Localization of Human Papillomavirus Type 16 DNA Using the Polymerase Chain Reaction in the Cervix Uteri of Women with Cervical Intraepithelial Neoplasia

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SUMMARY

The localization of human papillomavirus type 16 (HPV-16) DNA throughout the cervix uteri of women with cervical intraepithelial neoplasia (CIN) was studied by utilizing the polymerase chain reaction technique directly on histologically defined sections of paraffin-embedded cervical tissue obtained by conizations. HPV-16 DNA was detected only in the sections that contained CIN lesions and/or koilocytes. No HPV-16 DNA was detected in sections that contained only normal epithelium. This is in accordance with HPV-16 playing a role in the development of CIN lesions.

INTRODUCTION

Infection by human papillomaviruses (HPVs) has been widely recognized as a risk factor for the development of squamous and glandular neoplasia of the genital tract (zur Hausen et al., 1987; Wilbur et al., 1988a, b). More than 50 HPV types have been defined on the basis of DNA heterology. Relatively few, predominantly HPV-16 and HPV-18, have been identified in cervical squamous cell carcinomas. Additionally the genital types HPV-6 and HPV-11 are mainly associated with benign koilocytotic lesions (Pfister et al., 1987; Muñoz et al., 1988).

The majority of the data relating the HPV type to specific pathology has been collected by Southern blot hybridization. This technique does not allow an exact correlation between the hybridization data and the histology of the tissue because of the destruction of tissue during the DNA extraction. In situ DNA hybridization has allowed the precise cellular localization of probe attachment (Walboomers et al., 1988; Crum et al., 1986), but the sensitivity of this technique is rather low. Recently a promising new method, the polymerase chain reaction (PCR), was described by Saiki et al. (1985). Shibata et al. (1988a) described an adaptation of the PCR for the detection of HPV DNA in paraffin-embedded tissue. We employed this method to correlate the site of histologically classified cervical intraepithelial neoplasia (CIN) lesions with the distribution of HPV-16 DNA in the cervix uteri.

METHODS

Tissue specimens. Tissue specimens were selected from 12 patients who underwent conization for intraepithelial neoplasia. These cones contained, in addition to the neoplastic changes, areas of normal squamous epithelium. Some of the cones also showed viral changes indicated by the presence of koilocytosis. All the cones were cut into five to 12 segments, depending on the size of the cone. From all tissue blocks of each cone 5 μm sections were cut. To prevent contamination from one paraffin block to another, the knife was cleaned with 70% ethanol after each tissue block. Moreover, after cutting one tissue block, a paraffin block without tissue was cut. These paraffin sections without tissue were also analysed with the PCR for β-globin and HPV-16 DNA. From all the paraffin blocks one single section (with or without tissue) was placed in a microfuge tube and sequentially washed with

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PCR. Twenty nucleotide primers shown in Fig. 1 were synthesized (Eurosequence Groningen) on a DNA synthesizer. β-Globin primers were as described by Saiki et al. (1985). The primers were purified by thin-layer chromatography. About 50 μg of each primer was spotted on a Merck Kieselge160 F254 plate and resolved in a mixture of 40% propanol, 15% butanol, 30% ammonia and 15% water. The primers were scraped off the plate and eluted with water from the Kieselgel. The PCR reaction mixture was 10 mM-Tris-HCl pH 9.2, 50 mM-NaC1, 10 mm-MgC12, 0.2 mM of each deoxynucleoside triphosphate (Pharmacia), 0.01% gelatin and 150 ng of each primer. One unit Taq polymerase (Perkin-Elmer Cetus) was used in a reaction of 100 μl. The samples were overlaid with several drops of paraffin oil to prevent evaporation and subjected to 25 or 32 cycles of amplification as follows. The samples were heated at 95 °C for a 1.5 min period (to denature the DNA), cooled to 55 °C for 2 min (to allow annealing) and finally heated to 71 °C for 2.5 min (to activate the polymerase). After the last cycle all samples were incubated for an additional 7 min at 71 °C to ensure that the final extension step was complete. One-tenth of the PCR products was analysed by electrophoresis in a 2% agarose gel and transferred to Zeta-Probe by alkaline blotting as recommended by the manufacturer (Bio-Rad). The filters were hybridized with a 32P end-labelled 50-mer or 60-mer insert probe (see Fig. 1). The end labelling procedure was performed as described by Maniatis et al. (1982).

Selection of the primers and insert hybridization probes. For amplification we selected segments of the E7 and L1 open reading frames (ORFs) of the HPV-16 genome (Seedorf et al., 1985). The E7 segment was selected, since E7 appears to be invariably present in cervical carcinomas (Wilczynski et al., 1988). Amplification of HPV-16 DNA using the E7 primers would result in a 142 bp fragment. To exclude the possibility that positive reactions were due to low levels of pHPV16 (HPV-16 DNA cloned into the BamHI site of pSP65 or pBR322) contamination, we used PCR primers (L1 primers) spanning the BamHI site. Using the L1 primers the PCR would produce an amplified fragment of 3150 bp in the case of pHPV 16 DNA contamination and a 152 bp fragment for the collinear L1 ORF.

Southern blot analysis. Southern blot hybridization was performed as described previously (Cornelissen et al., 1988). Five μg of DNA digested with PstI was subjected to electrophoresis in an 0.8% agarose gel, denatured and transferred to nitrocellulose membranes. The filters were hybridized with a 32P-labelled HPV-16 DNA probe (specific activity 2 × 10^6 to 5 × 10^6 c.p.m./μg). Labelling was performed by the random primer labelling method (Feinberg & Vogelstein, 1983). Hybridization was carried out at 68 °C in 10% dextran sulphate, 6 × SSC (1 × SSC is 0.15 M-sodium chloride, 0.015 M-sodium citrate, pH 6-8), 0.1% SDS and 100 μg sheared salmon sperm DNA/ml. Filters were washed at Tm — 10 °C.

RESULTS

HPV-16 DNA localization

We used the PCR adapted to paraffin-embedded tissue for the localization of HPV-16 DNA in cervical cones, containing CIN lesions and normal epithelium. One paraffin section stained...
Localization of HPV-16 DNA using PCR

Fig. 2. PCR analysis of a paraffin-embedded tissue section from patient 3. PCR analysis of nine tissue blocks of the cone from patient no. 3 (see Table 1) using the E7 primer pair. (a) One-tenth of the reaction mix after 32 amplification cycles was applied to an agarose gel. The positive control (+) was 10 ng HPV-16-positive cervical tumour DNA and the negative control (−) was a section of a paraffin-embedded pancreas. (b) Hybridization under stringent conditions with the E7-specific insert probe. The autoradiogram was exposed for 3 h (−70 °C). (c) A second agarose gel showing the result of the PCR analysis of the same nine sections with the β-globin primer pair.

Table 1. Histological classification correlated with HPV-16 DNA

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* K, normal epithelium with koilocytes; N, normal; I, CIN grade I; II, CIN grade II; III, CIN grade III; −, not applicable.
† Entries in boxes are PCR positives.
‡ Only positive after hybridization.

with haematoxylin and eosin (HE) was histologically analysed. The next two paraffin sections from the same block were used for the detection of HPV-16 DNA and β-globin DNA by the PCR. The next section was again stained with HE for the histological diagnosis of the PCR analysed sections.

A double blind analysis was performed on cervical cones from 12 women and the results obtained from the cone of one of the women (no. 3, see Table 1) are presented in detail. Fig. 2
Fig. 3. HE staining of paraffin-embedded tissue section from patient no. 3. (a) Normal squamous epithelium (section F of Table 1). (b) Area with koilocytotic changes. There is a moderate dysplasia in the lower layer (CIN grade I) (section H of Table 1). (c) Area of dysplastic epithelium, which shows abnormal differentiation in all layers (CIN grade III) (section K of Table 1).
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(a) Tumour DNA

(b) Tumour DNA + PstI

Fig. 4. Reliability of the PCR primers. (a) Agarose gel showing PCR products of six cervical tumours. About 50 ng cellular DNA was used in the PCR (25 and 32 amplification cycles) with the L1 primer set. The positive control (+) is plasmid DNA (10 pg) containing cloned HPV-16 DNA. (b) Southern blot hybridization results from the same cervical squamous cell carcinoma DNAs.

shows the PCR products analysed by agarose gel electrophoresis. A 142 bp DNA fragment specifically hybridizing to the HPV-16 insert probe (see Fig. 1), was detected only in sections H and K. The β-globin primers were used as a positive control for the PCR. The HE-stained sections showed vacuolated cells (koilocytes) in section H and dysplasia in section K. Other sections characterized as normal sometimes showed other histological features such as inflammation, metaplasia, but no koilocytes. The HE staining of sections F, H and K of patient no. 3 is shown in Fig. 3.

Seven of the 12 cones (58%) were positive for HPV-16 DNA after 32 amplification cycles. No differences were observed using either set of primers, thus excluding contamination with pHpv-16 DNA. Carry-over from one tissue block to another was negligible, since all paraffin sections without tissue (see Methods) were negative with the β-globin and HPV-16 primers. HPV-16 DNA was detected virtually only in the sections containing CIN and/or koilocytes. One section
apparently containing only normal epithelium was positive after hybridization, indicating the presence of a low amount of HPV-16 DNA (see Table 1). The five HPV-16 DNA-negative cones have not been analysed for the presence of other HPV types. Three biopsies containing only normal epithelium collected from sites outside HPV-16-positive cones were analysed in order to confirm the negative results. These biopsies were taken immediately after conization and no HPV-16 DNA was detected although the β-globin control was positive (data not shown).

Reliability and efficiency of the PCR in detection of HPV-16 DNA

To evaluate whether sequence differences between HPV-16 genomes, ‘intratypic variation’, could interfere with the detection of HPV-16 DNA by the PCR we analysed 15 cervical squamous cell carcinomas by Southern blot hybridization (see Fig. 4). In seven of these 15 carcinomas (some of which are shown in Fig. 4) HPV-16 DNA could be detected. All seven showed the PstI digestion patterns of the HPV-16 DNA prototype. By comparing the intensity of the hybridization signals of the PstI fragments with standard amounts of PstI-cut pHVP16 DNA the mean HPV-16 copy number per cell could be estimated. The detection level of the Southern blot hybridization was about one copy per cell.

Fifty ng of DNA from each tumour was subjected to 25 and 32 cycles of PCR amplification using both sets of primers. The PCR products were analysed by agarose gel electrophoresis. The seven carcinomas that were positive by Southern blot hybridization were also positive in the PCR after 25 cycles. In addition the DNAs of three other tumours became positive after 32 cycles, so that in total 10 of the 15 tumours were HPV-16-positive.

These results showed that ‘intratypic variation’ did not interfere with HPV-16 DNA detection with either set of primers and we conclude that the PCR is a reliable method for the detection of HPV-16. Also the efficiency of the PCR amplification using purified DNA could be calculated from the estimated amounts of PCR products present on the agarose gel and the HPV-16 DNA copy number detected by the Southern blot hybridization. The efficiency appeared to be 90% of the theoretical value. This shows that the conditions used in the PCR were nearly optimal.

DISCUSSION

The distribution of HPV-16 DNA was studied in the cervical cones of 12 women who were treated for CIN grade III. Depending on the size of the cone, five to 12 tissue blocks were analysed by the PCR adapted for paraffin-embedded tissue (Shibata et al., 1988a). The presence of HPV-16 DNA correlated in all but one cases with cervical dysplasia or the presence of koilocytes. A high copy number of HPV-16 DNA in koilocytes detected by in situ hybridization has been described by Schneider et al. (1987). Despite the destruction of the morphology of the cells necessary for the employment of this technique, the ‘sandwich’ way of analysing sections (HE staining – PCR – HE staining) makes it possible to evaluate the relationship between the presence of HPV-16 DNA and morphological changes, specifically dysplasia and koilocytes.

DNA-DNA in situ hybridization enables exact localization of viral DNA within the cells of one tissue section, but the sensitivity of this technique is rather low (25 to 50 HPV DNA copies per cell; Walboomers et al., 1988). The amount of viral DNA per cell is important for HPV detection by this technique. If the total amount of virus present is distributed over a large number of cells resulting in a relatively low number of copies per cell DNA-DNA in situ hybridization would be unable to detect the viral DNA.

Virtually all sections containing normal epithelium were negative for HPV-16 DNA. The question arises as to how many HPV-16 DNA molecules should lead to a detectable signal. The efficiency of the PCR in paraffin-embedded tissue was estimated by performing 32 cycles using β-globin primers on a tissue section containing about 10⁵ cells. Two μg of the β-globin amplification product (length 100 bp) were synthesized. From these data (not shown) we calculated that the mean amplification efficiency with the β-globin primer set was 80% of the theoretical optimum. A control PCR experiment, analysing purified SiHa cell DNA containing one or two copies of HPV-16 DNA per cell (Baker et al., 1987) with the HPV-16 and the β-globin primer set, indicated that both sets were fully comparable with respect to their efficiency in the
amplification reaction (data not shown). We concluded that the amplification efficiency with the HPV-16 primer set also was 80% of the theoretical optimum.

We concluded that a negative result after 32 PCR cycles indicated that less than 100 HPV-16 DNA molecules could be present, since after hybridization under our conditions (see Methods) at least 0.1 to 1 ng of the HPV amplification product was detectable (Fig. 2). A possible damping effect on the PCR efficiency by the amount of DNA per sample (Abbott et al., 1988) should be taken into account, because the analysed sections mostly contained more cells than were used in the standardization experiment.

The presence of viral DNA outside CIN, cervical squamous cell carcinomas (Macnab et al., 1986), laryngeal papillomas (Steinberg et al., 1983) and genital warts (Ferenczy et al., 1985) has been described. These studies were performed by Southern blot hybridization for which whole tissue blocks had to be used in order to obtain sufficient amounts of DNA and the possibility exists that in the studies of cervical lesions dysplastic lesions or koilocytes were present in tissues classified as normal. We regularly observed that the histological diagnosis can vary from normal to grade III CIN in one single section.

The results described in this article are not necessarily in opposition with recently published PCR results detecting HPV-16 DNA in women with a normal papilloma smear (Young et al., 1989) because microlesions in the cervix could have been present.

In a similar study Shibata et al. (1988b) found that three of the 10 normal sections also contained HPV DNA. The reason for this apparent discrepancy is not known. Since all tissue blocks analysed in their study were known to contain HPV-16 or HPV-18 by in situ hybridization, the infected cells present probably contained a relatively high HPV DNA copy number. The tissue sections we analysed were taken at random from women who underwent a conization for the detection of CIN grade III and were not selected by in situ hybridization.

In vitro transformation studies recently have shown that HPV-16 induced histological abnormalities which closely resembled those seen in CIN (McCance et al., 1988). The early passage culture of those transformed keratinocytes showed besides mild dysplasia, cells resembling koilocytes (vacuolated cells). This would be in agreement with the presence of HPV-16 in CIN and in koilocytes. The HPV-16-positive sections did not always contain dysplastic cells, but koilocytes were always present. Our results support the hypothesis that an HPV infection is one of the factors involved in the development of a cervical carcinoma.

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