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Distribution of HPV 16 E6 gene variants in screening women and its associations with cervical lesions progression

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ABSTRACT

The aim of this study is to investigate distribution of human papillomavirus (HPV) 16 variants in screening healthy women and the potential association between HPV 16 variants and progression of cervical lesions. For this study a total of 2000 healthy women in Tianjin urban area and 212 patients who were HPV 16 positive and underwent colposcopy were analyzed for HPV 16 variants by pyrosequencing. The results show that the HPV 16 was the most prevalent genotype in Tianjin healthy women and five HPV 16 variant types were detected. The HPV 16 variants were determined by sequencing parital E6 region and the detected variants were European prototype E-T350 (E-p), E-G350, E-C109 G, Asian (As) and Asian-American (AA), among which the E-p variant was the most prevalent (82.76%) followed by As variant. Interestingly, in patients with suspected cervical lesions the most prevalent variant was As variant (54.9%) by increasing significance with severity of cervical diseases (OR 4.337; 95% CI 1.248–15.067; P = 0.021), and followed by HPV 16 E-p variant while E-G350 variant only appeared in HSIL and cervical cancer. Our results show that HPV 16 E-p variant was more prevalent than As in Tianjin healthy screening women while As variant was the most frequently type in HSIL and cervical cancer. It is suggested that the mutation of HPV 16 Asian variants, comparing with HPV 16 E-p variants, might contribute to the transformation from HPV 16 persistent infection to cervical cancer.

1. Introduction

Cervical cancer ranks the fourth frequent cancer in women worldwide and there are 528,000 new cases every year, mainly in developing countries accounting for 88% of global incidence of which 1/3 occurred in china (Chen et al., 2016; Torre et al., 2015). Epidemiological studies have confirmed that persistent HPV infection, especially high-risk HPV (HR-HPV) types, is the central cause and prerequisite of cervical cancer. HPV 16 accounts for approximately 65% of cervical cancer worldwide and is the most prevalent HPV type in China (Smith et al., 2007). The DNA virus oncogenic and persistence properties are due to expression of two viral oncoproteins, E6 and E7, binding with tumor suppressor genes p53 and pRB, which contributes to carcinogenesis by inhibiting apoptosis and the entry of cell into the S phase. Previous studies have identified that HPV 16 E6 variants are associated with boosted risks of CIN and invasive cervical cancer (Zehbe et al., 1998a; Andersson et al., 2000; Ding et al., 2010; Richard et al., 2010). Currently, HPV 16 E6 intra-typic variants, which have ≤2% nucleotide sequence variations as compared to HPV 16 prototype, are classified into four major variant groups based on geographical distribution and whole-genome sequencing: A, which includes A1-3(namely European) and A4(Asian) variants; B[African-1 (Af-1)]; C[African-2 (Af-2)] ;and D[North American (NA) and Asian-American(AA)] (Park et al., 2016; Huertas-Salgado et al., 2011a; Cornet et al., 2012, 2013). Furthermore, the European A1-3 variants are classified into subgroups such as E-T350 (E-p), E-G350 (T350 G), E-G131 (A131 G), E-C109 (T109C). Infections with non-A1-3

Abbreviations: HR-HPV, high-risk human papillomavirus; AA,LCR, long control region; EUR, European-Prototype; As, Asian variant; AA, Asian-American variant; Af-1, African-1 variant; Af-2, African-2 variant; NA, North American variant; OR, Odds Ratio; LSIL, Low-grade squamous intraepithelial lesions; HSIL, High-grade squamous intraepithelial lesions; CIN, cervical intraepithelial neoplasia; HLA, Human Leukocyte Antigen

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variants of HPV 16 tend to be more persistent and are associated with a higher risk of cervical neoplasia than infections with A1-3

variants (Hildesheim et al., 2001; Sichero et al., 2007; Hang et al., 2016). Sun et al has previously reported HPV16 EUR-L83 V and As were the two major variants of HPV16 in China (Sun et al., 2012). More in-depth researches identified examples showed D25E variant in Hubei (Cai et al., 2010), Asian and European lineages in Hong Kong (Chan et al., 2002), Asian variant in Taiwan、Zhejiang and Beijing (Ding et al., 2010; Hang et al., 2016; Chang et al., 2013), A4 or D sub-lineage in Korea (Park et al., 2016) confer higher carcinogenicity. Mutations in specific gene loci make it easier for the virus to improve viral fitness by escaping host immune surveillance or increase the chances of persistent infection and malignancy (Zehbe et al., 2001; Gheit et al., 2011; Sun et al., 2013). The specific variants may have effects on immunologic responses, carcinogenesis potential, P53 degradation, apoptosis, immortalization and the regulation of transcription (Pande et al., 2008; Hang et al., 2014; Zehbe et al., 2011). This may partly explain the fact that only a small percentage of patients with HPV 16 infection develop cervical cancer and the majority of HPV infected women clear HPV within a timeframe (Picconi et al., 2003). Moreover, there is significant evidence demonstrating that particular HPV16 variants vary widely in different regions and populations due to diversity in the distribution of HLA-class alleles of various populations (Ding et al., 2010; Park et al., 2016). Meanwhile, the carcinogenic risk of the same variants in different regions and populations in many studies showed a significant difference and even contrary to each other (Chan et al., 2002; Mosmann et al., 2015). Although the polymorphism of HPV 16 E6 gene and its mechanisms associated with cervical lesions progression have been studied in many parts of the world, the impact of HPV 16 E6 gene variation of Tianjin on the carcinogenic mechanism of HPV 16 remains to be investigated.

In this study, we performed HPV 16 variant analysis by Pyrosequencing for HPV 16 E6 intra-typic variants to investigated distribution of HPV 16 variants in screening healthy women and characterized the relationship between HPV 16 variants and the severity of cervical lesions.

2. Methods and materials

2.1. Study population and clinical specimens

Samples from 2000 healthy women aged 24–72 years in Tianjin were collected to screen for precancerous lesions and cervical cancer during March to October 2013. In parallel, 212 cases of cervical exfoliated cells from patients with suspicious cervical lesions who underwent colposcopy for the first time were collected from October 2013 to April 2014. 212 samples were biopsied and classified into three groups according to pathology (namely as inflammation, LSIL, HSIL and cervical cancer). All pathological results were reviewed by two experienced pathologists. Each patient gave signed informed consent and filled in the questionnaire of high-risk factors related to HPV infection. All procedures performed in studies involving human participants were approved by the Ethics Committee of the Tianjin Central Hospital of Gynecology Obstetrics(2015KY031).

2.2. DNA extraction, purification and amplification

The genomic DNA of all specimens was extracted from the Cervical exfoliated cells using the commercial DNA Mini Kit according to the manufacturer's instructions (TianGen, Beijing, China). In order to ensure the specificity of PCR amplification, the nested PCR/MGP-PCR for HPV amplification was then performed for HPV positive samples with the MY09/MY11, GP5+/GP6+, modified MGP consensus primers (Yang et al., 2012; Hu et al., 2011).

2.3. The determination of HPV DNA genotypes

The DNA sequencing of positive HPV samples were sequenced by pyrosequencing——a high-throughput DNA sequencing technology (Lavebratt and Sengul, 2006; Gharizadeh et al., 2001) according to manufacture's instructions and the sequence results were analyzed by sequence analysis (SQA) software(PyroMark Q96 software, QIAGEN, Germany). HPV genotypes were determined by comparing the sequence results with 20 known HPV sequence pyrograms (Wanram et al., 2009). The E6 region of the HPV 16 positive samples were amplified with specific E6 primers (forward primer

5′- CGAAACCGGTTAGTATAA-3′ and reverse primer 5′- GTATCTC CATGCATGATT-3′) (Ding et al., 2010; de Oliveira et al., 2017).The amplified products were tested by 2% gel electrophoresis.

2.4. Single-strand template preparation and pyrosequencing

30 μl GP5+/6+ or MGP + PCR products above were transferred to a 96-well microplate and pyrosequencing was performed according to manufacturer's instructions. Sequencing primers (Table S1) were synthesized by Shanghai Biotechnology Service Co. Ltd, China. Intra-type variants of E6 oncogene were classified based on E6 sequences as previously described (Huertas-Salgado et al., 2011b).

2.5. Statistical analysis

Data were analyzed using SPSS 17.0 software (SPSS, Chicago, IBM) with contingency table analysis. HPV 16 E6 Mutation detection rate and Odds Ratio (OR) predicting risk of cervical lesions progression was calculated among different groups by using chi-square test and Risk analysis, respectively. Considering the relatively small sample size, Fisher's exact probability method is adopted. P value was bilateral and $P < 0.05$ showed statistically significant difference.

3. Results

3.1. HPV prevalence in healthy screening women of Tianjin and HPV genotyping

271 of 2000 healthy women were HPV positive, and the total HPV infection rate in the screening of Tianjin healthy women was 13.55% (271/2000). Among the positive sample, 107 (5.35%) of 271 were HPV 16 positive, which was the most prevalent type. Of the 107 HPV 16 positive samples, 87 cases (81.3%) were successfully amplified for E6 region.

3.2. E-p variant is the most common variant of HPV 16 E6 in screening

We detected seven mutation sites of E6 in HPV 16 positive samples in screening healthy women, the results were shown in Table 1. The sequencing photographs of detected variants are shown in Fig. S1. The results indicated that T-178 G variant is second only to E-p variant, which is the most prevalent. In addition, we further analyzed the variation of HPV 16 E6 in different age groups. The frequency of HPV 16 E-p variant (50%) is the highest in the age group under 30, and the same applies for As (25%). However the variant rate of HPV 16 E6 among four age groups below were slightly different ($\chi^2 = 0.381$; $p = 0.944$). No significant difference in the variation rate compared between non-prototype and prototype among different age stages ($\chi^2 = 5.295$; $p = 0.151$) were observed (Tables 2, S3). The total variation trend of HPV 16 E6 variants in the age distribution was slightly decreasing with age increase among screening women ($p = 0.667$) (Table S2).

3.3. HPV 16 infection rate increased with the severity of cervical diseases

212 patients with suspected cervical lesions were divided into three

Table 1
Distribution of HPV16 E6 gene mutation sites and variants in census women.

Variant	HPV 16 E6 variation sites							Predicted-amino acid change	n	Proportion(%)
	109	131	132	143	145	178	350			
E-p	T	A	G	C	G	T	T	–	72	82.76
E-G350	–	–	–	–	–	–	G	L83V	3	3.45
E-C109	C	–	–	–	–	–	–	L83V	1	1.15
A4/As	–	–	–	–	–	G	–	D25E	10	11.49
D/AA	–	–	–	–	T	–	G	R10 T/Q14 H/H78Y	1	1.15

Notes: Red capital letters denote mutated bases and "–" denotes no mutation occurred.

groups according to pathology: inflammation and LSIL (91 cases), HSIL (90 cases) and cervical cancer (31 cases). In our study, HPV 16 was detected in 82 samples totally. The detection rates of HPV 16 among HPV-positive cases in inflammation/LSIL, HSIL and invasive cervical cancer were 25.4% (15/59), 49.4% (43/87), 77.4% (24/31), respectively. There were statistical differences among the groups ($\chi^2 = 22.756$, $P = 0.000$). HPV 16 infection rate increased with the severity of the lesion, indicating that HPV 16 infection is closely associated with progression of cervical lesions.

3.4. Distribution of HPV 16 E6 intra-typic variation in patients with different cervical lesions and age groups

Three kinds of variants were detected in total 82 HPV 16 positive samples and the most common variant was As variant (45/82,54.9%), followed by HPV 16 E variant (37/82,45.1%) (see Table 3). HPV 16 E6 variations in distinct lesions groups are shown in Fig. 1. In order to study the distribution of E6 variation in different age groups and find out the high-risk age groups, according to the age division, this experiment classified 35 years old as the demarcation line of young women, ≤35 years old as the young group, > 35 years old as the middle-aged group (Hu et al., 2009; Lewis et al., 2008). The most common variation in both groups was As variation, and the incidence of which in young group was 75.9% (22/29) and 50.0% (19/38) in middle-aged group. There was a significant difference in the incidence of As variant between the two groups (Fisher's exact test; $P = 0.044$).

4. Discussion

HPV 16 genomic variants have geographic diversity (Zehbe et al., 1998a; Andersson et al., 2000; Ding et al., 2010; Richard et al., 2010). Previous studies concluded HPV 16 A1- 3 were by far the most prevalent variants, with an overall prevalence of 95% in Europe, 86% in Central/South America, and 61% in Asia. However several studies among Chinese population before were inconsistent with each other, for instance, Asian variant was the most prevalent variant of HPV 16 in Zhejiang (Ding et al., 2010), Southern China (Chan et al., 2002), Taiwan (Chang et al., 2013) and Heilongjiang (Shang et al., 2011), while Dong Hang et.al (Hang et al., 2016) reported the most predominant variant sub-lineages among Chinese women infected with HPV16 were A1–3 (67.1%), followed by A4/Asian (32.9%). In our HPV infection screening of urban females in Tianjin, E6 variants were mainly European variants (A1-3,82.76%), with the highest proportion of standard prototype(E-p) followed by A4/As T178 G(D25E, 11.49%),which is consistent with previous studies performed by Dong Hang et.al. Moreover, we also detected four other variants in HPV screening, namely As T178 G (10/87,11.49%)、E-G350(3/87, 3.45%)、T-C109 (E-T350)(1/87, 1.15%)、AA (1/87,1.15%). We didn't detect B(Af-1) and C(Af-2) variants which were frequently reported in Africa. Additionally, many international studies manifested that there is little correlation between HPV 16 E-p variant and the progress of cervical cancer (Webster et al., 2000; Cricca et al., 2009), the high detection rate of E6 E-p lineage among healthy population in our study also confirms this opinion, which further proves why only a small number of women infected with HPV 16 will develop cervical cancer, and most of them are only viral carriers and do not progress to cervical cancer.

Later we selected 212 patients with suspected cervical lesions to characterize the relationship between HPV 16 variants and severity of cervical cancer. Some studies demonstrated Af 1/2, As, AA and E-350 G variants were more common in HSIL and invasive cervical cancer, suggesting that the risk of malignant cervical lesions was higher in Non-European types than in European types (Sun et al., 2013; Zehbe et al., 1998b). Other studies showed that HPV 16 As variant was the main risk factor for cervical precancerous lesion progression and cervical cancer among Japanese (Matsumoto et al., 2003) and Chinese (Beijing (Hang et al., 2016), Hubei (Cai et al., 2010), Taiwan(Chang et al., 2013), Heilongjiang (Shang et al., 2011) and Zhejiang (Ding et al., 2010) women, but not in Southern China, Honduras and Korean populations (Chan et al., 2002; Kang et al., 2005; Tabora et al., 2010). In addition, this research conducted in Southern China indicated both A4/As (50.6%) or European (44.3%) lineages showed no risk associations for HSIL and ICC. Nevertheless, our present research implicated A4/As variant was the most prevalent variant in women with cervical lesions, followed by type E, which has critical significance for the control of HPV16 infection and prevention of cervical lesions development. Table 3 demonstrates A4/As is significantly higher in HSIL and cervical cancer group compared with the E-prototype ($P = 0.015$, $OR = 4.337$), indicating As to be closely related to HSIL and cervical cancer. Namely, As variant can be considered as a high-risk factor that leads to development and progression of cervical lesions in Tianjin. However, E-p is also present in a small number of patients with HSIL and cervical

Table 2
Age distribution of HPV 16 E6 gene mutations among HPV 16 positive samples.

Age	cases	HPV 16+	Variants type					Variant rate (%)	*P value*
			E-p	As	E-G350	E-C109	AA	(variants/HPV 16+)	
≤30	37	4	2	1	0	0	0	3 (75.0)	
31-40	545	29	17	4	3	0	0	24 (82.8)	
41-50	663	36	28	2	0	0	0	30 (83.3)	0.944
≥51	755	38	25	3	0	1	1	30 (78.9)	
Total	2000	107	72	10	3	1	1	87 (81.3)	

Table 3
Distribution of HPV 16 E6 variants in different grade of cervical lesions.

HPV16 variants[a]	Inflammation and LSIL n (n = 15)			HSIL and cervical cancer (n = 67)			OR	95%CI	*P value*
	n	%	95%CI	n	%	95%CI			
A4/As	4	26.7	4.3-49.1	41	61.2	49.5-72.9	4.337	1.25-15.06	0.021
E-p	11	73.3	50.9-95.7	20	29.9	18.9-40.9	0.155	0.04-0.55	0.003
E-G350	0	0.0	N/A	6	9.0	2.1-15.9	N/A	N/A	1.000
A[b]1-3	11	73.3	50.9-95.7	26	38.8	27.1-50.5	0.231	0.07-0.80	0.021

LSIL: Low-grade Squamous Intraepithelial Lesion; HSIL: High-grade Squamous Intraepithelial Lesion; CI: Confidence interval.
[a] OR: crude Odds Ratio; 95%CI: 95% confidence intervals; N/A: not applicable. A[b]1-3 : E-p + E-G350.

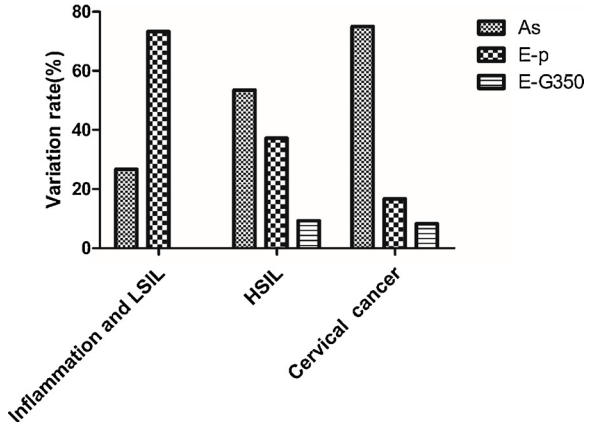

Fig. 1. Variations of HPV 16 E6 in different lesion groups.
As variant increases with the progression of cervical cancer from Inflammation and LSIL to HSIL and cervical cancer, while E-p variant is opposed to As variant and common in normal cytology and LSIL. E-G350 can only be found in HSIL and cervical cancer.

cancer, which may be related to host immunity microenvironmental factors.

Among the HPV 16 E6 variants, the most common studied variant in A1-3 is E-G350 which is a substitution T350 G changing leucine to proline (L83 V). Interestingly, E-G350 variant belonged to A1-3 was reported closely related with increased risks of cervical cancer. Zacapala-Gomez, A.E. et al found HPV 16 E6 variants altered the expression of 387 different genes involving in cellular processes related to the development of cervical carcinoma, such as adhesion, angiogenesis, apoptosis, differentiation, cell cycle, proliferation, transcription and protein translation in comparison with E-Prototype (Zacapala-Gomez et al., 2016). Recent in vitro studies reported oncogenic mechanisms of variants causing an amino acid change, for example, A4/As variant (D25E) down-regulated E-cadherin to facilitate an epithelial-to-mesenchymal transition (EMT) and E-G350 variant (L83 V) may give E6 greater efficiency in the degradation of P53 (Togtema et al., 2015). Therefore, the E-G350 variant is considered to be more pathogenic than other European variants. A result of the Swedish population suggest that E-G350 mutations are associated with the development of invasive cervical cancer (Sichero et al., 2007; Gheit et al., 2011; De Boer et al., 2005; Grodzki et al., 2006). However, previous studies reached inconsistent conclusions on whether A4/Asian and E-G350 variants represent a higher risk for cervical cancer. A study in Mexico showed that E-G350 mutants accounted for 93.5% of ICC, while the incidence of As in women with cervical cancer in Asian countries such as Japan and Thailand was higher than that of E-G350 (Ishizaki et al., 2013; Vaeteewoottacharn et al., 2003). No increased risk was observed by Chan, P.K.S. et al for the subclasses A1-3, especially E-350 G variants, which carry a higher risk for invasive cancer in some Western populations (Chan et al., 2002). In our study, E-G350 mutants were only detected in HSIL and ICC specimens but not in the inflammation and LSIL, suggesting that E-G350 variants might be an independent high-risk factor of HSIL and cervical cancer in Tianjin. However, in other European population surveys, different results have been reported. For example, Studies in countries such as Poland and Italy have shown that E-G350 mutations are more frequent in patients with CIN than in ICC. Mayrand MH and Matsumoto K et al (Matsumoto et al., 2000; Tu et al., 2006) found that HPV 16 E6 variant E-G350 was closely related to HPV 16 persistent infection and cervical lesions transformation from LSIL to HSIL while Tu JJ et al reported E-G350 variant is related to the progression of CIN (Sichero et al., 2007; Gheit et al., 2011; De Boer et al., 2005; Grodzki et al., 2006). Although our results further demonstrated the oncogenic role of E350 G, however, we failed to find any statistical difference of E-G350 between the two groups, which could partially be due to the small samples size of this study. A larger-scale systematic multicenter study is needed to verify the relationship between E-G350 variation and the development of cervical lesions.

At the same time, we further analyzed Age distribution of HPV 16 E6 gene mutations among HPV 16 positive samples of Screening populations. And noted the slightly higher variation rate in the young population, which may be possibly related to the peaks of HPV infection (namely 19–25) and the higher occurrences of A4/As variant, of which is common in China, in young women (Yang et al., 2012). But no significant difference in the total variation rate($P = 0.944$) and the variation rate compared between non-prototype and prototype among different age stages($P = 0.151$) were observed. As for case subjects, this experiment found the distribution frequency of As variant in young group was significantly higher than the middle-aged group (Fisher's exact test; $P = 0.044$), indicating that As variant may be a risk factor for cervical cancer rejuvenation, given the number of specimens and population selection restrictions. Both age distribution of variations and the oncogenic impact of As require more investigation with larger sample numbers.

In conclusion, HPV 16 E6 gene has sequence variations in patients with cervical lesions, which may be related to changes in viral carcinogenic potential and the severity of cervical cancer. As variant and E-350 G strains in patients with HSIL and invasive cervical cancer in Tianjin may be the two most dangerous variants of HPV 16. As variants were more strikingly correlated with cervical lesions progression.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The current study was approved by the Ethics Committee of the Tianjin Central Hospital of Gynecology Obstetrics (2015KY031).

Informed consent

Informed consent was obtained from all individual participants included in the study.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.virusres.2019.197740.

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