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Original Article

Relationship between HPV-16 infection and the progression of vaginal intraepithelial neoplasia

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Abstract: Objective: To explore the relationship between infection with human papillomavirus (HPV)-16 and the development of vaginal intraepithelial neoplasia (VaIN). Methods: This is a retrospective study. 78 patients with suspected VaIN admitted to the gynecologic clinic of Affiliated Hospital of Guilin Medical College from August 2016 to December 2020 who were confirmed to have HPV-16 infection by HPV rapid flow-through hybridization method were selected as the research subjects. The copy numbers of HPV-16 early genes E2 and E6 were detected by quantitative real-time PCR amplification to analyze the integration status of the virus. Results: The episomal form of HPV-16 exists in all levels of VaIN. As the pathological level of VaIN increased, the episomal form of HPV-16 gradually decreased, and a disruption of the E2 gene became more frequent. However, there was no significant difference between different levels of VaIN ($P>0.05$). With the increased severity of cytology results, the percentage of the episomal form of HPV-16 decreased from 76.47% in atypical squamous cells of undetermined significance (ASC-US) to 44.44% in the high-grade squamous intraepithelial lesion (HSIL) ($\chi^2=4.780$, $P<0.05$). However, the integrated form of HPV-16 did not increase significantly as the severity of cytology increased ($\chi^2=2.215$, $P>0.05$). Conclusion: HPV gene integration may occur before the onset of VaIN. However, the occurrence of HPV-16 integration is not a risk factor leading to the malignant progression of VaIN. An E2 gene disruption is more common in the early events after HPV-16 infection. HPV-16 gene integration may be the main reason for persistent HPV-16 infection.

Keywords: Human papilloma virus, old age, vaginal intraepithelial neoplasia, cytology

Introduction

Vaginal intraepithelial neoplasia (VaIN) refers to atypical hyperplasia of varying degrees just in the vaginal epithelial layer, mostly before the onset of vaginal epithelial cancer [1]. VaIN is more common in middle-aged and elderly women [2], which may be related to factors such as the thinning, atrophy, and damage susceptibility of the vaginal epithelium due to changes in hormone levels with age. The annual incidence of VaIN is (0.2-2.0)/100,000, which is much lower than that of cervical intraepithelial neoplasia (270/100,000) [3, 4]. Therefore, VaIN is often neglected and the probability of missed diagnosis is high. Most VaINs have a long and reversible precancerous stage, so patients can be cured completely after early diagnosis and treatment [5]. However, if not diagnosed and treated at an early stage, about 10% of high-level VaIN will progress to vaginal invasive carcinoma, making treatment difficult [6]. Studies have confirmed that most patients with VaIN had a history of cervical-related surgery and that VaIN in most patients was accidentally detected during screening for cervical lesions or follow-up [7]. An indicator is urgently needed to identify groups at high-risk for cancer.

The persistent infection of human papillomavirus (HPV) is the main factor leading to vaginal lesions [8], and high-risk HPV has been used as a detection index for precancerous screening, among which the HPV-16 subtype is the most common. However, simple HPV typing detection lacks specificity, resulting in excessive examination and increasing economic burden to

patients [9]. In some studies, it was found that the detection of HPV DNA status seems to be more valuable than simple DNA typing [10]. HPV mainly exists in three forms after infection of host cells, including an episomal form, integrated form, and mixed form [11]. A free state mainly exists in some benign lesions, and integration and mixed state in some high-risk HPVs causing malignant lesions is more common [12]. In cervical lesions, Melsheimer et al. confirmed that HPV gene integration events may lead to abnormal expression of host genes [13]. Few studies have analyzed whether the presence of HPV-DNA in host cells is involved in the progression of VaIN. Therefore, in this study, 78 patients with different levels of VaIN and HPV-16 positivity were selected as the research subjects. PCR was used to analyze the presence of HPV16-DNA in vaginal epithelial cell specimens of patients, and to explore a relationship between the presence of HPV16-DNA and the occurrence and development of VaIN, so as to provide an effective screening method for VaIN.

Materials and methods

Study subjects

78 patients with suspected VaIN admitted to the gynecologic clinic of Affiliated Hospital of Guilin Medical College from August 2016 to December 2020 who were confirmed to have HPV-16 infection by HPV rapid flow-through hybridization method were selected as the research subjects. Inclusion criteria: (1) VaIN diagnosed by biopsy; (2) aged ≥60; (3) HPV-16 infection positive; (4) without a history of cervical and vaginal surgery; (5) without uterine prolapse; (6) cervical and uterine integrity. Exclusion criteria: patients (1) with dysfunction of important oranges such as liver and kidney; (2) with mental abnormalities or communication difficulties; (3) with other sexually transmitted diseases; (4) with dysfunction of blood coagulation; (5) with immune system disease; (6) suffering from other malignant tumors; (7) Lack of relevant information. This study has been approved by the Affiliated Hospital of Guilin Medical College Medical Ethics Committee.

Study method

Cytologic examination of vagina: Cell samples were collected by applying a cell sampler to the vaginal wall for 1 to 2 circular movements and then it was sent for testing. The samples were detected by ThinPrep cytologic test and Pap staining. The results of the diagnosis report were classified into 3 levels according to the diagnostic criteria of the Bethesda system (2014) [14]: atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL).

Colposcopy biopsy: A speculum was used to expose the vagina, and excess secretions were wiped off. Iodine solution and 4% acetic acid solution was applied to the suspected lesion, then the samples of the basal layer of the epithelium were taken, and a pathologic examination of the samples was performed. According to the criteria for dividing the levels of VaIN lesions, the VaIN lesions were graded into 3. (i) VaIN I: the lesion has mild atypical hyperplasia, and the lesion area is 1/3 of the thickness of the vaginal epithelium, with few mitotic figures and the emergence of cell polarity. (ii) VaIN II: the lesion has moderate atypical hyperplasia, and the lesion area is 2/3 of the thickness of the vaginal epithelium, with common mitotic figures and the emergence of cell polarity. (iii) VaIN III: the lesion is severe atypical hyperplasia, and the lesion area is more than 2/3 of the thickness of the vaginal epithelium, with a complete basement membrane, increased mitotic figures, and no cell polarity.

PCR detection: (1) DNA extraction: tissue samples were first fixed with 10% formaldehyde fixative. After paraffin embedding, samples were made into 5 μm thick slices. 5 slices were put into centrifuge tubes with the addition of extract (purchased from Chaozhou Hybribio Biochemistry Ltd.) and incubated at room temperature (for 10 minutes at 50°C). Then well-suspended DNA modification reagent was added, and the supernatant was removed after centrifugation. 0.5 ml TE buffer was used for the elution of DNA. The DNA was extracted, dissolved in 60 μL of free water and stored at a low temperature (-20°C).

(2) PCR reactions: HPV-16 type integration status detection was carried out by PCR. HPV-16 E2 and E6 genes were amplified with primers listed in **Table 1**. The reaction conditions were as follows: pre-denaturation at 94°C for 3 min; 35 cycles (94°C 30 sec, 55°C annealing 30 sec, 72°C extension 45 sec); full extension at

Table 1. Primers used for PCR experiments

Gene	Primer sequence	fragment	Tm
HPV 16 E2			
F	5'-ACGGAAATCCAGTGTATGAG-3'	19 bp	55°C
R	5'-AAAGCACGCCAGTAATGTTG-3'	21 bp	56°C
HPV 16 E6			
F	5'-CGGAGCTCAGCAGACA-TTTTATGCACCAAAAGAG-3'	22 bp	55°C
R	5'-GCAAGCTTCATGCATGATTACAGCT-GGG-3'	25 bp	56°C

Note: F, forward; R, reverse.

Table 2. Basic characteristics of 78 patients

Characteristic	N [(%)]/ $\bar{x}$ ±s
BMI (kg/m^2)	21.45±0.85
Age	
60-70	40 (51.28)
71-75	21 (26.92)
>75	17 (21.80)
Complication	
Hypertension	16 (20.51)
Hyperlipidemia	8 (10.26)
Hyperglycemia	10 (12.82)
None	44 (56.41)

Note: BMI, body mass index.

72°C for 5 min. The PCR product was finally obtained.

(3) Semi-quantitative analysis of gene amplification products: 5 uL PCR products were taken for 2% agarose gel electrophoresis, Under the condition of 120 V voltage, electrophoresis 30 min. Image Lab software was used to measure and analyze the gray value of the electrophoresis band area of PCR products, and the ratio of E2 and E6 was calculated. The E2/E6 ratio was used to determine the state of HPV-I6 infection in vivo. (i) Episomal form: E2/E6>0.9, see a clear band at 100 bp; (ii) Integration form: E2/E6 was 0-0.15, and a clear band was seen at 250 bp; (iii) Mixed form: E2/E6 was 0.15-0.9, at 250 bp and 100 bp see two clear bands.

Statistical methods

SPSS19.0 software was used for statistical analysis. The enumerated data were described by composition ratio or rate (%), and the trend chi-square test was used for comparison between groups. When $P<0.05$, the difference was considered significant.

Results

Basic characteristics of the patients

The basic characteristics of 78 patients are shown in **Table 2**. The average body mass index (BMI) was (21.45± 0.85) kg/m^2, ranging from 19 to 25 kg/m^2. The median age was 66 years old (ranging from 60 to 78 years old), and the rate of hypertension was high (20.51%).

Relationship between the presence status of HPV 16 DNA and VaIN

The results of the colposcopic biopsy in 78 VaIN patients (**Table 3**) showed that there were 7 cases of simple vaginal inflammation, 16 cases of VaIN I, 32 cases of VaIN II, and 23 cases of VaIN III. HPV-16 in 7 patients with vaginitis was free-state. The free state HPV-16 also existed in all grades of VaIN, and with the increase of pathologic grade of VaIN, free state HPV-16 gradually decreased and E2 gene breakage increased, but there was no significant difference between the groups ($P>0.05$). Mixed-state HPV-16 infection rate increased with the increase of VaIN grades, and there was a significant difference in the mixed-state HPV-16 infection rate between VaIN II and VaIN III groups ($\chi^2=4.093$, $P<0.05$).

Relationship between the presence of HPV 16 DNA and the results of cytological examination

According to the results of the cytological examination (**Table 4** and **Figure 1**), 17 of the 78 patients with VaIN were ASC-US, 25 were LSIL, and 36 were HSIL. HPV 16-DNA in vaginal cells of VaIN patients mainly existed in episomal form. With the increase in cytological judgment level, the proportion of episomal form HPV-16 decreased from 76.47% of ASC-US to 44.44% of HSIL ($\chi^2=4.780$, $P<0.05$). The proportion of Integrated form HPV-16 increased with the increase in cytological severity ($\chi^2=2.215$, $P>0.05$).

Discussion

HPV is a common DNA double-stranded virus. After entering the body, it can cause vaginal

Table 3. Relationship between the Status of HPV 16 DNA and VaIN in the elderly

Pathology	*n*	Status of HPV 16			
		Episomal form (%)	Integrated form (%)	Mixed form (%)	E2 gene disruption (%)
Inflammation	7	7 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)
VaIN I	16	11 (68.75)	2 (12.50)	3 (18.75)	5 (31.25)
VaIN II	32	21 (65.63)	4 (12.50)	7 (21.88)	11 (34.37)
VaIN III	23	10 (43.48)	2 (8.70)	11 (47.83)*	13 (56.52)
Total	78	49 (62.82)	8 (10.26)	21 (26.92)	29 (37.18)

Note: VaIN, vaginal intraepithelial neoplasia; *Compared with VaIN II, $P<0.05$.

Table 4. Relationship between the status of HPV 16 DNA and the results of cytologic examination

Results of cytologic examination	*n*	Episomal form (%)	Mixed form (%)	Integrated form (%)
ASC-US	17	13 (76.47)	2 (11.76)	2 (11.76)
LSIL	25	14 (56.00)	5 (20.00)	6 (24.00)
HSIL	36	16 (44.44)*	9 (25.00)	11 (30.56)
Total	78	43 (55.13)	16 (44.44)	20 (24.36)

Note: ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; *Compared to ASC-US, P<0.05.

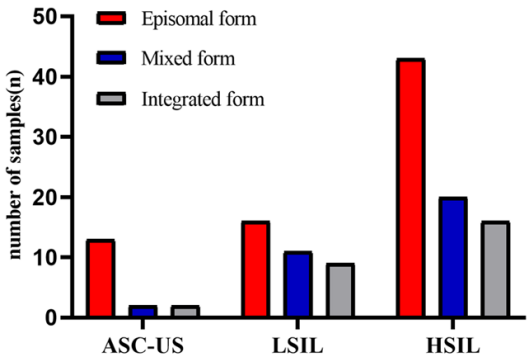

Figure 1. HPV 16 DNA status and cytological examination results. Note: ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

mucosal hyperplasia, a disorder of the body's immune system, so that a large number of inflammatory factors, and increased tissue damage, prompt a large area of tumor cell spread to promote disease development [15]. After checking, HPV has 3 coding regions on the DNA coding chain of the open reading frame, namely, early protein coding region, late protein coding region, and non-coding long control region. In the open reading frame in the early regional coding, the product E2 protein is involved in the integration and regulation of HPV DNA. E2 inhibits the replication and transcription of the E6 in viruses and maintains the episome [16], suggesting that the disruption or deletion of the E2 gene will relieve the inhibitory effect on the E6 gene, resulting in overexpression of the E6 gene [17]. Studies have found that the binding of E6 to cancer suppressor genes pRb and p53 will lead to inactivation of PRB protein, resulting in the continuous proliferation of infected HPV cells [18]. When the virus progresses to an integrated state, it is difficult for immune cells in the host to clear it.

In a previous study of cervical intraepithelial neoplasia, it was found that the E2/E6 ratio decreased with the increase in lesion grade, indicating that E2/E6 was correlated with the degree of cervical lesions [19]. Vaginal cancer, as a synchronous or metachronous tumor, is often associated with cervical cancer, and the two are closely related [20]. In addition, vaginal cancer has been reported to frequently occur after a hysterectomy for cervical cancer [21]. However, vaginal lesions are often neglected. In this study, the E2/E6 ratio of the HPV-16 gene was used as an indicator of gene status. The results showed that HPV-16 E2 gene breakage accounted for 31.25% of VaIN I and 12.50% of complete integration. It may be that in HPV-infected vaginal epithelial cells, the chromosomes of the host cell were distorted at an early stage, resulting in host cell dysplasia. It cannot be ruled out that these cells that have developed chromosomal aberrations are present in low-level vaginal lesions and extend into normal cells, becoming the basis for the malignant progression of vaginal lesions. Studies have shown that persistent infection with HPV-16 often exists in the integrated form, and after the virus integrates with the host genome, the genetic structure will change, and the gene function will also be reduced, so vaginal lesions

can worsen [22]. Some studies have observed that HPV integration occurs near certain oncogenes and tumor suppressor genes in cervical cancer, indicating that integration can aggravate and destroy normal human gene expression [23, 24]. Gallo et al. demonstrated that HPV16-DNA integration status detection can help predict the possibility of LSIL evolving into HSIL or becoming cancerous [25]. This study also found that as the level of VaIN increased, there are more HPV-16 E2 gene disruptions. Especially in the VaIN III tissue, the HPV-16 E2 gene disruption rate was as high as 56.52%. It is possible that the integrated form of HPV-16 formed a super-enhancer element that drives transcription of viral oncogenes, prompting E6 overexpression, transcribing adjacent genes, and improving the persistence of viral infections [26]. Kulmala et al. suggested that only the episomal form of HPV was present in diseased cells below the LSIL level, and there was no HPV in only episomal form in HSIL cytological changes [27]. However, in the analysis of cytology and HPV presence status, this study found that 11.76% of vaginal cytology ASC-US cases were in integrated form, which was very different from the above findings that there were episomal or integrated forms of HPV in all levels of cytology changes. We believe that this is another manifestation of the integration of viral genes into host cells as an early event of vaginal lesions, such as an increase in the number of E2 gene disruptions or deletions, which leads to an increase in the level of cytological pathology, resulting in a decrease in the episomal form of HPV-16, but some HPV-16 is still in the episomal form at various stages of vaginal lesions [28].

In summary, HPV gene integration can occur before, and develop with the occurrence of VaIN. HPV-16 integration is not a risk factor for the malignant progression of VaIN. However, E2 gene disruption is more common in early events after HPV-16 infection, and HPV-16 gene integration may be the main cause of persistent HPV infection. Therefore, HPV-16 integration is molecular biologic process necessary for the occurrence and development of VaIN.

Disclosure of conflict of interest

None.

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