Simultaneous detection and typing of genital human papillomavirus DNA using the polymerase chain reaction

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A simple method has been developed for detecting a broad range of genital human papillomavirus (HPV) types using the polymerase chain reaction (PCR). We utilized two consensus sequence primer pairs within the E6 and E7 open reading frames to amplify HPV DNA; malignant HPV DNA (from HPV-16, -18, -31, -33, -52b and -58) was amplified using the pU-1M/pU-2R primer pair whereas benign HPV DNA (from HPV-6 and -11) was amplified using the pU-31B/pU-2R primer pair. Identification of the amplification product was confirmed by restriction enzyme digestion.

In this study, a pU-1M/pU-2R-mediated PCR was successfully applied to 39 cervical carcinoma specimens; HPV-16 was detected in 19 cases, HPV-18 in five cases, HPV-31 in two cases, HPV-33 in two cases, HPV-52b in one case, HPV-58 in three cases, and an unknown type(s) was detected in four cases. Overall, the prevalence of HPV was 84.6%. The results indicate that this detection system is useful for the detection of HPVs not only of known types but also of new types.

Introduction

In recent years, a linkage between human papillomavirus (HPV) infection and cervical carcinoma has been suggested. Of the more than 60 different types of HPV identified (de Villiers, 1989), individual HPV types which infect the cervix show varying degrees of oncogenic association. In general, HPV-6 and HPV-11 are associated with benign cervical lesions (Lorincz et al., 1987), whereas HPV-16, HPV-18 and HPV-31 (Lorincz et al., 1987), HPV-33 (Beaudenon et al., 1986), HPV-52b (Shimoda et al., 1988; Yajima et al., 1988), HPV-58 (Matsukura & Sugase, 1990) and other types (de Villiers, 1989) have been found mainly in cervical intraepithelial neoplasia and cervical carcinoma. The accurate and efficient detection and typing of various HPV types in normal and diseased tissue samples are useful for understanding the role they play in the development of cancer.

We previously reported an HPV detection system based on a type-specific polymerase chain reaction (PCR) (Shimada et al., 1990). This system was simpler, more sensitive and more specific than the Southern blot hybridization method, although detectable HPV genotypes were limited to HPV-16, -18 and -33. A newly developed PCR-based method described here provides a simple accurate means of detecting a broad range of genital HPVs. From the nucleotide sequences of genital HPVs [HPV-6 (Schwarz et al., 1983), -11 (Dartmann et al., 1986), -16 (Seedorf et al., 1985), -18 (Cole & Danos, 1987), -31 (Goldborough et al., 1989), -33 (Cole & Streeck, 1986), -52b (Y. Ito, unpublished results), and -58 (T. Matsukura, unpublished results)], two pairs of consensus primers were designed, which were similar in sequence to sequences in the E6 and E7 open reading frames (ORFs).

In cervical carcinoma, the genome of integrated HPV has been often found to contain deletions but the long control region (LCR), and the E6 and E7 ORFs are preferentially conserved (Schwarz et al., 1985; Shirasawa et al., 1987; Pater & Pater, 1985; Matsukura et al., 1986; Choo et al., 1988). Recently developed PCR methods which have been used to identify HPV DNA in clinical samples have utilized primers with sequences homologous to regions of the E1 (Gregoire et al., 1989; van den Brule et al., 1990) and the L1 ORFs (van den Brule et al., 1990; Manos et al., 1989; Snijders et al., 1990). Our method is thought to be more reliable than these as it is less likely to produce false negative results.

Methods

DNA preparation from clinical samples. Tissue specimens from cervical carcinomas obtained from Sapporo Medical College Hospital, Sapporo, Japan, were frozen in liquid nitrogen and stored at -70 °C.
until use. Further processing and DNA extraction were carried out as described by Davis et al. (1986); extracted DNA (1 μg) was used in the amplification reaction.

**Primer synthesis.** Primers were synthesized on a DNA synthesizer (MidiGen 7500) and purified on an Oligonucleotide Purification Cartridge (Applied Biosystems). β-Globin primers (sense primer, ACACAAACTGTTCTACAGC; antisense primer, GAAAAATAGCCTAGGAGCAG) from Takara Shuzo were used.

**PCR.** Amplification reactions were done using the heat-stable *Thermus aquaticus* (Taq) polymerase (Saiki et al., 1988). Cloned pHPV DNA (1 ng), or cellular DNA (1 μg) was subjected to PCR. To assess the sensitivity of the assay, dilutions of several pHPV DNAs mixed with 1 μg normal human cervical DNA were also subjected to PCR. These DNA solutions were incubated for 10 min at 94 °C and chilled quickly for DNA denaturation. The reaction mixture of 100 μl contained 50 mM-KCl, 10 mM-Tris-HCl pH 8.3, 1.5 mM-MgCl₂, 200 μM of each dNTP (dATP, dGTP, dCTP and dTTP), 100 μg/ml gelatin, 2.5 units Taq polymerase (Perkin-Elmer Cetus) and 100 pmol of each consensus primer (pU-1M/pU-2R or pU-31B/pU-2R). The mixture was overlaid with 70 μl paraffin oil and subjected to 30 cycles of amplification using a DNA Thermal Cycler (Perkin-Elmer Cetus). Each cycle included a denaturation step at 94 °C for 1 min, an annealing step at 55 °C for 2 min, and a chain elongation step at 72 °C for 2 min. To avoid false positives, a reagent control (no template DNA) was included with each amplification. PCR product (10 μl) was electrophoresed on a composite gel consisting of 3% NuSieve agarose (FMC Bioproducts) and 1% Seakem agarose (FMC Bioproducts), to obtain adequate resolution of low M₁ DNA fragments, and then stained with ethidium bromide and photographed under u.v. light.

**Restriction enzyme analysis.** PCR product (90 μl) was purified by phenol–chloroform–isoamyl alcohol (25:24:1, v/v) extraction followed by ethanol precipitation, and suspended in 34 μl H₂O. A quarter of the p-I reaction mixture for 2 h at 37 °C. Digestion products were analysed on composite gels consisting of 3% NuSieve agarose (FMC Bioproducts) and 1% Seakem agarose (FMC Bioproducts), to obtain adequate resolution of low M₁ DNA fragments, and then stained with ethidium bromide and photographed under u.v. light.

**Results**

**Design of consensus primers**

Our aim was to design amplification primer pairs that would yield PCR products from any genital HPV. Of the genital HPVs, HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-52b, HPV-58 have been completely sequenced. Homology analysis of the nucleotide sequences of these HPVs was done using the DNASIS program (DNA Sequence Input System; Hitachi Software Engineering), specifically on the LCR, and E6 and E7 ORFs. Based on the similar sequences identified, two pairs of consensus primers of 20 nucleotides were designed (pU-1M/pU-2R and pU-31B/pU-2R); Fig. 1 shows the location of the consensus primers on the HPV-16 genome. The sense primers, pU-1M and pU-31B, are located in the middle of ORF E6 and the antisense primer, pU-2R, is located in the middle of the E7 ORF. The nucleotide sequences of the primers are shown in

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**Fig. 1.** Location of consensus primers in the HPV-16 genome. Open boxes indicate ORFs; NCR is the non-coding region between the L1 and E6 ORFs. The nucleotide numbering and the ORFs are based on published DNA sequences (Seedorf et al., 1985). Arrowheads represent the 3' end of each primer.

**Fig. 2.** Alignment of the consensus primers with the corresponding sequences of several HPV types. The symbol represents identical bases; mismatched bases are indicated. The 5' nucleotide position of the sequences and the number of mismatches are given.
**Application of consensus primers in the PCR on pHPV DNAs**

The PCR was performed under the conditions described in Methods on DNA from cloned pHPVs using both primer pairs (Fig. 3). The pU-1M/pU-2R primer pair yielded approximately 230 to 270 bp PCR products with HPV-16, -18, -31, -33, -52b and -58 DNAs, but no amplification was observed with HPV-2, -6 and -11 DNAs (Fig. 3a). When the pU-31B/pU-2R primer pair was used, fragments of about 230 bp were seen with HPV-6 and HPV-11 DNAs only (Fig. 3b). The sensitivity of the PCR was determined by examination of different concentrations of pHPV-16 DNA diluted in normal human cervical DNA (HPV-negative). Fig. 4 shows that 0.1 copies of the HPV-16 genome per cell could be detected by ethidium bromide staining.

**Restriction enzyme analysis of PCR products**

Typing of the products generated by pU-1M/pU-2R and pU-31B/pU-2R was performed by restriction enzyme digestion. In Table 1 (a, b) the size of each restriction

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**Table 1. Restriction fragment sizes of consensus PCR products**

(a) pU-1M/pU-2R products

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Virus</th>
<th>HPV-16</th>
<th>HPV-18</th>
<th>HPV-31</th>
<th>HPV-33</th>
<th>HPV-52b</th>
<th>HPV-58</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length</td>
<td></td>
<td>238</td>
<td>268</td>
<td>233</td>
<td>244</td>
<td>231</td>
<td>244</td>
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<tr>
<td>AvaII</td>
<td></td>
<td>157, 81</td>
<td>172, 96</td>
<td>NC*</td>
<td>136, 108</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>RsaI</td>
<td></td>
<td>NC</td>
<td>NC</td>
<td>119, 114</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>BglII</td>
<td></td>
<td>NC</td>
<td>NC</td>
<td>176, 55</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>AccI</td>
<td></td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>126, 118</td>
</tr>
</tbody>
</table>

(b) pU-31B/pU-2R products

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Virus</th>
<th>HPV-6</th>
<th>HPV-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length</td>
<td></td>
<td>228</td>
<td>228</td>
</tr>
<tr>
<td>AvaII</td>
<td></td>
<td>132, 96</td>
<td>166, 62</td>
</tr>
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</table>

* NC, No cut.
fragment is given for the predicted PCR products; Fig. 5 shows the results of restriction enzyme digestion of the PCR products. Digestion patterns were in agreement with the sizes estimated from the sequence data. The HPV-16, -18 and -33 products each contained an \textit{AvaII} site but gave different digestion patterns; other HPV types contained no \textit{AvaII} site. HPV-31 contained an \textit{RsaI} site, HPV-52b contained a \textit{BglII} site and HPV-58 contained an \textit{AccI} site which was not present in any other HPV type. HPV-6 and HPV-11 could be identified by \textit{RsaI} digestion. Using these restriction enzymes, we could identify each type of HPV clearly and efficiently.

**HPV prevalence in cervical carcinomas**

Using amplification with the pU-1M/pU-2R primer pair and subsequent restriction enzyme analysis, we analysed 39 cervical carcinomas. Specimens were prepared for PCR as described in Methods; an aliquot representing about $10^5$ cells (1 μg DNA) was used for each amplification reaction. PCR products were first analysed by agarose gel electrophoresis and all positive samples were analysed for their HPV type by \textit{AvaII}, \textit{RsaI}, \textit{BglII}, and/or \textit{AccI} digestion; examples are shown in Fig. 6. Carcinoma specimens 113 and 127 showed an
Fig. 6. Typing of clinical samples by restriction enzyme analysis. Carcinoma specimens (1 μg) and 1 ng of various pHPV DNAs were amplified using the pU-1M/pU-2R primer pair. (a) PCR products digested with AvaII; lanes 1 to 8, Mr standards (HaeIII-digested φX174 DNA), HPV-16, HPV-18, HPV-33, and specimens 113, 127, 128 and 132, respectively. (b) PCR products digested with AvaII and AccI; lanes 1 and 4, Mr standards (HaeIII-digested φX174 DNA); lanes 2 and 3, HPV-58 and specimen 132 respectively.

HPV-16 pattern; specimen 128 showed HPV-16 and -18 patterns; specimen 132 showed an HPV-18 pattern and a no-cut fragment (Fig. 6a). AvaII/AccI digestion of specimen 132 showed HPV-18 and -58 patterns (Fig. 6b). Table 2 shows the HPV type of the 39 cervical carcinomas; HPV-16 was detected in 19 cases, HPV-18 in five cases, HPV-31 in two cases, HPV-33 in two cases, HPV-52b in one case, HPV-58 in three cases and an unknown type(s) was detected in four cases. Overall the prevalence of HPV was 84.6%. A number of positive cases involved mixed infections (HPV-16 and -18, -18 and -33, and -18 and -58). Four cases classified as being of 'unknown type(s)' did not have AvaII, RsaI, BglII or AccI sites. The six HPV-negative samples were amplified using the PCR with β-globin primers (data not shown), indicating that sufficient cellular DNA was present in the samples and that the amplification reaction was not inhibited.

Discussion

The PCR technique (Saiki et al., 1985) has made possible the specific amplification of viral DNA sequences present in clinical samples. HPV type-specific primers have been used for amplification of HPV-6, -11, -16, -18 and -33 nucleotide sequences (Sibata et al., 1988; Melchers et al., 1989; Young et al., 1989), but the number of primers necessary to amplify DNA specifically from every HPV type would make PCR impractical for the typing of clinical specimens. Consequently, efforts were made to use the PCR for the detection of HPVs of different genotype. In addition, it would be useful for clinical diagnosis if benign HPV (HPV-6 and -11) and malignant HPV (HPV-16, -18, -31, -33, -52b and -58) could be amplified separately. Therefore, we designed two sense primers, pU-1M (specific for malignant HPV) and pU-31B (specific for benign HPV), homologous to regions in the E6 ORF, and one antisense primer, pU-2R (common to malignant and benign HPV), homologous to a region in the E7 ORF. Malignant HPV DNA (HPV-16, -18, -31, -33, -52b and -58) was successfully amplified using the pU-1M/pU-2R primer pair (Fig. 3a) and benign HPV DNA (HPV-6 and -11) using the pU-31M/pU-2R primer pair (Fig. 3b). Digestion of PCR products with restriction enzymes could identify HPV genotypes efficiently in clinical samples as well as in cloned pHPV DNAs (Fig. 5 and 6). Furthermore, we identified three cases of mixed infection (HPV-16 and -18, -18 and -33, and -18 and -58) in carcinoma specimens. In the same way, any combination of mixed infection could be identified because restriction fragments from each HPV type are of different sizes.

Recently, a similar PCR/restriction enzyme typing method which detects HPV-16, -18, -33 and -58 DNA using a single primer pair homologous to sequences in the LCR and the E6 ORF was presented by Yoshikawa et al. (1990).

The analysis described here does not need isotopes and is therefore convenient, especially where facilities to handle radioactivity are not available. The procedure is simpler than dot blot hybridization and can be performed within a day, although this system is less sensitive than a dot blot hybridization assay using isotopes. The dot blot hybridization system in conjunction with an HPV-16-, -18-, or -33-specific PCR which we developed previously could detect 10⁻⁵ to 10⁻⁶ copies of the HPV genome-cell (Shimada et al., 1990), but the system described here could detect only more than 0.1 copies/cell (Fig. 4). However, when samples are limited to a carcinoma, this sensitivity is sufficient to determine the presence of HPV. Carcinoma cells are thought of as clonal and the DNA sample derived from them contains very little normal tissue-derived DNA.
This restriction enzyme analysis method is particularly useful for identifying potentially new HPV types; we detected four cases of unknown type using this system. Three of these cases were subjected to direct sequence analysis; these cases had the same sequence in the region amplified using pU-1M and pU-2R as primers and were most closely related to HPV-31 of the HPVs sequenced. Cloning of this type is in progress and will permit a more detailed comparison with HPVs of known type.

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References


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