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Original Article

Variant sublineages of human papillomavirus type 16 predispose women to persistent infection characterized by a sequence analysis of the E6, L1, and LCR regions

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Abstract: Background: One of the precursors of cervical cancer is persistent infection with human papillomavirus (HPV), especially high-risk HPV. The aim of this study was to verify the relationship between HPV16 variants and persistent viral infection. Methods: Three-hundred and eighty-six Chinese women who had a low-grade squamous intraepithelial lesion (LSIL) or a lesion below LSIL with normal cellular morphology were selected and enrolled in this study. Flow-through hybridization and gene chip technology were applied to identify the HPV type, and a PCR-sequencing assay was performed to detect HPV16 *E6*, *L1*, and long control region (*LCR*) gene variants. The relationship between HPV16 variants and persistent infection was analyzed using Fisher's exact test. Results: In this population, 74.09% of HPV16 isolates belonged to the A4 sublineage, 24.87% to the A1/A2 sublineages, and 3.13% to B1/B2 sublineages. In addition, the A4 sublineage T178G ($P<0.001$) and the A1/A2 sublineages T350G and A442C ($P<0.001$) were associated with persistent HPV16 infection. *L1* and *LCR* variants were found to be common in this population. Nonetheless, no significant relation was identified between the *L1* or *LCR* variants and the persistence of infection ($P>0.05$). Conclusion: HPV16 E6 variants in the Shanghai Pudong District mainly belong to the A4 sublineage, and detection of the specific HPV *E6* T178G genotype may be considered a risk factor for viral persistence and progression to other cervical diseases.

Keywords: Human papillomavirus, E6, long control region, variant, cervical diseases

Introduction

Cervical cancer is the most common female genital malignant tumor and one of the reasons why women are often screened worldwide. Persistent infection with human papillomavirus (HPV), especially high-risk HPV, is a risk factor for the development of cervical cancer [1-3]. Approximately 500,000 women develop invasive cervical cancer yearly worldwide, and 250,000 women die from it, mainly in developing countries [4]. In spite of their high prevalence, most cervical HPV infections are transient and asymptomatic, with approximately 70% of new infections resolving within a year [5], and the median duration of infection is generally 8-12 months. Hence, detection of the same subtype of HPV twice or more than twice within 10-12 months is regarded as persistent HPV infection [6].

HPVs are small, nonenveloped DNA viruses, and there are over 150 types, many of which are capable of infecting either mucosal or cutaneous tissues [7]. In terms of cervical cancer, 13 types of HPV have been determined to be carcinogenic, among which HPV16 and HPV18 are responsible for ~80% of cervical-cancer cases [8]. HPV16 is the most carcinogenic type and is classified as high-risk HPV owing to the expression of oncogenic proteins E6 and E7, which can degrade p53 rapidly and downregulate Rb products [9, 10]. Nevertheless, the role of the HPV16 genetic variation remains unclear.

Based on genomic analysis, HPV16 variants have previously been detected and subdivided according by geographical area: Eur (European), As (Asian lineage), AA (Asian American lineage), Af1 (African lineage1), Af2 (African lineage2) [11], and the recently discovered Java (Javanese

lineage) in Indonesia [12]. Recently, with the use of multiple sequence alignments of complete viral genomes and phylogenetic analyses, HPV16 can be divided into four main variant lineages (A, B, C, D) and ten sublineages (A1, A2, A3, A4, B1, B2, C, D1, D2, D3), according to differences in the L1 sequence by 1.0-10.0% for lineages and 0.5-1.0% for sublineages respectively [11]. The variants of HPV16 differ in carcinogenicity, geographical distribution, and affected populations [13]. Nevertheless, the epidemiology of HPV16 variants and their involvement in cervical cancer in Shanghai, China are poorly investigated.

Some studies have focused mainly on the analysis of mutations in oncogenes *E6* and *E7* of HPV16, but studies on capsid proteins and regulatory proteins and sequences are rare [14-16]. The aim of this study was to analyze the mutations of genes *E6*, *L1* and *LCR* sequences of HPV16 in the female population of the Pudong District, Shanghai, to reveal a possible relationship between these variants and persistent cervical HPV infection. We found that the HPV16 *E6* As variant T178G was strongly related to persistent infection. In addition, the A1/A2 sublineages T350G and A442C may be associated with persistent HPV16 infection. In contrast, the *L1* and *LCR* variants turned out to be unrelated to persistent infection despite being common among the tested samples.

Methods

Subject recruitment and sample collection

This was a prospective cohort study on 35,968 patients who visited the gynecological clinic of the People's Hospital in the Shanghai Pudong District or who participated in the gynecological census from May 2011 to October 2013. Those who were enrolled in this study were selected according to the following criteria: 1) only HPV16 positive; 2) low-grade squamous intraepithelial lesions (LSIL) or lesions below LSIL with normal cellular morphology in the cervix as assessed by the ThinPrep cytological test; 3) no aberrant pathological morphology (such as a high-grade squamous intraepithelial lesion, cervical squamous carcinoma, or cervical adenocarcinoma); 4) age between 30 and 65 years and having lived in the Pudong District, Shanghai, for at least 2 years; 5) Chinese Han women; and 6) participating in the study voluntarily. Patients were excluded from this study as follows: 1) pregnant women; 2) patients infected with the human immunodeficiency virus; 3) patients coinfected with other subtypes of HPV; 4) patients with negative HPV sequencing results; 5) patients with a history of cervical intraepithelial neoplasia or cervical cancer. There were 386 female patients aged between 30 and 64 years who were enrolled. All the participants provided written informed consent and took part in the study voluntarily. The study protocol was approved by the Ethics Committee of the Hospital.

Sample collection and HPV genotyping

Cervical epithelia were collected using the special ThinPrep cervix brush. Flow-through hybridization and gene chip technology were applied to identify the HPV types and subtypes in the exfoliated cervical cells of the samples. The genotyping assay kit purchased from Hybribio (Chaozhou, Guangdong, China) was used to classify the HPV subtypes in accordance with the principle of reverse dot blot hybridization. The test can identify eight HPV types: HPV16, -18, -31, -33, -56, -58, -52, and HPV53.

PCR sequencing to determine HPV variants

The samples that were HPV16 positive and achieved a successful amplification of the B-globin DNA were analyzed by polymerase chain reaction (PCR) with type-specific primers (primers for E6, L1, or LCR). The whole-genome sequence of HPV16 was retrieved from GenBank, and the primers (**Table 1**) synthesized by the China National Biochip base of Shanghai (Shanghai, China) were designed to be specific to HPV16 *E6*, *L1*, or the *LCR* ORF. The PCR products were purified by the sodium acetate/ethanol method recommended by the ABI operating manual (Applied Biosystems, Foster City, California, United States). The sequencing reaction was conducted with the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). The sequenced and amplified products were purified again. After purification, the final products were sequenced on an ABI 3100 DNA sequencer (Applied Biosystems). A sequencing chromatogram with visible bases can be obtained directly by means of the ABI 3100 sequencer. The sequencing results were compared to the corresponding HPV16 *E6*, *L1*, and *LCR* prototype sequences in

Table 1. The primers used to amplify the target genes

Gene types	Primer sequence	Annealing temperature °C	Product length (bp)
HPV16 E6	F: 5' TATAAAACTAAGGGCGTAAC 3'	48	573
	R: 5' CATGCAATGTAGGTGTATCT 3'		
HPV16 L1	F: 5' TATAGTTCCAGGGTCTCCAC 3'	51	1705
	R: 5' TTTACAAGCACATACAAGCA 3'		
HPV16 LCR	F1: 5' CACCTCCAGCACCTAAAGAAGATCC 3'	55	1079
	R1: 5' GCAGCTCTGTGCATAACTGTGG 3'		
	F2: 5' TCCTGTGGGTCCTGAAACATTGCAG 3'		
	R2: 5' GACCTAGATCAGTTTCCTTTAGGAC 3'		

Table 2. The classification of the HPV16 E6 variant sublineages system of the studied population

Sublineages	Nucleotide sequence	No. (%) of women with HPV16 infection (386)
A4	T178G, A131C	286 (74.09)
A1/A2	T350G, A442C, G176A	96 (24.87)
B1/B2	C335T	12 (3.13)

GenBank to identify any differences in the sequences with the assistance of the BLAST website (http://blast.ncbi.nlm.nih.gov/Blast).

Follow-up to identify women with persistent HPV infection

The HPV subtypes were detected again after 12 months of follow-up. At that time, gene sequencing was performed again on the persistent HPV16-positive cases (HPV16 infection detectable for at least 12 months) to confirm the HPV variants. The correlations of the HPV16 variants with persistent viral infection or cleared infections were analyzed using various statistical methods.

Statistical analysis

The SPSS software (Version 18.0; SPSS Inc., Chicago, IL) was used for this study. The association between persistent HPV16 infections and the variants was assessed using Fisher's exact test. Data with *P* values less than 0.05 were considered statistically significant.

Results

Patients

A total of 35,968 cervical-epithelium samples collected in this study were subjected to flow-through hybridization and a gene chip assay. Among them, 3,865 samples tested positive for a single HPV16 infection. Among these HPV16-positive samples, 3,463 cases were excluded owing to disordered cytological or histological characteristics. An additional 16 samples were excluded due to target gene amplification failure. In the end, 386 samples remained and were assessed using PCR and sequencing analysis.

Classification of HPV16 variation modes

The full-length *E6* gene in the 386 samples was successfully amplified and then sequenced for comparison with an HPV16 prototype (HPV16.P, NC 001526), which belongs to the A1/A2 sublineages lineage. At least one amino acid change in a protein is considered a variant. We found that 74.09% (286/386) of the HPV16 *E6* variants belong to the A4 sublineage (T178G, A131C), 3.11% (12/386) of HPV16 *E6* variants belong to the A1/A2 sublineages lineage (G176A), 24.87% (96/386) were A1/A2 sublineages (T350G, A442C, and G176A), and 3.13% (12/386) were B1/B2 sublineages (G335T; **Table 2**).

E6 variants

Six single-nucleotide variants were detected in the *E6* gene of HPV16: G176A (D25N), T178G (D25E), C335T (H78Y), T350G (L83V), A442C (E113D), and A131C (R10R). Each single-nucleotide variant resulted in a missense mutation except for A131C. The most frequently observed substitution was T178G (278/386, 72.02%). Both A131C and T178G were simultaneously present in eight of the cases. The relationship between these single-nucleotide variants and persistent HPV16 infection was analyzed. The results showed that the variants T178G, T350G and A442C of the E6 gene were positively associated with persistent HPV16 infection, whereas the other variants were negatively associated with persistent HPV16 infection (**Table 3**).

Table 3. The variants among the HPV16 E6 gene

Nucleotide sequence	Amino acid	No. (%) of women with HPV16 infection		*P*
		Non-persistent (261)	Persistent (125)	
A131C	R10R	4 (1.53)	4 (3.2)	-
G176A	D25N	8 (3.07)	4 (3.2)	1.000
T178G	D25E	217 (83.14)	61 (48.8)	<0.001
C335T	H78Y	7 (2.68)	5 (4)	0.536
T350G	L83V	11 (4.21)	27 (21.6)	<0.001
A442C	E113D	18 (6.9)	28 (22.4)	<0.001

Fisher's exact test. -, as this is a synonymous mutation, the *P* value is meaningless.

Table 4. The variants among the HPV16 L1 gene

Nucleotide sequence	Amino acid	No. (%) of women with HPV16 infection		*P*
		Non-persistent (261)	Persistent (125)	
C6240G	H228D	189 (72.41)	98 (78.4)	0.216
A6432G	T292A	189 (72.41)	98 (78.4)	0.216
6902insATC	449insS	189 (72.41)	98 (78.4)	0.216
G7061A	--	144 (55.17)	79 (63.2)	-

P, Fisher's exact test. -, as this is a synonymous mutation, the *P* value is meaningless.

Table 5. The variants among the HPV16 LCR gene

Nucleotide sequence	No. (%) of women with HPV16 infection		*P*
	Non-persistent (261)	Persistent (125)	
G7059A	152 (58.24)	85 (68)	0.074
A7174C	131 (50.19)	72 (57.6)	0.192
T7176C	147 (56.31)	80 (64)	0.185
G7192T	261 (100.0)	125 (100.0)	-
T7200C	146 (55.94)	77 (61.6)	0.322
C7269T	146 (66.67)	77 (61.6)	0.322
A7286C	131 (50.19)	72 (57.6)	0.192
A7288C	79 (30.27)	49 (39.2)	0.084
A7728C	120 (45.98)	66 (52.8)	0.232
G7840A	131 (50.19)	62 (49.6)	1.000

P, Fisher's exact test.

L1 variants in L1 variability

In the HPV16 *L1* gene, four single-nucleotide variants were found, including two missense mutations [C6240G (H228D) and A6432G (T292A)], one insertion [6902insATC (449insS)], and one synonymous mutation (G7061A). Single-nucleotide variants H228D, T292A, and 449insS were detected in the vast majority of samples (287/386, 74.35%). There were no statistically significant relationships (*P*>0.05) between the *L1* variants and persistent infection or viral clearance (**Table 4**).

LCR variants

LCR mutations were detected in all of the samples, altogether 14 mutations, among which G7192T was present in each sample. There was no statistically significant (*P*>0.05) relationship between any of the 14 LCR variants and persistent infection or viral clearance (**Table 5**).

Discussion

The aim of this study was to test whether there is an association between genetic HPV16 sublineages and persistent HPV infection. We found that the A4 sublineage *E6* T178G and the A1/A2 sublineages *E6* T350G and A442C significantly correlated with persistent HPV16 infection. Furthermore, the majority (74.09%) of the HPV16 *E6* variants were found to belong to the A4 sublineage, 24.87% to A1/A2 sublineages and 3.1.3% to the B1/B2 sublineages. Nonetheless, the *L1* and *LCR* variants we identified showed no relationship with persistent HPV16 infection. These results indicate that the HPV16 *E6* variant T178G is strongly associated with persistent HPV16 infection and may play a major role in the progression to malignancy in women in Shanghai.

Yang *et al.* reported that the A4 sublineage of HPV16 is predominant in Yunnan, China [17]. Guo *et al.* identified two HPV lineages: the A1/A2 sublineages and the A4 sublineage in Shanghai, China [18]. Zheng *et al.* demonstrated that only two lineages are present in Liaoning: the A1/A2 sublineages and the A4 sublineage (48.2%) [19]. Similarly, we found three main HPV16 sublineages: A4 (74.09%), A1/A2 (24.87%), and B1/B2 (3.13%) in our samples. The above data indicate that the A4 sublineage is prevalent in some regions of China. By contrast, He *et al.* demonstrated that the most prevalent HPV16 variant type in Xinjiang is the A1/A2 sublineages (81%) [20]. The discrepancies between

these results on the HPV16 variant distribution in China suggest that the variant distribution in the Chinese population is highly affected by geography and/or ethnicity.

HPV is a DNA virus that infects the cutaneous and mucosal epithelium and induces epithelial proliferation PMID: 24244682. Persistent infection with high-risk HPV can lead to cervical intraepithelial lesions and cervical cancer PMID: 23360813. The development of alternative triage strategies for women with LSILs would be valuable because it could help differentiate women with LSILs that have high probabilities of progression to HSILs from women with LSILs that have spontaneously regressed PMID: 17060938. E6 of high-risk HPVs plays a key role in carcinogenesis. A previous study determined that the E6 viral load and its change contributed to the risk of LSILs progressing to HSILs during the 6-month interval after the baseline diagnosis of LSIL PMID: 17060938. The HPV16 A4 sublineage E6D25E shows a specific proteomic pattern correlating in cell transformation and suppressive innate immune response PMID: 27392712. HPV16 E6 polymorphisms have been reported to be associated with cervical intraepithelial neoplasia (CIN) or cervical cancer inconsistently [21, 22]. The HPV16 variants D25E and N29S were found to be the most common types across the genome in cervical cancer in Shanghai [23], and D25E (46.3%) accounted for most of the incidences of amino acid substitutions in *E6* in Liaoning [19]. Moreover, the dominant HPV16 *E6* variant was found to be D25E (68%) in Korean women [24], and D25E is strongly related to the development of cervical cancer in Japan [25]. In this study, we found that the HPV16 *E6* variant T178G ($P<0.001$) is negatively associated with persistent HPV16 infection, whereas T350G and A442C ($P<0.001$) are positively associated with persistent HPV16 infection. Considering the number of variants, we believe that T178G (D25E) is significantly related to persistent HPV16 infection.

Mutations in the *L1* regions of the HPV genome may be crucial for distinguishing different variants and for vaccine design. We found that the mutation hot spots of the HPV16 *L1* gene in the tested samples are C6240G, A6432G, and 6902insATC, accounting for 94.86% of the analyzed samples; this result is in agreement with the findings in India [26] and Canada [27]. On the other hand, the mutation hot spots in northern India, Greece, and Spain were reported to be A6695C and A6803T [28-30]. Hence, there are regional and ethnic differences in HPV16L1 variants, and they reveal the relevance of germline origin. Nevertheless, we found no statistically significant correlation between any of the HPV16 L1 variants and persistent infection or viral clearance, suggesting that the HPV16 *L1* gene polymorphism may be unrelated to persistent infection with this virus.

The HPV16 *LCR* polymorphism is closely related to persistent viral infections, high levels of cervical lesions, and cervical cancer [31, 32]. *LCR* is a variable noncoding segment of the HPV genome [33], and such segments are less likely to accumulate and tolerate sequence variations [34]. Single-nucleotide variants in *LCR* were detected in all the tested HPV16-positive cervical samples in Liaoning, in which the most commonly observed mutations were G7193T, 7434CIns, G7521A, and 7863ADel (100%) [19]. In Korea, the *LCR* single-nucleotide variants G7521A (91.5%), A7730C (59.6%), and G7842A (59.6%) have been observed [35]. In our study, altogether 14 mutations were detected, among which G7192T and G7519A were detected in each sample, indicating that the single-nucleotide variants in HPV16 LCR occur frequently. Nevertheless, we found no statistically significant correlation between any of the 14 *LCR* variants and persistent infection or viral clearance.

Conclusion

Our aim was to investigate mutations in genes *E6* and *L1*and the *LCR* sequence of HPV16 in a female population in Shanghai to reveal a possible relationship between these variant lineages and persistent cervical HPV infection. We found that the HPV16 *E6* variant T178G, which belongs to the A4 sublineage, is strongly related to persistent infection. In contrast, the *L1* and *LCR* variants are not related to persistent infection despite being common among the tested samples. These data are important for the early prediction of the potential risk of cervical cancer and may improve the efficiency and accuracy of cervical cancer screening. Moreover, these data lay the foundation for the development of diagnostic techniques and vac-

cine design for the eradication of cervical cancers in southeast China.

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Informed consent was obtained from each patient involved in the study.

Disclosure of conflict of interest

None.

Abbreviations

HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesion; LCR, long control region; A4, sub-lineage A4 for the E variants (European variant); A1/A2, sub-lineage A1, A2 for the E variants (European variant); B1/B2, B with B1 and B2 for Af1 variants (African-1 variant); PCR, polymerase chain reaction.

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