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ORIGINAL ARTICLE

Radiotherapy alone as a possible de-intensified treatment for human papillomavirus-related locally advanced oropharyngeal squamous cell carcinoma

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Abstract

Background Human papillomavirus (HPV)-related oropharyngeal squamous cell carcinoma (OPSCC) is defined by p16 positivity and/or HPV DNA positivity. Because survival of patients with HPV-related OPSCC after chemoradiotherapy is favorable, a de-intensified treatment is expected to lead to less morbidity while maintaining low mortality. The association of tumor p16 and HPV DNA status with survival after radiotherapy alone remains unknown.

Methods We retrospectively examined survival of 107 patients with locally advanced OPSCC after radiotherapy alone ($n=43$) or chemoradiotherapy ($n=64$) with respect to tumor p16 and HPV DNA status, using Cox's proportional hazard model.

Results Survival after radiotherapy alone was significantly worse in p16-positive/HPV DNA-negative locally advanced OPSCC than in p16-positive/HPV DNA-positive locally advanced OPSCC. In bivariable analyses that included T category, N category, TNM stage, and smoking history, the survival disadvantage of p16-positive/HPV DNA-negative locally advanced OPSCC remained significant. There was no significant difference in survival after chemoradiotherapy between p16-positive/HPV DNA-positive locally advanced OPSCC and p16-positive/HPV DNA-negative locally advanced OPSCC. Survival in p16-positive/HPV DNA-positive locally advanced OPSCC after radiotherapy alone was similar to that after chemoradiotherapy, which stayed unchanged in bivariable analyses after adjustment of every other covariable. Survival of p16-negative/HPV DNA-negative locally advanced OPSCC was poor irrespective of treatment modality.

Conclusions Survival in p16-positive locally advanced OPSCC differs depending on HPV DNA status. Radiotherapy alone can serve as a de-intensified treatment for p16-positive/HPV DNA-positive locally advanced OPSCC, but not for p16-positive/HPV DNA-negative locally advanced OPSCC.

Keywords Human papillomavirus · Oropharyngeal carcinoma · De-intensified treatment · Radiotherapy alone

Introduction

Head and neck squamous cell carcinoma (HNSCC) is associated with excessive tobacco and alcohol consumption, while human papillomavirus (HPV) is another causal factor of HNSCC, especially that arising in the oropharynx. Oropharyngeal squamous cell carcinoma (OPSCC) is divided into two entities: HPV-related and HPV-unrelated, and these two entities show extremely distinct clinicopathological characteristics [1]. A definite diagnosis of HPV-related OPSCC should be based on the detection of high-risk HPV viral oncogene expression. Viral oncogene expression is evidenced by reverse transcriptase PCR of E6/E7 mRNA or

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E6/E7 immunohistochemistry, although the performance of these procedures is profoundly limited from a practical point of view [2]. Given that HPV protein E7 degrades pRb, which in turn leads to p16 overexpression, p16 overexpression is used as a surrogate marker for transcriptionally active HPV infection on routine workup [3]. However, p16 overexpression is not always equal to HPV DNA positivity [2]. The majority of p16-positive OPSCCs are HPV DNA-positive, while a subset are HPV DNA-negative. p16-positive/HPV DNA-positive OPSCC expresses HPV E6/E7 mRNA without exception [4]. Moreover, p16-positive/HPV DNA-negative OPSCC is genetically more similar to p16-negative/HPV DNA-negative OPSCC than to p16-positive/HPV DNA-positive OPSCC [5]. Collectively, p16-positive/HPV DNA-positive OPSCC is "truly" HPV-related OPSCC, whereas p16-positive/HPV DNA-negative OPSCC is not.

Accumulating evidence has established that patients with p16-positive locally advanced OPSCC survive significantly longer than those with p16-negative locally advanced OPSCC when treated with chemoradiotherapy, the standard treatment for locally advanced OPSCC [6–8]. Likewise, patients with HPV DNA-positive locally advanced OPSCC survive significantly longer than those with HPV DNA-negative locally advanced OPSCC [6–8]. Given that younger patients are more prone to be affected by HPV-related OPSCC [1], given their long life expectancy and the severe late toxicity associated with chemoradiotherapy [9], the hypothesis has emerged that patients with HPV-related locally advanced OPSCC could be managed with a de-intensified treatment, resulting in less long-term morbidity without compromising survival. This hypothesis is under verification in a series of clinical trials [10], in which HPV-related OPSCC is often defined by p16 overexpression. As a meta-analysis has unveiled that survival of patients with p16-positive/HPV DNA-negative OPSCC is worse than survival of patients with p16-positive/HPV DNA-positive OPSCC [11], it is likely that patients with p16-positive/HPV DNA-negative locally advanced OPSCC would not be suitable candidates for the de-intensified treatment.

In a de-intensified treatment, it is of interest to know how the omission of chemotherapy would affect survival of patients. The impact of tumor p16 and HPV DNA status on survival after radiotherapy alone remains unknown. Accordingly, we sought to assess the possibility of radiotherapy alone to serve as a de-intensified treatment for HPV-related locally advanced OPSCC. To this end, we retrospectively examined survival of patients with locally advanced OPSCC after radiotherapy alone or chemoradiotherapy with respect to tumor p16 and HPV DNA status.

Materials and methods

Patients

Patients with newly diagnosed, distant metastasis-negative, stage III/IV OPSCC who were treated for curative intent with radiotherapy alone at Osaka University Medical School Hospital (Suita, Japan), Osaka International Cancer Institute (Osaka, Japan), and Toyonaka Municipal Hospital (Toyonaka, Japan) between 1995 and 2013 or with concurrent chemoradiotherapy at Osaka University Medical School Hospital between 2003 and 2012 were eligible. Patients who received less than 60 Gy and patients whose formalin-fixed paraffin-embedded (FFPE) biopsy specimens of primary tumor were not available were excluded. We performed a data review, and identified 110 patients who met the criteria. Although patients with stage III/IV OPSCC were generally managed by concurrent chemoradiotherapy or surgery followed by radiotherapy in our institutions at that time, a subset of patients received radiotherapy alone due to medical contraindications against chemotherapy and/or general anesthesia, and/or patients' refusal of chemotherapy and/or surgery. As the number of patients receiving radiotherapy alone at Osaka University Medical School Hospital was small, we added patients who received radiotherapy alone at the two affiliated hospitals. Concurrent chemotherapy consisted of six cycles of weekly docetaxel (10 mg/m^2) and cisplatin (20 mg/m^2) [12], while all patients received conventionally fractionated radiation (2 Gy/fraction/day). Tumors were staged according to the International Union Against Cancer TNM staging system (seventh edition) [13]. The Institutional Review Board approved the protocol of the present study, and waived informed consent of patients due to the retrospective nature of the study.

Immunohistochemistry of p16

Immunohistochemical analysis of p16 was carried out using 4 μm FFPE sections, with an appropriate positive control. Following antigen retrieval using a pressurized heating chamber (Pascal®, DAKO, Glostrup, Denmark), the sections were incubated with anti-p16 antibody at a dilution of 1 : 500 (clone LC8: Santa Cruz Biotechnology, Inc., Dallas, TX, USA). After incubation, anti-p16 antibody was detected using a kit (ChemMate EnVision kit®, DAKO) and visualized by means of diaminobenzidine as a chromogen. Negative control staining was done in the absence of primary antibody. Strong and diffuse nuclear and cytoplasmic immunostaining in > 75% of the cancer cells was classified as p16-positive.

Detection and genotyping of HPV

DNA was extracted from FFPE biopsy specimens using a commercial kit (DNeasy tissue kit®, QIAGEN Inc., Valencia, CA, USA). The presence of HPV DNA was screened by means of a nested polymerase chain reaction (PCR) using the PGMY09/11 primer set (for primary PCR) and the GP5+/6+ primer set (for secondary PCR) as previously reported [14]. These primers targeted the conserved L1 region of the virus genome, enabling the detection of a broad range of HPV types. The secondary PCR products were purified and sequenced directly using a genetic analyzer (3100®, Applied Biosystems, Foster City, CA, USA). Typing was achieved by comparing the sequence with those of known HPV types using the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). When the consensus PGMY/GP+ nested PCR was negative or equivocal though p16 immunostaining was positive, type-specific PCR for HPV16 was carried out using primer sets of E6 and E7 genes [15], except for E6 forward primer, for which we used 5′-AATGTTTCAGGA CCCACAGG-3′.

Statistical analysis

The endpoints were overall survival (OS) and progression-free survival (PFS). OS was defined as the time from the date of radiotherapy initiation to death from any cause. PFS was defined as the time from the date of radiotherapy initiation to death or the first documented relapse, which was categorized as local–regional recurrence or distant metastasis. Actuarial rates of OS and PFS were estimated using the Kaplan–Meier method. The log-rank test was used for comparison between the two groups. The Cox proportional hazards models were used to estimate hazard ratio (HR) and 95% confidence interval (CI). A p value <0.05 was considered to indicate statistical significance.

Results

p16/HPV DNA status and patient characteristics

Immunostaining of p16 scored positive in 59 (54%) of 110 tumors. Consensus nested PCR detected HPV DNA in 46 (42%) of 110 tumors, of which 43, 2, and 1 were genotyped as HPV16, HPV35 and HPV58, respectively, by subsequent sequencing. Nineteen tumors showed discordant phenotypes: 16 were p16-positive/HPV DNA-negative, and 3 were p16-negative/HPV DNA-positive. HPV16-specific PCR was applied to the subgroup that was p16-positive/HPV DNA-negative, of which 2 were revealed to be HPV16 E6/E7 DNA-positive. Collectively, 45, 48, 14, and 3 tumors were classified as p16-positive/HPV DNA-positive, p16-negative/HPV DNA-negative, p16-positive/HPV DNA-negative, and p16-negative/HPV DNA-positive, respectively. The three patients with p16-negative/HPV DNA-positive tumor were excluded from the analysis because of low statistical power. Baseline characteristics of patients included in the analysis ($n=107$) are summarized in Table 1 according to treatment modality. Baseline characteristics of patients with p16-positive/HPV DNA-positive locally advanced OPSCC are shown in Table S1 in the Supplement. Of note, the majority of patients (28 of 45, 62%) were heavy smokers (> 10 pack-years).

Survival after radiotherapy alone

The median dose of radiotherapy was 70 Gy (range 60–70). The median follow-up of surviving patients was 83 months (range 56–201). There were 18 deaths, including 9 deaths due to the index cancer, 4 deaths from a second primary cancer, and 5 deaths from other causes. Local, regional, and/or distant failures were identified in eight, eight, and three patients, respectively. The 5-year OS and PFS rates of the whole population were 67% (95% CI 52–79), and 53% (95% CI 38–68), respectively.

Patients with p16-positive/HPV DNA-positive locally advanced OPSCC had significantly better OS and PFS compared with patients with p16-negative/HPV DNA-negative locally advanced OPSCC ($p=0.03$ for OS, $p=0.004$ for PFS) and patients with p16-positive/HPV DNA-negative locally advanced OPSCC ($p=0.04$ for OS, $p=0.008$ for PFS) (Fig. 1a, b). There was no difference in OS and PFS between the p16-negative/HPV DNA-negative subgroup and the p16-positive/HPV DNA-negative subgroup ($p=0.68$ for OS, $p=0.61$ for PFS). The 5-year OS rates were 84% (95% CI 61–95) in the p16-positive/HPV DNA-positive subgroup, 51% (95% CI 28–74) in the p16-negative/HPV DNA-negative subgroup, and 57% (95% CI 23–86) in the p16-positive/HPV DNA-negative subgroup. The 5-year PFS rates were 79% (95% CI 56–92) in the p16-positive/HPV DNA-positive subgroup, 33% (95% CI 15–58) in the p16-negative/HPV DNA-negative subgroup, and 29% (95% CI 7–67) in the p16-positive/HPV DNA-negative subgroup.

Univariable analysis revealed that p16/HPV DNA status was a significant determinant of OS with smoking history being a marginal determinant, while smoking history and p16/HPV DNA status were significantly associated with PFS (Table S2 in the Supplement). As there were only 18 events for OS and 22 events for PFS, we assessed the association of p16/HPV DNA status with OS or PFS by bivariable analysis controlling for each covariable individually. Both the p16-negative/HPV DNA-negative status and the p16-positive/HPV DNA-negative status remained independent adverse prognostic factors when adjusted for every other variable ($p<0.05$) (Table 2), except for the less significant

Table 1 Baseline characteristics of patients

Variable	Level	Radiotherapy alone ($n=43$) No. of patients (%)	Chemoradiotherapy ($n=64$) No. of patients (%)
Age (years)	Median	70	65
	Range	29–89	38–80
Gender	Male	34 (79)	57 (89)
	Female	9 (21)	7 (11)
Subsite	Lateral wall	30 (70)	32 (50)
	Anterior wall	11 (26)	22 (34)
	Posterior wall	2 (4)	8 (13)
	Superior wall	0 (0)	2 (3)
T category	T1	9 (21)	5 (8)
	T2	23 (54)	26 (41)
	T3	7 (16)	12 (20)
	T4	4 (9)	21 (31)
N category	N0	6 (15)	3 (5)
	N1	10 (23)	11 (17)
	N2	23 (53)	47 (73)
	N3	4 (9)	3 (5)
TNM stage	III	14 (33)	12 (20)
	IV	29 (67)	52 (80)
Dose of radiation (Gy)	Median	70	66
	Range	60–70	60–70
Duration of radiotherapy(days)	Median	50	47
	Range	42–58	42–57
Cycles of chemotherapy	Median	–	6
	Range	–	1–6
Smoking history	Median	32	38
No. of pack-years	Range	0–90	0–157
	Never	8 (19)	13 (20)
	≤ 10	4 (9)	3 (5)
	> 10	31 (72)	48 (75)
p16/HPV DNA status	p16+/HPV+	19 (44)	26 (41)
	p16-/HPV−	17 (40)	31 (48)
	p16+/HPV−	7 (16)	7 (11)

association of p16-positive/HPV DNA-negative status with OS after adjustment for age ($p=0.05$). Smoking history remained marginally and significantly associated with worse OS ($p=0.09$) and PFS ($p=0.002$), respectively, after adjustment for p16/HPV DNA status (Table 2).

Survival after chemoradiotherapy

The median dose of radiotherapy was 66 Gy (range 60–70), and the median number of cycles of chemotherapy was 6 (range 1–6). The median follow-up of surviving patients was 71 months (range 29–167). There were 23 deaths, including 14 deaths from the index cancer, 4 deaths from a second primary cancer, and 5 deaths from other causes. Local, regional and/or distant failures were identified in 15 patients (local failure in 8 patients, regional failure in 8 patients, and distant failure in 3 patients). The 5-year OS and PFS rates of the whole population were 68% (95% CI 56–79) and 47% (95% CI 35–58), respectively.

Patients with p16-positive/HPV DNA-positive locally advanced OPSCC had significantly better OS and PFS than patients with p16-negative/HPV DNA-negative locally advanced OPSCC ($p=0.003$ for OS, $p=0.02$ for PFS), as after radiotherapy alone. In contrast, the survival advantage of patients with p16-positive/HPV DNA-positive locally advanced OPSCC over patients with p16-positive/HPV DNA-negative locally advanced OPSCC, which was observed after radiotherapy alone, disappeared ($p=0.33$ for OS, $p=0.81$ for PFS) (Fig. 1c, d). The 5-year OS rates were 88% (95% CI 69–96) in the p16-positive/HPV DNA-positive subgroup, 51% (95% CI 35–68) in the p16-negative/HPV DNA-negative subgroup, and 71% (95% CI 33–93) in

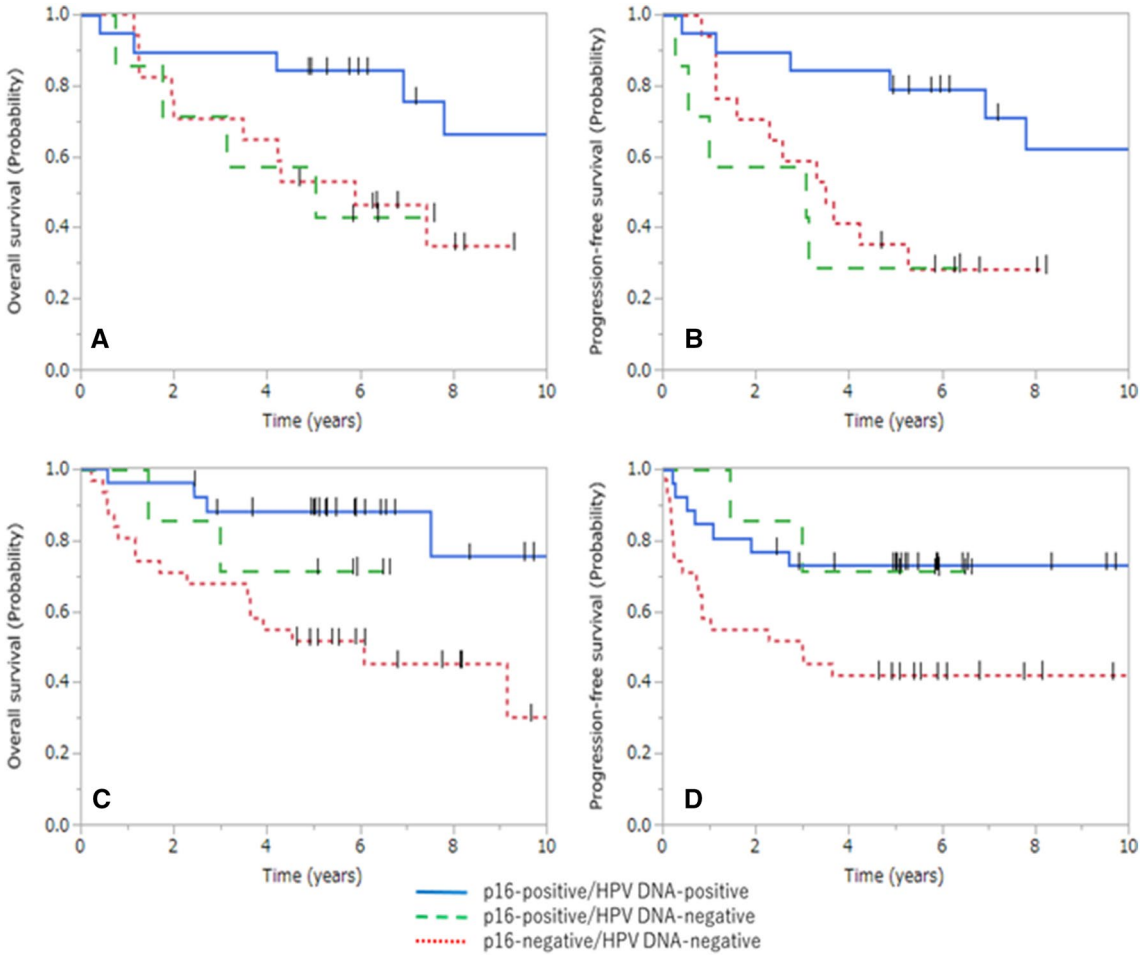

Fig. 1 Kaplan–Meier estimates of overall survival (**a, c**) and progression-free survival (**b, d**) in patients treated with radiotherapy alone (**a, b**) and chemoradiotherapy (**c, d**)

the p16-positive/HPV DNA-negative subgroup. The 5-year PFS rates were 73% (95% CI 53–86) in the p16-positive/HPV DNA-positive subgroup, 42% (95% CI 26–60) in the p16-negative/HPV DNA-negative subgroup, and 71% (95% CI 33–93) in the p16-positive/HPV DNA-negative subgroup.

Univariable analysis revealed that age and T category were significant and marginal determinants of OS, respectively, while p16/HPV DNA status was a significant determinant of both OS and PFS (Table S3 in the Supplement). As there were only 23 events for OS and 27 events for PFS, bivariable analysis was used to assess the association between p16/HPV DNA status and OS or PFS while controlling for each covariable individually. The p16-negative/HPV DNA-negative status remained an independent adverse prognostic factor when adjusted for every other variable ($p < 0.05$ for both OS and PFS) (Table 3). The difference in the risk of death and/or disease progression remained insignificant between the p16-positive/HPV DNA-positive subgroup and the p16-positive/HPV DNA-negative subgroup (Table 3). In contrast to the outcome after radiotherapy alone, smoking history was associated with neither OS nor PFS, after adjustment for p16/HPV DNA status (Table 3).

Survival of patients with p16-positive/HPV DNA-positive locally advanced OPSCC

OS and PFS in patients with p16-positive/HPV DNA-positive locally advanced OPSCC were compared between the subgroup receiving radiotherapy alone and the subgroup receiving chemoradiotherapy. Univariable analysis revealed that neither treatment modality was associated with OS or PFS (Table S4 in the Supplement). In bivariable analysis, the difference between the two remained insignificant after adjustment of every other covariable (Table 4). Of interest, although 3 of 19 patients treated with radiotherapy alone received no more than 60 Gy, all these patients showed complete response and stayed disease free thereafter. The details are shown in Table S5 in the Supplement.

Table 2 Bivariable analysis for p16/HPV DNA status in patients treated with radiotherapy alone

Variable	Overall survival			Progression-free survival		
	Hazard ratio*	95% CI	*p* value	Hazard ratio*	95% CI	*p* value
p16-negative/HPV DNA-negative	3.7	1.1–11.8	0.03	4.3	1.6–12.8	0.005
p16-positive/HPV DNA-negative	4.2	1.0–17.5	0.05	5.6	1.4–22.4	0.01
Age (per 10 years)	1.1	0.8–1.7	0.54	1.0	0.7–1.4	0.97
p16-negative/HPV DNA-negative	3.9	1.2–17.6	0.02	4.9	1.6–15.2	0.002
p16-positive/HPV DNA-negative	4.2	1.0–19.4	< 0.05	6.4	1.7–21.0	0.008
Gender (male vs. female)	0.1	0.01–0.8	0.01	0.2	0.05–0.9	0.02
p16-negative/HPV DNA-negative	4.0	1.1–15.4	0.01	4.8	1.5–16.0	0.003
p16-positive/HPV DNA-negative	5.4	1.3–27.2	0.02	7.6	1.7–31.4	0.007
T category (T1-T2 vs. T3-T4)	1.5	0.4–4.2	0.42	1.9	0.4–5.1	0.36
p16-negative/HPV DNA-negative	3.4	1.2–13.0	0.01	4.7	1.5–14.5	0.003
p16-positive/HPV DNA-negative	4.8	1.2–28.0	0.02	7.5	1.8–31.5	0.007
N category (N0-N1 vs. N2-N3)	1.9	0.6–4.9	0.32	0.9	0.6–2.5	0.89
p16-negative/HPV DNA-negative	3.2	1.2–14.1	< 0.05	4.0	1.8–15.2	0.005
p16-positive/HPV DNA-negative	4.8	1.1–21.7	0.02	6.0	1.4–21.0	0.009
TNM stage (III vs. IV)	0.7	0.4–2.1	0.28	1.2	0.6–3.6	0.84
p16-negative/HPV DNA-negative	3.2	1.1–12.8	0.03	4.3	1.6–12.6	0.006
p16-positive/HPV DNA-negative	4.0	1.2–17.4	0.04	4.8	1.5–23.4	0.02
Radiation dose(≥ 66 Gy vs. < 66 Gy)	0.8	0.1–2.4	0.73	0.9	0.2–2.8	0.72
p16-negative/HPV DNA-negative	3.3	1.1–10.5	0.04	4.2	1.4–12.1	0.005
p16-positive/HPV DNA-negative	5.0	1.2–21.0	0.03	10.5	2.6–42.0	0.001
Smoking history (per 10 pack-years)	1.2	1.0–1.4	0.09	1.3	1.1–1.5	0.002

*Hazard ratios of p16/HPV DNA status were estimated with p16-positive/HPV DNA-positive status being the reference

Discussion

We retrospectively analyzed patients with locally advanced OPSCC treated with radiotherapy or chemoradiotherapy, and found that, when treated with chemoradiotherapy, survival of patients with p16-positive/HPV DNA-negative locally advanced OPSCC was as favorable as that of patients with p16-positive/HPV DNA-positive locally advanced OPSCC. In contrast, when treated with radiotherapy, survival of patients with p16-positive/HPV DNA-negative locally advanced OPSCC was less favorable than that of patients with p16-positive/HPV DNA-positive locally advanced OPSCC. Survival of patients with p16-negative/HPV DNA-negative locally advanced OPSCC was poor irrespective of treatment intensity. These results suggest that patients with p16-positive/HPV DNA-positive, p16-positive/HPV DNA-negative, and p16-negative/HPV DNA-negative locally advanced OPSCC are at low, intermediate, and high risk of treatment failure, respectively, and that patients with p16-positive/HPV DNA-negative locally advanced OPSCC are not suitable candidates for the de-intensified treatment.

We also found that survival of patients with p16-positive/HPV DNA-positive locally advanced OPSCC after radiotherapy alone was as favorable as that after chemoradiotherapy. This finding encouraged us to assume that the de-intensified treatment of radiotherapy alone would be effective for patients with p16-positive/HPV DNA-positive locally advanced OPSCC. To address this assumption, we are currently doing a single-arm phase II clinical trial (UMIN000008953) evaluating the effectiveness of intensity-modulated radiotherapy at a prescribed total dose of 70 Gy for p16-positive/HPV DNA-positive locally advanced OPSCC, the results of which will be reported in the future. On the other hand, of interest was the finding that all 3 patients with p16-positive/HPV DNA-positive locally advanced OPSCC receiving radiotherapy alone at a total dose of 60 Gy stayed disease free after treatment. It is suggested that radiotherapy alone is effective for the subset of patients with p16-positive/HPV DNA-positive locally advanced OPSCC, even at a reduced dose, although the development of a biomarker to identify such patients, is mandatory.

We evaluated HPV DNA status by two-step PCR in an attempt to minimize the false negative rate of HPV DNA detection: first, consensus nested PCR for L1 region followed by genotyping; second, type-specific PCR for HPV16 E6/E7 when HPV DNA was negative or equivocal at the first step although p16 immunostaining was positive. This concept is based on the findings that breakpoints could occur in any part of the viral genome [16] and that HPV16

Table 3 Bivariable analysis for p16/HPV DNA status in patients treated with chemoradiotherapy

Variable	Overall survival			Progression-free survival		
	Hazard ratio*	95% CI	*p* value	Hazard ratio*	95% CI	*p* value
p16-negative/HPV DNA-negative	3.6	1.2–11.0	0.02	2.5	1.0–6.1	0.04
p16-positive/HPV DNA-negative	2.1	0.4–11.6	0.39	0.9	0.2–4.5	0.94
Age (per 10 years)	1.6	0.9–3.3	0.13	1.2	0.7–2.2	0.46
p16-negative/HPV DNA-negative	4.2	1.7–17.2	0.002	2.8	1.3–7.8	0.009
p16-positive/HPV DNA-negative	2.1	0.3–10.9	0.64	1.2	0.3–4.5	0.98
Gender (male vs. female)	2.2	0.7–5.4	0.27	2.3	0.6–5.6	0.34
p16-negative/HPV DNA-negative	3.6	1.3–11.4	0.007	3.0	1.4–8.3	0.008
p16-positive/HPV DNA-negative	1.9	0.3–8.3	0.32	1.2	0.2–4.9	0.89
T category (T1-T2 vs. T3-T4)	1.7	0.9–4.4	0.23	1.1	0.5–3.9	0.58
p16-negative/HPV DNA-negative	4.4	1.7–11.7	0.001	3.8	1.3–9.6	0.006
p16-positive/HPV DNA-negative	1.6	0.2–10.2	0.58	1.2	0.2–4.3	0.87
N category (N0-N1 vs. N2-N3)	1.4	0.7–4.6	0.31	1.3	0.8–4.7	0.38
p16-negative/HPV DNA-negative	3.8	1.6–10.0	0.004	3.6	1.4–7.8	0.004
p16-positive/HPV DNA-negative	1.7	0.2–8.8	0.34	1.1	0.1–4.9	0.65
TNM stage (III vs. IV)	2.0	0.6–8.3	0.25	1.7	0.5–8.6	0.37
p16-negative/HPV DNA-negative	4.0	1.5–11.3	0.004	2.9	1.2–8.3	0.01
p16-positive/HPV DNA-negative	1.5	0.3–10.3	0.48	0.8	0.1–3.7	0.67
Radiation dose(≥ 66 Gy vs.<66 Gy)	1.0	0.3–2.1	0.98	1.1	0.8–2.6	0.59
p16-negative/HPV DNA-negative	3.8	1.4–11.2	0.003	2.8	1.1–8.3	0.01
p16-positive/HPV DNA-negative	1.5	0.3–10.2	0.48	0.9	0.2–5.7	0.54
Chemotherapy (≥ 5 cycles vs. <5 cycles)	1.3	0.4–3.6	0.44	1.5	0.6–3.6	0.41
p16-negative/HPV DNA-negative	5.2	1.6–16.7	0.005	3.4	1.3–9.1	0.01
p16-positive/HPV DNA-negative	2.7	0.4–17.7	0.27	1.3	0.2–6.8	0.78
Smoking history (per 10 pack-years)	0.9	0.8–1.1	0.51	0.9	0.8–1.1	0.37

*Hazard ratios of p16/HPV DNA status were estimated with p16-positive/HPV DNA-positive status being the reference

Table 4 Bivariable analysis for treatment modality in patients with p16-positive/HPV DNA-positive LA-OPSCC

Variable	Overall survival			Progression-free survival		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
Chemoradiotherapy vs. radiotherapy	0.6	0.1–3.0	0.50	0.5	0.1–1.7	0.24
Age (per 10 years)	2.2	0.9–6.0	0.09	1.8	0.9–3.9	0.08
Chemoradiotherapy vs. radiotherapy	0.8	0.3–4.8	0.72	0.9	0.2–2.4	0.68
Gender (male vs. female)	1.2	0.1–3.8	0.93	0.9	0.3–4.1	0.73
Chemoradiotherapy vs. radiotherapy	0.8	0.4–8.3	0.52	0.8	0.2–5.9	0.67
T category (T1–T2 vs. T3–T4)	1.2	0.5–8.4	0.53	1.1	0.4–8.6	0.87
Chemoradiotherapy vs. radiotherapy	1.3	0.3–5.3	0.71	0.9	0.3–2.8	0.81
N category (N0–N1 vs. N2–N3)	2.1	0.2–16.9	0.50	3.2	0.4–24.8	0.27
Chemoradiotherapy vs. radiotherapy	1.3	0.3–5.3	0.71	0.9	0.3–2.8	0.81
TNM stage (III vs. IV)	2.1	0.2–16.9	0.50	3.2	0.4–24.8	0.27
Chemoradiotherapy vs. radiotherapy	0.7	0.2–3.5	0.71	0.6	0.2–2.1	0.47
Radiation dose(≥ 66 Gy vs. < 66 Gy)	0.2	0.1–1.5	0.10	0.4	0.1–1.5	0.15
Chemoradiotherapy vs. radiotherapy	1.2	0.3–4.9	0.78	0.7	0.2–2.3	0.57
Smoking history (per 10 pack-years)	1.0	0.8–1.4	0.80	1.1	0.8–1.4	0.27

is responsible for > 90% of HPV-related OPSCC [17]. When breakpoints occurred in L1 on the occasion of viral genome integration into the host genome, the consensus nested PCR is unable to detect HPV DNA. Even when it is the case, PCR specific for HPV16 E6/E7 is usually able to detect HPV DNA. In our series 2 of 16 OPSCCs negative for HPV DNA,

when evaluated by consensus nested PCR for L1, turned out to be positive for HPV DNA when further evaluated by type-specific PCR for HPV16 E6/E7. Nonetheless, of the 59 p16-positive OPSCCs, 14 (24%) tumors were HPV DNA-negative. This incidence was high compared with the report by Nauta et al. in which they evaluated HPV DNA by the same two-step PCR and found that 12% of the p16-positive OPSCCs were HPV DNA-negative [18]. We believe that this relatively high incidence happened by chance, most probably due to the small sample size. Of note, approximately 10% of p16-positive OPSCCs are HPV DNA-negative in our recent series (data not shown).

It may be argued that radiotherapy alone is an excessively de-intensified treatment and thus less effective than expected. This argument might come from the following findings: O'Sullivan et al. found that radiotherapy for p16-positive stage IV OPSCC resulted in comparable disease control but in less favorable survival, compared with chemoradiotherapy [19]. Rosenthal et al. found that patients with p16-positive/HPV DNA-positive locally advanced OPSCC receiving radiotherapy plus cetuximab survived longer than those receiving radiotherapy alone, although the difference was not statistically significance [20]. It is important to note that patients with p16-positive/HPV DNA-negative tumor, who were at intermediate risk of treatment failure, were most probably included in O'Sullivan et al.'s series. Rosenthal et al. used in situ hybridization for HPV DNA detection, which is less sensitive than two-step PCR. It is likely that a subset of p16-positive/HPV DNA-positive tumor were not included because they were misclassified as p16-positive/HPV DNA-negative.

Ang et al. analyzed patients with locally advanced OPSCC treated with chemoradiotherapy in the setting of a clinical trial, and found that tobacco abuse was an independent predictor of poor survival [7]. We found no association of tobacco smoking with survival after chemoradiotherapy. This was at least in part because the majority of patients (74%) had history of heavy smoking (> 10 pack-years). Rietbergen et al. analyzed an unselected cohort of patients with OPSCC, the majority of whom had smoking history of > 10 pack-years. They found that comorbidity was a stronger prognostic factor than smoking [21]. It is likely that the prognostic value of tobacco smoking is less significant in the cohort where patients with history of tobacco abuse account for the majority.

There were some limitations to the present study, such as a retrospective analysis of a relatively small cohort. The small number of events precluded us from making multivariable analysis. Instead, we employed bivariable analyses. Certain factors correlating with outcome, including comorbidity and performance status, were not available. In addition, chemotherapy concurrent with radiotherapy was weekly low-dose cisplatin/docetaxel, instead of the standard chemotherapy of tri-weekly high-dose cisplatin [22]. This is because a clinical trial of weekly low-dose cisplatin/docetaxel chemoradiotherapy was in progress at that time [12].

In conclusion, survival of patients with p16-positive locally advanced OPSCC differs depending on tumor HPV DNA status. Tumor HPV DNA status needs to be determined in addition to tumor p16 status, preferably by two-step PCR, to define "truly" HPV-related OPSCC. It is likely that patients with p16-positive/HPV DNA-positive locally advanced OPSCC would benefit from de-intensified treatment such as radiotherapy alone, while patients with p16-positive/HPV DNA-negative locally advanced OPSCC would not.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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