

Prognostic significance of human papillomavirus 16 viral load level in patients with oropharyngeal cancer

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Abstract

Human papillomavirus (HPV) infection in patients with oropharyngeal squamous cell carcinoma (OPSCC) is a major determinant for better prognosis. However, there remain HPV-positive patients who have poor outcomes. The stratification strategy for detecting high-risk patients among those with HPV-positive OPSCC has not been well delineated, especially for Asian patients. We undertook a retrospective cohort study on the survival rate of 89 Japanese patients diagnosed with primary OPSCC. The tumors were concurrently analyzed for the presence of HPV E6 DNA/mRNA, viral DNA load, p16 expression, viral physical status, and viral variant lineage. Human papillomavirus 16 viral DNA was found in 45 (51%) OPSCCs. Human papillomavirus 16 DNA-positive OPSCCs with higher viral load (classified as HPV16 DNA-medium/high OPSCCs) showed significantly favorable overall survival and progression-free survival compared with HPV16 DNA-positive OPSCCs with lower viral load (<10 copies/cell; HPV16 DNA-low OPSCCs) and HPV16 DNA-negative OPSCCs. E6 mRNA expression was observed in all HPV16 DNA-medium/high OPSCCs but not in HPV16 DNA-low OPSCCs. Notably, p16-positive and HPV16 DNA-negative/low OPSCCs showed significantly worse survival than p16-positive and HPV16 DNA-medium/high OPSCCs and resembled HPV-unrelated OPSCCs with regard to survival and risk factor profile. Although not significant, a trend toward shorter survival was observed for HPV16-integrated OPSCCs. Phylogenetic analysis revealed two major types of HPV16 variants termed Asian (A4) and European (A1/A2/A3) variants, but no difference in survival between these variants was observed. Altogether, these findings suggest that HPV viral load is a potentially informative factor for more accurate risk stratification of patients with OPSCC.

KEYWORDS

human papillomavirus, Japanese cohort, oropharyngeal cancer, prognosis, viral load

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1 | INTRODUCTION

Human papillomavirus (HPV), a circular dsDNA virus, is one of the leading causes of cancer worldwide.¹ Human papillomavirus is associated with not only cervical cancer but also certain subtypes of cancer in the head and neck, especially oropharyngeal squamous cell carcinoma (OPSCC).² The incidence of OPSCC has increased, particularly in North America and Western Europe, primarily due to the dramatic increase in the number of HPV-associated OPSCC cases.^{3,4} Risk factors for HPV-positive OPSCC are related to sexual behavior, whereas those for HPV-negative OPSCC are associated with tobacco smoking and alcohol consumption.⁵ Human papillomavirus-positive OPSCC has been established as a distinct biological entity, and this finding is significant because it indicates a more favorable prognosis of this cancer compared with HPV-negative OPSCC.^{5,6} Moreover, this finding has led to a recent change in the tumor classification, which now incorporates HPV status, and clinical trials on treatment deescalation for HPV-positive OPSCCs.^{5,6} Therefore, HPV testing, which is routinely carried out by immunostaining the tumor tissue for p16^{INK4A}, is now mandatory for predicting the prognosis of patients with OPSCC. The tumor suppressor protein p16 is often overexpressed in HPV-driven carcinomas; thus, its expression has been widely used as a surrogate marker for HPV status.⁷ Accordingly, p16-positive OPSCCs are considered HPV-positive. However, a subset of HPV-positive patients, determined using this method, have poor outcomes.⁸⁻¹³ Therefore, it is imperative to refine tools for improved risk stratification of HPV-positive patients.

The prevalence of HPV infection in OPSCC cases since the year 2000 was highly variable across different countries.³⁻⁶ In contrast to North American and European countries, relatively limited information on the prevalence of HPV-positive OPSCC in Asian countries is available. Moreover, a geographical difference in the HPV rates for OPSCC cases appears to exist, with persistently lower rates in Asian countries compared with the United States and Western Europe.¹⁴⁻¹⁶ This finding could contribute to survival disparities by race. In Japan, studies showed the prevalence rate of HPV in OPSCC cases to be 40%–50% over the last two decades,^{16,17} suggesting an increasing trend of HPV-positive OPSCC cases in this region. Thus far, knowledge about the risk stratification of OPSCC in populations with diverse racial/ethnic backgrounds remains limited. Studies based on patients from different geographic regions, especially Asian countries, are needed to identify significant prognostic factors and thereby enable the provision of more or less aggressive treatment, as appropriate, for patients.

Based on this background, we undertook a study on Japanese patients with OPSCC to assess the association of HPV-related factors, including viral DNA load, viral mRNA expression level, and viral physical status, with clinical outcome of patients. Our findings showed that HPV DNA-positive patients with high viral load or viral mRNA expression had improved overall survival (OS) and progression-free survival (PFS), whereas patients with either low viral load or no viral genome had a poor prognosis even when p16 immunostaining yielded positive results. Furthermore, we assessed

the association of HPV load level with demographics and clinical variables of patients. Finally, based on the geographic distribution of HPV variants that could be related to viral persistence and a risk for advanced HPV-related carcinomas,^{18,19} we also examined the distribution of HPV variants in our cohort and its impact on patient survival.

2 | MATERIALS AND METHODS

2.1 | Patients

Ninety-one consecutive Japanese patients (persons of Japanese descent who resided in Japan) diagnosed with a primary OPSCC at the Kochi University Hospital from 2009 to 2020, whose formalin-fixed, paraffin-embedded (FFPE) tumors were available for evaluation of HPV status, were enrolled in this study. Information within the institute's files was supplemented by a review of the patients' demographics and clinicopathologic characteristics. The clinical tumor and nodal stages were determined based on the 7th edition of the American Joint Committee on Cancer/UICC TNM classification to compare them between HPV DNA-positive and HPV DNA-negative OPSCCs. This study was approved by the Ethics Committees of the Kochi Medical School, Kochi University, and patients provided written informed consent. The Institutional Review Board waived the requirement for informed consent of patients diagnosed before this approval was granted due to the retrospective nature of the present study, under the condition that information regarding this study was disclosed on the website so that patients could apply for refusal of study enrolment.

2.2 | Detection of HPV DNA and viral genotyping

DNA was extracted from three 10- μ m-thick slices of FFPE tumors using a WaxFree DNA Extraction Kit (TrimGen). Human papillomavirus DNA was detected using PCR and the consensus primer set GP5⁺/GP6⁺ that targets the HPV L1 sequence, as previously described.²⁰ The obtained sequences were compared with those of known HPV genotypes in the GenBank database using the Basic Local Alignment Search Tool.

2.3 | Evaluation of HPV16 viral load

Two-hundred-nanogram aliquots of extracted DNA were analyzed for the quantification of HPV16 E6 gene (81 bp in size) using TaqMan-based real-time quantitative PCR (qPCR), as described elsewhere.²¹ To calculate viral copy numbers, the PCR product was cloned into the pMD20 T-vector (Takara Bio), and 10-fold serial dilutions of the cloned plasmid DNA were used to generate a standard curve. Results are expressed as the number of viral copies per cell. *RNase P* was used as a housekeeping gene to regulate the quality

of the DNAs and PCR assays. The PCR-targeted regions of the viral genomes and the sequences of primers and probes used in this study are listed in Table S1.

2.4 | Evaluation of HPV16 mRNA expression

The Quick-DNA/RNA FFPE Kit (Zymo Research) was used to extract RNA. The total RNA was treated with DNase to avoid amplification of genomic DNA. Absence of amplifiable β -actin DNA in the extracted RNA was confirmed. An aliquot of cDNA was subjected to real-time semiquantitative RT-PCR using Power SYBR Green Master Mix (Thermo Fisher Scientific), as previously described.²² The primers were designed to amplify the HPV16 E6* II and E6* I transcripts.²³ Relative HPV16 E6 mRNA expression level was calculated using the $2^{-\Delta\text{Ct}}$ method, with β -actin mRNA used as a housekeeping control.

2.5 | Evaluation of p16 expression

Expression of p16 was evaluated by immunohistochemistry using rabbit anti-p16 mAb (EPR1473; Abcam) following the manufacturer's instructions. Positive p16 expression was defined as strong diffuse nuclear and cytoplasmic staining in 70% or more of tumor cells, according to previously described criteria.²⁴

2.6 | Assessment of HPV16 physical status

The physical status of HPV16 was evaluated based on the ratio of E2 and E6 copy numbers, which was determined using real-time qPCR. The primers and probe were designed for specific amplification of the E2 region that is known to be disrupted during the process of viral integration.²⁵ An E2/E6 ratio of 1 or more indicates the presence of the episomal form only, while a ratio of 0 indicates the presence of integrated forms only, and greater than 0 and less than 1 indicate a mixed result of both episomal and integrated forms.²⁵ The samples were analyzed two or three times independently.

2.7 | Human papillomavirus 16 variant and phylogenetic analyses

The HPV16 sequences of the long control region (LCR) and E6 gene (nucleotide positions 6264-6673 [410 bp] and 7263-7642 [380 bp] based on the GenBank HPV16 sequence, respectively; accession number NC_001526) were amplified using PCR with the primer sets listed in Table S1. The purified PCR products were sequenced directly. Phylogenetic trees were constructed using the maximum likelihood method in MEGA X software. Bootstrap values for the tree were based on 1000 replicates. Representative HPV16 LCR and E6 sequences obtained in this study have been deposited in the GenBank database under accession numbers LC637899-LC637932.

2.8 | Statistical analysis

Overall survival and PFS were calculated using the Kaplan-Meier method and compared using the log-rank test. Differences in demographic and clinical characteristics among patient groups stratified by HPV viral load level and those stratified by both p16 expression and viral load level were assessed using Fisher's exact test, Student's *t* test, Welch's *t* test, Mann-Whitney *U* test, or Pearson's χ^2 test. Univariate and multivariate analyses were carried out using the Cox proportional hazards model to identify independent prognostic factors through backward elimination. *P* values less than .05 were considered significant.

3 | RESULTS

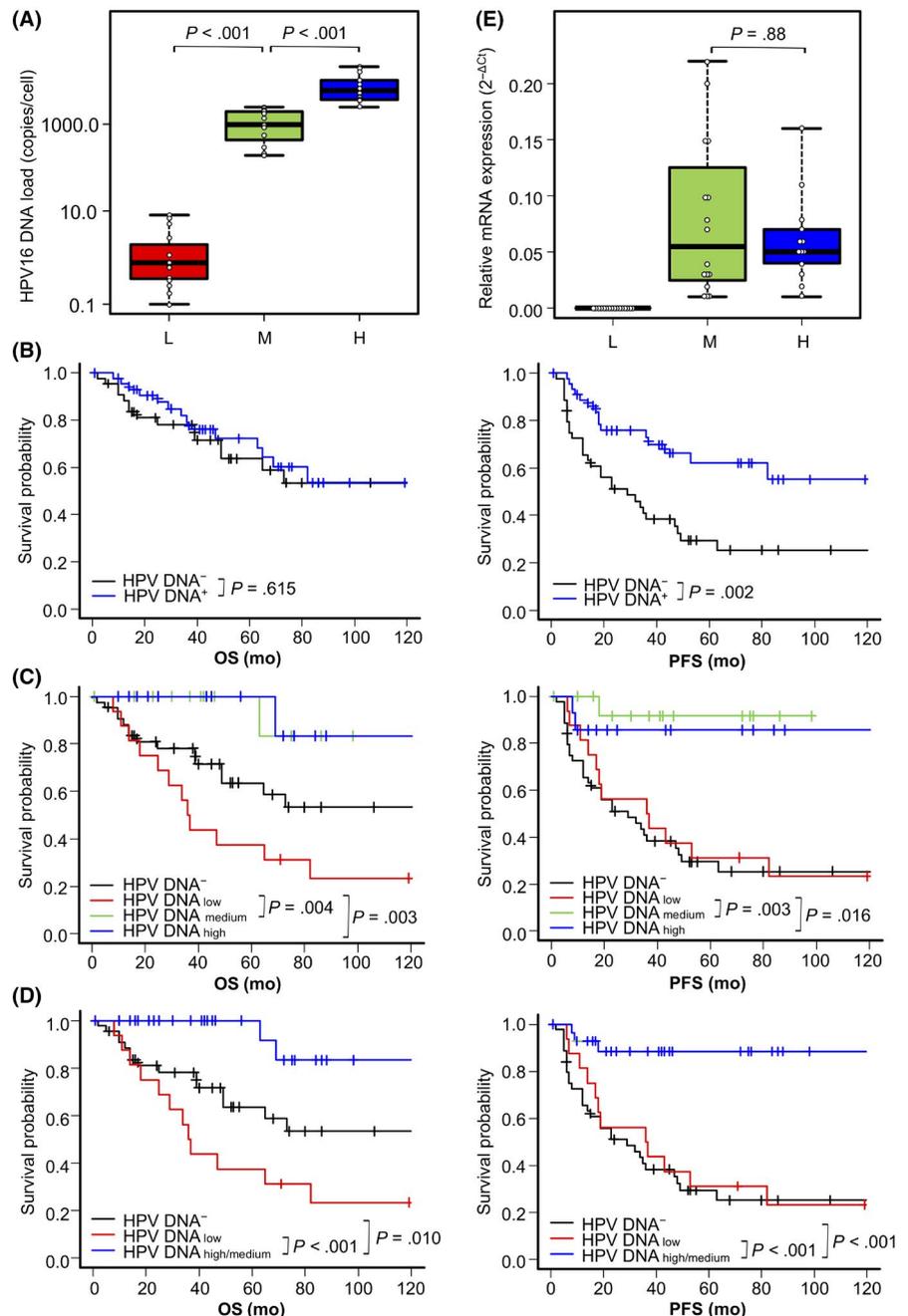
3.1 | Human papillomavirus DNA status and viral genotype

Of 91 Japanese patients with OPSCC, 44 (48%) yielded HPV DNA-negative results and 47 (52%) yielded HPV DNA-positive results. Human papillomavirus 16 genotype was found in 45 (96%) patients and HPV58 genotype in two (4%) patients. Because a vast majority of the HPV-positive tumors in our cohort showed the HPV16 genotype, we designed a retrospective study of a total of 89 patients, including 44 patients with HPV DNA-negative tumors and 45 with HPV16 DNA-positive tumors.

3.2 | Association between increased HPV16 DNA load and improved patient survival

Viral loads of the HPV16 DNA-positive tumors were tested two or three times independently. The results showed that the viral copy numbers were reproducible, demonstrating the reliability of our qPCR system for determination of viral load. The mean values were calculated and ranked from the smallest to largest. The viral DNA loads ranged from 0.1 to 20 820 copies/cell with a median of 910 copies/cell. A sharp difference in the distribution of viral copy numbers between less than 10 and 10 or more copies/cell was observed. The viral loads were further separated into three groups by dividing the group with a viral load of 10 or more copies/cell into two groups using the median value (Figure 1A): low, less than 10 ($n = 16$); medium, 10-2600 ($n = 15$); and high, more than 2600 copies/cell ($n = 14$). Based on this definition, the patients were categorized into four groups: those having no viral genome (referred to as HPV DNA⁻), low viral load (HPV DNA_{low}⁺), medium viral load (HPV DNA_{medium}⁺), and high viral load (HPV DNA_{high}⁺). The survival rates of these groups were compared. The median follow-up time was 72 months. Overall survival and PFS were first compared between the DNA⁻ and DNA⁺ groups (Figure 1B). Progression-free survival was significantly better in the HPV DNA⁺ group than in the HPV DNA⁻ group ($P = .002$), whereas no statistical difference was observed in the OS ($P = .615$). When the HPV16

FIGURE 1 Assessment of viral DNA load and mRNA expression of human papillomavirus 16 (HPV16) in oropharyngeal squamous cell carcinoma. A, Distribution of HPV16 viral load. Tumors were categorized by viral load level as follows: L, HPV DNA_{low}; M, HPV DNA_{medium}; and H, HPV DNA_{high}. Horizontal lines in box plots indicate median values, and the bars extend to the maximum and minimum values. *P* values were obtained using the Mann-Whitney *U* test. B, Kaplan-Meier curves showing overall survival (OS) and progression-free survival (PFS) of tumors categorized by HPV16 DNA status. C, Kaplan-Meier curves of tumors after being further categorized into four subgroups by viral load. D, Kaplan-Meier curves of tumors after being recategorized by combining the HPV DNA_{high} group with the HPV DNA_{medium} group (HPV DNA_{high/medium}). Note that the OS was not significantly different when only HPV16 DNA status was assessed. Grouping by viral load revealed a significant difference in both OS and PFS. *P* values were calculated using the log-rank test. E, Relative HPV16 E6 mRNA expression level stratified by viral load. *P* value was obtained using the Mann-Whitney *U* test



DNA-positive patients were stratified by viral load, Kaplan-Meier analysis revealed that both the HPV DNA_{high} and DNA_{medium} groups had significantly better OS and PFS than the HPV DNA_{low} group (all $P < .02$; Figure 1C), indicating that higher viral load was associated with improved survival in our patients. Given the low rate of disease recurrence and death in the HPV DNA_{high} and DNA_{medium} groups, comparative survival analysis was also undertaken for the combined HPV DNA_{high/medium} group (Figure 1D). This analysis also showed significant survival differences between the HPV DNA_{high/medium} and DNA_{low} group ($P < .001$ for both OS and PFS) and between the HPV DNA_{high/medium} and DNA₋ groups (OS, $P = .010$; PFS, $P < .001$).

Patient variables and tumor characteristics were analyzed among the three groups stratified by HPV status and viral load (Table 1).

No significant differences were observed between the HPV DNA_{low} and DNA₋ groups. The HPV DNA_{high/medium} group differed significantly from the HPV DNA₋ group with regard to sex, age, smoking pack-years, sake index units, tumor location, and clinical nodal stage. Moreover, no such differences were observed between the HPV DNA_{high/medium} and DNA_{low} groups, with the exception of the number of pack-years ($P = .004$).

Multivariable Cox regression analysis confirmed that the favorable prognosis of HPV DNA_{high/medium} group patients was maintained after adjusting for other independent variables that might also be related to the outcome compared with that of HPV DNA₋ group patients (OS, $P = .036$; PFS, $P = .003$) or HPV DNA_{low} group patients (OS, $P = .004$; PFS, $P = .004$) (Table 2).

TABLE 1 Clinicopathologic characteristics of patients with oropharyngeal cancer stratified by human papillomavirus 16 (HPV16) DNA load

HPV DNA ⁻	HPV DNA _{low}		HPV DNA _{high/medium}						
	n	(%)	n	(%)	P value ^a	n	(%)	P value ^b	P value ^c
Sex					.328			.004	.322
Male	41	(93)	13	(81)		19	(66)		
Female	3	(7)	3	(19)		10	(34)		
Age					.940			.027	.081
Mean (range)	68.6 (54-87)		68.8 (59-85)			62.2 (37-90)			
Smoking					.743			.003	.004
<20 pack-years ^d	3	(7)	0	(0)		8	(28)		
≥20 pack-years	34	(77)	14	(88)		11	(38)		
Never	7	(16)	2	(13)		10	(34)		
Alcohol consumption					.129			.001	.582
<60 units of sake index ^e	9	(20)	5	(31)		9	(31)		
≥60 units of sake index	29	(66)	6	(38)		7	(24)		
Never	6	(14)	5	(31)		13	(45)		
Tumor localization					.849			.003	.072
Palatine tonsil	22	(50)	10	(63)		26	(90)		
Base of tongue	13	(30)	3	(19)		2	(7)		
Soft palate and uvula	4	(9)	1	(6)		1	(3)		
Posterior wall of oropharynx	5	(11)	2	(13)		0	(0)		
cT stage ^f					.108			.211	.463
Tis	1	(2)	0	(0)		0	(0)		
T1	5	(11)	0	(0)		4	(14)		
T2	14	(32)	10	(63)		16	(55)		
T3	16	(36)	2	(13)		5	(17)		
T4a	8	(18)	4	(25)		4	(14)		
T4b	0	(0)	0	(0)		0	(0)		
cN stage ^f					.360			.01	.080
N0	16	(36)	4	(25)		2	(7)		
N1	8	(18)	1	(6)		8	(28)		
N2a	1	(2)	0	(0)		2	(7)		
N2b	8	(18)	5	(31)		13	(45)		
N2c	9	(21)	3	(19)		3	(10)		
N3	2	(5)	3	(19)		1	(3)		
Treatment					.341			.471	.732
Surgery	11	(25)	1	(6)		4	(14)		
Surgery with RT or CRT	13	(30)	8	(50)		9	(31)		
RT	7	(16)	2	(13)		3	(10)		
CRT	13	(30)	5	(31)		12	(41)		
Palliative care	0	(0)	0	(0)		1	(3)		

Note: Bold indicates significant values.

Abbreviations: cN, clinical nodal; CRT, chemoradiation therapy; cT, clinical tumor; RT, radiation therapy.

^aP value, HPV DNA-negative group compared with HPV DNA low-positive group.

^bP value, HPV DNA-negative group compared with HPV DNA high/medium-positive group.

^cP value, HPV DNA low-positive group compared with HPV DNA high/medium-positive group.

^dOne pack-year is defined as the equivalent of smoking one pack of 20 cigarettes per day for 1 year.

^eOne unit of sake index is defined as the equivalent of drinking 22 g alcohol per day for 1 year.

^fDetermined based on the 7th edition of the American Joint Committee on Cancer/UICC TNM classification.

TABLE 2 Univariate and multivariate Cox regression analyses of overall survival (OS) and progression-free survival (PFS) in patients with oropharyngeal cancer

	OS				PFS			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
HPV16 DNA load								
High/medium vs negative	5.53 (1.26-24.2)	.023	5.67 (1.12-28.82)	.036	8.36 (2.55-27.42)	< .001	7.70 (2.04-29.12)	.003
High/medium vs low	11.47 (2.56-51.27)	.001	11.8 (2.19-63.66)	.004	7.28 (2.05-25.8)	.002	7.72 (1.91-31.24)	.004
Sex								
Female vs male	3.73 (0.89-15.73)	.073			4.28 (1.32-13.81)	.015	2.89 (0.88-9.54)	.081
Age								
<Mean vs >mean	1.29 (0.62-2.69)	.493			1.40 (0.78-2.52)	.266		
Smoking								
Never vs ever	1.09 (0.42-2.88)	.855			1.73 (0.73-4.10)	.212		
Alcohol consumption								
Never vs ever	0.59 (0.27-1.32)	.199			1.14 (0.56-2.32)	.711		
Tumor localization								
Nonpalatine tonsil vs palatine tonsil	0.51 (0.24-1.07)	.075			0.55 (0.30-0.99)	.048	0.88 (0.48-1.62)	.676
cT stage								
Tis-T1 vs T2-T4	2.3 (0.55-9.70)	.257			1.71 (0.61-4.78)	.306		
cN stage								
N0-N1 vs N2-N3	1.20 (0.56-2.58)	.645			0.94 (0.52-1.70)	.838		
Treatment								
Surgery (with RT or CRT) vs RT or CRT	0.86 (0.41-1.80)	.686			1.09 (0.60-1.95)	.785		
p16								
Negative vs positive	0.38 (0.16-0.88)	.025	1.02 (0.40-2.70)	.942	0.44 (0.23-0.85)	.014	1.26 (0.60-2.62)	.542
HPV16 physical status								
Integrated vs episomal	0.30 (0.06-1.57)	.155			0.46 (0.11-1.93)	.286		
Integrated vs mixed	0.56 (0.18-1.77)	.324			0.59 (0.19-1.87)	.370		
Integrated vs no viral DNA	0.70 (0.25-1.93)	.490			1.66 (0.64-4.30)	.296		

Note: Bold indicates significant values.

Abbreviations: CI, confidence interval; cN, clinical nodal; CRT, chemoradiation therapy; cT, clinical tumor; HPV, human papillomavirus; HR, hazard ratio; RT, radiation therapy.

3.3 | Association between viral mRNA expression and viral DNA load

Because HPV *E6* expression is essential for initiation and maintenance of the transformed phenotype in HPV-driven tumors,²⁶ we

also examined the HPV16 DNA-positive tumors for the presence of a spliced version of the *E6* transcript. The transcript was found in 29 (64%) of 45 HPV16 DNA-positive tumors (hereafter referred to as HPV DNA⁺/RNA⁺). For the HPV DNA⁺/RNA⁺ tumors, the median value of HPV16 DNA copy numbers was 2560 copies/cell, while it

was 0.9 copies/cell for the HPV DNA⁺/RNA⁻ tumors. E6 mRNA expression was observed in all HPV DNA_{high} and DNA_{medium} tumors, whereas none of the 16 HPV DNA_{low} tumors harbored this transcript. Accordingly, the survival curves of the HPV DNA⁺/RNA⁺ and DNA⁺/RNA⁻ groups were the same as those of the HPV DNA_{high/medium} and DNA_{low} groups, respectively, as shown in Figure 1D. Semiquantitative RT-PCR analysis revealed that the expression level of E6 mRNA did not differ significantly between the HPV DNA_{high} and DNA_{medium} groups ($P = .88$) (Figure 1E).

3.4 | Impact of p16 expression and viral load on patient survival

Immunostaining of p16 yielded positive results for 40 (45%) of 89 cases and negative results for 49 (55%) cases. Notably, 11 (28%) of 40 p16-positive cases were HPV DNA-negative. Overall survival and PFS were first compared among four groups stratified by HPV DNA status and p16 expression (Figure 2A). The p16⁺/HPV DNA⁺ group had significantly better PFS than the other three groups: p16⁺/HPV DNA⁻, p16⁻/HPV DNA⁺, and p16⁻/HPV DNA⁻, but the difference in OS did not reach statistical significance between the p16⁺/HPV DNA⁺ and p16⁺/HPV DNA⁻ groups ($P = .231$). Next, the patients were stratified by viral DNA load and p16 expression, which led to six subgroup combinations: p16⁺/HPV DNA_{high/medium} ($n = 27$), p16⁺/HPV DNA_{low} ($n = 2$), p16⁺/HPV DNA⁻ ($n = 11$), p16⁻/HPV DNA_{high/medium} ($n = 2$), p16⁻/HPV DNA_{low} ($n = 14$), and p16⁻/HPV DNA⁻ ($n = 33$). Because the numbers of patients in the p16⁺/HPV DNA_{low} and p16⁻/HPV DNA_{high/medium} subgroups were too small to undertake a comparative survival analysis, the analysis was restricted to a total number of 85 patients among the other four subgroups (Figure 2B). The p16⁺/HPV DNA⁻ subgroup showed a significantly less favorable PFS compared with the p16⁺/HPV DNA_{high/medium} subgroup ($P < .001$), and the difference in OS between these two subgroups approached significance ($P = .071$). Based on the findings that the HPV DNA_{low} tumors did not express HPV16 E6 mRNA, and they were considered not to have a transcriptionally active HPV infection, the HPV DNA_{low} group was combined with the HPV DNA⁻ group (referred to as HPV DNA_{negative/low}). Then survival analysis was undertaken among the three subgroups: p16⁺/HPV DNA_{negative/low}, p16⁺/HPV DNA_{high/medium}, and p16⁻/HPV DNA_{negative/low} (Figure 2C). This analysis revealed that both OS ($P = .013$) and PFS ($P < .001$) were significantly worse for the p16⁺/HPV DNA_{negative/low} subgroup than for the p16⁺/HPV DNA_{high/medium} subgroup, and the survival curve of the p16⁺/HPV DNA_{negative/low} subgroup almost converged on the survival curve of the p16⁻/HPV DNA_{negative/low} subgroup.

Clinicopathologic characteristics of the patient subgroups stratified by p16 expression status and viral load are shown in Table 3. No statistical differences were observed between the p16⁺/HPV DNA_{negative/low} and p16⁻/HPV DNA_{negative/low} subgroups. The p16⁻/HPV DNA_{negative/low} subgroup differed significantly from the p16⁺/HPV DNA_{high/medium} subgroup with regard to sex, smoking pack-years, sake index units, tumor location, and clinical nodal stage. The

sake index units ($P = .037$) and tumor location ($P = .035$) were significantly different between the p16⁺/HPV DNA_{negative/low} and p16⁺/HPV DNA_{high/medium} subgroups.

3.5 | Impact of viral physical status on patient survival

Among the 45 HPV16-positive tumors, exclusively integrated viral form was found in eight (18%), exclusively episomal form in 15 (33%), and mixed form in 22 (49%) cases. A trend toward patients with integrated HPV16 having a worse survival than those with episomal HPV16 was observed, but the difference was not statistically significant (Figure 3A; OS, $P = .161$; PFS, $P = .293$). Overall, no significant differences were noted in survival among the three groups.

Viral DNA loads stratified by viral physical status are shown in Figure 3B. The viral loads were significantly higher in the episomal ($P = .008$) and mixed ($P = .002$) groups than in the integrated group. This finding was also confirmed when the proportions of the physical viral states were stratified according to the viral load (Figure 3C).

3.6 | Determination of HPV16 variant types

The prevalence of HPV16 variant types was also analyzed in our cohort. We attempted to amplify the 790-bp-long concatenated sequences of the E6 and LCR, which contain informative sequences for determining the type of HPV16 variant.²⁷ As a result, 24 sequences could be successfully amplified from our HPV16 DNA-positive specimens: three from the HPV DNA_{low} tumors, nine from the HPV DNA_{medium} tumors, and 12 from the HPV DNA_{high} tumors. We constructed a phylogenetic tree using these sequences and 50 GenBank-retrieved sequences obtained from cervical specimens, whose HPV16 variant types were known (Figure 4). All the HPV16 strains in our cohort belonged to lineage A (European-Asian lineage), with the exception of two strains that belonged to lineage D (Asian-American/North American lineage). Twelve (50%) isolates were of the Asian variant type (sublineage A4) and eight (33%) of the European variant type (sublineages A1/A2/A3). Intriguingly, two isolates formed a novel cluster referred to as A5, which was recently proposed by a study on cervical specimens from Japanese women.²⁸ Neither African-1 (lineage B) nor African-2 (lineage C) variants were found. No difference in viral load was observed between the A4/Asian and A1-3/European variant types ($P = .792$). In addition, no significant survival difference was found between the variant types (OS, $P = .286$; PFS, $P = .566$).

4 | DISCUSSION

To our knowledge, the present study is the first to concurrently investigate the prognostic values of HPV16 DNA copy number, viral mRNA expression, p16 expression, and viral physical status in

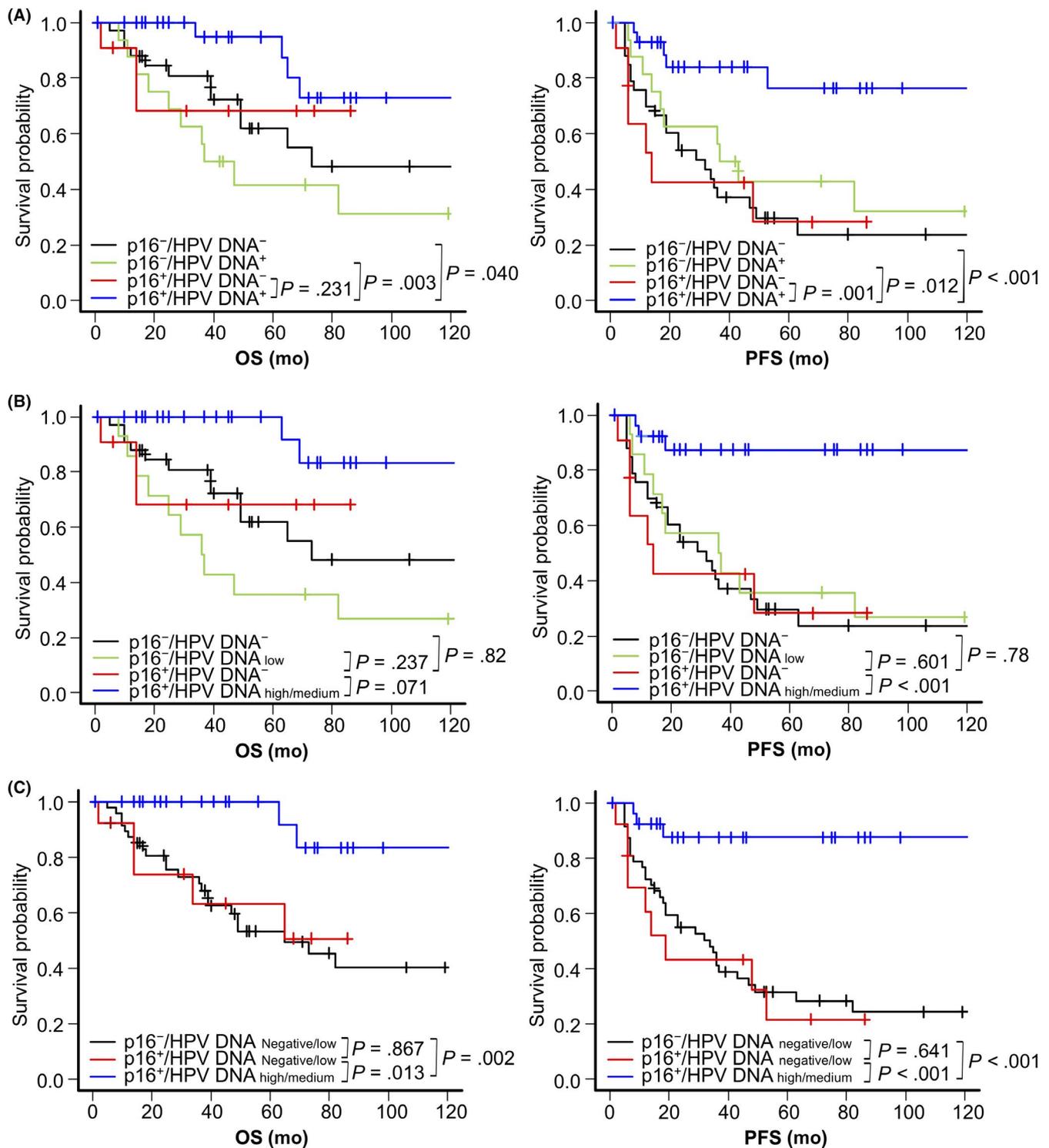


FIGURE 2 Kaplan-Meier curves showing overall survival (OS) and progression-free survival (PFS) of patients with oropharyngeal squamous cell carcinoma. A, Tumors were stratified by p16 expression and human papillomavirus 16 (HPV16) DNA status. B, Tumors were further categorized by p16 expression and HPV DNA viral load. C, Tumors were recategorized into three groups by combining the HPV DNA_{low} group with the HPV DNA⁻ group. P values were calculated using the log-rank test

patients with OPSCC. This study also evaluated the types of HPV16 variants prevalent in this Japanese cohort. Thus far, the data regarding prognosticators for HPV-positive OPSCC are predominantly derived from Caucasian populations; therefore, it is important to properly identify the high- and low-risk HPV-positive OPSCCs in

populations other than populations of European descent. In this context, our study significantly contributes to the understanding of risk stratification of patients with OPSCC.

Our findings indicated that patients with higher HPV16 E6 DNA load (≥ 10 copies/cell; HPV DNA_{high/medium}) showed more favorable

TABLE 3 Clinicopathologic characteristics of patients with oropharyngeal cancer stratified by p16 status and human papillomavirus 16 (HPV16) DNA load

	p16 ⁻ /HPV DNA _{negative/low}		p16 ⁺ /HPV DNA _{negative/low}		p16 ⁺ /HPV DNA _{high/medium}				
	n	(%)	n	(%)	P-value ^a	n	(%)	P-value ^b	P-value ^c
Sex					.602			.024	.451
Male	43	(91)	11	(85)		19	(70)		
Female	4	(9)	2	(15)		8	(30)		
Age					.289			.060	.053
Mean (range)	68 (54-85)		71 (54-87)			62.3 (37-90)			
Smoking					.360			<.001	.134
< 20 pack-years ^d	2	(4)	1	(8)		8	(30)		
≥ 20 pack-years	39	(83)	9	(69)		10	(37)		
Never	6	(13)	3	(23)		9	(33)		
Alcohol consumption					.598			<.001	.037
<60 units of sake index ^e	2	(4)	1	(8)		9	(33)		
≥ 60 units of sake index	37	(79)	9	(69)		7	(26)		
Never	8	(17)	3	(23)		11	(41)		
Tumor localization					.516			.010	.035
Palatine tonsil	25	(53)	7	(54)		24	(89)		
Base of the tongue	14	(30)	2	(15)		2	(7)		
Soft palate and uvula	3	(6)	2	(15)		1	(4)		
Posterior wall of oropharynx	5	(11)	2	(15)		0	(0)		
cT stage ^f					.796			.777	.297
Tis	1	(2)	0	(0)		0	(0)		
T1	5	(11)	0	(0)		4	(15)		
T2	19	(40)	5	(38)		14	(52)		
T3	13	(28)	5	(38)		5	(19)		
T4a	9	(19)	3	(23)		4	(15)		
T4b	0	(0)	0	(0)		0	(0)		
cN stage ^f					1			.024	.22
N0	16	(34)	4	(31)		2	(7)		
N1	7	(15)	2	(15)		6	(22)		
N2a	1	(2)	0	(0)		2	(7)		
N2b	10	(21)	3	(23)		13	(48)		
N2c	9	(19)	3	(23)		3	(11)		
N3	4	(9)	1	(8)		1	(4)		
Treatment					.504			.608	.886
Surgery	11	(23)	1	(8)		4	(15)		
Surgery with RT or CRT	17	(36)	4	(31)		9	(33)		
RT	6	(13)	3	(23)		3	(11)		
CRT	13	(28)	5	(38)		10	(37)		
Palliative care	0	(0)	0	(0)		1	(4)		

Note: Bold indicates significant values.

Abbreviations: cN, clinical nodal; CRT, chemoradiation therapy; cT, clinical tumor; RT, radiation therapy.

^aP value, p16-negative/HPV DNA-negative and low-positive group compared with p16-positive/HPV DNA-negative and low-positive group.

^bP value, p16-negative/HPV DNA-negative and low-positive group compared with p16-positive/HPV DNA high/medium-positive group.

^cP value, p16-positive/HPV DNA-negative and low-positive group compared with p16-positive/HPV DNA high/medium-positive group.

^dOne pack-year is defined as the equivalent of smoking one pack of 20 cigarettes per day for 1 year.

^eOne unit of sake index is defined as the equivalent of drinking 22 g alcohol per day for 1 year.

^fDetermined based on the 7th edition of the American Joint Committee on Cancer/UICC TNM classification.

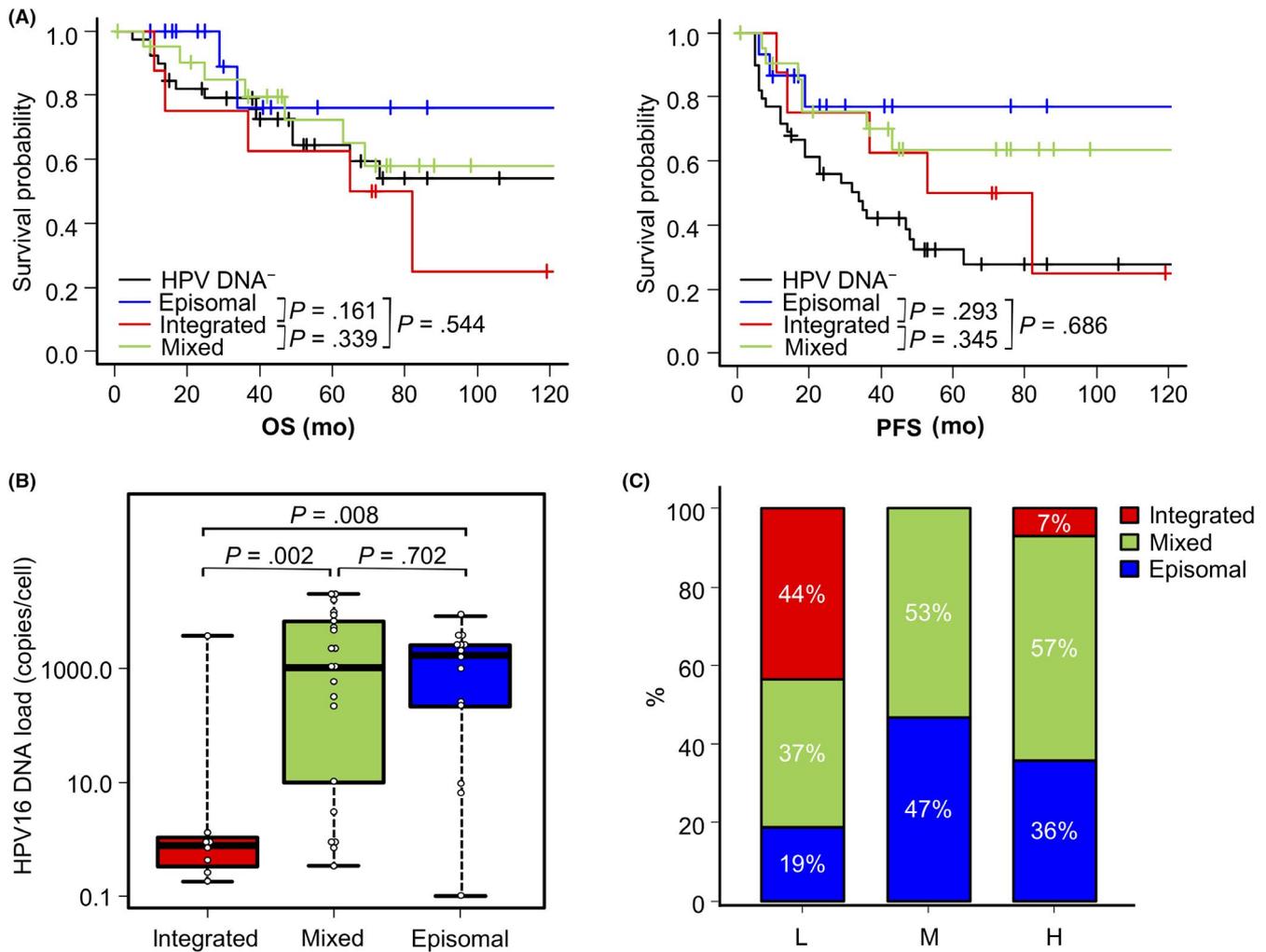
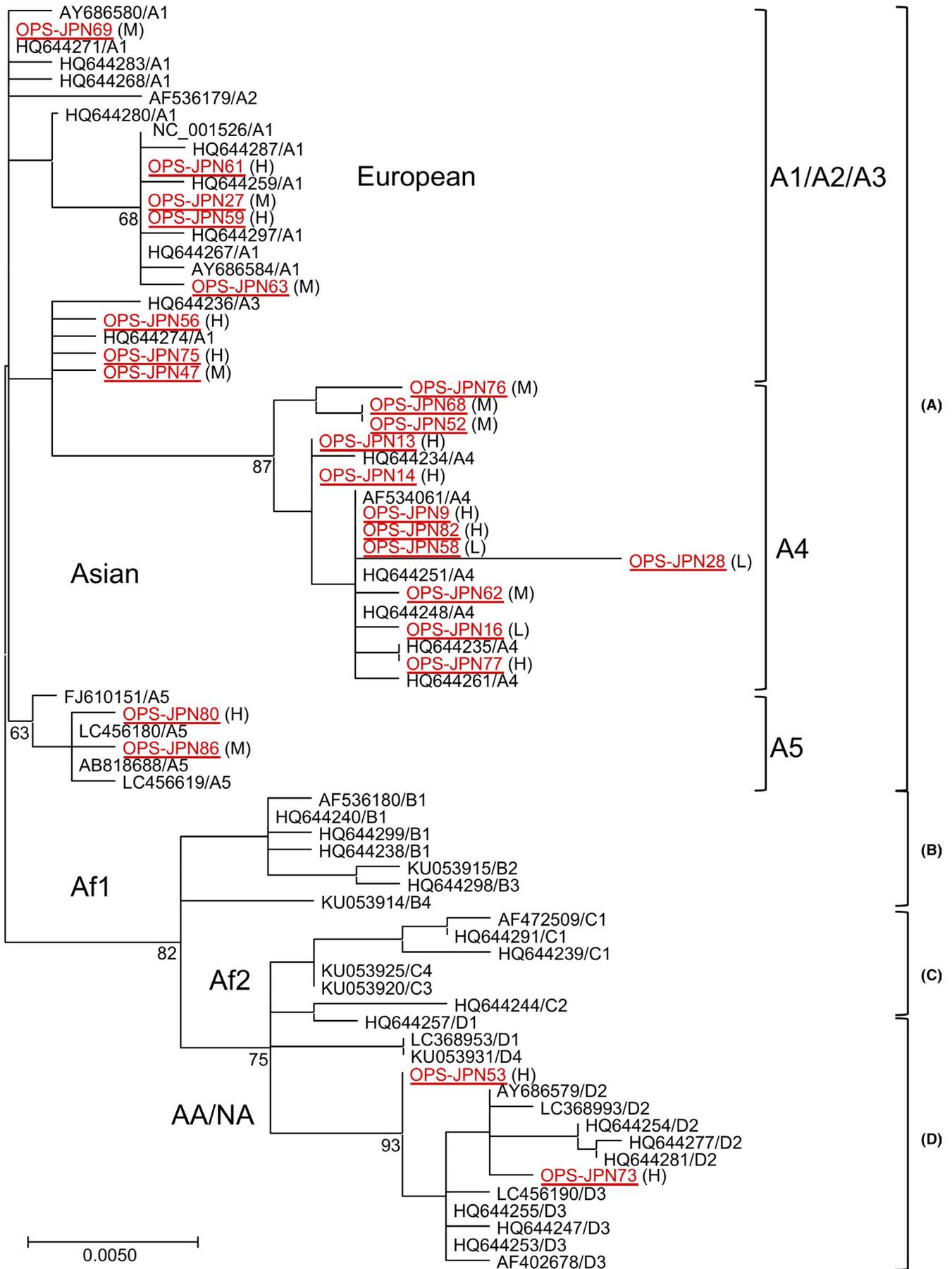


FIGURE 3 Assessment of the viral physical status of human papillomavirus 16 (HPV16) in oropharyngeal squamous cell carcinoma (OPSCC). A, Kaplan-Meier curves showing overall survival (OS) and progression-free survival (PFS) of patients with OPSCC tumors by viral physical status. *P* values were calculated using the log-rank test. B, Box plot showing HPV16 DNA loads stratified by viral physical status. *P* values were calculated using the Mann-Whitney *U* test. C, Proportion of viral physical status stratified by viral load. H, HPV DNA_{high}; L, HPV DNA_{low}; M, HPV DNA_{medium}

OS and PFS than patients with lower viral DNA load (<10 copies/cell; HPV DNA_{low}). The demographic and clinical behaviors of the HPV DNA_{low} group were closer to those of the HPV DNA⁻ group than the HPV DNA_{high/medium} group. A relationship between viral load and clinical outcome has also been reported in some studies from the United States and Europe.^{29,30} However, these studies did not define the cut-off value for risk stratification. Consequently, one challenge in standardizing a qPCR-based assessment for clinical application is the stipulation of the threshold separating low and high viral loads. Stevenson et al³¹ set the threshold at 8.7 copies/cell between “low” and “medium/high” HPV16 *E6* viral load and reported a better survival of patients with “medium/high” viral load, although

this was not statistically significant. Moreover, although additional studies with different detection methods have to be undertaken to support their and our observations, it is tentative to speculate that approximately 10 copies/cell could be a cut-off value for a low viral load. Our qPCR assay detected HPV16 DNA load as low as 0.1 copies/cell, and some studies have detected even lower loads of HPV16 DNA (0.001–0.003 copies/cell).^{31,32} However, low viral load might result from past infection that had not progressed to malignancy, thus representing biologically inactive HPV infection.³³ Indeed, we could not detect HPV16 *E6* mRNA in any of the HPV DNA_{low} tumors and showed that tumors in which the HPV16 mRNA was expressed had elevated viral loads. It appears that detection of viral mRNA is

FIGURE 4 Phylogenetic analysis of human papillomavirus 16 (HPV16). A phylogenetic tree was generated using the maximum likelihood method. It was constructed on the basis of concatenated 790-bp fragments of the *E6* and long control region. It included 24 sequences that were successfully recovered from tumor specimens of our patients with oropharyngeal squamous cell carcinoma and 50 GenBank-retrieved sequences from cervical specimens of various geographic origins. Sequences analyzed in this study are colored in red and underlined. HPV16 viral load levels in our specimens are shown in parentheses: H, high; L, low; M, medium. Scale bars represent the numbers of substitutions per site. AA, Asian-American; Af, African; NA, North American



the golden standard for clinically relevant HPV,²⁴ but the mRNA detection procedure is technically laborious, and the use of the method is mainly restricted to the research laboratory. Therefore, our data presented here would be helpful for future studies to develop a quantitative cut-off for viral copy number for routine clinical testing. It remains unknown why prognosis of patients with higher viral load was far better than those with lower viral load. A potential explanation might be the higher host immune response directed against viral antigens in tumors harboring high viral copy number.³⁴ Further work is needed to test this possibility.

One of the remarkable findings in this study was the significantly worse OS and PFS of patients with p16-positive OPSCC lacking HPV DNA or with low viral load (p16⁺/HPV DNA_{negative/low}) compared with patients with p16⁺/OPSCC with high viral load. Furthermore, p16-positive/HPV DNA_{negative/low} OPSCC showed more similarities to HPV-unrelated OPSCC than to p16-positive/HPV DNA_{high/medium} OPSCC with regard to risk factor profile and survival. Based solely on p16 expression status in a clinical setting, p16⁺/HPV DNA_{negative/low} OPSCC could be unnoticed and incorrectly staged as HPV-associated OPSCC. In this study, the proportion of HPV DNA-negative cases to p16-positive cases was 28%. When the HPV DNA_{low} cases were added to the HPV DNA⁻ cases, this proportion increased to 33%. Previous studies also showed less favorable survival of p16⁺/HPV DNA⁻ cases.⁸⁻¹³ However, information on the prognostic value of p16⁺/HPV DNA⁻ OPSCC is limited, especially in Asian populations. To our knowledge, only one study on Japanese patients showed that p16⁺/HPV DNA⁻ cases displayed OS similar to that of p16⁺/HPV DNA⁺ cases.³⁵ This discrepancy in our findings might be explained, in part, by the notion that HPV DNA detected using PCR could contain a low level of the transcriptionally inactive form of HPV DNA, which could lead to the tumor being spuriously labeled as HPV-driven OPSCC, as suggested by our study and other studies.³⁶ Indeed, after stratification of patients by viral load, we observed a greater difference in OS between the p16⁺/HPV DNA_{high/medium} and p16⁺/HPV DNA⁻ cases. In this context, our data suggest that measurement of HPV copy number in combination with evaluation of p16 expression is valuable for identification of "clinically relevant" HPV DNA-positive OPSCCs and for accurate HPV testing that enables better stratification of patients for treatment deescalation.

Studies regarding the impact of HPV16 physical status on the clinical outcome among Asian patients with OPSCC are scarce. In our cohort, patients with HPV-integrated form had a shorter survival duration than patients with episomal/mixed forms, although this was not statistically significant. However, as reported by previous studies on American and European patients, the influence of HPV integration on patient survival has been controversial. Studies reported that patients with HPV integration had a survival disadvantage over patients without HPV integration,³⁷⁻⁴⁰ and vice versa.⁴¹ These inconsistent findings could be due to different approaches being used for the identification of viral physical status. Therefore, studies are warranted worldwide to determine whether viral integration could be incorporated into the risk stratification strategy of patients with HPV-associated OPSCC.

Human papillomavirus 16 variants have been studied extensively in cervical cancer. However, few studies focused on HPV variants in OPSCC.⁴² To our knowledge, our study provides the first data on variant lineages of HPV16 isolates recovered from OPSCCs of Japanese patients, and it has shown the A4/Asian and A1-3/European types as two major variants. Human papillomavirus 16 variants are distributed differently among different geographic regions.^{18,19} For example, the A4 variants are mainly located in Southeast Asia and the A1-3 variants in all regions other than Africa. Our data confirmed that lineage A accounts for a majority of HPV16 isolates in Asian populations. Moreover, in case of cervical cancer, the occurrence of specific variants correlates with the racial background of the patients, and the A4 variants are associated with a higher risk for cervical cancer development in East Asian populations.^{28,43} Interestingly, the A5 variant was detected in our cohort, although only a small number of A5 isolates were obtained. Hirose et al²⁸ have indicated that the A5 variant is prevalent in the cervical lesions of Japanese women, and this unique variant might have different biological behaviors compared with other lineage A variants. Nevertheless, there is so far no indication that certain types of HPV variants would be clinically relevant in OPSCC. Although we failed to detect an association between HPV16 variant types and survival rate, our findings should stimulate further studies on the HPV16 genetic diversity in patients with OPSCC in different geographic regions. Such analyses will improve the understanding of the biological and epidemiological impact of HPV16 genetic changes.

A limitation of this study is that our study population was relatively small (n = 89), which limited the detection of modest differences in survival. Another limitation is that this study involved a retrospective case series with data collection from records of patients in a single institution in Japan.

In summary, we have analyzed a consecutive cohort of Japanese patients with OPSCC to elucidate the risk factors for survival and progression of the disease. The tumors predominantly harbored HPV16 with two major variants of Asian (A4) and European (A1-3) types and a novel variant termed A5 that is prevalent in the Japanese population. We found that OPSCCs with higher viral load displayed improved survival compared with OPSCCs with lower viral load (<10 copies/cell). Notably, among the p16-positive OPSCCs, those with low viral load displayed significantly worse prognosis. A trend toward worse survival was also found in OPSCCs with HPV-integrated form. These findings suggest that HPV16 viral load is a potentially useful clinical biomarker for accurate stratification of clinical outcomes in patients with OPSCC. Further larger, prospective validation studies are needed worldwide to confirm these findings.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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