A Novel Deletion within the Upstream Regulatory Region of Episomal Human Papillomavirus Type 16

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SUMMARY

The pattern of human papillomavirus type 16 (HPV-16) integration was studied in 23 invasive carcinomas of the cervix using subgenomic probes. Seventeen tumours contained integrated HPV-16 and in 13 of these there was evidence of disruption within the E1–E2 open reading frames (ORFs). In all cases the upstream regulatory region (URR)—E6–E7 ORFs was maintained intact. Two independently derived tumours were infected with episomal wild-type HPV-16 and an episomal variant of HPV-16 containing a 325 bp deletion within the URR (positions 7598 to 17) and a point mutation at position 20 (A to C). This is the first report of a variant HPV-16 which is likely to be both defective and transmissible. Loss of E2 expression and deletion of a large portion of the URR may be two of the mechanisms leading to altered HPV-16 early gene expression in cervical tumours.

The prevalence of human papillomavirus (HPV) infection in the normal female population suggests that HPV may be a relatively ubiquitous agent (de Villiers et al., 1987). This would imply that secondary events following HPV infection, probably combined with cellular factors, are of importance in the development of cervical neoplasia. One of the secondary events that may be relevant to this process is the integration of HPV DNA into the cellular genome. The consequence of integration could be the alteration of both HPV and cellular gene expression.

HPV-16 and -18 sequences have been shown to immortalize and transform cells in culture (Bedell et al., 1987; Kanda et al., 1988; Storey et al., 1988; Vousden et al., 1988). Genetic analysis has localized these activities to a region of the HPV genome containing the E6 and E7 open reading frames (ORFs) and transcription of these ORFs appears to be regulated by several responsive elements within the upstream regulatory region. A cell-specific enhancer effect has been demonstrated in some cell lines, perhaps explaining the tissue tropism of these viruses (Cripe et al., 1987). A glucocorticoid-responsive element has been found at position 7641 and may contribute to the regulation of early gene expression (Gloss et al., 1987). In common with other papillomaviruses, the product of the E2 ORF may positively or negatively regulate E6 and E7 expression by directly binding to the upstream regulatory region (URR) (Moskaluk & Bastia, 1988). Alteration in the control of E6 and E7 expression via either interruption of the E2 ORF (Choo et al., 1987) or deletion of the E2 binding sites in the URR is likely to contribute to the neoplastic process, but the roles of the other enhancer elements in the URR are unknown. We have studied a series of cervical carcinomas to determine whether any structural alteration to the URR–E6–E7 or E1–E2 region can be detected.

DNA was prepared, by phenol extraction and ethanol precipitation, from invasive carcinomas of the cervix and analysed for the presence of HPV-16 DNA. Twenty-three carcinomas contained HPV-16 DNA. Twenty of these tumours were squamous cell carcinomas
and three were adenocarcinomas. The physical state of the HPV DNA, either episomal or integrated, was determined using various endonuclease digestions, typically with BamHI, HincII, KpnI, SphI and HindIII, with separation of the fragments by field inversion gel electrophoresis and Southern blotting. The presence of HPV fragments that could not be predicted from endonuclease digestion of episomal HPV-16 in all digests of a sample indicates integration into the host genome. In 70% of the cervical carcinomas examined there was evidence of HPV DNA integrated within the host genome; in seven tumours only integrated HPV sequences were present, and a further nine tumours contained both episomal and integrated sequences. In seven tumours only episomal HPV DNA was detectable.

We tested whether HPV16 DNA was integrated into the host genome in a manner which preferentially opens the episomal DNA within the E1–E2 ORFs while selectively maintaining intact the URR–E6–E7 ORFs. DNA from the tumours was digested and hybridized with the subgenomic probes p16EN and p16NS (Fig. 1). Endonuclease digestion of HPV-16 DNA with PstI releases a 2-7 kb fragment which encompasses the E1–E2 ORFs if the viral DNA is episomal or integration has occurred within another ORF but if these ORFs are disrupted due to integration, fragments of different sizes are released (Fig. 2). The E1–E2 region was interrupted in six of the seven tumours that contained only integrated HPV DNA and in seven of the nine tumours having both integrated and episomal HPV DNA. The URR–E6–E7 region, contained in a 1-7 kb PstI fragment, was conserved in all 23 tumours examined (Fig. 3). A second PstI fragment, 1-4 kb in length, which hybridized to the p16EN probe was found in two different tumours (Fig. 3). Previous restriction enzyme analysis of the two tumours suggested that the detectable HPV DNA was entirely episomal. Further studies with the endonucleases TaqI, NarI, DraI and HaeIII revealed that the 1-4 kb PstI fragment contained an approximately 300 bp deletion within the URR located 3’ of position 7424 and 5’ of position 112. To map the deletion further and so determine which functional regions of the URR were no longer present, both the full-length URR and the shortened URR were amplified using the polymerase chain reaction (PCR) and the amplified DNA was subsequently cloned and sequenced. The region of HPV-16 DNA between positions 7435 and 75 was amplified using oligonucleotides constructed to include BamHI restriction sites at the 5’ end of each oligonucleotide to facilitate cloning into the M13 vector. Following amplification, two bands from each tumour were seen on an ethidium bromide-stained gel. One band, approximately 560 bp, was from the full-length segment of the URR, the second band, approximately 230 bp, was from the deleted segment of the URR. Clones, in both orientations, from the full-length segment of the URR and the shortened...
Fig. 2. Autoradiograph of cervical tumour DNA samples digested with endonuclease PstI and probed with 32P-labelled nick-translated p16NS. Lanes 1 to 5, cervical tumours showing evidence of interruption or deletion within the E1–E2 ORFs. Lanes 6 and 7, cervical tumours with intact E1–E2 ORFs. Lane S, PstI/BamHI double digest of pUC19. HPV16 (cloned via the BamHI site). The band at 2.8 kb represents the E1–E2 ORFs. Markers (kb) are a lambda BstEII digest. DNA was separated by gel electrophoresis using a 1.0% agarose gel. DNA was transferred to nylon filters (Hybond, Amersham) by the method of Southern (1975). Filters were washed under stringent conditions (2 × SSC twice for 15 min, 2 × SSC, 0.1% SDS for 30 min, 0.1 × SSC for 10 min at 65 °C). Filters were subsequently washed and reprobed with 32P-labelled nick-translated vector DNA.

Fig. 3. Autoradiograph of seven different cervical tumour DNA samples digested with the endonuclease PstI and probed with 32P-labelled nick-translated p16EN. Lanes 1, 2, 5, 6 and 7, cervical tumours with intact URR–E6–E7 ORFs. Lanes 3 and 4, cervical tumours with both intact and deleted URR–E6–E7 ORFs. Lane S, PstI/BamHI double digest of pUC19. HPV16 (cloned via the BamHI site). The band at 1.8 kb represents the intact URR–E6–E7 ORFs. Markers (kb) are a lambda BstEII digest. Method as for Fig. 2.
Fig. 4. Sequence gel illustrating (a) the deleted HPV-16 DNA and (b) wild-type HPV-16 DNA. The DNA strand illustrated is complementary to the published sequence. T-G, point mutation; d, breakpoint of deletion. The region between positions 7435 and 80 of HPV-16 was amplified in the tumours using the PCR (Saiki et al., 1988). The oligonucleotide sequences used were 5' GCGGATCCATTTTGTAGCTTCAA 3' and 5' GCGGATCCCTGCTTTTATACTAACGGG 3'. The reaction contained 50 pmol of each primer, 0.2 mM each dATP, dCTP, dTTP, dGTP, 50 mM-KCl, 10 mM-Tris-HCl pH 8.3, 0.01% (w/v) gelatin and 1 μg of genomic DNA in a total volume of 100 μl. The mixture was heated to 94 °C for 7 min before 2.5 units Taq DNA polymerase was added. Amplification was performed in a Cetus DNA thermal cycler and the cycle profile was 94 °C for 1 min, 55 °C for 2 min and 72 °C for 4 min repeated for 25 cycles. Following PCR the DNA was further purified by chloroform extraction. For subsequent cloning of the amplified products, 25 μl of the amplified DNA mixture was digested with BamHI for 120 min at 37 °C. The DNA was precipitated using ethanol and redissolved in 7.5 μl 10 mM-Tris-HCl pH 7.5, 1 mM-EDTA pH 7.5. The total genomic DNA, including the amplified region, was ligated into the cloning vector M13mp8, at the BamHI site using T4 DNA ligase at 15 °C for 16 h. Single-stranded DNA from M13 plaques containing inserts (white plaques) was prepared according to Bankier & Barrell (1983). To test the orientation and size of the inserts the primer sequence and the Klenow enzyme were used to synthesize a radioactive second strand of DNA which was then digested with either EcoRI or SphI and the size of the fragments was determined on 6% polyacrylamide gels. The inserts were sequenced using the dideoxynucleotide chain termination method (Sanger et al., 1977).

The change in the physical state of HPV DNA from episomal DNA to integration within the host genome has been proposed as an important event in the subsequent development of neoplasia. Integrated HPV-16 DNA has been found in normal cervical tissue (Murdoch et al., 1988), cervical intraepithelial neoplasia (Lehn et al., 1988), immortalized keratinocyte cell lines (Schneider-Maunoury et al., 1987) and cervical carcinomas (Dürst et al., 1985). Integration of
HPV DNA is believed to be an early event in the neoplastic process. Integrated HPV DNA was present in the majority (70%) of tumours examined in this study; however, we also found episomal HPV-16 DNA coexisting with integrated HPV-16 DNA in 60% of the tumours. Using subgenomic probes we have confirmed that the integrated HPV-16 DNA is preferentially opened within the E1–E2 ORFs while maintaining the URR–E6–E7 ORFs intact (Choo et al., 1987).

The effect of integration of HPV DNA may be to alter both viral and cellular gene expression. At present there is little evidence that HPV integrates at specific sites within the human genome and insertion near known oncogenes has been described only occasionally (Dürst et al., 1987). Viral gene expression in cells containing integrated HPV DNA is limited predominantly to the E6 and E7 ORFs. In the majority of cases studied, either from invasive tumours or cell lines, there is little evidence of E2 gene expression (Shirasawa et al., 1988). Proteins derived from the E2 reading frame are able either to activate or to repress the promoter for the E6 and E7 proteins located in the URR (Thierry & Yaniv, 1987; Chin et al., 1988). The effects of the E2 protein are mediated through defined DNA elements in the URR which bind the E2 protein (Hirochika et al., 1988; Dostatni et al., 1988). The loss of the E2 proteins, as a result of the interruption of the E1–E2 ORF due to integration, may allow aberrant expression of the E6 and E7 proteins, which have been shown to have the ability to transform and immortalize cells in culture.

There is evidence that when both episomal HPV DNA and integrated HPV DNA coexist in the same cell, transcription of the integrated HPV predominates over the expression of the episomal HPV DNA (Smotkin & Wettstein, 1986). The low level of E2 expression from the episomal HPV may not be sufficient to regulate the E6–E7 expression from the integrated HPV so allowing aberrant E6–E7 expression in these cells.

We have shown that deletion and rearrangement of HPV DNA may occur as a result of events other than integration. We have described a novel deletion and point mutation in episomal HPV-16 from two tumours obtained from different patients undergoing surgery on different dates and analysed on separate occasions. In both tumours the deleted viral DNA (ΔCR-16) was present with the wild-type viral DNA suggesting that the ΔCR-16 might be a defective virus requiring the wild-type virus to be present for replication. ΔCR-16 must, however, still retain the papillomavirus origin of replication. The deletion (positions 7598 to 17) includes the keratinocyte-dependent enhancer, the glucocorticoid-responsive element, a CAAT box and a TATAA box all of which influence early gene expression. Mutants of HPV-16 containing a similar deletion have little constitutive expression in keratinocytes or cervical tumour cells compared with wild-type HPV-16; however, expression may still be enhanced in the presence of E2 protein (Cripe et al., 1987). The finding of episomal HPV-16 containing a major deletion within the upstream regulatory region in two independently derived tumours demonstrates that considerable structural variation can occur within an HPV type and the presence of precisely the same variation in tumours from two unrelated women implies that the deleted HPV-16 is a transmissible variant. The loss of the regulatory unit might be one of the many mechanisms leading to altered HPV early gene expression which may contribute to the neoplastic process.

REFERENCES


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