

Polymerase Chain Reaction Study of Anogenital Condylomas for High Risk HPV Types

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Abstract

Background: Anogenital wart is a common sexually transmitted disease affecting both genders. The aim of this study was to evaluate human papilloma virus types of anogenital warts with Polymerase Chain Reaction (PCR).

Method: 65 formalin fixed paraffin embedded (FFPE) tissue blocks of the anogenital warts obtained from the pathology lab of Loghman Hakim hospital were included in this study.

Results: In 27 samples (41.5%), human papilloma virus was detected by PCR. In addition, low risk HPV types (6, 11) were identified in all samples without any positivity for high risk HPV types (16, 18, 31 and 33).

Conclusion: By finding low risk HPV types in all samples, it could be concluded that Anogenital warts seems not to be precursors of anogenital malignancies. (*Iran J Dermatol 2009;12:52-55*)

Keywords: anogenital condyloma, human papilloma virus, polymerase chain reaction

Introduction

Papilloma viruses are small DNA viruses that infect human epithelial tissues, causing cell proliferation. The most common manifestation of human papilloma virus infection is the development of warts¹. Anogenital warts (condyloma acuminatum) are common and often asymptomatic with an estimated 1.3 million new cases per year in the USA². This disease is seen in 1% of sexually active adults³. More than 100 type of HPV have been identified but they fall into two main groups: oncogenic and non-oncogenic. High risk types cause cervical cancer and cervical precancerous lesions

(CINII/III). Of high risk HPV types, type 16 and 18 are the most common types associated with cervical cancer in women¹. High risk HPV types have been implicated in vaginal, vulvar and anal cancers⁴. Of low risk HPV types, 6 and 11 are the most common types associated with condyloma acuminatum⁵. They are unlikely to progress to precancerous state or cancer. However, a patient with anogenital warts caused by low risk HPV types can also be coinfecting with high risk HPV genotypes, especially in immunocompromised cases⁶. CIN is often associated with a personal history of genital wart and with previous penile wart in the partner^{7,8}.

Our aim was to evaluate the frequency of HPV positivity of FFPE tissue blocks and also to determine HPV types 6,11,16,18,18,31 and 33 of anogenital warts.

Patients and Methods

Specimens

The study was conducted according to the principles of the declaration of Helsinki and was approved by the medical ethics review board of the Skin Research Center of Shahid Beheshti University of Medical Sciences. A written consent was obtained from all patients.

Sixty five formalin fixed paraffin embedded (FFPE) tissue blocks diagnosed with condyloma by H&E staining (received from pathology laboratory of Loghman Hakim hospital) were used in this study. Data was collected from patients' records. All tissues excised from the blocks (even small pieces of tissues or 10 to 20 μm thick sections) were submitted to the molecular diagnosis laboratory for nucleic acid extraction and purification, HPV PCR and typing.

Nucleic acid extraction and purification

BioRobot EZ1 workstation and EZ1 DNA tissue kit were used for DNA extraction and purification. Magnetic-particle technology was used.

Primarily, a small (about 25 mg) piece of paraffin embedded tissue with minimum peripheral wax contamination was lysed in lysis buffer of the kit and treated with Proteinase K in an Eppendorff thermomixer overnight according to kit instruction for paraffin embedded tissue. Two hundred μL of the homogenized sample was automatically purified using EZ1 DNA tissue kit (Qiagen, Hilden) on Qiagen BioRobot EZ1 workstation. As the starting samples were paraffin embedded tissues, EZ1 DNA paraffin section card had to be used on the machine.

HPV PCR

Conventional PCR for HPV was carried out on each DNA template. MY09 (5'-CGT CCM ARR GGA WAC TGA TC-3') and MY11 (5'-GCM CAG GGW CAT AAY AAT GG-3') primers (TIB MOLBIOL, Berlin), designed to amplify a 450 bp conserved L1 region of HPV genome, were used for PCR to amplify and detect HPV. This pair of degenerate primers amplifies at least 25 different types of anogenital HPV. The final concentration of HPV PCR master mix components for a 25 μL reaction were 2.5 mM MgCl_2 , 0.2 mM each dNTP (Qiagen, Hilden), 0.5 μM each primer, 1.25 U Taq

DNA Polymerase (Qiagen, Hilden), 1X PCR buffer (10X). Five μL of extracted and purified DNA was added to each PCR reaction as template. The thermocycling profile was holding at 95°C for 5 minutes, as pre-denaturation step, then 35 cycles at 94°C to denature for 1 minute, ramp 10 seconds to 55°C to anneal for 1 minute, ramp 10 seconds to 72°C to extend for 1 min., ramp 10 seconds back to 94°C. The amplification cycles were followed by an extra-extension segment at 72°C for 5 minutes^{9,10}. For run validation and quality control, positive and contamination (H_2O) controls were included in each HPV PCR run. To prevent false positive results, standard precautions such as working in distinct pre- and post-PCR sites, working in PCR workstation, using disposable blades to cut tissues from the paraffin blocks and changing it from one block to another, frequent use of UVC lamp and surface decontaminants, recruiting filter tips, waste management and safe handling of PCR products were strictly followed. According to in run control results, no carry over or failure of the test system was observed.

Globin PCR

To investigate false negative results due to the presence of PCR inhibitors, an independent PCR reaction for β -globin was done for each extracted DNA template. PC04 (5'-GAA GAG CCA AGG ACA GGT AC-3') and GH20 (5'-CAA CTT CAT CCA CGT TCA CC-3') primers were used to amplify a 286 bp region of β -globin gene. The PCR master mix and thermocycling profile, as well as standard precautions, were the same as HPV PCR¹⁰.

Agarose Gel Electrophoresis of PCR products

Post-amplification agarose gel electrophoresis detection was carried out by applying 10 μL of each PCR product in a 1.5 agarose gel. Ethidium bromide stained gels were examined on UV transilluminator and photographed for documentation. The DNA templates that amplified β -globin (286 bp PCR product) but had no HPV PCR product were considered HPV negative. Templates positive for HPV PCR specific product (approx. 450 bp products) were considered HPV positive, whether or not β -globin was amplified. Templates failing to amplify any PCR product could not be interpreted due to the possible presence of PCR inhibitors. Such samples may require further optimization (e.g. dilution to decrease the inhibitor concentration) or purification.

HPV Typing

All HPV positive templates were subject to HPV genotyping by HPV type 3.5 LCD Array kit (Chipron GmbH, Berlin). The pre-labeled PCR primer mixes were provided with the kit generated labeled fragments of the viral DNA. The non-fluorescent labeled PCR fragments were combined with the provided hybridization buffer and hybridized to the individual array fields of one chip. During hybridization, the labeled PCR fragments bound to the specific capture probes and were immobilized as spots on the bottom of each field. Following a short washing procedure, each field was incubated with a secondary label solution (enzyme-conjugate). After a second washing step, those positions (spots) where PCR fragments and secondary labels were bound could be visualized by a blue precipitate formed by the enzyme substrate provided as "BLUE stain". The data read-out could either be done by simple optical examination, using the pattern matrix provided by the kit or, alternatively, by the scanner.

Two primer sets for PCR amplification were provided by the kit, one based on the published and commonly used MY09/MY11 system and a second pair, producing a shorter PCR product of 125 bp in length. According to the instruction of the kit manufacturer, two independent PCR reactions were carried out on each HPV positive template; then, to follow the post-PCR hybridization steps on polymer LCD-chip, 5 μ L of each PCR product was mixed together.

HPV kit 3.5 LCD Array is capable of detecting 32 HPV types including 06, 11, 16, 18, 31, 33, 35, 39, 42, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68, 70, 72, 73, 81, 82, 83, 84, 90 and 91.

Results

In this study, 65 tissue blocks of anogenital wart from 32 males and 33 females were investigated for HPV typing. Mean age of the patients was 31.3 ± 7.2 years (min. 19 and max. 53). Mean duration of the disease was 9.7 ± 7 months (min. 1 and max. 48 months). There were similar lesions in their sexual partners in 20 patients. Previous treatments (e.g. cryotherapy, cautery, topical podophyllin) were mentioned in 15 cases. Warts were present in the genital area of 44 cases and in the perianal area of 6 cases. Simultaneous involvement of genital and perianal was observed in 15 cases. Extent of involvement was mild (less than 10 lesions), moderate (10 to 20 lesions) and severe (more than 20 lesions) in 36, 15 and 14 cases, respectively. Condyloma acuminatum was

noted in 42 cases and flat condyloma in 23 cases. None of patients were immunosuppressed. In all cases, clinical diagnosis was confirmed by pathological finding. PCR findings in 27 out of 65 specimens (41.5%) were positive for human papilloma virus. HPV type 6 was present in 21 specimens (77.8%). HPV type 11 in 5 specimens (18.5%), and HPV types 6 and 11 simultaneously in 1 specimen (3.7%). None of the samples were positive for high risk HPV genotypes (e.g. 16, 18, 31 and 33). Among 65 FFPE specimens, 38 (58.5%) samples were positive for inhibitory effects at first PCR for both HPV and β -globin (neither HPV nor β -globin PCR products were seen on agarose gel electrophoresis). Nineteen out of 28 DNA templates, optimized by 1:5 to 1:20 dilution of DNA templates and repeating the PCR for HPV as well as β -globin after DNA template dilution, were positive for either HPV or β -globin, or both. This observation is suggestive of a high degree of inhibitory effects on FFPE specimens.

Discussion

In this research, 41.5% of samples were positive for HPV. In two other studies performed by Syrjanen et al. and Handly, the rate of HPV positivity were 44.45 and 53.3%, respectively 12,13. Unsuccessful HPV and β -globin PCR observed in a number of samples in this study is believed to be due to several reasons such as DNA damage or degradation due to delay in tissue fixation, type and quality of fixative, duration of fixation, or tissue block storage condition (temperature and duration). Formalin is known to be a cause of DNA fragmentation. The samples showing strongest inhibitory effects, particularly the templates resistant to optimization by dilution, were generally older than 36 months. Several researches have been done ^{9,10,11} to evaluate the effect of fixation methods on PCR detection of viral or bacterial DNA. For successful and efficient detection of HPV, as well as HPV typing in tissue biopsy, fresh or frozen tissues or tissue biopsy specimens fixed in a proper and PCR compatible fixative is preferred versus formalin fixed or FFPE tissues.

In our study, HPV type 6 was found in 21 samples and HPV type 11 in 5 samples; these are low risk viruses for malignancy. However, high malignancy risk types 16,18,31 and 33 were not identified in any samples. Similarly, in previous studies, low risk viruses have been reported to be much more frequent in comparison with high risk types in most anogenital wart samples ¹⁴. In a study by Skerlev et al ¹⁵, types 6 and 11 were identified

in 79% of the samples and type 16 and 18 in 21%. In another study by Grce, 80% of the identified viruses were of low risk type, HPV type 16 was detected in 4.6% of the samples, and type 18 in 1.7%¹⁶. In contrast, in a previously published study, type 6 and 11 were detected in 41.6% of the samples and types 16 and 18 in 58.3%³. Findings of this study are in obvious contradiction with other researches, probably because of the method used in this study.

In our study, similar to other studies, age distribution of patients was correlated with age of maximum sexual activity. In 20 patients, there were similar lesions in sexual partners, indicating the high rate of infection through sexual contact. Also, as in previous studies, the most commonly affected area was the genital area and most common clinical form was condiloma acuminatum^{12,15}.

The findings of our study suggest that anogenital warts might not associated with an increased risk for the development of the subsequent anogenital malignancies, but further study with larger sample size would be necessary to confirm these findings.

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