In situ evidence for HPV 16, 18, 33 integration in cervical squamous cell cancer in Britain and South Africa

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Abstract
In a previous study three types of HPV signal were described in CIN. It was suggested that a type 1 signal represented episomal HPV while a type 2 signal represented integrated HPV; and a type 3 signal was indicative of both episomal and integrated HPV. To test this hypothesis 91 squamous cell cancers (SCC) of the cervix from Britain and South Africa were examined for HPV 6, 11, 16, 18, 31, 33, 35. Of the South African group (n = 69) 64% contained HPV types 16 (n = 29) and 18 (n = 15). The SCC in the British group (n = 22) contained HPV 16 and HPV 33 in 12 and three cases, respectively. Of the HPV positive biopsy specimens, 86% showed a type 2 signal in keratinising and non-keratinising tumours and the remainder a type 3 signal. Type 3 signal was present only in keratinising tumours. The presence of punctate signal in 100% of HPV containing SCC, together with localisation of HPV signal to sister chromatids in tumour cell mitotic figures in vivo, provides further evidence for type 2, and the punctate component of type 3 signal representing viral integration.

The presence of HPV 16, 18, 31, 33, 35 and other types is associated with cervical intraepithelial neoplasia (CIN). It is clear, however, that the presence of these HPV types is not sufficient per se to trigger the entire invasive neoplastic process in the cervix. Other cofactors are required. One of these may be HPV integration into the host genome at an early stage in pathogenesis. Viral integration is indirectly inferred from Southern blot analysis and directly by cloning junction fragments. Recently, we have shown that HPV 16, 18, 33, and 35 are present in three morphologically distinct forms in the nuclei of CIN tissue. These forms are referred to as NISH (non-isotopic in situ hybridisation) signal types 1–3. A type 1 signal is diffuse throughout the nuclei; type 2 signal is punctate; and type 3 is a combination of types 1 and 2. We postulated that a type 1 signal represents episomal virus, type 2 integrated virus, and type 3 a combination of both.

It has been shown that HPV 16 and 18 are integrated into the host genome in invasive squamous cell cancer (SCC) and tumour derived cell lines of the cervix. To test the hypothesis that type 2/3 NISH signals represent integrated HPV we examined 91 invasive SCC from Britain and South Africa by NISH using digoxigenin labelled HPV probes for HPV 6, 11, 16, 18, 31, 33, and 35.

Methods
Archival, formalin fixed, paraffin wax embedded cervical biopsy specimens of squamous cell cancer (SCC) were obtained from the surgical files of King Edward VIII Hospital, Durban, South Africa (n = 69). The British group (n = 22) of SCC biopsy specimens came from the archival files of this Department. Both sets were from the years 1988–90. Parallel haematoxylin and eosin stained slides of SCC were reviewed and classified according to morphological subtype.

Non-isotopic in situ hybridisation was performed using digoxigenin labelled probes for HPV 6, 11, 16, 18, 31, 33, 35, as previously described for HPV detection in CIN.

Age correlations were compared with the two-tailed χ² test.

Results
NISH HPV GENOTYPING
In the South African group 44 of 69 (64%) of the SCC contained HPV types 16 (n = 29) and 18 (n = 15). HPV 6, 11, 31, 33, and 35 were not present in any of the cancers in this group. The cervical cancers in the United Kingdom population contained HPV DNA in 68% (15 of 22) of the biopsy specimens. HPV 16 and 33 was present in 12 and three cases, respectively.
HPV 6, 11, 18, 31, and 35 were not detected in this group (table 1). No lesion was infected with multiple HPV types.

**CORRELATION OF CANCER MORPHOLOGY WITH HPV TYPE**

Of the 66 keratinising tumours, 50 (76%) contained HPV DNA. Of the 19 well differentiated 14 (74%) contained HPV DNA. Thirty six of the 47 (77%) moderately differentiated tumours contained HPV DNA. Of the 25 poorly differentiated non-keratinising large cell tumours, nine (36%) were positive for HPV (table 2).

Sixty per cent of well differentiated SCC from South Africa contained HPV DNA compared with 89% from the United Kingdom. The difference between the groups for moderately differentiated was less noticeable, with 78% of South African tumours containing HPV DNA compared with 70% of those from the United Kingdom. Only 41% of South African poorly differentiated non-keratinising large cell tumours, nine (36%) were positive for HPV (table 3).

**NISH SIGNAL TYPE**

Most HPV positive biopsy specimens (n = 51) from the combined South African and United Kingdom groups had a type 2 signal, as previously defined (table 4). This took the form of a red punctate/dot signal in the nucleus (fig 1). Thirty five of these biopsy specimens contained HPV 16, 14 contained HPV 18, and two HPV 33. The number of signals per nucleus ranged from one to five and varied between and within cases. In most cases the NISH signal was present in focal collections of cells. In a minority the signal was present in almost all tumour cells (n = 7). This type 2 pattern of signal was present in both keratinising (well differentiated/moderately differentiated) and non-keratinising tumours.

A type 3 signal, comprising a combined diffuse/granular intranuclear signal (type 1) and a type 2 signal was present in eight cases (fig 2). A type 3 signal was associated only with keratinising tumours; three were well differentiated and five moderately differentiated. Six of these cases contained HPV 16, one HPV 18, and one HPV 33.

Either a type 2 or type 3 NISH signal for HPV 16, 18, or 33 was found in 100% of all HPV positive SCC from the United Kingdom and South Africa (table 5).

A novel observation was the presence of a type 2 HPV 16 signal within mitotic figures in a keratinising (moderately differentiated) cervical carcinoma. These signals were paired suggesting that they were on sister chromatids (figs 3A and B). The signal number ranged from 1–5 per mitotic figure.

**CORRELATION BETWEEN AGE AND HPV DNA**

There was no significant difference in the overall HPV DNA detection rate in both groups combined when the age groups below and above 40 years were considered (p > 0.10) (table 6). Nor was there a significant difference (p > 0.10) in HPV DNA detection rate when the groups were divided above and below 30 years. In these age groups the HPV 16 detection rate for either the South African, United Kingdom, or combined groups was the same (p > 0.10).

**Discussion**

In CIN 3 without morphological wart virus infection HPV DNA occurs as a punctate/dot-like (type 2) NISH signal in 60%. Because a qualitatively similar signal is detected in CaSki and HeLa cells, we hypothesised that a type 2 NISH signal may represent in vivo evidence of integration of HPV 16 and 18; CaSki and HeLa cells contain integrated HPV 16 and 18, respectively. In cervical SCC HPV 16 integration has been shown in a small number of cases by Southern blotting. It would follow, therefore, that if a type 2 NISH signal did indeed represent in vivo evidence of HPV integration, this signal type would be found in squamous cell cancers from more that one geographical location. In this study we have shown that a punctate (type 2 or 3) signal is present in 100% of HPV positive SCC. A type 2 signal was present in 67, 85, and 93% of SCC in which
HPV 33, 16, and 18 sequences, respectively, were detected. A type 3 NISH signal was present in the remainder of the HPV positive cases. We have suggested that a type 3 signal represents the morphological counterpart of a combination of integrated and episomal HPV sequences, a situation which has been documented in cervical SCC by Southern blotting. All the HPV cervical SCC had a type 2 or 3 NISH signal, suggesting integration of HPV in 100% of this cancer type where HPV sequences are detected. It is important to emphasise that the latter statement applies to three different HPV types (16, 18, 33) in two widely separated geographic locations. Further evidence for the hypothesis that a punctate signal represents integration of HPV derives from the observation that NISH signals were found in mitotic figures in one of the SCC containing HPV 16. This signal consisted of two closely adjacent dots on chromosomes suggesting that HPV was present on sister chromatids. We are now quantifying the number of cases in which this phenomenon occurs and determining whether type 2/3 signals correlate with Southern blot evidence of integration.

A type 2 signal was detected in well, moderately, and poorly differentiated non-keratinising SCC of cervix, but a type 3 signal was only present in keratinising tumours. This argues that persistence of HPV in these cancers is independent of cellular differentiation. A type 3 signal, representing putative integrated and episomal HPV, correlated with keratinisation. This is consistent with the idea that the regulation of episomal viral replication may be associated with mechanisms linked to keratin differentiation. There is some in vitro evidence that HPV infection is an early event in cervical carcinogenesis. This, together with the current hypothesis that a type 2/3 signal indicates integration, may be clinically relevant in CIN because there is some evidence that severe dysplasias may regress or progress. If HPV integration is a clinically important early event in cervical carcinogenesis then a type 2/3 signal may indicate those HPV positive cases which will progress to a more severe degree of CIN or invasive cancer. This concept is supported by a case studied here. The patient presented initially with CIN 3. At cone biopsy this was found to be microinvasive and contained HPV 16 and 18 sequences by NISH. After treatment, the CIN recurred rapidly and both HPV 16 and 18 were present as type 2 signals in non-dividing cells and in mitotic figures in the CIN 3 lesion (C S Herrington et al, unpublished observations).

The detection of HPV DNA in 68% of SCC biopsy specimens in the British group is similar to that of other British prevalence studies. Although HPV 16 dominated in this group, the presence of HPV 33 in three cases is novel. The overall detection of HPV DNA sequences in cases of United Kingdom and South African cervical cancers (66%) compares favourably with other studies using the polymerase chain reaction (PCR) (74%) and Southern blots with radiolabelled probes (63%). Multiple HPV infection was not found in our series of 91 cases of SCC of the cervix by NISH. Studies using the PCR are inconsistent; in one report, multiple HPV infections were extremely common but the reverse has been documented by others. The sensitivity of the PCR in paraffin wax sections has been estimated at about 400 copies/sample compared with an NISH sensitivity of 10 copies/cell.

The HPV DNA prevalence in the South
Table 6 Correlation between HPV type and age in British and South African cases of SCC

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Overall HPV positivity</th>
<th>HPV genotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 30</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>30-39</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>40-59</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>&gt; 60</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>22</td>
</tr>
</tbody>
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*HPV types 6, 11, 31, 35, were not detected. HPV 18 and HPV 33 were not present in the British and South African groups, respectively.

African group (64%) in our study is higher than that of other African (sub-Saharan) studies. A combined Kenyan/Brazilian series identified HPV 16 in eight of 23 (35%) cervical cancers. A study of cervical cancers in Uganda showed the presence of HPV 16 and HPV 18 in five (15%) and 12 (35%) cases, respectively, in a series of 34 patients. Fifty of 57 (88%) Algerian patients with cervical cancers contained HPV DNA sequences in the biopsy specimens: 56% of these were HPV 16 and 18% HPV 18 positive.\(^6\) The ethnic groups in the Algerian cases were not specified. Interestingly, the incidence of HPV 18 and 19 was 42% and 22% in our South African patients. As noted in this study, the prevalence of HPV 18 in SCC is higher than in both the West\(^8\) and East.\(^21\) The detection of HPV 18 in the South African group is also higher in SCC (34%) compared with CIN (19-4%).\(^4\) It has been suggested that cervical lesions with HPV 18 may progress rapidly through the precursor stage (CIN) and hence account for the lower detection rate in CIN than SCC.\(^21\) It was therefore postulated that HPV 18 may play a role in the development of rapidly progressive cervical cancer.\(^2\) This is supported by the case referred to above (C S Herrington et al., unpublished observations). The absence of HPV 18 in the present United Kingdom group may be due to the small number of cases studied, or alternatively, this virus is endemically less common in the United Kingdom.

HPV 16, 18, and 33 are linked with keratin differentiation in SCC. HPV 16 is strongly associated with keratinising tumours (well differentiated, moderately differentiated), being detected in 50%, but in only 32% of poorly differentiated SCC (poorly differentiated non-keratinising large cell tumours). These data are comparable with those reported from the USA\(^5\) and Algeria.\(^19\) Of those tumours which contained HPV 18 or 33, 93% of the HPV 18 positive cases were keratinising and all three cases with HPV 33 also formed keratin.

No correlation was found between age and rate of HPV DNA detection in the groups combined. Similarly, HPV 16 DNA also correlated with age in either the combined or individual South African and United Kingdom groups. Age does not therefore seem to have a role in the actual rate of detection of HPV DNA in cervical preneoplasia and neoplasia. This is contrary to a previous suggestion that prevalence of HPV 16 detection increases with age.\(^12\)