大韓바이러스學會誌 : 第 29 卷 第 1 號 1999
J. Korean Soc. Virology, Vol. 29, No. 1, 1999

PCR Approach for Detection and Typing of Epidermodysplasia Verruciformis-associated Human Papillomavirus Types

Yong-Jae Shin, Hyun-Chul Choi[1], Chan-Gil Kim and Kwang-Ho Lee*

Department of Molecular Biology, College of Natural Science, Department of Dermatology[1], College of Medicine, Konkuk University, Chung Ju 380-701, Korea

=Abstract=

Warts, or verrucae, are benign epithelial proliferations of the skin and mucosa caused by infection with human papillomaviruses (HPV). It is now recognized that there are many different HPV types. Especially type3 is most frequently observed in flat wart. Other types, such as type2, 10, 14, 27, 28, 29, 38, and 41 are rarely encounted in flat wart.

We describe here a simple and economic method for detection and identification of epidermodysplasia verruciformis-associated HPV. The method is based on polymerase chain reaction (PCR) amplification and restriction analysis. The method has been developed with cloned HPV DNA and DNA from clinical samples. Clinical samples are from either frozen tissue or paraffin-embedded tissue. Genomic fragments were obtained from two different HPV types (3 and 10). The amplification fragments were identified by a form of miniature fingerprinting, with a set of restriction enzymes that gave a unique digestion pattern for each HPV type. We have tested 74 clinical samples. Only type3 among these clinical samples is detected, and one sample is involved in neither type3 nor type10.

Key Words: Wart, PCR, HPV type3, HPV type10

INTRODUCTION

Flat wart is known to be caused by the infection of HPV. These viruses do not produce acute signs or symptoms but induce slow-growing lesions that can remain subclinical for long periods of time. A subset of the human papillomaviruses has been associated with the development of epithelial malignancies.

Numerous HPVs have been found associated with benign epidermodysplasia verruciformis (EV) lesions. Some of these viruses, such as HPV3, HPV10, and related viruses, are also found associated with flat warts in the general population [1,3,4,5] whereas other viruses so far have been found only in lesions of EV patients [2,6,7,8,9].

The papilloma virus (PV) genome is present within the viral particle as a covalently closed, supercoiled circle of double-stranded DNA. The genome is composed of about 8000 nucleotide base pairs. The various HPV types are currently discriminated according to the relatedness of their DNA, using molecularly cloned HPV DNAs of known type as standards.

HPVs do not infect laboratory animals, rodent models for laboratory investigation are

접수 : 1999년 3월 2일

*Corresponding Author: Kwang-Ho Lee, Department of Molecular Biology, Konkuk University, Chung Ju 380-701, KOREA. Phone: 0441) 840-3613, Fax: 0441) 851-5235, e-mail: kwangho@kcucc.cj.konkuk.ac.kr 소속기관명; Kon Kuk University

not available, and it is not yet possible to propagate the PVs in tissue culture. These obstacles stand in the way of determining the mechanisms and biologic consequences of PV infection. However, recent applications of recombinant DNA technology have partially circumvented these problems and have provided new insights into the molecular mechanisms of PV pathogenesis. However, the study on the flat wart in our country so far is not carried out. Particulary the frequency of EV patients in Chung Ju area is much higher than that of other areas and also higher than average frequency in the world. Therefore, the study on the regional relationship to the EV might be very attractive.

We therefore have developed an approach for the PCR amplification and subsequent typing of verruciformis-associated HPVs. The approach relies on the fact that although the EV HPV types are each unique, they share interspersed regions of considerable DNA sequence homology. Taking advantage of these homologous regions, we have designed consensus oligonucleotide primer pairs that should aneal to both type3 and type10 which are most extensively found in flat wart lesions. Furthermore, the high conservation of the selected primer regions suggests that other known or as yet unidenfied EV HPVs would also serve as efficient templates for these primers. The amplification products can be distinguished to be type3 or type10 after suitable restriction enzyme digestion. Here we present the detection and typing of HPVs in clinical specimens using amplification with consensus primers and subsequent typing with restriction enzymes.

MATERIALS AND METHODS

Preparation of clinical samples

Seventy-four tissue specimens of common warts located on hands, feet and face were obtained from patients who were attending the Department of Dermatology of the Konkuk University. After surgical removal, part of each specimen was stored frozen at -70℃ or was fixed in formalin and embedded in paraffin.

DNA-extraction from frozen tissue

The frozen tissue specimens were crushed and homogenized in 720 μl of SET solution (150 mM NaCl, 50 mM Tris-Cl [pH 8.0], 10 mM EDTA [pH 8.0]) and 80 μl of 0.5 M EDTA. Proteins were removed by incubation in 20 μl of 10% SDS, 40 μl of 5 M $NaClO_4$, 400 μl of phenol and 400 μl of chloroform for 2 hrs at room temperature. After a second phenol-chloroform extraction and ethanol precipitation, the DNA was resuspended in water. About 50ng of DNA was used for each PCR reaction.

DNA extraction from paraffin-embedded tissue

Section of various biopsy samples were sliced and placed in 1.5 ml microfuge tubes. 500 μl of DEXPAT solution (Takara Co) was added and heated at 100℃ for 10 min prior to centrifugation at 4℃ for 10 min. The supernatant was used for PCR. Approximately 1/100 diluted DNA from extracted tissue DNA was used for each PCR reaction.

PCR amplification

In order to detect a wide range of different HPV types, we designed the general primer pair by sequence comparison of HPV3 and HPV10. The plasmid DNA of HPV type3 and type10 were kindly provided by Dr. Gerard Orth at Pasteur Institute in France, and used for positive control.

The polymerase chain reaction was performed in a total volume of 50 μl with primers having a concentration of 20 pM for DNA extracted from both frozen tissue and paraffin-embedded tissue, 2.5 U Ex Taq DNA polymerase (Takara Co), and the following buffer: 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-HCl

pH 8.3 and 200 μM of each dNTP. At first, a denaturation step at 94℃ for 3 min was performed. Amplification was carried out through 30 cycles with each cycle running 1 min. Each cycle was consisted of denaturation temperature at 94℃, annealing temperature at 54℃ for paraffin-embedded tissue and at 58℃ for frozen tissue, and extension at 72℃. The program terminated with an extension step of 5 min.

Typing of PCR products

After amplification, a 20 μl sample was digested with several restriction enzymes; *AccI, AluI, ScaI, StuI* and *StyI*. The location of restriction enzyme site in PCR products from both HPV type3 and 10 are shown in Figure 1. The predicted sizes of the digested fragments are shown in Table 1. Enzyme digested PCR products were separated on 1% agarose gels and stained with ethidium bromide.

Table 1. Sizes of expected fragments after restriction enzyme digestion of PCR products. Expected sizes are deduced from the complete nucleotide sequence of the HPV3 and HPV10. The fragments without enzyme sites are shown with asterisks

Enzyme	Fragment size (bp)	
	HPV3	HPV10
AccI	548*	251, 294
AluI	11, 83, 134, 320	11, 534
ScaI	61, 168, 319	545*
StuI	548*	204, 341
StyI	195, 353	186, 359

RESULTS

Design of a nested PCR to detect type3 and type10

PCR primers in this experiment are consensus primer set, which is intended for the detection of both type3 and type10 those are most frequently found in EV patients. We have designed primers to detect and easily identify two types of HPV after restriction enzyme digestion of the PCR products. The length of type3 and type10 are 7,820 bp and 7,919 bp, respectively. The location of primers of both type3 and type10 are shown in Table 2. Although 2 nucleotide sequences in 5' consensus primer are unmatched between type3 and type 10, the nucleotide sequences for 3' primer are completely matched between two types (Table 2). The predicted length of PCR product with this primer set are 548 bp with type3 and 545 bp with type10.

The DNA concentration for PCR reaction (primer and template)

We have checked the optimal DNA concentration for PCR reaction (Figure 2). Primer set was tested for on a pannel of HPV type3 and the optimum concentration was found to be either 20 pM or 50 pM per reaction mixture (50 μl) (data not shown). Assay sensitivity for template was of the order of diluted concentration for DNA extracted from both frozen tissue and paraffin-embedded tissue (Figure 2).

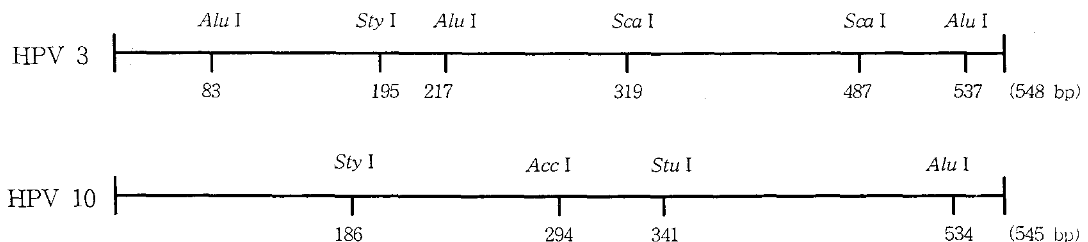

Figure 1. Restriction site map of the PCR product. The length of PCR products are 548 bp for type3 and 545 bp for type10, respectively. The correst position of every enzyme from 5' terminus are shown under enzyme site.

Table 2. Primer sequence and its location in HPV3 and HPV10. Multiple sequence alignment of primers with HPV3 and HPV10 and their location in each HPV are shown. Only differences are specified; periods indicate no change with respect to the sequence above

Primer & HPV	Sequence (5'-3')	Location
Primer (PV 5)	ATGGCCGCTGAGCCCTATGGCGACAGCATG	
HPV 3	....	6455-6485
HPV 10	........G.....T...............	6527-6560
Primer (PV 3)	GTGTCCTCCAAGCTAGTGGACGGTGGC	
HPV 3	....	7003-6976
HPV 10	....	7072-7045

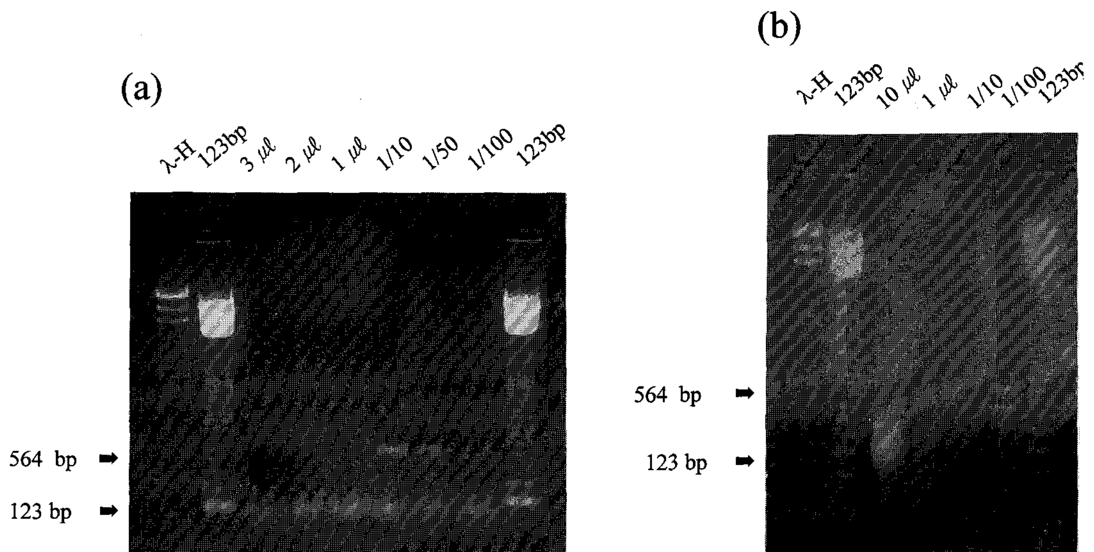

Figure 2. Optimum concentrations of template DNA. DNAs from frozen tissues **(a)** and paraffin-embedded tissues **(b)** were diluted in linear order and used for PCR reaction. Each amplification reaction was analyzed by gel electrophoresis of 1% agarose. Size markers were used: λ-H; λ-HindIII, 123 bp; 123 bp ladders. Diluted samples were used at 1 µl for PCR reaction.

Amplification efficiency for template was most effective at 1/10 diluted DNA from frozen specimen (Figure 2 (a)), and 1/100 diluted DNA from paraffin-embedded tissue (Figure 2 (b)). The reason why less amount of DNA from paraffin-embedded tissue are more effective in PCR reaction might be due to the inhibition effect of paraffin in PCR reaction mixture.

Detection and typing of HPV from samples

The degenerate consensus primers used in this study were able to direct the amplification of DNA from both HPV type3 and type10, giving a size of approximately 548 bp length from type3 and 545 bp from type10. Plasmid DNAs of both type3 and type10 (Figure 3 (a)), and DNA from frozen specimens (Figure 3 (b)) were used for template DNA for the test reaction. The specificity of the detected products was tested by restriction enzymes (Figure 3). Restriction enzymes gave fragments of the expected size, demonstrating that the amplified DNA was specific for the different HPVs. Type3 was cut at position 319 bp and 487 bp

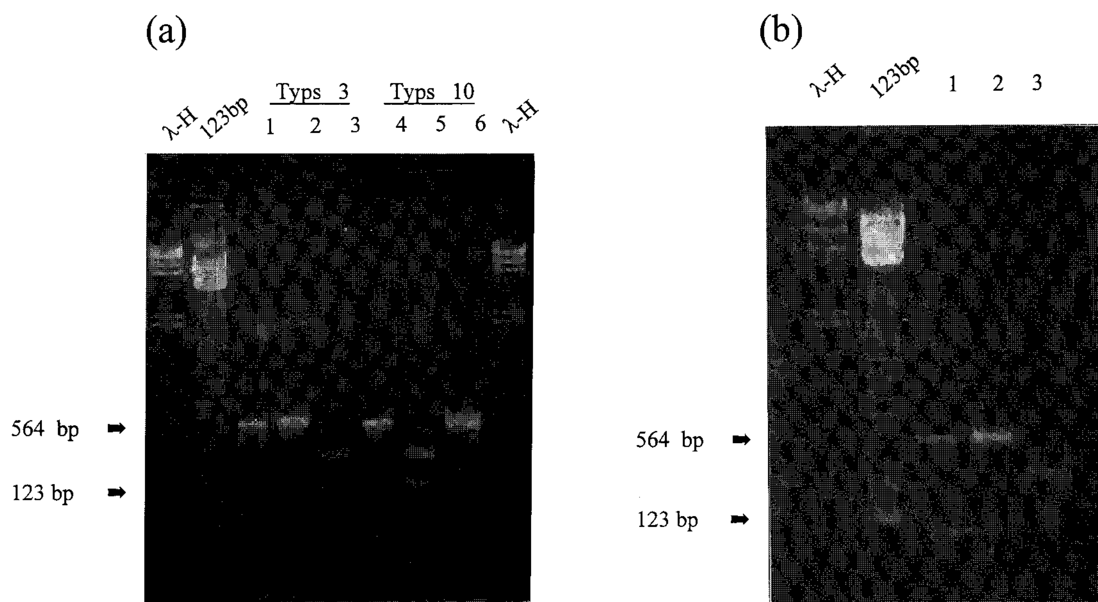

Figure 3. Electrophoretic analysis of restriction enzyme digests of PCR products. Plasmid DNA of HPV3 and HPV10 **(a)** and DNAs extracted from frozen tissues **(b)** were use for PCR amplification. PCR products were digested with restriction enzymes and analyzed by 1% agarose gel electrophoresis. Used enzymes were *StuI* (lane 1) and *ScaI* (lane 2). Uncleaved controls were also loaded (lane 1). Size markers were applied: λ-H; λ-HindIII, 123 bp; 123 bp ladders.

with *ScaI*, giving three bands of 61 bp, 168 bp and 319 bp (Figure 3 (a)). 61 bp fragment was hardly detected. For type10, the pattern is opposite to type3 (Figure 3 (a)). As shown in Figure 3 (a), the PCR fragments was cut at position 341 bp with *StuI*, giving two bands of 204 bp and 341 bp, which are shown cleary in lane 5. Further analysis with *StyI, AccI* and *AluI* gave fragments of the expected sizes (data not shown). However, these enzymes are more or less expensive than *StuI* and *ScaI*. Further more, we can easily distinguish between type3 and 10 by using *StuI* and *ScaI*. Thus we have used these two enzymes to typing viruses with EV specimens in this experiment. The same also holds true for DNA from either frozen specimens (Figure 3 (b)) or paraffin-embedded tissue (data not shown).

To investigate the incidence of different HPV types, DNA from 53 frozen tissues and 21 paraffin-embedded tissues from different skin lesions were tested. From our experiments, only type3 was detected. No other samples harboured type10 except an unidentified sample, which exhibited different pattern from both type3 and 10 (data not shown). We are now analyzing this unidentified virus.

SUMMARY

We have established the PCR approach to detect both HPV type3 and type10, which are most extensively found in EV patients. By using developed PCR reaction, 74 clinical samples were tested, and only type3 among these is detected, and only one sample was not involved in either type3 or type10.

Acknowledgements

We thank Dr. Gerard Orth in Pasteur Institute for his gift of plasmid DNAs of HPV3 and HPV10 together with their full length sequences.

신용재 등: PCR, typing, EV, HPV

REFERENCES

1) **Green M, Brackmann KH, Sanders PR, Loewenstein PM, Freel JH, Eisinger M and Switlyk SA:** Isolation of a human papillomavirus from a patient with epidermodysplasia verruciformis: presence of related viral DNA genomes in human urogenital tumors. *Proc Natl Acad Sci USA* **79:** 4437-4441, 1982.
2) **Kremsdorf D, Jablonska S, Favre M and Orth G:** Biochemical characteriztion of two types of human papillomaviruses specifically associated with epidermodysplasia verruciformis. *J Virol* **43:** 436-447, 1982.
3) **Orth G, Favre M, Breitburd F, Croissant O, Jablonska S, Obalek S Jarzabek-Chorzelska M and Rzesa G:** Epidermodysplasia verruciformis: a model for the role of papillomaviruses in human cancer. 259-282. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1980.
4) **Orth G, Jablonska S, Favre M, Croissant O, Jarzabek-Chorzelska M and Rzesa G:** Characterization of two types of human papillomaviruses in lesions of epidermodysplasia verruciformis. *Proc Natl Acad Sci USA* **75:** 1537-1541, 1978.
5) **Orth G, Jablonska S, Jarzabek-Chorzelska M, Rzesa G, Obalek S Favre M and Croissant O:** Characterization of the lesions and risk of malignant conversion as related to the type of the human papillomavirus involved in epidermodysplasia verruciformis. *Cancer Res* **39:** 1074-1082, 1979.
6) **Ostrow RS, Bender M, Niimura M, Seki T, Kawashima M, Pass F and Faras AJ:** Human papillomavirus DNA in cutaneous primary and metastasized squamous cell carcinoma from patients with epidermodysplasia verruciformis. *Proc Natl Acad Sci USA* **79:** 1634-1638, 1982.
7) **Pfister H, Gassenmaier A, Nurnberger F and Stuttgen:** Human papillomavirus 5-DNA in carcinoma of an epidermodysplasicia verruciformis patient infected with various human papillomavirus types. *Cancer Res* **43:** 1436-1441, 1983.
8) **Pfister H, Nurnberger F, Gissmann L and zur Hausen H:** Characterization of a human papillomavirus from epidermodysplasia verruciformis lesions of a patient from Upper Volta. *Int J Cancer* **27:** 645-650, 1981.
9) **Yutsudo M, Tanigaki T, Tsumori T, Watanabe S and Hakura A:** New human papillomavirus isolated from epidermodysplasia verruciformis lesions. *Cancer Res* **42:** 2440-2443, 1982.