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ORIGINAL RESEARCH

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Clinical impact of p16 positivity in nasopharyngeal carcinoma

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Abstract

Purpose: The clinical characteristics and prognosis of HPV-related nasopharyngeal cancer (NPC) remain controversial. The relationship between p16 status and outcome was retrospectively investigated in the NPC patients.

Materials and Methods: Between May 2009 and May 2019, 81 NPC patients who received definitive radiation therapy, in a hospital in Japan, were identified and the prognosis was investigated. p16, p53, and Epstein–Barr virus (EBV) status were assessed. Also, circumferential tumor extent in the nasopharyngeal cavity was assessed on a 5-point scale.

Results: Nine and 72 patients were p16-positive and p16-negative, respectively. Fewer patients were EBV-encoded RNA in situ hybridization (EBER-ISH)-positive in the p16-positive group than in the p16-negative group ($p < .01$). Seventy-five patients were nonkeratinizing NPCs, and six patients were keratinizing NPCs. There were two p16-positive patients among the keratinizing NPCs.

The mean circumferential tumor extent scores of 16-positive and p16-negative NPCs were 4.2 and 3.2, respectively with a statistically significant difference ($p = .02$). Two-year progression-free survival (PFS) of p16-positive and p16-negative patients undergoing chemoradiation therapy were 100% and 69%, respectively ($p = .13$).

Conclusion: In this study conducted in Japan, p16-positive NPC patients are minor but not very low, and the proportion of keratinizing NPCs was small. p16-positive NPCs were seen both in keratinizing and nonkeratinizing NPCs. P16-positive NPC had a tendency of better PFS than p16-negative NPC. This better prognosis might be due to the higher radiosensitivity of the p16-positive cell. Additionally, p16-positive NPCs seemed to spread more extensively in circumference along the nasopharyngeal mucosa than p16-negative NPCs.

KEYWORDS
head and neck, HPV, nasopharyngeal cancer, p16, radiation therapy

1 | INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a rare head and neck malignancy in most parts of the world including Japan, whereas it is seen frequently in Southern China, Southeast Asia, the Middle East, North Africa, and the Arctic region.[1] Epstein–Barr virus (EBV) plays a major role in the pathogenesis of NPC,[2,3] and the amount of plasma EBV-DNA is known to be an independent prognostic marker for NPC.[4] On the other hand, several studies suggested that high-risk human papillomavirus (HR-HPV) is also involved in the pathogenesis of some NPC.[5–7] HR-HPV infection is a well-known cause of oropharyngeal squamous cell carcinoma (OPSCC). HPV 16 is found most often in HPV-related OPSCC.[8–10] HPV-related OPSCC is responsive to chemoradiotherapy (CRT) and has a better prognosis than HPV-unrelated OPSCC.[11,12] There have been several reports on the association between HPV and NPC with different results between endemic and non-endemic regions.

The clinical characteristics and prognosis of HPV-related NPC remain controversial to date. In this single institutional retrospective study, the relationship between p16 status, which is a surrogate marker of HPV infection, clinical characteristics, and the outcome were investigated in NPC patients in Japan.

2 | MATERIALS AND METHODS

From May 2009 to May 2020, 95 consecutive NPC patients were treated in the Department of Radiation Oncology, National Cancer Center Hospital. In all patients, NPC was diagnosed by confirming that the lesion was located mainly in the nasopharynx based on local findings by fiberoptic pharyngolaryngoscopy, computed tomography (CT), and magnet resonance imaging (MRI) findings. Fourteen patients were excluded because of the following reasons and the remaining 81 were included in the analysis: 1 patient had a prior history of radiation therapy in the head and neck region and 13 were p16-status unknown. Fiberscope, whole-body CT with/without contrast enhancement, MRI of head and neck area, and positron emission tomography (PET)-CT or PET-MRI were employed for the staging. For TNM classification, the 8th edition was used.

2.1 | Histopathologic analysis

All 81 patients were diagnosed as having squamous cell carcinoma of nonkeratinizing or keratinizing subtypes. Pathological diagnosis was done exclusively by a single expert head and neck pathologist (TM) using hematoxylin–eosin staining with a help of a panel of immunohistochemistry (IHC) staining. IHC was performed for p53 and p16. Strong expression of nuclear p53 or no expression (missense of exon 5–9 of p53, where most known abnormalities occur) was considered to have a p53 gene mutation. Otherwise, it was considered not to have a p53 mutation. For p16, only tumor cells with at least moderate staining intensity both in the nucleus and cytoplasm were classified as positive. The tumor was classified as p16-positive if more than or equal to 75% of the tumor cells were p16-positive. For EBV detection, the RNA in situ hybridization (ISH) technique of the paraffin-embedded section was used for demonstrating EBV-encoded small RNAs (EBERs).

2.2 | Radiation therapy

Radiation therapy was delivered in a conventional fractionation with intensity-modulated radiation therapy (IMRT).

Before radiation therapy, every patient went through a dental checkup and appropriate dental treatment, and a mouthpiece was created. Thermoplastic fixation masks were produced with the mouthpiece in place. Contrast-enhanced CT was performed for planning. The initial clinical target volume ($CTV_{46\ Gy}$) included primary lesion, nasopharynx, enlarged lymph nodes, bilateral level II, III, IV, V, supraclavicular, and retropharyngeal lymph node areas, and they were irradiated up to 46 Gy. Secondary boost radiation was delivered to the $CTV_{70\ Gy}$ including clinically involved lymph nodes, primary lesion, and nasopharynx with margins. $CTV_{70\ Gy}$ was further radiated up to 70 Gy. Supportive care measurements such as gargling with an anti-inflammatory drug, acetaminophen, opioids, and liquid nutrition were prescribed to cope with radiation mucositis. Oral nutritional supplements are prescribed to help maintain the patient's body weight. When a weight reduction of more than 10% from the baseline was found, percutaneous gastrostomy was encouraged.

2.3 | Chemotherapy

Three cycles of cisplatin (80 mg/m^2, every 3 weeks) were administered concurrently with the IMRT. In the patients with renal dysfunction, carboplatin was used instead of cisplatin. In the patients who had a good tumor shrinkage at the end of concurrent chemoradiation therapy (cCRT), the merits and demerits of adjuvant chemotherapy were explained to the patients, and the decision to perform adjuvant chemotherapy was made based on the patient's decisions. Adjuvant chemotherapy was administered in 37 patients, which consisted of 1–3 cycles of cisplatin (70 mg/m^2 on day 1) and 5-fluorouracil (5-FU) (700 mg/m^2 on days 1–5) every 4 weeks (FP).

Because a phase III clinical trial demonstrated better 3-year progression-free survival (PFS) with induction TPF (cisplatin,

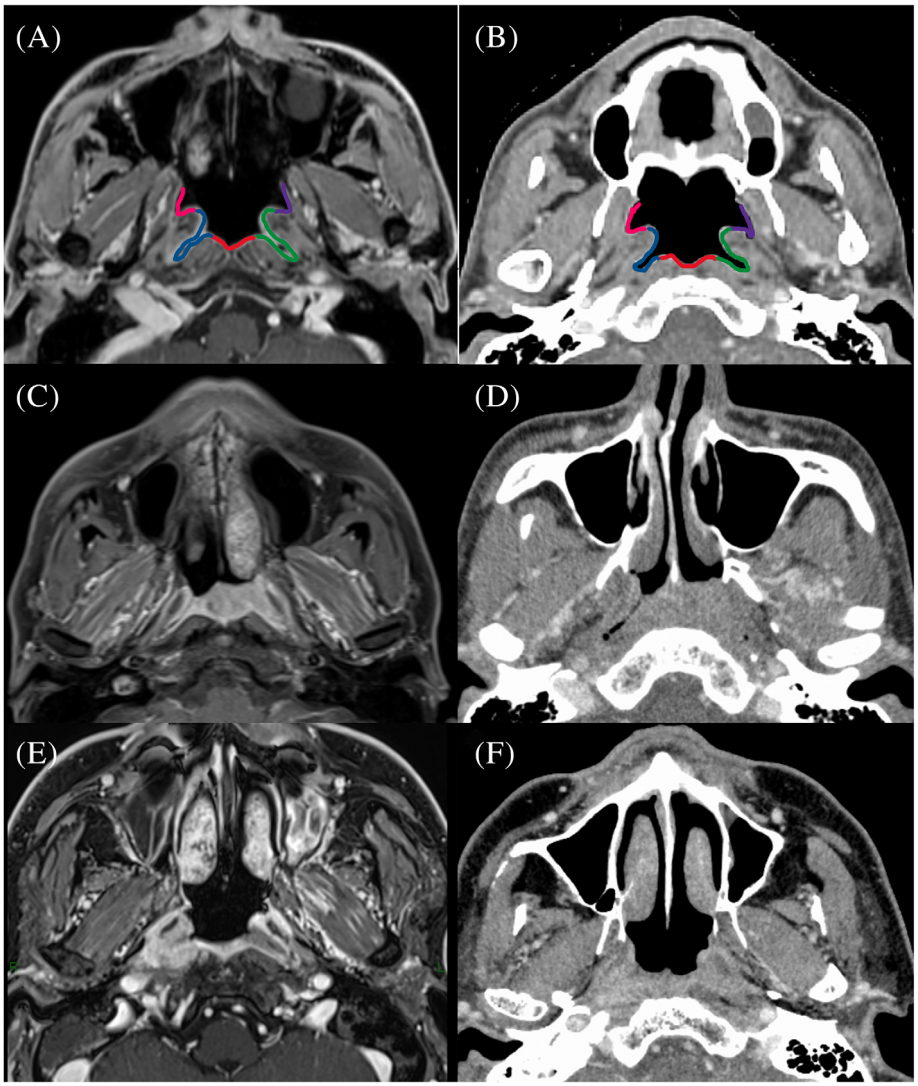

FIGURE 1 (A) shows a T1-weighted contrast-enhanced magnet resonance imaging (MRI) axial section of the normal nasopharynx. (B) shows enhanced computed tomography (CT) of the normal nasopharynx. The colored lines in images A and B demonstrate five divided areas of the normal nasopharynx of the same patient. The red line shows the posterior wall, the blue line the right Rosenmüller fossa, the green line the left Rosenmüller fossa, the pink line the right Eustachian tube opening, the purple line the left Eustachian tube opening (C) shows a T1-weighted contrast-enhanced MRI axial section of a nasopharyngeal cancer (NPC) patient with tumor invasion in the posterior wall, the bilateral Rosenmüller fossae, and the left Eustachian tube mouth, and a circumferential tumor extent score of 4. (D) shows enhanced CT of the same patient as C. (E) shows a T1-weighted contrast-enhanced MRI axial section of a NPC patient with tumor invasion in the right Rosenmüller fossa, and the right Eustachian tube mouth, and a circumferential tumor extent score of 2. The (F) shows enhanced CT of the same patient as image E

fluorouracil, and docetaxel) followed by cCRT compared with cCRT alone,[13] since July 2017, patients with T4 or N3 were managed by 2 or 3 cycles of induction TPF (docetaxel 70 mg/m^2 on day1, cisplatin 70 mg/m^2 on day 1, and 5-FU 750 mg/m^2 on days 1–5, every 3 weeks) followed by cCRT. Patients who underwent induction TPF did not receive adjuvant chemotherapy after cCRT.

2.4 | Circumferential tumor extent score

Circumferential tumor extent along the nasopharyngeal mucosa was assessed by the number of anatomical landmarks involved by NPC in axial CT and/or MRI. The nasopharynx was divided into five areas: left and right Rosenmuller fossae, left and right Eustachian tube openings, and the posterior wall of the nasopharynx. Circumferential tumor extent score was defined by the number of involved areas in the nasopharynx which was scored from 1 to 5, with 5 representing the circumferential involvement of the whole nasopharyngeal cavity mucosa. Figure 1 demonstrates the scoring method of circumferential tumor extent score in this report.

2.5 | Statistics

Overall survival (OS) was calculated from the start of radiation therapy until the date of death and living patients at the last follow-up visit were censored. Progression-free survival (PFS) was calculated from the start of radiation therapy with any disease relapses and death from any cause considered as an event. Locoregional control (LRC) was calculated from the start of radiation therapy until the date of locoregional relapse. Dead patients without locoregional relapse were censored on the day of death. Survival curves were calculated by the Kaplan–Meier method with a difference evaluated by the log-rank test. Survival analyses were conducted only in the patients treated by chemoradiation therapy. The patients undergoing only radiation therapy were excluded from the survival analyses. Differences between continuous and categorical variables were tested with t-tests and chi-square tests, respectively. Categorical variables containing 5 or fewer patients were tested with Fisher's exact test. All analyses were performed using Excel Statistics for Windows.

This study was approved by the Institutional Review Board of our hospital (approved number 2017–091). Written informed consent to

TABLE 1 Patient Characteristics and Treatment ($n = 81$)

	Number		*p*-value	
	ALL	p16-positive	p16-negative	(p16-positive vs p-16 negative)
Sex				
Male	59	5	54	0.25
Female	22	4	18	
Median Age (range)	56 years (12–85)	54 years (39–73)	57 years (12–85)	0.82
Smoking History				
yes	44	6	38	0.46
no	32	2	30	(yes vs. no)
unknown	5	1	4	
Histology				
nonkeratinizing	75	7	68	0.13
keratinizing	6	2	4	
Stage				
I	4	1	3	0.20
II	15	3	12	(I–II vs. III–IV)
III	30	3	27	
IV	32	2	30	
T stage				
T1	23	4	19	0.49
T2	20	2	18	(T1-2 vs. T3 -4)
T3	15	2	13	
T4	23	1	22	
N stage				
N0	9	2	7	0.06
N1	20	4	16	(N0-1 vs. N2-3)
N2	28	1	27	
N3	24	2	22	
P53				
Wild-type	50	6	44	1.00
mutated	31	3	28	(wild-type vs. mutated)
EBER-ISH-				
infected pattern	65	3	62	<0.01
uninfected pattern	15	6	9	(infected pattern vs. uninfected pattern)
unexamined	1	0	1	
Treatment				
Radiation therapy alone	9	1	8	1.00
Concurrent chemotherapy Cisplatin	66	8	57	(Concurrent chemotherapy vs. Radiation alone)
Carboplatin	7	0	7	
Induction chemotherapy				
yes	12	2	10	0.62
no	69	7	62	
Adjuvant therapy				
yes	40	1	36	0.03
no	41	8	36	
Median irradiation duration (range)	51 days (48–61)	51 days (48–55)	52 days (50–61)	

(Continues)

TABLE 1 (Continued)

	Number		*p*-value	
	ALL	p16-positive	p16-negative	(p16-positive vs p-16 negative)
Median total radiation dose (range)	70 Gy (59.4–82)	70 Gy (70–72)	70 Gy (59.4–82)	
Tumor Extent Score				
1	8	0	8	
2	10	0	10	
3	29	3	26	
4	18	1	17	
5	16	5	11	
mean	3.3	4.2	3.2	0.02
mean of T1-2	3.2	4.7	2.9	<0.01

Abbreviations: EBER-ISH: Epstein–Barr virus-encoded small RNA in situ hybridization.

participate in this retrospective study were obtained from all the patients, and this study complied with the institutional review board protocols.

3 | RESULTS

The median follow-up period was 48.0 months (range 4.2–141.9 months). Patients' demographics were summarized in Table 1. Nine patients were p16-positive, and the remaining 72 patients were p16-negative. As for the clinical stage, T stage, and N stage of NPC, there could not be seen any statistically significant differences between p16-positive and p16-negative tumors. There were no significant relationships between p16 status and p53 mutation and histological classification (keratinizing or nonkeratinizing). EBER-ISH was examined in 80 patients.

Seventy-five patients were nonkeratinizing NPCs, and the remaining six patients were keratinizing NPCs in this study. Whereas the nonkeratinizing type showed positive EBER-ISH in 66 of 75 patients, none of the six patients with keratinizing type was EBER-ISH-positive ($p < .01$). Although only two, there were p16-positive patients among the keratinizing NPCs (Table 1).

The positivity of EBER-ISH was significantly fewer in the p16-positive patients in contrast to the p16-negative patients ($p < .01$). Double positivity of EBER-ISH and p16 was seen in three patients.

The mean circumferential tumor extent score of p16-positive and p16-negative NPCs were 4.2 and 3.2, respectively with a statistically significant difference ($p = .02$) (Table 1). The mean circumferential tumor extent scores of T1-2 and T3-4 were 4.7 and 4, respectively, for p16-positive patients. For p16-negative patients, the mean circumferential tumor extent scores of T1-2 and T3-4 were 2.9 and 3.7, respectively. In T1-2 cases, there was a significant difference in tumor extent scores between p16-positive and p16-negative patients ($p < .01$). The p16-positive patients showed a more extensive circumferential spreading along the nasopharyngeal mucosa, in comparison to the p16-negative patients.

With respect to the treatment, concurrent chemotherapy regimens, the conduct of neoadjuvant chemotherapy, and radiation methods were not different between p16-positive and negative patients. However, the adjuvant chemotherapy was performed on only one of the p16-positive patients, whereas about half of the p16-negative patients underwent adjuvant chemotherapy ($p = .03$). As adjuvant chemotherapy after cCRT, one p16-positive patient received 3 cycles of FP. Among p16-negative patients, 34 patients received 1–3 cycles of FP and 2 patients received carboplatin+5-FU without cisplatin due to renal function and comorbidity.

Two-year OS, PFS, and LRC rate of 72 patients treated by chemoradiation therapy were 92%, 77%, 97%, respectively (Table 2). Two-year PFS for the patients with p16-positive and p16-negative was 100% and 69%, respectively ($p = .13$) (Figure 2). Two-year LRC rate for the patients with p16-positive and p16-negative was 100% and 97%, respectively ($p = .47$) (Figure 3). In the patients who did not undergo adjuvant chemotherapy, 2-year OS, PFS, and LRC were all 100% for p16 positive and 96%, 89%, and 96% for p16-negative patients. Statistically significant differences were not observed because of the small number of patients.

4 | DISCUSSION

Most undifferentiated NPC is related to infection of EBV, but it has been reported that some NPCs are related to HPV infection, even after meticulous examinations excluding the possibility of OPSCC invading the nasopharynx. HPV-related OPSCC has been confirmed to have a better prognosis in comparison to the HPV-non-related OPSCC.[11,12] Several studies also suggested that patients with HPV-related head and neck squamous cell cancers (HNSCCs) of the oral cavity, hypopharynx, or larynx have favorable outcomes in comparison to the patients with HPV-unrelated counterparts.[14,15] These findings

TABLE 2 PFS, OS, and LRC for patients who received cCRT with or without adjuvant chemotherapy

	Adjuvant chemotherapy	p16 status	*n*		Two-year OS	Two-year PFS	Two-year LRC
All patients			72		92%	77%	97%
		positive	8		100%	100%	100%
		negative	64		89%	69%	97%
				p-value	.39	.13	.47
	Performed	positive	1		100%	100%	100%
		negative	36		85%	58%	97%
				p-value	.82	.62	.87
	Not performed	positive	7		100%	100%	100%
		negative	28		96%	88%	96%
				p-value	.62	.17	.24

Abbreviations: LRC, locoregional control; PFS: progression-free survival; OS, overall survival.

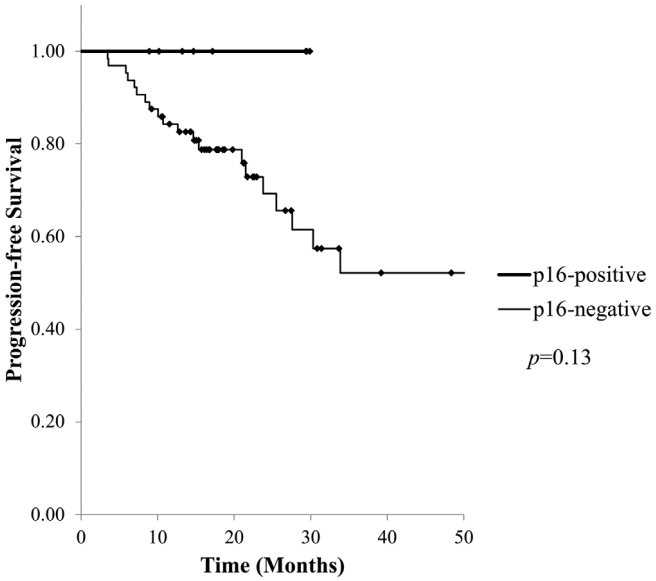

FIGURE 2 Progression-free survival (PFS) curves comparing 72 p16-positive and negative nasopharyngeal cancers (NPCs) treated by chemoradiation therapy. Two-year PFSs for patients with p16-positive and p16-negative NPCs were 100% and 69%, respectively ($p = .13$)

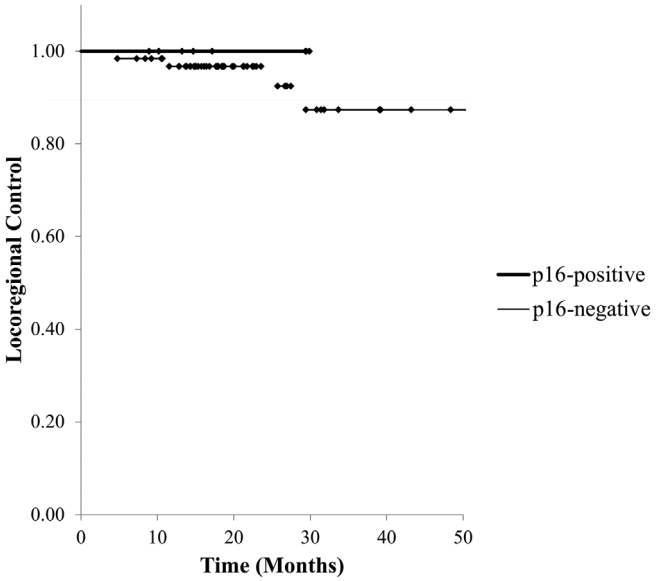

FIGURE 3 Locoregional control (LRC) curves comparing 72 p16-positive and p16-negative nasopharyngeal cancers (NPCs) treated by chemoradiation therapy. Two-year LRCs for patients with p16-positive and p16-negative NPCs were 100% and 97%, respectively ($p = .47$)

raise the question of whether HPV may play a role in the pathogenesis and prognosis of NPC.

Although HPV status in tumors can be determined by several assays, including HPV DNA detection by ISH or polymerase chain reaction (PCR), p16 protein expression diagnosed by IHC is used frequently as a surrogate marker of HPV infection. P16 is a cyclin-dependent kinase inhibitor, which is overexpressed as a compensatory phenomenon of the inactivation of p53 and retinoblastoma protein by HPV infection. Therefore, the existence of HPV infection is indirectly demonstrated by the expression the of p16 protein.[11,16] Although there are some reports which showed a discrepancy in HPV infection detected by DNA analysis and p16 expression in NPC,[17–19] numerous reports have demonstrated concordance between p16 IHC and DNA analysis.[7,20–22] Therefore, p16 expression by IHC was used as a surrogate for the HPV infection in this study.

The rate of double infection of HPV and EBV appeared to be rare[23,24] and the rate in Asian people seems to be 0.6%–10%.[21,25] In the current study, double infection of HPV and EBV was observed in 4% of the patient. Because it is known that EBV latent membranous protein 1 can block p16 expression,[26] the actual number of patients with double infection could be larger in this series.

There seems to be a different incidence rate of HPV-related NPC in endemic and non-endemic areas of NPC. In endemic areas such as Southern China, where type II (differentiated nonkeratinizing carcinoma) and type III (undifferentiated nonkeratinizing carcinoma) are predominant, the prevalence of HPV(+) was relatively low (7.7%), and

HPV(+)/EBV(−) patients showed better prognosis after radiotherapy.[22]

In non-endemic areas, where type I (keratinizing squamous cell carcinoma) occurs with a relatively high incidence, many reports show that the proportion of p16 positivity is relatively high (5%–90%).[7,17,18,22,27–29] In non-endemic areas, it appears that HPV-related NPC of keratinizing squamous cell carcinoma is included predominantly, but the prognosis of HPV-related NPC has been variously reported.[19,23,24]

The current study showed that 11% of NPC patients are p16-positive, which corresponds to the character of non-endemic NPC. The proportion of keratinizing NPCs in this study was small (7.4%) and deviated from the typical for non-endemic areas.

Although the number of cases is only nine, all p16-positive NPC patients remained without disease progression, even though only one patient underwent adjuvant chemotherapy after cCRT (Table 2). The prognosis of p16-positive NPC seems to be favorable in this study. The increased intrinsic radiosensitivity in HPV-positive HNSCC is reported as specifically due to impaired repair capacity of radiation-induced double-strand breaks. Also, irradiation of HPV-positive cell lines induces a G2/M arrest, and can thereby effectively repress cell proliferation and oxygen consumption.[30–32] Therefore, favorable 2-year PFS of p16-positive NPC in this current study may be due to the good radiosensitivity of p16-positive cells.

There could not be seen any significant relationships between p16 expression and T stage. Whereas T stage in TNM classification is mainly dealing with the depth of tumor involvement, circumferential tumor extent score expresses extent of the circumferential growth along the nasopharyngeal mucosa. The circumferential tumor extent score in this study revealed that p16-positive NPC was likely to involve the nasopharyngeal mucosa more extensively in contrast to p16-negative NPC. The mean tumor extent score of p16-positive and -negative NPCs was 4.2 and 3.2, respectively, with a statistically significant difference ($p = .02$) (Table 1). Also, the p16-positive patients showed more extensive circumferential involvement of nasopharyngeal mucosa from the early T stages. Patterns of tumor spread might be different between p16-positive and -negative NPCs.

There are several limitations to our retrospective study. Because of the paucity of NPCs, and especially, p16-positive NPCs, we could not demonstrate favorable survivals and LRC of p16-positive NPCs with a statistical significance. Additionally, the patients were treated with inhomogeneous treatment strategies, radiation therapy with or without several chemotherapy regimens. In this study, only the patients with a good tumor shrinkage at the end of cCRT and who gave a consent, were treated by adjuvant chemotherapy. As a result, there was only one patient treated by adjuvant chemotherapy in the p16-positive patients in this study. It may be treatment selection bias.

For HPV infection analysis, p16 protein IHC was utilized instead of DNA analysis in this study, and concordance between p16 expression and HPV DNA analysis was not studied.

5 | CONCLUSION

In this study conducted in Japan, a non-endemic area for NPC, p16-positive NPC patients are minor but not very low, which corresponds to the character of non-endemic NPC. The proportion of keratinizing NPCs in this study was small and deviated from the typical for non-endemic areas. p16-positive NPCs were seen both in keratinizing and nonkeratinizing NPCs.

P16-positive NPCs have a tendency of better PFS than p16-negative NPCs. This better prognosis might be due to good radiosensitivity of p16-positive cell. Additionally, there seems to exist a difference in the pattern of tumor spread in circumference along the nasopharyngeal mucosa between p16-positive and -negative NPCs.

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CONFLICT OF INTEREST

Dr. Itami reports personal fees from HekaBio, AlphaTAU, and Palette Science, research grants from Elekta, and ITOCHU outside the submitted work. Dr. Igaki reports grants and personal fees from HekaBio, grants from CICS, grants from Elekta KK, personal fees from AstraZeneca, personal fees from Itochu, personal fees from HIMEDIC, personal fees from Varian, outside the submitted work. Dr. Inaba reports personal fees from Boston Scientific Japan, outside the submitted work. Dr. Kashihara reports personal fees from Astra Zeneca, outside the submitted work. The other authors have no conflicts of interest.

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