RESEARCH COMMUNICATION

Distribution of HPV-16 Intratypic Variants among Women with Cervical Intraepithelial Neoplasia and Invasive Cervical Cancer in Mongolia

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Abstract

Objectives. Infection with high-risk human papillomavirus (HPV) is a critical factor associated with carcinogenesis of the uterine cervix. HPV-16 is most frequently found, and is further subclassified into intratypic variants based on the nucleotide sequences of the viral genes. Although certain HPV-16 variants are reported to be associated with the progression of cervical lesions, these relationships remain controversial with different results for different populations. To provide data for another population, we investigated the prevalence of HPV-16 and distributions of its intratypic variants among Mongolian women with cervical intraepithelial neoplasia (CIN) and invasive cervical cancer. Materials and Methods. We analyzed samples from 374 randomly selected women who attended the National Cancer Center of Mongolia between January 2002 and July 2007, including 147 invasive cervical cancer patients, 127 CIN patients and 100 age-matched controls who were cytologically normal. HPV genotyping was initially conducted, followed by variant analysis for HPV-16-positive samples by nucleotide sequencing of the E6 gene. The HPV data were evaluated statistically for correlations with the patients' clinical data. Results. HPV genotyping detected 101 HPV-16-positive samples. Among these samples, 92 were available for subsequent variant analysis, including 66 invasive cervical cancer samples, 25 CIN samples and 1 cytologically normal sample. A total of 14 different variants were identified. All 14 variants belonged to the European lineage, and the European prototype was detected in 66% (61/92) of the samples. Among the remaining 31 variants, variants with the T350G nucleotide change were predominant (13/31, 42%), followed by variants containing G94A (11/31, 35%), G176A (4/31, 13%) and G274T (2/31, 7%) . There were no significant differences among all the variants regarding their distributions in CIN and invasive cervical cancers. Conclusions. HPV-16 variants of the European lineage were exclusively distributed among the Mongolian women examined, and the European prototype was overwhelmingly predominant. Since no significant differences were found between the types of variants and severities of the cervical lesions, it is possible that racial or geographic factors may have some influences on these relationships.

Key Words: Human papillomavirus - HPV-16 variants - E6 gene - cervical intraepithelial neoplasia - cancer

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Introduction

Human papillomavirus (HPV) infection is one of common sexually transmitted infections among sexually active people. More than 150 types of HPV have been found, and a certain group of high-risk HPV types is strongly and consistently associated with cervical intraepithelial neoplasia (CIN) and invasive cervical cancers (zur Hausen et al., 2000). In particular, HPV-16 is the most common type found in patients with CIN and invasive cancers, and is reported to be present in >50% of such lesions (Schlecht et al., 2001; Woodman et al., 2001; Munoz et al., 2003). We previously reported at the 22nd International Papillomavirus Conference and Clinical Workshop in 2005 that HPV-16 is the most commonly detected type among Mongolian women with CIN or invasive cervical cancers, followed by HPV-33 and HPV-31. These findings were confirmed in a recent report (Dondog et al., 2008).

HPV-16 is also the most commonly distributed type in the normal population, and therefore only a certain proportion of women with this viral infection are considered to develop CIN and cervical cancers (Bosch et al., 2002). The issues of why the infection persists and leads to the development of malignant cervical lesions in certain women are key questions for current researchers

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in this field. Both host factors and viral factors need to be considered to answer these questions, and HPV-16 intratypic variation is a candidate for one of the viral risk factors. HPVs vary genetically not only among the >150 types but also within each type. Intratypic variants are defined as HPVs that vary by ≤2% from the reference viral prototype in the nucleotide sequences of specific viral genes, such as E6, E7, L1 and L2 (Zehbe et al., 1999; Bernard et al., 1994). Owing to the predominant distribution of HPV-16, its intratypic variants have been extensively investigated, and can be clustered into five major geographical variant lineages, namely the European (E), Asian (A), Asian-American (AA), African-1 (Af-1) and African -2 (Af-2) lineages (Ho et al., 1993; Yamada et al., 1995; Yamada et al., 1997). Several researchers have reported correlations between specific HPV-16 variants and persistent viral infection followed by the development of malignant lesions (Xi et al., 1997; Villa et al., 2000; Hildesheim et al., 2001; Berumen et al., 2001; Xi et al., 2007; Sichero et al., 2007). The biological activities of the viral oncogenic proteins and antigenicities of the viral epitopes are considered to be responsible for the differences among variants (Ho et al., 1993; Zehbe et al., 2001).

In particular, an HPV-16 intratypic variant carrying one nucleotide alteration at position 350 (T-to-G transition) in the E6 gene (designated the T350G variant) was reported to be strongly associated with the oncogenicity and persistency of the viral infection in certain European populations, including French, Swedish and British populations (Londesborough et al., 1996; Zehbe et al., 1998; Grodzki et al., 2006). On the other hand, some investigations found that the T350G variant was not correlated with malignant progression of uterine cervical lesions in different populations (Nindl et al., 1999; Andersson et al., 2000; Chan et al., 2002). To understand the virological and clinical characteristics of HPV-16 variants, we need further information from different countries and populations. Therefore, we investigated the distribution patterns of intratypic variants of HPV-16 among women with CIN and invasive cervical cancers in Mongolia.

Materials and Methods

Study population and sample collection

We analyzed 374 women randomly selected from patients who attended the National Cancer Center of Mongolia from January 2002 to July 2007. The women comprised 147 histologically confirmed invasive squamous cell carcinoma patients, 127 histologically confirmed CIN patients and 100 age-matched cytologically normal women. All the study subjects were of Mongolian nationality and provided written informed consent to participate in the study.

DNA detection and genotyping

Cervical scrapes were collected into an appropriate amount of phosphate-buffered saline during clinical examination. After collection of the cellular contents, DNA was extracted with a DNA Mini Kit (Qiagen Sciences, Valencia, CA) according to the manufacturer's viral DNA extraction protocol for fresh cells. Detection of HPV-16 was carried out by amplifying a 374-bp segment of the E6 and E7 genes by polymerase chain reaction (PCR) with a type-specific primer (HVPpF/16R) (Shimada et al., 1990) and a PCR Human Papillomavirus Detection Set (Takara, Tokyo, Japan).

HPV-16 variant analysis

Variants of HPV-16 were further discriminated based on the nucleotide sequences of the E6 gene as previously described (Tu et al., 2006). Briefly, the entire region of the E6 gene was amplified using a proofreading High Fidelity Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) and a pair of PCR primers that amplified a 455-bp segment corresponding to nucleotides 78-532. The nucleotide sequences of both strands of the amplified E6 gene were directly determined using the PCR primers as sequencing primers, a Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a Prism 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. The obtained nucleotide sequences were analyzed with the sequence analysis software programs GENETYX 7.0 (Genetyx Corporation, Tokyo, Japan) and MEGA4.0 (Tamura et al., 2007). The nucleotide sequence of the HPV-16 prototype (GenBank accession number K02718) was used as the HPV-16 reference sequence in this study. The amino acid positions are stated numerically from 1 to 158, according to the amino acid sequence provided under GenBank accession number AAA46939.

Statistical analyses

The correlations between HPV-16 variants and the clinical data of the patients were evaluated using SPSS version 15 for Windows (SPSS Inc., Chicago, IL). The correlations between factors were analyzed by the chi-square test. For the analysis of Mongolian ethnic groups, the Yates 2x2 chi-square test was applied. Values of $p<0.05$ were considered to indicate statistical significance.

Results

Distribution of HPV-16

Overall, 27% (101/374) of the study participants were positive for HPV-16. HPV-16-positivity was identified in 48% (71/147) of the invasive cervical cancer samples, 23% (29/127) of the CIN samples and 1% (1/100) of the cytologically normal samples. Among the 101 HPV-16-positive samples, 92 (66 invasive cancers, 25 CIN and 1 normal cytology) were available for subsequent nucleotide sequencing analysis to determine the intratypic variants.

HPV-16 variant analysis

Based on the E6 gene nucleotide sequence, all the HPV-16 variants detected belonged to the European lineage, and a total of 14 different intratypic variants were identified (Table 1). The variant identified as the European prototype (EP) accounted for 66% (61/92) of the samples. There were 11 nucleotide positions that differed from the European prototype among the remaining 31 variants.

Table 1. HPV-16 variants Determined by E6 Gene Nucleotide Sequencing Among Mongolian women

Variant	Amino acid	Number	94G	176G	183T	188G	241T	245C	246G	274G	295T	350T	426A
EP	Prototype-	61	-	-	-	-	-	-	-	-	-	-	-
E(G94A)	M1K	7	A	-	-	-	-	-	-	-	-	-	-
E(G176A)	D25N	4	-	A	-	-	-	-	-	-	-	-	-
E(G176T)	D25Y	1	-	T	-	-	-	-	-	-	-	-	-
E(G188C)	E29Q	1	-	-	-	C	-	-	-	-	-	-	-
E(T241G)	Silent mutation	1	-	-	-	-	G	-	-	-	-	-	-
E(G246A)	N58S	1	-	-	-	-	-	-	A	-	-	-	-
E(G274T)	Silent mutation	2	-	-	-	-	-	-	-	T	-	-	-
E(A426C)	K108T	1	-	-	-	-	-	-	-	-	-	-	C
E(T350G)	L83V	5	-	-	-	-	-	-	-	-	-	G	-
E(T350G/G94A)	L83V/M1K	4	A	-	-	-	-	-	-	-	-	G	-
E(T350G/T183C)	L83V/I27R	1	-	-	C	-	-	-	-	-	-	G	-
E(T350G/T295G)	L83V/D64E	2	-	-	-	-	-	-	-	-	G	G	-
E(T350GT295G/ C245T)	L83V/D64E/R48E	1	-	-	-	-	-	T	-	-	G	G	-

The types of HPV-16 intratypic variants are shown in the first column according to the order of the nucleotides of the prototype, the nucleotide positions in the E6 gene and the nucleotides of the variants. The amino acid alterations caused by the nucleotide changes are shown in the second column in the order of the amino acids of the prototype, the amino acid positions (the first amino acid encoded by the E6 gene is number 1) and the amino acids encoded by the variants. The amino acids are written in the one-letter code. The third row shows the numbers of cases with the variants. The nucleotide positions and nucleotides of the European prototype are shown across the top. The absence of genetic variations relative to the prototype is marked with a dash, and any variant nucleotides are indicated. EP, European prototype; E, European lineage variant

Table 2. HPV-16 Intratypic Variants and the Severity of Cervical Lesions

Sample	EP	T350G	G94A	Others	P value
Normal	1	-	-	-	
CIN1	-	-	1	-	
CIN2	2	-	1		
CIN3	15	4	1	1	
Invasive cancer	46	9	4	7	
Total	64	13	7	8	0.131

Statistical analyses were performed using the chi-square test. Descriptions of the variants are presented in the subscript to Table 1. CIN, cervical intraepithelial neoplasia; T350G, all variants carrying the E(T350G) nucleotide change; G94A, all variants carrying the E(G94A) nucleotide change; Others, all variants except for the EP, T350G and A94G variants

Nine variants had a single nucleotide change from the prototype and accounted for 25% (23/92) of the samples. A further 2 and 1 variants had 3 nucleotide changes and accounted for 8% (7/92) and 1% (1/92) of the samples, respectively. Among the non-prototype variants, the European (E) T350G variant with or without other nucleotide changes was predominant (13/31, 42%), followed by the E(G94A) variant (11/31, 35%). Concerning the coding amino acids, two single nucleotide changes at positions 241 (E(T241G)) and 274 (E(G274T)) were silent while the other nucleotide changes were accompanied by alterations in coding amino acids (Table 1). Thirteen E(T350G)-containing variants were found in 4 (31%) CIN and 9 (69%) invasive cancer samples but not in the CIN1, CIN2 or normal specimens. This pattern of distribution did not differ significantly from those of EP and the other variants (Table 2). Relations to cllinical data are shown in Table 3.

Population description

The patients and the woman with normal cytology examined in the HPV-16 variant analysis were distributed in 25 Mongolian administration units and 7 kinds of Mongolian small ethnic groups. A total of 28 patients (30%

Table 3. HPV-16 Intratypic Variants in Relation to Clinical Data

Variables		EP	T	G	O	P value
Age (year)	≤20	7	1	1	-	
	21-30	19	4	1	3	
	31-40	25	4	4	4	
	41-50	10	2	0	1	
	51-60	3	2	1	-	0.858
Age first intercourse	>18	28	5	2	3	
	≤18	36	8	5	5	0.94
Number sex partners	≥3	54	9	6	7	
	<3	10	4	1	1	0.585
Tobacco smoking	No	60	12	7	8	
	Yes	4	1	-	-	0.783
Alcohol consumption	No	61	12	7	7	
	Yes	3	1	-	1	0.711
Oral contraceptives	No	53	11	6	7	
	Yes	11	2	1	1	0.985
Education	Primary	15	6	2	2	
	High school	31	4	2	5	
	Secondary+	18	3	3	1	0.528
Ethnic group	Bayad	2	-	-	-	
	Durvud	4	-	-	-	
	Khalkh	56	12	6	7	
	Zahchin	2	-	-	-	
	Kazak	-	1	-	-	
	Myangad	-	-	-	1	
	Urianhai	-	-	1	-	*0.045
Menopausal status	Pre	43	7	6	7	
	Post	21	6	1	1	0.303
Number pregnancies	None	1	-	-	-	
	≤3	16	3	-	2	
	4 to 6	32	3	3	2	
	≥7	15	7	4	4	0.337
Medical abortions	1	27	8	4	3	
	≥2	37	5	3	5	0.528

The correlations between factors were analyzed by the chi-square test. Abbreviations: T, T350G;G,G94A; O, others. *Among the Mongolian ethnic groups, the Bayad, Durvud, Khalkh and Zahchin groups only harbored the EP variant and were considered as one group for comparisons with the Kazak, Myangad and Urianhai groups by the Yates 2x2 chi-square test

of all participants) in our study lived in the capital city Ulaanbaatar. Statistical analyses of data collected by questionnaires given to the patients revealed that the distributions of the HPV-16 intratypic variants in Mongolian women were not correlated with patient age, education status, some gynecological and sexual histories (such as age at first intercourse, number of sexual partners, number of pregnancies and pre- or postmenopausal state), oral contraceptive usage, smoking and alcohol drinking status (p=0.3-0.9). There was a significant correlation (p=0.045) between the HPV-16 variants and small ethnic groups of Mongolia. Khalkh is the predominant ethnic group in Mongolia, and accounts for almost 90% of the population. All the variants identified were detected among the Khalkh ethnic group, while the overwhelmingly predominant EP was exclusively found in the Bayad, Durvud and Zahchin ethnic groups. On the other hand, EP was not detected in the Urianhai, Kazak and Myangad ethnic groups.

Discussion

In the present study, we have shown the distribution patterns of intratypic variants of HPV-16 among Mongolian women for the first time. A total of 374 samples were examined in the study, and most of the HPV-16-positive samples (92/101, 91%) were subjected to intratypic variant analysis by nucleotide sequencing of the E6 gene. Our results clarified that only the European lineage of HPV-16 is distributed in Mongolia, and that the European prototype is the most dominant variant among the lineage.

Mongolia is one of the most sparsely populated, but independent, countries in the world. It has a population of around 2.9 million people and is geographically located in the heart of Central Asia. Only two neighboring counties, Russia and China, that can be considered as significant representatives of Europe and Asia, respectively, surround Mongolia. We expected that the Asian lineages of HPV-16 variants might be dominant in our samples owing to the geographic location of the country. Unexpectedly, however, the E6 gene sequencing analyses clarified that all the HPV-16 variants detected belonged to the European lineage. Although HPV-16 European lineage variants are most commonly distributed around the world (Watt et al., 2001; Picconi et al., 2002), the distributions of HPV-16 variants were reported to show geographic specificities (Matsumoto et al., 2003; Tornesello et al., 2004; Pista et al., 2007; Choi et al., 2007;). A variant analysis in Russia, the northern neighbor of Mongolia, demonstrated all HPV-16 variants identified in samples from CIN and invasive cancer patients belonged to the European lineage (Hu et al., 2001). In China, the southern neighbor of Mongolia, the Asian and Asian American lineages are circulating in the central area while the Asian and European lineages are circulating in the southern area of the country (Chan et al., 2002; Qui et al., 2007). The exclusive circulation of the European lineage in Mongolia could be partly explained by the former political system, which only allowed communication with Russia for the last seven decades. Therefore, this circulation picture may change in the near future owing to recent increases in migration and movement of Mongolians throughout the world, especially into Asian countries.

Based on human leukocyte antigen (HLA) polymorphisms, it has been reported that Mongolians show close similarities to European populations (Munkhbat et al., 1997; Chimge et al., 1997; Chimge et al., 1999) rather than Asian populations. To the best of our knowledge, no previous reports have described the relationships between infections with HPV-16 variants and the HLA subtypes of patients. However, it is possible that the exclusive circulation of the European lineage in Mongolian women might be caused by their HLA subtypes. Further investigations are required to clarify the relationships between HPV-16 infections and HLA subtypes in Mongolians.

Other than EP, E(G94A) was the second most frequent variant after E(T350G)-containing variants and was detected in 1 CIN1, 1 CIN2, 1 CIN3 and 4 invasive cancer samples. Qui et al. (2007) recently reported E(G94A) as a novel mutation found in South China. The reason for Mongolian and South Chinese populations sharing this new variant should be further investigated in the interest of public health governance against HPV-16 propagation. We also encountered 1 case with three nucleotide changes, namely E(T350G), E(T295G) and E(C245T), and 2 cases with two nucleotide changes, namely E(T350G) and E(T295G). These results could be caused by mixed infections of two or three different European lineage viruses in the individual patients. The HPV-16 viruses should be isolated from these patients to confirm the existence of these variants.

The E(T350G) variant was reported to be strongly associated with the oncogenicity and persistency of HPV-16 infection in certain European populations (Londesborough et al., 1996; Zehbe et al., 1998; Anserson et al., 2000; Grodzki et al., 2006). In the present study, however, E(T350G), EP and the other variants showed similar distribution patterns in the patients with invasive cervical cancers and CINs, and there was no significant association between E(T350G) and invasive cancers, the highest degree of malignancy in uterine cervical lesions caused by HPV. There may be racial differences in the relationships between HPV-16 variant infections and the severities of cervical lesions.

There was a significant correlation (p=0.045) between HPV-16 variants other than EP and the Urianhai, Kazak and Myangad small ethnic groups. However, the sample number was small and we need to collect more samples to confirm this correlation.

In the present study, we have described the distribution patterns of intratypic variants of HPV-16 among Mongolian women for the first time. In Mongolia, however, there is still no well-organized cervical cancer screening program and the capacity of laboratories to analyze HPV infections is still developing. Continuous efforts are required to collect more samples to clarify the relationships of HPV infections, especially those for the intratypic variants and advanced cervical lesions. These data are essential for an effective HPV vaccination

program, which may start in the near future, and for eradication of cervical cancers in Mongolia.

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