Original Article
Human papillomavirus genotype prevalence in the women of Shanghai, China and its association with the severity of cervical neoplasia

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Abstract: Aims: Human papillomavirus (HPV) viral load and genotype are the primary determinants for the development of cervical neoplasia. We aim to identify the prevalent HPV genotypes in the women of Shanghai, China and investigate the association between the HPV viral load and the severity of cervical neoplasia. Methods: Formalin-fixed, paraffin-embedded tissue samples were obtained from 20 cases of histologically normal cervix, 52 cases of low grade squamous intraepithelial lesion (LSIL), 46 cases of high grade squamous intraepithelial lesion (HSIL), and 29 cases of cervical squamous cervical cancer (SCC). A polymerase chain reaction reverse dot blot (PCR-RDB) genotyping chip was used to examine 23 HPV genotypes. Real-time quantitative PCR was used to detect the viral load of HPV in the fresh tissue of 80 cases. Results: The HPV infection rate in the 147 cases of cervical biopsies was 73.5%. Fourteen HPV genotypes were detected, including 12 high risk (HR)-HPVs and 2 low-risk (LR)-HPVs. HPV-16 (33.3%), HPV-31 (6.1%), HPV-52 (6.1%), and HPV-58 (5.4%) were the most popular genotypes of HR-HPV. Significant differences were found in HPV viral load between histologically normal cervix and cervix tissues with epithelial dysplasia (P<0.05). There was a statistically insignificant trend of gradual increase of viral load as the epithelial lesion progresses from LSIL to HSIL and to SCC (P>0.05). Conclusions: HPV 16, 31, 52, and 58 are the most prevalent genotypes in women of Shanghai, China. HPV viral load is an indicator of the presence of cervical neoplasia but not an accurate predictor for the severity of cervical neoplasia.

Keywords: Cervical cancer, neoplasia, genotype, HPV, viral load

Introduction

Cervical cancer is the fourth most common cancer of women in the world with an estimated 528,000 new cases in 2012 and nearly 300,000 deaths annually [1]. Human papillomavirus (HPV) infection is the primary cause of cervical cancer in 95 to 100% of the cases [2]. HPV is a group of more than 200 related viruses distinguished by genetic variability. These are further categorized into two groups: low-risk HPV types (LR-HPV) and high-risk HPV types (HR-HPV), depending on their relative risk of causing human malignancy. Based on meta-analyses of HPV prevalence by specific cervical disease performed by the International Agency for Research on Cancer (IARC) and updated by the Institute Catalan Oncology (ICO)/IARC Information Center on HPV and Cancer until

2015, HPV is detected in 52.5% (51.6-53.3) of atypical squamous cells of undetermined significance (ASCUS) neoplasia, 74.8% (74.3-75.3) of low grade cervical neoplasia, and 88.9% (88.5-89.3) of high grade cervical neoplasia worldwide. However, regional differences are observed [3]. Data from different regions of China could contribute to the world literature and help to find more targeted preventive measures for the local population.

HPV viral load has been variably associated with the cervical disease. In some studies, HR-HPV viral load was positively associated with the severity of cervical neoplasia [4, 5]. Higher HPV16 and HPV18 viral loads have been found in women with cervical intraepithelial neoplasia (CIN) compared to women with normal cytology or without CIN neoplasia [6, 7]. On
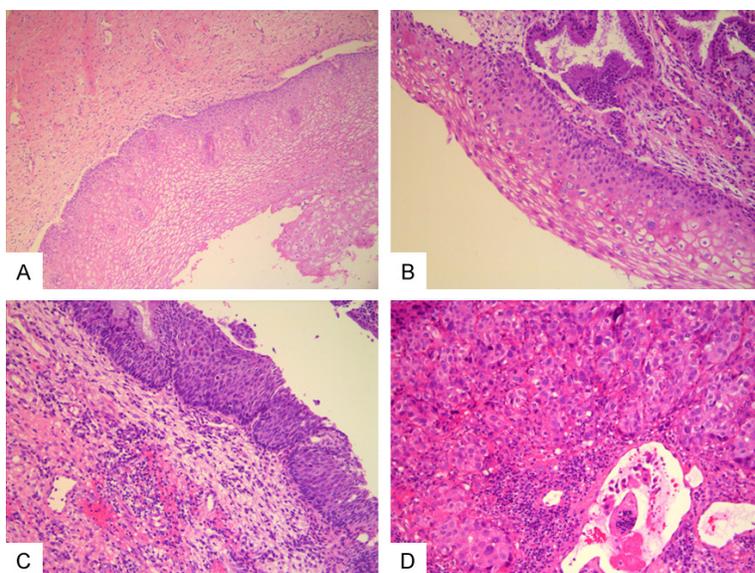
Figure 1. Representative H&E images in various cervical neoplasias. A. Normal cervical tissue (H&E, ×100); B. Low grade squamous intraepithelial neoplasia (H&E, ×200); C. High grade squamous intraepithelial neoplasia (H&E, ×200); D. Squamous cell carcinoma (H&E, ×200).

Methods

Tissue samples

Formalin-fixed, paraffin-embedded tissue blocks of cervical neoplasia from 147 patients were obtained from the archives (2014-2016) of the Department of Pathology, the Fifth People's Hospital of Shanghai, Fudan University (Shanghai, China). The clinical diagnoses included 20 cases of normal cervical tissues, 52 cases of low grade squamous intraepithelial lesion (LSIL), 46 cases of high grade squamous intraepithelial lesion (HSIL), and 29 cases of cervical squamous cervical cancer (SCC). Patient ages ranged between 17 and 75 years (mean 48.6 years). None of the patients had been treated for cervical abnormalities prior to the biopsy. All slides were reviewed by at least 3 pathologists (Li XJ, Liu XP, and Gao ZH), discrepancies were resolved by reevaluation and discussion at the multi-head microscope setting. Images were viewed under a light microscope (BX45, Olympus, Tokyo, Japan). Only one final diagnosis was made for each case. Representative images of H&E from tissues with increasing grades in cervical neoplasia are shown in **Figure 1**, Among the 147 cases, fresh specimens were collected from 80 patients in order to detect the HPV viral load in the tissue by quantitative real-time PCR, including 10 cases of normal cervical tissues, 28 cases of LSIL, 25 cases of HSIL, and 17 cases of SCC. The study was approved by the ethics committee of the fifth people's hospital of Shanghai, Fudan University (Shanghai, China). Written informed consent was obtained from the patients or the patients' direct relatives.

PCR-RDB HPV genotyping for 23 types

PCR-RDB was performed using HPV Genotyping Kit for 23 Types (Yaneng Bioscience Co., Ltd., China), according to the manufacturer's instructions, as previously described by Zhang et al. [13] and Sun et al. [14]. The kit can identify 17 high-risk HPVs, HPV-16, -18, -31, -33, -35,-39, -45, -51, -52, -53, -56, -58, -59, -66, -68, -73, and -82, and 6 low-risk HPVs, HPV-6, -11, -42,

the other hand, low HPV16 and HPV18 viral loads have been associated with the clearance of HPV infection [8]. However, some studies could not find any association between HPV16 and cervical disease [9, 10]. Deng et al. suggested that low initial HPV viral load may be a poor prognostic factor for cervical cancer patients who have undergone radical hysterectomy [11]. In a study from Colombia [12], Del Rio-Ospina et al. found a significant association, and that low HPV16 and high HPV31 viral loads are associated with higher CIN frequency, which might be related to infection duration and immune system response [12]. Regarding neoplasia among all different variants, HPV16 has been the most frequently detected genotype in all stages of the disease [3]. HPV16 is detected in 19.3% (18.9-19.7) of low grade cervical neoplasias and 45.1% (44.6-45.5) of high-grade cervical neoplasias [3]. To date, the link between viral loads of other HPV subtypes with cervical cancer has not been well established.

The present study aimed to determine the frequencies of various HPV genotypes in women of Shanghai, China and to evaluate the contribution of viral load of 23 HPV genotypes to the histological grading of cervical neoplasia. This study provides more insight on the association of viral load of different subtypes of HPV with the development and severity of cervical neoplasia.

Table 1. Kits used for RNA isolation and RT-PCR analysis

Kit	Catalog	Function	Source
RNAiso Plus	9108	Total RNA extraction	TaKaRa (Dalian, China)
PrimeScript® RT Master Mix	DRR036A	Reverse transcription	TaKaRa (Dalian, China)
SYBR® Premix Ex Taq™	RR420A	Real-time PCR	TaKaRa (Shiga, Japan)

Table 2. The results of various HPV type infections in 147 cases of cervical neoplasia

HPV subtype	Normal	LSIL	HSIL	SCC	Total
1. HR-HPV single infection					
HPV-16	0	1	16	10	27
HPV-18	0	1	0	2	3
HPV-31	0	2	6	1	9
HPV-33	0	3	2	1	6
HPV-45	0	1	0	1	2
HPV-51	0	1	0	1	2
HPV-52	0	3	6	0	9
HPV-53	0	1	0	0	1
HPV-56	0	4	0	0	4
HPV-58	0	3	5	0	8
HPV-66	0	1	0	0	1
HPV-68	0	1	0	0	1
2. HR-HPV multiple infection					
HPV-16 and other HR-HPV	0	5	7	10	22
H-HR HPV (exclude HPV-16)	0	3	2	1	6
3. HR-LR HPV multiple infection					
HPV-16 and LR-HPV	0	2	0	0	2
HR-HPV (exclude HPV-16) and LR-HPV	0	0	0	1	1
4. LR-HPV single infection					
HPV-6	1	2	0	0	3
HPV-11	0	1	0	0	1
5. HPV negative	19	17	2	1	39
Total	20	52	46	29	147

Note: HPV: Human papillomavirus; HR: High risk; H-HR: High-high risk; H-LR: High-low risk; LR: Low risk; LSIL: low grade squamous intraepithelial lesion; HSIL: low grade squamous intraepithelial lesion; SCC: Squamous cervical cancer.

-43, -81, and -83, all of which are common in the Chinese population. Briefly, total cellular DNA was extracted as described in our previous study [15]. An aliquot of 5 μL of extracted an DNA sample or a control (positive or negative) was used in the 24 μL reaction system. HPV was amplified in a thermal cycler (PTC-100, program thermal controller, MJ research Inc., America) under the following conditions: an initial 50°C for 15 min, 95°C for 10 min; 40 cycles of 94°C for 30 s, 42°C for 90 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. After amplification, the PCR products were immobilized onto a nitrocellulose membrane and hybridized with fixed 23 different type-specific probes. Final results were recorded by direct visualization of the location of blue spots on the membrane.

HPV viral load detection

We used real-time quantitative PCR (qPCR) to detect HPV viral loads, as described in our previous study [16]. Briefly, total RNA was isolated from tissues using RNAiso plus according to the manufacturer's instructions and then reverse transcribed to cDNA using PrimeScript® RT master mix. The following program: 37°C for 15 min, 85°C for 5 s, and 4°C. qPCR for Pit-1 and GAPDH was performed in a 10-μL reaction volume using SYBR® premix Ex Taq™ and an ABI7900HT Real-Time PCR System (Life Technologies, Singapore). The thermal cycling conditions consisted of one cycle at 95°C for 30 s and 40 cycles of amplification at 95°C for 5 s and annealing/elongation at 60°C for 30 s. HPV mRNA expression was normalized to the geometric mean mRNA expression of the housekeeping gene (GAPDH) and was calculated using the formula $2^{-\Delta Ct}$ [ΔCt = Ct (HPV) - Ct (GAPDH)], where Ct represents the threshold cycle for each transcript. The kits used in the study are shown in **Table 1**. The PCR primer sequences were as follows: GP5+, 5'-TTTG TTACTGTGGTAGATACTAC-3', GP6+: 5'-GAAAAA TAAACTGTAAATCATATTC-3', as previously described by Erhart et al. [17].

Statistical analysis

Statistical analyses were performed using SPSS software (version 13.0; SPSS, Inc.,
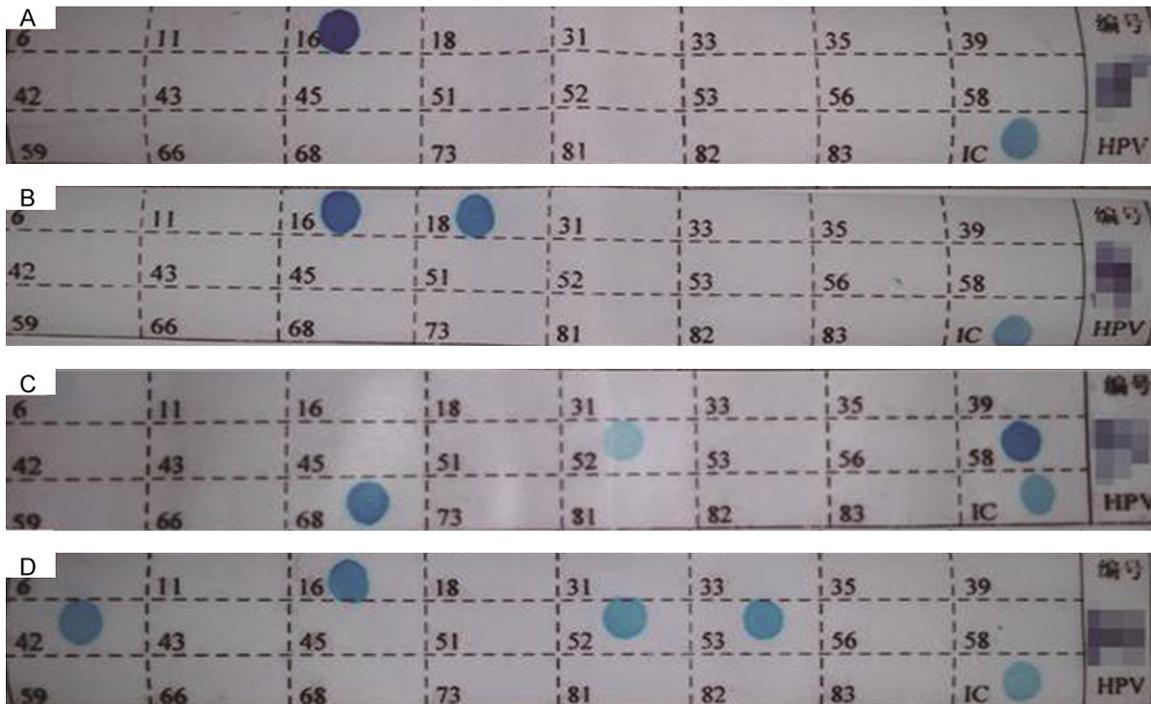
Figure 2. PCR-RDB genotyping results of a part of cervical neoplasia. A. Single HPV16 infection; B. Mixed infection of HPV16 and HPV18; C. Mixed infection of HPV52, HPV58, and HPV68; D. Mixed infection of HPV16, HPV42, HPV52, and HPV53.

Chicago, IL, USA). The statistical significance in HPV frequency was determined with the Chi-squared test or Fisher's exact test. Kruskal-Walli rank test was used for the evaluation of the significant differences between HPV viral loads of each subtype with the severity of cervical neoplasia. A *P* value <0.05 was considered statistically significant.

Results

Prevalence of HPV infection

This study cohort consisted of 147 samples from women who had undergone PCR-RDB genotyping. The total proportion of the HPV infection rate was 73.5% (108/147). As shown in **Table 2**, 14 HPV subtypes were detected, including 12 HR-HPV subtypes (HPV16, 18, 31, 33, 45, 51, 52, 53, 56, 58, 66, 68,) and 2 LR-HPV subtypes (HPV 6, 11). The positivity of single-type HR-HPV infection was 49.7% (73/147), accounting for 67.6% of the positive specimens (73/108). The positivity of single-type LR-HPV infection was 2.7% (4/147), accounting for 3.7% of the positive specimens (4/108). The positivity of multiple-type HPV infections was 21.1% (31/147), accounting for

19.4% of the positive specimens (21/108). Among the multiple-type HPV infection cases, the positivity of mixed HR-HPV and HR-HPV (H-HR HPV) infection was 19.0% (28/147), the positivity of mixed HR-HPV and LR-HPV (H-LR HPV) infection was 2.0% (3/147), but no mixed LR-HPV and LR-HPV infection cases were identified. HPV16 (33.3%, 49/147) was the most prevalent genotype in the HPV-positive cases, followed by HPV31 (6.1%, 9/147), 52 (6.1%, 9/147), 58 (5.4%, 8/147). The remaining less prevalent genotypes were HPV33, 56, 18, 6, 45, 51, 53, 66, 68, 11, accounting for 4.1%, 2.7%, 2.0%, 2.0%, 1.4%, 1.4%, 0.7%, 0.7%, 0.7% and 0.7% respectively. Representative PCR-RDB genotyping results are shown in **Figure 2**.

HPV infections in patients with different grades of cervical neoplasia

The proportion of HPV infection was 5.0% (1/20) in normal patients, 67.3% (35/52) in patients with LSIL, 95.7% (44/46) in patients with HSIL, and 96.6% (28/29) in patients with SCC. No HR-HPV genotype was identified in the normal cases. HR-HPV was detected in 91.4% (32/35) cases with LSIL, 100% (44/44) cases

with HSIL, and 100% (28/28) cases with SCC. With an advance in the histological grade of cervical neoplasia, the HPV detection rate also increased. Statistical significant differences in HPV infection rate were found between normal and LSIL, normal and HSIL, LSIL and HSIL (*P*<0.05). There was no statistical significant difference in neoplasia HPV infection rate between the HSIL group and the SCC group (*P*>0.05). Only 1 LR-HPV6 infection was identified in the histologically normal cases. All identified HPV genotypes were detected in cases with LSIL. HR-HPV (16, 18, 31, 33, 52, and 58) were only identified in 95.8% (69/72) cases with HSIL and SCC.

HPV viral load of real time qPCR assay

Quantitative real-time PCR was performed in 10 cases of normal cervical tissues, 28 cases of LSIL, 25 cases of HSIL, and 17 cases of SCC. In the normal cervical tissue group, only 1 case was detected with an HPV viral load 0.67281E-05. The HPV viral load was 2.63152 ± 0.68173 E-05 in cases with LSIL, 3.08763 ± 0.59262 E-05 in cases with HSIL, and 3.35171 ± 0.57651 E-05 in cases with SCC. The HPV viral load increased gradually with the advance in the histological grade of cervical neoplasia. The HPV viral load of the LSIL group, the HSIL group, and the SCC group were all significantly higher than the normal group (*P*<0.05). No significant HPV viral load difference was identified between the LSIL group and the HSIL group (*P*>0.05), or between the HSIL group and the SCC group (*P*>0.05).

Discussion

HPV infection, the main etiological factor of cervical cancer, has been extensively studied worldwide. Approximately a dozen HPV types has been found to be associated, in different prevalences, with the development of cervical cancer. Persistent HPV infection is required for the development of precancerous lesions that ultimately progress to cervical cancer. Infections with HPV are common and generally transient, and about 20% of HPV infections persist and can develop into cervical intraepithelial neoplasia (CIN). The duration of this development can take as long as 15-30 years [18].

The prevalence of HPV infection varies from country to country. It is a sexually transmitted disease and most often spread through genital

skin-to skin contact. According to the latest data performed by the ICO/IARC Information Center on HPV and Cancer [3], the global prevalence of HPV infection with normal cervical cytology is estimated as 11.7% (95% confidence interval: 11.6-11.7), and HPV16 is the most frequent oncogenic type, detected in 3.2% of women, followed by HPV 18 (1.4%), HPV 52 (0.9%), HPV 31 (0.8%), and HPV 58 (0.7%). In a study by Rogovskaya et al. [19], based on scarce data from 12 countries, HR-HPV prevalence ranged from 0.0% to 48.4% with normal cytology, 29.2%-100% with LSIL, 77.2%-100% with HSIL, 89.8%-100% with cervical cancer, and HPV 16 was the most commonly detected HPV genotype in all categories. In Weihai China [20], the HPV detection rate was reported as follows: CINI (74.11%), CINII (84.31%), CINIII (90.32%), and SCC (100%). In our present study, the HPV infection rate was 73.5%, of which the HPV detection rate was 5.0% in normal cervical tissue, 67.3% in the LSIL group, 95.7% in the HSIL group, 96.6% in the SCC group. With an advance in the grading of cervical neoplasia, the HPV infection rate also showed a gradual increase. Consistent with most published studies, we found HPV16 to be the most prevalent subtype (33.3%) in our study population, followed by HPV 31, HPV52, HPV58, et al. Of all HR-HPV subtypes, HPV 16, 18, 31, 33, 52, and 58 were the most popular, accounting for 95.8% of the infected cases.

Quantitative real-time PCR methods are currently considered to be the gold standard for evaluating HPV DNA load. HPV viral load has been described as a contributing factor for the persistence of infection and the development of cervical neoplasia [21]. For patients with persisting HPV infections, it is conceivable to speculate that viral load might have some predicative value for the histological severity of the disease. [22, 23]. However, the relationship between the histological severity of cervical neoplasia and the viral load of HPV remains elusive. Some authors have suggested that with the increase in the severity of cervical neoplasia, HPV viral load also increased [24-28]. Other investigators believe that there is no inevitable link between them, and that cervical disease progression is only related to the integration of HPV DNA with host squamous epithelial cells, rather than virus replication [29-31]. Further, some studies suggest that the prognosis of cervical cancer is related to the expression of HPV

oncogene mRNA but not to the HPV viral load [32]. Van der Weele et al. [21] investigated higher HPV16/18 viral load levels in multiple HPV-type infections and found that they were not associated with significantly more persistent HPV 16 or HPV18 infections. We have found that the HPV virus is required for the development of cervical neoplasia. There is also a trend of increasing HPV viral load as the cervical lesion progresses from LSIL to HSIL, and then to invasive carcinoma. Based on our observations, a high HPV viral load contributes, at least in part, to the progression of cervical neoplasia.

Studies have found that the relationship between HPV viral load and the histological severity of cervical neoplasia differs among different HPV genotypes. Saunier et al. [33] and Carcopino et al. [34] showed that only HPV16 and the severity of cervical disease was related. A recent publication demonstrated that high viral load is associated with prevalent cervical cancer precursors for most HR-HPV genotypes, but only the HPV16 load predicts the development of incident disease [35]. Boulet et al. [36] suggest that all HPV genotypes that can cause cervical cancer in addition to HPV16 and their virus load might be associated with the severity of the disease. Kim et al. [37] showed that a low initial HPV viral load is a strong independent prognostic factor associated with a higher overall relapse rate. In our present study, we found that the HPV viral load increased gradually with the grading of cervical neoplasia. Significant differences were only found in HPV viral load between a histologically normal cervix and cervix tissues with epithelial dysplasia. However, we found a statistically insignificant trend of a gradual increase in viral load as the epithelial lesion progresses from LSIL to HSIL and to SCC. Therefore, HPV viral load cannot be reliably used to predict the grading of cervical neoplasia.

In summary, our study showed that the most prevalent high-risk HPV genotypes of HPV in Shanghai were HPV16, 31, 52, and 58. HPV high-risk or low-risk virus mixed infections occurred frequently. Although HPV viral load contributes to the development and progression of cervical neoplasia, it does not accurately predict the histological severity of the disease. Therefore, HPV testing can be used for the detection and follow up of treated cervical neoplasia but cannot reliably predict the severity of the disease. Due to the sample size limita-

tion, the conclusion derived from this study needs to be further validated in a larger cohort of patients.

Disclosure of conflict of interest

None.

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