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HEAD & NECK

Roles of Ki-67 and p16 as biomarkers for unknown primary head and neck squamous cell carcinoma

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Abstract

Purpose Treatment guidelines have not been established for unknown primary head and neck squamous cell carcinoma (SCC). For these patients, chemoradiotherapy (CRT) can provide a better prognosis than that for patients with other head and neck cancers. The presence of HPV in the tumor is associated with a better outcome. However, not all patients with HPV-positive unknown primary head and neck SCC experience good treatment outcomes in actual clinical settings.

Methods We thus retrospectively determined the Ki-67 proliferation index and p16 expression status to assess the associations of these parameters with treatment outcomes of patients with unknown primary head and neck SCC.

Results The subjects were 13 patients who underwent CRT after surgery or excision biopsy between 1999 and 2016. The 2- and 5-year overall survival (OS) rate was 76.9% and 68.4%, respectively. The prognostic factor was age. There was no significant difference in survival between patients with a high Ki-67 vs. low Ki-67 or between patients with p16-positive vs. p16-negative metastases OS. However, all p16-positive patients with low Ki-67 showed good locoregional control.

Conclusions The combination of ki67 expression and p16 expression status may allow prediction of local control more accurately than p16 expression status alone.

Keywords Unknown primary head and neck squamous cell carcinoma · Chemoradiotherapy · Ipsilateral oropharynx and neck irradiation · p16 · Ki-67

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Introduction

Treatment guidelines have not been established for unknown primary head and neck squamous cell carcinoma (SCC). Recently, the presence of human papillomavirus (HPV) in unknown primary head and neck SCC has been reported to be a prognostic factor, as is the case with other HPV-positive head and neck cancers [1]. Furthermore, because HPV-positive head and neck cancers are sensitive to radiation [2] and have a relatively good prognosis [3], the treatment approach is expected to shift to radiotherapy, which is associated with fewer adverse events [4, 5].

With regard to treatment method, numerous studies have investigated the methods of radiotherapy and radiation fields [6–13], controversy remains as to whether comprehensive irradiation is necessary or ipsilateral irradiation is equally effective in some patients [11, 14]. The efficacy of ipsilateral irradiation has recently been reported for HPV-positive unknown primary head and neck SCC [15]. However, one study demonstrated a significant difference in survival between p16-positive and p-16 negative patients with unknown primary head and neck SCC [16], while another did not [16]. On the other hand, the HPV-positive rate is reportedly very low in patients with unknown primary head and neck SCC [17], making it difficult to plan prospective studies. Thus, in our view, factors other than the presence of HPV infection merit retrospective investigation. As one of the factors warranting investigation, we focused on Ki-67 expression, a factor reportedly predicting a poor response to treatment of head and neck cancer [18, 19]. We also investigated other factors including p16-positivity, smoking status, age, and radiation techniques. To our knowledge, this is the first study to focus on the associations of various factors, including Ki-67 expression, with the outcomes of patients with unknown primary head and neck SCC.

Materials and methods

Patient characteristics

We retrospectively investigated 13 patients with unknown primary head and neck SCC who underwent chemoradiotherapy (CRT) after surgery at our hospital between 1990 and 2016. The subjects comprised 11 men and 2 women aged 36 to 79 years (median 70 years). Endoscopic observation of the area around the oropharynx and random biopsies of the area from the tongue base to the tonsil were performed in all patients undergoing surgery under general anesthesia. Histopathologic findings confirmed squamous cell carcinoma in all patients. The degree of differentiation was assessed in four patients, which included one patient with well-differentiated, three with moderately differentiated, and five with poorly differentiated cancer. The disease stage was N2a in five patients, N2b in five, and N3 (unilateral) in three. Extranodal invasion was observed in four patients.

Seven patients underwent chemoradiotherapy (CRT) after radical neck dissection (RND) and six underwent CRT after excisional biopsy (the lymph nodes identified in the images were completely resected in all patients except those with N3 disease). The chemotherapy regimens based on cisplatin-based multidrug therapy or docetaxel (DOC) monotherapy (Table 1).

Statistical analysis

Survival was calculated by the Kaplan–Meier method and differences were expressed at a 5% significance level with a two-tailed log-rank test. Multivariate analysis of the data was performed using a Cox proportional hazards model. All calculations and survival displays were conducted using the SPSS 21.0J statistical software (SSPS Inc., Chicago, IL, USA). Acute and late complications were graded according to the National Cancer Institute-Common Terminology Criteria (NCI-CTC), Version3.0 [20].

Results

Radiotherapy

Radiotherapy was administered using a 4-MV X-ray Siemens Primus KD2 (Siemens Oncology Care Systems, Concord, CA, USA). Treatment planning was performed using XiO version 4.4.0-4.6.0 software (Elekta, Hamburg, Germany), with the isocenter calculated using the Clarkson method.

Regarding the radiation field, comprehensive irradiation of the whole nasopharynx and neck (bilateral opposing portal irradiation) and subclavicular region (anterior single irradiation), or of the ipsilateral oropharynx and neck (anteroposterior opposing portal irradiation) including the IIA and IV (according to the American Joint Committee on Cancer) fields, was performed. Irradiation using a 4MV X-ray beam (2 Gy/f/day) was performed in all patients. The total doses ranged from 50 to 70 Gy (median, 66 Gy). To optimize the irradiation field, we routinely consulted with several specialists in the fields of otorhinolaryngology and radiology (Table 1). The total dose of postoperative irradiation was 50 Gy for most patients who underwent RND. If extracapsular extension was identified, the use of boost irradiation was considered (Table 1).

Table 1 Patient characteristics

	n = 13
Follow-up (months)	
Median	86
Range	7–195
Sex	
Male	11
Female	2
Age	
Median	70
Range	36–79
Pathology	
SCC	4 (30.8)
W/D SCC	1 (7.7)
M/D SCC	3 (23.1)
P/D SCC	5 (38.5)
p16 expression	
Positive	5 (38.5)
Negative	8 (61.5)
Ki 67	
Median	61.2
Range	30.9–96.6
Smoking status	
Smoking	9 (69.2)
Non-smoking	4 (30.8)
Chemotherapy	
CDDP + 5-FU + DOC	3 (23.1)
CDDP + DOC	4 (30.8)
DOC	5 (38.5)
Cetuximab	1 (7.7)
Radiation therapy	
Dose (Gy)	
2 Gy/fraction, total 50 Gy	4 (30.8)
2 Gy/fraction, total 60–70 Gy	9 (69.2)
Radiation field	
Ipsilateral oropharynx and neck	6 (46.2)
Comprehensive neck	7 (53.8)
Stage (UICC 7th)	
N2a	5 (38.5)
N2b	5 (38.5)
N3 (ipsilateral huge LN metastasis)	3 (23.1)

Follow-up periods and age are presented as median values, with the corresponding ranges given in parentheses. Other data are presented as the number of patients, with percentages given in parentheses

SCC squamous cell carcinoma, *W/D* well differentiated, *M/D* moderately differentiated, *P/D* poorly differentiated, *DOC* docetaxel, *CDDP* cisplatin, *5-FU* 5-fluorouracil

Chemotherapy

The chemotherapy regimens were 5-fluorouracil (5-FU) (750 mg/m^2) plus cisplatin (CDDP) (60 mg/m^2) plus docetaxel (DOC) (60 mg/m^2) (DCF) in three patients, CDDP (80 mg/m^2) plus DOC (10 mg/m^2) (DC) in four, double DOC (10 mg/m^2) in five, and cetuximab (250 mg/m2) in one. Although the standard chemotherapy regimen at our hospital is platinum-based combination chemotherapy, DOC monotherapy is administered twice a week during the irradiation period to patients with poor renal function and those who do not actively wish to receive chemotherapy (Table 2).

Overall survival

The 2-year OS survival rate was 76.9%, while the 5-year OS survival rate was 68.4% (Fig. 1a). Univariate analysis showed age < 70 years to be a significant prognostic factor (p = 0.0009). There was no significant difference in OS rates between patients with Ki-67 proliferation index ≥ 61.2% vs. <61.2% (p = 0.3921) (Fig. 2a) or between patients with p16-positive vs. p16-negative metastases (p = 0.3638) (Fig. 2b). There was no significant difference in survival between patients who underwent ipsilateral vs. comprehensive irradiation (p = 0.4716). All p16-positive patients with a high Ki-67 expression level survived (Fig. 3a).

The 2-year PFS rate was 83.9%, while the 5-year PFS rate was 58.7% (Fig. 1b). Univariate analysis showed age < 70 years to be a significant prognostic factor (p = 0.0334). There was no significant difference in PFS rates between patients with Ki-67 proliferation index ≥ 61.2% vs. <61.2% (p = 0.6743) (Fig. 2c) or between patients with p16-positive vs. p16-negative metastases (p = 0.2474) (Fig. 2d). There was no significant difference in PFS between patients who underwent ipsilateral vs. comprehensive irradiation (p = 0.1869). All p16-positive patients with a low Ki-67 expression level achieved locoregional control (Fig. 3b).

Ki 67 proliferation index, p16 expression and smoking status

Patients were divided into two groups, one with a Ki-67 proliferation index ≥ 61.2% (high Ki-67 group) and the other with a Ki-67 proliferation index < 61.2% (low Ki-67 group). The median Ki-67 proliferation index was 61.2% (range 30.9–96.6%); seven patients had a Ki-67 proliferation index ≥ 61.2% and six had a Ki-67 proliferation index < 61.2%. p16 immunostaining was classified as positive (≥ 50% of the cells) or negative (< 50%). In this study, classification was not difficult because the majority of tumor cells were positive in the p16-positive patients, whereas no more than 20% were positive in the p16-negative patients.

Five patients were positive for p16 (5/13, 38.5%), including two receiving ipsilateral oropharynx and neck irradiation and three receiving comprehensive irradiation. None of the p16-positive patients died in the early stages,

Table 2 Eight patients without p16-positive and five patients with p-16 positive

No	Age (years)/sex	*N* stage	RND or biopsy	Pathology	Radiation field	Radiation dose	Chemotherapy	Adverse event	Smoking status	p-16 status	Ki 67 (%)	Survival time (months)
1	62/M	N2b	RND	P/D SCC	Comprehensive irradiation	50 Gy	DOC	Grade 2 Pharyngitis	Non-Smoking	(−)	30.9	112
2	51/M	N2b	Biopsy	P/D SCC	Comprehensive irradiation	68 Gy	DOC + CDDP+ 5FU	Grade 2 Pharyngitis Grade 3 dermatitis	Non-Smoking	(−)	47.3	86 Lung metastasis at 3 years Death due to pharyngeal ca
3	72/M	N2b	Biopsy	SCC	Comprehensive irradiation	66 Gy	DOC + CDDP	Grade 2 Pharyngitis	Smoking	(−)	31.9	8 Death due to pneumonia
4	63/M	N3	Biopsy	SCC	Comprehensive irradiation	66 Gy	DOC + CDDP	Grade 2 Pharyngitis	Smoking	(−)	77	103
5	79/M	N3	Biopsy	SCC	Ipsilateral oropharynx and neck irradiation	70 Gy	DOC + CDDP	Grade 3 Pharyngitis	Smoking	(−)	61.2	7 Died from disease
6	47/M	N2a	RND	M/D SCC Extra- capsular invasion	Ipsilateral oropharynx and neck irradiation	50 Gy	DOC	Grade 2 Pharyngitis and dermatitis	Grade 2	(−)	71.3	152 Esophageal cancer at 11 years
7	70/M	N2b	RND	P/D SCC Extra- capsular invasion	Ipsilateral oropharynx and neck irradiation	50 Gy	DOC + CDDP + 5FU	Grade 2 Pharyngitis and dermatitis	Smoking	(−)	51.4	142 Oropharyngeal cancer at 4 years
8	71/M	N2a	Biopsy	M/D SCC	Ipsilateral oropharynx and neck irradiation	66 Gy	DOC	Grade 2 Pharyngitis	Smoking	(−)	96.6	25
9	36/F	N2b	Biopsy	P/D SCC	Ipsilateral oropharynx and neck irradiation	66 Gy	DOC+ CDDP	Grade 3 Pharyngitis	Smoking	(+)	69.6	130
10	40/F	N2a	RND	W/D SCC Extra- capsular invasion	Ipsilateral oropharynx and neck irradiation	50 Gy	DOC+ CDDP + 5FU	Grade 2 Pharyngitis and dermatitis	Non-Smoking	(+)	35.3	116
11	76/F	N2a	RND	SCC	Comprehensive irradiation	66 Gy	DOC	Grade 2 Pharyngitis	Smoking	(+)	31	66 Death due to pneumonia
12	62/M	N2b	RND	M/D SCC	Comprehensive irradiation	66 Gy	DOC	Grade 2 stomatitis	Smoking	(+)	81.1	79
13	74/M	N2a	Biopsy	P/D SCC	Comprehensive irradiation	66 Gy	Cetuximab	Grade 3 Pharyngitis and dermatitis	Non-Smoking	(+)	95.8	30 Oropharyngeal cancer at 2.5 years

RND radical neck dissection, *SCC* squamous cell carcinoma, *P/D* poorly differentiated, *W/D* well-differentiated, *M/D* moderate-differentiated, *DOC* docetaxel, *CDDP* cisplatin, *5-FU* 5-fluorouracil

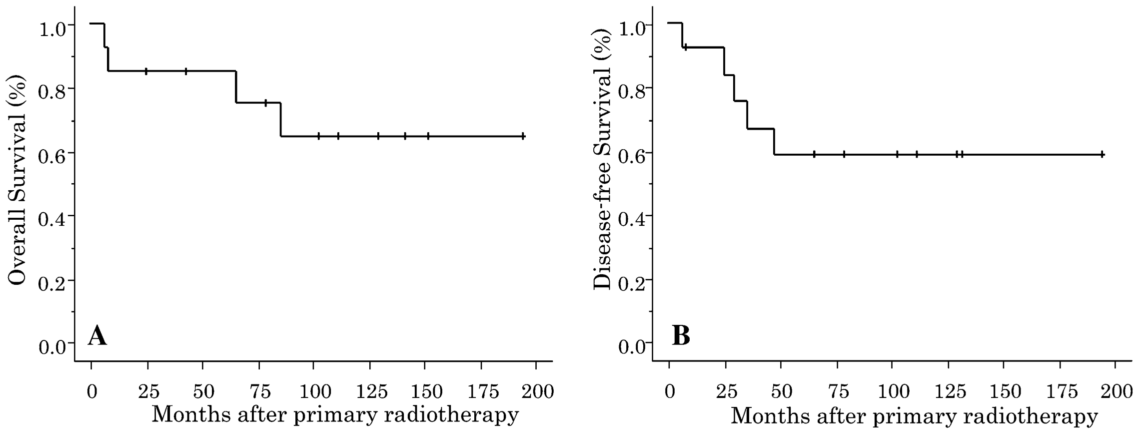


Fig. 1 **a** Overall survival curve of all 13 patients. **b** Disease-free survival curve of all 13 patients

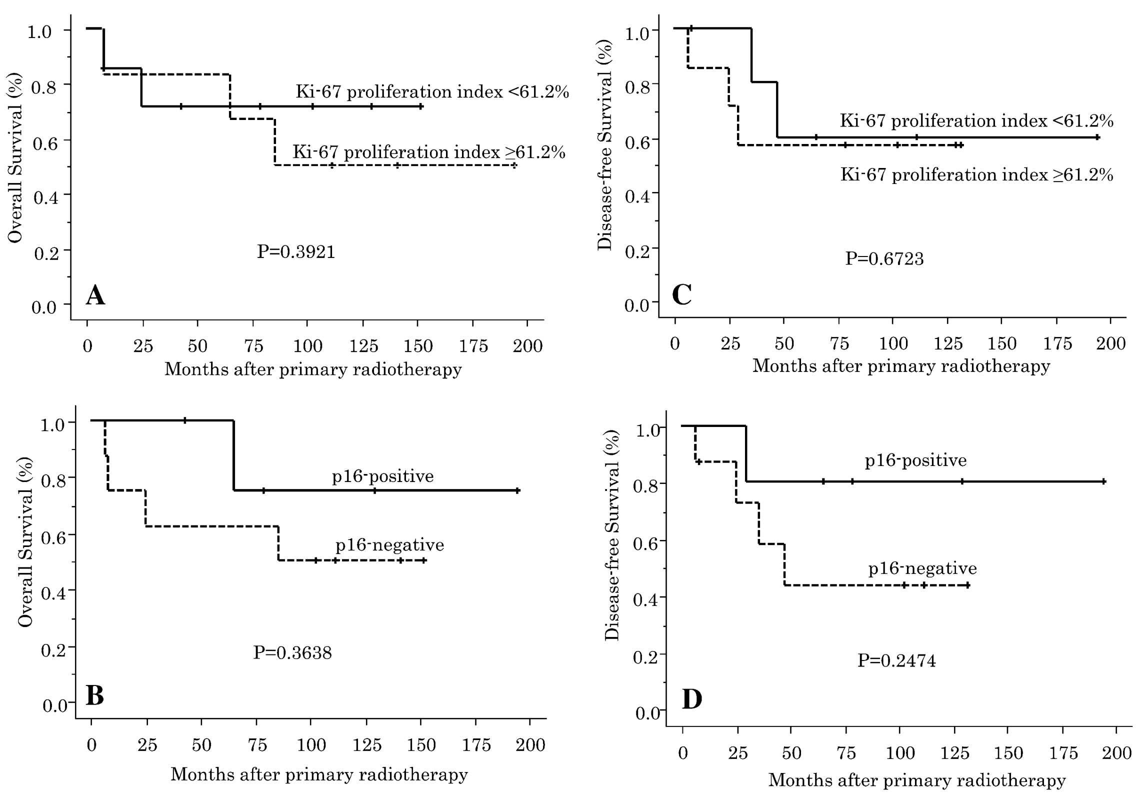
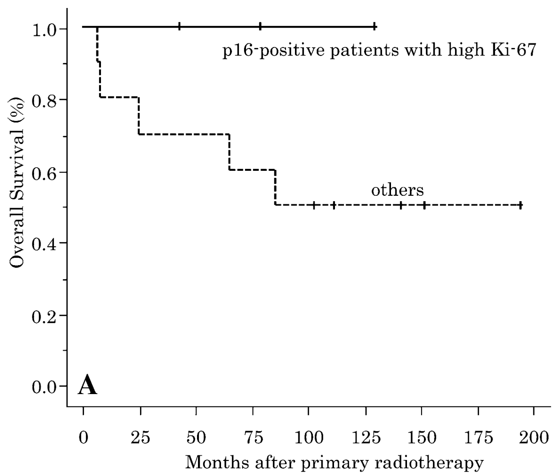


Fig. 2 **a** Overall survival and Ki-67 proliferation index. *p* values were calculated by the log-rank test stratified according to the radiation therapy treatment group. **b** Overall survival of cases developing p16-negative and p16-positive metastases. *p* values were calculated by the log-rank test, with stratification according to the radiation therapy group. **c** Disease-free survival and Ki-67 proliferation index. *p* values were calculated by the log-rank test stratified according to the radiation therapy treatment group. **d** Disease-free survival of cases developing p16-negative and p16-positive metastases. *p* values were calculated by the log-rank test, with stratification according to the radiation therapy group

but one receiving comprehensive irradiation subsequently died of aspiration pneumonia. Four of the five patients with emphysematous changes in the lungs were smokers and the other was a non-smoker.

Three p16-positive patients had a Ki-67 proliferation index ≥ 61.2%; of these, two received comprehensive irradiation and the other ipsilateral irradiation. In the one patient (the sole nonsmoker) receiving comprehensive irradiation,



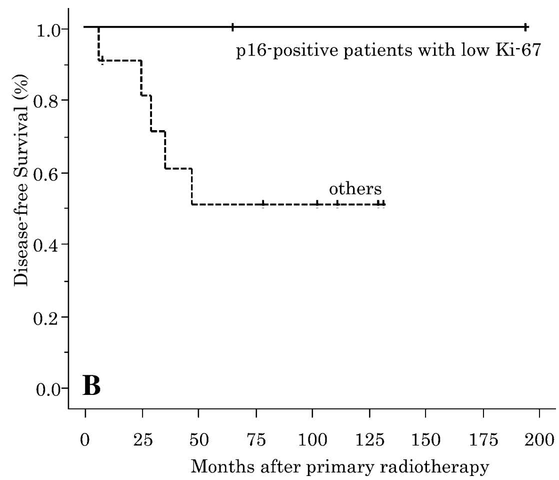


Fig. 3 **a** Overall survival of cases developing p16-positive patients with high Ki-67 and others. *p* values were calculated by the log-rank test, with stratification according to the radiation therapy group. **b** Disease-free survival of cases developing p16-positive patients with low Ki-67 and others. *p* values were calculated by the log-rank test, with stratification according to the radiation therapy group

oropharyngeal cancer developed 2.5 years after completion of the initial treatment. Two of the p16-positive patients had a Ki-67 proliferation index < 61.2%; one received comprehensive irradiation and the other ipsilateral irradiation. Both patients survived for more than 5 years. The one patient receiving comprehensive irradiation died of aspiration pneumonia.

Smokers were defined as those with emphysematous changes detectable on imaging studies. Nine (69.2%) of the 13 patients were smokers and neither their OS rate ($p = 0.6714$) nor their PFS rate ($p = 0.7413$) differed significantly from that of nonsmokers. The *t* test showed a significant association ($p = 0.000$) between smoking and the Ki-67 proliferation index level. The *t* test also showed significant associations of the Ki-67 proliferation index level with both p16-positive status ($p = 0.004$) and p16-negative status ($p = 0.000$) in patients who smoked.

Adverse events

Thirteen patients (13/13, 100%) developed stomatitis or pharyngitis (acute adverse events ≥ grade 2) including three (3/13, 23.1%) with Grade 3 stomatitis and pharyngitis. All three of these patients developed pharyngitis, necessitating hospitalization for medical therapy, and two also developed dermatitis (grade 2 severity) requiring medical therapy. Thus, there were no occurrences of adverse events ≥ grade 4 (Table 2). Hematotoxicity ≥ grade 2 developed in seven patients (8/13, 61.5%) including three with grade 3 (Table 2). However, long-term follow-up revealed that two patients who had received comprehensive irradiation (2/7, 28.6%) died after treatment, because of aspiration pneumonia, which can be neither confirmed nor ruled out as a therapy-related late adverse event.

Primary tumor emergence

We routinely perform random biopsies of the tonsil and tongue base under general anesthesia.

However, three (23.1%) patients had primary tumor emergence after treatment completion; oropharyngeal cancers were detected in two patients 2.5 years and 4 years, respectively, after treatment completion. One patient achieved locoregional control with CRT administered for cancer outside of the radiation field. The other also achieved locoregional control with surgery performed to excise a malignancy that had developed within the radiation field. The one remaining patient had the emergence of a primary tumor in the hypopharynx within the radiation field 3 years after treatment completion. This patient received chemotherapy but died of systemic metastases 50 months later.

Discussion

Treatment guidelines for unknown primary head and neck SCC have not been established. Among patients with head and neck SCC, especially of the oropharynx, those with HPV-positive tumors have a better prognosis. Thus, a clinical study aiming to reduce the intensity of the standard therapy was conducted in such patients [21]. It was demonstrated that patients with HPV-positive unknown primary head and neck SCC also had a better outcomes [1, 22, 23] and studies of less intensive radiotherapy in such patients have recently obtained excellent treatment results [15, 24].

However, one study found a significant difference in survival rate between p16-positive patients with unknown primary head and neck SCC and their p16-negative counterparts [16], while another study did not [17]. Our results showed a trend toward favorable outcomes in p16-positive patients but the difference did not reach statistical significance. This may partially be explained by the small number of p16-positive patients with unknown primary head and neck SCC in this study. Nevertheless, to explore other factors, we examined the associations of treatment outcomes with other factors including Ki-67 expression level, smoking status, age, and radiation techniques. Age was the only significant prognostic factor, i.e., none of the other factors was significantly associated with outcomes. However, all p16-positive patients with a low Ki-67 expression level achieved locoregional control. To date, five studies have examined p16 and Ki-67 expressions, in combination, in patients with head and neck carcinoma [25–29]. Two of these studies identified associations with treatment outcomes [28, 29].

Of two p16-positive patients with a low Ki-67 expression level, only one, who had received comprehensive irradiation, died of aspiration pneumonia. Among late adverse events, serious late toxicities including persistent dysphagia after comprehensive irradiation are common. Moreover, treatment-related deaths due to aspiration pneumonia and other adverse events are not uncommon in some patients receiving comprehensive irradiation. Thus, there are a few reports describing comprehensive irradiation as not being a minimally invasive approach, i.e., that such irradiation strategies are not beneficial to all patients [30, 31]. Ritta et al. [29] reported that HPV-positive head and neck cancer patients with low Ki-67 expression have a good prognosis. Unlike our study, they evaluated HPV status employing HPV DNA in situ hybridization. The NCCN guidelines [32] recommend the use of p16 immunostaining and HPV DNA in situ hybridization as surrogate markers in HPV testing of tumor tissue. The sensitivity of p16 for HPV testing of tumor tissue is reportedly high [33]. However, p16-positive patients with oropharyngeal SCC are rarely negative for HPV [34, 35; 35]. In this study, only two p16-positive patients were regarded as being positive for HPV, thus hampering further speculation regarding the association between these two parameters. However, a review of the literature on treatment outcomes of HPV-positive head and neck cancer patients with low Ki-67 expression suggested that HPV-positive unknown primary cervical lymph node metastasis with low Ki-67 expression may also have a good prognosis. For these patients, it might be feasible to reduce treatment intensity and thereby decrease the risk of late adverse events [15, 24].

All p16-positive patients with a high Ki-67 expression level survived; one of them received comprehensive irradiation and subsequently developed oropharyngeal cancer, which was surgically resected. The patient was still alive without evidence of recurrence or metastasis 6 months after surgery. This patient was initially treated with chemotherapy including cetuximab. The oropharyngeal cancer may have been related to the decrease in the intensity of chemotherapy. Our institution uses chemotherapy regimens mainly consisting of CDDP and DOC in patients with head and neck cancer. The efficacy of DOC has been reported in patients with breast cancer who have a high Ki-67 expression level [36]. Thus, our regimens may offer a better treatment outcome. Although firm conclusions cannot be drawn based on a single case, reduced-intensity chemotherapy appears not to be an appropriate choice for p16-positive patients with a high Ki-67 expression level. Liu et al. [28] reported that the locoregional control rate after CRT appears to be high in HPV-positive head and neck cancer patients with high Ki-67 expression. Two studies analyzing both HPV and Ki-67 in relation to treatment outcomes obtained conflicting results for HPV positivity and Ki-67 expression. Therefore, further studies are needed to examine the association between HPV positivity and Ki-67 expression in not only patients with unknown primary cervical lymph node metastasis but also those with head and neck cancer.

Our results showed a significant association between the Ki-67 expression level and smoking, regardless of p16-positivity or negativity. HPV-associated head and neck cancer can reportedly develop even in patients with smoking habits [37, 38]. As mentioned above, our one patient with oropharyngeal cancer developing after CRT had a high Ki-67 expression level and was positive for p16; however, he was not a smoker, and his disease was not related to smoking. For four patients who smoked, two had a high Ki-67 expression level and were positive for p16, and the other two had a low Ki-67 expression level and were positive for p16. These results are consistent with those of previous studies.

Four patients failed to respond to treatment; three developed a primary tumor after treatment completion and one experienced locoregional failure involving a cervical lymph node. Of the three patients who had primary tumor emergence, two developed oropharyngeal cancer and the other hypopharyngeal cancer. The median Ki-67 expression level was relatively high (56.3% range, [47.3–95.8%]) in these four patients. Our results suggest that the Ki-67 expression level can serve as a guide to considering treatment intensity even in elderly patients, although the treatment methods differed among our cases, such that firm conclusions regarding treatment regimens cannot be drawn based on this study.

Four patients who had particularly low Ki-67 expression levels, ranging from 30 to 39%, responded well to treatment, although one patient died of aspiration pneumonia after completion of comprehensive irradiation. It may be possible to decrease the intensity of treatment in elderly patients with a low Ki-67 expression level, although we cannot analyze this issue in detail due to the small number of such patients in the present study. We plan to further investigate this possibility by accumulating more cases. At present, it is difficult to consider changes in treatment strategies based only on the Ki-67 expression level; however, combined use of biomarkers is a promising strategy which may contribute to selecting a reduced-intensity therapeutic regimen for elderly patients with p16-positive unknown primary head and neck SCC.

Conclusions

This is the first study, to our knowledge, to examine the associations of treatment outcomes with Ki-67 and p16 expressions in patients with unknown primary head and neck SCC. The combination of ki67 expression levels and p16 expression status may allow better prediction of local control than p16 expression status alone. In the future, we intend to accumulate more cases to allow more comprehensive analysis of Ki-67 and p16 as biomarkers for unknown primary head and neck SCC.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Ethical approval retrospective studies This study was approved by the institutional review board, and patient informed consent was obtained. (Trial registration number: Nihon University Itabashi Hospital Clinical Research Center RK-170214-9).

Informed consent Informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article. Documentation of informed consent for treatment was signed by each patient and placed in the patient's medical record.

Research data policy The dataset supporting the conclusions of this article is included within the article.

Consent for publication copy of the written consent is available for review by the Editor-in-Chief of this journal.

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