Distribution of HPV infection and tumour markers in cervical intraepithelial neoplasia from cone biopsies of Mozambican women

C Carrilho, L Cirnes, M Alberto, L Buane, N Mendes, L David

ORIGINAL ARTICLE

Cervical carcinoma is a major cause of cancer death in Africa. The strong causal association between cervical carcinoma and infection by human papillomavirus (HPV) is well established, and it was recently proposed that HPV infection is a necessary cause of cervical cancer development. Both our group, and that of Castellsague et al, have previously described the profile of HPV infection in Mozambican women. In both studies, the types of HPV identified coincided with those previously described in other sub-Saharan countries, with a high prevalence of HPV types 16, 31, 33, 35, and 58.

With the improvement of methods to detect HPV at the molecular level there is increasing evidence of the presence of HPV in cervical intraepithelial neoplasia (CIN) lesions. The detection of multiple infections has also increased, probably because of the higher sensitivity of the techniques that are currently used.

"Cervical carcinoma is a major cause of cancer death in Africa"

Very few studies have explored the relation between the topographical distribution of HPV infection and HPV types and the different grades of CIN lesions in the uterine cervix. To the best of our knowledge, no studies relating the presence or absence of HPV infection and HPV types in CIN lesions, which have thoroughly evaluated the whole cervix, have been undertaken.

CIN and HPV infection are accompanied by several alterations in molecular structures that are expressed de novo or are lost. The putative usefulness of these markers for diagnostic and prognostic purposes is still controversial, and the results so far obtained are frequently conflicting.

In our present study, using serial samples from total inclusions of whole cervixes with intraepithelial lesions, we aimed to determine the distribution and types of HPV infection, in addition to the association between infection and the presence of different grades of intraepithelial lesion and with different markers associated with infection/lesions in previous studies (Ki-67, p53, cytokeratin 8 (Ck8), Ck10, Ck13, Ck17, Gp230 glycoprotein, simple mucin-type carbohydrates Tn, sialyl-Tn, T, and sialyl-T).

METHODS

Tissue
Cervical cone specimens from five consecutive patients with CIN diagnosed at the department of pathology of the Maputo Central Hospital in Mozambique were studied. For each cone specimen, several samples obtained by serial radial sections encompassing the whole cone were collected, varying from seven to 13 samples for each cone—52 samples in total. All CIN lesions were located in the transformation zone. Figures 1–5 depict the location of the lesions, and serial sections are numbered clockwise starting from the anterior lip of the cervix at 12 hours (section 1 in all cases). All samples were fixed in 10% formalin, embedded in paraffin wax, and cut into 4 µm thick sections. Haematoxylin and eosin stained sections were used to grade CIN lesions according to the World Health Organisation recommendations.

Immunohistochemistry
Immunohistochemistry was performed to study the expression of Ck8, Ck10, Ck13, Ck17, Gp230 glycoprotein, p53, Ki-67, and mucin-type carbohydrates—Tn, sialyl-Tn, T, and sialyl-T. The detection of the sialyl-T antigen was performed using antibodies directed at the T antigen, after treating the sections with neuraminidase.

Abbreviations: CIN, cervical intraepithelial neoplasia; Ck, cytokeratin; EIA, enzyme immunoassay; HPV, human papillomavirus; PCR, polymerase chain reaction
Table 1 Monoclonal antibodies, isotypes, working dilutions, and sources

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ck8</td>
<td>NCL-Ck8-TS1</td>
<td>IgG1</td>
<td>1/100</td>
<td>Novocastra Laboratories, Newcastle, UK</td>
</tr>
<tr>
<td>Ck10</td>
<td>NCL-Ck10</td>
<td>IgG1, k light chain</td>
<td>1/50</td>
<td>Novocastra Laboratories</td>
</tr>
<tr>
<td>Ck13</td>
<td>NCL-Ck13</td>
<td>IgG1</td>
<td>1/200</td>
<td>Novocastra Laboratories</td>
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<tr>
<td>Ck17</td>
<td>NCL-Ck17</td>
<td>IgG2b</td>
<td>1/20</td>
<td>Novocastra Laboratories</td>
</tr>
<tr>
<td>Tn</td>
<td>HB-Tn</td>
<td>IgM</td>
<td>1/15</td>
<td>H Clausen, S Hakamori, unpublished</td>
</tr>
<tr>
<td>Sialyl-Tn</td>
<td>HB-STn</td>
<td>IgG1</td>
<td>1/8</td>
<td>Kjeldsen and colleagues</td>
</tr>
<tr>
<td>T</td>
<td>HB-T</td>
<td>IgM</td>
<td>1/10</td>
<td>Clausen and colleagues</td>
</tr>
<tr>
<td>Gp230</td>
<td>PANH4</td>
<td>IgM</td>
<td>1/10</td>
<td>Nielsen and colleagues</td>
</tr>
<tr>
<td>p53</td>
<td>D07</td>
<td>IgG2b</td>
<td>1/50</td>
<td>Novocastra Laboratories</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MM1</td>
<td>IgG1</td>
<td>1/100</td>
<td>Novocastra Laboratories</td>
</tr>
</tbody>
</table>

Ck, cytokeratin.

Antibodies

Table 1 lists the mouse monoclonal antibodies used, their respective isotypes, working dilutions, and sources.

Immunostaining

Serial sections (4 μm thick) from 10% formalin fixed, paraffin wax embedded material were used for immunostaining. Immunohistochemistry was carried out according to the avidin–biotin–peroxidase complex method after dewaxing. Before immunostaining for Ck10, the specimens were trypsin digested at room temperature in Tris buffered saline. For the remaining keratins—Ck8, Ck13, and Ck17—and for p53 and Ki-67, specimens were submitted to high temperature (pressure cooker) using a 10mM sodium citrate buffer solution, pH 6. Sections to be treated with neuraminidase were preincubated with type VI neuraminidase from Clostridium perfringens (Sigma, Poole, Dorset, UK), diluted in 0.2M sodium acetate buffer, pH 5.5, to a final concentration of 0.1 U/ml. In all sections, endogenous peroxidase was blocked by incubation in 0.3% H2O2 in methanol for 10 minutes. Sections were incubated for 20 minutes with normal non-immune serum to eliminate non-specific staining. Excess normal serum was removed from the slides. The sections were then incubated for 30 minutes with the primary antibodies (dilutions specified in table 1), at room temperature for all Cks, p53, and Ki-67, and overnight for simple mucin-type carbohydrate antigens and Gp230 glycoprotein. This step was followed by incubation with a 1/200 dilution of biotin labelled antimouse secondary antibody (Dako, Copenhagen, Denmark) for 30 minutes and avidin–biotin–peroxidase complex for a further 30 minutes.

Careful rinses with Tris buffered saline were performed between each step of the procedure. The slides were then treated with 3,3-diaminobenzidinetetrahydrochloride, counterstained with Mayer’s haematoxylin, dehydrated, and mounted.

All series included positive controls. Negative controls were carried by omission of the primary antibodies. The presence of 0.1 U/ml. In all sections, endogenous peroxidase was blocked by incubation in 0.3% H2O2 in methanol for 10 minutes. Sections were incubated for 20 minutes with normal non-immune serum to eliminate non-specific staining. Excess normal serum was removed from the slides. The sections were then incubated for 30 minutes with the primary antibodies (dilutions specified in table 1), at room temperature for all Cks, p53, and Ki-67, and overnight for simple mucin-type carbohydrate antigens and Gp230 glycoprotein. This step was followed by incubation with a 1/200 dilution of biotin labelled antimouse secondary antibody (Dako, Copenhagen, Denmark) for 30 minutes and avidin–biotin–peroxidase complex for a further 30 minutes.

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Abnormal immunostaining profiles and scoring of the staining

To detect abnormal expression, the expression of Cks, mucin-type carbohydrates, and Gp230 glycoprotein was compared with the normal profile of expression defined in previous studies (table 2). For p53 and Ki-67 the number of positive cells was obtained by counting 100 cells in each section.

Molecular study for HPV detection

Sections for the detection and characterisation of HPV were obtained between two haematoxylin and eosin stained sections to control for the presence of representative sampling of the lesions. To avoid contamination, the section knife was replaced after cutting each section, and an empty paraffin wax block was used alternately. All negative samples were re-tested after DNA extraction and polymerase chain reaction (PCR) by cutting new sections, followed by a haematoxylin and eosin stained section to control for the presence of the lesion.

DNA preparation

DNA was extracted and purified according to standard techniques and the protocol used in our previous study.7 Each tissue section was digested with 10 mg/ml proteinase K in a buffered solution. For the PCR reaction 10 μl aliquots of DNA were used.

PCR

To check the quality of the target DNA all samples were tested by PCR using α actin specific primers to amplify a
RESULTS
HPV infection, CIN lesions, and expression of p53 and Ki-67

We studied a total of 52 samples from the five patients, encompassing the whole cervical cone specimen from each patient (seven to 13 samples for each cone specimen) (figs 1–5).

All the patients showed HPV infection with high risk HPV types. Low risk HPV types were never detected. Four of the five patients were infected with multiple HPV types. We found coinfection by HPV-33 and HPV-58 in two patients; by HPV-33, HPV-35, and HPV-58 in one patient; by HPV-16, HPV-33, and HPV-35 in one patient; and by HPV-16 alone in another patient (table 3). HPV-33 was seen in four cases, HPV-58 was seen in three cases, and HPV-16 and HPV-35 were seen in two cases each (table 3).

Taking all samples together, we found coinfection with multiple HPV types in 32 of 52 samples (32 of 36 HPV positive samples). Seventeen of all 27 samples (17 of 19 HPV positive samples) with CIN III lesions showed infection with multiple HPV types (tables 3 and 4). Table 4 shows the association between the presence or absence of CIN lesions of variable grade according to the presence or absence of HPV and of HPV types/combinations in all the samples from the five patients. All the HPV positive samples had CIN. However, 14 of the 16 HPV negative samples also had CIN lesions. HPV-58 alone or in combination with HPV-33 was always found in CIN III lesions. HPV-35 alone or in combination with HPV-33 was more frequently seen in CIN I lesions (table 4).

The percentage of cells positive for Ki-67 or p53 was similar in HPV negative and positive samples and in samples with different HPV types (table 5). A higher proportion of Ki-67 and p53 positive cells was seen in CIN III lesions (table 5) (fig 6A).

Patterns of expression of Cks, Gp230 glycoprotein, and simple mucin-type carbohydrates in the whole specimen cone sets

Abnormal expression of Sialyl-Tn was not detected.

- Case 1 (fig 1): abnormal expression of Ck8 (fig 6B), Ck17, Ck10, and Tn antigen largely coincides with both CIN III lesions and HPV infection. Ck13 (fig 6C, D) and Gp230 glycoprotein (fig 6E, F) are rarely expressed with an abnormal pattern. Abnormal expression of Sialyl-T antigen does not co-localise with either CIN lesions or the presence of HPV.
- Case 2 (fig 2): abnormal expression of Ck8, Ck10, and Ck17 largely coincides with both CIN III lesions and HPV infection. An exception is seen in section 2, with abnormal expression of the three markers in the absence of HPV. Ck10, in contrast to Ck8 and Ck17, is also expressed in CIN I (sections 10 and 11) and CIN II lesions (section 9). Ck13, Gp230 glycoprotein, and Tn antigen are rarely expressed with an abnormal pattern. Abnormal expression of Sialyl-T antigen does not co-localise with either CIN lesions or the presence of HPV.
- Case 3 (fig 3): abnormal expression of Ck10, Tn, T, and Sialyl-T is seen in almost all the tissue sections.
- Case 4 (fig 4): abnormal expression of Ck8, Ck10, and Tn antigen is seen in all sections with CIN III lesions, independent of the presence of HPV. Both Ck17 and Sialyl-T antigen have a more limited abnormal expression pattern.
- Case 5 (fig 5): abnormal expression of Ck8 and Ck17 coincides with both CIN III lesions and HPV infection. Ck10 and Tn antigen are abnormally expressed in almost

Table 2  Normal profile of expression of Ck and mucin markers in human cervical epithelium defined previously

<table>
<thead>
<tr>
<th>Markers</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ck8</td>
<td>Absent</td>
</tr>
<tr>
<td>Ck17</td>
<td>Absent</td>
</tr>
<tr>
<td>Ck10</td>
<td>Present (superficial and intermediate layers)</td>
</tr>
<tr>
<td>Ck13</td>
<td>Present (superficial and intermediate layers)</td>
</tr>
<tr>
<td>Gp230</td>
<td>Present (superficial and intermediate layers)</td>
</tr>
<tr>
<td>Tn</td>
<td>Present (superficial and intermediate layers)</td>
</tr>
<tr>
<td>Sialyl-Tn</td>
<td>Present (superficial and intermediate layers)</td>
</tr>
<tr>
<td>T</td>
<td>Absent</td>
</tr>
<tr>
<td>Sialyl-T</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Ck, cytokeratin.
all the tissue sections. Sialyl-T antigen is rarely expressed with an abnormal pattern.

Relation between HPV infection and CIN lesions in the whole specimen cone sets

In most cases, CIN lesions coincided with the presence of HPV. In cases 1, 2, 4, and 5 we saw CIN lesions in the absence of detectable HPV. We never detected HPV in sections without CIN lesions (cases 1 and 2).

DISCUSSION

Our present study showed a high frequency of HPV infection by multiple viral types in cervical specimens with CIN lesions. In fact, infection by a single viral type (HPV-16) was found in only one case. All the viral types identified were high risk HPVs and included HPV types 16, 33, 35, and 58.

To the best of our knowledge, our study is the first to evaluate thoroughly the presence and viral types of HPV in whole cone specimens. This is probably the reason why we found such a high frequency of multiple infections (four of five cases). The prevalence of HPV infection by multiple viral types reported in the literature varies from 24.5%11 to 40–50%.391023

With the improved methods to detect HPV at the molecular level there is increasing evidence of the presence of HPV in CIN lesions.2 The detection of multiple infections has also increased, probably because of the higher sensitivity of the techniques that are currently used.9–11 We used a GP5+/bio-GP6+ PCR-EIA method, which has a high sensitivity and specificity for HPV/HPV type detection, as demonstrated in previous studies.2122

Two of the five patients were human immunodeficiency virus negative when the cone specimen was taken and both had multiple infections (cases 3 and 5), so that we can assume that the high rate of multiple infections in our cases is not the result (at least exclusively) of human immunodeficiency virus infection.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Presence or absence of CIN according to the presence or absence of HPV and HPV types/combinations in all samples of whole cones from each patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case (no of samples)</td>
<td>No of samples/sections*</td>
</tr>
<tr>
<td>Case 1 (n = 9)</td>
<td>1/section 7</td>
</tr>
<tr>
<td>Case 2 (n = 11)</td>
<td>7/sections 1, 2, 3, 5, 6, 8, 9</td>
</tr>
<tr>
<td></td>
<td>2/sections 10, 11</td>
</tr>
<tr>
<td></td>
<td>1/section 9</td>
</tr>
<tr>
<td></td>
<td>3/sections 1, 6, 7</td>
</tr>
<tr>
<td>Case 3 (n = 13)</td>
<td>9/sections 1, 2, 3, 4, 5, 7, 11, 12, 13</td>
</tr>
<tr>
<td></td>
<td>2/sections 6, 8</td>
</tr>
<tr>
<td></td>
<td>1/section 10</td>
</tr>
<tr>
<td>Case 4 (n = 7)</td>
<td>6/sections 1, 2, 3, 4, 5, 7</td>
</tr>
<tr>
<td></td>
<td>1/section 6</td>
</tr>
<tr>
<td></td>
<td>1/section 9</td>
</tr>
<tr>
<td>Case 5 (n = 12)</td>
<td>4/sections 8, 9, 11, 12</td>
</tr>
<tr>
<td></td>
<td>7/sections 1, 2, 3, 4, 5, 6, 7</td>
</tr>
<tr>
<td></td>
<td>1/section 10</td>
</tr>
</tbody>
</table>

*Sections are numbered in figs 1–5.

CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Presence or absence of CIN according to the presence or absence of HPV and HPV types/combinations in all the sections from the five patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cervix (n/%)</td>
<td>CIN I (n/%)</td>
</tr>
<tr>
<td>HPV negative (n = 16)</td>
<td>2/12.5</td>
</tr>
<tr>
<td>HPV-33/58 (n = 14)</td>
<td>0/0</td>
</tr>
<tr>
<td>HPV-33/35 (n = 16)</td>
<td>0/0</td>
</tr>
<tr>
<td>HPV-16/33/35 (n = 2)</td>
<td>0/0</td>
</tr>
<tr>
<td>HPV-35 (n = 1)</td>
<td>0/0</td>
</tr>
<tr>
<td>HPV-58 (n = 1)</td>
<td>0/0</td>
</tr>
<tr>
<td>Total HPV positive (n = 36)</td>
<td>0/0</td>
</tr>
</tbody>
</table>

CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Percentage of Ki-67 and p53 positive cells according to the presence or absence of HPV and the different HPV types/combinations and CIN lesions in all sections from the five cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>p53</td>
</tr>
<tr>
<td>HPV negative (n = 16)</td>
<td>23.9 (23.6)</td>
</tr>
<tr>
<td>HPV positive (n = 36)</td>
<td>25.9 (13.7)</td>
</tr>
<tr>
<td>HPV-33/58 (n = 14)</td>
<td>26.1 (10.7)</td>
</tr>
<tr>
<td>HPV-33/35 (n = 14)</td>
<td>23.0 (16.2)</td>
</tr>
<tr>
<td>HPV-16/33/35 (n = 2)</td>
<td>29.0 (1.4)</td>
</tr>
<tr>
<td>HPV-16 (n = 2)</td>
<td>35.5 (27.6)</td>
</tr>
<tr>
<td>HPV-58 (n = 1)</td>
<td>24</td>
</tr>
<tr>
<td>Normal (n = 2)</td>
<td>0.5 (0.7)</td>
</tr>
<tr>
<td>CIN I (n = 20)</td>
<td>19.3 (13.7)</td>
</tr>
<tr>
<td>CIN III (n = 27)</td>
<td>31.6 (17.4)</td>
</tr>
</tbody>
</table>

Values are mean (SD).

*Three HPV positive sections (1 with HPV-35 and 2 with HPV-33 and HPV-35) could not be used for Ki-67 and p53 evaluation because of a lack of material.

CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.
The small number of cases in our study does not allow us to make definitive conclusions regarding the frequency of multiple infections, and this should be confirmed by larger studies in the future. Despite this, the amount of information we collected should be taken into account for designing both primary prevention strategies, namely vaccines, and for building the best approach in screening programmes.24 25

"We cannot rule out the possibility that at least a proportion of cervical intraepithelial neoplasia lesions progress in the absence of human papillomavirus”

The low number of cases evaluated in our present series also does not allow us to make comparisons with previous studies with respect to the relative frequencies of viral types. In any case, the viral types we identified in our study (HPV types 16, 33, 35, and 58) are the most frequently reported in African populations.2–6

As expected, we found a positive relation between the presence of HPV in the individual samples and the presence of CIN lesions. In fact, all the specimens in which HPV was detected showed CIN lesions. However, CIN lesions were also seen in HPV negative samples. We cannot exclude the possibility that the characteristics of the specimens (low representativity of neoplastic tissue, relatively low quality of the preservation method (10% unbuffered formalin), and possible low viral load) might explain the HPV negative results in our cases.

However, because most of the HPV negative samples had CIN lesions—mainly CIN III lesions—we cannot rule out the possibility that at least a proportion of CIN lesions progress in the absence of HPV, thus favouring the concept that CIN can be a bona fide neoplastic lesion. Supporting this interpretation are data showing that HPV is lost/disintegrated during cancer progression.26 Another explanation is the “hit and run concept”.27 28 We think that it is unlikely that our screening process missed a rare or even yet unknown viral type.

Although our study has obvious limitations regarding the number of cases (five), it is interesting that HPV-58 was always detected in samples with CIN III lesions (15 of 27) and HPV-35 was detected more frequently in samples with CIN I lesions (15 of 22). This observation may be partly explained by different capabilities of the E6 protein to inactivate p53 according to the type of high risk HPV.29

The high percentage of cells positive for Ki-67 and p53 in CIN III lesions agrees with previous reports.30–33 The similar degree of Ki-67 and p53 expression in HPV positive and
negative samples seen in our study and in previous publications, is probably the result of the high prevalence of CIN lesions in HPV negative sections.

In previous studies, we evaluated the usefulness of different markers for the identification of preinvasive and invasive cervical lesions: Ck8, Ck10, Ck13, Ck17, Gp230 glycoprotein, and simple mucin-type carbohydrates—Tn, Sialyl-Tn, T, and Sialyl-T. We identified the expression of Ck8 and Ck17 and the loss of Ck10 and Ck13 as good indicators of malignant transformation in the human cervix, in agreement with other publications. We also found that loss of expression of Gp230 glycoprotein is associated with malignant transformation at preinvasive stages. Investigation of the expression of simple mucin-type carbohydrates showed that Tn and ST were useful markers of invasive carcinomas. In an attempt to expand our study in preinvasive cervical lesions and to map precisely their pattern of expression we evaluated their profile of expression in whole cervical cone biopsies.

The expression of Ck8 and Ck17 showed a complete (Ck8) or almost complete (Ck17) overlap with CIN III lesions, reinforcing their practical usefulness for the detection of CIN III lesions, namely in cytology specimens. Loss of expression of both Ck10 and Ck13 is associated with preinvasive cervical lesions, more consistently for Ck10. However, the lack of a consistent profile of alterations hampers their usefulness for diagnostic purposes.

Altered expression of Gp230 glycoprotein, Tn, and sialyl-T was frequently seen in all low grade and high grade cervical lesions, showing that mucin and mucin glycosylation changes are early events in cervical neoplasia, as reported previously by our group and by others. However, the lack of consistency between their expression profile and the cervical lesions in our whole cone specimens limits their usefulness for diagnostic purposes. Altered expression of sialyl-Tn was not detected in our present series, in agreement with our previous report, showing that sialyl-Tn has little value as a marker for malignancy in the human cervix.

None of the putative diagnostic markers showed a clear association with HPV infection, indicating that they are markers of cervical lesions and not of cervical infection by HPV.

In conclusion, our study shows that when whole cone biopsies are evaluated the rate of infection by multiple HPV types is very high, and combines the most frequent high risk HPV types described in African populations. We also show that Ck8 and Ck17 are useful markers for CIN III lesions, independent of HPV infection.

ACKNOWLEDGEMENTS

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Authors’ affiliations

C Carrilho, M Alberto, L Buane, Department of Pathology, Medical School, Eduardo Mondlane University, and Maputo Central Hospital, Maputo, Mozambique

C Carrilho, L Cirmes, N Mendes, L David, IPATIMUP-Institute of Molecular Pathology and Immunology, University of Porto, Rua Roberto Frias, s/n, 4200, Porto, Portugal

L David, Medical Faculty of the University of Porto

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