Human Papillomavirus Type 16 DNA in Genital Tumours: A Pathological and Molecular Analysis

By DARIO DI LUCA, 1 SILVANA PILOTTI, 2 BERNARDINA STEFANON, 3 ANTONELLA ROTOLA, 1 PAOLO MONINI, 1 MAURO TOGNON, 1 GIUSEPPE DE PALO, 3 FRANCO RILKE 2 AND ENZO CASSAI 1.

1 Institute of Microbiology, University of Ferrara, Via L. Borsari 46, 44100 Ferrara and Divisions of 2 Pathology and Cytology and 3 Clinical Oncology 'A', Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, 20100 Milano, Italy

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SUMMARY

The presence of human papillomavirus type 16 (HPV16) DNA in 34 genital tract tumours of Italian female patients was investigated by Southern blot hybridization in high stringency conditions. HPV16 DNA was detected in 16 neoplasias, including cervical invasive and intraepithelial lesions as well as vulvar intraepithelial neoplasias and, to a lesser extent, vulvar invasive carcinomas. Appropriate control tissues included in the study were negative. The data suggest that integration of viral DNA had occurred in most tumours, both in invasive and in intraepithelial lesions. HPV16 variants or defective genomes, lacking the BamHI restriction site, were detected in three tumours.

In recent years several epidemiological studies have suggested that the aetiology of human genital cancer may have an infectious component (Melnick & Adam, 1978). The significant association with sexual promiscuity and the higher risk for sexual partners of cancer patients to develop genital neoplasia, indicate the involvement of transmissible factors in the development of this disease (zur Hausen et al., 1981). Among the various infectious agents considered as candidates in the aetiology of carcinoma of the uterine cervix, the role of human papillomaviruses (HPV) has been supported by a number of studies (Gissmann, 1984; Della Torre et al., 1978). Direct and circumstantial evidence implicating HPV infection in genital neoplasia includes: (i) histological observations that as many as 70% of cervical dysplasias may be associated with so-called koilocytotic atypia, a hallmark of HPV infection (Fu et al., 1983; Pilotti et al., 1981, 1982); (ii) HPV structural proteins may be detected in cervical intraepithelial neoplasias (CIN) by immunohistochemical techniques (Dyson et al., 1984; Syrjanen, 1983; Pilotti et al., 1984); (iii) HPV lesions of the genital tract may undergo malignant transformation (Syrjanen et al., 1984); (iv) animal papillomaviruses have oncogenic potential (Gissmann, 1984); (v) antibodies against a papillomavirus group-specific antigen were detected in sera of cancer patients but not of controls (Baird, 1983).

Molecular hybridization studies revealed that four HPV types are commonly associated with lesions of the lower genital tract. Types 6 and 11 are mainly detected in condylomata acuminata and rarely in carcinomas (Gissmann et al., 1983; McCance et al., 1983), whereas types 16 and 18 are mostly associated with neoplastic processes. HPV18 DNA was revealed by Southern hybridization under high stringency conditions in about 25% of cervical tumours (Boshart et al., 1984). Sequences homologous to HPV16 DNA have been found in cervical carcinoma samples at a variable frequency in different countries. Indeed, HPV16 DNA was detected in 17% of invasive cervical tumours analysed in the United States (Fukushima et al., 1985), but in 60% of cervical cancers collected in Germany (Durst et al., 1983); furthermore, cervical cancer biopsies from Kenya and Brazil revealed HPV16 DNA in about 35% of cases (Durst et al., 1983), while the incidence of HPV16 DNA-positive samples in England (Scholl et al., 1985) is intermediate.
Short communication

(45%) between those reported for German patients and African or South American patients. Also, vulvar and penile cancer biopsies hybridized to HPV16 DNA, with an incidence of about 25% of the cases (Durst et al., 1983).

Since some geographical difference seems to exist in the incidence of HPV16 infections in human genital cancer, we studied the prevalence of HPV16 DNA in invasive and intraepithelial cervical and vulvar neoplasias of the Italian population. Southern blot hybridization under high stringency conditions was used throughout our study. All samples were collected in Milan (National Tumour Institute) and consisted of either biopsies or surgically removed material. In order to select the proper areas of neoplastic and non-neoplastic tissue of each sample, frozen cryostatic sections were taken for microscopical examination and the selected tissues were stored at -80 °C for subsequent hybridization analysis. Adequate material immediately adjacent to each selected site was taken for permanent histological sections which were stained with haematoxylin and eosin to provide a careful histological diagnosis. Whenever possible, adjacent normal cervical or vulvar tissues were processed and examined in the same way as the tumour tissues. DNA was extracted as previously described (Cassai et al., 1981); approximately 10 μg of DNA was digested for 4 h with 60 units of the restriction endonucleases HindIII or BamHI, subjected to electrophoresis on 0.6% agarose gels (1.5 V/cm for 15 h) and transferred to hybridization membranes (Gene Screen Plus, New England Nuclear). The filters were hybridized with an HPV16 probe labelled with 32P by nick translation (Rigby et al., 1977); the specific activity obtained was 1.5 × 10^8 to 5 × 10^8 c.p.m./μg. HPV16 cloned at the BamHI site of pBR322 was the kind gift of Dr L. Gissmann (Durst et al., 1983). Hybridization was carried out for 15 to 18 h at 42 °C in the presence of 50% deionized formamide, 1% SDS, 1 M-sodium chloride and 10% dextran sulphate. The hybridized filters were extensively washed in decreasing concentrations of SSC (2× to 0.1×) at 65 °C and autoradiographed, usually for 15 days. HindIII-digested lambda DNA fragments were used in each gel as molecular weight markers. Reconstruction experiments with known amounts of HPV16 DNA showed that the sensitivity of the technique allowed detection of 0.1 copies of HPV16 DNA per diploid genome (Fig. 1). Since HPV16 DNA used as probe was not purified from plasmid DNA before labelling,
Table 1. *HPV16 DNA* in neoplastic and normal specimens from cervix and vulva

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>No. of cases</th>
<th>Positive cases</th>
<th>HPV copies per diploid cell genome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cervix</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive squamous cell carcinoma</td>
<td>13</td>
<td>6</td>
<td>2–200</td>
</tr>
<tr>
<td>CIN II or CIN III associated with HPV infection</td>
<td>4</td>
<td>3</td>
<td>2–50</td>
</tr>
<tr>
<td>Mixed adenocarcinoma and squamous cell carcinoma</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>19</td>
<td>9 (47.4%)</td>
<td></td>
</tr>
<tr>
<td><strong>Vulva</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive squamous cell carcinoma</td>
<td>10</td>
<td>2</td>
<td>2–10</td>
</tr>
<tr>
<td>VIN III associated with HPV infection</td>
<td>5</td>
<td>5</td>
<td>2–200</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>15</td>
<td>7 (46.7%)</td>
<td></td>
</tr>
<tr>
<td>Condylomata acuminata</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Flat condylomata</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervix</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Vulva</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

all the samples were also separately hybridized to a pBR322 probe, in order to exclude the possibility of false positive results due to cross-hybridization of labelled plasmid DNA to host DNA or to DNA from bacteria of the genital flora. None of the tumour or control DNA showed homology to pBR322 under our conditions of hybridization (data not shown).

We found that 16 out of 34 (47%) genital neoplasias contained sequences homologous to *HPV16 DNA*, which was detected at the same frequency both in cervical and vulvar specimens; in contrast, none of the condylomata or normal tissues analysed contained viral DNA (Table 1). The amount of viral DNA detected in tumours is shown in Table 1. No correlation was found between the amount of *HPV16 DNA* and the stage of tumour growth or the location (cervical or vulvar) of neoplasia. The number of *HPV16 DNA* copies per diploid cell genome was determined in each sample on the basis of reconstruction experiments run in parallel.

While many studies have focused upon the presence of *HPV16 DNA* in cervical preinvasive lesions or invasive carcinomas, less attention has been paid to vulvar lesions. All the biopsies of intraepithelial neoplasia of the cervix grades II and III and of vulva grade III examined in this study showed the most typical histological features of wart virus infection and eight of nine were positive for *HPV16 DNA*. Since *HPV16 occurs infrequently in condylomatous lesions* (Durst *et al.*, 1983 and Table 1), the significant association already established between invasive cervical tumours and *HPV16 can be extended also to preinvasive lesions.* On the other hand, in our study only about 20% of invasive vulvar carcinomas harboured *HPV16 DNA*, in agreement with previous reports (Durst *et al.*, 1983). This observation contrasts with the frequency of *HPV DNA* present in five of five vulvar intraepithelial neoplasias (VIN) and six of 13 (46%) invasive cervical carcinomas. Our data show that *HPV16 is not consistently detected in invasive squamous cell vulvar carcinomas,* despite its frequent association with vulvar intraepithelial neoplastic lesions. One could speculate that cervical and vulvar invasive carcinomas have different aetiologies and that the positive results from vulvar intraepithelial neoplasias merely reflect the influence of squamous differentiation on HPV replication in precancerous vulvar epithelium.

The state of HPV DNA was analysed after cleavage of tumour DNA with *BamHI* which cuts viral DNA once producing a full-length linear molecule (FIII) or *HindIII* which does not cut viral DNA. Digestion of invasive carcinoma DNAs with *BamHI* showed eight positive tumours (Fig. 2a, b); in five samples a prominent band co-migrating with HPV DNA FIII was observed. Of the other three tumour DNAs one showed bands co-migrating mostly with FII (relaxed circular form) and to a lesser extent with FI (supercoiled form) and FIII of HPV DNA, suggesting that in this tumour only free monomeric viral DNA is present (Fig. 2b, lane 8). In this
Fig. 2. Autoradiograms of Southern blots containing BamHI-digested DNAs (10 μg/lane) from genital invasive carcinomas hybridized in high stringency conditions to a 32P-labelled HPV16 DNA probe. Copy number cannot be determined from the hybridization pattern presented in the figure because, in order to show the presence of lower intensity bands clearly, exposure times were different for each sample. The size markers are HindIII fragments of phage lambda DNA (kbp). (a) Samples from five genital tumours (lanes 1 to 5) showing a prominent band co-migrating with HPV DNA FIII. (b) Samples from invasive genital carcinomas showing lack of a band corresponding to HPV DNA FIII (lanes 6 and 7) or a prominent band corresponding to HPV DNA FII (lane 8).

In the case of the sample in lane 7, the HindIII cleavage pattern does not show viral monomeric molecules (compare this lane with Fig. 4, lane 6) and is indicative of integrated sequences. Intraepithelial neoplasia DNAs digested with BamHI showed a prominent band co-migrating with HPV DNA FIII in all the positive samples (Fig. 3). Furthermore, six intraepithelial neoplasia positive DNAs showed additional bands of variable size and lower intensity (Fig. 3), probably representing, at least in some instances (see below), junctions between viral DNA sequences and cellular DNA in viral integration sites. Similar bands were present also in four invasive carcinoma DNAs (Fig. 2a). In particular, the detection of high molecular weight bands in intraepithelial neoplasias (Fig. 3, lanes 3, 4, 5 and 7), suggestive of integration sites or of defective oligomeric forms, is an interesting finding, since previous work has shown HPV16 to be free and monomeric in intraepithelial lesions (Durst et al., 1983; Crawford, 1984).

Tumour DNAs digested with HindIII showed a prevalence of free viral genomes (Fig. 4, lanes 1 to 5). Indeed, a prominent band was detected corresponding in size to HPV DNA FII, while...
Fig. 3. Autoradiograms of seven out of eight genital neoplasias positive for HPV16 DNA. With the exception of the sample electrophoresed in lane 6, showing only a band co-migrating with HPV-16 DNA FIII, all the others showed additional bands which appeared after exposures different for each sample. The size markers are as in Fig. 2.

Fig. 4. Blot hybridization to $^{32}$P-labelled HPV16 DNA of HindIII-cleaved DNA from vulvar and cervical neoplasias. Lanes 1 to 5 represent a pattern found in most HindIII digests of HPV16 DNA-positive tumours, with FII being the more prominent band. Lanes 6 to 8 show samples from three tumours in which free viral monomeric forms are not detectable. The size markers are as in Fig. 2.
the remaining viral sequences were located in the position of viral DNA FI or FIII. Minor bands migrating in the high molecular weight region of the gel could represent either integrations of viral sequences into cellular DNA or free polymers of the viral genome. Three invasive carcinomas lacked evidence of free viral genomes, the only hybridizing bands being about 16 to 18 kb (Fig. 4, lanes 6 to 8). Such bands could hardly be indicative of viral oligomers since they lack any counterpart in the other tumours analysed (Fig. 4, lanes 1 to 5); instead, they are suggestive of viral insertions into cellular DNA.

Even if from our work no direct evidence is available for integration of viral DNA into the host cell genome, the combined BamHI and HindIII cleavage patterns suggest that both free and integrated viral sequences are present in invasive tumours, in agreement with previous studies (Gissmann, 1984), and in intraepithelial neoplasias. Furthermore, the incidence of HPV16 in genital tumours of the Italian population (47%) is lower than that detected in Germany (60%) (Durst et al., 1983), but similar to that detected in England (45%) (Scholl et al., 1985).

Of further interest is the observation that one peritumoural non-neoplastic cervical tissue with histological evidence of HPV infection but without any sign of malignancy or intraepithelial neoplasia contained HPV16 DNA in a free state (data not shown). Since HPV16 is seldom found in condylomata (Gissmann et al., 1984), its presence in non-neoplastic tissue adjacent to a carcinoma of the lower genital tract may represent a valuable prognostic sign of tumour progression and extension.

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REFERENCES


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