Analysis of HPV16, 18, 31, and 35 DNA in pre-invasive and invasive lesions of the uterine cervix

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Abstract

Aims—To analyse the physical state of different human papillomavirus (HPV) DNAs in 55 intraepithelial and invasive HPV associated cervical neoplasms.

Methods—Restriction analysis, using a panel of five HPV type specific enzymes, was carried out for each sample; this was followed by Southern blot analysis.

Results—Six (25%) of 24 cervical intraepithelial neoplasms had integrated DNA of different HPV types. In contrast, integration was detected in 25 (81%) of 31 cervical carcinomas. Tumour samples revealed differences in the integration profile of HPV16 and the other HPV types. Six (26%) of 23 HPV16 associated cancers contained only episomal DNA. In contrast, all eight tumours containing HPV18, 31, or 35 revealed integrated DNA exclusively.

Conclusions—The results suggest that in advanced cervical intraepithelial neoplasia lesions, a subset of lesions can be identified in which the viral genome is integrated and there is a greater risk of malignant progression. In addition, HPV16 DNA was not present in the integrated form in 26% of tumours, suggesting that integration and subsequent inactivation of the transcriptional regulator, E2, are not essential steps for the development of HPV16 associated carcinoma. In this respect, the behaviour of HPV16 associated tumours is different from HPV18, 31, and 35 associated tumours, where the viral genome is always present in the integrated form.

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Keywords: uterine cervix; human papillomavirus; HPV DNA physical state

Molecular biology approaches have demonstrated the presence of certain human papillomavirus (HPV) types in precancerous and cancerous lesions of the genital tract. Cervical cancer is one of the most common malignant diseases in women worldwide. An element that appears to be important in the development of this cancer is infection with certain types of HPV including types 16, 18, 31, 33, and 35. In addition to the type of HPV present, another factor to consider is the physical state of the viral genome. Generally, it is accepted that HPV DNA is maintained as an episome in benign infections, whereas integrated HPV sequences have been identified in cervical cancer, in cell lines isolated from cervical neoplasms, and in immortalised human keratinocytes. The integration event was proposed as an activation mechanism for the progression from pre-invasive advanced lesions to cervical carcinomas. HPV encode two proteins, E6 and E7, which are expressed in HPV positive cancer and in derived cell lines. The viral genome integration occurs usually in connection with the E1/E2 open reading frame (ORF), causing loss of expression of these genes. The E2 protein is an important transcriptional regulator of viral gene expression and its loss due to integration can result in overexpression of the E6 and E7 oncoproteins. HPV E6 forms complexes with wild-type p53 protein. As p53 appears to play a role in the regulation of entry into the S-phase of the cell cycle, elimination of this function by complexing with E6 and subsequent degradation via the ubiquitin system could be an important event for the malignant progression of HPV associated lesions. Recent studies conducted on viral integration in cervical intraepithelial neoplasia and in cervical cancers have given contrasting results. Several studies have reported the detection of integrated HPV genomic sequences in the majority of cancers and cell lines isolated from cervical neoplasms; on the other hand, Matsukura et al observed only episomal HPV16 DNA in 70% of cervical cancers, and Fuchs et al reported that in 36% of cancer specimens HPV16 DNA was present only in its episomal form. However, two groups showed integrated HPV genomic sequences in over 50% of cervical intraepithelial neoplasia, suggesting that this event could be of interest, given its ability to identify a subgroup of lesions with increased risk of malignancy. Recently, other authors have observed the simultaneous presence of episomal and integrated HPV16 DNA in squamous cell carcinomas of the cervix. The aim of the present study was to investigate the role of integration during neoplastic progression in cervical intraepithelial neoplasia, grade 2–3, and in cervical cancer using Southern blot analysis after digestion with an HPV type specific panel of restriction enzymes.

Methods

PATIENTS

Both the presence and type of HPV DNA were determined in 41 samples from consecutive patients affected by uterine cervix carcinoma...
(mean age, 48 years; range 23–80 years) and in 34 patients affected by cervical intraepithelial neoplasia, grade 2 and 3 (mean age, 34.3 years; range 24–67 years). Samples were collected by the Obstetric and Gynecologic Clinic, Florence University, Italy.

TISSUE SAMPLES
Cervical intraepithelial neoplasia samples were obtained surgically. Also, cervical tissue specimens were taken under colposcopic control from areas with no apparent disease at ¬5 mm distance from the lesion. Cancer samples were obtained from surgical specimens. The samples were bisected and one portion was submitted for standard histopathological diagnosis while the other was used for molecular analysis.

DNA EXTRACTION AND QUANTITATIVE DETERMINATION
DNA was extracted from tissue specimens by proteinase K digestion and phenol extraction. Because of the small size of the specimens, the DNA concentration could not be quantified spectrophotometrically. Therefore, it was necessary to set up a method for DNA quantification that required the least possible amount of material and allowed a high level of precision. The DNA content of a small size RNA free sample was measured using a two-dimensional approach. Briefly, 2 μl aliquots of the DNA samples were spotted on to the surface of a 0.7% agarose gel containing ethidium bromide; the ultraviolet induced low light fluorescence emitted by ethidium bromide molecules intercalated into the DNA was evaluated using an advanced analysis system (UVP; Cambridge, UK) capable of distinguishing low light fluorescent patterns from the background. The use of an internal standard was necessary because the intensity of the signal is determined by the aperture of the camera diaphragm and the gel conditions. Using this method, it is possible to obtain precise quantification of as little as 2 ng of DNA. DNA from tumours was extracted and measured using the same conditions.

SOUTHERN BLOTTING AND HPV TYPING
Genomic DNA (5 μg) was electrophoresed in 0.8% agarose gel and transferred to Hybond N (Amersham, Bucks, UK) filters by Southern blotting. To assess the probe performance, hybridisation stringency, and molecular size known amounts of gel purified HPV DNA were included. Virus typing was accomplished by hybridising the Southern blots with probes specific for HPV types 11, 16, 18, 31, 33, and 35. The analysis of type 6 HPV was not performed because of the homology with type 11 HPV and the low probability of finding it in premalignant and malignant lesions. Probes were prepared by radiolabelling gel purified HPV whole DNA with 32P using a multiprime DNA labelling system (Amersham). Prehybridisation was carried out in hybridisation solution without the probe for two hours, followed by hybridisation overnight at 65°C in hybridisation buffer containing 5 × saline sodium citrate (SSC), 5 × Denhardt’s solution, and 0.5% sodium dodecyl sulphate (SDS). Blots were washed twice in 2 × SSC containing 0.1% SDS at room temperature, then once in 1 × SSC, 0.1% SDS at 65°C, and finally once in 0.1 × SSC, 0.5% SDS at 65°C. The filters were subjected to autoradiography with intensifying screens at ¬80°C.

ANALYSIS OF THE PHYSICAL STATE OF HPV DNA
Genomic DNA was digested with a panel of five restriction enzymes for each virus type, as described by Cullen et al., chosen in order that the first two enzymes would not cleave the corresponding HPV genome and the last three would cut the HPV DNA once or twice. Biopsy specimens were analysed undigested and after digestion with the appropriate enzymes using the conditions described above.

STATISTICAL METHODS
Owing to the low frequency of certain events, it is not reliable to use the χ² test; therefore, statistical significance was assessed by an algorithm which applies the test described by Fisher in contingency tables with R rows and C columns (R × C). The calculations were carried out using STACT-XAT software (Cytel, Massachusetts, USA).

Results
ANALYSIS OF THE PHYSICAL STATUS OF VIRAL DNA IN LESIONS WITH DIFFERENT HISTOPATHOLOGICAL DIAGNOSES
The presence of HPV DNA type 11, 16, 18, 31, 33, and 35 was investigated in 34 cervical intraepithelial neoplasias (grade 2–3), in related cervical tissue with no apparent disease, and in 41 cervical carcinomas. Twenty four (71%) cervical intraepithelial neoplasms and 31 (76%) carcinomas were positive, while viral DNA was not revealed in any of the healthy tissue samples. Restriction analysis followed by Southern blot (typical results are shown in fig.

Table 1 The physical state of different types of HPV DNA in cervical intraepithelial cancer (CIN) grade 2–3 and uterine cervix carcinoma

<table>
<thead>
<tr>
<th>HPV type and physical state</th>
<th>CIN</th>
<th>Tumours</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HPV16</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Episomal</td>
<td>13</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>Episomal + integrated</td>
<td>3</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Integrated</td>
<td>3</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td><strong>HPV18</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Episomal</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Episomal + integrated</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Integrated</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>HPV31</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Episomal</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Episomal + integrated</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Integrated</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>HPV35</strong></td>
<td></td>
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</tr>
<tr>
<td>Episomal</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Episomal + integrated</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Integrated</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>24</td>
<td>31</td>
<td>55</td>
</tr>
</tbody>
</table>

Physical state & histopathology: HPV16, p < 0.02.
1) allowed the physical state of viral DNA to be highlighted in the 55 positive samples. The results are shown in fig 2. Both episomal and integrated DNAs were detected in three (12.5%) of 24 cervical intraepithelial neoplasms (grade 2–3); three (12.5%) cervical intraepithelial neoplasms contained integrated HPV DNA only, and the remaining 18 (75%) cervical intraepithelial neoplasm samples contained episomal HPV DNA sequences exclusively. In contrast, integration was detected in 25 (80.6%) of the 31 cancers: 20 (64.5%) showed only integrated sequences and five (16.1%) contained both episomal and integrated DNAs; the remaining six (19.4%) cancer samples contained episomal HPV sequences exclusively. The differences in the physical state of the viral genome in lesions with different histopathological diagnoses were highly significant (p < 0.0001); the differences were also significant (p < 0.02) when only HPV16 associated lesions were considered, as shown in table 1.

**Figure 1** Integration analysis of HPV16 DNA in three different cervical cancer samples (A–C). In each panel, lanes 1 and 2 contained DNA digested with restriction endonucleases that do not have cleavage sites in HPV16 DNA (BglII and EcoRI, respectively); lanes 3, 4, and 5 contained DNA samples that were digested with enzymes with one cleavage site in the HPV16 genome (BamHI, Ncol, and Spel, respectively). Panel A is characteristic of only integrated HPV16 DNA: in lanes 3, 4, and 5 more than two HPV-human junction fragments per lane are present and no band is seen at 8 kb. Panel B exhibits only episomal forms of HPV16 genome: the multimeric circle (mc), open circle (oc), and supercoil (sc) DNA forms visible in lanes 1 and 2 are converted totally to an 8 kb linear form when DNA samples are digested with enzymes which cleave HPV16 DNA once (lanes 3, 4, and 5). Panel C is representative of a complex coexistence of episomal monomeric, multimeric, and integrated HPV16 DNA.

**Figure 2** Analysis of the physical state of HPV DNA in lesions with different histological diagnoses. The difference in frequency of viral genome status between cervical intraepithelial neoplasia (CIN) (grade 2–3) and cervical carcinoma is statistically significant (p < 0.0001).

**The Physical State of Different Types of HPV DNA**

The results of the analysis of the physical state of different types of HPV in cervical intraepithelial neoplasia (grade 2–3) and invasive carcinomas of the cervix are shown in table 1. Differences were observed between cervical intraepithelial neoplasia (grade 2–3) lesions containing HPV16 DNA and those containing the other types of HPV analysed. Of the 19 cervical intraepithelial neoplasia (grade 2–3) samples containing HPV16, episomal sequences only were seen in 13, three had both episomal and integrated HPV DNA, and the other three specimens revealed only integrated HPV DNA. In contrast, HPV18 and 35 DNA was not found in any cervical intraepithelial neoplasia (grade 2–3) samples; in addition, the five cervical intraepithelial neoplasia samples containing HPV31 showed viral DNA only in the episomal form. To date no correlation has been observed between these findings and the clinical pathological parameters.

Cervical cancers revealed differences in the integration profiles of HPV16 and HPV18 DNA. Of the 23 cancers containing HPV16 DNA, 12 revealed only integrated HPV DNA, five specimens showed both episomal and integrated DNA, and the remaining six had only episomal HPV16 DNA. In contrast, all four cancers containing HPV18 sequences revealed integrated viral DNA exclusively. Although this appeared to be a real phenomenon, the difference between the DNA integration frequency of HPV16 and 18 was not statistically significant, owing to the low frequency of HPV18 DNA. The two tumours containing HPV31 DNA and the two cases containing HPV35 DNA showed integrated sequences only.

**Discussion**

The presence of both episomal and integrated HPV DNAs is the major problem confounding the analysis of the physical state of the viral genome because the monomeric and multi-
meric episomal forms can mask the relatively small amount of integrated DNA. In order to resolve this problem we used the principal method described by Cullen et al., which uses restriction analysis with a panel of HPV type specific restriction endonucleases followed by Southern blot analysis. The use of five different enzymes for each specimen reduced the chance of faint HPV–human junction fragments being obscured by episomal HPV DNA in the same sample. The study permitted the clear definition of the physical state of the viral DNA in 55 samples. In 81% of the cervical cancers, integrated HPV DNA was revealed in comparison with 25% of the cervical intraepithelial neoplasia (grade 2–3) samples. The results suggest that there is a temporal relationship between the integration event and the acquisition of invasive characteristics. These data are in agreement with those reported by Cullen et al.1 who observed the presence of integrated viral DNA in 81% of the cancers analysed. Generally, it is assumed that integration into the host chromosome leads to the disruption of the E1/E2 ORF.10,11 with consequent malignant progression due to the possible overexpression of the E6 and E7 sequences. From the results obtained in this study, where 19% of the cervical carcinomas analysed contained only episomal HPV DNA, it can be hypothesised that integration is a fundamental, although not obligatory, element in the majority of tumours. A large heterogeneity in the frequency of integration has been reported in HPV16 associated tumours11141516; this could be due to the epide-
imiological differences in the populations considered. The heterogeneity does not exist in the case of tumours containing HPV18, where integration is observed consistently.14 In the majority of early studies, the physical state of lesions containing HPV16 were investiga-
d11151617; subsequently, the analysis was extended to other types of HPV.14 From the comparative analysis of the physical state of HPV16, 18, 31, and 35 significant differences were found between the integration profiles of HPV16 with respect to the other HPV types. In fact, all eight carcinomas containing HPV18, 31, or 35 showed integrated DNA exclusively, whereas of the 23 cancers containing HPV16 genomes, six (26%) revealed only episomal sequences. Moreover, HPV18 and 35 DNAs were not observed in any of the cervical intraepithelial neoplasia samples analysed. It has been hypothesised that HPV18 is clinically more aggressive than HPV16 because it has been found to be associated with cervical intraepithelial neoplasia less frequently than with cervical carcinoma, and also because it is considered to be more efficient at immortalising human keratinocytes in vitro.7 In addition, HPV16 has been found to be associated with well differentiated cancers much more often than HPV18.18 The data obtained in this study indicate the existence of tumours in which HPV16 DNA is not present in the integrated form, suggesting that the spatio-
temporal inactivation of E2 are not essential for the development of HPV16 associated carcino-
mas; in this respect they are different from HPV18, 31, and 35 tumours where the viral genome is present in the integrated form.

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