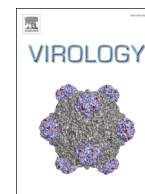




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Analysis of human papillomavirus 16 variants and risk for cervical cancer in Chinese population

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ARTICLE INFO

Article history:

Received 6 September 2015

Returned to author for revisions

13 November 2015

Accepted 16 November 2015

Available online 30 November 2015

Keywords:

Human papillomavirus 16

Variant

Cervical cancer

Case-control study

Meta-analysis

ABSTRACT

HPV16 is the most carcinogenic HPV type, but only a minority of HPV16 infections progress to cancer. Intratype genetic variants of HPV16 have been suggested to confer differential carcinogenicity. To investigate risk implications of HPV16 variants among Chinese women, a case-control study was conducted with 298 cervical cancer patients and 85 controls (all HPV16-positive). HPV16 isolates were predominantly of the A variant lineage, and variants of A4 (previously named “Asian”) sublineage were common. A4/Asian variants were significantly associated with increased risk of cervical cancer compared to A1–3 (OR=1.72, 95% CI=1.04–2.85). Furthermore, a meta-analysis including 703 cases and 323 controls from East Asia confirmed the association (OR=2.82, 95% CI=1.44–5.52). In conclusion, A4 variants appear to predict higher risk of cervical cancer among HPV16-positive women, which may provide clues to the genetic basis of differences in the carcinogenicity of HPV16 variants.

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Introduction

Cervical cancer ranks as the fourth most common malignancy in females worldwide, with a relatively high burden in developing countries, including in China (Ferlay et al., 2015). Infection with a subset of human papillomavirus (HPV), termed high-risk types, is a necessary pre-requisite for this disease (zur Hausen, 2002). Notably, although approximate 80% of women will have acquired cervical HPV infection by age 50, less than 1% of persistent infections will ultimately lead to invasive cancer (Myers et al., 2000; Schiffman et al., 2011). Factors that influence the outcome of HPV infection are not fully understood, but HPV intratype variants have been suggested to play a critical role in cervical carcinogenesis, and are recognized as an important marker for research on viral transmission, persistence, and carcinogenicity (Wang and Hildesheim, 2003; Xi et al., 2014).

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Of the 13 key genital high-risk human papillomavirus types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), HPV16 is the most prevalent type, and causes more than half of cervical cancer cases worldwide (Guan et al., 2012). Based on whole HPV genome sequencing, HPV16 variants have been classified into four major lineages: (1) A, that includes A1–3 (previously named European), and A4 (Asian) sublineages; (2) B (African 1); (3) C (African 2); and (4) D (including Asian–American [AA] and North-American [NA]) (Burk et al., 2013).

Epidemiological studies have suggested that certain HPV16 variants, particular D lineages, might promote viral persistent infection and cervical cancer development (Berumen et al., 2001; Schiffman et al., 2010; Xi et al., 2007). However, D lineages are rare in East Asia, where A lineages predominate (Cornet et al., 2013; Yamada et al., 1997). In particular, the prevalence in cervical cancer of A4/Asian variants (most commonly classified by the polymorphism T178G in E6) is much higher in Asia (65.5% in China, 85.2% in Korea, and 44% in Japan) than in Europe (2%) and North America (3%) (Kang et al., 2005; Matsumoto et al., 2000; Wu et al., 2006; Yamada et al., 1997). However, in comparison to a number of relevant studies in European and American populations, little is known about the oncogenic potential of HPV16 variants in Asian women. A4/Asian variants were associated with a higher risk of

Table 1
Nucleotide polymorphisms identified at HPV16 E6 gene.

Variant ^a HPV16 reference	94	109	111	131	132	136	145	154	168	173	176	178	184	188	193	219	241	267	276	286	295	310	315	335	350	442	516	Predicted amino acid change	Case (N=298)	Control (N=85)	Total
	G	T	A	A	G	G	G	A	C	C	G	T	A	G	T	G	T	G	A	T	T	T	C	C	T	A	C				
A1-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	98	35	133
a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	6	18	
a	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D25N	1	2	3	
a	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	G	-	-	D25N/L83V	1	0	1		
a	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	D25E	10	1	11		
a	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	T	-	-	D25E/H78Y	1	0	1		
a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	S71C	1	0	1		
a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	H78Y	1	2	3		
a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	L83V	0	1	1		
c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	L83V	0	1	1		
-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K11N	1	0	1		
-	-	-	-	-	-	-	-	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	T22S/L83V	1	0	1		
-	-	-	-	-	-	-	-	-	-	T	C	-	-	C	-	-	-	-	-	-	-	-	-	-	-	H24Y/D25H/E29Q	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D25N	9	0	9		
-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D25H	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D25Y	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	g	-	-	-	-	-	-	-	-	-	D25N	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	I27M	0	1	1		
-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	E29Q	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	R39Q	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	g	-	-	-	-	-	-	-	-	-	-	7	4	11		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	N58S	1	2	2		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	D64E	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	G	-	D64E/L83V	2	1	3		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	F69L	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	G	-	H78Y/L83V	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	C	H78Y/E113D	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	L83V	3	1	4		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	C	-	L83V/E113D	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	G	-	L83V/S138C	0	1	1		
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	E113D	1	0	1		
A4/Asian	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D25E	117	24	141	
c	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D25E	0	1	1	
C	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q3P/D25E	1	0	1	
c	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D25E	11	2	13	
c	-	-	-	-	-	-	-	-	-	-	G	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	D25E/E29Q	1	0	1	
-	-	-	-	-	-	-	-	G	-	-	G	-	-	-	-	-	-	-	-	-	-	-	G	-	-	T22S/D25E/L83V	2	0	2		
-	-	-	-	-	-	-	-	-	-	-	G	-	-	c	-	-	-	-	-	-	-	-	-	-	-	D25E	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	g	-	-	-	-	-	-	-	-	-	D25E	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	A	-	-	-	-	-	-	-	-	D25E/R55L	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	T	-	-	D25E/H78Y	1	0	1		
-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	G	D25E/S138C	0	1	1		
D	-	-	-	-	C	-	T	-	-	-	-	-	-	-	-	-	-	a	-	-	-	-	-	-	-	-	R10T/Q14H/H78Y	1	0	1	

^a Variant classification based on the E6 sequences.

cervical cancer among Chinese and Japanese women (Matsumoto et al., 2000; Sun et al., 2013), but not in Hongkong and Korean populations (Chan et al., 2002; Kang et al., 2005).

To clarify the association between HPV16 A4/Asian variants and cervical cancer risk, we conducted this case-control study with 298 cervical cancer patients and 85 healthy controls (all HPV16-positive) from Chinese women. In addition, we performed a meta-analysis to evaluate associations in East Asian populations.

Results

A total of 298 cases and 85 controls are included in subsequent analyses (after exclusion of two cases co-infected with more than one HPV16 variant). In cases, 289 (97.0%) were diagnosed with squamous cell carcinoma, 7 (2.3%) with adenocarcinoma, and 2 (0.7%) with adenosquamous carcinoma. The mean ages of cases and controls were similar (49.2 ± 8.5 years for cases and 48.4 ± 11.2 years for controls, $P=0.478$).

HPV-16 E6 polymorphisms and variants

The most predominant variant sublineages among Chinese women infected with HPV16 were A1–3 (56.9%, 218/383), followed by A4/Asian (42.8%, 164/383) and D (0.3%, 1/383) (Table 1). Of 30 nucleotide polymorphisms identified in E6, 23 (76.7%) were non-synonymous, including T178G (42.8%, 164/383), T350G (4.2%, 16/383), and G176A (3.7%, 14/383) as the three most frequent changes.

Association of HPV16 variants with cervical cancer risk

A4/Asian variants were significantly associated with a higher risk of cervical cancer compared with A1–3 (Odds ratio (OR)=1.72, 95% confidence interval (CI)=1.04–2.85; $P=0.036$) (Table 2). The association did not change materially after adjusting for age.

A1–3 variants were further stratified into 350T and 350G (based on polymorphism at position 350, regardless of sequences at other positions), and no significant difference in the distribution of these variants was observed between cases and controls (OR=0.62, 95% CI=0.20–1.92; $P=0.404$).

Meta-analysis

A total of six studies evaluating the risk of HPV16 A4/Asian variants for invasive cervical cancer (ICC) in East Asia were included (Table 3). Cervical samples were derived from women in China (Ding et al., 2010; Sun et al., 2013), Japan (Matsumoto et al., 2000), Korea (Cornet et al., 2013; Kang et al., 2005), and Thailand (Chopjitt et al., 2009; Cornet et al., 2013). All studies used PCR plus sequencing to detect viral variants and HPV16 E6 gene was sequenced in each study, A4/Asian variants being predominantly defined by the presence of the polymorphism T178G.

Table 2
Association between HPV16 variants and cervical cancer.

Variant ^a	Case (N=298)	Control (N=85)	OR (95% CI)	OR (95% CI)
A1–3	161 (54.0)	57 (67.1)	Reference	
350T	152 (51.0)	52 (61.2)		Reference
350G	9 (3.0)	5 (5.9)		0.62 (0.20, 1.92)
A4/Asian	136 (45.6)	28 (32.9)	1.72 (1.04, 2.85)	
D	1 (0.3)	0 (0.0)		

OR, odds ratio; CI, confidence interval.

^a Variant classification based on the E6 sequences.

Table 3
Studies included in meta-analysis.

First author, year	Region	Gene tested	Control (normal cytology/low grade lesions)		Case (ICC)	
			A1–3	A4	A1–3	A4
Present study	China	E6	57	28	161	136
Sun, 2013	China	E6, LCR	113	24	19	31
Ding, 2010	China	E6, E7	10	29	6	42
Matsumoto, 2000	Japan	E6	16	4	22	19
Kang, 2005	Korea	E6	0	8	3	23
Cornet, 2013	Korea	E6	2	2	7	35
Cornet, 2013	Thailand	E6	3	18	30	146
Chopjitt, 2009	Thailand	E6	7	2	6	17
Total			208	115	254	449

ICC, invasive cervical cancer.

Together with our present study, a total of 703 women with ICC (HPV16-positive) and 323 with normal cytology or low-grade lesions (HPV16-positive) were included in the meta-analysis. Overall, A4/Asian variants were significantly associated with ICC compared to A1–3 variants (OR=2.82, 95% CI=1.44–5.52; $P=0.003$) (Fig. 1). Sensitivity testing was conducted by omitting each study in turn to evaluate its influence on the meta-analysis. No single study altered the pooled ORs substantially, indicating that the results of meta-analysis were robust (Fig. 2). No obvious asymmetry of the funnel plots was observed for included studies (Fig. 3) and the Egger's test was nonsignificant ($P=0.896$), thus suggesting that no significant publication bias existed.

Discussion

Although the burden of cervical cancer in China is substantial, there are limited data on the epidemiology of HPV variants among Chinese population. In this study, we investigated the sequence polymorphisms of HPV16 E6 oncogene and evaluated their oncogenic implications among Chinese women. We found that A1–3 and A4/Asian were the most common HPV16 variant sub-lineages in China. Compared with A1–3, A4/Asian variants were significantly associated with elevated risk of cervical cancer. A systematic meta-analysis further supported that A4/Asian variants confer higher carcinogenicity among East Asian women.

It is well known that HPV16 variants display differential geographic distributions and our study confirmed that A lineages account for a majority of HPV16 isolates in Asian populations (Cornet et al., 2013; Yamada et al., 1997). However, previous studies reached inconsistent conclusions on whether A4/Asian variants represent a higher risk for cervical cancer (Kang et al., 2005; Matsumoto et al., 2000; Sun et al., 2013). In the present study, that was by far the largest to date, we found a significant association of A4/Asian variants with cervical cancer in China, consistent with previous studies in Japan and Thailand (Chopjitt et al., 2009; Matsumoto et al., 2000). Given that HPV16-positive controls need to be derived from large population-based screening programs, relatively small number of controls is the inherent problem in many studies. Thus, we pooled data from six studies together with ours, and confirmed that HPV16 A4/Asian variants were more oncogenic than A1–3 variants. Although significant heterogeneity between studies was observed, there was no evidence of publication bias, and the association was statistically robust in sensitivity testing.

We also studied the most common polymorphism within A1–3 variants, namely 350T/G, but found no significant association with cervical cancer in our Chinese population. However, 350G was rare, confirming observations from other East Asian studies (Chang

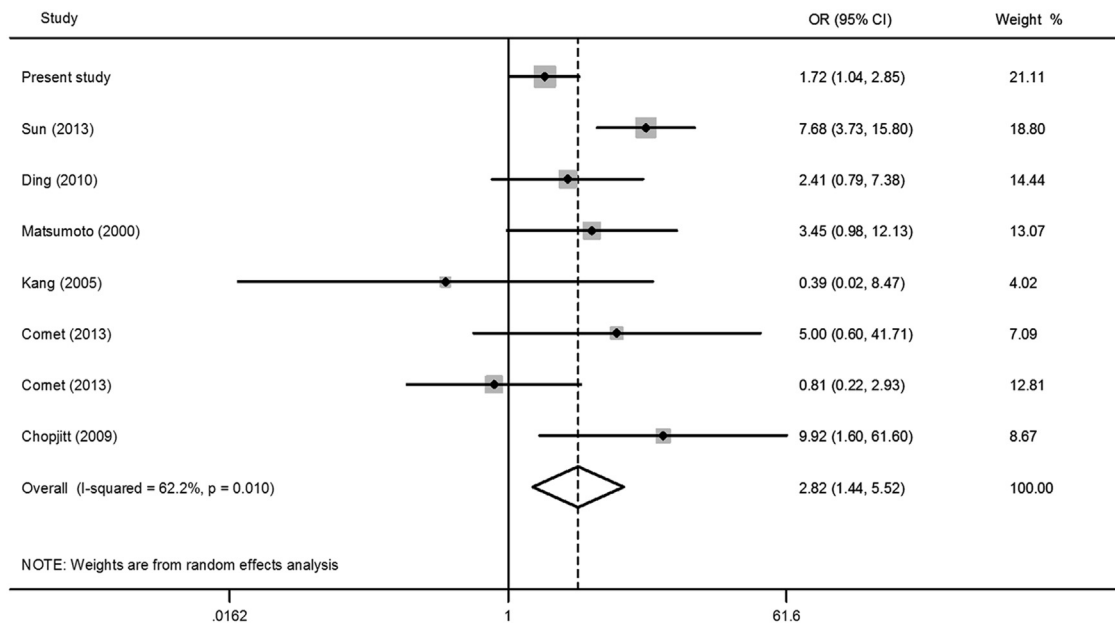


Fig. 1. Forest plots with odds ratios (ORs) of cervical cancer for HPV16 A4/Asian variants.

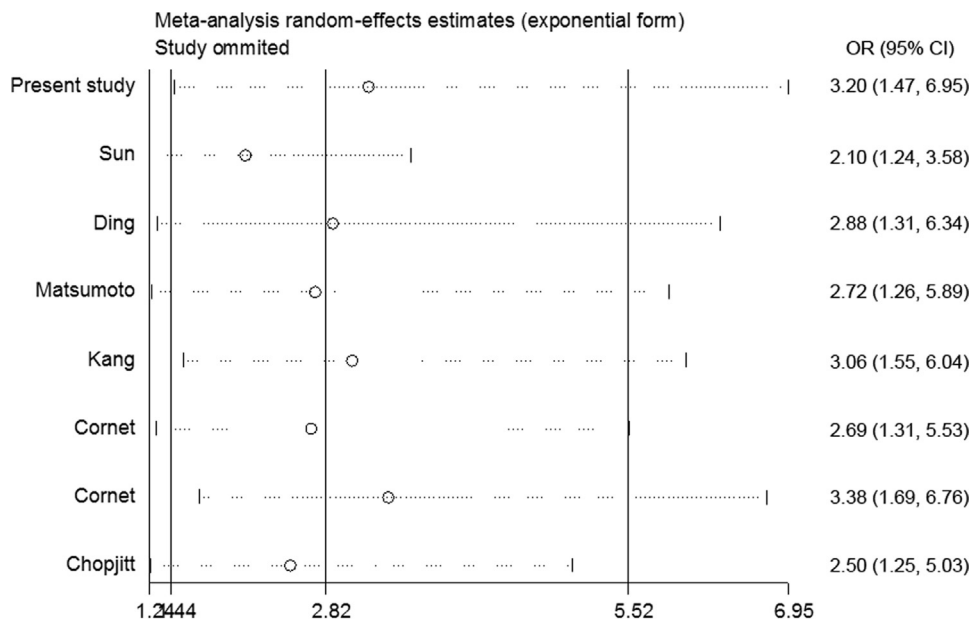


Fig. 2. Sensitivity analysis of the pooled odds ratios (ORs) of cervical cancer for HPV16 A4/Asian variants.

et al., 2013; Kang et al., 2005), in comparison to those from Europe and the Americas where it is more frequent (Cornet et al., 2013; Yamada et al., 1997). The risk implications of this common polymorphism have been widely studied, with discrepant results (Chan et al., 2002; Grodzki et al., 2006; Zehbe et al., 2001, 1998), leading some authors to suggest that the carcinogenicity of 350T vs 350G might be population-dependent (Cornet et al., 2013; Zehbe et al., 2001). Host genetic factors, which differ by population, might have a role in the association between a particular HPV16 variant and cervical cancer development. However, differences by population might be simply explained by residual genetic heterogeneity within HPV16 genomes classified solely upon position 350, which, although highly polymorphic, does not seem to robustly define phylogenetic sublineages (Chen et al., 2005). Unfortunately, we were not able to compare the phylogenetically

meaningful sublineages of A1, A2 and A3, due to lack of resolution based solely on E6.

The association of HPV16 E6 variants with cervical cancer risk may be explained by improved viral fitness (i.e. ability to evade host immune system and establish a persistent infection) and/or enhanced carcinogenicity. Functional studies suggested that T350G may have biological advantages over the reference to abrogate the serum/calcium-dependent differentiation of primary human foreskin keratinocytes (PHFKs) (Asadurian et al., 2007), to promote cellular immortalization and down-regulate E-cadherin (Togtema et al., 2015), as well as to enhance E6-mediated MAPK signaling and cooperative transformation with deregulated Notch1 pathway (Chakrabarti et al., 2004; Slichero et al., 2012). However, there are relatively less functional data on T178G, the diagnostic E6 polymorphism for A4/Asian variants. It was reported that T178G induced degradation of tumor suppressor p53 at a similar level to

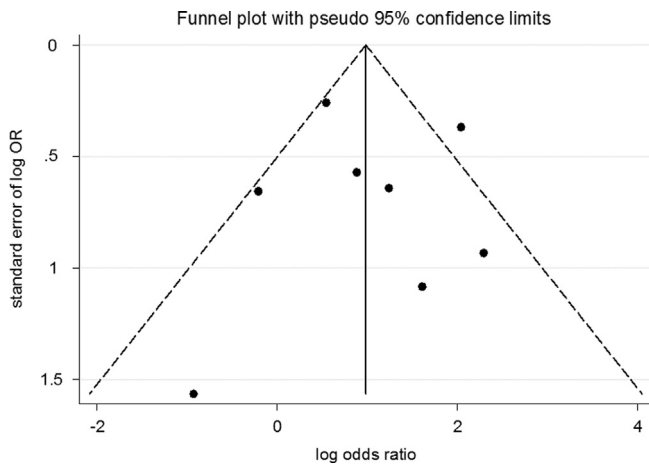


Fig. 3. Funnel plots of included studies on the association of HPV16 A4/Asian variants with cervical cancer risk.

the reference E6 (Hang et al., 2014; Yi et al., 2013), but a microarray experiment identified 14 other genes affected differentially by this variation (Jang et al., 2011). Thus, more functional studies of T178G and other linked polymorphisms throughout the HPV16 genome of A4/Asian variants are necessary to explore the biological evidence of increased carcinogenicity.

In summary, our study provides evidence that HPV16 A4/Asian variants may predict higher cervical cancer risk among Chinese women. These findings provide the basis for HPV16 genome wide studies to better determine the exact genetic determinants of differences in carcinogenicity of HPV16 variants.

Materials and methods

Study population

Cervical cancer cases were consecutively recruited from Cancer Institute and Hospital, Chinese Academy of Medical Sciences in Beijing, China, since January 2010 to July 2012. All cases were newly diagnosed and histologically confirmed invasive cancer. Control subjects were from the population who participated in a hospital-based screening program for cervical cancer in Beijing conducted by Department of Cancer Prevention, Cancer Institute and Hospital, Chinese Academy of Medical Sciences, since January 2010–July 2012. Controls had no abnormal intraepithelial lesions identified by liquid-based cytology (LBC) and were frequency-matched to the cases by age (± 5 years). Written informed consent was obtained from each subject. This study was approved by the ethics committees of Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Nanjing Medical University.

HPV genotyping

The β -actin gene (forward primer, 5'-GAAATCGTGCCTGACATTA-3'; reverse primer 5'-AAGGAAGGCTGGAAGAGTG-3') was amplified by polymerase chain reaction (PCR) to confirm the quality of DNA extracted from exfoliated cervical cells. All β -actin positive specimens were tested for HPV DNA by using a HPV GenoArray Test Kit (HybriBio Ltd., Beijing, China), which could identify 21 HPV types simultaneously (13 high-risk types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68; two intermediate-risk types 53 and 66; and six low-risk HPV types: HPV6, 11, 42, 43, 44, and 81). In total, 300 of 607 cancer cases and 85 controls from the cohort of 5066 participants were HPV16-positive and included in this study.

Variant identification

HPV16-positive samples were analyzed for E6 polymorphisms by PCR amplification of 545-bp product followed by sequencing. Briefly, type-specific primers applied were flanking outside of the coding region of HPV16 E6 (nucleotides 21–565): forward primer 5'-AAACTAAGGGCGTAACCGAA and reverse primer 5'-GCATGAT-TACAGCTGGGTTTCTC. The PCR mixture contained 50 ng template DNA, 0.2 μ M each primer, 0.2 mM deoxynucleoside triphosphate, 1 \times PCR buffer containing 1.5 mM $MgCl_2$, and 0.8 U HotStarTaq DNA polymerase (Qiagen, Germany). After a 15 min enzyme activation at 95 $^{\circ}C$, 35 cycles of amplification (94 $^{\circ}C$ for 40 s, 58 $^{\circ}C$ for 50 s, and 72 $^{\circ}C$ for 40 s) and a final extension at 72 $^{\circ}C$ for 10 min were carried out. For quality control, a negative control reaction without DNA template and a positive control reaction with HPV16 plasmid DNA were included in each PCR test. Double-stranded sequencing of the amplified product was performed using the same primers as in PCR. E6 polymorphisms were identified by comparison to the reference HPV16 sequence (GenBank: K02718), and intratype variants were classified based on E6 sequences as previously described (Cornet et al., 2012; Huertas-Salgado et al., 2011).

Meta-analysis

We searched all epidemiological studies on the association between HPV16 variants and cervical cancer in East Asian populations. Eligible studies, published in English, up to June 2015, were identified by using the search terms “Human papillomavirus 16, Asian variant, T178G, cervical cancer” in Pubmed. Additional relevant references cited in retrieved articles were also evaluated. If data were published more than once, only the publication with the largest sample size was included. The raw data were abstracted independently by two reviewers. If different results were generated, they were discussed until a consensus was reached. Odds ratios (ORs) and relevant 95% confidence intervals (CIs) were calculated in logistic regression models. The Cochran's Q-test and I^2 statistic were performed to assess the heterogeneity between studies (Higgins and Thompson, 2002). If there was significant heterogeneity ($P < 0.05$ or $I^2 > 50\%$), a random-effects model was applied instead of a fixed-effect model to pool the data of included studies. Publication bias was examined by funnel plots and the Egger test (Sterne and Egger, 2001).

Statistical analyses

Differences in demographic characteristics between cases and controls were evaluated by using the Student's *t*-test and the χ^2 -test for continuous and categorical variables, respectively. The association between HPV16 variants and cervical cancer risk was estimated by computing the ORs and 95% CIs in logistic regression models. All *P* values presented were two-sided and were assumed significant as $P < 0.05$. Statistical analyses were conducted using Stata version 11.0 (Stata Corporation, College Station, TX, USA).

Acknowledgments

This work was supported by Jiangsu Province Clinical Science and Technology Projects (Clinical Research Center, BL2012008), the National Natural Science Foundation of China (Grant numbers 81172757, 81373079, 81502873, and 30901236), Natural Science Foundation of Jiangsu Province for Youth (Grant number BK20150997), Beijing Natural Science Foundation (Grant number 7123225), Beijing Nova Program (Grant number xx2012067),

China Postdoctoral Science Foundation (Grant number 2015M570467), and Jiangsu Planned Projects for Postdoctoral Research Funds (Grant number 1402017A).

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