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Pathogenicity of high risk HPV infection in pseudocondyloma of vulvae and its carcinogenicity in inducing cervical lesions

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Abstract. – OBJECTIVE: To identify high risk human papillomavirus (HPV) infection in pseudocondyloma of vulvae (PV) and the causal relationship between high risk HPV infection and cervical cancer.

PATIENTS AND METHODS: The patients were divided into condyloma acuminatum group, PV groups and PV high-risk HPV infection group according to the clinical data and morphological features. Condyloma acuminatum group and PV group were two control groups. The exfoliated cells were detected and typed by human HPV nucleic acid typing kit. The gene fusion site of HPV and its potential gene integration mechanism were investigated using genome-wide sequencing and high-throughput virus integration screening techniques. The HPV integration frequencies of some key gene integration sites were calculated and some novel genes integration sites were identified.

RESULTS: The samples from PV high-risk HPV infection group showed both the pathologic manifestations of PV and the koilocytes caused by the virus infection. Suspected HPV virus particles with a density different from chromatin were observed from the samples of PV high-risk HPV infection group under transmission electron microscopy (TEM). The intercellular desmosomes were regularly connected, and autophagosomes can also be observed in some cases. HPV genome was not detected in PV groups and PV high-risk HPV infection group due to the low copy number. HPV genome was only detected in condyloma acuminatum group.

CONCLUSIONS: PV high-risk HPV infection showed both the symptoms of PV and HPV infection with suspected HPV virus particles in cells.

Key Words:

Condyloma acuminatum, Human papillomavirus (HPV), Pseudocondyloma of vulvae (PV), Pseudocondyloma of vulvae (PV), High-risk HPV infection.

Abbreviations

PV = Pseudocondyloma of vulvae, HPV = Human papillomavirus, TEM = Transmission electron microscopy, HE = hematoxylin and eosin, PBS = phosphate-buffered solution, SD = Standard deviation, BWA = Burrows-wheeler alignment, GATK = Genome analysis toolkit, SNP = single nucleotide polymorphism, INDEL = Insertion-deletion, CREST = Clipping reveals structure, SV = structure variation.

Introduction

Pseudocondyloma of vulvae (PV), which is also called as vulvopapillary hyperplasia, was firstly reported by Altmeyer et al[1]. PV usually occurs in the inner side of the labia minora with the granular pale red pimples and mild itching[2]. To date, the pathogenesis of PV is still unknown. It is well accepted that atavistic deformity and the abnormal physical conditions are responsible for the onset as well as the development of PV. In 2008, studies have also shown that chronic infection and long-term inflammation may contribute to the progression of PV[3]. Xu et al[4] have reported that some diseases with the same symptoms of PV could be misdiagnosed as PV, delaying the treatment and affecting the treatment outcomes.

HPV infection, which is usually transmitted by sexual behavior, causes no symptoms or they can be resolved spontaneously[5]. However, in some cases, the chronic HPV infection can cause warts or even precancerous lesions[6]. It has shown that the precancerous lesions caused by human papillomavirus (HPV) infection can significantly increase the risk of a variety of cancers, including oropharyngeal, cervical, head, neck cancer and so on[7-9]. In clinical practice, PV was sometimes mis-

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diagnosed as condyloma acuminatum or genital warts because they share similar symptoms[10,11]. During our clinical practice, we found that a variety of subtypes of high-risk HPV infection can be detected in more than 20% of all the cases of PV, among whom the HPV positive rate is much higher than the normal people group. Those cases of HPV infection were not the latent infection of HPV combined with PV. They were also not the early manifestations of the low-risk HPV infection. It was believed that the disease was caused by certain subtypes of high-risk HPV infection, which can induce similar symptoms of PV. So this disease can be misdiagnosed as PV. Therefore, we named it PV high risk HPV infection. Xu et al[4] also found that patients with a special type of HPV infection can be misdiagnosed as PV, which in turn delayed the treatment. Then, it will be with significant clinical values to identify the specific pathological features of this disease, and to avoid the misdiagnosis.

In our work, the patients with suspected PV high-risk HPV infection were included with the condyloma acuminatum and PV patients as control. The tissue morphological features of the lesion sites of all the patients were observed, and the type of the virus was identified. In addition, the virus integration sites were analyzed.

Patients and Methods

Patients

The patients were divided into condyloma acuminatum group, PV group and PV high-risk HPV infection group, 10 patients in each group. Typical skin lesions and HPV infection were identified in patients of condyloma acuminatum group. The patients in PV group showed HPV negative. The inclusion criteria for the patients in PV high-risk HPV infection group: 1, patients had impurity sexual life with more than one sexual partners or sexual partners have been suffering from HPV infection-related diseases; 2, symmetrical distribution of fresh and wet sandy papules in bilateral pairs of labia minora with slow growth, itching or no symptoms but showed no bleeding; 3, patients with or without visible vaginal and cervical lesions, cervical cytology and pathological examination were performed; 4, patients with vacuolization of stratum spinosum and stratum granulosum; 5, acetic acid test weak positive; 6, HPV positive. The tissue samples were collected from the lesion sites of the patients in all the three groups. Each sample was divided into two parts. The first part was used for viral classification and DNA extraction. The other was immobilized by 4% formalin and 2.5% glutaraldehyde for normal histopathological and transmission electron microscopy (TEM) observations. The sample collection was approved by the patients, and the study was approved by the Ethics Committee of our hospital.

Clinical Morphological Characteristics

The general clinical data of the patients in the three groups were analyzed. The clinical data included age, duration of illness, skin lesions, the number of sexual partners, the previous and current status of condyloma acuminata of themselves and their sexual partners. Colonoscopy was performed on the patients in each group to understand the vaginal and cervical lesion; biopsy was also done. The information about the symptoms and distribution of skin lesions, surface traits, the presence of vaginal inflammation or cervical erosion or hyperplasia and so on were recorded and statistically analyzed. Using healthy people and PV patients as control, the clinical features and the general morphological characteristics of the patients in this group were summarized.

Histopathological Observation

The tissue was fixed in 4% neutral formaldehyde solution for 24 h, followed by dehydration by passing a series of graded concentrations of ethanol and paraffin-embedding. The embedded tissue was then sliced into 5 μm to make tissue sections. Hematoxylin and eosin (HE) staining was performed using a kit (Beyotime Institute of Biotechnology, Jiangsu, China), followed by histopathological observation under an optical microscopy (Nikon, Chiyoda, Tokyo, Japan).

TEM Observation of Ultrastructure of the Labia Minora Clinical Samples

The tissue was cut into small pieces (less than 1 mm^3 for each piece) and fixed in 2.5% glutaraldehyde phosphate-buffered saline (PBS) overnight. After washing twice with 0.1 M PBS (15 min for each time), 1% osmium acid was added for fixation for 1 h. The tissue, after been washed twice with 0.1 M PBS (15 min for each time), was stained in 2% uranyl acetate solution for 30 min. After that, dehydration was performed by passing a series of graded concentrations of ethanol (50%, 70%, 90%, 100% and 100%, 15 min for each) and

100% acetone (20 min × 2 times). After that, the tissue was implanted in the embedding agent and sliced into ultrathin slices with a thickness of 120 nm to prepare tissue sections. The staining was performed using 4% uranyl acetate for 20 min and lead citrate for 5 min. The stained tissue was transferred to single-hole copper mesh, TEM (Japan Electronics Co., Ltd., Tokyo, Japan) was used to observe the cell morphology.

Virus Detection and Typing

The exfoliated cells were collected and the DNA was extracted from the cells using a DNA Extraction Kit (Invitrogen, Carlsbad, CA, USA). The virus typing was performed using human HPV nucleic acid typing kit with sensitivity of 10 x 10^3 copies/ml (Yaneng Bio, Shenzhen, China). This Kit can detect 17 high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 83) and 5 low-risk types (6, 11, 42, 43, and 44). The pre-processed data were compared with all HPV reference genomes using the Burrows-Wheeler Alignment (BWA) tool to obtain basic typing information with an average sequencing depth greater than 10 and coverage rate greater than 50%. The corresponding HPV reference genome was selected according to the typing information. The pre-processed data were compared using BWA, picard-tools was used to label repeat, and Genome Analysis Toolkit (GATK) Haplotype Caller was used to detect single nucleotide polymorphism (SNP) and insertion-deletion (INDEL). GATK variant filtration was used to filter SNP and INDEL results; finally, annovar was used to annotate the SNP and INDEL results. The consistency of the cervical infection HPV subtype and infection subtype of PV skin lesion were further analyzed. The consistency of PV high-risk HPV infection and cervical local HPV infection were also statistically analyzed.

Detection of HPV Gene Integration Site

DNA was extracted from the skin and mucous membrane specimens collected form the area around the lesions. The gene integration site of HPV and its potential gene integration mechanism were analyzed by whole genome sequencing and high-throughput virus integration screening technique. The specific experimental steps were as follows: according to the typing information, the strain with the depth > 4X percentage was selected as the reference genome for HPV-integration analysis. The filtered results were compared with hg19 and HPV-Integration reference genome bwa mem, and the results were compared with bam. The results were compared with hg19 and HPV-integration reference genome bwa mem. Based on the comparison, structure variation (SV) detect was used to analyze structural variations to get the preliminary results of integration regions. Clipping reveals structure (CREST) was used to analyze the HPV integration site. The CREST analysis results and SV detect analysis results were combined. CREST analysis provided high-confidence results, while the SV detect analysis results without CREST detection were low-confidence results.

Statistical Analysis

SPSS19.0 statistical software (IBM, Armonk, NY, USA) was used to analyze the variance of all the monitoring data. All the data were expressed as mean ± standard deviation (± SD). The *t*-test was used to compare the data between the two groups. $p < 0.05$ was considered to be statistically significant.

Results

Clinical Morphological Characteristics Identified in Different Group

The basic clinical information of all the subjects was summarized and analyzed (Table I). Our data suggested that the skin lesion of PV high-risk HPV infection was similar as that of PV, but is different from that of condyloma acuminatum.

Histopathological Observation Showed Different Tissue Structures in Different Groups

The histopathological features of PV showed different degrees of hyperkeratosis and spine cell layer thickening. Mild PV-like hyperplasia, scattered vacuolar cells, normal basal cell morphology and arrangement, dermal capillary hyperplasia and hyperemia, and Inflammatory cell infiltration was observed in various degrees around the lesion (Figure 1A). The histopathological features of condyloma acuminatum showed different degrees of hyperkeratosis and spine cell layer thickening, or mild PV-like hyperplasia, normal shape and arrangement of basal cells, dermal capillary hyperplasia and congestion, different degrees of inflammatory cell infiltration surrounding the lesions and the appearance of koilocytes (Figure 1B). The koilocytes, which

Table I. The basic clinical information of the subjects.

Groups	Characteristics	Acetic acid test
PV	Lesions mainly occurred on the inner side of both side of minora medial side, but occurred less on introitus. The main disease characteristics were the roe-like clustered pimples (1-2), grainy feeling with touch, fluffy protrusions could be observed in some cases and the lesion surface was smooth.	Negative
Condyloma acuminatum	Skin lesions showed large number of small and soft verrucous pale red papules. In some cases, the lesions were papillary or cauliflower-like. Rough wart surface, easy to break bleeding, accompanied by itching and other feeling.	Positive
PV high-risk HPV infection	The skin lesions were similar as PV. Symmetrical distribution of sand-like pimples on both sides of labia minora. Slow growth without changes for many years, accompanied by vaginal and cervical lesions, it's usually misdiagnosed or diagnosed as PV.	Weak positive

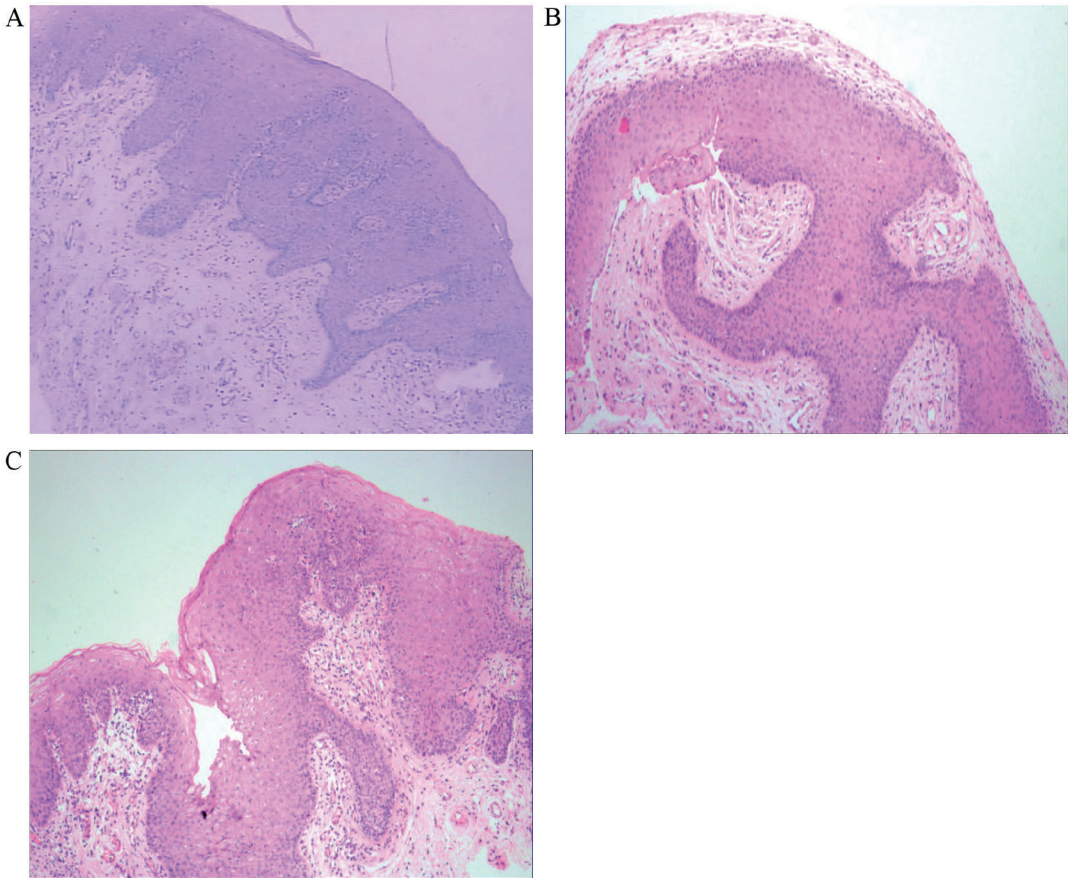

Figure 1. Histopathological observation of tissues from different groups after HE staining (x100). **A,** Representative results of PV group; **B,** Representative results of condyloma acuminatum group; **C,** Representative results of PV high-risk HPV infection group.

were bigger than normal cells, showed cat eye-like shape, concentrated nucleus and translucent halo around the nucleus. The histopathological features of PV high-risk HPV infection showed both the symptoms of PV and the appearance of koilocytes caused by HPV infection (Figure 1C).

TEM Observation Showed Different Ultrastructure in Different Groups

Condensed chromatin and cell apoptosis can be observed in the nucleus of PV group. In condyloma acuminatum group, the intercellular desmosomes were regularly connected, and autophagosomes could be observed in some cases. Suspected HPV virus particles with a density different from chromatin were observed from the samples of PV high-risk HPV infection group. In addition, the intercellular desmosomes in PV high-risk HPV infection group were regularly connected, and autophagosomes were observed in some cases. Our results showed that ultrastructure of the labia minora in three groups were different from each other (Figure 2).

Virus Typing Failed to Identify the Virus Strains in PV high-risk HPV Infection Group

The exfoliated cells were detected and typed using the human HPV nucleic acid typing kit. In each sample, the highest reading number of the strain was used as the result of typing (Table II). The results showed that HPV infection levels in PV group and PV high-risk HPV infection group were below the detectable level, so HPV genome was not identified in those two groups. The virus stain was only identified in condyloma acuminatum group.

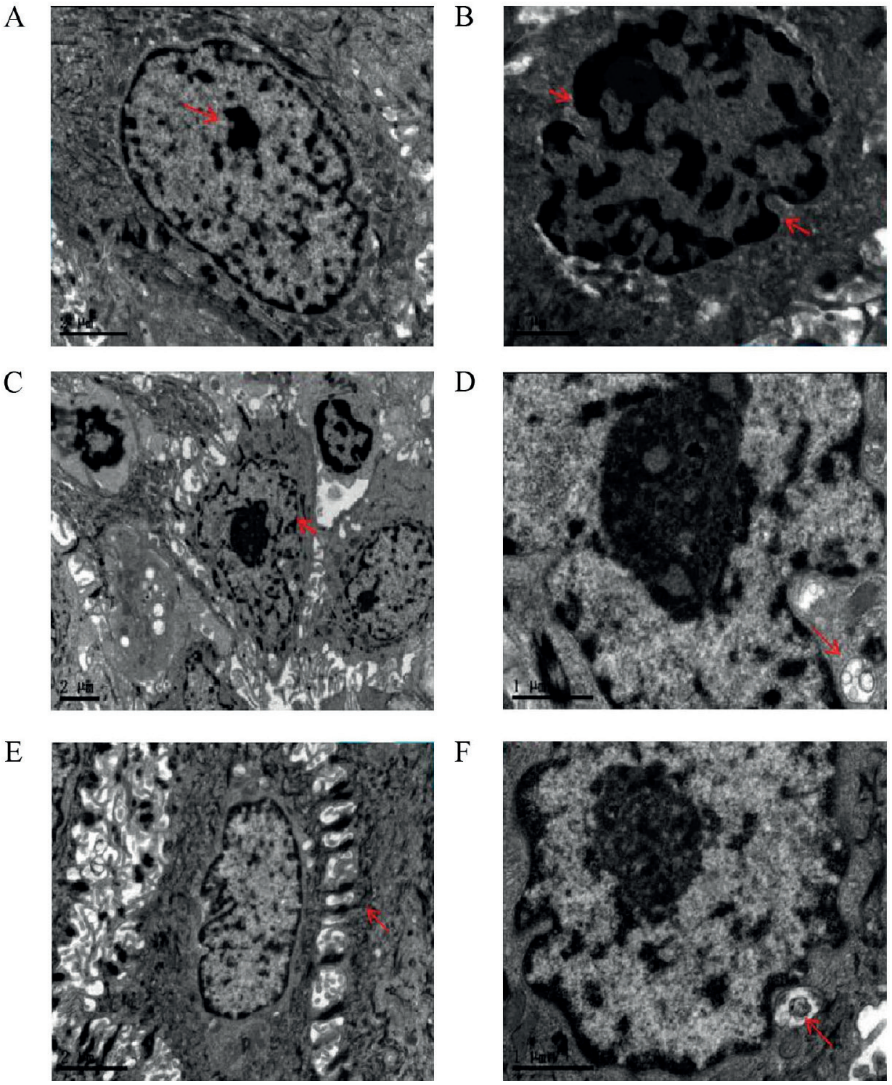

Figure 2. TEM observation of labia minora tissues from different groups. ***A-B,*** Representative results of PV group (***A,*** X 4200, ***B,*** X 8300); ***C-D,*** Representative results of condyloma acuminatum group (***C,*** X 2500, ***D,*** X 10000); ***E-F,*** Representative results of PV high-risk HPV infection group (***E,*** X 7400, ***F,*** X 10000).

Table II. The results of virus typing.

Sample	PV	Condyloma acuminatum	PV high-risk HPV infection
Type	NO	6	NO
Reads-Count		297530	
Reads-Percentage		97.26	
Ref-Name		FR751334.1	
Length		8031	
Average Depth		5195.8	
Reads-Covered-Length		8031	
Covered-percentage		100	
Depth>4X percentage		99.99	
Depth>10X percentage		99.96	
Depth>20X percentage		99.9	
Annotation		Human papillomavirus type 6 complete genome, isolate CAC11	
Capture-type		YES	

The Detection of HPV Gene Integration Site

HPV genome was only detected in condyloma acuminatum group, so the HPV gene integration site was only analyzed in condyloma acuminatum group; however, the results showed low confidence of the integration site.

Discussion

HPV infection can induce the onset and progression of a variety of cancers[7-9]. However, most of the patients with HPV infection showed no symptoms. It has been reported that 1-2% of HPV infection cases showed significant symptoms such as cancer and genital warts[12]. Therefore, it is important to detect the HPV infection and infection types of the patients with high-risk of HPV[13]. Morphological studies have shown that the symptoms induced by HPV infection were similar to the clinical manifestations of PV, leading to the misdiagnosis of HPV infection[14]. We also found that patients with PV high-risk HPV infection showed similar skin lesions of the patients with VP. So, patients with this type of HPV infection were usually misdiagnosed. The acetic acid test was widely used in distinguishing condyloma acuminatum. However, previous studies have shown that the efficiency of acetic acid test in the identification of small condyloma was unsatisfied. In our studies, all the patients with PV showed negative signals in acetic acid test, and all the patients in condyloma acuminatum group showed positive signals in the test. Weak positive signals were detected in the patients with PV high-risk HPV infection, indicating that acetic acid test may be a way to distinguish PV high-risk HPV infection from other disease.

It is generally believed that the patients with genital warts usually show peri-nuclear vacuolization. And the common vacuolization could always be observed in tissue specimens of patients with pseudocondyloma acuminatum[15]. In our work, histopathological observation showed that the patients with PV high-risk HPV infection have both the symptoms of PV and the appearance of peri-nuclear vacuolization. However, a recent study has reported that this method is insufficient to make a solid diagnosis[4]. Therefore, TEM technique was adopted to observe the ultrastructure of the lesion areas of the patients in all the three groups. We observed that the suspected HPV virus particles with a density different from chromatin were observed from the samples of the patients with PV high-risk HPV infection, which are significantly different form the ultrastructure of the samples of patients with PV. Our data suggest that TEM technique based ultrastructure observation can be a way to distinguish PV high-risk HPV infection from other diseases with similar histological features. HPVs are a group of double-stranded non-enveloped DNA viruses; to date, more than 100 subtypes of HPV have been identified[16,17]. Different subtypes of HPV have been shown to be involved in the pathogenesis of different diseases. Scholars[13,18] have shown that HBV subtypes in papillomavirus genus are mainly responsible for the onset and development of cervical anal, penile, vulvar, oral and vaginal cancers. However, the benign genital condylomas are mainly caused by the HPV subtypes in the Alpha papillomavirus genus[19]. Therefore, the identification of the subtypes of HPV infection

will definitely benefit the diagnosis of HPV infection-induced disease. Xu et al[4] has shown that 3 non-oncogenic subtypes and 10 oncogenic subtypes of HPV were involved in special type of oncogenic HPV infection. In our research, HPV type 6 was identified in patients with condyloma acuminatum, which is consistent with previous study[20]. However, HPV genome was not identified in patients with PV high-risk HPV infection, possibly due to the low copy number.

The integration of HPV genome into the host DNA can significantly promote the progression of related diseases. Bodelon et al[21] have shown that integration sites of HPV were mainly distributed in CpG sites, gene-rich regions, open chromatin regions and repetitive elements. That research indicated that HPV integration can alter the expression of host genes. However, the HPV integration sites were not identified in our study due to undetectable infection levels of specific HPV subtypes.

Conclusions

PV high-risk HPV infection showed both the symptoms of PV and HPV infection, which are different from PV. In addition, suspected HPV virus particles and autophagosomes were also observed in PV high-risk HPV infection. Our data suggested that PV high-risk HPV infection may be accompanied with HPV infection and cannot be treated as PV in clinical practice. However, the limitation of this work was the low copy number of HPV in PV high-risk HPV infection, which only provides undetectable virus levels. Therefore, further studies with higher sensitivity to identify the virus strains and virus integration sites are needed.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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