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Original Article

Variants of human papillomavirus type 16 predispose toward persistent infection

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Abstract: A cohort study of 292 Chinese women was conducted to determine the relationship between human papillomavirus (HPV) type 16 variants and persistent viral infection. Enrolled patients were HPV16 positive and had both normal cytology and histology. Flow-through hybridization and gene chip technology was used to identify the HPV type. A PCR sequencing assay was performed to find HPV16 *E2*, *E6* and *E7* gene variants. The associations between these variants and HPV16 persistent infection was analyzed by Fisher's exact test. It was found that the variants T178G, T350G and A442C in the *E6* gene, as well as C3158A and G3248A variants in the *E2* gene were associated with persistent HPV16 infection. No link was observed between *E7* variants and persistent viral infection. Our findings suggest that detection of specific HPV variants would help identify patients who are at high risk for viral persistence and development of cervical neoplasia.

Keywords: Human papillomavirus, persistent infection, variants

Introduction

It is well known that persistent infection with high risk human papillomavirus (HPV) is the primary cause of cervical cancer, especially type 16 [1-3]. To date, about one hundred HPV types have been identified and characterized. For a given HPV type, viral isolates that differ by less than 2% of their DNA sequence for the L1 gene are defined as variants [4]. The sequencing analysis revealed the existence of numerous natural variants that differ from the original prototype sequence by up to 2% in the coding region and up to 5% in the noncoding region. Therefore, HPV16 variants were divided into five major phylogenetic clusters: Asian, Asian American, North American, European and African [5]. Multiple studies have documented that HPV16 E6 variants contribute to persistent viral infection and the development of cervical neoplasia [6-15].

Persistent infection plays a key role in the development of cervical cancer; therefore, prediction of persistent HPV16 infection is an important step for cervical cancer prevention. To our knowledge, there is no study exploring the association between persistent HPV16 infection and variants within the Chinese Han population. The purpose of this study was to determine the distribution of HPV16 variants among women who live in Shanghai, China. We collected exfoliated cervical cells and sequenced the HPV16 DNA, in order to detect HPV16 variants associated with persistent infection.

Materials and methods

Subject recruitment and sample collection

Patients who came to the gynecologic clinic at the People's Hospital of Shanghai Pudong District between May 2011 and October 2012

Table 1. Primers used to amplify the target genes

Name	Sequence	Annealing Tempreture (°C)	Product length (bp)
HPV16 E6	F: 5' TATAAAACTAAGGGCGTAAC 3'		
	R: 5' CATGCAATGTAGGTGTATCT 3'	48	573
HPV16 E2	F: 5' CGGAAATCCAGTGTATGAGC 3'		
	R: 5' AAAGCACGCCAGTAATGTTG 3'	56	1240
HPV16 E7	F: 5' TTGCAGATCATCAAGAACAC 3'		
	R: 5' TACAGCCTCTACATAAAACC 3'	50	417

for cervical disease were enrolled in this study. Patients were excluded from this study for the following reasons: 1) confirmed cervical intraepithelial neoplasia (CIN), cervical cancer or other malignancies, 2) previous therapeutic procedure to cervix, 3) pregnancy. In order to be eligible for this study patients had to meet the following criteria: 1) positive for only HPV16, confirmed by flow-through hybridization and gene chip technology, 2) no histologic abnormality on cytology or biopsy, 3) be between the ages of 30 and 70 years and have lived in Shanghai for at least 2 years, 4) be of Chinese Han ethnicity. The infectious profile of enrolled participants with HPV16 infection was detected at one year intervals. All participants provided written, informed consent. The study was approved by the Ethics Committee of the Hospital and conducted in accordance with the 2008 Declaration of Helsinki.

Molecular HPV16 variant analysis

An aliquot of 5 μl from each sample was amplified for β-globin DNA. Specimens that were positive for both β-globin and HPV16 were used to amplify E2, E6 and E7 genes with specific primers. Briefly, the template (5 μl) was used in a 100 μl reaction volume with ampliTag Gold DNA polymerase for PCR. The PCR primers are listed in **Table 1**. Amplification was carried out for 36 cycles. The PCR products were purified with the QIAquick gel extraction kit (Qiagen). PCR sequencing was performed by the fluorescent cycle-sequencing method (BigDye terminator ready-reaction kit; Perkin-Elmer) on an ABI Prism 3100 Genetic Analyzer system. The variations were confirmed by a second PCR sequencing.

Statistical analysis

Patients with isolated HPV16 infection were considered to have a persistent infection if they had the same HPV type infection after one year. SPSS software (Version 18.0; SPSS Inc, Chicago, IL) was used for this study. The association between persistent HPV-16 infections and variants was assessed by Fisher's exact test. *P*-values less than 0.05 were considered statistically significant.

Results

A total of 32,686 women were enrolled in this study and underwent flow-through hybridization and gene chip assay. 3,363 women came back positive for an isolated HPV16. Among the HPV16 positive women, 3,063 cases were further excluded due to aberrant cytological and pathological morphology. An additional eight cases were excluded due to target gene amplification failure. A total of 292 women underwent PCR amplification and sequencing analysis for this study (**Figure 1**).

199 of the 292 study participants (68.15%) had the E6 HPV16 T178G Asia prototype variant (As.P). Of the remaining participants 4.79% had the European prototype (EP), 9.25% Asian variants, 15.07% European variants, and 2.75% had the Africa 1 variant (Af1). As for European variants, we detected both T350G and A442C at the same time. Further analysis demonstrated that both the Asian variant of E6 T178G and the European variant of E6 T350G and A442C were associated with HPV16 persistent infection (P=0.007 and P<0.001 respectively; **Table 2**).

Six variants were found in the E6 gene of HPV16, including G176A, T178G, C335T, T350G, A442C and A131C. Among these variants, A131C is a silent mutation, the remainder of the variants resulted in missense mutations (**Table 3**). 189 of the 292 total cases were identified as transient HPV16 infections, while 103 cases were confirmed as persistent HPV16 infections (**Table 3**). The T178G (P=0.007), T350G (P<0.001) and A442C (P<0.001) variants in E6 were found to be associated with persistent infection. In the E7 gene, three variants were identified, including A646C, A647G, and T846C. However, there was no link between the variants and persistent HPV 16 infection (**Table 4**). As for the E2 gene, 43 cases had an E2 deletion. Among the other 249 cases, seven

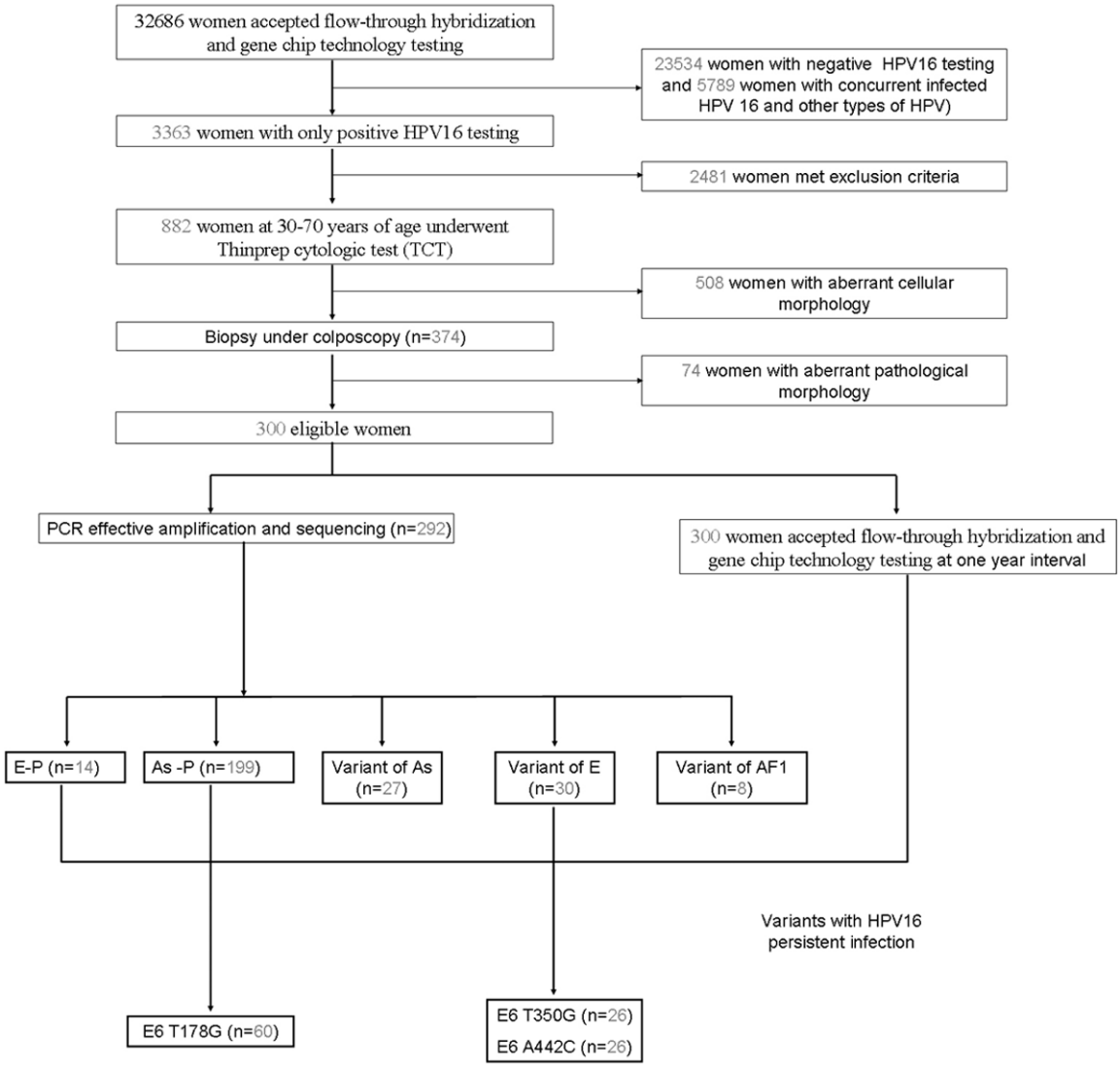

Figure 1. Test results and outcome. A total of 32,686 women were enrolled in this study and underwent flow-through hybridization and gene chip assay. 3,363 women came back positive for an isolated HPV16. Among the HPV16 positive women, 3,063 cases were further excluded due to aberrant cytological and pathological morphology. An additional eight cases were excluded due to target gene amplification failure. A total of 292 women underwent PCR amplification and sequencing analysis for this study.

missense mutation and two silent mutation variants were identified. Further analysis demonstrated that among the E2 variants the C3158A (P<0.035) and G3248A (P<0.035) were associated with persistent HPV16 infection (**Table 5**).

Discussion

Cervical cancer is the third most common gynecologic neoplasm, with the highest incidence seen in less developed countries. The incidence is primarily influenced by human papillomavirus (HPV) infection. Persistent infection with high risk human papillomavirus is a key factor in the development of cervical cancer. High risk HPV DNA screening is an effective method to define the association between HPV infection and cervical cancer. Currently, exfoliated cervical cells are the ideal sample and have been widely used in cervical cancer screening, both for HPV testing and Pap testing [16]. While the majority of HPV infections are transient and eliminated by the immune system, persistent high risk HPV infection can progress into cervical intraepithelial neoplasia

Table 2. Variants among HPV16 E2, E6 and E7 genes

Nucleotide substitutions																				Predicted amino acid substitution			N	Percentage (%)
	E6						E7			E2										E6	E7	E2		
Variant classes	1	1	1	3	3	4	6	6	8	2	2	3	3	3	3	3	3	3	3					
	3	7	7	3	5	4	4	4	4	8	9	1	2	3	4	4	5	6	7					
	1	6	8	5	0	2	6	7	6	2	2	5	4	8	0	4	2	8	8					
										7	5	8	8	3	9	8	3	3	6					
HPV16-R(E-P)	A	G	T	C	T	A	A	A	T	G	A	C	G	T	C	G	T	C	C					
E-P	-	-	-	-	-	-	C			-	G	-	-	-	-	-	-	-	-	-	N29H	-	14	4.79
As-A131C	c	-	A	-	-	-	C			a	G	-	-	-	-	-	-	-	A	-/D25E	N29H	-/-/D344E	27	9.25
E-G176A	-	A	-	-	-	-				-	G	-	-	-	-	-	-	-	-	D25N	-	-	14	4.79
As-T178G	-	-	G	-	-	-		G	c	-	G	-	-	-	T	-	-	A	-	D25E	N29S/-	-/P219S/T310K	75	25.68
Af1-C335T	-	-	-	T	-	-				-	G	-	-	-	-	-	-	-	-	H78Y	-	-	8	2.75
E-T350G	-	-	-	-	G	C				-	G	-	-	-	-	-	-	-	-	L83V/E113D	-	-	30	10.27
As	-	-	G	-	-	-				a	G	A	A	-	-	-	-	-	A	D25E	-	-/-/T135K/R165Q/D344E	73	25.00
As	-	-	G	-	-	-		G	c	-	G	-	-	C	-	A	c	-	-	D25E	N29S/-	-/I210T/E232K/-	50	17.13
As	-	-	-	-	-	-		G			G	-	-	C	-	A	c	-	-	D25E	N29S	-/I210T/E232K/-	1	0.34

Table 3. Relationship between E6 variants and HPV16 persistent infection

Nucleotide sequence variation	Amino acide variation	Infection		*P*
		Transient (n=189)	Persistent (n=103)	
A131C	R10R	10 (5.29)	17 (16.50)	–
G176A	D25N	6 (3.17)	8 (7.77)	0.091
T178G	D25E	139 (73.54)	60 (58.25)	0.007*
C335T	H78Y	3 (1.59)	5 (4.85)	0.135
T350G	L83V	4 (2.15)	26 (25.24)	<0.001*
A442C	E113D	4 (2.15)	26 (25.24)	<0.001*

Fisher exact test. *, p<0.05, E6 variants associate with HPV16 persistent infection.

Table 4. Relationship between E7 variants and HPV16 persistent infection

Nucleotide sequence variation	Amino acide variation	Infection		*P*
		Transient (n=189)	Persistent (n=103)	
A646C	N29H	24 (12.70)	17 (16.50)	0.085
A647G	N29S	67 (35.45)	59 (57.28)	0.074
T846C	S95S	98 (51.85)	27 (26.21)	–

Fisher exact test.

Table 5. Relationship between E2 variants and HPV16 persistent infection

Nucleotide sequence variation	Amino acid variation	Infection		*P*
		Transient (n=155)	Persistent (n=94)	
G2827A	D25D	27	22	0.254
C3158A	T135K	28	8	0.035*
G3248A	R165Q	28	8	0.035*
T3383C	I210T	14	11	0.504
C3409T	P219S	23	15	0.824
G3448A	E232K	14	12	0.356
T3523C	L257L	14	12	0.356
C3683A	T310K	14	11	0.504
C3786A	D344E	26	10	0.175

Fisher exact test. *, p<0.05, E2 variants associate with HPV16 persistent infection.

or invasive cancer. As such persistent high risk HPV infection is a critical step in the development of cervical neoplasia.

Recent studies have shown that HPV16 E6 variants are associated with persistence of viral infection and development of cervical lesions in western developed countries. In the current study, a large Chinese cohort was utilized to elucidate whether HPV16-specific variants are predictors for persistent HPV infection within the Chinese Han population. We sequenced samples from 292 patients and found six E6 gene variants, three E7 gene variants and nine E2 gene variants. Among the E6 variants, the variant T178G was detected in 139 transient HPV infections and 60 persistent HPV infections. Two other variants, T350G and A442C were detected in 4 transient HPV cases and 26 persistent HPV cases. Both variants were associated with persistent HPV16 infection. The rest of the E6 gene variants had no association with persistent HPV16 infection. To further investigate the relationship of HPV16 variants with persistent infection, the E2 and E7 genes were screened. There was no association between E7 variants and HPV16 infection. Among the nine variants in the E2 gene, both the C3158A and G3248A variants were associated with persistent HPV16 infection.

Although we identified several variants associated with persistent HPV16 infection, how these genetic mutations influence viral persistence is not clear at this time. One way in which these variants may influence persistence is that the missense mutations may allow for infected cells to evade the host's immune system or alter their functional ability. It is likely that HPV variants in concert with HLA and other immune-genetic polymorphisms play a role in persistence and progression.

We have demonstrated that HPV16 variants are associated with the persistence of HPV16 infection in a population of women at risk for HPV infection. Additional prospective studies

are being planned to use the identified variants to predict persistent HPV16 infection. Further studies on other HPV16 gene variants are necessary to determine whether the associations found in this study are explained by a direct effect that the E2 and E6 variations have on HPV16 infection, or if there is an interaction between these gene variants and other variations present within the genome. Future functional studies of mutated isolates would help evaluate the impact that these mutations have on HPV infection. Our findings demonstrate that genetic mutations in HPV are important in the progression of HPV infection. In clinical practice, the presence of these variants may argue for early treatment because of the likelihood of persistent HPV infection and possible progression to cervical neoplasia.

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Disclosure of conflict of interest

None.

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References

[1] Moscicki AB, Schiffman M, Kjaer S and Villa LL. Chapter 5: Updating the natural history of HPV and anogenital cancer. Vaccine 2006; 24 Suppl 3: S3/42-51.

[2] Rodriguez AC, Burk R, Herrero R, Hildesheim A, Bratti C, Sherman ME, Solomon D, Guillen D, Alfaro M, Viscidi R, Morales J, Hutchinson M, Wacholder S and Schiffman M. The natural history of human papillomavirus infection and cervical intraepithelial neoplasia among young women in the Guanacaste cohort shortly after initiation of sexual life. Sex Transm Dis 2007; 34: 494-502.

[3] Schlecht NF, Platt RW, Duarte-Franco E, Costa MC, Sobrinho JP, Prado JC, Ferenczy A, Rohan TE, Villa LL and Franco EL. Human papillomavirus infection and time to progression and regression of cervical intraepithelial neoplasia. J Natl Cancer Inst 2003; 95: 1336-1343.

[4] Lizano M, Berumen J and Garcia-Carranca A. HPV-related carcinogenesis: basic concepts, viral types and variants. Arch Med Res 2009; 40: 428-434.

[5] Ho L, Chan SY, Burk RD, Das BC, Fujinaga K, Icenogle JP, Kahn T, Kiviat N, Lancaster W, Mavromara-Nazos P, et al. The genetic drift of human papillomavirus type 16 is a means of reconstructing prehistoric viral spread and the movement of ancient human populations. J Virol 1993; 67: 6413-6423.

[6] Xi LF, Kiviat NB, Hildesheim A, Galloway DA, Wheeler CM, Ho J and Koutsky LA. Human papillomavirus type 16 and 18 variants: race-related distribution and persistence. J Natl Cancer Inst 2006; 98: 1045-1052.

[7] Xi LF, Koutsky LA, Hildesheim A, Galloway DA, Wheeler CM, Winer RL, Ho J and Kiviat NB. Risk for high-grade cervical intraepithelial neoplasia associated with variants of human papillomavirus types 16 and 18. Cancer Epidemiol Biomarkers Prev 2007; 16: 4-10.

[8] Schiffman M, Rodriguez AC, Chen Z, Wacholder S, Herrero R, Hildesheim A, Desalle R, Befano B, Yu K, Safaeian M, Sherman ME, Morales J, Guillen D, Alfaro M, Hutchinson M, Solomon D, Castle PE and Burk RD. A population-based prospective study of carcinogenic human papillomavirus variant lineages, viral persistence, and cervical neoplasia. Cancer Res 2010; 70: 3159-3169.

[9] Aho J, Hankins C, Tremblay C, Forest P, Pourreaux K, Rouah F and Coutlee F. Genomic polymorphism of human papillomavirus type 52 predisposes toward persistent infection in sexually active women. J Infect Dis 2004; 190: 46-52.

[10] Khouadri S, Villa LL, Gagnon S, Koushik A, Richardson H, Ferreira S, Tellier P, Simao J, Matlashewski G, Roger M, Franco EL and Coutlee F. Human papillomavirus type 33 polymorphisms and high-grade squamous intraepithelial lesions of the uterine cervix. J Infect Dis 2006; 194: 886-894.

[11] Chan PK, Ho WC, Yu MY, Pong WM, Chan AC, Chan AK, Cheung TH, Wong MC, To KF and Ng HK. Distribution of human papillomavirus types in cervical cancers in Hong Kong: current situation and changes over the last decades. Int J Cancer 2009; 125: 1671-1677.

[12] Lo KW, Wong YF, Chan MK, Li JC, Poon JS, Wang VW, Zhu SN, Zhang TM, He ZG, Wu QL, Li GD, Tam JS, Kahn T, Lam P, Cheung TH and Chung TK. Prevalence of human papillomavirus in cervical cancer: a multicenter study in China. Int J Cancer 2002; 100: 327-331.

[13] Wu Y, Chen Y, Li L, Yu G, He Y and Zhang Y. Analysis of mutations in the E6/E7 oncogenes and L1 gene of human papillomavirus 16 cervical cancer isolates from China. J Gen Virol 2006; 87: 1181-1188.

[14] Grodzki M, Besson G, Clavel C, Arslan A, Franceschi S, Birembaut P, Tommasino M and Zehbe I. Increased risk for cervical disease progression of French women infected with the human papillomavirus type 16 E6-350G variant. Cancer Epidemiol Biomarkers Prev 2006; 15: 820-822.

[15] Gillison ML and Shah KV. Chapter 9: Role of mucosal human papillomavirus in nongenital cancers. J Natl Cancer Inst Monogr 2003; 57-65.

[16] Tian Q, Li Y, Wang F, Li Y, Xu J, Shen Y, Ye F, Wang X, Cheng X, Chen Y, Wan X, Lu W and Xie X. MicroRNA detection in cervical exfoliated cells as a triage for human papillomavirus-positive women. J Natl Cancer Inst 2014; 106.