Detection of Human Papillomavirus (HPV) DNA Sequences in Normal Oral Scrapes Using the Nested PCR.

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ABSTRACT

We investigated the prevalence rate of HPV DNAs in normal mucosa in the oral region. The nested PCR method was utilized to detect target DNA sequences using HPV E6/E7 consensus primer pairs. Of 56 patients examined, HPV 6 and HPV 16 DNA sequences were detected in a 46-year-old male and a 35-year-old female, respectively. These results suggest that HPVs are uncommon in normal oral epithelium, and that we should carry out careful follow-up in HPV DNA-positive cases.

Key words: HPV, Nested PCR, Normal oral epithelium

INTRODUCTION

Clinico-pathological and in vitro studies indicate that human papillomavirus (HPV) DNAs are closely associated with the tumorigenesis of human epithelial lesions. Among the 67 presently known types of HPVs, HPV-6 and -11 DNAs have been identified mostly in benign lesions, while HPV-16, -18, -33 and some other HPVs such as HPV -31, -33, -52b and -58 have been detected in malignant

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lesions of the uterine cervix(1). The oral region is covered with squamous epithelium similar to the uterine cervix. We have detected HPV-16 and -18 DNA in about 30% of oral squamous cell carcinomas (SCC) with the polymerase chain reaction (PCR)(2). Thus, HPV DNAs are closely associated with tumorigenesis in oral as well as cervical carcinoma and it is important to investigate the prevalence rate of HPV DNAs in normal epithelium as a risk factor for tumorigenesis. The ability of a set of primers to detect a series of HPV DNA is of practical importance for the purpose of screening. This study attempted to determine the prevalence rate of HPV DNAs in normal oral epithelial scrapes using consensus primers in PCR.

MATERIALS AND METHODS

Extraction of DNA

Specimens were collected from patients who consulted the Department of Oral Surgery at the Dental Hospital in Hokkaido University from September 1991 to April 1992. Clinical examinations were carried out and patients without any epithelial lesions were selected. Fifty-six oral scrapes were obtained from buccal mucosa of these patients by sterilized cotton swab. The scrapes were immersed in 2 m*l* of sterilized phosphate buffered saline (PBS). After vigorous shaking, 1.5 m*l* of scraping suspension was transferred to a 1.5 m*l* centrifuge tube and centrifuged (3000 rpm, 3 min). The cell pellets were digested overnight with 500 μl of 10 mM Tris-HCl (pH8.0), 5 mM EDTA, 0.5% SDS and 50 μg/m*l* proteinase K at 37℃. The DNA was purified by two extractions with a mixture of phenol equilibrated with 10 mM Tris-HCl (pH8.0) and chloroform : isoamyl alcohol (24 : 1), then precipitated with ethanol and dissolved in distilled water. Care was taken to avoid cross contamination of samples throughout the procedure.

PCR and gel electrophoresis

HPV DNAs in DNA samples were amplified by PCR. We used 4 primers as essentially described by Fujinaga *et al.*(3). pU-0 (5'-AGGGAGTGACCGAAAA CGGT-3') is a sense primer that corresponds to the LCR sense sequences of HPV-6, -11, -16, -18, -31, -33, -52b and -58 with some mismatches. pU-1M (5'-TGTCAAAAACCGTTGTGTCC-3') and pU-31B (5'-TGCTAATTCGGTGCT ACCTG-3') are inner sense primers homologous to the E6 sense sequences of malignant type HPV-16, -18, -31, -33, -52b, -58 , and benign type HPV-6 and -11, respectively. pU-2R (5'-GAGCTGTCGCTTAATTGCTC-3') is an antisense primer that corresponds to the E7 antisense sequences of the above HPVs with some mismatches. The nested PCR was carried out similarly to the method

described by Fujinaga *et al.*(3). In brief, the first amplification of HPV DNAs were carried out using pU-0 and pU-2R. One-hundredth of those reaction products was then subjected to the second PCR using 2 sets of primers pU-1M/pU-2R and pU-31B/pU-2R in the same concentration of PCR media. After amplification, 10-μl aliquots of the reaction mixture were subjected to electrophoretic analysis on 3% NuSieve and 1% Seakem agarose (FMC, Rockland, ME) and stained with ethidium bromide. Samples of HPV DNA-positive cases were purified by ethanol precipitation, and subjected to restriction enzyme analysis using Ava II, Rsa I, Bgl II and Acc I to define HPV types. Each HPV DNA cloned into plasmids was also investigated by the same method and the sensitivity of the method was estimated.

RESULTS

HPV DNAs were successfully amplified using consensus primers for both malignant and benign types. HPV-16, -18, -31, -33, -52b and -58 DNAs were detected using pU-1M/ pU-2R (Fig. 1a), and HPV-6 and -11 were amplified only when pU-31B/pU-2R primer pairs were used for the second step of the nested PCR (Fig. 1b). Restriction enzyme analysis showed that these amplified DNA sequences could be used to classify specific HPV types. HPV-16, -18 and -33 can be distinguished according to the Ava II cutting patterns. HPV-31 can be

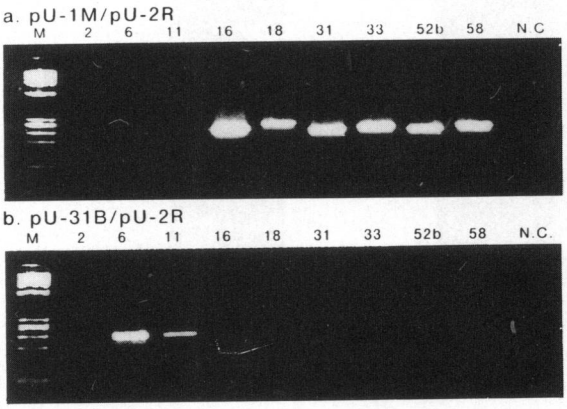


Fig. 1 Results of PCR amplification by PU1M/PU2R (Fig 1a) and PU31B/PU2R (Fig. 1b) primers pair.
The number of each lane indicates the type of HPV DNA cloned into plasmid (1 copy/cell as a concentration) examined.
M; ϕX174 RF DNA/Hae III fragments, N. C.; negative control (no DNA).

Table 1 *Total length and fragmented sizes of amplified HPV DNA*

Primers	pU−31 B/pU− 2 R		pU− 1 M/pU− 2 R					
HPV type	6	11	16	18	31	33	52 b	58
Total length	228	228	238	268	233	244	231	244
Ava II	NC	NC	157,81	172,96	NC	136,108	NC	NC
Rsa I	132,96	166,62	NC	NC	119,114	NC	NC	NC
Bgl II	NC	NC	NC	NC	NC	NC	176,55	NC
Acc I	NC	NC	NC	NC	NC	NC	NC	126,118

NC ; No cut

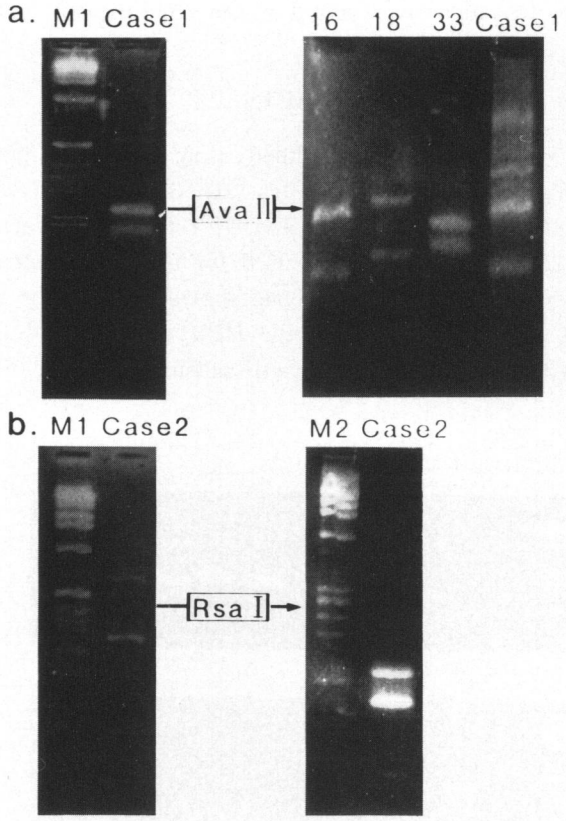
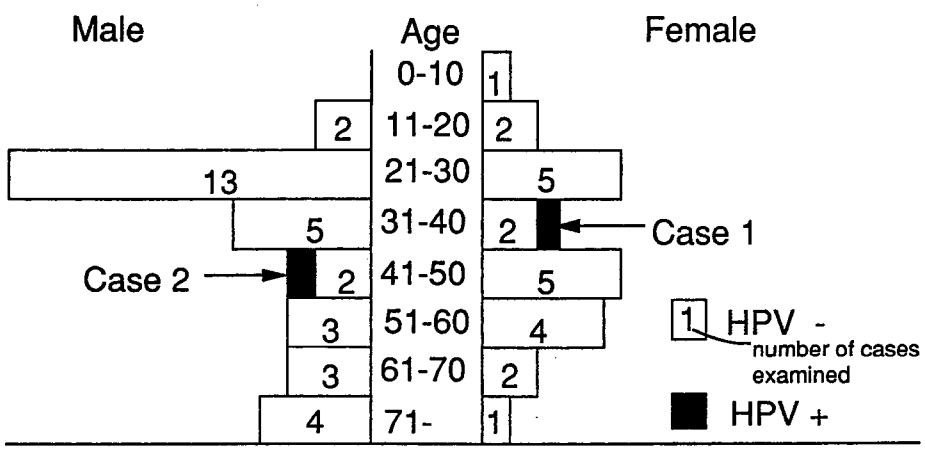


Fig. 2a Case 1 ; Amplified product by the nested PCR (pU-0/pU-2R, pU-1M/pU -2R) is in the left, and Ava II cutting pattern of amplified products are in the right. The number of the lane indicates each type of HPV examined. An additional band is seen, however, the cutting pattern is related to HPV 16 DNA (Lane 16).

2b Case 2 ; Amplified product by pU-0/pU-2R and pU-31B/pU-2R. Rsa I cutting pattern is seen in the right. M1 ; 1 Kb DNA ladder, M2 ; ϕX174 RF/Hae III fragments

Table 2



fragmented only when Rsa I is used, and HPV-52b and -58 were fragmented by Bgl II and Acc I, respectively. HPV-6 and HPV-11 can be divided using Rsa 1 as the restriction enzyme.

Table 1 shows the lengths of amplified products and the sizes of DNAs fragmented by restriction enzymes. We assessed the sensitivity of this method using serially diluted HPV DNAs. By the single set of amplification using the primer pairs pU-1M/pU-2R or pU31-B/pU-2R, 10^{-1} copy/cell of HPV DNAs could be detected, while the nested PCR method made it possible to detect 10^{-2} copy/cell of each HPV DNA(3).

For this study, 33 males and 23 females were examined. Patients' ages ranged from 7 to 76 years old. Clinical examination showed that these patients had no epithelial lesions and cytological examination revealed no atypical cells in any the cases. Among the 56 cases of normal oral scrapes, we could detect HPV DNAs in only 2 cases. The restriction enzyme cutting pattern revealed HPV-16 DNA in a 35-year-old female (Case 1: Fig. 2a) patient and HPV-6 DNA in a 46-year-old male (Case 2: Fig. 2b) patient (Table 2).

DISCUSSION

HPV DNAs have been detected in cervical carcinoma and cervical intraepithelial neoplasia (CIN) with high incidences (4, 5), and also in other locations such as laryngeal(6), lung(7), penile(8) and oral regions(2, 9). Besides in vivo detection of HPVs in various human epithelial lesions, HPV DNAs have the ability to immortalize or transform human fibroblasts and keratinocytes(10) as well as

rodent cell lines in vitro(11, 12, 13). Thus, it is now widely accepted that HPV DNAs are closely associated with tumorigenesis of human epithelial lesions.

HPV DNAs are divided into high- to low-risk groups according to their detection rates in malignant-to-benign epithelial lesions and biological characteristics in vitro(14). In brief, HPV-16 and -18 DNAs are classified as high-risk HPVs as they are frequently detected in carcinomas and have the ability to transform various cells in vitro, while HPV-6 and -11 are detected mainly in condyloma and other benign lesions and are of weak transforming ability so that they are categorized into the low-risk group. HPV-31, -33, -52b, and -58 are categorized into the intermediate-risk group. Since HPV DNAs are tumorigenic virus sequences, once epithelium is infected by the virus, it might have a latent potential for tumorigenesis, whether changes are manifested or not, especially in the case of high-risk HPVs.

The capability to detect various types of viral DNAs is of practical importance as a screening system. Fujinaga *et al.*(3, 4) showed that a series of HPV DNA sequences were able to be amplified using E6/E7 consensus primers for several HPV types cloned into plasmids. Other investigators also looked for HPV sequences using consensus primers(15, 16) oriented to amplify the L1 region of HPVs, which is, however, conserved less often than the E6/E7 region(17). This is the reason that we used the E6/E7 consensus primers developed by Fujinaga *et al.*(3, 4). Nested PCR method is a newly developed method which contributes to an increase in the sensitivity to detect specific DNA sequences(18). We used 2-step PCR (nested PCR) in order to increase the sensitivity(3) because not all cells in the tissue are thought to harbor HPV DNAs, and scrapings contain relatively small amounts of total DNAs.

A rather wide variety of detection frequences of HPV DNAs in normal tissues have been reported in cervices, perhaps due to differences of race, sample variety and the techniques employed. But the reliability rate for HPV identification was only a few percent in reports that surveyed relatively large numbers of cases(19, 20, 21). Few reports have addressed HPV identification in oral epithelium. Kashima *et al.*(22) reported that HPV DNAs were detected in normal epithelium adjacent to HPV DNA positive carcinomas in many patients. These results are similar to the findings in the cervical region(23), and related to the carcinogenesis of normal epithelial cells. Our present results indicate that HPV infection in normal oral epithelium is very rare; nonetheless, our earlier results showed that high-risk HPV DNAs were increasingly identified in oral carcinoma(2) so that it is suggested that HPV DNA positive epithelial cells have a latent potential for tumorigenesis if high-risk HPV DNAs harbored in oral epithelium. Although no lesional changes were observed in the 6 months after

initial treatment of the patients in whom HPV DNAs were detected, our results urge systematic follow-up for such patients.

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