EJGO European Journal of Gynaecological Oncology

The relationship between HPV16 integration and cervical lesions

Shuang Liang[1*], Yulei Wei[1*], Xiujie Chen[1], Meng Duan[2], Pengpeng Qu[1]

[1]Tianjin Central Hospital of Gynecology Obstetrics, Tianjin, [2]Tianjin Medical University, Tianjin (China)

Summary

Aim: Cervical cancer is closely associated with persistent high-risk human papillomavirus (HR-HPV) infection. However, infections will persist in only 20% of the HR-HPV. The integration of the HPV DNA into the host DNA has been proposed as an early event and a risk factor for the progression of cervical lesions. Therefore, the purpose of this study is to evaluate the relationship between the physical status of HPV16 and the severity of cervical lesions based on both cytology and pathology results. *Materials and Methods:* A total of 150 patients with cervical lesions were enrolled in this study. All patients were HPV-positive, and corresponding pathology results were acquired by biopsy. HPV infection and the expression of HPV16 E2 and E6 forms were analyzed using multiplex-PCR, while the physical status of HPV16 was evaluated using the E2/E6 ratio. *Results:* The analysis of Spearman rank correlation showed that there were correlations between the integration ratio for HPV16 and the severity of cervical lesions based on both cytology and pathology results (both $p < 0.05$). *Conclusion:* The integration ratio for HPV16 is closely associated with the severity of cervical lesions based on pathology and cytology and it is also an important risk factor for the persistence and progression of cervical lesions.

Key words: Human papillomavirus; Cervical lesions; Cervical cancer; HPV16; integration; E2 gene; E6 gene.

Introduction

Cervical cancer is the second most common cancer among women, and it is closely associated with persistent high-risk human papillomavirus (HR-HPV) infection [1, 2]. The incidence of cervical cancer has been significantly reduced thanks to the cytology and HPV-based screening. However, most infections are transient, and only 20% of HR-HPV infections will persist and develop into high-grade squamous intraepithelial lesions (HSILs), ultimately leading up to cervical cancer [3]. Relative specificities and positive predictive values (PPV) were comparable at 81.1% and 16.9% for HR-HPV [4]. Therefore, due to the low specificity and PPV of testing for HR-HPV, new parameters are needed to assess the risk for persistence and the progression of cervical lesions.

Of the various HR-HPV viruses, HPV16, 18, 58, 31, 33, and 52 are most commonly associated with cervical cancer and the cancer precursors. However, HPV16 is the predominant HR-HPV and causes more than 50% of all cervical cancers [5]. Studies have demonstrated that populations of cells with integrated HPV16 possess a selective growth advantage compared to cells that maintain HPV16 episomes [6, 7]. Interestingly, the HPV16 genome includes six regulatory proteins (E1, E2, E4, E5, E6, and E7) that regulate viral life cycle, gene expression, and cell function [8]. The E6 and E7 genes are considered oncogenes in cervical cancer, where their expression can be upregulated by the disruption of the E2 gene during viral integration into the host DNA [9]. HPV16 integration and E2 gene disruption represent an early important event in HPV-infected lesions, which is also closely associated with the severity and clinical outcome of the cervical lesions.

In this study, the authors evaluated the physical status of HPV16 by the ratio of HPV16 E2/E6 using multiplex-PCR in HPV positive cervical samples from a cross-sectional study. The aim of this study was to evaluate the relationship between the physical status of HPV16 and the severity of cervical lesions based on both cytology and pathological results.

Materials and Methods

Plasmids for HPV16, HPV18, and HPV31 were donated by the Department of Structural Biology, Stanford University School of Medicine. The viral load for each plasmid was approximately 10^6 copies/μL.

A total of 150 cervical lesion cell samples were collected from patients who were treated at Tianjin Central Hospital of Gynecology and Obstetrics (China) from June 2010 to December 2011. All samples were confirmed to be HPV-positive using Hybrid Capture 2 (HC2) test and were analyzed using ThinPrep cytological test.

The samples were grouped in accordance with the Bethesda system as within normal limits (WNL, n = 58), atypical squamous cell (ASC, n = 47), low-grade squamous intraepithelial lesion (LSIL, n = 21), and high-grade squamous intraepithelial lesion (HSIL, n = 24). In addition, the diagnosis of each patient was pathologically confirmed by biopsy. Based on the WHO criteria for cervical cancer, the pathological results were classified as normal cervix (n = 50), LSIL (n = 15), HSIL (n = 35), and cervical cancer (n = 50). The age of the patients ranges from 22 to 60 years, with a mean of 44 years. No patients had a history of cervical disease prior to sampling.

Complete genomes were extracted from the cervical lesion

*Contributed equally.

Revised manuscript accepted for publication August 10, 2017

Table 1. — *β-globin and HPV16 primers.*

Gene	Primer	Sequence (5′–3′)	Amplimer length (bp)
β-globin	PC04	CAACTTCATCCACGTTCACC	286
	GH20	GAAGAGCCAAGGACAGGTAC	
HPV LI	GP5+	TTTGTTACTGTGGTAGAACTAC	150
	GP6+	GAAAAATAAACTGTAAATCATATTC	
HPV16 E2	E2(F)	CGACTATCCAGCGACCAAG	257
	E2(R)	CCAATGCCATTCGACGTAGACGAC	
HPV16 E6	E6(F)	ACCCAGAAAGTTACCACAGT	397
	E6(R)	GCAACAAGACATACATCGAC	

samples using a DNA extraction kit according to the manufacturer's instructions. To verify the quantity and quality of the isolated DNA, all DNA concentrations were determined using a spectrophotometer. To monitor the DNA extraction protocol, a 286-bp sequence of the β-globin gene (PC04 and GH20 primers, Table 1) was amplified by PCR as an internal control.

Multiplex-PCR was used to simultaneously amplify three HPV16 genes: L1, E2, and E6. All primers are listed in Table 1. PCR runs were performed in a 20-μL reaction volume, containing 1 μL of DNA, 0.25 μM of all six primers, and 10 μL of PCR master mix. The PCR conditions were as follows: initial denaturation at 96°C for three minutes, followed by 40 cycles of 94°C for 45 seconds, 60°C for 45 seconds, 72°C for one minute, and a final extension at 72°C for five minutes. Five microliters of the PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide, and were visualized using electrophoresis image analysis software. The quantities of the E2 and E6 genes were measured and analyzed by calculating the gray scale area of the PCR products.

The L1 and E6 genes were also amplified to confirm the HPV infection, and the E2/E6 ratio was used to detect the physical status of HPV16. The episomal state was defined as positive E2 and E6 with the E2/E6 ratio of 1 (or close to 1), the mixed state was defined as positive E2 and E6 with the ratio < 1, and the integrated state was defined as negative E2 and positive E6.

Statistical analysis was performed using SPSS software (version 17.0). The authors used trend Chi-squared test(χ^2) for the relationship between the integration ratio for HPV16 and the severity of cervical lesions based on both cytology and pathology results. Frequency tables were evaluated using Fisher's exact test or χ^2 test. *P*-values < 0.05 were considered statistically significant.

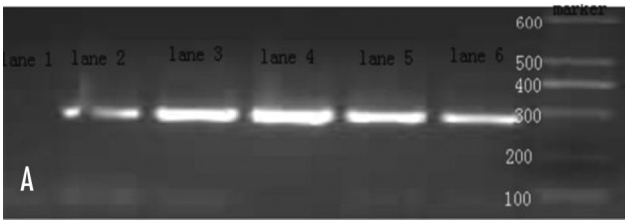

Figure 1A. — Electrophoresis image of PCR performed using extracted DNA and the β-globin primers. Lane 1 is the negative control and lanes 2–6 are samples.

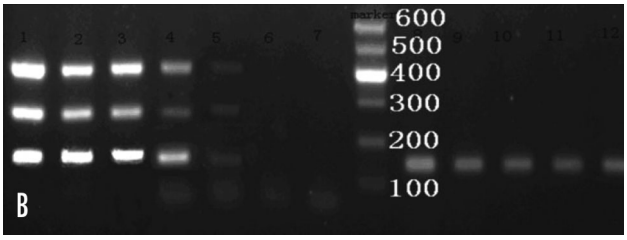

Figure 1B. — Results of the multiplex-PCR and GP5+/GP6+ PCR for HPV16 plasmids in the following concentrations: 106, 105, 104, 103, and 102 copies/μL. Multiplex-PCR: lanes 1–5, respectively. Lanes 6 and 7 are negative controls. Lanes 8–12 are GP5+/6+ PCR (106, 105, 104, 103, and 102 cells/μL, respectively).

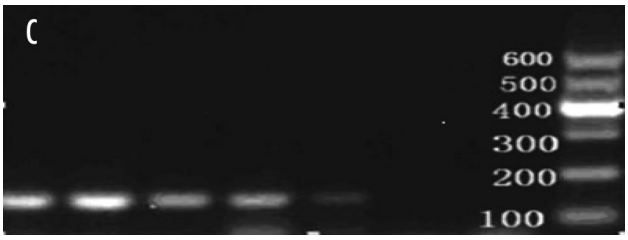

Figure 1C. — Multiplex-PCR results for HPV18 with the following concentrations: 106, 105, 104, 103, and 102 copies/μL (left to right).

Results

The average concentration of the isolated DNA was 200 ng/μL. All portions of the amplified β-globin gene were clearly visible after electrophoresis (Figure 1A). Varying concentrations of three HPV plasmids (HPV16, 18, and 31) were tested by multiplex-PCR and general PCR using the $GP5^+/GP6^+$ primers. Positive results were obtained using multiplex-PCR at a concentration of 100 copies/μL, which was consistent with the results of GP5+/GP6+ PCR (Figures 1B-1D). Multiplex-PCR was also specific for multiple HPV infections, including HPV16 with HPV18/31, and HPV18 with HPV31 (Figure 1E).

All 150 clinical samples were confirmed to be HPV-positive using multiplex-PCR (Figure 1F showing a subset of the samples tested), and 115 (76.67%) were HPV16-positive. Table 2 shows the physical status of HPV16 infection for the various cytological results.

The trend via χ^2 test showed that there were correlations between the integration ratio for HPV16 and the severity of cervical lesions ($\chi^2 = 44.89$, $p < 0.01$). As the cytological severity increased, an increase was also observed in the integrated form, with a concurrent decrease in the episomal form.

Table 3 showed the physical status of HPV16 infection for the various pathology results. The trend via χ^2 test showed that the integration ratio for HPV16 was correlated with the severity of cervical lesions ($\chi^2 = 57.99$, $p < 0.01$). As the severity of the cervical lesions increased,

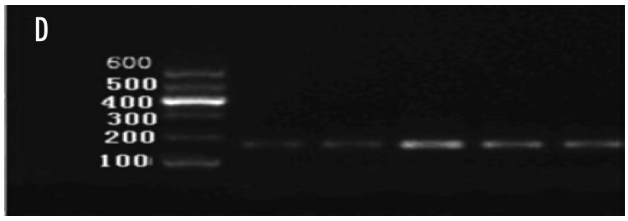

Figure 1D. — Multiplex-PCR results for HPV31 with the following concentrations: 102, 103, 104, 105, and 106 copies/μL (left to right).

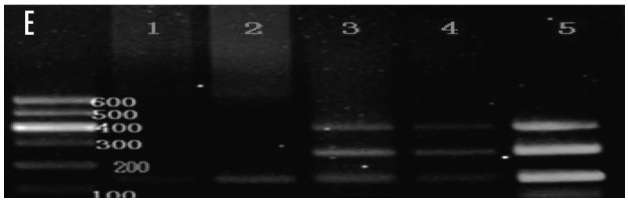

Figure 1E. — Multiplex-PCR results for mixed HPV plasmids. Lanes 1 and 2: HPV18 and HPV31 with 102 and 103 copies/μL, respectively. Lanes 3–5 HPV16 and HPV18/31 with 102, 103, and 104 copies/μL, respectively.

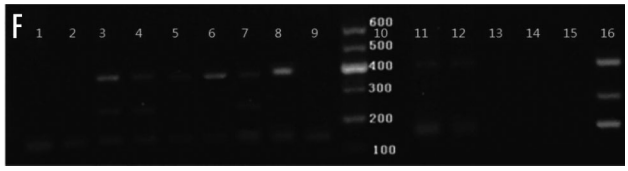

Figure 1F. — Multiplex-PCR results for clinical DNA samples. Lanes 1–12 are samples, lanes 13–15 are negative controls, and lane 16 is positive control.

an increase was observed in the proportion of the integrated form, with a concurrent decrease in that of the episomal form.

Discussion

In this study, the authors assessed simultaneously cytology results, pathology results and physical status of HPV16, and they found that the integration ratio for HPV16 was strongly associated with the severity of cervical lesions based on both pathology and cytology results. This finding suggests that the physical status of HPV16 can be used as potential predictive biomarkers to assess the risk for persistence and the risk for progression, which can increase the PPV of testing for HR-HPV.

Several studies have demonstrated that the integration of HPV DNA into the host genome is an important mechanism for cervical cancer progression [10-13]. The integration of HPV DNA into the host genome was observed in the majority of invasive cancers [14], with integration frequencies for HPV16 ranging from 50% to 83% in cervical cancers [15]. In this study, 115 out of 150 cases were HPV16-positive, and 36 (31.30%) of these cases showed the integrated form. The frequency of HPV16 integration was 0%, 28.20%, 38.10% and 70.83%, respectively, in WNL, ASC, LSIL, and HSIL cytological groups and 2.94%, 10.00%, 20.69%m and 66.67%, respectively, in normal cervix, LSIL, HISL, and cervical cancer pathological groups. Moreover, it is worth noting that the integration ratio for HPV16 was strongly associated with the severity of cervical lesions. As the severity of the cervical lesions increased, an increase was observed in the proportion of the integrated form, with a concurrent decrease in that of the episomal form in both cytological and pathological groups.

Although previous studies have reported that HPV integration is a significant molecular event during the transformation from LSIL to cervical cancer [14], limited data is available regarding the relationship between HPV integration and cytological results. In the present study, the authors observed HPV integration frequencies of 0%, 28.20%, 38.10%, and 70.83% for the WNL, ASC, LSIL, and HSIL cytological groups, respectively. The frequency of HPV16 integration was increased with the increase of cytological severity, with a concurrent decrease in the frequency of the episomal form. The integration rate was significantly different between the various cytology groups ($p < 0.01$), which indicated that the authors could detect HPV16 and its physical status using cytological samples, thereby predicting the severity of cervical lesions at an early stage.

Among cervical lesions, HPV16 is the predominant virus, and women who are infected with HPV16 have a higher likelihood of progressing to CIN and cancer [13, 16, 17]. As with other HPV viruses, HPV16 can exist as episomes, or can be fully integrated into the human chromosomes, or can be a combination of both states. Given the close relationship between HPV16 and cervical cancer, HPV16 infection and its physical status could provide an early indication for cervical cancer. Although HC2 has been globally accepted as a method for detecting HPV infection [18], it cannot identify the specific virus responsible for the infection or its physical status. Moreover, the hybridization method can detect the episomal or integrated form of HPV, although it is labor-intensive and relatively expensive, and the hybridization results cannot detect the combined physical state (episomal and integrated) [19]. Previous studies have also shown that the E2 gene plays various roles in HPV, including regulation of E6 expression, which is critical in the development of HPV-related cervical cancer. Therefore, it is thought that the E2/E6 ratio can provide an accurate estimate for the degree of HPV integration [12, 20, 21]. In

Table 2. — *HPV16 physical status for the various cytological results.*

	n	HPV16 infection	Episomal form	Mixed form	Integrated form
WNL	58	31 (53.45%)	20 (64.52%)	11 (35.48%)	0 (0%)
ASC	47	39 (82.98%)	8 (20.51%)	20 (51.28%)	11 (28.21%)
LISL	21	21 (100%)	1 (4.76%)	12 (57.14%)	8 (38.10%)
HISL	24	24 (100%)	0 (0%)	7 (29.17%)	17 (70.83%)
Total	150	115 (76.67%)	29 (25.22%)	50 (43.48%)	36 (31.30%)

WNL: within normal limits, ASC: atypical squamous cell, LISL: low-grade squamous intraepithelial lesion, HISL: high-grade squamous intraepithelial lesion.

Table 3. — *Physical status of HPV16 infection for the various pathology results.*

	n	HPV16 infection	Episomal form	Mixed form	Integrated form
Normal	50	34 (68.00%)	25 (73.53%)	8 (23.53%)	1 (2.94%)
LISL	15	10 (66.67%)	1 (10.00%)	8 (80.00%)	1 (10.00%)
HSIL	35	29 (82.86%)	1 (3.45%)	22 (75.86%)	6 (20.69%)
CC	50	42 (84.00%)	2 (4.76%)	12 (28.57%)	28 (66.67%)
Total	150	115 (76.67%)	29 (25.22%)	50 (43.48%)	36 (31.30%)

LISL: low-grade squamous intraepithelial lesion, HISL: high-grade squamous intraepithelial lesion, CC: cervical cancer.

addition, PCR detection of the HPV gene and its integration state can produce results that are as reliable as hybridization [22, 23]. In the present study, multiplex-PCR was used to detect HPV infection, HPV16, and its physical status, and the present results indicate that this technique was sensitive and specific. Moreover, multiplex-PCR results were consistent with the HC2 results regarding the detection of HPV DNA.

Detection of HPV infection and HPV16 physical status using multiplex-PCR appears to be a promising biomarker of persistent infection and cervical precancerosis, and it is also a supernumerary marker for screening programs aiming to reduce the rate of unnecessary referrals for colposcopy. This technique could provide more reliable diagnostic data for the prevention, treatment, and prognosis of cervical cancer at an early stage. Further research should focus on following up those with no detectable abnormalities in pathology or cytology.

Acknowledgement

This study was supported by grants (09ZCZDSF03900) from the Tianjin Science and Technology Plan of China and (15KG140) from the Tianjin Health Industry Key Research Project.

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Corresponding Author:
PENGPENG QU, M.D.
Tianjin Central Hospital of
Gynecology and Obstetrics
No.156 Sanma Road, Nankai District
Tianjin (China)
e-mail: qu.pengpeng@hotmail.com