, which showed an increasing trend of HPV prevalence over time ( $P < 0.001$ ), HPV infection was inversely correlated with tobacco smoking, alcohol drinking, *p53* mutations, and a disruptive mutation ( $P = 0.003$ ,  $<0.001$ ,  $<0.001$ , and  $<0.001$ , respectively). The prevalence of *p53* mutations differed significantly between virus-unrelated HNSCC and virus-related HNSCC consisting of nasopharyngeal and HPV-positive oropharyngeal carcinomas (48.3% vs 7.1%,  $P < 0.001$ ). Although *p53* mutations were associated with tobacco smoking and alcohol drinking, this association disappeared in virus-unrelated HNSCC. A disruptive mutation was never found in virus-related HNSCC, whereas it was independently associated with primary site, such as the oropharynx and hypopharynx ( $P = 0.01$  and  $0.03$ , respectively), in virus-unrelated HNSCC. Moreover, in virus-unrelated HNSCC, G:C to T:A transversions were more frequent in ever-smokers than in never-smokers ( $P = 0.04$ ), whereas G:C to A:T transitions at CpG sites were less frequent in ever-smokers than in never-smokers ( $P = 0.04$ ). In conclusion, HNSCC is etiologically classified into virus-related and virus-unrelated subgroups. In virus-related HNSCC, *p53* mutations are uncommon with the absence of a disruptive mutation, whereas in virus-unrelated HNSCC, *p53* mutations are common, and disruptive mutagenesis of *p53* is related with oropharyngeal and hypopharyngeal carcinoma.

Head and neck squamous cell carcinoma (HNSCC) is etiologically correlated with tobacco smoking and alcohol abuse, whereas a subset of oropharyngeal carcinoma is caused by infection of high risk human papillomavirus (HPV), predominantly HPV type 16 (HPV16).<sup>(1,2)</sup> The *p53* protein, as a tumor suppressor, has a highly conserved role as a guardian of the genome and is central to cellular anticancer mechanisms.<sup>(3)</sup> One of the most common molecular alterations in HNSCC is the abrogation of *p53* function, which is mediated by mutations of its gene, *p53*, loss of heterozygosity of *p53*, or interaction with HPV oncoprotein E6.<sup>(4–6)</sup> Tobacco smoking increases the frequency of *p53* mutations in a dose-dependent manner,<sup>(7)</sup> whereas E6 binds and degrades *p53*.<sup>(8)</sup>

Crystal structural analyses of the effect of *p53* mutations on DNA binding classifies *p53* mutations into two distinct types, disruptive mutations and non-disruptive mutations, according to their ability to knock out *p53* function.<sup>(9,10)</sup> Disruptive mutations are non-conservative mutations occurring within the core DNA binding domain L2/L3, stop mutations, or frameshift

mutations, and thus entirely abrogate *p53* function. All other mutations defined as non-disruptive mutations affect *p53* function in a limited fashion. It has been previously shown that HPV-positive oropharyngeal carcinoma is less likely to harbor *p53* mutations as compared with HPV-negative oropharyngeal carcinoma.<sup>(11)</sup> Recently, an inverse relationship between HPV infection and disruptive mutations in oropharyngeal carcinoma has been reported, though in a small number.<sup>(12)</sup>

Human papillomavirus-positive oropharyngeal carcinoma is an entity clinically and epidemiologically different from HPV-negative oropharyngeal carcinoma. Oropharyngeal carcinoma that is HPV-positive has risk factors related to sexual behavior, whereas HPV-negative oropharyngeal carcinoma is linked with tobacco smoking and alcohol drinking.<sup>(2)</sup> Patients with HPV-positive oropharyngeal carcinoma survive better than patients with HPV-negative oropharyngeal carcinoma.<sup>(13)</sup> Of note is an increasing HPV prevalence in oropharyngeal carcinoma over time, which currently exceeds 70% in the USA and 90% in Europe.<sup>(14,15)</sup> However, there is a report showing the presence

of a racial disparity in HPV prevalence.<sup>(16)</sup> Human papillomavirus-positive tumors are significantly less frequent in black patients than in white patients. Despite these findings, the prevalence and pattern of HPV infection and *p53* mutations in HNSCC among Japanese patients remain less well established. We designed the present study to address this issue in a large patient group and to give a further insight into the association between HPV status, *p53* mutations, and clinicopathological parameters.

## Materials and Methods

**Patients.** A consecutive series of 493 patients with newly diagnosed HNSCC at Osaka University Medical School Hospital (Suita, Japan) between October 1995 and December 2012 were examined for tumor HPV status, and its subset of 283 patients were also examined for tumor *p53* mutations. All tumors were staged according to the 2002 International Union Against Cancer TNM staging system. The protocol of the present study was approved by the Institutional Review Board in December 2004, and thereafter patients provided written informed consent. The Institutional Review Board waived informed consent of patients before December 2004 due to the retrospective nature of the study, under the condition that information on the present study was disclosed on the website so that patients could apply for refusal of the enrolment.

**DNA extraction.** Formalin-fixed, paraffin-embedded biopsy specimens of untreated tumors were cut into 5- $\mu$ m serial sections, one of which was stained with H&E, and examined using light microscopy. The other sections were used for DNA extraction by means of the DNeasy tissue kit (Qiagen, Valencia, CA, USA) when tumor cells accounted for at least 70% of the tissue sample. Tissues with <70% tumor cells were microdissected to enrich the tumor cell content of the specimen prior to DNA extraction.

**Detection and typing of high-risk HPV DNA and assessment of HPV16 physical status.** Human papillomavirus DNA from HPV subtypes 16, 18, 31, 33, 35, 52, or 58 was detected by PCR to amplify DNA fragments within the ORFs of *E6* and *E7* using consensus primers, pU-1M and pU-2R, as described previously.<sup>(17,18)</sup> The amplified products were typed by direct sequencing. Direct sequencing was carried out by the dideoxy chain termination method using the Big Dye Terminator cycle sequencing kit (Perkin-Elmer, Forester City, CA, USA). After ethanol precipitation, the samples were analyzed by the autoanalyzer (ABI PRISM 3100 genetic analyzer; Perkin-Elmer). The physical status of HPV16 was addressed according to ratio of *E2* to *E6* copy numbers, which was determined by real-time PCR amplification of the *E2* and *E6* ORFs as described previously.<sup>(18)</sup> Primers for the *E2* ORF were designed to amplify the *E2* hinge region, which is usually deleted after viral integration into the host genome.<sup>(19)</sup> The *E2* and *E6* ORFs were quantified with the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). CaSki cells, containing 500 integrated copies of HPV16 DNA per cell, were used as a positive control.<sup>(20)</sup>

**Detection of *p53* gene mutations.** Polymerase chain reaction amplification followed by SSCP analysis and direct sequencing was carried out as previously described<sup>(21)</sup> to detect *p53* mutations in exons 4–8, containing 94% of all mutations described in HNSCC (International Agency for Research on Cancer TP53 Database, <http://p53.iarc.fr/>). The PCR primer pairs for the amplification are described elsewhere.<sup>(22)</sup> The SSCP bands that showed altered mobility as compared to a wild-type control

(human lymphocyte DNA), were extracted from the gel and reamplified by PCR to enrich mutated alleles. The mutated alleles were purified using the QIAquick PCR purification kit (Qiagen). Direct sequencing was carried out as described above.

**Statistical analysis.** Associations between HPV infection, *p53* mutations, and a disruptive mutation with clinicopathological parameters were assessed by univariate analyses involving logistic regression model or Clopper–Pearson's exact test, as appropriate. Multivariate analyses were made by forward stepwise logistic regression model. Akaike's information criterion<sup>(23)</sup> was used to evaluate the relative usefulness of the model. Differences in HPV prevalence, *p53* mutation prevalence, and pattern of *p53* mutations between groups were examined by Fisher's exact test. Trend in HPV prevalence over calendar time was evaluated by the Cochran–Armitage test. All statistical tests were two-tailed at a significant level of 0.05. All data were analyzed using SAS Version 9.3 (SAS Institute, Cary, NC, USA).

Patients were classified as light and heavy smokers if their lifetime cigarette exposure was below and equal to or above the median pack-years in each analyzed group, respectively. Alcohol abuse was estimated by alcohol index, which was calculated by the formula: number of alcoholic units per day multiplied by year-duration. Twenty-eight grams of alcohol contained in alcoholic beverages corresponded to one unit of alcohol. Patients were classified as light and heavy drinkers if their lifetime alcohol consumption was below and equal to or above the median alcohol index in each analyzed group, respectively. Ever smokers and drinkers were defined as former plus current smokers and drinkers, respectively.

## Results

**Prevalence and pattern of HPV in HNSCCs.** There was a striking difference in HPV prevalence between oropharyngeal carcinoma and non-oropharyngeal carcinoma (Table 1). Fifty-six of 163 (34.4%) oropharyngeal carcinomas were HPV-positive, whereas 12 of 330 (3.6%) non-oropharyngeal carcinomas were HPV-positive ( $P < 0.001$ ). Prevalence of HPV in oral, hypopharyngeal, and laryngeal carcinomas was drastically decreased compared with that in oropharyngeal carcinoma ( $P < 0.001$ ). Of note is nasopharyngeal carcinoma, 12.0% of which was HPV-positive, although the prevalence was still significantly less than in oropharyngeal carcinoma (odds ratio (OR), 0.3; 95% confidence interval (CI), 0.05–0.9;  $P = 0.04$ ). As summarized in Table 2, HPV16 was the most prevalent, accounting for 62 of 68 (91.1%) HPV-positive tumors. All HPV-positive tumors were positive for a single type of high-risk HPV. An *E2* to *E6* transcript ratio consistent with complete viral integration was detected in 24 of 62 (38.7%) HPV16-positive tumors. The remaining 38 (61.3%) HPV16-positive tumors showed the presence of both episomal and integrated viral forms.

**Association of HPV status with clinicopathological factors in oropharyngeal carcinoma.** As HPV-positive tumors were highly predominant in oropharyngeal carcinoma, we addressed the relationship between HPV status and clinicopathological factors in the single group of patients with oropharyngeal carcinoma (Table 3). Older patients, heavy smokers, and heavy drinkers were less likely to have HPV-positive tumors than younger patients (OR, 0.4; 95% CI, 0.2–0.8;  $P = 0.009$ ), light smokers (OR, 0.4; 95% CI, 0.2–0.7;  $P = 0.003$ ), and light drinkers (OR, 0.3; 95% CI, 0.1–0.6;  $P < 0.001$ ), respectively. The prevalence of HPV infection was significantly decreased

**Table 1. Prevalence of human papillomavirus (HPV) in head and neck squamous cell carcinomas according to primary tumor site (n = 493)**

Primary site	Total no.	HPV-positive n (%)	Odds ratio†	95% CI	P-value
Oropharynx	163	56 (34.4)	Reference		
Non-oropharynx (whole)	330	12 (3.6)	0.1	0.0400–0.1000	<0.001
Oral cavity	57	0 (0.0)	0.0	0.0008–0.2000	<0.001
Nasopharynx	25	3 (12.0)	0.3	0.0500–0.9000	0.035
Hypopharynx	171	6 (3.5)	0.1	0.0200–0.2000	<0.001
Larynx	77	3 (3.9)	0.1	0.0200–0.3000	<0.001

†Univariate analysis was made by logistic regression model, except for oral cavity that was made by the Clopper–Pearson method. CI, confidence interval.

**Table 2. Subtype of human papillomavirus (HPV) and physical status of HPV16 (n = 68)**

Type of HPV	No.	%	Physical status		
			Integrated no. (%)	Mixed no. (%)	Episomal no. (%)
HPV16	62	91.1	24 (38.7)	38 (61.3)	0 (0.0)
HPV18	1	1.5			
HPV33	1	1.5			
HPV35	3	4.4			
HPV58	1	1.5			

in the posterior wall and soft palate (OR, 0.1; 95% CI, 0.02–0.4;  $P < 0.001$ ) as compared with the palatine tonsil. In contrast, poorly differentiated or undifferentiated tumors were more likely to be HPV-positive than well differentiated tumors (OR, 3.7; 95% CI, 1.3–11.0;  $P = 0.01$ ). In the multivariate analysis, age, alcohol drinking, and subsite remained as significant determinants of HPV infection.

**Trend of HPV prevalence in oropharyngeal carcinoma.** As HPV-positive tumors were specific to the palatine and lingual tonsils among subsites of the oropharynx, we addressed the chronological change of HPV prevalence in oropharyngeal carcinoma originating from these subsites. Prevalence of HPV was increased from 36.8% during 1995 to 1999, 39.0% during 2000 to 2009, to 48.3% during 2010 to 2012 (Fig. 1). This increasing trend across calendar periods was statistically significant ( $P < 0.001$ ).

**Association of HPV status with p53 mutations.** In oropharyngeal carcinoma, 28 of 57 (49.1%) tumors carrying wild-type p53 were HPV-positive, whereas 2 of 25 (8.0%) tumors harboring any p53 mutation and none of 10 tumors harboring a disruptive mutation were HPV-positive (Table 4). In turn, 2 of 30 (6.7%) HPV-positive tumors harbored p53 mutations. Accordingly, tumors harboring any p53 mutation, and particularly a disruptive mutation, were less likely to be HPV-positive as compared with tumors carrying wild-type p53 (for any p53 mutation: OR, 0.09; 95% CI, 0.01–0.3;  $P < 0.001$ ; for a disruptive mutation: OR, 0.05; 95% CI, 0.003–0.9;  $P < 0.001$ ), indicating an inverse relationship between HPV infection and any p53 mutation and a disruptive mutation. After adjustment for tobacco smoking and alcohol drinking in the multivariate

analysis, p53 mutations remained inversely associated with HPV infection ( $P = 0.005$ ). As none of the tumors harboring a disruptive mutation was HPV-positive, multivariate analysis of a disruptive mutation at a risk of HPV infection was prohibited. We also examined whether there was an inverse relationship between HPV infection and p53 mutations in non-oropharyngeal carcinoma as observed in oropharyngeal carcinoma. As shown in Table 4, 5 of 117 (4.3%) tumors carrying wild-type p53 and 4 of 84 (4.8%) tumors harboring any p53 mutation were HPV-positive, indicating the absence of the inverse relationship. Of note, all the mutations observed in HPV-positive tumors were non-disruptive. Human papillomavirus infection and p53 disruptive mutation did not coexist even in non-oropharyngeal carcinoma.

**Prevalence and pattern of p53 mutations in HNSCCs.** One hundred and nine of 283 (38.5%) tumors were found to harbor p53 mutations. Of 109 tumors with p53 mutations, 76 (69.7%) and 33 (30.3%) were classified to harbor a non-disruptive mutation and a disruptive mutation, respectively. Table 5 summarizes the prevalence of any p53 mutation and a disruptive mutation in relation to clinicopathological factors and HPV status. Tumors in heavy smokers and heavy drinkers were more likely to harbor p53 mutations than tumors in light smokers (OR, 1.6; 95% CI, 1.0–2.6;  $P = 0.06$ ) and light drinkers (OR, 2.0; 95% CI, 1.2–3.3;  $P = 0.006$ ), respectively. Although the difference between light and heavy smokers was marginal, there was an increasing trend of p53 mutations in proportion to the amount of tobacco smoking (OR, 1.0; 95% CI, 1.0–1.1;  $P = 0.04$ , per 5 pack-years). Of note is a striking difference between virus-unrelated HNSCCs and virus-related HNSCCs. Human papillomavirus-positive oropharyngeal carcinoma and nasopharyngeal carcinoma that is caused by Epstein–Barr virus<sup>(24)</sup> constitute virus-related HNSCCs. Only one of 21 (4.8%) nasopharyngeal carcinomas and 2 of 30 (6.7%) HPV-positive oropharyngeal carcinomas harbored p53 mutations. In contrast, the frequency of p53 mutations ranged from 44.2% to 48.7% in virus-unrelated HNSCCs. The frequency of any p53 mutation was significantly higher in virus-unrelated HNSCCs than in virus-related HNSCCs (45.7% vs 5.9%,  $P < 0.001$ ). Compared with oral carcinoma, nasopharyngeal and HPV-positive oropharyngeal carcinomas showed a significantly lower prevalence of p53 mutations (for nasopharyngeal carcinoma: OR, 0.1; 95% CI, 0.003–0.3;  $P < 0.001$ ; for HPV-positive oropharyngeal carcinoma: OR, 0.1; 95% CI, 0.01–0.3;  $P < 0.001$ ). Moreover, as in HPV-positive oropharyngeal carcinoma, no disruptive mutation was found in nasopharyngeal carcinoma.

**Association of p53 mutations with clinicopathological parameters in virus-unrelated HNSCCs.** As p53 mutations were extremely uncommon in virus-related HNSCCs, we sought to address an association of p53 mutations and a disruptive mutation with clinicopathological parameters in virus-unrelated HNSCCs (Table 6). Association of p53 mutations with tobacco smoking and alcohol drinking, which was evident in the whole study group, disappeared in the subpopulation of virus-unrelated HNSCCs. No clinicopathological parameter was associated with p53 mutations, whereas a disruptive mutation significantly correlated with tobacco exposure and primary site. The proportion of tumors with a disruptive mutation in tumors with any mutation was more frequent in ever-smoker patients than in never-smoker patients (7.1% vs 34.1%; OR, 6.7; 95% CI, 1.2–124.8;  $P = 0.02$ ). As compared with oral carcinoma, HPV-negative oropharyngeal carcinoma and hypopharyngeal carcinoma were more likely to harbor a disruptive mutation

**Table 3.** Association of human papillomavirus (HPV) status with clinicopathological factors in oropharyngeal carcinoma (*n* = 163)

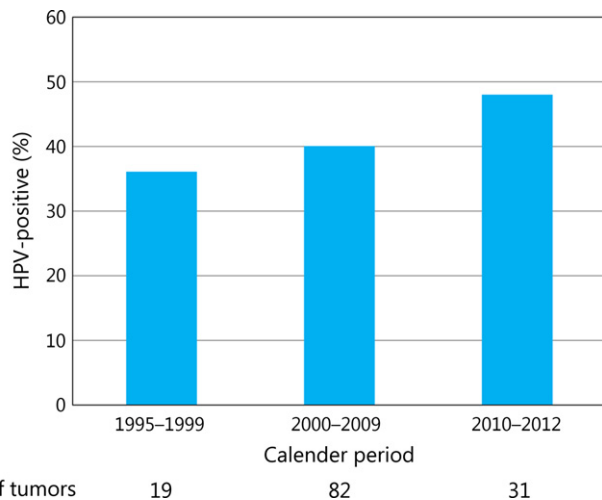
Factor and level	Total no.	HPV-positive No. (%)	Univariate analysis†			Multivariate analysis†		
			Odds ratio	95% CI	<i>P</i> -value	Odds ratio	95% CI	<i>P</i> -value
<b>Sex</b>								
Male	140	46 (32.9)	Reference					
Female	23	10 (43.5)	1.6	0.6–3.8	0.327			
<b>Age at diagnosis‡</b>								
<65 years	73	33 (45.2)	Reference			Reference		
≥65 years	90	23 (25.6)	0.4	0.2–0.8	0.009	0.4	0.2–0.7	0.005
Per 10 years			0.7	0.5–1.0	0.030			
<b>Cigarette smoking‡,§</b>								
<42 pack-years	81	37 (45.7)	Reference					
≥42 pack years	81	19 (23.5)	0.4	0.2–0.7	0.003			
Per 5 pack-years			0.9	0.8–1.0	<0.001			
Never	22	13 (59.1)	Reference					
Ever	140	43 (30.7)	0.3	0.1–0.8	0.011			
<b>Alcohol drinking‡,§</b>								
<69 units of sake index	81	39 (48.2)	Reference			Reference		
≥69 units of sake index	81	17 (21.0)	0.3	0.1–0.6	<0.001	0.3	0.1–0.6	0.001
Per 5 units of sake index			0.9	0.9–1.0	<0.001			
Never	37	20 (54.1)	Reference					
Ever	125	36 (28.8)	0.3	0.2–0.7	0.005			
<b>Subsite</b>								
Palatine tonsil	93	42 (45.2)	Reference			Reference		
Lingual tonsil	43	12 (27.9)	0.5	0.2–1.0	0.053	0.4	0.2–0.9	0.040
Posterior wall and soft palate	27	2 (7.4)	0.1	0.02–0.4	<0.001	0.1	0.02–0.4	<0.001
<b>Tumor stage</b>								
T1–T2	96	36 (37.5)	Reference					
T3–T4	67	20 (29.9)	0.7	0.4–1.4	0.310			
<b>Nodal stage</b>								
N0–N1	59	17 (28.8)	Reference					
N2–N3	104	39 (37.5)	1.5	0.8–3.0	0.300			
<b>Cell differentiation</b>								
Well	34	7 (20.6)	Reference					
Moderate	72	23 (31.9)	1.8	0.7–5.1	0.214			
Poor–undifferentiated	39	19 (48.7)	3.7	1.3–11.0	0.011			
Unknown	18	7 (38.9)	2.5	0.7–8.9	0.163			

†Univariate analysis was carried out using the logistic regression model; multivariate analysis was by the forward stepwise logistic regression model. ‡Median of age at diagnosis, cigarette smoking, and alcohol abuse was 65 year, 42 pack-years, and 69 units of sake index, respectively. §One pack-year and one unit of sake index are defined as the equivalent of smoking one pack of cigarettes per day for 1 year and drinking 28 g alcohol per day for 1 year, respectively. CI, confidence interval.

(for HPV-negative oropharyngeal carcinoma: OR, 6.2; 95% CI, 1.3–44.9; *P* = 0.02; for hypopharyngeal carcinoma: OR, 4.6; 95% CI, 1.1–31.5; *P* = 0.03). After adjustment for age and tobacco exposure, HPV-negative oropharyngeal carcinoma and hypopharyngeal carcinoma remained independently associated with a disruptive mutation compared with oral carcinoma (for HPV-negative oropharyngeal carcinoma: OR, 7.6; 95% CI, 1.5–60.0; *P* = 0.01; for hypopharyngeal carcinoma: OR 4.9; 95% CI, 1.1–34.7; *P* = 0.03). Tobacco exposure also independently correlated with a disruptive mutation after adjustment for age and primary site (OR, 6.0; 95% CI, 1.0–115.3; *P* = 0.04).

**Pattern of *p53* mutations in virus-unrelated HNSCCs.** The prevalence of any *p53* mutation was not associated with tobacco smoking in virus-unrelated HNSCCs, as shown in Table 6, whereas tobacco carcinogen is known to link with a specific type of mutagenesis.<sup>(25)</sup> We addressed the pattern of *p53* mutations in virus-unrelated HNSCCs, in relation to tobacco exposure. One hundred and twelve of 232 (48.3%) tumors harbored a total of 165 *p53* mutations (22 tumors with two

distinct mutations, 6 with three, 2 with four, 2 with five, and 1 with six), including 118 missense mutations, 12 nonsense mutations, 8 frame-shifts, and 27 silent mutations (detail of the mutations in Table S1), although six tumors harbored only silent mutations. The codon distribution of *p53* mutations differed depending on tobacco exposure. In ever-smokers, 19 of 143 (13.2%) mutations were detected at codons described as “hotspots” for transversions (codon 157, one mutation; codon 158, six mutations; codon 245, six mutations; codon 248, five mutations; codon 273, one mutation),<sup>(26)</sup> whereas, in never-smokers, mutations at hotspots were found only at codon 245, accounting for 2 of 22 (9.1%) mutations. Figure 2 shows the types of mutations according to smoking status. Transversions accounted for 39.2% of mutations in ever-smokers, whereas the vast majority (81.8%) of mutations in never-smokers were transitions. Two types of mutations showed consistent difference in relation to smoking. G:C to T:A transversions were more prevalent in ever-smokers than in never-smokers (22.4% vs 4.5%, *P* = 0.04), whereas G:C to A:T transitions at CpG sites were less



**Fig. 1.** Prevalence of human papillomavirus infection in carcinomas of the palatine and lingual tonsils across three calendar periods (1995–1999, 2000–2009, and 2010–2012). An increasing trend was statistically significant ( $P < 0.001$ ). The number of specimens evaluable for each assay is shown below the x-axis.

prevalent in ever-smokers than in never-smokers (10.5% vs 27.3%,  $P = 0.04$ ).

## Discussion

In the present study we established the prevalence and pattern of HPV in Japanese patients with HNSCC. The most prevalent oropharyngeal carcinoma tumors were HPV-positive, especially those arising in the palatine and lingual tonsils, where HPV-positive tumors accounted for 45.2% and 27.9%, respectively, although this prevalence is still lower than in North America and Europe.<sup>(14,15)</sup> In the USA, HPV prevalence in oropharyngeal carcinoma significantly increased over calendar time, reaching up to 72% during 2000 to 2004.<sup>(14)</sup> We also showed the increasing trend in Japan. However, there is a racial disparity in HPV prevalence.<sup>(16)</sup> The prevalence is significantly lower in black patients than in white patients. Sexual behavior, which is significantly associated with the pathogenesis of HPV-positive oropharyngeal carcinoma,<sup>(2,26)</sup> might differ among races or nations. Additionally, the rate of smoking, which is the other

major cause of oropharyngeal carcinogenesis, is higher in Japan than in the USA.<sup>(27)</sup> Given this knowledge, it remains uncertain whether HPV prevalence in oropharyngeal carcinoma among Japanese will reach as high as that among white populations. As for HPV subtypes, HPV16 commanded a vast majority (91.1%) of HPV-positive tumors in Japan, which is consistent with a worldwide systematic review.<sup>(28)</sup>

We addressed factors associated with HPV infection in oropharyngeal carcinoma. In accordance with previous reports,<sup>(11,29)</sup> we found that tobacco smoking, alcohol drinking, and older age were inversely related with HPV infection. Likewise, HPV-positive tumors were predominant in the palatine and lingual tonsils, and differentiation grade of HPV-positive tumors was poor. Although HPV-positive tumors present mostly at an early tumor stage and advanced nodal stage,<sup>(15)</sup> we failed to show such findings, the reason for which remains unknown. An additional discordance from previous reports was the absence of a gender gap. We did not find a higher rate of HPV-positive tumors in men than in women. The higher rate in men in North America and Europe is considered to be attributed to HPV prevalence in cervical rather than penile tissue, which might facilitate the chance of HPV infection when performing oral sex on a woman. Differences in oral sex behavior might be responsible for the absence of a gender gap in Japan.

Gillison *et al.*<sup>(11)</sup> examined HPV status and *p53* mutations in 34 oropharyngeal carcinomas, and showed that HPV-positive tumors as compared with HPV-negative tumors were less likely to harbor *p53* mutations. Westra *et al.*<sup>(12)</sup> showed that 12 of 20 (60.0%) tonsillar carcinomas without a disruptive mutation were HPV-positive, whereas none of 7 tonsillar carcinomas carrying a disruptive mutation was HPV-positive. In the present study we further verified these findings in a larger scale, and established the inverse relationship between HPV infection and any *p53* mutation or a disruptive mutation in oropharyngeal carcinoma. However, we found the absence of that inverse relationship in non-oropharyngeal HNSCCs, as previously reported.<sup>(11)</sup> This finding, given the report that *p53* mutations of HNSCCs are associated with poor outcome,<sup>(30)</sup> might partially explain why a prognostic advantage of HPV-positive tumors disappear in non-oropharyngeal HNSCCs.<sup>(31)</sup> We also found that HPV infection and a disruptive mutation did not coexist, irrespective of primary tumor site, indicating that the coexistence of HPV infection and *p53* mutations occurs only when the mutations are non-disruptive. Disruptive

**Table 4.** Relationship between human papillomavirus (HPV) status and *p53* mutations in head and neck squamous cell carcinomas ( $n = 283$ )

<i>p53</i> mutations			Total no.	HPV-positive No. (%)	Univariate analysis†			Multivariate analysis‡,§		
					Odds ratio	95% CI	<i>P</i> -value	Odds ratio	95% CI	<i>P</i> -value
Oropharyngeal carcinoma	<i>p53</i> status	Wild-type	57	28 (49.1)	Reference			Reference		
		Mutant	25	2 (8.0)	0.09	0.01–0.3	<0.001	0.1	0.02–0.5	0.005
	Mutation category	Wild-type	57	28 (49.1)	Reference					
		Non-disruptive	15	2 (13.3)	0.2	0.02–0.6	0.008			
		Disruptive	10	0 (0.0)	0.05	0.003–0.9	<0.001			
Non-oropharyngeal carcinoma	<i>p53</i> status	Wild-type	117	5 (4.3)	Reference					
		Mutant	84	4 (4.8)	1.1	0.3–4.4	0.868			
	Mutation category	Wild-type	117	5 (4.3)	Reference					
		Non-disruptive	61	4 (6.6)	1.6	0.4–6.2	0.516			
		Disruptive	23	0 (0.0)	0.4	0.02–7.4	0.176			

†Univariate analysis was carried out using the logistic regression model for *p53* status and by the Clopper–Pearson method for mutation category. ‡Multivariate analysis was carried out using the forward stepwise logistic regression model. §Adjusted for cigarette smoking and alcohol drinking. CI, confidence interval.

**Table 5.** Association between *p53* mutations and clinicopathological factors in Japanese patients with head and neck squamous cell carcinoma (*n* = 283)

Factor and level	Total no.	<i>p53</i> status				Mutation category			
		Mutant No. (%)	Univariate analysis†			Disruptive No. (%)	Univariate analysis†		
			Odds ratio	95% CI	<i>P</i> -value		Odds ratio	95% CI	<i>P</i> -value
<b>Sex</b>									
Male	240	94 (39.2)	Reference			31 (33.0)	Reference		
Female	43	15 (34.9)	0.8	0.4–1.6	0.593	2 (13.3)	0.3	0.05–1.2	0.101
<b>Age at diagnosis‡</b>									
<63 years	131	45 (34.4)	Reference			19 (42.2)	Reference		
≥63 years	152	64 (42.1)	1.4	0.9–2.3	0.181	14 (21.9)	0.4	0.2–0.9	0.023
Per 10 years			1.2	0.9–1.5	0.140		0.7	0.5–1.1	0.120
<b>Cigarette smoking‡,§</b>									
<45 pack-years	131	43 (32.8)	Reference			11 (25.6)	Reference		
≥45 pack years	146	64 (43.8)	1.6	1.0–2.6	0.060	21 (32.8)	1.4	0.6–3.4	0.421
Unknown	6	2 (33.3)	1.0	0.2–5.8	0.979	1 (50.0)	2.9	0.1–77.8	0.472
Per 5 pack-years			1.0	1.0–1.1	0.040		1.0	1.0–1.1	0.310
Never	47	15 (31.9)	Reference			1 (6.7)	Reference		
Ever	230	92 (40.0)	1.4	0.7–2.8	0.295	31 (33.7)	7.1	1.3–131.8	0.017
<b>Alcohol drinking‡,§</b>									
<80 units of sake index	135	41 (30.4)	Reference			13 (31.7)	Reference		
≥80 units of sake index	142	66 (46.5)	2.0	1.2–3.3	0.006	19 (28.8)	0.9	0.4–2.1	0.749
Unknown	6	2 (33.3)	1.1	0.2–6.1	0.878	1 (50.0)	2.2	0.08–57.4	0.601
Per 5 units of sake index			1.0	1.0–1.0	0.210		1.0	1.0–1.0	0.240
Never	59	14 (23.7)	Reference			3 (21.4)	Reference		
Ever	218	93 (42.7)	2.4	1.3–4.8	0.007	29 (31.2)	1.7	0.5–7.7	0.445
<b>Primary site</b>									
Oral cavity	37	18 (48.7)	Reference			2 (11.1)	Reference		
Nasopharynx	21	1 (4.8)	0.1	0.003–0.3	<0.001	0 (0.0)	2.2	0.1–70.4	0.632
HPV-negative oropharynx	52	23 (44.2)	0.8	0.4–2.0	0.680	10 (43.5)	6.2	1.3–44.9	0.019
HPV-positive oropharynx	30	2 (6.7)	0.1	0.01–0.3	<0.001	0 (0.0)	1.3	0.05–36.3	0.505
Hypopharynx	108	49 (45.4)	0.9	0.4–1.9	0.730	18 (36.7)	4.6	1.1–31.5	0.030
Larynx	35	16 (45.7)	0.9	0.3–2.2	0.803	3 (18.8)	1.8	0.3–15.7	0.526
<b>Tumor stage</b>									
T1–T2	138	55 (39.9)	Reference			15 (27.3)	Reference		
T3–T4	145	54 (37.2)	0.9	0.6–1.4	0.652	18 (33.3)	1.3	0.6–3.1	0.491
<b>Nodal stage</b>									
N0–N1	132	51 (38.6)	Reference			12 (23.5)	Reference		
N2–N3	151	58 (38.4)	1.0	0.6–1.6	0.969	21 (36.2)	1.8	0.8–4.4	0.148
<b>Cell differentiation</b>									
Well	79	37 (46.8)	Reference			12 (32.4)	Reference		
Moderate	119	50 (42.0)	0.8	0.5–1.5	0.504	16 (32.0)	1.0	0.4–2.5	0.966
Poor–undifferentiated	69	19 (27.5)	0.4	0.2–0.9	0.015	4 (21.1)	0.6	0.1–1.9	0.364
Unknown	16	3 (18.8)	0.3	0.1–0.9	0.031	1	1.0	0.05–11.9	0.975
<b>HPV status</b>									
Negative	244	103 (42.2)	Reference			33 (32.0)	Reference		
Positive	39	6 (15.4)	0.2	0.09–0.6	<0.001	0 (0.0)	0.2	0.009–0.8	0.034

†Univariate analysis was carried out using the logistic regression model. ‡Median of age at diagnosis, cigarette smoking, and alcohol abuse was 63 years, 45 pack-years, and 80 units of sake index, respectively. §One pack-year and one unit of sake index are defined as the equivalent of smoking one pack of cigarettes per day for 1 year and drinking 28 g alcohol per day for 1 year, respectively. CI, confidence interval; HPV, human papillomavirus.

mutations are selectively associated with allelic loss of wild-type *p53*,<sup>(32)</sup> and confer “gain of function” phenotype.<sup>(33,34)</sup> It seems likely that disruptive mutations lead directly to the acquisition of malignant phenotype, whereas non-disruptive mutations, in concert with other events targeting the *p53* pathway such as transcriptionally active HPV, might produce a synergistic effect of *p53* functional abrogation.

Of interest is the finding that, although there was a positive association between *p53* mutations and tobacco smoking in the whole patient group, this association disappeared in the

subpopulation of virus-unrelated HNSCCs. This is most probably because as high as 76.6% of virus-related HNSCC patients with known history of tobacco smoking were light or never-smokers (data not shown), and because no <94.1% of virus-related HNSCCs carried wild-type *p53* (Table 5). There has been a series of studies showing that the frequency of *p53* mutations in HNSCCs increases in proportion to the level of exposure to tobacco smoking.<sup>(35–37)</sup> In these studies, oropharyngeal carcinoma accounted for a substantial portion of the analyzed population, and HPV status of oropharyngeal

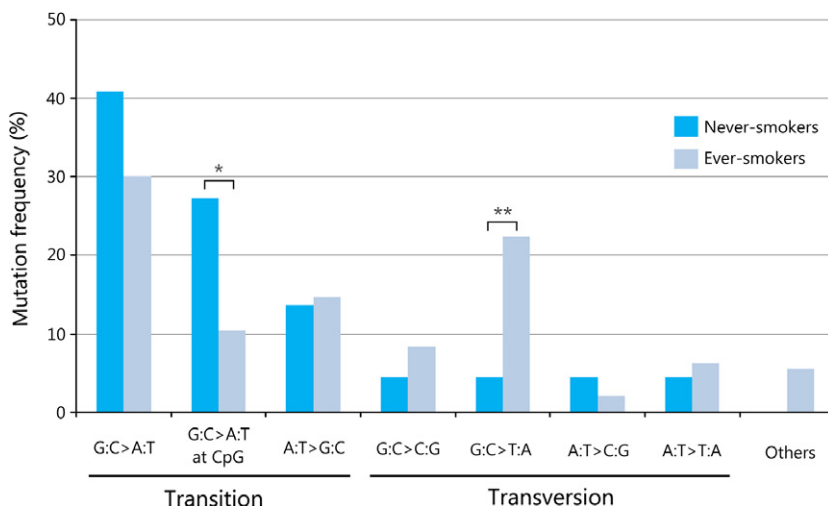
**Table 6.** Association between *p53* mutations and clinicopathological factors in virus-unrelated head and neck squamous cell carcinomas (*n* = 232)

Factor and level	<i>p53</i> status					Mutation category						
	Mutant		Univariate analysis†			Disruptive No. (%)	Univariate analysis†			Multivariate analysis†		
	Total no.	No. (%)	Odds ratio	95% CI	P-value		Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
<b>Sex</b>												
Male	202	93 (46.0)	Reference			31 (33.3)	Reference					
Female	30	13 (43.3)	0.9	0.4–1.9	0.781	2 (15.4)	0.4	0.05–1.5	0.166			
<b>Age at diagnosis‡</b>												
<63 years	102	45 (44.1)	Reference			19 (42.2)	Reference			Reference		
≥63 years	130	61 (46.9)	1.1	0.7–1.9	0.670	14 (23.0)	0.4	0.2–0.9	0.035	0.4	0.1–1.0	0.042
per 10 years			1.1	0.8–1.4	0.710		0.7	0.5–1.2	0.180			
<b>Cigarette smoking‡,§</b>												
<45 pack-years	95	42 (44.2)	Reference			11 (26.2)	Reference					
≥45 pack years	135	63 (46.7)	1.1	0.7–1.9	0.713	21 (33.3)	1.4	0.6–3.4	0.434			
Unknown	2	1 (50.0)	1.3	0.08–22.2	0.831	1 (100.0)						
Per 5 pack-years			1.0	1.0–1.0	0.670		1.0	1.0–1.1	0.340			
Never	31	14 (45.2)	Reference			1 (7.1)	Reference			Reference		
Ever	199	91 (45.7)	1.0	0.5–2.2	0.953	31 (34.1)	6.7	1.2–124.8	0.023	6.0	1.0–115.3	0.045
<b>Alcohol drinking‡,§</b>												
<80 units of sake index	97	40 (41.2)	Reference			13 (32.5)	Reference					
≥80 units of sake index	133	65 (48.9)	1.4	0.8–2.3	0.250	19 (29.2)	0.9	0.4–2.0	0.724			
Unknown	2	1 (50.0)	1.4	0.08–1.4	0.830	1 (100.0)						
Per 5 units of sake index			1.0	1.0–1.0	0.740		1.0	1.0–1.0	0.260			
Never	36	13 (36.1)	Reference			3 (23.1)	Reference					
Ever	194	92 (47.4)	1.6	0.8–3.4	0.207	29 (31.5)	1.5	0.4–7.2	0.527			
<b>Primary site</b>												
Oral cavity	37	18 (48.7)	Reference			2 (11.1)	Reference			Reference		
HPV-negative oropharynx	52	23 (44.2)	0.8	0.4–2.0	0.680	10 (43.5)	6.2	1.3–44.9	0.019	7.6	1.5–60.0	0.014
Hypopharynx	108	49 (45.4)	0.9	0.4–1.9	0.730	18 (36.7)	4.6	1.1–31.5	0.030	4.9	1.1–34.7	0.033
Larynx	35	16 (45.7)	0.9	0.3–2.2	0.803	3 (18.8)	1.8	0.3–15.7	0.530	1.4	0.1–14.1	0.763
<b>Tumor stage</b>												
T1–T2	107	54 (50.5)	Reference			15 (27.8)	Reference					
T3–T4	125	52 (41.6)	0.7	0.4–1.2	0.175	18 (34.6)	1.4	0.6–3.2	0.447			
<b>Nodal stage</b>												
N0–N1	106	50 (47.2)	Reference			12 (24.0)	Reference					
N2–N3	126	56 (44.4)	0.9	0.5–1.5	0.678	21 (37.5)	1.9	0.8–4.5	0.132			
<b>Cell differentiation</b>												
Well	73	37 (50.7)	Reference			12 (32.4)	Reference					
Moderate	106	49 (46.2)	0.8	0.5–1.5	0.557	16 (32.7)	1.0	0.4–2.5	0.981			
Poor–undifferentiated	41	17 (41.5)	0.7	0.3–1.5	0.343	4 (23.5)	0.6	0.2–2.3	0.500			
Unknown	12	3 (25.0)	0.3	0.07–1.2	0.091	1 (33.3)	1.0	0.05–11.9	0.975			

†Univariate analysis was carried out using the logistic regression model; multivariate analysis was carried out using the forward stepwise logistic regression model. ‡Median of age at diagnosis, cigarette smoking, and alcohol abuse was 64 years, 49 pack-years, and 100 units of sake index, respectively. §One pack-year and one unit of sake index are defined as the equivalent of smoking one pack of cigarettes per day for 1 year and drinking 28 g alcohol per day for 1 year, respectively. CI, confidence interval.

carcinoma was unknown in spite of the inverse relationship of HPV infection with *p53* mutations and tobacco smoking.<sup>(35–37)</sup> This background behind the studies might explain, at least in part, the divergence from our finding. Of note, in virus-unrelated HNSCCs a disruptive mutation was independently associated with primary site (the oropharynx and hypopharynx). This finding is potentially interesting because hypopharyngeal carcinoma is the most fatal among

HNSCCs,<sup>(29)</sup> and because *p53* disruptive mutations serve as an adverse prognostic factor in HNSCCs.<sup>(29)</sup> Another interesting finding is that in virus-unrelated HNSCCs, smoking was associated with increased prevalence of G:C to T:A transversions as reported for carcinomas of the lung<sup>(38)</sup> and upper aerodigestive tract.<sup>(35)</sup> This type of mutagenesis is caused by bulky carcinogen from tobacco smoke, such as polycyclic aromatic hydrocarbons.<sup>(25)</sup> Conversely, we found that smoking



**Fig. 2.** Pattern of p53 mutations in human papillomavirus-unrelated head and neck tumors from never- and ever-smokers. One hundred sixty-five mutations are represented in 112 patients. Never-smokers showed a higher prevalence of G:C to A:T transitions at CpG sites than ever-smokers (\* $P = 0.04$ ). Ever-smokers showed a higher prevalence of G:C to T:A transversions than never-smokers (\*\* $P = 0.04$ ). Others represent insertion and deletion.

was associated with decreased prevalence of G:C to A:T transitions at CpG sites, a type of mutation commonly associated with endogenous mutagenesis processes, such as spontaneous deamination of methylated cytosine into thymine. This phenomenon has also been described in carcinomas of the upper aerodigestive tract.<sup>(35)</sup>

In conclusion, HNSCC is etiologically classified into virus-related and virus-unrelated subgroups. In virus-related HNSCC, p53 mutations are uncommon with the absence of a disruptive mutation. In virus-unrelated HNSCC, although p53 mutations are common, the prevalence is not associated with tobacco and

alcohol exposure. Disruptive mutagenesis of p53 is closely related with primary site.

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### Disclosure Statement

The authors have no conflict of interest.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Table S1.** Details of *p53* mutations in virus-unrelated head and neck squamous cell carcinomas.