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Full Length Research Paper

Detection and typing of human papillomavirus (HPV) in condyloma acuminatum and bowenoid papulosis HybriBio HPV GenoArray test kit, real-time polymerase chain reaction (PCR) and sequencing

Juan Du*, Xiaonian Lu, Jun Liang, Yongsheng Yang, Jinran Lin, Xiaohua Zhu and Jinhua Xu

12th, Wulumuqi Middle Road, Shanghai, China, 200040, China.

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A new method using HybriBio HPV GenoArray test kit was developed for detection of human papillomavirus (HPV) in anogenital non-tumor diseases, which was compared with the real-time PCR, and sequencing for specificity and sensitivity. All initial validation studies with the control DNA proved to be type-specific. Tissue specimens were obtained from 66 patients with external genital condylomata acuminate (n = 28) and bowenoid papulosis (n = 28) and 10 negative controls. HPV 6 DNA and HPV 11 DNA were detected in 14 and 9 of 28 condylomata acuminate patients, respectively and simultaneous infection of HPV 6 and HPV 11 DNA, HPV 11 and HPV 18 DNA were detected in 3 and 1 of 28 condylomata acuminate patients. HPV 16 DNA and HPV 6 DNA were detected in 17 and 1 of 28 bowenoid papulosis patients, and the other ten bowenoid papulosis patients were detected negative. The sensitivity of HybriBio HPV GenoArray test and real-time polymerase chain reaction (PCR) were 97.8% (45/46) and 95.7% (44/46), and the specificity was 100% (20/20) and 95% (19/20). The overall agreement between HybriBio HPV GenoArray test and sequencing was 98.5% (Cohen's kappa value = 0.96), while real-time PCR and sequencing was 95.5% (Cohen's kappa value = 0.89). HPV genotyping was performed by using the newly commercially introduced HPV GenoArray test kit which makes use of both DNA amplification and HybriBio's proprietary flowthrough hybridization technique to simultaneously identify 21 HPV genotypes, with 3 common subtypes of HPV 53, HPV 66 and cp8304 in Chinese, which can potentially be a valuable tool for the detection of HPV DNA.

Key words: Human papillomavirus, condylomata acuminate, bowenoid papulosis.

INTRODUCTION

Human papillomavirus (HPV) is a small DNA virus belonging to the family Papovaviridae which infect squamous cell mostly. More than 100 types of HPV have been identified (Syrjanen et al., 2003). The HPV infection of the vulva and anogenital region is reflected in a spectrum of histological changes. Condylomatous verrucous lesions, smaller papular or plaque-like changes with vulvar intraepithelial neoplasms (VIN) histological features, as well as infiltrative malignant neoplasia with certain histological properties, could be induced by different HPV types. A typical morphologically well-differentiated change is condyloma acuminatum, with the pathological finding of acanthosis, hyperkeratosis, parakeratosis, dyskeratosis and koilocytosis (Boon et al., 2005).

Bowenoid papulosis, the lesions which consists of multiple red or brown papules in the genitoanal region, histologically, show alterations of the epidermis similar to those in Bowen's disease. The course of the disease is benign; however, malignant transformation has been documented (Palefsky and Joel, 2007). Female sexual partners of men with this disease may be at increased risk of cervical cancer (Gesierich et al., 2008). The aetiopathogenesis of the disorder is not well defined but it

*Corresponding author. E-mail: Dujuan08_hs@yahoo.com.

may be linked to HPV infection. Therefore, clinical diagnosis may not be sufficient and histopathological diagnosis and virological tests are necessary. Moreover, since high-risk types of HPV associated with cervical cancer and Bowenoid papulosis have been detected, identification of genotypes is preferable. The purpose of this study was to confirm diagnosis and to detect the type of HPV associated with vulvar condyloma acuminate and Bowenoid papulosis by three methods of HybriBio HPV GenoArray test, real-time polymerase chain reaction (PCR) and sequencing.

MATERIALS AND METHODS

Clinical samples

Fifty-six patients (31 males and 25 females; mean age, 34 [range, 18 to 75 years]) with external genital polypoid lesions entered the study at Huashan Hospital outpatient clinic between 2008 and 2010. The study was conducted in accordance with the guidelines of the Ethical Review Board of the Fudan University School of Medicine. For each patient, biopsy specimens measuring 3 mm in diameter were taken from two lesions. The specimens were used both for routine histopathological diagnosis and for HPV detection and genotyping.

Diagnostic criteria

The people whose anogenital pathologic diagnosis is condylomata acuminate or Bowenoid papulosis could enter the study.

Exclusion criteria

The patients who have any kinds of treatments or any complications could not enter the study and specimens biopsied from cervix would be excluded.

DNA extraction

DNA was extracted from the specimen using QIAamp DNA Kit (Qiagen, Chatsworth, CA). After extraction, DNA was eluted in 200 µl distilled water and was stored at -20°C.

HybriBio HPV genoarray

HPV genotyping was performed by using the HPV GenoArray test kit (Hybribio Limited, Hong Kong), which makes use of both DNA amplification and HybriBio's proprietary flowthrough hybridization technique to simultaneously identify 21 HPV genotypes genotypes, including 5 low-risk types (types 6, 11, 42, 43, and 44), 14 high-risk types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and 2 intermediate-risk types (types CP8304 and 53) (Grisaru D et al., 2008). The test employs a macroarray format with a nylon membrane onto which HPV genotype-specific oligonucleotides probes have been immobilized.

DNA extraction

DNA was extracted from the specimen using QIAamp DNA Kit (Qiagen, Chatsworth, CA). After extraction, DNA was eluted in 200 µl distilled water and was stored at -20°C.

Real-time polymerase chain reaction (PCR)

Type-specific real-time PCR was used to measure the quantity of the DNAs of HPV 6, 11, 16, and 18 in each sample. The sequences of primers and probes for E6/E7 region used have been listed below (Table 1). PCR reactions were carried out using the TaqMan PCR Kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's directions. Standard curves measuring HPV 6, 11, 16, and 18 DNA concentrations were constructed using cycle threshold (CT) values obtained from serially diluted plasmids, pBR322, respectively, which contain the target DNA sequences. The CT value from each sample was plotted on a standard curve, allowing automatic calculation of the copy number using Sequence Detector v1.6 software (PE Applied Biosystems). Each sample was tested in duplicate; the copy number of each sample is represented as the mean of the two values.

Direct HPV DNA sequencing

All samples were amplified with E6/E7 primers and purified with QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. After purification, 2 µl of each amplicon was tested by electrophoresis on 2% agarose gel for the evaluation of the quality and the quantity of amplified DNA. The purified amplicons were sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). The reaction products were analyzed with an ABI Prism 310 Genetic Analyzer (Applied Biosystems) and the resulting sequences were compared with HPV sequences of known types using the basic local alignment search tool from the NCBI website (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Statistical analysis

The degree of agreement between HybriBio HPV GenoArray test, real-time PCR and sequencing in detecting HPV infection was calculated by Cohen's kappa values. Kappa values of less than 0, 0 to 0.2, 0.21 to 0.4, 0.41 to 0.6, 0.61 to 0.8, 0.81 to 0.99 and 1.0 designate no, slight, fair, moderate, substantial, almost perfect and perfect agreements, respectively.

RESULTS

The results detected by HybriBio HPV GenoArray test

HPV 6 and 11 DNA were detected in 14(50%) and 9(32.1%) of 28 condylomata acuminata, respectively, and simultaneous infection of HPV 6/11 DNA, HPV 11/18 DNA were detected in 3(10.7%) and 1(3.6%) of 28 condylomata acuminate, 1 (3.6%) detected negative. HPV 6 DNA and HPV 16 DNA were detected in 1 (3.5%) and 17 (60.7%) of 28 Bowenoid papulosis. 10 (35.7%) Bowenoid papulosis were detected negative. The controls were detected negative (Table 2 and Figure 1).

The results detected by real-time PCR

HPV 6/11 DNA were detected in 26 (92.9%) of 28 condylomata acuminata, respectively, and 2 (7.1%) specimens were detected negative. HPV 16 DNA, HPV 6/11 DNA and HPV 18 DNA were detected in 17 (60.7%),

Table 1. Primers and probes used for real-time PCR detection of HPV types.

HPV type	Primer and probe sequences	Target gene
6	F: 5'-CGGTTGATAAAGCTAAATTGTACGT-3' R: 5'-AGGGTAACATGTCTTCCATGCA-3' P: 5'-AAGGGTCGCTGCCTACACTGCTGG-3'	E6
11	F: 5'-GCTTCATAAAACTAAATAACCAGTGGAA-3' R: 5'-GTCAGGAGGCTGCAGGTCTAGTA-3' P: 5'-CTATATCCTTTAGGGTAACAAGTCTTCCATGCATGTTG-3'	E6
16	F: 5'-TTGCAGATCATCAAGAACACGTAGA-3' R: 5'-CAGTAGAGATCAGTTGTCTCTGGTTGC-3' P: 5'-AATCATGCATGGAGATACACCTACATTGCATGA-3'	E7
18	F: 5'-AGAGGCCAGTGCCATTCGT-3' R: 5'-GTTTCTCTGCGTCGTTGGAGT-3' P: 5'-TCCTGTCGTGCTCGGTTGCAGC-3'	E6
Control	F: 5'-CTGGCACCCAGCACAATG-3' R: 5'-GCCGATCCACACGGAGTACG-3' P: 5'-ATCAAGATCATTGCTCCTCCTGAGCGC-3'	beta-actin

F, forward primer; R, reverse primer; P, probe.

Table 2. The results detected by HybriBio HPV GenoArray test.

Specimen	HPV 6	HPV 11	HPV 16	HPV 18	HPV 6, 11	HPV 11, 18	Negative
CA	14	9	0	0	3	1	1
BP	1	0	17	0	0	0	10
Controls	0	0	0	0	0	0	10

Table 3. The results detected by real-time PCR.

Specimens	HPV 6/11	HPV 16	HPV 18	Negative
CA	26	0	0	2
BP	1	17	1	9
Controls	0	0	0	10

1 (3.6%) and 1 (3.6%) of 28 Bowenoid papulosis, respectively. 9 (32.1%) Bowenoid papulosis were detected negative. The controls were all detected negative (Table 3 and Figure 2).

The results detected by sequencing

All specimens were detected by sequencing as the gold standard, with the same results by HybriBio HPV GenoArray test and real-time PCR, except for the five, in which one had pathologic diagnosis to be bowenoid papulosis and the other four were condyloma acuminatum (Table 4).

Sensitivity and specificity and the overall agreement between sequencing and HybriBio HPV GenoArray test or real-time PCR

The sensitivity of HybriBio HPV GenoArray test and real-time PCR were 97.8% (45/46) and 95.7% (44/46), and the specificity was 100% (20/20) and 95% (19/20), regarding sequencing as golden standard. The overall agreement between HybriBio HPV GenoArray test and sequencing was 98.5% (Cohen's kappa value = 0.96), while the agreement between real-time PCR and sequencing was 95.5% (Cohen's kappa value = 0.89).

DISCUSSION

HPV pertain to the Papillomaviridae family, a highly diverse group of viruses that infect the skin and mucosal epithelia of several vertebrate species. Forty HPV types

Table 4. The five cases with the different results detected by the three methods.

Pathologic diagnosis	HybriBio	Real-time PCR	Sequencing
BP	Negative	HPV18	Negative
CA	HPV6	Negative	HPV6
CA	HPV11	Negative	HPV11
CA	Negative	HPV6/11	HPV6
CA	HPV11, HPV18	HPV6/11	HPV11, HPV18

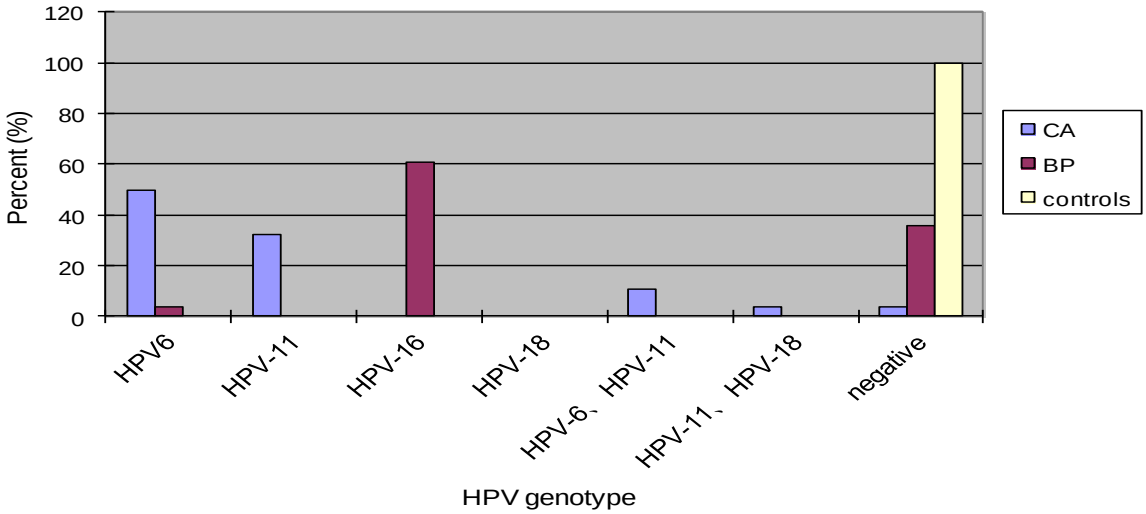

Figure 1. The percents of each HPV genotype detected by HybriBio HPV GenoArray test in the specimens of CA and BP.

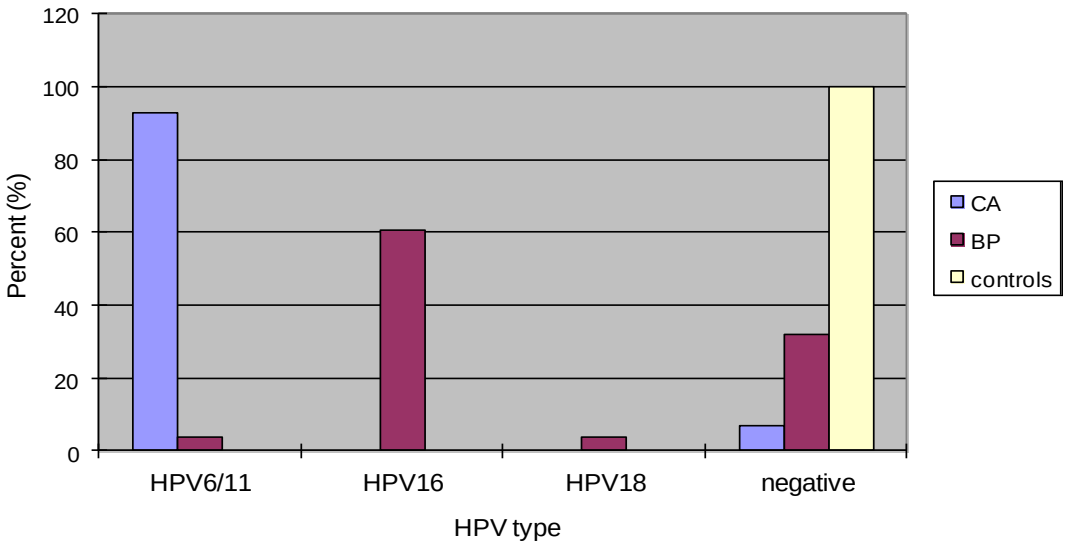

Figure 2. The percents of each HPV genotype detected by real-time PCR in the specimens of CA and BP

belonging to the a-papillomavirus genus have been detected infecting the epithelium and mucosa lining the anogenital tract (Boccardo et al., 2010). HPV types have been further classified based on their oncogenic potential as low- or high-risk HPV types. Low-risk HPV types (that is, HPV 6 and HPV 11) cause common genital warts,

such as condyloma acuminatum, benign hyperproliferative lesions with limited tendency to malignant progression. In our test, 92.8% (26/28) condylomata acuminate were detected HPV 6 or 11 or both, and 3.6% (1/28) was detected HPV 11/18 DNA simultaneous infection by both HybriBio HPV GenoArray Test and sequencing.

Several HPV genotypes are detected in the infection of Bowenoid papulosis, including HPV 16, 18, 31, 35, 39, 42, 48 (Gross and Pfister, 2004), especially HPV 16 infection, which is strongly associated with the external genital squamous cell carcinoma *in situ* such as Bowen's disease and Queyrat erythroplasia (Naoto et al., 2006). The results that 60.7% Bowenoid papulosis infected by HPV 16 detected by the means of both HybriBio HPV GenoArray test and real-time PCR consist in the published data (Naoto et al., 2006), while 3.5% (1/28) was detected HPV 6 corresponding to the spontaneous remission of BP.

HPV detection is important to identify patients who may be at high risk of developing squamous cell carcinoma, though it is clinically difficult to determine whether it is necessary to treat HPV associated anogenital tumors. Virological testing includes *in situ* hybridization (ISH), Southern blot hybridization method, dot blot hybridization, PCR, and real-time PCR. However, detection power using ISH is low. In addition, PCR and real-time PCR need specific expensive equipment such as a thermal cycler, and these methods have not yet become common procedures in hospital laboratories (Masanori et al., 2007). Now we used the HybriBio GenoArray test for HPV genotyping, this assay is a commercial kit which was recently introduced into the market. Although it has already been used for some time in China, some literatures are available to support its value in the high-risk HPV detection (Wang et al., 2012; Liu et al., 2010; Dan et al., 2008). There is little study about its use in condyloma acuminatum and Bowenoid papulosis. Nevertheless, the results of this study show a high level (98.5%) of agreement between the results of that assay and those of sequencing, while the agreement between real-time PCR and sequencing is 95.5%, but the two Cohen’s kappas show “almost perfect” agreement between them. This high level of concordance is encouraging, since regarding sequencing as the golden standards and real-time PCR had gained a good reputation over the past few years as a sensitive, specific, and reliable test for the detection of infections with high risk and low risk (Guo et al., 2002) HPV types.

Conclusion

This study indicates that the HybriBio HPV GenoArray test is shown as reliable for clinical performance as does the real-time PCR test. The HybriBio HPV GenoArray method is superior in terms of sensitivity, specificity, speed, and simplicity, and can be a valuable tool for the detection of HPV DNA in laboratories.

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