Human papillomavirus genotype affects metastatic rate following radiotherapy in patients with uterine cervical cancer

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Abstract. Human papillomavirus (HPV) infection is well known as a major etiological risk factor associated with carcinogenesis in uterine cervical cancer. However, few reports have investigated the association between HPV genotype and outcome in patients with uterine cervical cancer following radiotherapy (RT). The aim of the present study was to investigate the association between the HPV genotype and clinical outcome following RT in Japanese patients with uterine cervical cancer. Between November 2001 and August 2006, 157 Japanese women with uterine cervical cancer were treated with RT or concurrent chemoradiotherapy with curative intent. Pretreatment, formalin-fixed, paraffin-embedded biopsies were obtained from 83 patients. HPV genotypes were determined using the polymerase chain reaction method. Patients were categorized, according to HPV L1 protein sequence homology, into the HPV α-9 (HPV 16, 31, 33, 52, and 58), HPV α-7 (HPV 18, 39, 45, 59, and 68) or 'other' (HPV 51 and 56) groups. Associations between HPV genotype and clinical outcome following RT were evaluated. A total of 54 (65.1%) tumors were HPV α-9-positive, 13 (15.7%) were HPV α-7-positive, 2 (2.4%) were categorized under 'other' and 14 (16.9%) were HPV-negative. There were no significant differences in age, FIGO stage, regional lymph node metastases rate at diagnosis, or concurrent chemotherapy administration between the HPV α-9 and α-7 groups. The median follow-up period was 52 months (range, 2-156 months). The 5-year disease-free survival rates were 54.5 and 30.8% in the HPV α-9 and α-7 groups, respectively (P=0.034), and the 5-year distant metastasis rates were 38.0 and 69.2%, respectively (P=0.015). There were no significant differences in the 5-year local control or overall survival (OS) rates between the two groups. HPV genotype affected the 5-year distant metastatic rate, however not the 5-year local control or OS rate in patients with uterine cervical cancer following RT.

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Introduction

Uterine cervical cancer (CC) is the second most common cancer among women, worldwide (1). The annual global incidence of CC in 2012 was 528,000 cases, and the annual global mortality rate was 266,000 deaths, with 85% of cases occurring in developing countries, where CC is a leading cause of cancer-related death in women (2). Oncogenic type human papillomavirus (HPV) infection is a major etiologic risk factor (3) associated with carcinogenesis (4). More than 100 HPV types have been identified, with a subset of these being classified as high risk. HPV 16 and HPV 18 are the most commonly detected genotypes occurring in 71% of invasive CCs (5). Current detection methods have uncovered a HPV prevalence of 95-100% in women with CC (6,7).

The primary treatment strategy for uterine CC consists of surgery or radiotherapy (RT). Surgery is typically reserved for early-stage disease and small lesions including stage IA, IB1, and selected IIA1 diseases (8). Based on the results of randomized clinical trials, concurrent chemoradiotherapy (CCRT) has become the primary treatment for stage IB2 to IVA disease (9,10). Only a few studies have assessed specific treatments for cervical adenocarcinomas, but they are typically treated in a similar manner to cervical squamous cell carcinomas (11).

Several studies have demonstrated a relationship between HPV subtype and outcome in patients who underwent primary

surgery (12-15). These studies showed that, of all HPV genotype infections, HPV 18 infection was associated with more aggressive CC. By contrast, some studies showed that patients with HPV 33-related tumors had favorable outcomes (16,17). Few reports have investigated the relationships between HPV genotype and outcome in patients with uterine CC after RT.

Oncogenic HPV types can be classified phylogenetically according to their L1 open reading frame (18). When HPVs share 60-70% nucleotide identity, they are clustered into the same species. Two HPV species, α-7 (HPV 18, 39, 45, 59, and 68) and α-9 (HPV 16, 31, 33, 35, 52, and 58), account for >80% of all CCs (19). Wang *et al* reported that patients with HPV α-7-positive CC had worse local control (LC) after RT compared with HPV α-9-positive patients (20). However, by performing assays in clonal CC cell lines, Hall *et al* revealed that poor prognosis associated with HPV species might not be explained by intrinsic radiosensitivities because cells harboring the HPV α-9 and α-7 species had similar radiosensitivities (21). In the present study, we investigated the frequencies of HPV genotypes and species distribution in Japanese CC patients who underwent RT at our institution. We then evaluated the relationships between LC after RT and HPV species in these patients.

Materials and methods

Patient characteristics. Between November 2001 and August 2006, 157 patients with uterine CC were treated with RT or CCRT with curative intent in our institution. Pretreatment, formalin-fixed, paraffin-embedded biopsies were obtained from 83 patients. All 83 patients provided written informed consent according to the institutional regulations. The concept of the present study was approved by the Institutional Review Board (ID: NIRS-06-004). The patients' characteristics are listed in Table I.

HPV genotyping. DNA was extracted from formalin-fixed paraffin-embedded tumors using the DEXPATTM system (Takara Bio, Inc., Otsu, Japan). Extracted DNA were further purified using the QIAamp DNA Micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA was also isolated from biopsies frozen in RNAlater (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using the Genomic-tip 100/G kit (Qiagen GmbH). HPV genotypes were determined using the polymerase chain reaction (PCR) method (22) and the Linear Array HPV Genotyping test according to the manufacturer's instruction (Roche Molecular Diagnostics, Pleasanton, CA, USA) (23,24). Samples that contained more than about 70% of tumor cells were used in our study (22). The genomic DNA samples analyzed for the presence of HPV DNA were also used for searching structural variations of tumor suppressor gene candidates including p53. The DNA samples were either analyzed directly or after mixing with equal amounts of reference DNA, which was obtained commercially (Promega Corporation, Madison, WI, USA). This reference DNA that did not contain HPV genome but had p53 gene could be used to test the HPV detection manners used here. More specifically, mixing the reference DNA can provide us information about how the DNA sample is amplifiable or the DNA sample does not contain any inhibitor for the reactions. The reference DNA also work as a negative control of the detection of HPV genome. We used some cervical cancer patients' tumor DNA containing HPV genome, which were detected in our previous experiments, as positive controls. The data indicated that the DNA samples used here showed good quality for PCR (24). Sixteen oncogenic HPV genotypes were evaluated including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 71, and 82 (23,24).

Treatment. Patients were treated using a combination of external beam RT and high-dose rate brachytherapy. External whole pelvis irradiation was performed using the anteroposterior-posteroanterior field or box techniques. The median external beam RT dose was 50.0 Gy with 1.8-2.0 Gy/fraction. Central shielding (3-cm width) was used, yielding total doses of 19.8-20.0 Gy for stage IB1 and II disease (tumor diameter ≤4 cm) or 30.0-30.6 Gy (or 39.6-40.0 Gy for bulky cases) for stage IB2 and II, IIIB, and IVA disease (tumor diameter >4 cm). Pelvic irradiation with central shielding was performed to a total dose of 49.8-50.6 Gy. In patients with gross lymph node metastases, an additional 6.0-10.0 Gy boost was applied to the lesion.

High-dose rate brachytherapy was performed using the Ir-192 remote after loading system (microSelectron HDR; Elekta Instrument AB, Stockholm, Sweden). A Fletcher-Suit Asian Pacific applicator set (tandem and half-size ovoids) was used in the majority of the patients. If a patient had severe vaginal invasion, a vaginal cylinder was used in place of the tandem and ovoids. An in-room CT on-rail brachytherapy system was installed in 2001 at our institution, and CT-based brachytherapy was introduced for advanced cases that year. According to dose distribution generated by radiography-based 2D planning, the dose was administrated to Point A in 4 fractions with 6.0 Gy/fraction. Dose adaptation was performed based on dose changes at Point A for advanced cases. We modified the dose at Point A so that a 6 Gy isodose line could cover the tumor. For patients with FIGO stage IB-II or III-IVA tumors >4 cm in diameter or those with pelvic lymph node metastasis, cisplatin-based chemotherapy was administered concurrently during RT. Exclusion criteria for chemotherapy included being >70 years old or having severe concomitant diseases.

Follow-up. Patients were followed-up every 1-3 months for the first 2 years and every 3-6 months for the subsequent 3 years. Disease status was assessed at every follow-up examination by a physical examination, with or without appropriate laboratory and radiologic tests. Suspected recurrent CC was confirmed by biopsy whenever possible.

Statistical analyses. LC was measured from the date of therapy initiation to the date of the first local recurrence or the most recent follow-up. Distant metastasis-free survival (DMFS) was measured from the date of therapy initiation to the date of detection of the first distant metastasis. Disease-free survival (DFS) was measured from the date of initiation of therapy to the date of the first recurrence was detected regardless of recurrent site. Overall survival (OS) was measured from the date of the therapy initiation to the date of death from any cause or the

Table I. Patient characteristics.

Characteristics (n=83)	Number (%)
Age, years (range)	59 (32-83)
Histology	
Squamous cell carcinoma	61 (73.5)
Adenocarcinoma	21 (25.3)
Small cell carcinoma	1 (1.2)
FIGO stage	
IB	8 (9.6)
II	13 (15.7)
III	45 (54.2)
IV	17 (20.5)
Pelvic LN metastasis	
Negative	40 (48.2)
Positive	43 (51.8)
PALN metastasis	
Negative	69 (83.1)
Positive	14 (16.9)
Concurrent chemotherapy	
No	36 (43.4)
Yes	47 (56.6)

FIGO, International Federation of Gynecology and Obstetrics; LN, lymph node; PALN, Para-aortic lymph node.

Table II. Frequency of HPV genotypes in 83 patients.

Parameter	Type	n	%
Single HPV	16	26	32.3
	18	6	7.2
	31	5	6.0
	33	2	2.4
	35	1	1.2
	39	2	2.4
	45	2	2.4
	51	1	1.2
	52	6	7.2
	56	1	1.2
	58	10	12.0
	59	1	1.2
	68	1	1.2
Multiple HPV	16, 58	1	1.2
	16, 66	1	1.2
	18, 71	1	1.2
	56, 58	1	1.2
	58, 82	1	1.2
HPV negative		14	16.9

HPV, human papillomavirus.

most recent follow-up. The actuarial rates of LC, DMFS, DFS, and OS were calculated using the Kaplan-Meier method and compared using the log-rank test. The Mann-Whitney U test was used to evaluate associations with and between clinico-pathological variables. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 23.0 for Mac (SPSS, Inc., Chicago, IL, USA).

Results

HPV genotype frequencies. Among 83 patients, 14 patients (16.9%) had HPV-negative tumors. For the 69 HPV-positive patients, 16 HPV genotypes were detected. HPV genotypes are summarized in Table II. The 5 most prevalent genotypes included HPV 16, 58, 18, 52, and 31. The patients were categorized into the HPV α-7 (HPV 18, 39, 45, 59, and 68 genotypes), HPV α-9 (HPV 16, 31, 33, 35, 52, and 58 genotypes), or 'other' (HPV 51 and 56) groups. Patients with multiple HPV infections, but who had at least one HPV α-7 or α-9 species were categorized into the HPV α-7 or α-9 groups, respectively. Fifty-four patients comprised the HPV α-9 group, 13 comprised the HPV α-7 group, and 2 were included in the 'other' group.

Associations between HPV genotype and RT outcomes. The median follow-up time after treatment initiation was 52 months (range, 2-161 months). There were no significant differences between the HPV α-9 and α-7 groups regarding age, FIGO stage, the lymph node metastases rate at diagnosis, or concurrent chemotherapy administration, whereas CC histology was significantly different between the two groups (P<0.01). The comparison of patients' characteristics between the HPV α-9 and α-7 groups are shown in Table III.

By the end of the study, among all 83 patients, 40 (48.2%) had no recurrence, and 43 (51.8%) had experienced treatment failure including 4 local failures and 34 distant relapses, and 5 patients had both. Forty patients were alive without disease, 6 patients were alive after successful salvage, and 37 patients were dead. Of the 37 patients who died, 34 (91.8%) died due to CC. The 5-year LC, DFS, DMFS, and OS rate in all cases were 97.3, 45.2, 49.5, and 61.0%, respectively (Fig. 1). There were no significant differences in the 5-year LC (P=0.242) and OS rates (P=0.352) between the HPV α-7 and α-9 groups (Fig. 2). By contrast, the HPV α-7 group had significantly inferior 5-year DFS and DMFS rates compared with the HPV α-9 group (P=0.027 and 0.016, respectively). The 5-year DMFS in patients with squamous cell CC showed a tendency toward inferiority in the HPV α-7 group compared with the HPV α-9 group, although the difference was not significant (P=0.108).

The comparison of patients' characteristics between the HPV positive and negative groups are shown in Table IV. There were no statistical differences in age, FIGO stage, presence of LN metastases, or administration of chemotherapy. However, there was statistical difference in histology; more than 70% patients who were judged as HPV negative had adenocarcinoma. Although relatively poorer outcomes in the HPV negative group were observed, there were no significant differences between the HPV positive and negative groups in the 5-year LC (P=0.620), OS (P=0.497), DFS (P=0.128), or DMFS rates (P=0.079) (Fig. 3).

Table III. Comparison of patients' characteristics between HPV α-9 and α-7.

Characteristics (n=83)	HPV α-7 (n=13)	HPV α-9 (n=54)	P-value
Age, years (range)	52 (38-74)	61 (36-82)	0.10
Histology			<0.01
Squamous cell carcinoma	7	48	
Adenocarcinoma	5	6	
Small cell carcinoma	1	0	
FIGO stage			0.22
IB	0	5	
II	1	12	
III	9	26	
IV	3	11	
Pelvic LN metastasis			0.19
Negative	3	28	
Positive	10	26	
PALN metastasis			0.09
Negative	10	46	
Positive	3	8	
Concurrent chemotherapy			0.09
No	3	30	
Yes	10	24	

HPV, human papillomavirus; FIGO, International Federation of Gynecology and Obstetrics; LN, lymph node; PALN, Para-aortic lymph node.

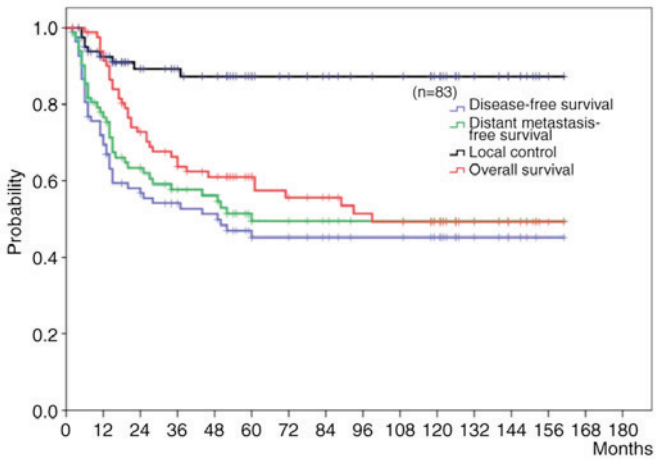

Figure 1. Survival outcome curves The Kaplan-Meier curves show local control (black line), disease-free survival (blue line), distant metastasis-free survival (green line), and overall survival (red line) rates in all cases.

Discussion

The present study is the first to show the relationship between HPV genotypes and clinical outcomes after RT in Japanese patients with uterine CC. The majority of patients in the present study were HPV-positive, with high proportions of HPV 16, 58, 18, 52. Although the number of patients in the present study was small, the findings were consistent with previous reports regarding the global incidence of HPV genotypes in uterine CC (5). De Sanjose *et al* found that HPV 16 and 18 were the most common genotypes for uterine CC worldwide (5). However, Salehi-Vaziri *et al* reported that the most common HPV genotypes in Iranian women were HPV 16 and 53 (25). García Muentes *et al* reported that the most common HPV genotypes in Ecuadorian women were HPV 16 and HPV 33 (26). Wang *et al* indicated that the most common HPV genotypes in Chinese women were HPV 16, 52, and 58 (27). Moreover, a high prevalence of HPV 52 and 58 genotypes in Southeast Asian countries has been reported previously (28-30). Therefore, HPV genotype distribution in uterine CC varies geographically.

Out of 83 cervical cancers analyzed, 14 (16.9%) were found to be HPV-negative. This fig. is higher than previously reported (20,21). Kusanagi *et al* reported that HPV has been rarely detected in some types of adenocarcinoma of the uterus cervix (31). The present study included a larger number of patients (25.3%) with adenocarcinoma. Therefore, relatively higher proportions of HPV-negative patients may contribute to the higher proportion of adenocarcinoma in the present study. However, there was no significant clinical effect of HPV presence on the outcomes of patients with CC who underwent RT.

Meanwhile, the present study showed the significant clinical effect of HPV genotypes on outcome in patients with CC patients who underwent RT. HPV α-7-positive patients had significantly inferior DFS and DMFS rates compared with α-9-positive patients. By contrast, HPV species had no impact on LC or OS rates in patients with uterine CC.

Table IV. Comparison of patients' characteristics between HPV positive and HPV negative.

Characteristics (n=83)	HPV positive (n=69)	HPV negative (n=14)	P-value
Age, years (range)	60 (36-82)	58 (37-83)	0.33
Histology			<0.01
Squamous cell carcinoma	57	4	
Adenocarcinoma	11	10	
Small cell carcinoma	1	0	
FIGO stage			0.20
IB	5	3	
II	13	1	
III	36	7	
IV	15	3	
Pelvic LN metastasis			0.66
Negative	32	8	
Positive	37	6	
PALN metastasis			0.91
Negative	58	11	
Positive	11	3	
Concurrent chemotherapy			0.11
No	34	3	
Yes	35	11	

HPV, human papillomavirus; FIGO, International Federation of Gynecology and Obstetrics; LN, Lymph node; PALN, Para-aortic lymph node.

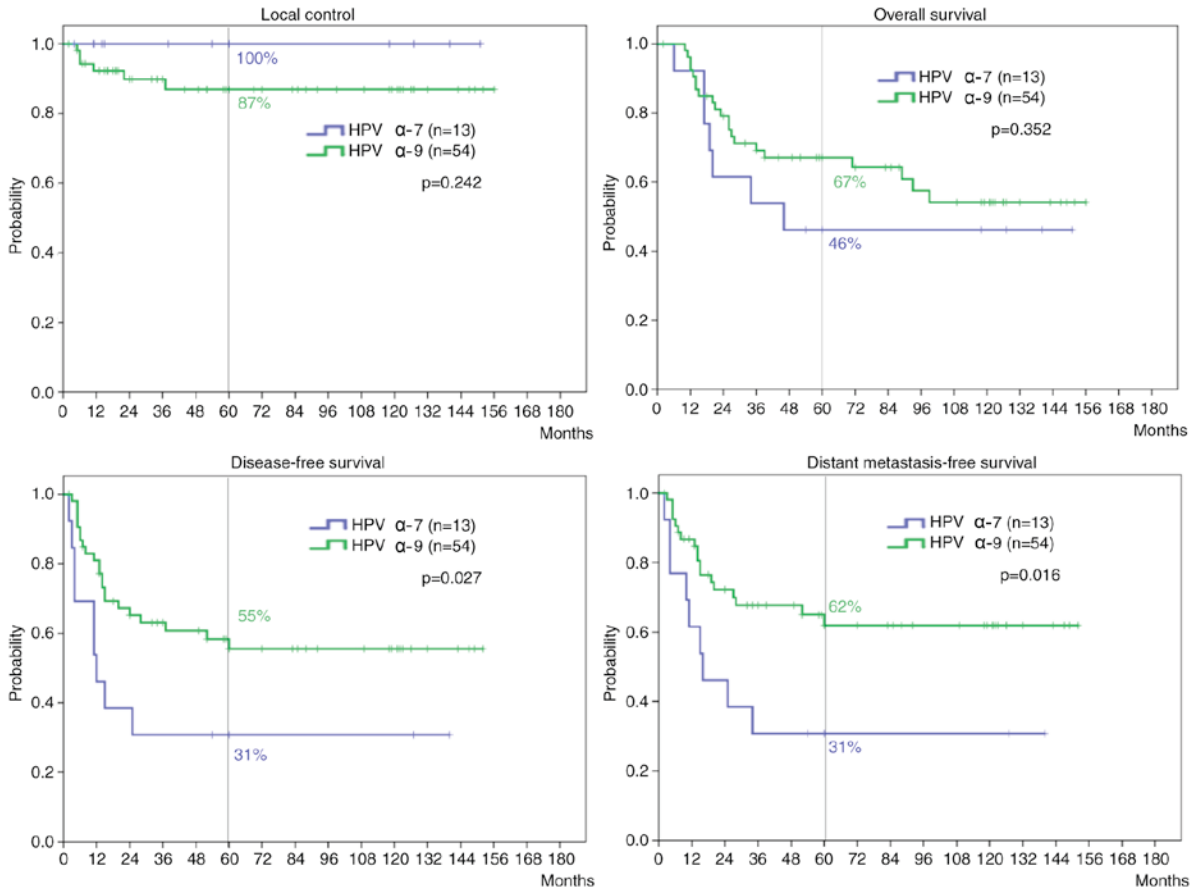

Figure 2. Comparison of the clinicopathological characteristics of the HPV α-7 and α-9 groups. HPV, human papillomavirus.

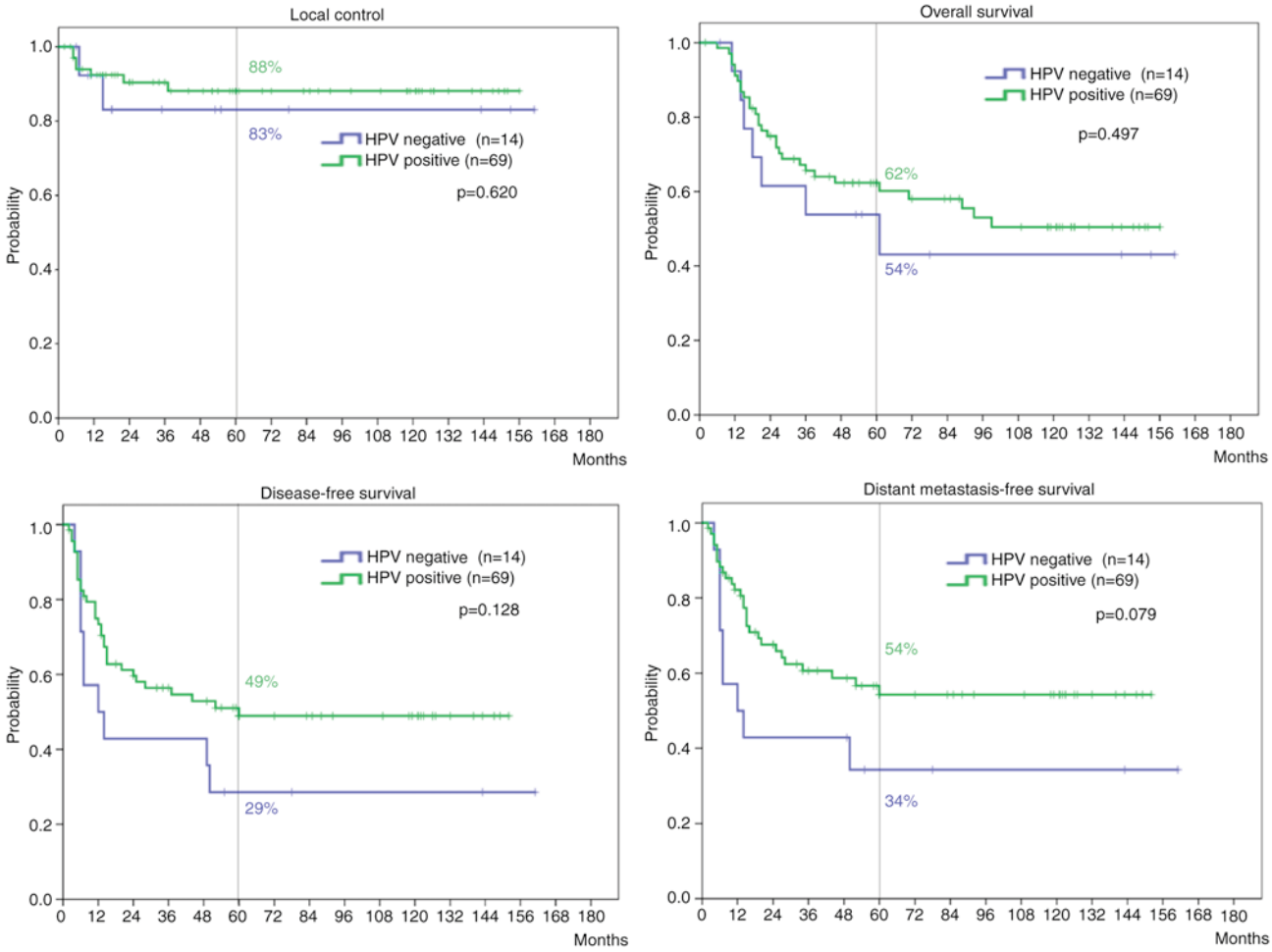

Figure 3. Comparison of the clinicopathological characteristics of the HPV positive and negative groups. HPV, human papillomavirus.

These findings contrast with those of Wang *et al* who found a significant effect of HPV species on LC and local progression-free survival rates, respectively (20). Meanwhile, Hall *et al* assessed that the HPV α-9 and α-7 species had similar radiosensitivity by performing assays in clonal CC cell lines (21). Taken together, HPV genotype may affect the DFS and DMFS rates, but not the LC or OS in patients with uterine cervical cancer after RT. Our present study showed superior local control rates when compared to Wang *et al* and Hall *et al* (20,21). The main reason for a higher local control rate could be attributed to our use of CT-based brachytherapy. In general, if the local control rate improves, DFS or DMFS should also improve. Nonetheless, statistically significant differences were still found in DFS or DMFS between HPV α-7-positive patients and α-9-positive patients in the present study. This fact supports that HPV genotype affected the distant metastatic rate. However, the underlying mechanisms associated with increased metastatic rates in patients with the α-7 species are unknown. The findings of Hall *et al* (21) in clonal CC cell lines, which, although preclinical, suggest that these species do not differ regarding radiosensitivities, implied than another, as yet undetermined factor could be responsible for outcome differences according to HPV species. Therefore, further studies are needed to identify these potential factors and determine the mechanisms involved.

HPV α-7 species, in particular, the HPV 18 and HPV 45 genotypes, are commonly associated with adenocarcinoma of the cervix; there is a significantly higher association of HPV 18 and HPV 45 with adenocarcinoma (44%) compared with squamous cell carcinoma (14%) (5). In the present study, the distribution of HPV α-7 and α-9 was similar between patients with adenocarcinoma, although the number of patients with adenocarcinoma was small. By contrast, there was a significant difference in the distribution of HPV species in patients with squamous cell carcinoma, for whom the HPV α-9 species was especially prevalent. A previous study showed that patients with adenocarcinoma had significantly poorer prognoses after radical RT compared to those with squamous cell carcinoma (32). However, in the present study, in patients with squamous cell carcinoma, although HPV α-7 was associated with inferior DMFS compared with HPV α-9, the difference was not observed in patients with squamous cell carcinoma. This suggested that higher metastatic rates in patients with HPV α-7 cannot be explained by tumor histology alone.

In vitro studies have shown that several features of HPV 18 infection that differ from those of HPV 16 infection, including enhanced E7 phosphorylation and increased transformation (33,34). Furthermore, in uterine CC cells, HPV 18 was associated with significantly lower levels of apoptosis compared with HPV 16 (35). E6 protein activities in CC

also differ between HPV 18 and HPV 16 infections (36). E6 protein plays a pivotal role in carcinogenesis by targeting the host proteins, such as p53 and PDZ domain proteins that are involved in proteasome-mediated degradation (36). The PDZ domains play a vital role in organizing and maintaining complex scaffolding formations (37). This difference also exists between the HPV α-9 and α-7 species. In uterine CC cells, these molecular effects mediated by HPV infection are thought to play a key role in carcinogenesis. In addition, it has been suggested recently that scaffolding proteins might regulate invasion and metastasis in some cancer types (38-40), which affords a possible explanation for the more aggressive nature of CC associated with the HPV α-7 species.

In conclusion, HPV species affected the DMFS and DFS rates but not the LC or OS rates in patients with uterine CC after RT. However, further large population studies on the usefulness HPV genotype information in Japanese patients with uterine CC are needed to determine if HPV genotypes could be considered a prognostic marker in patients with uterine CC after RT.

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