Molecular Cloning and Nucleotide Sequence Analysis of a Novel Human Papillomavirus (Type 82) Associated with Vaginal Intraepithelial Neoplasia

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Received 26 July 1999/Returned for modification 6 October 1999/Accepted 4 November 1999

The genome of a novel human papillomavirus (HPV-82) was cloned from a vaginal intraepithelial neoplasia grade I. In our series of 291 biopsy specimens, HPV-82 was identified in one case each of cervical intraepithelial neoplasia grade II and grade III by blot hybridization. The histological localization of HPV-82 DNA in the three lesions was confirmed by in situ hybridization. The results indicated that HPV-82 is an etiologic agent for vaginal and cervical intraepithelial neoplasia. By nucleotide sequence similarity of L1 open reading frame (ORF), HPV-82 was closely related to HPV-26, -51, and -69. To know the precise relationship between the HPV, we determined the complete sequence of HPV-82, as well as that of HPV-69. Sequencing revealed that the four HPVs had no initiation codon in the E5 ORF and had extensive nucleotide sequence similarities in all ORFs. In addition, they exhibited unique frame position patterns for ORFs, different from those of the other genital HPVs.

Human papillomavirus (HPV) has a genome of closed-circular double-stranded DNA and HPV type 1 (HPV-1), HPV-2, and HPV-4 were first molecularly cloned in 1980 (6). During the past 20 years, 79 distinct HPVs have been cloned (4), of which 39 HPVs are labeled as genital HPV, having the potential to induce genital tumors (17). Genital HPVs are pathologically classified into two groups, high-risk types associated with malignant tumors and low-risk types associated with benign tumors. HPV-16 and -18 were first classified as high-risk types in 1986 (21), and then eight other HPVs (HPV-31, -33, -35, -39, -45, -51, -52, -56, and -58) were added in 1992 (9). However, all 39 genital HPVs were not yet rationally classified in these groups, and HPVs designated as high-risk types were different from study to study (5, 7, 8, 15). For example, Jacobs et al. designated 14 high-risk types: HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68 (8). On the other hand, Gravitt et al. designated 15 high-risk types: HPV-16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -55, -56, -58, -59, and -68 (5). Surprisingly enough, HPV-66, classified as a high-risk type in the former study, was considered a low-risk type in the latter. It was not known how each HPV was classified in these studies. We speculated that any HPV detected in invasive cervical carcinoma (ICC) might be categorized as a high-risk type (1). However, the mere detection of viral fragment DNA in ICC is not sufficient to establish the causality of ICC. To be considered an etiologic agent for ICC, any HPV DNA should be histopathologically demonstrated in at least a single case of ICC by in situ hybridization (18).

In the present study, we report the cloning and nucleotide sequencing of a novel genital HPV, partial nucleotide sequences of which have been detected in ICC by PCR. In addition, we examined the histopathologic localization of the viral DNA in different genital lesions.

MATERIALS AND METHODS

Histopathology, immunohistochemistry, and in situ hybridization. The biopsy specimens were divided into two parts, one of which was fixed in 10% buffered formalin for routine histopathology. In addition, paraffin sections were tested by immunohistochemistry for the presence of viral capsid antigen with an anti-HPV capsid antibody, K1H8 (Dako Corp., Carpinteria, Calif.). Briefly, sections were incubated with the antibody at 4°C overnight and sequentially reacted with biotinylated anti-mouse immunoglobulin G antibody and the avidin-biotin complex solution (Vector Laboratories, Burlingame, Calif.). To examine the histological localization of HPV DNA in the lesion, paraffin sections were applied to in situ hybridization as reported previously (19). Briefly, after proteinase K treatment, the sections were fixed in 4% paraformaldehyde-0.1 M phosphate-buffered saline (pH 7.4) and dehydrated. The sections were then mounted with 20 μl of hybridization buffer containing 10 ng of biotinylated HPV DNA probe and denatured at 95°C for 5 min. After hybridization at 42°C for 14 h, the sections were washed three times in 0.2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 50°C and blocked with Blockace (Snow Brand, Tokyo, Japan) at room temperature. Finally, the biotinylated viral DNA was visualized by incubation with a streptavidin-alkaline phosphatase complex, followed by nitroblue tetrazolium–5-bromo-4-chloro-3-indolyphosphate treatment (Dako in situ detection kit; Dako, Kyoto, Japan). The sections were counterstained with 2% methyl green.

Molecular cloning and nucleotide sequencing of HPV genome. The other part of the biopsy specimens was used for HPV analysis by blot hybridization (12). Briefly, total DNA from biopsy specimens was digested with an appropriate restriction enzyme and electrophoresed in a 1% agarose gel, with 40 mM Tris-acetate–2 mM EDTA (pH 8.0) as running buffer. After transfer to a nitrocellulose filter, DNA was hybridized with 32P-labeled HPV-58 DNA probe at a Tm–40°C in 5× SSC–50 mM HEPES buffer (pH 7.0)–0.02% each polyvinylpyrrolidone and Ficoll–20% formamide at 42°C. The filter was then washed at a Tm–40°C and exposed to X-ray film with intensifying screens at –80°C.

Molecular cloning of the HPV genome was done as reported previously with some modifications (11). Total DNA was digested with a single-cut enzyme and electrophoresed in a 1% agarose gel. The 7- to 8-kb fragment was removed and purified by electrophoresis. One hundred nanograms of the DNA was ligated with 50 ng of plasmid DNA and used to transform Escherichia coli JM109. From the resulting colonies, positive clones were selected by hybridization analysis with an HPV-58 probe. HPV DNA was sequenced by the primer walking method (3) and the dye terminator cycle sequencing method. For the sequence comparison, the

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complete nucleotide sequences of HPV-26 (X744272) and HPV-51 (M62877) were obtained from the databases and analyzed with DNASIS software (Hitachi Software Engineering, Tokyo, Japan).

Nucleotide sequence accession numbers. The nucleotide sequences established in the present study have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession no. AB027020 for HPV-69 and AB027021 for HPV-82.

RESULTS

Molecular cloning of HPV-82 DNA and in situ hybridization. Under colposcopy, a lesion showing sharply demarcated white epithelium was biopsied (Fig. 1a). It was histopathologically diagnosed as vaginal intraepithelial neoplasia (VAIN) grade I (Fig. 1b), and HPV capsid antigen was detected within the nuclei of the superficial koilocytic cells by immunohistochemistry (Fig. 1c). Subsequently, we looked for HPV DNA in the lesion by blot hybridization with $^{32}$P-labeled HPV-58 DNA probe at a $T_m$ $-40^\circ$C. As shown in Fig. 2, the lesion harbored a possible HPV DNA with characteristic PstI, BamHI, and MspI cleavage patterns, different from those of any known HPVs. In addition, the positive bands were detected at the positions of forms I, II, and III of a circular double-stranded DNA of approximately 8 kb (FI, FII, and FIII in lanes BamHI, EcoRI, and EcoRV, respectively), although the intensity of form I DNA was extremely low. Furthermore, it was assumed that forms I and II were converted to form III with XbaI digestion. Accordingly, we cloned the DNA by using a plasmid vector, pBluescript KS (Stratagene, La Jolla, Calif.), and a possible single-cut restriction enzyme, XbaI. We then determined partial sequences of the cloned DNA, corresponding to the L1 open reading frame (ORF) of HPV, which showed less than 90% nucleotide sequence identity to L1 sequences of any known HPVs at that time. The clone was named HPV-82. The VAIN lesion was revealed to harbor more than 3,000 viral copies per cell by blot hybridization with the cloned HPV-82 DNA as probe. Furthermore, we examined the presence of other genital HPVs in the lesion by blot hybridization with different HPV probes and found no other HPV. Consequently, the histological localization of HPV-82 DNA in the lesion was examined by using paraffin sections and biotinylated HPV-82 DNA probe. As can be seen in Fig. 1d, viral DNA was abundantly found in the nuclei of superficial cells of the lesion as was the viral capsid antigen; moreover, it was disclosed in deeper intermediate cells than was the antigen. Together with the blot hybridization analysis, it was assumed that the whole genome of HPV-82 might replicate in most cells of the lesion and produce progeny virions in some superficial cells. In our series of 220 cervical intraepithelial neoplasias (CINs), HPV-82 was identified in one case each of CIN II and CIN III by blot hybridization, and the viral DNA was demonstrated in the lesions by in situ hybridization (data not shown). The results indicated that HPV-82 has a potential to induce VAIN and CIN.

Nucleotide sequences of HPV-69 and HPV-82. By L1 sequence analysis, HPV-82 was closely related to HPV-26, -51, and -69. In addition, it was noted that the MM4 sequence in the databases was nearly identical to a part of the L1 sequence.

FIG. 1. VAIN. (a) Colposcopic appearance of VAIN lesion from which HPV-82 was cloned. (b) Paraflin section of the lesion stained with hematoxylin and eosin. (c) Immunohistochemistry with anti-HPV capsid antibody. (d) In situ hybridization with biotinylated HPV-82 DNA probe. Scale bars = 500 μm.
of HPV-82 (442 of 455 bp [97%]). To know the precise relationship among the four HPVs, we determined the entire nucleotide sequence of HPV-82, as well as that of HPV-69, cloned originally in our laboratory (17). HPV-69 consists of 7,700 nucleotides with a 38.8% GC content, while HPV-82 consists of 7,871 nucleotides with a 40.2% GC content. It was notable that restriction enzyme cleavage patterns deduced from the sequences corresponded well to the patterns detected by blot hybridization. For example, PstI fragments of the HPV-82 sequence were 3,892, 3,621, and 358 bp, which were consistent with the fragment sizes seen in lane PstI of Fig. 2. It was assumed that the whole genomes of HPV-69 and HPV-82 were cloned without deletion. Both HPVs have all major ORFs on one strand, characteristic of papillomavirus. The positions of ORFs and the numbers of amino acids of their putative encoded proteins are summarized in Table 1. We then compared the sequences with those of HPV-26 (7,855 nucleotides; 38.6% GC) and HPV-51 (7,808 nucleotides; 39.1% GC). As shown in Table 1, the four HPVs had ORFs with nearly identical sizes and no initiation codon in the E5 ORF, although HPV-26 and -69 also had no initiation codon in the E4 ORF. By fixing the position of the E6 ORF at the first of three reading frames, it was revealed that the frame position patterns for ORFs of these HPVs were quite different from those of the other genital HPVs. For example, the E7 ORF of these four HPVs was positioned at the first or second frame, whereas the E7 ORF of 31 other genital HPVs, except HPV-52, -59, and -73, was in the third frame (data not shown). The percent similarities of nucleotide and amino acid sequences of each ORF among HPV-26, -51, -69, and -82 are summarized in Table 2. The HPVs had high similarities in all ORFs. To be precise, HPV-26 and -69 and HPV-51 and -82 were much more closely related to each other. Each set of two HPVs exhibited similar features, such as GC content, frame position patterns for ORFs, and ORFs without initiation codons.

**DISCUSSION**

By phylogenetic analysis of partial L1 sequences of papillomavirus, HPV-82, as MM4, had been classified in group A5 of supergroup A, together with HPV-26, -51, and -69 (2). As clustered in the group, the four HPVs had extensive nucleotide and amino acid sequence similarities for all the other ORFs (Table 2), suggesting that the HPVs might have similar biological activities in inducing genital lesions. Owing to analysis of whole genomes, it was revealed that this group has no initiation codon in E5 and/or E4 ORFs and exhibits distinct frame position patterns for ORFs (Table 1). Although there

**TABLE 1. Positions of ORFs of HPV-69 and HPV-82**

| ORF | HPV-69 | HPV-82 | HPV-26 | HPV-51 |
|-----|--------|--------|--------|--------|
|     | First nt* | First ATG | Last nt | No. of amino acids | Frame position | First nt | First ATG | Last nt | No. of amino acids | Frame position | No. of amino acids | Frame position | No. of amino acids | Frame position |
| E6  | 75      | 102     | 557    | 151     | 1 | 72 | 102 | 557 | 151 | 1 | 150 | 1 | 151 | 1 |
| E7  | 558     | 564     | 878    | 104     | 1 | 487 | 565 | 867 | 100 | 2 | 104 | 1 | 101 | 2 |
| E1  | 847     | 886     | 2790   | 634     | 2 | 738 | 876 | 2804 | 642 | 1 | 638 | 2 | 634 | 1 |
| E2  | 2666    | 2732    | 3838   | 368     | 3 | 2719 | 2746 | 3825 | 359 | 2 | 375 | 3 | 358 | 2 |
| E4  | 3309    | 3614    | 101    | 1b      | 1 | 3323 | 3335 | 3601 | 88  | 3 | 106 | 1b | 87  | 3 |
| E5  | 3846    | 4142    | 98     | 1b      | 1 | 3885 | 4139 | 84  | 41  | 1b | 85  | 3b | 84  | 2b |
| L2  | 4133    | 4157    | 5560   | 467     | 3 | 4072 | 4168 | 5547 | 459 | 2 | 472 | 1 | 468 | 3 |
| L1  | 5499    | 5541    | 7064   | 507     | 1 | 5469 | 5571 | 7082 | 503 | 1 | 503 | 2 | 504 | 1 |

*a* nt, nucleotide.

*b* Initiation codon absent.
was no explanation of the significance of the features, they might implicate some biological and functional effects.

This group might be distinct among HPVs, having tropism both for the skin and for the genital mucosa. HPV-26 was originally cloned from papillomas on the skin (14) and found in cervicovaginal lavage specimens (7). On the other hand, HPV-51 was cloned from a cervical condyloma (13) and was detected in basal cell carcinomas of the skin (16). In addition, HPV-69 and HPV-82, cloned from VAIN lesions, were found in a dysplastic wart or in squamous cell carcinomas of the skin (16; T. Matsukura, unpublished data).

HPV-82, as W13B/MM4, was first found in exfoliated cells of women with normal cytology by sequencing of PCR amplimer (10). Table 3 shows the distribution of HPV-82, as well as of HPV-26, -51, and -69, in different geographic areas. In study 1, we analyzed biopsy specimens by blot hybridization (12, 17). In contrast, smear and biopsy specimens were examined by PCR in study 2 (20) and study 3 (1), respectively. It was clear that HPV-82 is widespread. Since we confirmed the presence of HPV-51 and -69 DNA in our cases by in situ hybridization, this group should be etiologic agents for CIN or VAIN. However, we wondered whether the group might be responsible for inducing ICC (5, 7). To establish the causality for ICC, it would be essential to histopathologically demonstrate the viral DNA in ICC, as mentioned above.

In conclusion, we have cloned HPV-82, closely related to HPV-26, -51, and -69, and determined the complete nucleotide sequences of HPV-69 and -82. The four HPVs might be a distinct group among genital HPVs, exhibiting unique frame position patterns for ORFs.

### Acknowledgments

We gratefully acknowledge the help of H. Delius and E.-M. de Villiers in the identification of new HPV types.

This research was supported by grants from the Ministry of Health and Welfare for 2nd-Term Comprehensive 10-Year Strategy for Cancer Control, Japan.

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