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HPV16 variants and HLA alleles are associated with cervical carcinoma in Korean women

한국 여성의 자궁경부암과 사람유두종바이러스 16형 변이형, 사람백혈구항원 대립유전자의 관계

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Abstract

HPV16 variants and HLA alleles are associated with cervical carcinoma in Korean women

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Background: Persistent human papillomavirus type 16 (HPV16) is the major risk factor for cervical cancer. HPV16 intratypic variants differ in their geographical distribution and oncogenic potential. In addition, the susceptibility to cervical cancer is also known to be affected by ethnicity. This study aimed to analyze the distribution of HPV16 variants and human leukocyte antigen (HLA) polymorphism and their association with cervical lesion histopathology in Korean women.

Methods: In total, 133 HPV16-positive cervical samples from women admitted to Seoul National University Boramae Hospital were analyzed by sequencing E6, E7,

and L1 genes and the long control region (LCR), and the variant distribution according to cervical lesion grade was determined. Among them, the samples from invasive cancer patients were tested for the determination of allele frequencies of HLA-B, -DRB1, -DQA1 and -DQB1 of host cells. The association between the HLA allele frequency and HPV 16 variant specific invasive cancer was analyzed.

Results: Isolates were grouped into a phylogenetic lineage, and A1-3, A4, C, and D sublineages were detected in 54.1, 37.8, 0.7, and 7.4% of samples, respectively. The most commonly observed LCR variations were 7521G>A (91.5%), 7730A>C (59.6%), and 7842G>A (59.6%). Furthermore, A4 or D sublineage-positive women had a higher risk for invasive cancer than women who were positive for A1–3. Among HPV phylogenetic clusters, A1–3 was the predominant sublineage, and within A1–3, the 350G polymorphism was highly frequent. Although limited number of samples were included, HLA-B*13, B*51, DRB1*07, DRB1*09, DRB1*11, DQA1*2 and DQA1*4 were risk factor of invasive cancer for A4 infected patients. HLA-DRB1*15 was risk factor for cervical cancer for A1-3 and D infected patients.

Conclusions: The results differed from those of previous studies in Korea and other Asian countries. The findings suggest that cervical neoplasia incidence in HPV16-infected patients could be affected by the distribution of HPV16 variants and HLA type in the population.

Keywords: Human papillomavirus 16, variant, HLA, allele, cervical cancer, squamous intraepithelial lesion, case–control study

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목차

표 목차

그림 목차

Introduction

The incidence rate of cervical cancer in Korea is higher than that in other developed countries. In 2012, the reported incidence was 14.2 cases per 100,000 women, with 3,584 new cases and 889 deaths [1]. Persistent infection by oncogenic human papillomaviruses (HPV) is strongly associated with the development of cervical neoplasia. Oncogenic HPV type 16 (HPV16) accounts for approximately 65% of cervical cancer worldwide [2] and is the most common HPV type associated with cervical cancer in Korea [3].

The HPV oncogenes, E6 and E7, are consistently expressed in cervical cancers, and these genes are involved in HPV-mediated carcinogenesis [4]. Currently, HPV16 intratypic variants, which have nucleotide sequence variations of ≤2% as compared with the HPV16 prototype, are classified into four major lineages based on whole-genome sequencing: A, which includes the A1–3 (previously named European), and A4 (Asian) sublineages; B (African 1); C (African 2); and D [including Asian-American (AA) and North American (NA)] [5]. The long control region (LCR) adjacent to the downstream region of E6 contains the early promoter and regulatory elements involved in viral DNA replication and transcription [6]. Epidemiological studies have reported that HPV16 variants confer different risks of viral persistence and/or progression to pre-cancer and cancer. According to previous studies, infections with non-A1–3 variants tend to be more persistent and are associated with a higher risk of cervical neoplasia than infections with A1–3 variants [7-9].

Cervical cancer is one of the most frequent cancers among women in Korea;

however, there has not been adequate investigation regarding the distribution of HPV16 variants in Korea. Recent Korean studies showed inconsistent results regarding the proportions of HPV16 variants [10, 11], and the results were also different from those reported for other Asian countries [12, 13]. Previous studies have reported that the association between a certain HPV16 variant type and the occurrence of cervical cancer could vary according to geography and ethnicity. In a study in the USA, A1–3 variants were reported to be associated with persistent infection in white women, while infection with B or C variants tended to be persistent in African-American women [9]. In Japanese women, the D25E variant was reportedly more prevalent in invasive squamous carcinoma of the cervix, and this variant was associated with a decreased probability of cervical lesion regression [14]. However, these findings were not replicated in Korea, where the D25E variant has also been commonly detected [15]. These inconsistent results demonstrate the need to investigate the distribution of HPV16 variants and their oncogenicity according to geography and ethnicity.

In addition, human leukocyte antigen as a host immune factor could influence the infectivity and the potential to progress to cancer of HPV. Although there were reports about the association between HLA type and cervical cancer, little is known in Korea [16].

In this study, we performed genetic sequencing of HPV16 isolates obtained from Korean women with cervical lesions who visited a metropolitan hospital in Korea, and we determined the distribution of HPV16 variants according to the severity of the cervical lesions. From a part of the samples, host DNA was also extracted and determined for HLA allele.

Materials and Methods

1. Study subjects and design

Among 274 DNA samples extracted from HPV 16 positive cervical cytobrush specimen, 133 samples were selected excluding cases of duplication, other ethnicity and no pathology results (Fig 1). These samples were collected from women admitted to the Seoul National University Boramae Hospital from 2010 to 2012. HPV detection and typing, which can identify 40 HPV types, was performed using a GG HPV Genotyping Chip Kit (Goodgene, Seoul, Korea). Based on cytological and histological evaluations of fresh specimens, the cervical lesions were graded according to their severity as follows: no evidence of disease (NED), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and invasive cancer. The histological diagnosis of each case was reviewed by an experienced pathologist who was unaware of the HPV testing results. Through the medical chart review, it was ascertain whether the infection was persistent. This study was approved by the Institutional Review Board of Seoul National University Boramae Hospital (26-2013-31).

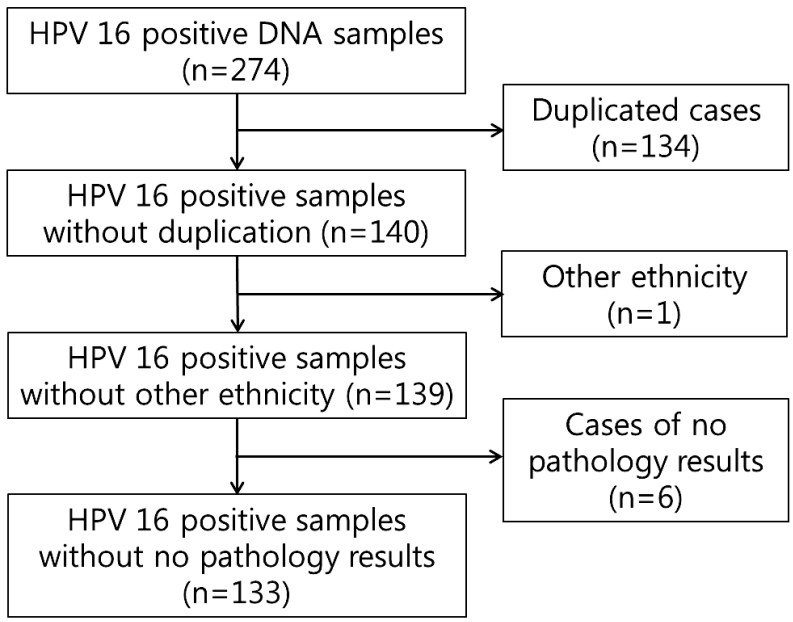

Fig 1. Study subjects and design

2. Amplification and sequencing of E6, E7, L1, and the LCR

The DNA samples remaining after HPV testing were stored at −80 °C and amplified for E6, E7, L1, and the LCR using the specific primers listed in Table 1 [17-19]. The PCR mixture (50 μL) contained 5 μL of template DNA, 10× PCR buffer, 2.5 mM dNTPs, 25 mM $MgCl_2$ (Takara Bio Inc., Shiga, Japan), 25 pmol primer, and 1.25 U of Taq polymerase (Takara Bio Inc). PCR was performed for 35 cycles in a PTC-200 (Bio-Rad, Hercules, CA, USA) at 94 °C for denaturation (15 s), 55 °C for annealing (45 s), and 72 °C for extension (45 s), including an initial denaturation step at 95 °C for 8 min and a final extension step at 72 °C for 10 min to obtain the amplified products of E6, E7, L1, and the LCR. PCR products were visualized in a 2% (w/v) agarose gel stained with ethidium bromide and purified using an AMPure® purification kit (Agencourt Bioscience, Beverly, MA, USA). For sequencing of E6, E7, L1, and the LCR, 150 ng of each purified product was mixed with 3.2 pmol of each PCR primer and a BigDye® Terminator Cycle Sequencing mix (Applied Biosystems, Foster City, CA, USA), and then loaded onto a PRISM® 3730xl analyzer (Applied Biosystems). All samples were sequenced from both directions to exclude PCR artifacts, and the obtained sequences were analyzed using CodonCode Aligner version 4.2.7 (http:// www.codoncode.com/aligner/archive.com) and compared with GenBank sequence NC_001526.

Table 1. Primers used for PCR amplification and sequence analysis

Gene	Position	Direction	Sequence	Reference
E6/E7	34-879	Forward	AAC/CGA/AAT/CGG/TTG/AAC/CG	17
		Reverse	TGC/AGG/ATC/AGC/CAT/GGT/AGA/T	
L1	6582-7033	Forward	GAG/CAC/AGG/GCC/ACA/ATA/AT	18
		Reverse	TCC/TAA/AGG/AAA/CTG/ATC/TAG/G	
LCR	7337-7866	Forward	CAA/CAC/CTA/CTA/ATT/GTG/TTG/TGG	19
		Reverse	AAA/TCG/GTT/TGC/ACA/CAC/CCA/TGT	

3. Identification of variants and LCR analysis

Variants were identified using the prototype sequence (HPV16R) as the standard for comparisons and nucleotide position numbering [20]. Variants were then classified into four lineages (A, B, C, and D) as described by Burk et al. [5]. TFSearch software was used to assess the effects of LCR variations on binding sites for cellular transcription factors (TFs) [21].

4. HLA typing

Genomic DNA was extracted from the cells of cervical scrapes using the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN, USA). Each sample was typed for HLA–B, DRB1 and DQB1 alleles at low-to-intermediate resolution using Luminex and LABType® B, DRB1, DQA1/B1 SSO typing kits (One Lambda inc., Canoga Park, CA, USA) according to the manufacturers' protocols. Two-digit or DNA group level typing results were converted to the serologic equivalents based on the World Health Organization HLA nomenclature [22] and the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/).

5. Statistical analyses

After classifying the isolates into phylogenetic lineages, the association between the variant types and the grades of cervical lesion severity was analyzed. The distribution of HPV16 variants was compared between the control group (NED or NED/LSIL) and case group (invasive cancer). The magnitude of the association between each HPV16 sublineage or variant and the case group was assessed by calculating the odds ratios (OR) and the respective 95% confidence interval (CI).

Linear trend and chi-squared tests were used to assess the significance of the association between the distribution of HPV16 variants and cervical lesion severity, and a Mann–Whitney test was used to assess the significance of the differences in patients' ages between the control group and the case group. The HLA–B, DRB1, DQA1 and DQB1 allele frequencies (AFs) were calculated by direct counting and deviations from the Hardy–Weinberg equilibrium were tested. The risk association for each allele was assessed by comparing the AFs between cases and published controls. HLA B-DRB1 haplotype frequencies (HFs) were estimated using the maximum likelihood method, which uses an expectation-maximization algorithm. The risk association for each haplotype was assessed by comparing the HFs between cases and controls from Seoul metropolitan government public cord blood bank data (not published). To clarify the contribution of independent HLA allele factors to the cervical pathology, we performed the $\chi 2$ test or Fisher's exact test after stratification by the HPV variants. All p-values were presented using Bonferroni correction to clarify the effect of the multiple comparisons. All statistical analyses were performed using R version 2.15.1 (http://www.r-project.org).

Results

1. Study subjects and clinical data

The average age of patients was 43 ± 15 years, with a range of 21–86 years (Table 2). Thirty-one women were histologically diagnosed as invasive cancer. Among the lower grade lesions, 21 women were categorized as NED, 48 as LSIL, and 33 as HSIL. The mean age of the invasive cancer patients was significantly lower than that of the NED group, while it was not significantly different between the invasive cancer patients and the NED/LSIL group. Through the medical chart review, persistence of infection was ascertained in 51 cases. Thirty-four cases were persistent and 17 cases were transient.

Table 2. Clinical data of HPV 16 positive patients

		N	%
Age			
	20-29	30	22.6%
	30-39	33	24.8%
	40-49	23	17.3%
	50-59	23	17.3%
	60-69	17	12.8%
	≥70	7	5.3%
	Mean ± S.D.	43 ± 15	
	Range	21 – 86	
Pathology of cervix			
	NED[1]	21	15.8%
	LSIL[2]	48	36.1%
	HSIL[3]	33	24.8%
	Invasive cancer	31	23.3%
Persistence of infection			
	Persistent	34	66.7%
	Transient	17	33.3%

[1]NED, no evidence of disease; [2]LSIL, low-grade squamous intraepithelial lesion; [3]HSIL, high-grade squamous intraepithelial lesion

2. Composition of HPV16 variants and nucleotide variations in E6, E7 and L1

Genetic variations in the E6, E7, and L1 genes of the 133 samples with complete data are shown in Table 3. In the E6, E7, and L1 genes, five samples contained a complete sequence that was homologous to that of the prototype. Among the 133 isolates available for variant analysis, the A1–3, A4, C, and D variants were detected in 72 (54.1%), 50 (37.6%), 1 (0.8%), and 10 (7.5%) samples, respectively (Table 3). Nucleotide variations in E6, E7, and L1 were observed in 128 isolates (96.2%) and resulted in 26 silent mutations and 26 missense mutations (Table 3). In A1–3 variants, 350T>G variation was most frequently observed (57/72, 79.2%). Except 2 isolates, 178T>G and 647A>G variations were observed in A4 variants. In all the D variants, 145G>T, 286T>A, 289A>G, 335C>T, 350T>G, 532A>G, 732T>C, 789T>C, were 795T>G were observed. In addition, 6695A>C, 6721G>A, 6803A>T, 6854C>T, 6865C>T, 6970C>T and 6994G>A were frequently observed in D variants. A1–3 and A4 had fewer nucleotide variations in L1 than E6 and E7. Amino acid changes occurred in 124 isolates (93.2%). In the E6 gene, L83V was the most common at 50.4%, followed by E113D (42.1%) and D25E (36.8%). In the E7 gene, N29S (41.4%) and L28F (37.6%) occurred frequently. L83V and E113D in E6 and L28F in E7 were most frequent amino acid changes in A1–3 variant. D25E in E7 and N29S in E7 were significantly related with A4 variant. Q14H/D and H78Y in E6 and T379P in L1 were frequent in C and D variants. Amino acid changes were associated with the grade of cervical lesions (Table 4). L83V, E113D and L28F were associated with the lower grade of cervical lesions. On the other hand, D25E, N29S, T379P and T415S were associated with the higher grade of cervical lesions.

Table 3. Nucleotide sequence variations in E6, E7 and L1 among the HPV16 isolates

Type of variant	N	E6																					E7												L1																	Grade of cervical lesion			
		94	109	131	132	135	143	145	178	181	183	286	289	295	310	335	350	403	442	463	490	532	645	647	663	666	712	732	757	758	760	789	790	795	6619	6634	6658	6687	6695	6721	6803	6826	6854	6862	6865	6883	6949	6956	6964	6970	6994	NED (n=21)	LSIL (N=48)	HSIL (N=33)	Invasive cancer (N=31)
HPV16R		G	T	A	G	A	C	G	T	A	T	T	A	T	T	C	T	A	A	C	G	A	A	A	G	G	C	T	C	G	T	T	C	T	C	T	A	A	A	G	A	C	C	T	C	A	A	C	A	C	G				
A1-3	1	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	1	0
A1-3	1	A	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	0	0	0	1
A1-3	1	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	1	0
A1-3	1	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	0	0	1	0
A1-3	1	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	0	0	0	1
A1-3	1	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	1	0	0
A1-3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	1
A1-3	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	1	4	0
A1-3	49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	C	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16	25	5	3
A1-3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	1	0	0	0
A1-3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	1
A1-3	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	1	0	1
A1-3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	1	0
A1-3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	1
A1-3	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	2	3	0
A4	1	-	-	C	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	1
A4	1	-	-	-	-	C	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0	0	0
A4	1	-	-	-	-	-	-	-	G	G	G	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	1
A4	3	-	-	-	-	-	-	-	G	-	G	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	2	0	1
A4	1	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	C	-	A	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	1
A4	5	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	C	-	-	-	-	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	2	1	2
A4	1	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	C	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	1	0
A4	1	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	1	0
A4	1	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	T	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	1	0	0
A4	1	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	1	0	0
A4	1	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	1
A4	1	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	0	0	1	0
A4	1	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	0	1	0	0
A4	1	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	0	0	0	1
A4	1	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	0	0	1	0
A4	27	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	7	10	8
A4	2	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	1	0	1
C	1	-	C	-	T	-	G	T	-	-	-	A	G	-	-	T	-	G	-	-	-	-	-	G	-	-	-	-	-	-	-	C	-	G	-	-	-	-	C	A	-	-	T	-	T	-	-	-	-	T	A	0	1	0	0
D	2	-	-	-	-	-	-	T	-	-	G	A	G	-	-	T	G	-	-	-	-	G	-	-	-	-	-	C	-	-	-	C	-	G	-	-	-	-	C	A	-	-	T	C	T	-	-	-	-	T	A	0	1	0	1
D	6	-	-	-	-	-	-	T	-	-	-	A	G	-	-	T	G	-	-	-	-	G	-	-	-	-	-	C	-	-	-	C	-	G	-	-	-	-	C	A	T	-	T	-	T	-	-	-	-	T	A	0	0	2	4
D	1	-	-	-	-	-	-	T	-	-	-	A	G	-	-	T	G	-	-	-	-	G	-	-	-	-	-	C	-	-	-	C	-	G	-	-	-	-	C	A	-	-	T	C	T	-	-	-	-	T	A	0	1	0	0
D	1	-	-	-	-	-	-	T	-	-	-	A	G	-	-	T	G	-	-	-	-	G	-	-	-	-	-	C	-	-	-	C	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0	0	0
Amino acid substitution				R10G	R10I	K11T	Q14D	Q14H/D	D25E	I27M	I27R			D64E	F69L	H78Y	L83V		E113D	D120E			L28F	N29S			H51N		R66W	R66Q			R77C			V359D			T379P		T415S							P466S	K468N						

(Continued)

NED, no evidence of disease; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion

E6, E7 and L1 nucleotide positions at which variations were observed are written vertically across the top of the image. The phylogenetic groupings based on the analysis of E6 and E7 are indicated along the left. The correct HPV16 DNA reference sequence is indicated as HPV16R. For each variant sequence, positions that do not vary relative to the HPV reference sequence are marked with dashes. Amino acid sequence variations are shown at the bottom; amino acid changes whose codons contain more than one nucleotide replacement are marked with /. The composition of cervical lesion grade for each sequence variation group was shown along the right.

Table 4. Association between amino acid changes in HPV 16 E6, E7, L1 and the grade of cervical lesions

Amino acid change	NED		LSIL		HSIL		Invasive cancer		P^1
	N	(%)	N	(%)	N	(%)	N	(%)	
H78Y	1	(4.8)	3	(6.3)	2	(6.1)	5	(16.1)	0.131
L83V	18	(85.7)	29	(60.4)	11	(33.3)	9	(29.0)	<0.001
E113D	16	(76.2)	26	(54.2)	7	(21.2)	7	(22.6)	<0.001
D25E	3	(14.3)	14	(29.2)	14	(43.8)	18	(58.1)	<0.001
Q14H	1	(4.8)	3	(6.3)	2	(6.3)	5	(16.1)	0.129
I27R	0	(0.0)	3	(6.3)	0	(0.0)	3	(9.7)	0.273
L28F	17	(81.0)	25	(52.1)	5	(15.2)	3	(9.7)	<0.001
N29S	3	(14.3)	17	(35.4)	15	(45.5)	20	(64.5)	<0.001
T379P	0	(0.0)	3	(6.1)	2	(5.9)	5	(15.6)	0.038
T415S	0	(0.0)	0	(0.0)	2	(5.9)	4	(12.5)	0.006

NED, no evidence of disease; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion

[1]Linear by linear association

3. Nucleotide variation analysis in long control region

LCR was not amplified in 49 samples. The sequence variations in the LCR of the remaining 84 isolates were analyzed. 35 nucleotide variations were observed in the LCR (Table 5). Among the 84 isolates, 7521G>A (92.9%) was the most frequent substitution, followed by 7730A>C (59.5%), 7842G>A (59.5%), and 7781T>C (29.8%). As shown at Table 6, several nucleotide variations, including 7730A>C, 7842G>A, 178T>G, and 647A>G, were co-linked in A4 sublineage as previously reported by Chang et al. [23]. We discovered seven novel nucleotide substitutions in the LCR. Following analysis with TFSearch, 23 substitutions were identified in the TF-binding site. The 7489G>A mutation results in the loss of the SRY TF-binding site, 7394T>C, 7395C>T, 7714T>G, and 7842G>A result in the creation of the SRY TF-binding site, and 7743T>G causes the loss of the Oct-1 TF-binding site, while 7764C>T results in the creation of the TATA TF-binding site. All of the nucleotide variations in the TF-binding sites were not significantly related with the grade of cervical lesions.

Table 5. Nucleotide sequence variations and related transcription factor binding sites in LCR among the HPV 16 isolates with different grades of cervical lesions.

Nucleotide variation	Transcription factors	No. of women with different grades of cervical lesion				Total n=84[a]
		NED n=4	LSIL n=24	HSIL n=29	Invasive cancer n=27	
[N]7377A>C	HFH-1, CF2-II, cdxA, XFD-2	0	1	0	0	1
7387G>C	HNF-3b, GRE-1	0	1	0	0	1
[N]7389T>A	c-Ets, HNF-3b	0	0	0	1	1
[N]7391C>T	c-Ets	0	0	1	0	1
7394C>T	c-Ets	0	2	3	5	10
7395C>A	c-Ets	0	1	0	0	1
7395C>T	c-Ets	0	2	2	5	9
[N]7424G>A		1	0	0	0	1
7428C>A		0	1	0	1	2
7434C>T		1	1	1	0	3
7435G>A		0	1	2	0	3
7441T>G		0	0	0	1	1
7450T>C		0	0	1	0	1
7458A>T	HSF, E2	0	1	0	0	1
7485A>C	YY-1, GRE-1	0	3	2	5	10
7489G>A	GRE-1	0	3	4	6	13
7507A>G	AP-1	0	2	0	1	3
7521G>A	YY-1	3	23	26	26	78
7669C>T	cap	0	3	2	5	10
7689C>A	TEF-1, E4BP4	0	2	2	5	9
[N]7700T>G		0	1	0	1	2
[N]7701A>G		0	0	1	0	1
7714T>G	NF-1, cdxA	0	0	1	1	2
7729A>C	S8	0	2	2	5	9
7730A>C	S8	3	15	15	17	50
7743T>G	S8, Oct-1, cdxA, TEF-1	0	0	2	4	6
7764C>T	HSF	0	3	2	5	10
7781T>C	YY-1	0	6	10	8	24
7786C>T	YY-1, cap	0	3	2	5	10

[N]7807T>C		0	0	0	1	1
7826G>A	Sox-5, HFH-1, HSF, HFH-2, YY-1, TEF-1	0	1	0	0	1
7834G>T	cap, cdxA	0	1	0	0	1
7837A>C	cap	0	1	0	0	1
7839A>G	cap	0	1	0	0	1
7842G>A	YY-1, SP-1, OCT-1	3	15	15	17	50

GRE, Glucocorticoid Response Element; NF1, Nuclear Factor 1; Oct-1, Octamer Binding Factor-1; TEF-1, Transcriptional Enhancer Factor-1; YY-1, Yin–Yang Factor 1; NED, no evidence of disease; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion

[a]No LCR sequences were available in 49 isolates; [N]Novel variation

Table 6. Nucleotide sequence variations in HPV 16 LCR among the HPV16 isolates.

Type of variant	N	7337	7387	7389	7391	7394	7395	7424	7428	7434	7435	7441	7450	7458	7485	7489	7494	7507	7521	7569	7589	7600	7701	7714	7729	7730	7743	7764	7781	7786	7801	7806	7823	7837	7839	7842	Grade of cervical lesion			
																																					NED	LSIL	HSIL	Invasive cancer
HPV16		A	G	T	C	C	C	G	C	C	G	T	T	A	A	G	T	A	G	C	C	T	A	T	A	A	T	C	T	C	T	G	G	A	A	G				
A1-3	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	0	3	1
A1-3	11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	4	5	2
A1-3	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	0	0	0	2
A1-3	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	A	0	1	0	0
A1-3	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	0	0	1	1
A1-3	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.	A	0	1	0	1
A1-3	1	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	0	1	0
A1-3	1	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1	0	0
A1-3	1	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	0	0	1
A1-3	1	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	0	1	0
A1-3	1	.	.	.	T	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	0	1	0
A1-3	1	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0	1	0	0
A4	16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	1	7	3	5
A4	17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	A	0	4	7	6
A4	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	C	.	.	C	.	.	.	.	.	.	A	0	0	1	0
A4	3	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	A	0	0	2	1
A4	1	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	0	0	0	1
A4	1	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	0	0	1	0
A4	3	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	1	1	1	0
A4	1	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	A	0	1	0	0
A4	1	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	A	1	0	0	0
A4	1	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	A	0	0	0	1
C	1	.	C	.	.	.	.	.	.	.	A	.	.	T	C	A	.	.	A	T	.	.	.	.	.	.	.	T	.	T	.	A	T	C	G	.	0	1	0	0
D	1	.	.	.	.	T	.	.	.	.	.	.	.	.	C	A	.	G	A	T	A	.	.	.	C	.	.	T	.	T	.	.	.	.	.	.	0	0	0	1
D	5	.	.	.	.	T	T	.	.	.	.	.	.	.	C	A	.	.	A	T	A	.	.	.	C	.	G	T	.	T	.	.	.	.	.	.	0	0	2	3
D	1	.	.	.	.	T	T	.	.	.	.	.	.	.	C	A	.	.	A	T	A	.	.	.	C	.	G	T	.	T	C	.	.	.	.	.	0	0	0	1
D	2	.	.	.	.	T	T	.	.	.	.	.	.	.	C	A	.	G	A	T	A	.	.	.	C	.	.	T	.	T	.	.	.	.	.	.	0	2	0	0

NED, no evidence of disease; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion

4. HPV 16 infection persistence according to variant type and cervical pathology

Associations between HPV16 infection persistence and variant type or the grade of the cervical lesions are outlined in Table 7. The infection of A4 and D sublineages persisted more than that of A1−3. However, the grades of cervical lesions were not associated with the persistence of infection.

Table 7. HPV 16 infection persistence according to variant type and cervical pathology

Parameters		Persistent		Transient		P^1
		N	%	N	%	
Type of variant	A1-3	13	52.0	12	48.0	0.030
	A4	18	78.3	5	21.7	
	D	3	100.0	0	0.0	
Cervical pathology	NED	2	66.7	1	33.3	0.910
	LSIL	16	66.7	8	33.3	
	HSIL	9	64.3	5	35.7	
	Invasive cancer	7	70.0	3	30.0	

NED, no evidence of disease; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion

[1]Linear by linear association

5. Association between HPV16 variants and cervical lesion severity

Associations between HPV16 variants and the grade of the cervical lesions are outlined in Table 3. The A1–3 sublineage tended to be associated with lower grades of cervical lesions, whereas A4 were significantly associated with severe lesions. An assessment of the risk of each sublineage and variant for invasive cancer, rather than lower grades of lesions, is shown in Table 8. When the NED group was considered as the control group, the A4 sublineage was significantly associated with invasive cancer, compared with A1–3 (age-adjusted OR = 9.72, 95% CI = 2.16–43.68; p = 0.003). The association did not change when the NED/LSIL group was considered as the control group (age-adjusted OR = 5.69, 95% CI = 2.05–15.76; p = 0.001). The D sublineage was also significantly associated with invasive cancer, compared with A1–3 (age-adjusted OR = 8.46, 95% CI = 1.70–42.15; p = 0.009) when the NED/LSIL group was considered as the control group, but the association was not significant when the NED group was considered as the control group. The A1–3 sublineage was further stratified into the 350T and 350G variants based on the polymorphism at position 350, regardless of sequences at other positions. The A1–3 350G variant was significantly associated with a lower risk of invasive cancer compared with the A1–3 350T variant (age-adjusted OR = 0.06, 95% CI = 0.01–0.32; p = 0.001).

Table 8. Association between HPV16 variants and the cervical lesion grade

HPV 16 variants	N (%)								P_{trend}[1]	P[2]	vs. NED		vs. NED/LSIL	
	NED (N=21)		LSIL (N=48)		HSIL (N=33)		Invasive cancer (N=31)				OR[3] (95% CI)	OR[3] (95% CI)	OR[3] (95% CI)	OR[3] (95% CI)
A1-3	17	(81.0)	30	(62.5)	16	(48.5)	9	(29.0)	0.002	0.002	(referent)		(referent)	
350T	0	(0.0)	3	(10.0)	7	(43.8)	5	(16.1)				(referent)		(referent)
350G	17	(81.0)	27	(52.5)	9	(4.7)	4	(12.9)						0.06 (0.01-0.32)
A4	3	(14.3)	15	(31.3)	15	(45.5)	17	(54.8)	0.020	0.017	9.72 (2.16-43.68)		5.69 (2.05-15.76)	
C	0	(0.0)	1	(2.1)	0	(0.0)	0	(0.0)						
D	1	(4.7)	2	(4.2)	2	(6.1)	5	(16.1)	0.308	0.219			8.46 (1.70-42.15)	

NED, no evidence of disease; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; CI, confidence interval

[1]linear trend test; [2]chi-squared test; [3]age-adjusted odds ratio

6. Association between HPV16 variant specific cervical cancer and HLA–B, –DRB1, –DQA1 and –DQB1 allele frequencies

Frequencies of HLA-B*13, B*51, DRB1*07, DRB1*09, DRB1*11, DQA1*02 and DQA1*04 alleles were significant higher in A4-infected cervical cancer patients than in controls (Table 9, 10). Frequencies of HLA-DRB1*15 was significant higher in A1-3-infected and D-infected cervical cancer patients than controls. In contrast, frequency of HLA-DRB1*04 was significant lower in A4-infected cervical cancer patients than in controls. Regardless of variant type, frequencies of HLA-B*51, DRB1*15, DQA1*02 and DQA1*04 were significant higher in cervical cancer patients than in controls. In contrast, frequencies of HLA-DRB1*04 and DRB1*12 were significant lower in cervical cancer patients than in controls. Frequencies of B*38-DRB1*15, B*51-DRB1*09 and B*13-DRB1*07 were significantly higher in A1−3, A4 and D-infected cervical cancer patients than in controls, respectively (Table 11, 12).

Table 9. Association of HLA-B, DRB1, DQA1 and DQB1 alleles with HPV16-variant specific cervical invasive cancer.

HLA alleles	Published controls[a]		HPV-16 variant from cervical invasive cancer patients						Total	
			A1-3		A4		D			
	n	%	n	%	n	%	n	%	n	%
B										
*07	42	4.33%	0	0.00%	1	2.94%	1	10.00%	2	3.23%
*08	4	0.41%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
*13	54	5.57%	0	0.00%	6	**17.65%**	0	0.00%	6	9.68%
*14	20	2.06%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
*15	141	14.54%	2	11.11%	2	5.88%	1	10.00%	5	8.06%
*27	24	2.47%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
*35	59	6.08%	2	11.11%	0	0.00%	0	0.00%	2	3.23%
*37	14	1.44%	1	5.56%	0	0.00%	0	0.00%	1	1.61%
*38	11	1.13%	1	5.56%	1	2.94%	0	0.00%	2	3.23%
*39	11	1.13%	0	0.00%	1	2.94%	0	0.00%	1	1.61%
*40	116	11.96%	2	11.11%	5	14.71%	2	20.00%	9	14.52%
*44	94	9.69%	3	16.67%	0	0.00%	1	10.00%	4	6.45%
*46	43	4.43%	0	0.00%	2	5.88%	2	20.00%	4	6.45%
*48	33	3.40%	1	5.56%	1	2.94%	1	10.00%	3	4.84%
*51	87	8.97%	3	16.67%	9	**26.47%**	0	0.00%	12	**19.35%**
*52	27	2.78%	2	11.11%	1	2.94%	0	0.00%	3	4.84%

*54	57	5.88%	0	0.00%	1	2.94%	1	10.00%	2	3.23%
*55	30	3.09%	0	0.00%	1	2.94%	0	0.00%	1	1.61%
*56	7	0.72%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
*57	2	0.21%	0	0.00%	1	2.94%	0	0.00%	1	1.61%
*58	63	6.49%	1	5.56%	2	5.88%	0	0.00%	3	4.84%
*59	20	2.06%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
*67	9	0.93%	0	0.00%	0	0.00%	1	10.00%	1	1.61%
DRB1										
*01	31	7.49%	0	0.00%	2	5.88%	1	10.00%	3	5.00%
*03	7	1.69%	0	0.00%	2	5.88%	0	0.00%	2	3.33%
*04	81	19.57%	1	6.25%	1	**2.94%**	1	10.00%	3	**5.00%**
*07	29	7.00%	1	6.25%	6	**17.65%**	1	10.00%	8	13.33%
*08	43	10.39%	1	6.25%	4	11.76%	2	20.00%	7	11.67%
*09	34	8.21%	1	6.25%	7	**20.59%**	1	10.00%	9	15.00%
*10	9	2.17%	1	6.25%	0	0.00%	0	0.00%	1	1.67%
*11	15	3.62%	1	6.25%	4	**11.76%**	0	0.00%	5	8.33%
*12	39	9.42%	1	6.25%	0	0.00%	0	0.00%	1	**1.67%**
*13	43	10.39%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
*14	38	9.18%	3	18.75%	2	5.88%	0	0.00%	5	8.33%
*15	45	10.87%	6	**37.50%**	5	14.71%	4	**40.00%**	15	**25.00%**
*16	0	0.00%	0	0.00%	1	2.94%	0	0.00%	1	1.67%
DQA1										

*01	398	42.61%	6	60.00%	10	35.71%	5	62.50%	21	45.65%
*02	68	7.28%	1	10.00%	6	**21.43%**	1	12.50%	8	**17.39%**
*03	302	32.34%	3	30.00%	6	21.43%	1	12.50%	10	21.74%
*04	15	1.61%	0	0.00%	3	**10.71%**	1	12.50%	4	**8.70%**
*05	108	11.57%	0	0.00%	3	10.71%	0	0.00%	3	6.52%
*06	41	4.39%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
DQB1										
*02	34	8.20%	1	8.33%	5	19.23%	0	0.00%	6	13.04%
*03	146	35.30%	3	25.00%	9	34.62%	2	25.00%	14	30.43%
*04	49	11.90%	1	8.33%	3	11.54%	1	12.50%	5	10.87%
*05	72	17.30%	3	25.00%	5	19.23%	1	12.50%	9	19.57%
*06	113	27.30%	4	33.33%	4	15.38%	4	50.00%	12	26.09%

Bold characters indicate significantly different data compared with published data.

[a]Lee et al.[24] for HLA-B; Song et al.[25] for HLA-DRB1 and DQB1; Lee et al. [26] for HLA-DQA1.

Table 10. Association of significant HLA alleles with HPV16-variant specific cervical cancer.

HLA alleles	Control		HPV16 variant	Cervical invasive cancer		*P*	P_c	OR	(95% CI)
	N	AF		N	AF				
B*									
13	54	5.57%	A4	6	17.65%	0.013	NS	3.63	1.44-9.13
51	87	8.97%	A4	9	26.47%	0.003	NS	3.65	1.65-8.06
51	87	8.97%	Total	12	19.35%	0.013	NS	2.43	1.25-4.74
DRB1*									
04	81	19.57%	A4	1	2.94%	0.011	NS	0.13	0.02-0.92
04	81	19.57%	Total	3	5.00%	0.004	NS	0.22	0.07-0.71
07	29	7.00%	A4	6	17.65%	0.039	NS	2.85	1.09-7.42
09	34	8.21%	A4	7	20.59%	0.026	NS	2.90	1.18-7.14
11	15	3.62%	A4	4	11.76%	0.047	NS	3.55	1.11-11.36
12	39	9.42%	Total	1	1.67%	0.045	NS	0.16	0.02-1.21
15	45	10.87%	A1-3	6	37.50%	0.007	NS	4.92	1.71-14.18
15	45	10.87%	D	4	40.00%	0.020	NS	5.47	1.49-20.11
15	45	10.87%	Total	15	25.00%	0.006	NS	2.73	1.41-5.30
DQA1*									
02	68	7.28%	A4	6	21.43%	0.016	NS	3.47	1.36-8.83
02	68	7.28%	Total	8	17.39%	0.021	NS	2.68	1.20-5.96
04	15	1.61%	A4	3	10.71%	0.014	NS	7.34	2.00-26.96

04	15	1.61%	Total	4	8.70%	0.010	NS	5.82	1.85-18.31

P_c, P value after Bonferroni correction; NS, not significant

Table 11. Association of HLA B-DRB1 haplotype with HPV16-variant specific cervical invasive cancer.

B-DRB1 haplotype	Controls (n=100)	HPV-16 variant from cervical invasive cancer patients						Total	
		A1-3		A4		D			
	%	n	%	n	%	n	%	n	%
*07-*01	1.68	1	5.56	0	0.00	1	10.00	2	3.33
*13-*07	2.91	0	0.00	4	12.50	2	**20.00**	6	10.00
*15-*04	1.03	0	0.00	1	3.13	0	0.00	1	1.67
*15-*15	1.78	0	0.00	3	9.38	0	0.00	3	5.00
*15-*16	0.23	0	0.00	1	3.13	0	0.00	1	1.67
*35-*04	2.28	0	0.00	1	3.13	0	0.00	1	1.67
*37-*10	1.94	0	0.00	1	3.13	0	0.00	1	1.67
*38-*15	0.97	2	**11.11**	0	0.00	0	0.00	2	3.33
*39-*01	0.49	0	0.00	1	3.13	0	0.00	1	1.67
*40-*08	2.37	0	0.00	1	3.13	0	0.00	1	1.67
*40-*09	1.58	1	5.56	0	0.00	1	10.00	2	3.33
*40-*11	1.44	2	11.11	1	3.13	1	10.00	4	6.67
*40-*15	1.21	1	5.56	1	3.13	0	0.00	2	3.33
*44-*07	2.67	2	11.11	0	0.00	0	0.00	2	3.33
*44-*12	0.24	1	5.56	0	0.00	0	0.00	1	1.67
*44-*14	0.28	0	0.00	0	0.00	1	10.00	1	1.67
*46-*08	3.69	0	0.00	2	6.25	0	0.00	2	3.33

*46-*09	0.92	1	5.56	0	0.00	0	0.00	1	1.67
*46-*15	0.32	0	0.00	0	0.00	1	10.00	1	1.67
*48-*14	0.97	0	0.00	1	3.13	0	0.00	1	1.67
*48-*15	0.97	1	5.56	0	0.00	1	10.00	2	3.33
*51-*08	3.34	2	11.11	1	3.13	0	0.00	3	5.00
*51-*09	2.31	1	5.56	4	**12.50**	0	0.00	5	8.33
*51-*14	2.20	0	0.00	3	9.38	0	0.00	3	5.00
*52-*15	2.43	2	11.11	0	0.00	0	0.00	2	3.33
*54-*09	0.53	0	0.00	1	3.13	0	0.00	1	1.67
*54-*11	0.59	0	0.00	0	0.00	1	10.00	1	1.67
*54-*15	0.95	0	0.00	2	6.25	0	0.00	2	3.33
*55-*09	1.16	0	0.00	1	3.13	0	0.00	1	1.67
*57-*08	0.24	0	0.00	0	0.00	1	10.00	1	1.67
*58-*03	1.70	1	5.56	0	0.00	0	0.00	1	1.67
*58-*15	0.26	0	0.00	1	3.13	0	0.00	1	1.67
*67-*04	0.24	0	0.00	1	3.13	0	0.00	1	1.67

Bold characters indicate significantly different data compared with control data.

Table 12. Association of significant HLA B-DRB1 haplotypes with HPV16-variant specific cervical invasive cancer.

HPV16 variants	B-DRB1 haplotypes	Controls (n=100)	Cervical invasive cancer		*P*	P_c	OR	95% CI	
		%	N	%				Lower	Upper
A1-3	*38-*15	0.97	2	11.11	0.044	NS	12.8	1.1	152.7
A4	*51-*09	2.31	4	12.50	0.035	NS	6.0	1.1	32.2
D	*13-*07	2.91	6	20.00	0.032	NS	8.3	1.2	58.0

P_c, P value after Bonferroni correction; NS, not significant

Discussion

In this study, genetic variations in the E6, E7, L1 genes and the LCR of HPV16 were simultaneously investigated for the first time in Korea. We determined the distribution of HPV16 variants in Korean women with cervical lesions who were infected with HPV16 but no other HPV types, and we discovered associations between specific HPV16 variants and the severity of cervical lesions.

The distribution of HPV16 variants differed from those reported in previous Korean studies. A previous study of HPV16 variants in 54 commercial sex workers demonstrated a variant distribution of 68% A4, 16% A1–3, and 14% D [10]. A recent hospital-based Korean study reported a distribution of 57.7% A4, 34% A1–3, and 8.3% D [11]. Here, the proportion of A4 was 37.6%, which was much lower than that of previous studies, and A1–3, at 54.1%, was the most frequently detected variant. Studies in other Asian countries have reported percentages of the A1–3 and A4 variants as follows: 39.7 and 60.3% (central China), 69.0 and 31.0% (southwestern China), 26.1 and 73.9% (Thailand), 55.8 and 44.2% (Japan), respectively [14, 27-29]. The percentages found in this study were comparable to those reported in Japan. A remarkable finding of the present study was that the percentage of the A1–3/350G variant (42.9%) was very high compared with all previous reports in Asia, which detected its proportion as 3.6% (China), 28% (Japan), and 6% (northeastern Asia) [30]. The proportion of A1–3/350G in this study was comparable to its proportions reported in North America and Europe (40 and 44%, respectively) [30]. The inconsistencies between these results and those of the previous reports on the HPV16 variant distribution in Korea suggest that the

variant distribution in the Korean population is not only affected by geography and/or ethnicity, but also other demographic factors. Further studies are needed to identify which factors affect the HPV16 variant distribution.

Several studies in Asian populations have demonstrated that non-A1–3 variants cause more persistent infection and have a higher risk of progression to invasive cervical cancer than A1–3 variants do [31-35]. It is remarkable that this trend is dependent upon ethnicity [9]. A1–3 variants lead to persistent infection in Caucasian populations, while infections with B variants are more likely to be persistent in African-American populations. Similarly, in most Asian studies, A4 variants have been most frequently observed in cervical cancer [13, 14, 36, 37]. In previous Asian studies, A1–3 variants showed a tendency toward a negative association with severe cervical lesions [6, 23, 36, 38]. This is concordant with our finding that A1–3 variants were negatively associated with cervical cancer compared to A4 or D variants. Further investigation showed that A4 and D variants conferred 5.7- and 8.5-fold higher risks of cervical cancer, respectively, compared to A1–3 variants, when NED/ LSIL was considered as the control group. In addition, the A1–3/350G variant was found to confer a significantly lower risk of cervical cancer compared to the A1–3/350T variant. To the best of our knowledge, this is the first Asian report concerning the associations of D variants and the A1–3/350G variant with invasive cancer.

HPV16 E6 polymorphisms have been shown, although inconsistently, to be associated with squamous intraepithelial lesion or invasive cancer [6]. The D25E and L83V variants are both associated with an elevated risk of cervical carcinoma and the risk that they confer appears to vary geographically based on genetic

differences between populations [14, 31, 39-44]. In Europe, L83V was reported to be associated with an increased risk of persistent infection and higher grades of cervical lesions [42, 45]. However, this finding was inconsistent with those observed in other European populations and in studies performed in other parts of the world [46, 47]. Currently, the functional implications of the L83V substitution are unknown. Here, the L83V variant was associated with a lower grade of cervical lesions. Additionally, it was notable that the L28F and L83V mutations were commonly associated (50/58, 86.2%). To date, a small number of reports about the L28F mutation have been generated in Japan, Thailand, India, and China [48-51]. L28F is known to affect DNA synthesis, E2F-pRb dissociation, pRb binding, and the nuclear localization signal [50]. The potential association between L28F and lower grades of cervical lesions should be investigated further, considering its known functional implications.

R10I and Q14D are prevalent amino acid substitutions observed in B variants [52]. Q14H, H78Y, and L83V show covariance in D variants and reflect combined differentiation and apoptosis patterns associated with a phenotype that promotes carcinogenesis and benefits the viral life cycle [53]. This is consistent with our association of D variants with higher grade lesions. Additionally, we found that 181A>G (missense mutation, I26M), 463C>G (missense mutation, D120E), and 490G>A in the E6 gene, and 757C>T (missense mutation, R66W) in the E7 gene were novel single-nucleotide substitutions found in A4 variants, where the corresponding cervical pathology was HSIL or worse, except for isolates bearing the 757C>T mutation.

Frequencies of HLA-B*13, B*51, DRB1*07, DRB1*09, DRB1*11, DQA1*02

and DQA1*04 alleles were significant higher in A4-infected cervical cancer patients than in controls. Frequencies of HLA-DRB1*15 was significant higher in A1-3-infected and D-infected cervical cancer patients than controls. There were no previous reports about the association between HLA-B*13 and HPV16 infection. The research of HLA class I related with HPV variant is very small number. A previous Sweden study reported that the presence of HLA-B*51 was associated with 4-fold higher risk of cervical cancer in L83V variant (currently A1-3) [43]. HLA class II has been studied more than HLA class I. From the data of meta-analysis, HLA-DRB1*04 and DRB1*07 had positive correlation with the cervical cancer, but DRB1*09 had negative correlation [54]. A Brasil study reported that HLA-DRB1*15 had positive correlation with A1-3-related cervical cancer [55]. In Japanese, genetically close with Korean, HLA-DRB1*1501 was significantly related with A1-3- and A4-infected cervical cancer patients [12]. Other studies also reported the association of HLA-DRB1*1501 with HPV16-positive cervical cancer [16, 56, 57]. However, this finding was not consistent in other studies [58, 59]. Wu et. suggested that this inconsistency is related with the distribution of E6 prototype (currently A1-3) [60]. In this study, A1-3 (previous European)-infected cervical cancer patients had significantly higher frequency of HLA-DRB1*15. There were no previous reports about the association of HLA-DQA1*02 and DQA1*04 with cervical cancer. In summary, a part of HLA alleles were correlated with HPV16-variant specific cervical cancer. However, limited number of patients could lead the result to miss significant alleles related with cervical cancer. In addition, the significances of all the association between HLA alleles and HPV16-variant specific cervical cancer were denied after Bonferroni correction. But, many

researchers pointed out that this kind of correction could overlook the significant correlation [61, 62].

Although the L1 gene is located in a conserved region, we found that 110 isolates (82.7%) had no mutation in the L1 gene and that 12 isolates had amino acid changes resulting from missense mutations (Table 3). We found that 6695A>C, 6721G>A, 6854C>T, 6865C>T, 6970C>T, and 6994G>A were common polymorphisms in D variants. T379P (6695A>C) and T415S (6803A>T) are known to occur in the region involving a B-cell epitope and were reported in 12 and 8% of HPV16 isolates from an Indian population, respectively [50]. In a previous study, T379P was reported to cause a reduction in the immunogenicity of epitopes in the vicinity of the mutation [63]. Here, we discovered that the number of samples bearing this variation was higher than that reported in previous Korean studies. Considering the high oncogenicity and low immunogenicity of the T379P variant, it appears that a HPV vaccine should be designed against this variant for use in Korea. Furthermore, 6619C>T, 6658A>G, 6721G>A, 6865C>T, 6949A>G, 6956C>T, and 6970C>T mutations in the L1 gene were not previously reported.

The LCR displays greater sequence variability than any other region in the HPV16 genome. This region includes several binding sites for cellular and viral TFs. In this study, the variation rate in the LCR was 94.6% (87/92), and 24 loci with a total of 35 mutations were located within TF-binding sites (data not shown). Additionally, 61 of 87 isolates bearing LCR variations exhibited mutations in TFbinding sites. However, no single mutation was associated with the grade of cervical lesions. Furthermore, seven novel single-nucleotide polymorphisms were found in the LCR. Transcriptional analysis of the HPV16 LCR is important to

evaluate the association between LCR sequence variations and the oncogenic potential of HPV16 variants [64]. Sequence variations in the HPV16 LCR may affect the carcinogenic potential of HPV16, as nucleotide substitutions within this regulatory region modulate replication and transcription through their effect on the formation of regulatory protein complexes on DNA [65]. Therefore, further study is needed to identify the effects of nucleotide changes in the LCR.

This study was the first extensive investigation of the genetic variations in the E6, E7, and L1 genes, and the LCR in Korea. We calculated the odds ratio of cervical invasive cancer in each HPV16 sublineage compared to A1–3. We also adjusted the data for the age discrepancy of the participants in each histopathologic group. In addition, we conducted the first analysis about the correlation of HLA polymorphism with HPV16-variant specific cervical cancer in Korea. One limitation of this study was that the LCR sequence was unavailable in 41 isolates, and most of these isolates (n = 39) were typed as the A1–3/350G variant. After our experiment, a study reported the high prevalence of a nucleotide deletion (g.7863delA) in the target area corresponding with one of the primers used in this study [6]. Although we could not conduct additional sequencing experiments due to sample scarcity, this deletion may have inhibited LCR amplification. Another limitation was the small sample size due to the hospital-based research design of the study. To obtain more generalizable results, further prospective and large-scale studies should be conducted. Additionally, experiments investigating the biological activities of each HPV16 variant should also be performed.

In conclusion, HPV16 A1–3 variants and, particularly, the 350G variant were clearly predominant in this study was remarkable. Additionally, we reported for the

first time in an Asian population a significant association of A4, D, and A1–3/350G variants with cervical cancer. This constitutes the first extensive investigation of the E6, E7, and L1 genes and the LCR in Korea, and 18 novel single-nucleotide polymorphisms (three in E6, one in E7, seven in L1, and seven in the LCR) were found in this study. Based on comparisons of our results with those of previous studies, it seems that the distribution of HPV16 variants can vary among populations, even those with the same ethnicity and geographical location. In addition, we could clarify the possible correlation of HLA polymorphism with HPV16-variant specific cervical cancer. These data will aid in the development of HPV vaccines and diagnostic techniques in Korea. Prospective and large scale studies to investigate the dynamics of HPV16 variants in Korean populations and their correlations with the severity of cervical lesions are needed.

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초록

배경: 사람유두종바이러스 16형(HPV16)의 지속 감염은 자궁경부암의 제일 주요한 위험요인이다. HPV16의 유전자변이형은 지리적으로 분포를 달리하고 발암성 측면에서도 차이를 보인다. 또한 자궁경부암에 대한 감수성은 인종별로도 다른 것으로 알려져 있다. 본 연구에서는 한국여성에서 HPV16 변이형의 분포와 HLA 다형성이 자궁경부병변의 조직병리학과 어떤 관계가 있는지 분석하였다.

방법: 총 133개의 HPV16 양성 자궁경부 검체를 서울대학교병원 보라매병원에 내원한 환자에서 채취하였으며, HPV16에 대해서 E6, E7, L1, LCR 유전자 및 유전자사이영역에 대한 염기서열 분석을 시행하였다. 그리고 자궁경부병변의 정도에 따른 변이형 분포를 확인하였다. 이 중에서 자궁경부암 환자의 세포에 대해서는 HLA-B, -DRB1, -DQA1, -DQB1에 대한 대립유전자 빈도를 확인하였다. HLA 대립유전자 빈도와 HPV16 유전자변이형에 따른 자궁경부암과의 관계를 분석하였다.

결과: 유전자변이형은 A1-3, A4, C, D가 각각 54.1%, 37.8%, 0.7%, 7.4%를 구성하고 있었다. 가장 흔한 LCR 영역의 변이는 7521G>A (91.5%), 7730A>C (59.6%), 7842G>A (59.6%)였다. 나아가, A4나 D에 감염된 여성은 A1-3에 감염된 경우보다 자궁경부암에 이환된 위험이 유의하게 높았다. A1-3가 가장 많은 변이형이었으며, A1-3 중에서도 350G 변이를 보이는 경우가 제일 많았다. 제한된 숫자를 분석하였지만, HLA-B*13, B*51,

DRB1*07, DRB1*09, DRB1*11, DQA1*2, DQA1*4 대립유전자가 A4에 감염된 환자에서 자궁경부암의 유의한 위험요인이었으며, HLA-DRB1*15는 A1-3, D에 감염된 환자에서 자궁경부암의 유의한 위험요인이었다.

결론: 본 결과는 이전의 한국 데이터나 다른 아시아 국가 데이터와 차이를 보였다. HPV16에 감염된 환자에서 자궁경부암이 발생할 확률은 해당 인구에서 HPV16 유전자변이형과 HLA형의 영향을 받는 것으로 보인다.

주요어: 사람유두종바이러스 16형, 유전자변이형, 사람백혈구항원, 대립유전자, 자궁경부암, 편평상피내병변, 환자-대조군 연구

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