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Human papillomavirus (HPV) 16-positive uterine cervical squamous cell carcinoma with co-infection of HPV 34 has lower incidence in lymph node metastasis than that without co-infection of HPV 34

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Running title: Significance of HPV 34 co-infection with HPV 16

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Abstract

Our earlier study demonstrated high prevalence of multiple human papillomavirus (HPV) infection in patients with invasive uterine cervical cancer, including squamous cell carcinoma (SCC). HPV 16 is the most predominant genotype related to SCC of the uterine cervix. The aim of this study is to reveal the biological significance of multiple HPV infection concerning the tumor progression of invasive uterine cervical SCC. In the present study, the effects of co-infection of genotypes other than HPV 16 on the tumor growth and lymph node metastasis of invasive uterine cervical SCC with HPV 16 infection were examined. Although co-infection of most genotypes did not influence the tumor progression, the clinical stage of patients co-infected with HPV 16 and HPV 34 was significantly lower than those without HPV 34 co-infection ($p=0.0038$). Moreover, no patient co-infected with HPV 16 and HPV 34 manifested lymph node metastasis but about half of the patient population without HPV 34 co-infection did ($p=0.0299$). These findings suggested that co-infection of HPV 34 could prevent the tumor progression of invasive uterine cervical SCC with HPV 16 infection.

Key words: Uterine cervical cancer; Invasive SCC; HPV; Multiple infection

Introduction

Uterine cervical cancer is the second most common cancer in women worldwide. Certain genotypes of human papillomavirus (HPV) have been shown to be critically associated with the development of uterine cervical cancer. Currently, more than 100 different HPV genotypes have been identified. In Japan, HPV 16, 18, 31, 33, 35, 39, 45, 52, and 58 are regarded as representative high-risk genotypes because they are identified in high-grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma (SCC) of the uterine cervix [1]. On the other hand, HPV 6 and HPV 11 are considered as low-risk genotypes. Initially cloned as a homologue of high-risk HPV 16 [2], HPV 34 is currently classified as a low-risk genotype [3]. Today, HPV genotyping information is clinically useful for follow-up of patients based on the risk [4].

Our recent study has demonstrated high prevalence of multiple HPV infection in Japanese patients with invasive uterine cervical cancer [5]. The biological significance of multiple HPV infection remains elusive. Ho *et al*. investigated the natural history of cervicovaginal HPVs and found an association between the presence of multiple HPV genotypes and the persistence (over 6 months) of infection [6]. On the contrary, other studies showed that persistent HPV infection was independent on co-infection with other HPV genotypes [7, 8]. The carcinogenic risk of multiple HPV infection is also controversial. Lee *et al*. reported that patients infected with multiple HPV genotypes had a 31.8-fold higher risk of uterine cervical cancer compared to women without HPV infection, while those infected with a single HPV genotype had a 19.9-fold increased risk [9]. On the other hand, Sasagawa *et al*. reported that the odds ratio of multiple HPV infection was 24 for low-grade squamous intraepithelial lesion, 16 for HSIL, and 8.3 for SCC of the uterine cervix [10]. This discrepancy could reflect the diverse interaction among HPV genotypes.

Tumor progression is evaluated clinically by local growth, metastasis to the regional lymph nodes, and distant metastasis. According to the International Federation of Gynecology and Obstetrics (FIGO) classification, invasive uterine cervical cancer which can be diagnosed only by microscopy is classified into stage Ia. Clinically visible lesions limited to the uterine cervix or pre-clinical cancers greater than stage Ia are classified into stage Ib. Cervical cancers invading beyond the uterus are classified into stage II or higher. In the present study, the correlation of multiple HPV infection with the FIGO stage (stage Ib or higher than stage Ib) and lymph node metastasis (negative or positive) was examined concerning clinically visible, not microscopic, invasive uterine cervical SCC.

Materials and Methods

Clinical samples

This study was conducted with permission of the Medical Ethics Committee of Hokkaido University Graduate School of Medicine. A total of 42 cases of invasive uterine cervical SCCs were obtained from the archives of fresh-frozen tissues between the period of 1999 and 2004 at the Department of Gynecology of Hokkaido University, Sapporo, Japan. Written informed consent was obtained from all of the patients. Tissues (5 mm^3) were obtained from the inside of the tumors to avoid contamination of extrinsic HPVs. Patients' age ranged from 20 to 71 years with an average of 47.6 years (Table 1). A gynecologic oncologist performed pelvic examination and staging of the carcinoma according to the FIGO classification. The status of lymph node metastasis of 36 patients who underwent radical hysterectomy was recorded.

HPV genotyping

Genomic DNA was extracted from fresh-frozen tissues of 42 cases of invasive uterine cervical SCCs using PureGene DNA isolation kit (Qiagen, Tokyo, Japan) and stored at -20 ºC until use. HPV genotyping was performed using HPV array as described [5]. The array was designed to detect 13 high-risk HPVs (HPV 16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), 8 low-risk HPVs (HPV 6, 11, 34, 40, 42, 53, 54, and 61), and 4 skin-type HPVs (HPV 17, 20, 21, and 47). The specificity and sensitivity of this array was described elsewhere [5].

Statistical analysis

The correlation between the status of HPV infection and the clinical factors, including the FIGO stage (stage Ib or higher than stage Ib) and lymph node metastasis (negative or positive), was examined. For this analysis, Fisher's exact test was employed. Next, the correlation between the HPV infection status and clinico-pathological factors, including the FIGO stage (Ib or higher than Ib), lymph node metastasis (negative or positive), differentiation of carcinoma cells (keratinizing or nonkeratinizing), counts of mitotic figures (0-5/10 high power fields of view (HPF) or more than 5/10 HPF), and lymphatic and vascular invasions (negative or positive), was examined. For this purpose, multivariate analysis was performed. In both analyses, p-values less than 0.05 were considered to be significant.

Results

Detection of HPV genotypes

Figure 1 shows the frequency of HPV genotypes detected in invasive SCC of

the uterine cervix. Among high-risk genotypes, HPV 16, 18, 31, 33, 35, 52, and 58 were detected. The most prevalent genotype was HPV 16 (37/42, 88.1%), followed by HPV 52 (17/42, 40.5%) and HPV 18 (13/42, 31.0%). Among low-risk genotypes, HPV 11 (14/42, 33.3%) and HPV 34 (8/42, 19.0%) were detected. The rate of multiple infection was 81.0% (Table 2, 34/42).

Effects of multiple HPV infection on tumor progression of invasive uterine cervical SCC

HPV 16 is the most predominant genotype that contributes to the development of SCC of the uterine cervix [1]. Correspondingly, HPV 16 was detected most frequently in this study. To determine the significance of multiple HPV infection on tumor progression, the effects of co-infection of genotypes other than HPV 16 on the FIGO stage (stage Ib or higher than stage Ib) and lymph node metastasis (negative or positive) of invasive SCC with HPV 16 infection were examined (Table 3). Prior to conduction of this analysis, we expected that co-infection of some genotypes would promote the tumor progression of SCC with HPV 16 infection. However, co-infection of most genotypes had no influence on either the FIGO stage or lymph node metastasis. Interestingly, contrary to our expectation, the FIGO stage of patients co-infected with HPV 16 and HPV 34 was significantly lower than those without HPV 34 co-infection ($p=0.0038$). Moreover, no patient co-infected with HPV 16 and HPV 34 manifested lymph node metastasis; whereas, about half of the patient population without HPV 34 co-infection experienced lymph node metastasis ($p=0.0299$).

To verify this relation, multivariate analysis was conducted between the HPV infection status (HPV 16 with HPV 34 or HPV 16 without HPV 34) and clinico-pathological factors, including the FIGO stage (Ib or higher than Ib), lymph node metastasis (negative or positive), differentiation of carcinoma cells (keratinizing or

nonkeratinizing), counts of mitotic figures (0-5/10 HPF or more than 5/10 HPF), and lymphatic and vascular invasions (negative or positive). As a result, the FIGO stage and the state of lymph node metastasis were significantly correlated to the HPV infection status ($p=0.0113$ and $p=0.0167$, respectively, Table 4). The next correlative factor was the state of lymphatic invasion, though the correlation did not reach the statistically significant level.

Discussion

The E6 and E7 of high-risk HPVs are critically involved in the carcinogenesis of uterine cervical cancer [11]. HPV E6 has 2 zinc-finger loops that can bind with host proteins, one of which is E6-associated protein (E6AP) and the other is tumor-suppressive cell-cycle regulator, p53 [12]. The E6AP is a ubiquitin ligase that can yield polyubiquitination of the related proteins [13]. High-risk HPV E6 can bind with E6AP and p53, then E6AP yields polyubiquitination of p53 [14]. The polyubiquitinated p53 is subsequently degraded by proteasome resulting in acceleration of the cell-cycle [15, 16]. On the other hand, high-risk HPV E7 can bind with retinoblastoma protein (pRB) that holds and inactivates the cell-cycle promoter, E2F1 [17]. The pRB bound to E7 is phosphorylated, and then E2F1 is released resulting in acceleration of the cell-cycle. In addition, high-risk E7 is suggested to act as a mitotic mutator, which can increase the chance of errors during each round of cell division [18].

In order to determine the mechanism that HPV 34 prevents the progression of invasive SCC with HPV 16 infection, the amino acid sequences of E6 and E7 regions were compared among high-risk genotypes HPV 16, 18, 31, 33, 35, 52, and 58, and low-risk genotypes HPV 6, 11, and 34. The E6 sequences of all genotypes include 2

zinc-finger loops (Figure 2, highlighted in green). There are characteristic differences in the E6AP-binding site and p53-binding site between high-risk genotypes and low-risk genotypes, including HPV 6 and HPV 11 (highlighted in light blue in the E6AP-binding site and highlighted in yellow in the p53-binding site). These diversities could reflect the difference in the carcinogenic risk of HPVs. Interestingly, although the critical p53-binding site sequences of HPV 34 are identical to high-risk genotypes (highlighted in yellow), the E6AP-binding site sequences are different (highlighted in light blue). These findings suggest that HPV 34 E6 can bind with p53 but may not bind with E6AP, or if it can bind with E6AP, the binding affinity may be weaker than high-risk HPVs. Accordingly, p53 bound to HPV 34 E6 might be difficult for polyubiquitination by E6AP compared with that bound to E6 of high-risk HPVs resulting in escape from degradation by proteasome. Regarding other low-risk genotypes, including HPV 6 and HPV 11, such protective potential for p53 is not likely because the critical p53-binding sequences (highlighted in yellow) are different from those of high-risk genotypes.

The present study demonstrated the possibility that co-infection of HPV 34 with HPV 16 infection prevented the tumor progression of invasive uterine cervical SCC. The FIGO stage and the state of lymph node metastasis were significantly correlated to the HPV infection status. The state of lymphatic invasion also tended to correspond with the HPV infection status. These findings supported our conclusion that HPV 16-positive uterine cervical SCC with co-infection of HPV 34 was not apt to invade lymphatic vessels compared to that without co-infection of HPV 34, which resulted in the lower FIGO stage. Although further study with larger cases and investigation into the molecular mechanism in which HPV 34 competes with HPV 16 for p53-binding are needed, the results of this study suggest that reagents which compete with high-risk HPVs for p53-binding could be novel candidates for treatment of invasive SCC of the uterine cervix.

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Table 1. Clinical findings of 42 cases of invasive SCCs

Clinical findings		Number
Age	-29	3
	30-39	8
	40-49	11
	50-59	14
	60-	6
FIGO stage	Ib	15
	II	15
	III	9
	IV	3
Lymph node metastasis	negative	24
	positive	12
	unknown	6

Table 2. HPV infection status of 42 cases of invasive SCCs

HPV infection status		Number
Multiple infection	more than 2 genotypes	19
	2 genotypes	15
Single infection		8
No infection		0

Table 3. Effects of HPV co-infection on tumor progression of invasive SCC

Infection status	FIGO stage		p-value	Lymph node metastasis		p-value
	Ib	>Ib		negative	positive	
HPV16 w/ 18	4	8	0.7235	6	5	0.4713
HPV16 w/o 18	11	14		15	7	
HPV16 w/ 31	4	3	0.4081	6	0	0.0648
HPV16 w/o 31	11	19		15	12	
HPV16 w/ 52	7	9	0.7486	11	4	0.4688
HPV16 w/o 52	8	13		10	8	
HPV16 w/ 58	2	5	0.6767	4	2	1.0000
HPV16 w/o 58	13	17		17	10	
HPV16 w/ 11	5	7	1.0000	8	3	0.7026
HPV16 w/o 11	10	15		13	9	
HPV16 w/ 34	7	1	0.0038**	8	0	0.0299*
HPV16 w/o 34	8	21		13	12	

**p<0.01, *p<0.05 in Fisher’s exact test.

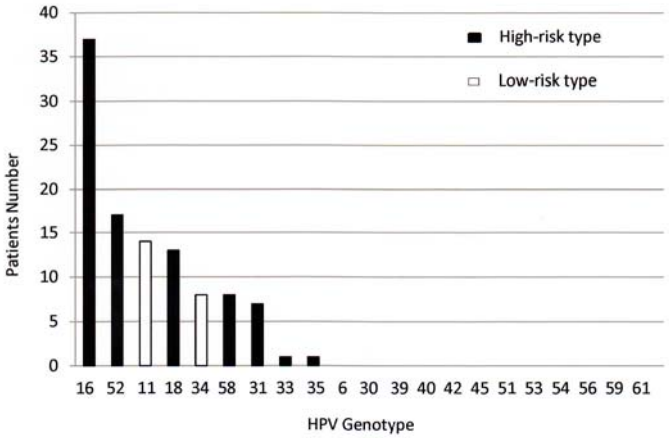

Figure 1. Frequency of HPV genotypes detected in 42 cases of invasive SCCs. The black and white bars represent high-risk and low-risk genotypes, respectively.

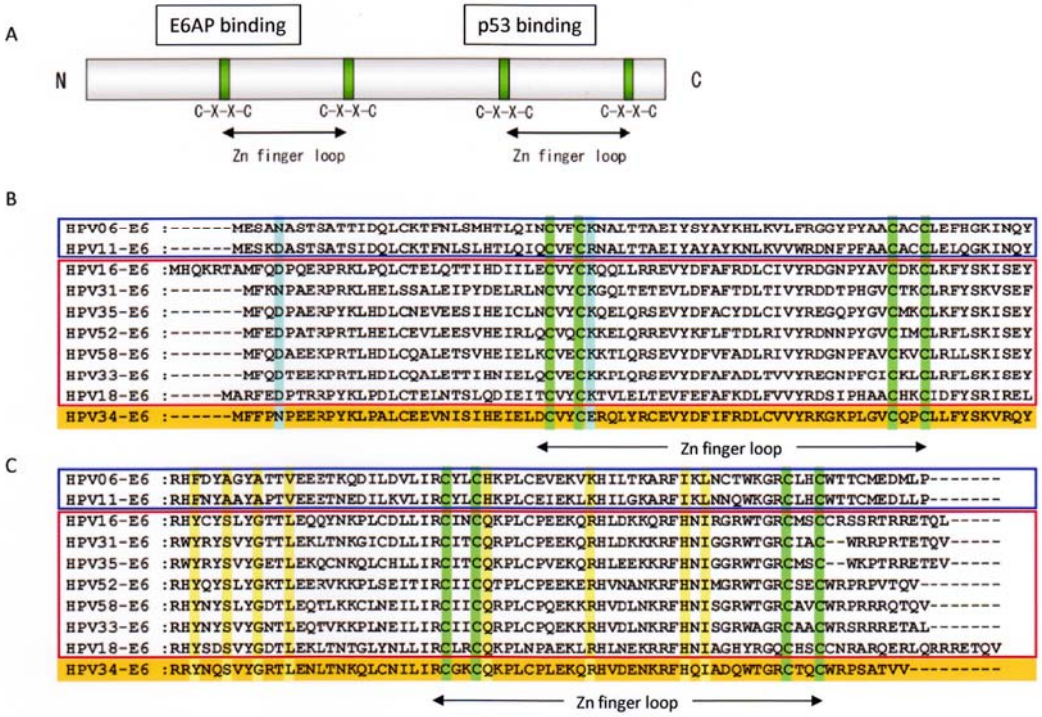

Figure 2. Comparison of E6 amino acid sequences between HPVs. (A) Schema of the E6 region. The E6AP-binding site and p53-binding site are presented. The zinc-finger loops are represented by arrows. The C-X-X-C motif in the zinc-finger loop is highlighted in green. (B) Comparison of the E6AP-binding sites. High-risk HPVs are surrounded by a red square, and low-risk HPV 6 and HPV 11 are surrounded by a blue square. HPV 34 sequence is highlighted in orange. Cysteine in the C-X-X-C motif in the zinc-finger loop is highlighted in green. The characteristic differences between high-risk genotypes and low-risk genotypes, including HPV 6 and HPV 11, are highlighted in light blue. (C) Comparison of the p53-binding sites. High-risk HPVs are surrounded by a red square, and low-risk HPV 6 and HPV 11 are surrounded by a blue square. HPV 34 sequence is highlighted in orange. Cysteine in the C-X-X-C motif in the zinc-finger loop is highlighted in green. The characteristic differences between high-risk genotypes and low-risk genotypes, including HPV 6 and HPV 11, are highlighted in yellow.