PCR analysis of the upstream regulatory region of human papillomavirus genomes in cervical intraepithelial neoplasia and cervical carcinoma

Y K Donaldson, M J Arends, E Duvall, C C Bird

Abstract

Aims—To test whether human papillomavirus (HPV) variants with large scale sequence alterations to the upstream regulatory region are present in cervical intraepithelial neoplasias (CIN) and cervical carcinomas.

Methods—New PCR based assays were designed specifically to detect large scale insertion/deletion alterations in the upstream regulatory region of HPV 16 and 18. The assays were applied to 24 cases of CIN and 34 cases of cervical carcinoma previously shown to contain these two high risk HPV types.

Results—No large scale sequence alterations were found in any of the HPV containing CIN or carcinomas.

Conclusions—These negative findings suggest that major upstream regulatory region variants of HPV 16 and 18 do not contribute to most cervical neoplasms.

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The common genital human papillomavirus (HPV) types differ in oncogenic potential. HPV types 6b and 11 are more frequently found in low grade cervical intraepithelial neoplasia (CIN 1) and benign lesions, whereas types 16 and 18 are found mostly in high grade dysplasia (CIN 2 and CIN 3) and invasive carcinoma.1-3 Similar properties have been shown by experimental reconstructions by introducing HPV DNA into human genital keratinocytes. HPV types 16 and 18, but not types 6b and 11, can induce immortalisation.4

Comparison of the two major high risk types has shown that HPV 18 is associated with the development of more rapidly progressive cervical neoplasia than HPV 16.5,6 There is also a 10 to 50-fold greater efficiency of keratinocyte immortalisation by HPV 18 compared with HPV 16. Experiments to investigate which sequences in the HPV genomes are responsible for this difference have shown that the viral upstream regulatory region determines this effect, presumably by controlling levels of expression of the viral transforming genes E6 and E7.7,8 This suggests that differences in upstream regulatory region sequence may in part account for the different oncogenic potentials associated with particular HPV types.

The upstream regulatory region differs between HPV types while retaining its regulatory functions for viral DNA replication and gene expression.9 Furthermore, the results of several studies have indicated a high degree of upstream regulatory region sequence divergence between HPV types.10-11 HPV variants with large scale changes to the upstream regulatory region have been discovered in a variety of genital cancers and unusual lesions. It has been suggested that pathogenicity of these HPV variants may be influenced as a result of the deletion or duplication of sequences encompassing some of the putative control elements for viral gene expression.12-15 There are several reports of sequence alterations in the upstream regulatory region of the low risk HPVs. Subtypes HPV 6vc and HPV 6T-70, both isolated from invasive vulvar carcinomas, had deletions or insertions in the 5’ region of the URR.11,14 Another subtype, HPV 6d, was isolated from a Buschke-Löwenstein tumour, which differed from HPV 6b by a 459 base pair duplication of part of the upstream regulatory region.13 There have been surprisingly few reports of large scale sequence alterations in the upstream regulatory region of high risk HPVs, although the first indication that viral variants may be transmissible came from the discovery of two independently derived cervical carcinomas infected with an HPV 16 variant.16 Despite the increasing number of variants being reported and evidence to suggest that they may be transmissible, very little is known about their prevalence in cervical lesions.

In this paper we present a systematic survey of CIN and cervical carcinomas using PCR based assays specifically designed to detect large scale insertions or deletions, such as those described above, in the upstream regulatory regions of high risk HPV types 16 and 18. Our aim was to look for correlation between genomic alterations and the grade of the lesion. Any such correlation might explain why some cases containing HPV 16 and 18 progress and others merely persist or regress.

Methods

PCR assays were designed to amplify the entire upstream regulatory region, from the end of the L1 gene to the start of the E6 gene, in four contiguous segments (A-D). Small deletions or insertions within this region would be seen as deviations in length of PCR products compared with the prototype. Alternatively, a deletion of part of the upstream regulatory region that includes one
Figure 1  Agarose gel electrophoresis of amplified DNA using cloned HPV DNA as template and primer pairs for HPV 16 segments A-D (gel i: tracks a-d), and HPV 18 segments A-D (gel ii: tracks e-h). Analysis of extracted DNA from eight cervical squamous cell carcinomas shows prototypic PCR product sizes for HPV 16 segment B (gel ii: tracks a-d), and HPV 18 segment D (gel iii: tracks a-d). Included are 1 kilobase ladder size marker (track M), template-free negative control (track N) and cloned HPV plasmid DNA positive control (track P).

### PCR analysis of upstream regulatory region of HPV genomes present in CIN and cervical carcinomas

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Nucleotide sequence (5' to 3') of the pairs of PCR primers (P1 and P2) for amplification of segments A-D of HPV 16 and HPV 18, their position within the HPV genome according to the EMBL database nomenclature, the lengths of amplified products and optimum annealing temperatures (Ta).
HPV in the upstream regulatory region in cervical lesions

or more of the primer binding sites will result in absence of amplified product. The clear separation of 142/154 and 200/220 base pair fragments in the 1 kilobase ladder size marker indicates that insertions or deletions of about 12–20 base pairs could be readily detected by this method (figure).

PCR analysis was performed on 24 cases of CIN comprising nine CIN 2 with HPV 16, three CIN 2 with HPV 18, eleven CIN 3 with HPV 16 and one CIN 3 with HPV 18 (punch biopsy frozen sections), and 34 cervical carcinomas comprising 15 squamous cell carcinomas (SCC) with HPV 16, six SCC with HPV 18, five adenocarcinomas with HPV 16, five adenocarcinomas with HPV 18, and three adenosquamous carcinomas—two with HPV 16 alone and one with both HPV 16 and 18 (DNA extracted from paraffin wax sections of resected tumours). Each case was histologically confirmed by two pathologists. Samples from the cases were also previously shown to contain either HPV 16 or 18 DNA by a type specific PCR assay. Clinical material was prepared and analysed by PCR, as described before, using oligonucleotide primers whose sequences, upstream regulatory regions positions (according to EMBL database nomenclature), and optimised annealing temperatures for PCR are shown in the table. Appropriate template-free negative controls were included in every analysis and particular care was taken to avoid carryover contamination as previously described. Amplified products were electrophoresed on 12%–15% agarose gels (5% NuSieve GTG; 1% Seakem GTG) for 8–10 hours at 100mA along with a size marker (MspI digest of pBR322, Biolas; or 1 kilobase ladder, Gibco-BRL). Optimisation of the reactions was performed using cloned HPV 16 or HPV 18 containing plasmids (figure).

Results
All 24 cases of CIN (20 HPV 16, four HPV 18) and 34 cases of cervical carcinoma (22 HPV 16, 11 HPV 18, one of both HPV 16 and HPV 18) produced upstream regulatory region (UVRG) PCR products, which were indistinguishable in length from cloned HPV prototypes (figure). Thus, no large scale insertions or deletions in HPV 16 and 18 upstream regulatory regions were found in these cases.

Discussion
These PCR based assays were designed specifically to look for large scale insertions or deletions in high risk HPV upstream regulatory regions which might influence the risk or rate of progression of neoplasia. The absence of HPV 18 upstream regulatory region variants was consistent with the lack of previous reports of such variants. The negative HPV 16 upstream regulatory region findings are also compatible with a recent report that looked for individual nucleotide sequence alterations in the 3′ portion of the HPV 16 upstream regulatory region. In this study of a total of 118 cases of HPV 16 (90 of which were isolated from cervical biopsy specimens and smears), 38 variants were found and most of these were point mutations that were unlikely to confer noticeable functional effects. These results suggest that large scale insertions or deletions in the upstream regulatory region of HPV 16 and 18 are likely to be rare and do not contribute to neoplastic progression in most patients with cervical lesions. It would also be possible to use this method to detect smaller scale alterations, involving 2–10 base pairs by analysis of the amplified products using non-denaturing polyacrylamide gel electrophoresis, or single nucleotide changes by single strand conformational polymorphism (SSCP) analysis. Despite these negative findings for HPV 16 and 18, the possibility remains that upstream region variants of low risk HPV types 6b and 11 (which were not analysed in this study) may contribute to neoplastic transformation.

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