

HPV-16 E2 gene disruption and sequence variation in CIN 3 lesions and invasive squamous cell carcinomas of the cervix: relation to numerical chromosome abnormalities

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Abstract

Aim—To test the hypothesis that, because the human papillomavirus (HPV) E2 protein represses viral early gene transcription, E2 gene sequence variation or disruption could play a part in the induction of the numerical chromosome abnormalities that have been described in squamous cervical lesions.

Methods—The integrity and sequence of the E2 gene from 11 cervical intraepithelial neoplasia (CIN) grade 3 lesions and 14 invasive squamous cell carcinomas, all of which contained HPV-16, were analysed by the polymerase chain reaction (PCR). The E2 gene was amplified in three overlapping fragments and PCR products sequenced directly. Chromosome abnormalities were identified by interphase cytogenetics using chromosome specific probes for chromosomes 1, 3, 11, 17, 18, and X.

Results—E2 gene disruption was present in significantly more invasive carcinomas (eight of 14) than CIN 3 lesions (one of 11) ($p = 0.03$). No association was found between E2 disruption and the presence of a numerical chromosome abnormality. The E2 gene from the non-disrupted isolates was sequenced and wild-type ($n = 5$) and variant ($n = 11$) sequences identified. Variant sequences belonged to European and African classes and contained from one to 15 amino acid substitutions. Although numerical chromosome abnormalities were significantly more frequent in invasive squamous cell carcinoma than CIN 3 ($p = 0.04$), there was no significant relation between the presence of sequence variation and either histological diagnosis or chromosome abnormality.

Conclusions—These data do not support the hypothesis that E2 gene disruption or variation is important in the induction of chromosome imbalance in these lesions. However, there is a relation between E2 gene disruption and the presence of invasive disease.

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Human papillomaviruses (HPV) are DNA tumour viruses that are associated with the formation of epithelial tumours. Over 80 types of HPV have now been described and are categorised as low risk (for example, HPV-6 and

HPV-11), intermediate risk (for example, HPV-31 and HPV-33), and high risk (for example, HPV-16 and HPV-18), based on their association with clinical disease.¹ The HPV genome encodes two classes of genes that have been divided into two functional groups; the early (E) genes (E1, E2, E4, E5, E6, and E7) and the late (L) genes (L1 and L2). The E6 and E7 genes encode oncoproteins that, among other properties, bind to the p53 and retinoblastoma (pRb) proteins, respectively. The E1 protein is involved in viral DNA replication, the E4 protein in productive viral infection, and the E5 protein has some transforming properties. The L1 and L2 genes encode the major and minor capsid proteins, respectively.

The papillomavirus E2 protein is a modulator of papillomavirus transcription and replication, having major roles in viral DNA replication and as a repressor of viral early gene transcription.^{2–6} It has three functional domains: the N-terminal transactivation domain, the hinge region, and the C-terminal DNA binding domain.^{7–9} In vitro studies have shown that the HPV-16 and HPV-18 early promoters, which regulate the transcription of the oncogenic E6 and E7 genes, are regulated by the viral E2 protein.^{10–11} The expression of both the E6 and E7 genes of the high risk HPVs is necessary for the efficient immortalisation of primary human keratinocytes,^{12–13} and the E2 protein is able to repress E6 and E7 expression by binding to sites adjacent to the major early promoter.¹⁴ The integration of HPV DNA into host cellular DNA is associated with neoplastic progression¹⁵ and, when HPV integrates, the viral DNA frequently breaks in the E1–E2 region.¹⁶ Consequently, disruption of the E2 open reading frame results in loss of E2 protein function, leading to uncontrolled E6 and E7 gene expression. This is also in keeping with the fact that E2 gene mutation or disruption can increase the immortalisation capacity of HPV-16.^{15–17}

In a previous study, we identified distinct patterns of numerical chromosome abnormality in high grade intraepithelial and invasive squamous lesions of the cervix.¹⁸ In view of the in vitro evidence that the E2 protein can abrogate a mitotic control checkpoint,¹⁹ we hypothesised that the presence of numerical chromosome abnormalities in these lesions might be associated with the retention of intact E2 genes. Similarly, because mutation of the E2 gene can alter viral immortalisation capacity,¹⁵ we analysed the DNA sequence of the E2 gene in those lesions in which it was not disrupted to

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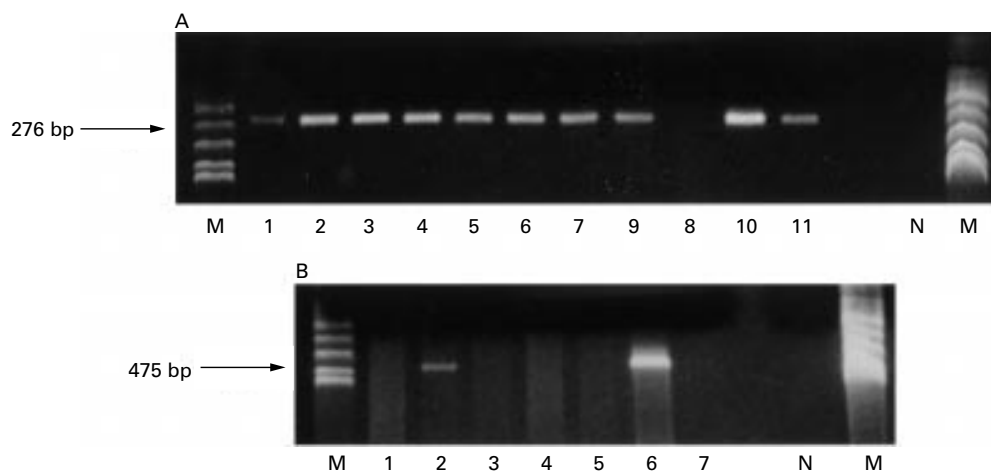


Figure 1 (A) Gel photograph of PCR performed on cervical intraepithelial neoplasia grade 3 (CIN 3) lesions with primers C1 and C2, demonstrating failure of amplification of this region of the E2 gene in one of 11 lesions. (B) Gel photograph of PCR performed on invasive squamous carcinomas with primers A1 and A2, demonstrating failure of amplification of this region of the E2 gene in five of seven lesions. N, water blank negative control; M, marker (*Msp* I digested pBR322).

ance) (table 1). Aneusomy was present in significantly more invasive carcinomas (nine of 14) than CIN 3 lesions (two of 11) ($p = 0.04$).

DISRUPTION OF THE E2 GENE

The overlapping primer pairs were validated initially using DNA extracted from CaSki and SiHa cells: all three fragments were amplifiable from CaSki cells but only one (amplimer C) could be amplified from SiHa cells, as predicted from the published sequences.²⁴ The 536 bp β -globin fragment was amplified in all 25 cases. Amplification of the HPV-16 E2 gene from these lesions demonstrated a failure of amplification of one or more amplimer, indicating disruption, in significantly more invasive squamous cell carcinomas (eight of 14) than CIN 3 lesions (one of 11) ($p = 0.03$; fig 1). There was, however, no significant relation between the presence of aneusomy and E2 gene disruption ($p = 1$; table 1).

E2 GENE SEQUENCE ANALYSIS

The HPV-16 isolates that did not show evidence of disruption were sequenced, as were amplimers from the HPV-16 clone and from CaSki cells, and compared with the wild-type HPV-16R E2 reference sequence²² to investigate variation within the E2 gene. Mutations were identified in a total of 24 positions, 18 of which resulted in amino acid changes (fig 2). A guanosine residue was also identified in position 2926 in all isolates, in addition to CaSki cells and the HPV-16 clone: this has been identified recently as a sequencing error in the HPV-16R sequence.²² Several consistent variants were identified. The most common individual base change was C to T at position 2410. This was present in isolates in which it was the only variant residue, or in which it was associated with other single base changes (group 1). It was also present in variants that showed more widespread variation (groups 2 and 3). Group 2 variants had the same sequence as the E2 gene in CaSki cells.²⁴ Group 3 variants showed a much greater variation, with a total of 20 variant positions. Overall,

there was no relation between the presence of E2 mutation and either histological diagnosis ($p = 1.0$) or chromosome imbalance ($p = 1.0$).

Variation in the transactivation domain was only identified in groups 2 and 3. In group 2, the single mutation in this domain is silent. In group 3, there are five mutations that lead to amino acid substitutions. Variation was also observed in the hinge and DNA binding regions, with a greater number of mutations again being present in the group 3 variants. Two different mutations were identified at position 3377: C to G was present in group 3, and produces a proline to arginine amino acid substitution; C to T, which produces a proline to serine substitution, was identified in a single isolate in group 1. Some mutations within the hinge region also affect the sequence of the E4 gene. These were identified only in group 3 variants and are as follows: L62T (3516, C to A and 3517, T to C); Q69P (3538, A to C); H78Q (3566, T to G).

Discussion

Disruption of the E2 gene was significantly more frequent in invasive squamous cell carcinomas than in CIN 3 lesions. Although this is in keeping with viral integration being important in neoplastic progression, disruption of the E2 gene does not necessarily equate to viral integration. E2 genes can be intact in the presence of viral integration—for example, when both episomal and integrated sequences are present within a lesion, or in the presence of integration of multiple copies of the viral genome, as occurs in CaSki cells.²³ No relation was found between E2 gene disruption and the presence of numerical chromosome abnormalities, as defined using pericentromeric repeat probes. More specifically, chromosome imbalance (aneusomy) was identified both in lesions with disrupted E2 genes and in those with intact E2 genes. These data do not support the hypothesis that the presence of an intact E2 gene is important in the induction of numerical chromosome abnormalities.

variants containing this mutation were shown to be capable of supporting viral DNA replication in carcinomas in which the E1–E2 region was retained,²⁸ indicating that this mutation does not abrogate this function. Mutations within the hinge region are of less clear functional importance, although they could potentially alter the three dimensional relation between transactivation and DNA binding domains. This possibility is of particular relevance to the P219S (3410 C to T) mutation, which occurred in all but one of the variant isolates, because the replacement of proline with serine at this position could significantly alter the secondary and tertiary protein structures. The frequency of this mutation raises the possibility that variants with this alteration could be involved in the development of high grade intraepithelial and invasive disease. However, an analysis of low grade intraepithelial lesions is required to assess this hypothesis.

The ability to bind to DNA, particularly that in the long control region of the HPV genome, is central to the function of the E2 protein. When binding to DNA, the E2 protein forms a homodimer, which interacts with the DNA molecule through its DNA binding helices formed from residues 292–309. Therefore, the T310K mutation, present both in group 2 and group 3 variants, might affect the three dimensional structure of this helix, and hence its ability to bind to DNA. However, a recent study showed very similar transcriptional activities when E and AA variants were compared. Although in vivo function cannot necessarily be inferred from in vitro functional studies, these data suggest that the amino acid sequence variation identified might not be relevant to this function of the protein.

Another possible effect of amino acid substitution in the E2 gene is an alteration of the host immune response to this protein. Linear epitopes have been described within the E2 protein: one in the transactivation domain (residues 121–140), three in the hinge region (residues 181–200, 241–260, and 271–290), and one in the DNA binding domain (residues 328–346).^{29–31} It is interesting that only the amino acid substitutions present in the group 3 variants, and that in the single group 4 isolate, affect these regions. Whether this is of biological relevance remains to be determined.

The lack of a relation between the presence of sequence variation and either histological diagnosis or chromosome imbalance suggests that any functional difference between the E2 variants is unlikely to be involved in determining either the development of aneuploidy or progression to invasive carcinoma. However, these data do not exclude the possibility that the variants might differ in their ability to induce progression of a productive HPV-16 infection to CIN 3.

In conclusion, E2 gene disruption appears to be related to stromal invasion, but not to the presence of numerical abnormalities of chromosomes 1, 3, 11, 17, 18, or X. A variety of E2 gene variants were identified in the lesions analysed, but there was no association between the

presence of these variations and either stromal invasion or chromosome abnormality. However, this does not exclude the possibility that E2 gene variation is important in determining the natural history of HPV-16 infection or the likelihood of progression to CIN 3. Further data are required regarding the prevalence of the E2 variants described across the spectrum of cervical squamous neoplasia.

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