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Absence of Human Papillomavirus DNA in Nongenital Seborrheic Keratosis

Seborrheic keratosis (SK) is a benign epidermal tumor of unknown etiology. Because of its wart-like morphology, human papillomavirus (HPV) has been suggested as a possible causative agent. Viral involvement, however, has not been confirmed yet despite extensive research. The aim of this study was to evaluate the presence of HPV 6/11, 31, 33 DNA in nongenital SK. We analyzed 40 biopsy specimens taken from patients with nongenital SK using in situ polymerase chain reaction (PCR) and PCR with tissue extracts. The SK specimens (n=40), analyzed by in situ PCR, were negative for all HPV probes tested (types 6/11, 31, 33). Control slides (condyloma acuminatum, n=3) were positive for type 6/11, 31, and 33 HPV probes tested. Melasma samples (n=4), the negative controls, were consistently negative. No HPV DNA band was detected by PCR with the tissue extracts from paraffin-embedded SK samples, while condyloma acuminatum, the positive controls, showed DNA bands of the correct molecular weights. Our results show that HPV type 6/11, 31, and 33 cannot be recognized as causative agents for nongenital SK, which is in contrast to the previous studies. Further studies are required to reveal the presence of other types (more than 90) of HPV DNA.

Key Words : *Keratosis, Seborrheic; Polymerase Chain Reaction; In Situ PCR; Papillomavirus, Human*

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INTRODUCTION

Human papillomavirus (HPV) has been detected in various benign or malignant epidermal tumors (1-5). Seborrheic keratoses (SK), which are benign epidermal tumors, are similar and often confused with warts in their clinical and/or histological appearance. These similarities have led investigators to study the role of HPV in the pathogenesis of seborrheic keratoses (6-10).

Zhao et al. (6) detected, by electron microscopy, HPV-like particles in 4 of 89 nongenital SK. In cases of seborrheic keratoses of cases of the genital area, HPV has been reportedly found in 42% (n=57) using polymerase chain reaction (PCR) from tissue extracts (7). However, the same group reported lack of HPV-DNA in nongenital SK (8), demonstrated by PCR from tissue extracts (n=27). Soler et al. (9) showed HPV type 5 DNA by in situ hybridization in a nongenital SK obtained from a renal transplant recipient. Recently, Tsambaos et al. (10) detected HPV-DNA in 20% (34/173) of nongenital SK specimens using in situ hybridization.

We believe that it is necessary to clarify the discrepancies of HPV-DNA detection in nongenital SK using accurate and sensitive methods. To demonstrate the presence of HPV in nongenital SK, we analyzed 40 biopsy specimens taken from patients with nongenital SK using in situ PCR and PCR from tissue extracts.

MATERIALS AND METHODS

To investigate the possibility that HPV might be a causative factor for SK, 40 nongenital SK tissue sections were examined for the presence of HPV 6/11, 31 or 33 DNA by PCR and in situ PCR.

Tissue samples

A series of 40 formalin-fixed and paraffin-embedded biopsy specimens were collected as tissue samples. Paraffin-embedded tissue samples were retrieved from archival diagnostic specimens. The specimens were taken from 40 patients, 22 men and 18 women, who had visited the Dermatology Department of Ajou University Hospital from October 1997 to October 1999. Biopsy sites included the face (n=15), trunk (n=8), scalp (n=7), leg (n=3), neck (n=2), arm (n=2), hand (n=1), back (n=1), and buttock (n=1). All specimens used in this study showed the typical histological features of SK.

Samples included 8 hyperkeratotic types and 32 acanthotic types of SK. The diagnosis was confirmed again after reexamination of clinical photos and histological slides by two dermatologists (WHK and ESL). We used 3 specimens of condyloma acuminatum as the positive controls and 5 specimens of melasma as the negative controls.

In situ PCR

In our study, in situ PCR was modified from Nuovo's method (11). Four μm tissue sections were prepared and dewaxed with xylene for 30 min and 100% ethanol for 5 min. The samples were gradually rehydrated using a graded series of alcohols and digested with 20 μg/mL proteinase K (Behringer Manheim Co., Indianapolis, IN, U.S.A.), in PBS for 10 min at 37℃. The samples were washed extensively in phosphate-buffered saline (PBS) to inactivate the enzyme. The slides were dehydrated in a graded series of alcohols and their PCR amplification was performed using the in situ thermal cyclers (M J Research, Waltham, MA, U.S.A.). PCR reaction was performed using type-specific primers. We synthesized the primers for HPV 6/11, 31, 33 as follows: HPV 6/11 (F) 5′-AAGGGCGTAACCGAAATCGGT-3′, (R) 5′-TGTCACAAACCGCTGTGTGA-3′; HPV 31 (F) 5′-TGTCAAAGACCGTTGTGTCC-3′, (R) 5′-GAG CTGTCGGGTAATTGCTC-3′; HPV 33 (F) 5′-AAGG-GCGTAACCGAAATCGGT-3′, (R) 5′-GTCTCCAAT-GCTTGGCACA-3′. Twenty-five μL PCR reaction mixtures contained *Taq* polymerase 0.8 μL, 10×PCR buffer 2.5 μL, 2.5 mM dNTPs 4 μL, 1 mM digoxigenin-11-dUTP 1 μL (Boehringer Mannheim Biochemicals, Indianapolis, IN), 20 pmol/μL HPVpF 1 μL, and 20 pmol/μL HPVpR 1 μL. The slides were warmed to 82℃ for 7 min in order to perform hot start PCR and then at 55℃, 25 μL PCR reaction mixture was placed directly on the tissue section and covered with slide seal (TaKaRa Biomedicals, Japan) and cycled as follows: 94℃ for 3 min (1 cycle), followed by 55℃ for 2 min and 94℃ for 1 min (15 cycles). Slide seals were removed and the tissue was washed in PBS for 5 min.

The samples were then incubated for 30 min with anti-digoxigenin-alkaline phosphatase-conjugated Fab antibody fragments (1:2,000 dilution; Boehringer Mannheim Biochemicals, Indianapolis, IN) prepared in blocking buffer. The slides were washed 3 times in tris buffer solution (pH 7.6). Freshly prepared BCIP/NBT (5′-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium; Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as the chromogenic substrate. Slides were covered with the substrate and monitored for color development. The sections were then counterstained with Mayer hematoxylin.

PCR analysis of tissue extracts

DNA amplification by PCR was carried out as previously described (12) on the tissue samples. Briefly, 8 μm thick sections were deparaffinized in 1.5 mL microfuge tubes with 500 μL xylene, followed by dehydration with 100% ethanol. The samples were dried in a vacuum centrifuge, which were digested overnight at 55℃ with proteinase K (Sigma Chemical Co., St. Louis, MO). Then, it was boiled in 95℃ heat block to stop the reaction. Amplification of HPV DNA was performed using the supernatant. We used PCR core kit (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.) and GeneAmp PCR System 9600 (Perkin Elmer Cetus, Norwalk, CT, U.S.A.). The PCR was implemented using 30 cycles with the primers; 30 sec at 94℃, 2 min at 55℃, and 2 min at 72℃. PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide, and photographed under UV light. Positive and negative control reactions were included with all amplifications.

RESULTS

In situ PCR

The 40 tissue samples, analyzed by in situ PCR, were negative for all HPV probes tested (6/11, 31, 33) (Fig. 1A). Control slides (condyloma acuminatum, n=3) were consistently positive for type 6/11, 31, and 33 HPV probes tested (Fig. 1B, C). Dark brown colored positive staining was observed on the nuclei of keratinocytes in the upper stratum malpighii and stratum corneum. Melasma samples (n=4), the negative controls, were consistently negative.

PCR analysis of tissue extracts

No HPV DNA band was detected by PCR with the tissue extracts from paraffin-embedded SK samples (n=40). However, condyloma acuminatum (n=3), which served as the positive controls, showed DNA bands of the correct molecular weights (Fig. 2).

DISCUSSION

The present study, in situ PCR or PCR with SK tissue extracts, could not demonstrate HPV genomes type 6/11, 31, or 33 in the nongenital SK tissue samples (n=40). This result is contrary to the result by Tsambaos et al. (10), which reported the presence of HPV genomes (6/11, 8.7%; 31/33/35, 8%; other types, 2.9%; n=173). At present, we cannot explain the discrepancy between the two studies.

We used very sensitive and specific methods to detect the tissue HPV (type 6/11, 31, and 33). We believe that our negative results are not technical errors for the following reasons: 1) in situ PCR method used in this study is more sensitive than in situ hybridization due to the advantages of

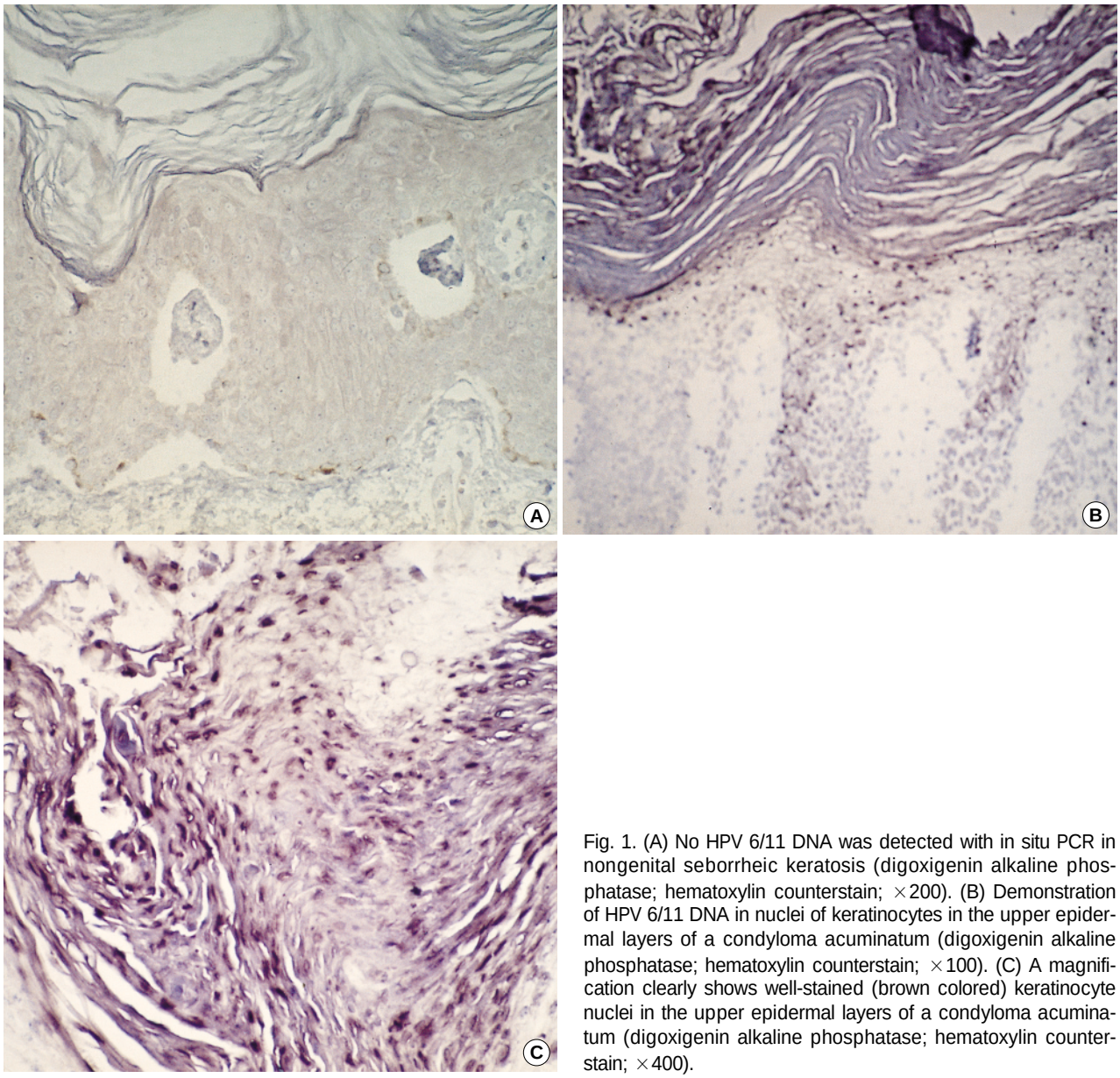

Fig. 1. (A) No HPV 6/11 DNA was detected with in situ PCR in nongenital seborrheic keratosis (digoxigenin alkaline phosphatase; hematoxylin counterstain; ×200). (B) Demonstration of HPV 6/11 DNA in nuclei of keratinocytes in the upper epidermal layers of a condyloma acuminatum (digoxigenin alkaline phosphatase; hematoxylin counterstain; ×100). (C) A magnification clearly shows well-stained (brown colored) keratinocyte nuclei in the upper epidermal layers of a condyloma acuminatum (digoxigenin alkaline phosphatase; hematoxylin counterstain; ×400).

both PCR and in situ hybridization and enabled us to visualize the cellular localization of HPV DNA; 2) to confirm the negative results of in situ PCR for the HPV DNA, we performed PCR using SK tissue extracts, which revealed no HPV DNA (type 6/11, 31, and 33); 3) the positive controls (condyloma acuminatum, n=3) run simultaneously showed definite dark brown colored staining of the nuclei (Fig. 1B, C), while the negative controls (melasma, n=4) did not show the staining pattern.

One possible explanation might be that the venereal HPV transmission would increase the risk of exposure of both sites (genital and periungual) to HPV infection (13). Therefore, despite the same disease entity, the affected site could be the source of such differences in virus detection. For example, Mitsuishi et al. (14) detected HPV DNA in Bowen's disease of the hands, while Lu et al. (4 hand from total 91) (13) failed to demonstrate the presence of HPV DNA in Bowen's disease of the skin. Similar results have also been reported with squamous cell carcinoma (15, 16). In our study, only one specimen was taken from the finger, which might partly explain our negative results.

In conclusion, we believe that HPV type 6/11, 31, and 33 can not be recognized as causative agents for nongenital SK in contrast to the previous study (10). The present study has checked 4 types (6/11, 31, and 33) of HPV DNA among more than 90 types. Further studies are required to reveal the presence of other types of HPV DNA, which would help to clarify the role of HPV in the pathogenesis of nongenital SK.

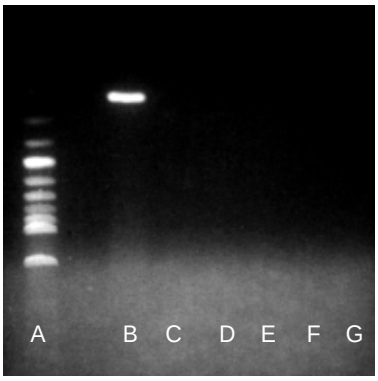

Fig. 2. Ethidium-stained 1.5% agarose gel demonstrating HPV 6/11 DNA amplimers. Lane A indicates molecular weight markers; lane B, condyloma acuminatum, Lane C through G, SKs. Lane B is positive for HPV 6/11 DNA. Lane C through G are negative.

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