Detection of Human Papillomavirus DNA in Normal Epithelium and in Squamous Metaplasia of the Uterine Cervix by the Polymerase Chain Reaction

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The presence of human papillomavirus (HPV) 16 was studied by using the polymerase chain reaction (PCR) directly on sections histologically defined as normal squamous epithelium and metaplastic squamous tissue of the uterine cervix. Ten specimens of normal epithelium were obtained at hysterectomy from women with uterine leiomyoma. Six specimens of squamous metaplasia were adjacent to the areas of dysplastic epithelium in which HPV 16 DNA had been detected previously by PCR. HPV 16 DNA was amplified specifically and detected in two of 10 normal cervical epithelium specimens and in all of 6 squamous metaplasia specimens adjacent to dysplastic lesions. However, HPV DNA could not be detected in the metaplasia by in situ hybridization. These results suggest that metaplastic squamous tissue adjacent to dysplastic lesions harbors fewer copies of HPV DNA than the dysplastic area and the carcinoma and that the HPV copy number per cell may be relevant to the pathogenesis of cervical carcinoma.

uterine cervix; normal epithelium; squamous metaplasia; human papillomavirus; polymerase chain reaction

In recent years, many studies were undertaken to determine the relationship between pathogenesis of cervical cancer and human papillomavirus (HPV) (Reid et al. 1980; Dürst et al. 1983; zur Hausen and Schneider 1987). We reported previously that HPV DNA was detected in only a few percent of healthy women, by the dot blot hybridization (Konno et al. 1991). Other researchers also detected HPV DNA more frequently in healthy women using the high sensitive polymerase chain reaction (PCR) method (Saiki et al. 1988; Shibata et al. 1988a, b; Young et al. 1989). To elucidate the natural history of cervical cancer, it would be important to determine whether HPV is present in the healthy cervix or its benign lesions. We conducted the present study on HPV in the normal and squamous...
metaplasia using PCR to investigate the role of the virus in the development of cervical carcinoma.

**Subjects and Methods**

*Subjects.* From patients who underwent total hysterectomy due to uterine myoma, 10 specimens of normal cervical epithelium, which were not abnormal on postoperative pathological examination, were selected. In 6 patients with squamous metaplasia of the cervix, the areas of squamous metaplasia were adjacent to the areas of cervical dysplasia in which positivity to HPV 16 DNA had been confirmed previously by in situ hybridization and PCR.

*In situ hybridization.* The hybridization was performed using the Viratype in situ kit (Life technology Inc., Gaithersburg, MD, USA) by the method described in our previous report (Konno et al. 1992). The lower limit of the detection of HPV 6/11, 16/18 and 31/33/35 DNA was about 20-50 copies per cell (Richart and Nuovo 1990).

*Preparation of samples for PCR.* By microscopic observation of the hematoxylin-eosin stained specimens of the subjects described above, target regions in formalin-fixed and paraffin-embedded tissues were selected. After removing of excess tissue and paraffin, new paraffin blocks consisting only of the tissues to be examined were prepared. Two 5 μm-thick sections were made from the block, deparaffinized by xylene, dehydrated by ethanol and dried in a 500 μl tube. After treatment with 100 μl of proteinase K solution (200 μg/ml in 50 mM Tris-HCL [pH 8.0]); Boehringer Mannheim Biochemical, Mannheim, FRG) for 3 hr at 55°C, the proteinase was inactivated by heating to 95°C for 8 min. For PCR, 10 μl of the supernatant after the centrifugation for 30 sec was used as a sample.

*Amplification of HPV DNA by PCR.* Two 20-mer oligonucleotides for PCR primers were synthesized with a Cyclone DNA synthesizer (Milli Gene/Biosearch, Milford, MA, USA). The sequences of the primers 1 and 2 corresponded to the E6/E7 region of open reading frame (Seedorf et al. 1985), so that a DNA sequence of 177 bp would be amplified if HPV 16 was present. Moreover, these primers for HPV 16 had high homology with HPV 52b, in which a DNA sequence of 171 bp would be amplified. HPV 16 and 52b were identified by the difference in the restriction enzyme cleavage pattern. HPV 16 was cleaved into 105 and 72 bp fragments using Pvu II (Takara Shuzo, Shiga), and HPV 52b was cleaved into 115 and 56 bp fragments using Bgl II (Takara Shuzo). It was confirmed previously that the PCR assay used in the present study could not amplify HPV 6, 11, 18, 31, 33 or 35 DNA, and a few as 10 copies of HPV 16 genomic DNA per tube could be detected (Yaegashi et al. 1991; Konno et al. 1992).

PCR was performed using a DNA amplification kit (GeneAmp, Perkin-Elmer Cetus, Norwalk, CT, USA). Briefly, 100 μl of a solution containing 10 mmol Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (W/V) gelatin, 2.5 units Taq polymerase, 200 μmol each of dNTP, and 1 μmol each of primers 1 and 2 together with 10 μl of the sample described previously were treated with 40 cycles of incubation using a DNA thermal cycler (Perkin-Elmer Cetus) at 95°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 2 min (extension) to amplify the target DNA. The PCR product was purified using phenol-chloroform, precipitated with ethanol, and subjected to electrophoresis on 2% agarose gel, in which bands were detected by ethidium bromide staining and brief ultraviolet illumination.

**Results**

Ten normal cervical epithelium and six squamous metaplasia epithelium were examined for the presence of HPV DNA by the PCR. Two (20%) of ten normal cervical epithelium were HPV 16 DNA-positive (Figs. 1 and 2). These positive
cases were asymptomatic and had no abnormal pathological findings. This result suggests that HPV causes silent infection.

All of six specimens of squamous metaplasia tissues adjacent to dysplasia which contained HPV 16 were also positive for HPV 16 (Table 2, Fig. 3). We determined the presence of HPV DNA in the metaplastic lesions by a more sensitive method, PCR, although we could not detect HPV 16 using in situ
TABLE 1. Sequences of oligonucleotide primers for PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Corresponding genomic location in HPV 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>5'-AATAAAAGGGTGCGGCTTAAAATTGC-3'</td>
<td>482-501</td>
</tr>
<tr>
<td>2</td>
<td>5'-CTGAGCTGTCATTTAATTGC-3'</td>
<td>639-658</td>
</tr>
</tbody>
</table>

*Primer 1 corresponds to the sense sequence of the HPV 16 E6 ORF (open reading frame) and primer 2 corresponds to the antisense sequence of the HPV 16 E7 ORF. The primers are highly homologous to the sequence of the HPV 52b, so that PCR with these primers also amplifies the 177bp fragment for HPV 16 and the 171bp fragment for HPV 52b. There are 3 (primer 1; A→T, G→T) and 2 (primer 2; T→C) base mismatches to HPV 52b. Mismatched nucleotides are underlined in the above table. PCR derived products of HPV 16 and HPV 52b carry the restriction sites for of Pst II and Bgl II, respectively.

TABLE 2. Presence of HPV DNA in metaplastic squamous epithelium and adjacent lesions

<table>
<thead>
<tr>
<th>Case No.</th>
<th>HPV DNA in squamous metaplasia by PCR*</th>
<th>Pathological diagnosis of adjacent lesions</th>
<th>HPV DNA in adjacent lesion by ISH*</th>
<th>HPV DNA in adjacent lesion by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPV 16</td>
<td>Severe dysplasia</td>
<td>HPV 16/18</td>
<td>HPV 16</td>
</tr>
<tr>
<td>2</td>
<td>HPV 16</td>
<td>Mild dysplasia</td>
<td>HPV 16/18</td>
<td>HPV 16</td>
</tr>
<tr>
<td>3</td>
<td>HPV 16</td>
<td>Moderate dysplasia</td>
<td>HPV 16/18</td>
<td>HPV 16</td>
</tr>
<tr>
<td>4</td>
<td>HPV 16</td>
<td>Severe dysplasia</td>
<td>—</td>
<td>HPV 16</td>
</tr>
<tr>
<td>5</td>
<td>HPV 16</td>
<td>Mild dysplasia</td>
<td>—</td>
<td>HPV 16</td>
</tr>
<tr>
<td>6</td>
<td>HPV 16</td>
<td>Moderate dysplasia</td>
<td>—</td>
<td>HPV 16</td>
</tr>
</tbody>
</table>

*PCR, polymerase chain reaction; *ISH, in situ hybridization; *HPV 16/18, positive for HPV 16 and/or 18.

Fig. 3. Hematoxylin-eosin stained formalin-fixed and paraffin-embedded tissue section from case 2 of squamous metaplasia adjacent to dysplasia containing HPV 16 DNA as detected by PCR. (original magnification ×240)
hybridization. These results indicate that there were fewer copies of HPV DNA in the area of squamous metaplasia than in the adjacent area of dysplasia or in the carcinoma.

**DISCUSSION**

PCR in extremely sensitive, but can amplify even minute amounts of a contaminant (Kwok and Higuchi 1989). We took great care, in the present study, to prevent extraneous contamination. For example, disposable forceps and gloves were used to prepare sections from paraffin blocks and microtome blades were changed for each specimen. Reagents were autoclaved as far as possible and aliquoted according to requirements. Sufficient care was taken in handling pipettes and tubes, and the PCR was carried out on a negative control which contained no template DNA.

While it was reported that DNA could not be amplified by PCR in some paraffin-embedded tissues (de Franchis et al. 1988), we were able to amplify β-globin genes directly from the paraffin-embedded tissues used in the present study, indicating that the method was also applicable to HPV in those tissues.

Since HPV DNA can be detected in cervical cancer tissues, there have been many studies on the mechanism by which it participates in carcinogenesis. The likelihood of detection of HPV DNA in squamous cell carcinoma of the cervix is increasing gradually as the method is improved and as the number of cases studied and the number of types of HPV DNA probes increase. zur Hausen stated that HPV 16 and 18 could be detected in 70%, HPV 33 in 10% and the other HPVs in the remainder of the cases to compromise a total of 100% eventually (zur Hausen 1989). We also detected HPV 16, 18, 31, 33, 35 or 52b in 92% of cervical cancer tissues by combining in situ hybridization with the PCR (Konno et al. 1992).

It is important to determine when and where HPV infection occurs to understand carcinogenesis of cervical cancer. HPV infection can be detected in squamous metaplasia adjacent to a dysplastic lesion which contains HPV DNA. We believe that there are fewer copies of HPV DNA in squamous metaplasia than in dysplasia or carcinoma in situ. This is because HPV DNA cannot be detected in squamous metaplasia by in situ hybridization for which the lower limit of HPV DNA detection is about 20-50 copies per cell from the examination of HeLa cells (Richart and Nuovo 1990); 3 out of 6 cases were examined by in situ hybridization in this study.

The presence of HPV DNA, however, is proven for the first time by the PCR, a more sensitive technique. Cornelissen et al. (1989) reported that HPV can not necessarily be detected by PCR in cervical tissues which surround malignant or dysplastic cells. HPV is not found in normal regions which are adjacent to cervical intraepithelial neoplasia (CIN) lesions, but it is present only in CIN or in koilocytosis. However, Shimano et al. (1990) reported that they detect HPV
DNA in squamous metaplasia by in situ hybridization. It is suggested, therefore, that a small number of HPV copies are present in squamous metaplasia which surrounds malignant or dysplasia which contains HPV.

In normal epithelium, HPV may be present at a low rate. In the present study, HPV DNA was detected in 20% of cervical tissues in which no abnormality was found by histological examination. Parakeratosis was found in 2 cases with HPV. It reported that parakeratosis, dyskeratosis and multinucleation were common in HPV infection, but this was not a specific finding and should be used only as a crude indicator of HPV infection (Takamura et al. 1991). Other researchers have also reported that HPV 16 DNA can be found in 30 to 70% of normal cervical tissues using PCR (Shibata et al. 1988b; Young et al. 1989). This would suggest that HPV infection is often silent. In future studies, we will clarify how HPV infection may cause squamous metaplasia.

References


