Changes in the physical state and expression of human papillomavirus type 16 in the progression of cervical intraepithelial neoplasia lesions analysed by PCR

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Using a PCR strategy which detects disruptions in the E2 reading frame we have analysed the progression of human papillomavirus type 16 (HPV-16)-positive cervical lesions. From a total of 192 samples analysed, we detected HPV-16 in 74. In samples from the spectrum of inflammatory states and cervical intraepithelial neoplasia (CIN) grade I lesions we detected episomal forms of the virus. In invasive tumours and in samples from CIN III lesions there were no episomes detected, suggesting that lesions with integrated HPV-16 precede the invasive stage. The RT-PCR analysis demonstrated the presence of E6 transcripts at all stages and E2 transcripts in all early lesions. The E2 transcripts were not detected in 26 out of 29 CIN III lesions and tumours in which there was a disruption in the E2 gene. In tumours with E2 gene disruptions, we used single-primer PCR to demonstrate the presence of E2 gene sequences.

We are interested in following the progression of human papillomavirus type 16 (HPV-16)-induced cervical lesions from the precursor stage to invasive tumours. Our emphasis is on determining the physical state in the evolution of HPV-16-induced cervical tumours and the associated changes in viral gene expression. There have been reports demonstrating a high percentage of episomes in tumours (Matsukara et al., 1989) and a fair number of integrations in dysplasias (Di Luca et al., 1986; Lehn et al., 1988). But the overall consensus that has emerged is that viral DNA is present as an episome in cervical intraepithelial neoplasia (CIN) lesions and the principal form of the viral DNA in invasive tumours is integrated into the genome (Dürst et al., 1985; Cullen et al., 1991; Das et al., 1992; for review see zur Hausen, 1991). The pattern of individual gene expression in the progression of cervical lesions has largely been analysed using the technique of in situ hybridization (Crum et al., 1988; Dürst et al., 1992; Stoler et al., 1992).

To study the stage and consequences of integration of HPV-16 DNA, we used an approach developed by Das and coworkers (Das et al., 1992; Gopalkrishna et al., 1992) using PCR to define the physical state of the virus. The absence of amplification with primers for the E2 gene in the presence of amplification with primers corresponding to the URR, E6 and E7 gene sequences correlated very well with the presence of integrated HPV-16 DNA (Das et al., 1992). Using this approach, we were unable to detect episomes in high grade dysplasias. In this study, we demonstrate that the integration of HPV-16 occurs in high grade dysplasias prior to any histopathological features of microinvasion. In addition, we have used these primers in RT-PCR reactions to determine the pattern of transcripts of the E6 and E2 genes at various stages of cervical lesions.

The clinical samples were analysed following DNA extraction, using three sets of HPV-16 primers. We followed the DNA extraction protocols, PCR primers and reaction conditions of Das and coworkers (Das et al., 1992; Gopalkrishna et al., 1992). The sizes of the amplified products from clinical samples (Fig. 1a, lanes 3, 6 and 9) and CaSki cells, a HPV-16-positive cell line (Fig. 1a, lanes 2, 5 and 8) were 217, 477 and 1139 bp using primers for the URR, E6 and E2 sequences, respectively. Samples which showed no amplification with the primers were considered HPV-16-negative (Fig. 1a, lanes 4 and 7). To assess the sensitivity of agarose gel electrophoresis in detecting PCR products, clinical samples were amplified using the E2 primers and...
Fig. 1. (a) Amplification of HPV-16 DNA using three different primer sets. DNA extracted from clinical samples and CaSki cells was subjected to PCR amplification with primers corresponding to the URR (217 bp) sequence and the E2 (1139 bp) and E6 (477 bp) ORFs. DNA extracted from CaSki cells following amplification with the three primers was run in lanes 2, 5 and 8, respectively. DNA from HPV-16-positive clinical samples after amplification with the primers was run in lanes 3, 6 and 9, respectively. DNA from HPV-16-negative clinical samples following amplification with primers was run in lanes 4 (URR) and 7 (E2). φX174 DNA digested with HaeIII was run in lane 1 as a marker. (b) Southern hybridization of undigested tumour samples. DNA was extracted from CaSki cells and tumours which were PCR-positive with the URR and E6 primers and negative with the E2 primers. DNA was separated on an agarose gel without any restriction enzyme digestion and detected by Southern hybridization with a 32P-labelled fragment from HPV-16. Lane 1 is from a tumour sample and lane 2 is CaSki DNA. (c) Two-dimensional gel electrophoresis of tumour DNA. DNA extracted from a tumour was digested with HindIII and run on 0.4% agarose gel. The lane was excised, rotated and run again on a 0.8% agarose gel prior to Southern blotting with a HPV-16-specific 32P-labelled probe. (d) RT-PCR of RNA extracted from clinical samples and CaSki cells. RNA was subjected to RT-PCR with primers corresponding to the E2 and E6 ORFs and the URR sequence. The products of RT-PCR using CaSki cell RNA with E2 and E6 primers were run in lanes 2 and 3, respectively. The products of a clinical sample which was positive on DNA PCR with both the E2 and E6 primers were run in lanes 4 and 5, respectively. The products from a tumour sample which was negative on DNA PCR with the E2 primers and positive with the E6 primers were run in lanes 6 (E2) and 7 (E6). (e) Southern blotting of E2 RT-PCR products. RNA obtained from clinical samples were subjected to RT-PCR with the E2 primers and the products were run on agarose gels prior to a Southern transfer. The filter was hybridized with a 32P-labelled E2 probe. The URR primers were used as negative controls for all reactions. RT-PCR products from a clinical sample negative with HPV-16 E2, E6 and URR primers were run in lane 1. Products from a clinical sample positive with HPV-16 E6 primers but negative with the E2 primers were run in lane 2. Products from samples positive with E2 and E6 primers were run in lanes 3 and 4. (f) Southern blot of E6 RT-PCR products. RNA obtained from clinical samples was subjected to RT-PCR with the E6 primers and the products run on agarose gels prior to Southern transfer. The filter was hybridized with a 32P-labelled E6 probe. The URR primers were used as negative controls for all RT-PCR
Table 1. HPV-16 DNA and RT–PCR

<table>
<thead>
<tr>
<th>Biopsy report</th>
<th>Number of samples</th>
<th>DNA PCR</th>
<th></th>
<th>RT–PCR</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>URR</td>
<td>E6</td>
<td></td>
<td>E2</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>CIN III*</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>CIN I</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>Inflammatory lesion†</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>12 (29 ND)</td>
</tr>
<tr>
<td>No lesions</td>
<td>120</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Two of the seven CIN III lesions had associated CIN II regions and two had small focal microinvasions. ND, Not done.
† This includes acute inflammation, epithelial hyperplasia, chronic cervicitis, squamous metaplasia and lesions with koilocytic changes.

To confirm that the disruption of the E2 gene detected by the PCR approach correlated with integrated HPV-16 DNA, we analysed a selected number of tumour samples by Southern hybridization without any PCR amplification. Undigested tumour DNA, from a tumour which showed amplification with the E6 and URR primers but not the E2 primers, and CaSki cell DNA were directly loaded on a gel without restriction enzyme digestion. Following Southern transfer the samples were hybridized with a HPV-16-specific 32P-labelled probe (Fig. 1 b, lanes 1 and 2, respectively). Both the samples showed the presence of high molecular mass DNA with retarded mobility and no episomal forms. In addition, a similar tumour sample and CaSki cell DNA (data not shown) were digested with HindIII, which does not cut in the HPV-16 genome. The DNA was run on a two-dimensional gel system and hybridized with a HPV-16-specific 32P-labelled probe following Southern transfer. The hybridization patterns in both the tumour sample (Fig. 1 c) and the CaSki cell line were identical with no spots off the diagonal, which would be suggestive of episomes.

To determine the pattern of transcripts in these lesions, an RT–PCR analysis was undertaken using the E2 and E6 primers with lesions which demonstrated the presence of HPV-16 virus DNA. The primers for the URR were used as negative controls in all the RT–PCR reactions. The reactions were undertaken using random hexamers for RT followed by the same conditions as above for PCR. A representative RT–PCR reaction is shown in Fig. 1 (d). The E2 RT–PCR products from CaSki cells (Fig. 1 d, lane 2) and a HPV-16 episome-positive clinical sample (Fig. 1 d, lane 4) were around 1-2 kb and no products were detected from a HPV-16-positive tumour with a disrupted E2 gene, as determined by DNA PCR (Fig. 1 d, lane 6). The E6 RT–PCR products from CaSki cells (Fig. 1 d, lane 3) and a HPV-16 episome-positive clinical sample (Fig. 1 d, lane 5) were around 0.5 kb. Similar 0.5 kb products were detected from a HPV-16 positive tumour with a disrupted E2 gene as determined by DNA PCR (Fig. 1 d, lane 7). On Southern hybridization, the E2 RT–PCR products from a HPV-16 episome-positive clinical sample (Fig. 1 e, lanes 3 and 4) were around 1.2 kb and no products were detected from a HPV-16-negative sample (Fig. 1 e, lane 1) or a sample with integrated HPV-16 (Fig. 1 e, lane 2). The E6 RT–PCR products from a HPV-16 episome-positive clinical sample (Fig. 1 f, lanes 3 and 4) and a HPV-16-integrated sample (Fig. 1 f, lane 2) were around 0.5 kb and no products were detected from a HPV-16-negative sample (Fig. 1 f, lane 1). We occasionally detected extremely faint levels of E2 RT–PCR products on Southern blots from such lesions, which may reflect either PCR spillovers or the presence of low level transcripts from residual episomes which are below the level of detection by DNA PCR.

A summary of our DNA PCR and RT–PCR analysis is presented in Table 1. Out of a total of 192 samples, we detected 74 HPV-16-positive samples. Out of the 120 samples which were histopathologically normal there were only two that were HPV-16-positive by PCR. In the other 72 samples, which had HPV-16 DNA and abnormal histopathology, there were 22 tumour samples.
and only three of these had integrations occurring without an E2 gene disruption (PCR positive only for the URR and E6 primers). There were also seven CIN III lesions with no detectable episomes (PCR positive only for the URR and E6 primers) and 43 lesions from a spectrum of low grade inflammatory lesions, including two samples with CIN I and detectable episomes (PCR positive for all three primers). The three tumour samples with intact E2 genes exhibited patterns of integrated HPV-16 genomes with no detectable episomes using a two-dimensional gel system, as in Fig. 1 (c). Out of the 19 tumour samples which had E2 gene disruptions, all the samples were negative for E2 by RT–PCR. However, E2 RT–PCR products were detected in the three tumours with intact E2 genes. All seven CIN III lesions were positive for E6 transcripts and negative for E2 transcripts. Out of the 12 samples analysed from the 41 inflammatory lesions, all were positive for both E2 and E6 transcripts. The overall RT–PCR analysis indicates that in the samples with integrated virus and E2 gene disruptions, there were E6 transcripts and no E2 transcripts.

Following the protocol of Parks et al. (1991) we used single-primer PCR reactions at 37 °C or 42 °C with individual E2 primers to demonstrate the presence of E2 sequences in several tumours with disrupted E2 genes. We detected E2-specific bands and smears on Southern hybridization only from samples with integrated HPV-16 genomes and were unable to detect them from either DNA samples or cell lines that were negative for HPV-16. We carried out single-primer PCR from a single tumour, with the first 10 cycles at 42 °C followed by 25 cycles with the usual parameters. The products were separated on an agarose gel (Fig. 2a) and also detected using Southern hybridization with a 32P-labelled E2 fragment (Fig. 2b). The PCR reactions from a HPV-16-negative cell line SW620 (Leibowitz et al., 1976) were negative with both E2 primers (Fig. 2a, b; lane 1) and negative with primers E2-1 and E-2, respectively (lanes 2 and 3). The PCR reactions from a tumour sample with a disrupted E2 gene sequences were negative with both E2 primers (lane 4) and positive with each primer individually (lanes 5 and 6), with multiple specific bands generated with E2-2 (lane 6). In several tumour samples using such reactions we have seen a band of approximately 500 bp which hybridized to an E2-specific probe and which we assume is a virus-specific band (data not shown).

It is not clear why we saw a differing pattern of integrations from previous studies. It is possible that we were detecting the progression of early cervical lesions at a clearly defined CIN II/CIN III stage with detectable integrations and the absence of episomes. Most of the samples with histopathological features of CIN I or inflammatory lesions which were HPV-16-positive had episomal copies of the virus and were principally drawn from a gynaecology outpatient department. All the clinical samples that were histopathologically indicative of CIN II/CIN III, or with features of microinvasive and frank-invasive tumours were from a cancer hospital. The E2 reading frame has been fairly extensively studied; it codes for DNA-binding proteins involved in the replication and transcription of the virus (for review see Souza et al., 1990; McBride et al., 1991) and is believed to function as a repressor of the URR. However, a recent
report (Hwang et al., 1993) suggests that one of the roles of the E2 gene products may be to suppress cell proliferation, independent of any repression of the URR.

Our data support the hypothesis that a disruption in the E2 reading frame would provide cells with some selective advantage to proliferate and we speculate that CIN III lesions reflect a clonal expansion and proliferation of cells with integrations. Cuzick et al. (1992) have made the interesting observation that the amount of HPV-16 DNA increases with the grade of CIN lesion, with the highest amounts of DNA present in CIN III lesions. The presence of integrated virus genomes with no detectable episomes in high grade dysplasias prior to progression towards an invasive tumour (for review see zur Hausen, 1991). Previous work using cell lines has demonstrated interesting changes in glucocorticoid-dependent regulation of the E6–E7 transcripts following integration of HPV-18 (Doeberitz et al., 1991).

In this study, the transcripts for the E6 gene were detected at all stages. Our RT–PCR analysis was not quantitative and did not measure the relative levels of E6 transcripts in precursor lesions versus invasive tumours or high grade dysplasias. Our transcript analysis correlates very well with a disruption of the E2 gene in CIN III lesions and tumours. Two recent reports showing that the HPV-16 E2 gene can function as a transcriptional activator (Bouvard et al., 1994; Ushikai et al., 1994) might be an explanation for our observed co-existence of E2 and E6 transcripts in early lesions.

In this report we have demonstrated the presence of E2 gene products using single-primer PCR reactions, from samples in which we did not detect a 1.2 kb band with both E2 primers. These reactions are very specific to cells which contain HPV-16 DNA sequences. We are currently cloning the E2 host-specific bands that we generated in these reactions.

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References


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