

Detection of Human Papilloma Virus DNA in Seborrheic Keratosis of Korean Skin

Young Il Jeong, M.D., Woo Jin Lee, M.D., Hana Bak, M.D., Sang Hyun Oh, M.D.,
Hae Jin Jung*, Sung Eun Chang, M.D., Jee Ho Choi, M.D.

Department of Dermatology and Research Institute of Dermatology, Asan Medical Center,
University of Ulsan College of Medicine, Seoul, Korea*

Background: DNA of epidermodysplasia verruciformis (EV)-associated human papillomaviruses (HPVs) has been detected in benign and malignant skin tumors and other proliferative diseases of epithelial origin. The objective of this study was to determine the association of EV-associated HPV DNA in nongenital seborrheic keratoses (SK) in Korean patients.

Subjects and Methods: Forty biopsy specimens were collected from patients with nongenital SK and cutaneous SCC and controls. All tissue samples were examined by PCR.

Results: By polymerase chain reaction (PCR), EV-associated HPV DNA was detected in 15 of 40 nongenital SK (37.5%) compared with 1 of 40 cutaneous squamous cell carcinomas (SCC) samples and 3 of 40 healthy controls. Detected viruses in SK included HPV 20 (n=6), HPV 23 (n=2). The rest tested positive for HPV 5, 16, 17, 22, 25, 37, and RTRX4.

Conclusion: Our findings suggest that EV-associated HPV may be involved in the pathogenesis of nongenital SK of Asian skin.

(Ann Dermatol (Seoul) 19(3) 99~105, 2007)

Key Words: Human papillomavirus, Seborrheic keratosis

INTRODUCTION

EV-associated HPV DNA has been found in benign tumors, cutaneous squamous cell carcinoma (SCC) and proliferative disorders of epithelial origin, including psoriasis, actinic keratosis, and Darier's disease, and even in normal skin¹⁻⁶. However, unlike cervical dysplasia and anogenital carcinoma, the presence of EV-associated HPVs in these nongenital lesions does not indicate that they act as pathogens⁷.

Nongenital SK is histologically similar to common warts, with pronounced hyperkeratinization, acanthosis and papillomatosis in the epidermis. In some lesions, focal vacuolated epithelial cells, a typical

characteristic of HPV infection, have been detected in the granular layer⁸. Using electron microscopy, HPV particles have been found in 4 of 89 nongenital SK lesions⁹. HPV DNA has also been detected in skin tags and stucco keratosis, two variants of SK¹⁰.

Most of the above observations were made in Western populations. In contrast, there have been few reports showing a positive association between EV-associated HPV and nongenital SK in Asian skin. However, in one pilot study, 0 of 40 nongenital SK samples were found to have EV-associated HPVs¹¹. Most nonmelanoma skin cancers in the general population were found to be only sporadically associated with HPV, whereas 60% of periungual SCC and palmoplantar Bowen's disease were found to be positive for HPV 16^{12,13}. The use of more sensitive HPV detective methods, especially PCR, has shown an increased rate of HPV positive SCC at other anatomical sites¹⁴. All of these observations prompted us to assay nongenital SK and cutaneous SCC in a Korean population for the presence of EV-associated HPV DNA.

Received June 2, 2006

Accepted for publication May 18, 2007

Reprint request to: Sung Eun Chang, M.D., Department of Dermatology, Asan Medical Center, University of Ulsan College of Medicine. Tel: 82-2-3010-3460, Fax: 82-2-486-7831, E-mail: cse@amc.seoul.kr

MATERIALS AND METHODS

Confirmed cases as nongenital SK or cutaneous SCC patients on biopsies in Asan Medical Center were selected for study. The local medical ethics committee approved this research. The patients were matched by age (± 10 years) and sex. The exclusion criteria for cases and controls included the presence of HPV-related skin lesions (warts and condylomas); history of SCC of the skin including the cervix; history or signs and symptoms of immunosuppression; and history of skin diseases for which UV radiation therapy was indicated, such as psoriasis or vitiligo, or where sun avoidance was recommended, such as connective tissue diseases.

Forty biopsy specimens each were collected from patients with nongenital SK and cutaneous SCC and controls. All tissue samples were formalin-fixed and paraffin-embedded. Each specimen was immersed in 500 μl of DEXPATTM (TaKaRa, Kyoto, Japan) and preincubated at 100°C for 10 min in a block heater. The tubes were centrifuged for 10 min at 12,000 rpm at 4°C and the supernatants were collected. The adequacy of the DNA preparations for PCR was tested by using β -globin primers.

For PCR amplification of the complete set of EV-associated HPV types (HPV-5b, -8, -9, -12, -14a, -15, -17, -19, -20, -20b, -21, -22, -23, -24, -25, -36, -37, -38, 47, and -49), we constructed a primer set consisting of degenerate primers that amplified a sequence located in the late L1 ORF. The forward primer sequence was 5' CAA GGT CAC AAC AAT GGC AT 3' (CP65) and the reverse primer sequence was 5' AAC TTT CGT CCC AAA GAA AAT TGA TC 3' (CP70), which corresponded to nucleotides 6832 to 6851 and 7273 to 7298 of the HPV-8 genomic sequence, respectively¹⁵. The primer set amplified a 452- to 467-bp product, depending on the target HPV type.

As a positive control, we used a mucosal wart, which had been previously shown to constitutively express EV-associated HPV. As a negative control, we used distilled water in place of DNA. All samples were amplified in 50 μl of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 3.6 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 0.2 mM of each deoxynucleoside triphosphate (Pharmacia, Uppsala, Sweden), 1 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, USA), 5 μl of template DNA and 300 ng of each primer¹⁶.

The amplification protocol consisted of five cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1.5 min, and extension at 72°C for 2 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, performed in a Peltier Thermal Cycler.

Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using an ABI PRISM[®] BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq[®] DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed on a positive template following PCR using the CP65/CP70 primer pair. The fluorescence-labeled fragments were purified from the unincorporated terminators by ethanol precipitation. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730 xl sequencer (Applied Biosystems). DNA sequences were compared with sequences in the EMBL/GenBank Database using the BLAST program.

The frequency of detection of HPV DNA sequences in nongenital SK and control samples was compared using the chi square test, and the frequency of detection in cutaneous SCC and control samples was compared using Fisher's exact test. A p value ≤ 0.05 was considered statistically significant.

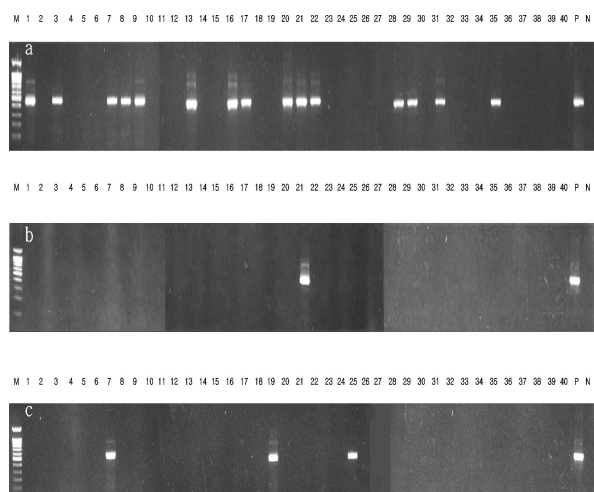
RESULTS

The baseline characteristics of each set of patients and matched controls are shown in Table 1. For each set, the mean age was 60.4 years and ranged from 36 to 86 years. Of each set of 40 patients, 29 (72.5%) were males and 11 (27.5%) were females, making the ratio of males to females 2.64 : 1. The location of nongenital SK and cutaneous SCC was the face and neck, the extremities, and the trunk according to the frequency, and biopsies were taken from controls in the appropriate locations.

Using a PCR assay for EV-associated HPV, we found that 15 of 40 (37.5%) specimens from patients with nongenital SK were positive, compared with 1 of 40 (2.5%) specimens from patients with cutaneous SCC, and 3 of 40 (7.5%) control specimens (Fig. 1). Compared with controls, there was a significant difference in the HPV DNA positive rate in

Table 1. Demographic characteristics of 40 nongenital SK, 40 cutaneous SCC, and 40 healthy controls

	Nongenital SK	Cutaneous SCC	Controls
Age (years)			
30-50	9	9	9
50-70	15	15	15
70-90	16	16	16
Sex			
Male	29	29	29
Female	11	11	11
Location			
Face and neck	19	31	6
Trunk	10	2	20
Extremities	11	7	14

**Fig. 1.** Polymerase chain reaction for HPV DNA detection in nongenital SK (a), cutaneous SCC (b), and healthy control (c) samples. PCR products were electrophoresed in agarose gels and stained with ethidium bromide. A 100-bp size marker (lane M), a positive control (mucosal wart) (lane P), and a negative control (lane N).

nongenital SK specimens ($P = 0.001$), but not in cutaneous SCC specimens ($P = 0.615$). The negative controls remained negative in the PCR, excluding the possibility of contamination or false positivity. All samples from nongenital SK, cutaneous SCC and controls were positive for the 110 bp β -globin gene fragment, as expected, thus excluding the possibility of false negativity (data not shown).

Direct sequencing identified 9 EV-associated HPV

genotypes, subtypes or related types in the nongenital SK specimens. Of the 15 specimens positive for HPV, 6 were positive for HPV 20, 2 were positive for HPV 23, and 1 each was positive for HPV 5, HPV 16, HPV 17, HPV 22, HPV 25, HPV 37 and RTRX4. The single HPV-positive cutaneous SCC sample was positive for HPV 5, whereas, of the 3 HPV positive specimens from healthy controls, one each was positive for HPV 5, HPV 20 and HPV 23 (Table 2).

DISCUSSION

Due to their histological similarity to plantar warts, SKs have been assayed for the presence of HPV. Using in situ hybridization, HPV 5 was detected in a nongenital SK obtained from renal transplant recipients¹⁷. Using general probes for HPV 6/11 and HPV 31/33/35, mucosal HPVs were subsequently detected in 34 of 173 (20%) nongenital SKs, suggesting that HPVs are involved in the pathogenesis of nongenital SK⁸. The low rate of detection of HPV DNA was probably due to the use of in situ hybridization, which is less sensitive and less specific than PCR. Using primers designed for mucosal HPVs (MY09/MY11), HPV DNA was detected in 1 of 29 (3%) nongenital SKs and in 23 of 43 (53%) genital SKs¹⁸. However, the use of in situ PCR with primers specific for HPV 6, 11, 31 and 33 failed to detect HPVs in nongenital SKs¹¹. These results do not indicate that EV-associated HPVs are rarely found in nongenital SK, because

Table 2. Frequency of individual HPV types in nongenital SK, cutaneous SCC, and healthy control specimens

HPV types	No. of nongenital SK (%)	No. of cutaneous SCC (%)	No. of controls (%)
20	6 (15%)	-	1 (2.5%)
23	2 (5%)	-	1 (2.5%)
5	1 (2.5%)	1 (2.5%)	1 (2.5%)
16	1 (2.5%)	-	-
17	1 (2.5%)	-	-
22	1 (2.5%)	-	-
25	1 (2.5%)	-	-
37	1 (2.5%)	-	-
RTRX4	1 (2.5%)	-	-
Negative	25 (62.5%)	39 (97.5%)	37 (92.5%)

the primers for mucosal HPVs are not suitable for detecting EV-associated HPVs.

Using PCR primers specific for EV-associated HPVs, we detected EV-associated HPV DNA in 15 of 40 (37.5%) nongenital SK lesions, a rate much higher than in healthy controls (3 of 40, 7.5%) ($P < 0.005$). These results suggest that EV-associated HPV infection may be a causative or coordinative factor in the pathogenesis of nongenital SK.

In our experiments, we used a modification of a previously described method and primers¹⁵, but we did not perform nested PCR. Nested PCR may increase the rate of detection of HPV in the nongenital SK and cutaneous SCC. In addition, an increased detection rate may occur using fresh frozen biopsies instead of paraffin-embedded archival samples.

Similar findings have been reported in renal transplant recipients¹⁸, where HPV DNA was detected more frequently in patients with multiple carcinomas (26 of 50), than in those with single carcinomas (6 of 22). The prevalence of EV-associated HPV may also correlate with age, as patients aged 60-80 years had a significantly higher prevalence of EV-associated HPV than patients aged 30-50 years¹⁹. However, in our study, HPV positive samples were distributed haphazardly, without relation to age, so additional experiments are needed to determine the impact of age on the prevalence of EV-associated HPVs.

The prevalence of EV-associated HPV in healthy controls has not been found to vary widely. For example, we detected HPV DNA in 7.5% of our controls, and Mahe et al. (2003) found HPV DNA in skin scrapings from 7% of 28 healthy adult controls using nested PCR, the CP 65/70 and CP 66/69 primer pairs, and PCR methods similar to ours. The variation in prevalence may be due to the difference in specimens (skin scrapings versus skin biopsies); the difference in DNA extraction methods (InViSorb Forensic Kit I (InViTek GmbH, Berlin, Germany) versus Tris-phenol and trichloromethane); and the difference in geographical populations (France versus South Korea).

Photoimmunosuppression has been shown to reduce the immune response to some infectious agents (Norval et al., 1994). For example, some viral warts can be exacerbated by UVB radiation. In renal transplant recipients (RTRs), nonmelanoma skin cancers and wart-like lesions predominantly develop in sun-exposed skin, and EV-associated HPV DNA was detected in most skin lesions in RTRs (de Jong-Tieben et al., 1995). The promoter of HPV 1, 5, 7, 20 and 23 can be activated under the influence of UV irradiation (de Viller et al., 1999). Recently, E5 and E6 proteins of HPVs were reported to be important in reducing the UVB-induced apoptosis of keratinocytes (El-Mahdy et al, 2000; Zhang et al., 2002). In our study, HPV 20 was the predominant genotype detected. HPV 20 shares a high degree of

DNA homology with HPV 5, which is one of the predominant genotypes in normal skin and hair bulbs. Thus, HPV may reside in normal skin, follicular bulbs and proliferative skin lesions as a latent infection. HPV replication may be activated when the immune response is damaged by cumulative photoimmunosuppression (Vermeer et al., 1998).

UV radiation is considered the most important inducer of skin cancer, because most of these cancers are located in sun-exposed areas. Moreover, more than 90% of these cancers contain UV signature mutations (Ziegler et al., 1993; Brash et al., 1996). Suppression of cellular immune responses is also an important pathogenic factor, as indicated by the increased incidence in transplant patients. The risk of skin cancer seems to depend on the degree of immunosuppression, because the incidence of SCC was reported to be higher in renal transplant recipients receiving a combination of three immunosuppressive drugs (cyclosporine A, azathioprine, and prednisolone) than in patients receiving only azathioprine and prednisolone (Jensen et al., 1999; Glover et al., 1997). In addition, infection with human papillomaviruses (HPV) may also be involved in skin carcinogenesis. HPV-induced warts (verruca vulgaris) occur in up to 90% of transplant recipients. Clinical and histologic analysis showed that viral warts progress to dysplastic lesions and invasive SCC in immunosuppressed patients (Blessing et al., 1989). Thus, viral warts, which are considered benign lesions in immunocompetent patients, may be of different prognostic importance in immunosuppressed patients.

In contrast to HPV-induced cutaneous warts, the role of these viruses in the development of non-melanoma skin cancers is less clear. Nonmelanoma skin cancers in the general population were reported to be only sporadically associated with HPV, with the exception of periungual SCC and palmoplantar Bowen's disease, more than 60% of which were positive for HPV16 (Moy et al., 1989; McGregor et al., 1996). Increasing rates of HPV positive SCC at other anatomical sites have been found using more sensitive HPV detection methods, mainly PCR assays (Pfister et al., 1997). HPV DNA has been detected in 70% to 90% of cutaneous SCC in organ transplant recipients (Berkhout et al., 2000; De Villiers et al., 1997; Harwood et al., 2000; Meyer et al., 2001). However, the prevalence of HPV is also high in

non-cancerous skin lesions, benign tumors, and normal oral mucosae of both immunosuppressed and immunocompetent people (Berg et al., 2002; Berkhout et al., 2000). And there are studies reporting low positive rate of EV-associated HPV in cutaneous SCC in renal transplant recipients (Tieben LM et al., 1994). Thus, while the presence of HPV is a cofactor in cutaneous carcinogenesis, its presence alone is not sufficient to induce skin cancer development.

HPV E6 protein in the skin of transplant patients has been found to inhibit apoptosis, both directly and by inhibiting p53, a major pro-apoptotic tumor suppressor protein important in cutaneous basal cell carcinoma and squamous cell carcinoma (Jackson et al., 2000). In addition, UV light has been shown to upregulate (via p53) cutaneous HPV in organ transplant patients (Purdie et al., 1999). Immunosuppression of organ transplant recipients may enhance the prevalence of HPV, inhibiting apoptosis and allowing abnormal clones of keratinocytes to persist and continue to receive further mutagenic UV radiation, eventually progressing to skin cancer.

In contrast to our findings in nongenital SK, we found that only 1 of 40 samples from patients with cutaneous SCC was positive for HPV. This may have been due to the population of patients with cutaneous SCC being immunocompetent, and not immunosuppressed.

In conclusion, we found that the presence of HPV was correlated with nongenital SK, but not cutaneous SCC, in a Korean population. Further studies with larger numbers of patients are needed to show an association between HPV and nongenital SK and cutaneous SCC.

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