Immunohistochemical analysis of p53 expression in anal squamous neoplasia

O A Ogunbiyi, J H Scholefield, J H F Smith, S V Polacarz, K Rogers, F Sharp

Aims—To determine the pattern of expression of the p53 tumour suppressor gene product in anal squamous neoplasia, and to determine if this could be used as a marker of disease progression. The association between p53 expression and human papillomavirus (HPV) 16 DNA status of the anal lesions was also investigated.

Methods—The presence and localisation of the p53 protein in formalin fixed, paraffin wax embedded specimens of anal squamous epithelium (normal and neoplastic) was examined using immunohistochemical staining with a panel of two monoclonal antibodies (DO-1, DO-7) and one polyclonal antibody (CM-1). Thirty nine normal anal epithelia, 14 anal intraepithelial neoplasia (AIN) grade 1, seven AIN 2, and 20 AIN 3 specimens were obtained from patients without demonstrable invasive disease; twelve AIN 3 specimens adjacent to invasive disease and 34 anal squamous cancers were also examined. Genomic DNA from all 126 specimens was extracted and analysed for HPV 16 DNA using the polymerase chain reaction (PCR).

Results—Nuclear p53 was strongly expressed in 67% (23/34) of invasive anal squamous tumours, 75% (9/12) of AIN 3 specimens adjacent to invasive disease, and in 60% (12/20) of AIN 3 specimens obtained from patients without demonstrable invasive disease. Two of the patients in the latter group with positively staining specimens subsequently developed invasive tumours which had staining characteristics similar to those of the AIN 3 specimens. p53 protein was expressed in very low concentrations in low grade AIN and not at all in normal anal squamous epithelium. In those specimens which stained positively for p53, HPV 16 DNA sequences were detected in 69-5% (16/23) of invasive disease, 77-7% (7/9) of AIN 3 adjacent to invasive disease, 75% (9/12) of AIN 3 obtained from patients without demonstrable invasive disease, 33-3% (2/6) of AIN 2, and in 40% (2/5) of AIN 1. There was no significant correlation between p53 immunostaining and HPV 16 DNA status (p < 0.05).

Conclusions—Aberrant expression of the p53 gene product is probably involved in the pathogenesis of anal squamous neoplasia. Long term follow up studies of all patients with AIN are required to determine if this could be used as a marker of likely disease progression from high grade AIN to invasive disease. There does not seem to be an association between the presence or absence of HPV 16 DNA sequences and mutant p53 proteins in anal squamous neoplasia.
might contribute to carcinogenesis. The conformational change produced by p53 gene mutation is thought to stabilise the protein, thereby prolonging the half-life of the protein which can be detected immunohistochemically by specific p53 antibodies. The increased expression of p53 protein has been shown in several human tumours and has also been correlated with mutation of the p53 gene. The accumulation of high concentrations of p53 is emerging as a potential marker for malignancy and in certain tumour types may be associated with a poor prognosis.

Methods

One hundred and twenty six specimens were studied. These comprised 39 normal anal epithelia, 14 AIN 1, seven AIN 2, 20 AIN 3—all obtained from patients without demonstrable invasive disease—12 AIN 3 specimens adjacent to invasive disease, and 34 anal squamous cancers. All specimens were obtained from the archival files of the histopathology departments of the Northern General Hospital, Sheffield, and St Mark's Hospital, London. The specimens had been previously fixed in 10% buffered formalin phosphate and paraffin wax embedded. The histopathological diagnosis in all specimens was reviewed by two independent observers (JHFS, SVP). There was good agreement between the two observers.

The specimens were analysed with a panel of two monoclonal antibodies DO-1 (courtesy of Professor DP Lane) and DO-7 (Novocastra Laboratories, England) and the rabbit polyclonal antibody CM-1 (courtesy of Professor DP Lane) produced against recombinant human p53 and permitting detection of p53 in routine formalin fixed histological material. The antibodies were used in dilutions of 1 in 100, 1 in 2000, and 1 in 4000 for DO-7, CM-1, and DO-1, respectively. DO-1 and DO-7 recognise epitopes between amino acids 1 and 45 at the N-terminal end of both mutant and wild type p53 protein.

Sections (5 μm) were cut, floated on poly-l-lysine coated glass microscope slides, and air dried overnight at room temperature. Sections were dehydrated in xylene and hydrated through graded alcohols to deionised water. Immunohistochemical analysis was performed using a conventional three-layered streptavidin horseradish peroxidase technique. Endogenous peroxidase activity was blocked by a 5 minute incubation in 3% hydrogen peroxide, followed by incubation of the specimens in non-immune goat serum to block non-specific binding. The specimens were then incubated with the appropriate dilution of the primary antibody for 1 hour.

Localisation of the primary antibody was achieved by means of the labelled streptavidin-avidin-biotin horseradish peroxidase technique (Dako LSAB kit, Dako Ltd, High Wycombe, Bucks) and 3',3-diaminobenzidine (activated with 3% hydrogen peroxide) as the chromogen. Washing in TRIS buffered saline (pH 7.6) was carried out between each step. All incubations were carried out at room temperature. The sections were then lightly counterstained with Harris's haematoxylin, dehydrated in ascending grades of alcohol, and then mounted. A positive result is indicated by brown staining of the nucleus.

Sections of a colon adenocarcinoma known to express strongly mutant p53 protein were used as known positive controls. Non-immune mouse serum was used in place of the primary antibodies in all sections of anal tissue and used as negative controls. Assessment of the specimens for p53 staining was made by two independent observers (OAO and SVP).

Specimens were considered to have stained positively for p53 if there was brown nuclear staining of the squamous epithelial cells with one or more of the p53 antibodies in at least 5% of the squamous epithelium. The intensity of staining was graded as 0 no staining; + minimal staining; ++ moderate staining; +++ strongly staining (table 1). There was complete agreement between the two observers with regard to those specimens that stained moderately or strongly for p53. In those specimens which stained less positively, and for which no agreement could be reached by the two observers, the specimens were graded as negatively staining.

Four 20 μm thick sections from 126 specimens were cut, dewaxed in xylene and hydrated through graded alcohols. Genomic DNA extraction was carried out by proteinase K digestion and phenol extraction. All DNAs were analysed for HPV 16 DNA by polymerase chain reaction (PCR) using a pair of primers to the E6 open reading frame. The primer sequences are described below. PCR was performed on 200 ng of genomic DNA in the presence of 20 mM MgCl₂ with 2 units of Taq polymerase (Promega, UK), 50 μM of each primer, buffer (Promega), and dNTPs to a reaction volume of 100 μl. Specific reaction conditions for PCR included 40 cycles of denaturation at 94°C for 1 minute, primer

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Total</th>
<th>No positive</th>
<th>Intensity of staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Normal anal epithelium</td>
<td>39</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AIN 1/HPV</td>
<td>14</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>AIN 2</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>AIN 3—no invasion</td>
<td>20</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>AIN 3—invasion</td>
<td>12</td>
<td>9</td>
<td></td>
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<tr>
<td>Anal cancer</td>
<td>34</td>
<td>23</td>
<td></td>
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</table>
Immunohistochemical analysis of p53 expression in anal squamous neoplasia

Immunohistochemical staining for p53 protein in (A) invasive anal squamous cancer; (B) AIN 3; (C) AIN 2, with the monoclonal antibodies DO-1, DO-7, and the polyclonal antibody CM-I, respectively. The reaction product is brown (counterstain Harris’s haematoxylin). Staining is intense, nuclear, and diffuse in anal cancer and AIN 3. Staining in AIN 2 is also nuclear, although it is much weaker and focally distributed.

Table 2  Staining with p53 antibodies in anal squamous cell neoplasia

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Total No positive (n = 55)</th>
<th>DO-7 positive (%)</th>
<th>DO-1 positive (%)</th>
<th>CM-I positive (%)</th>
<th>All antibodies positive (%)</th>
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<tr>
<td>Normal anal epithelium</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AIN 1/HPV</td>
<td>5</td>
<td>4 (80)</td>
<td>4 (80)</td>
<td>5 (100)</td>
<td>4 (80)</td>
</tr>
<tr>
<td>AIN 2</td>
<td>6</td>
<td>6 (100)</td>
<td>5 (83)</td>
<td>6 (100)</td>
<td>5 (83)</td>
</tr>
<tr>
<td>AIN 3—no invasion</td>
<td>12</td>
<td>12 (100)</td>
<td>11 (91)</td>
<td>11 (91)</td>
<td>11 (91)</td>
</tr>
<tr>
<td>AIN 3—invasion</td>
<td>9</td>
<td>9 (100)</td>
<td>9 (100)</td>
<td>9 (100)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>Anal cancer</td>
<td>23</td>
<td>21 (91)</td>
<td>20 (90)</td>
<td>22 (95)</td>
<td>20 (90)</td>
</tr>
</tbody>
</table>
exclusively nuclear, weak in intensity, involving at least 5% of the dysplastic squamous epithelium (Figure). At least 80% of the specimens stained positively with all 3 antibodies (table 2). There was no staining of the stroma and normal squamous epithelial cells in the specimens.

HIGH GRADE AIN
Analysis of p53 immunostaining in the two groups of AIN 3 specimens showed that a significant number stained positively with the p53 antibodies (table 2). In AIN 3 specimens obtained from patients without demonstrable invasive disease in the primary biopsy material, 60% (12/20) of the specimens stained positively for p53 protein. Staining was exclusively nuclear, involving over 50% of the squamous cells in seven specimens, and 10–15% of the squamous cells in five specimens (Figure). During a mean follow up period of 12 months (range 3–18 months), two patients with positive staining specimens subsequently developed invasive anal squamous cancer. The invasive lesions had similar staining characteristics to the AIN 3 specimens. Seventy five per cent (9/12) of the specimens of AIN 3 adjacent to invasive tumour stained very strongly for p53, with over 50% of squamous cells involved.

INVASIVE ANAL SQUAMOUS CELL CARCINOMA
Analysis of 34 invasive anal cancers show that about 67% (23/34) of the tumours stained strongly for p53 protein. Staining was nuclear and involved almost all the tumour cells (figure). At least 90% of the positively staining tumours reacted with all three p53 antibodies. The distribution of staining with the antibodies was similar, although the intensity of staining was slightly greater with DO-1 compared with CM-1 and DO-7.

HPV STATUS AND ABBERRANT p53 EXPRESSION
All 126 anal specimens were analysed for HPV 16 DNA status using the PCR technique. HPV 16 DNA sequences were detected in 76-5% (26/34) of invasive tumours, 66-6% (8/12) of AIN 3 specimens adjacent to invasive disease, 75% (15/20) of AIN 3 specimens from patients without demonstrable invasive disease, 42-8% (3/7) of AIN 2 and 28-5% (4/14) of AIN 1 specimens. None of the specimens of normal anal epithelium was positive for HPV 16 DNA (table 3). A total of 43-6% (55) specimens stained positively for p53. Of these, 36 were HPV 16 positive and 19 were HPV 16 negative (table 4).

Statistical analysis examining the correlation between p53 staining and the HPV DNA status in all the neoplastic anal specimens was performed using the $\chi^2$ test (with significance of $p < 0.05$). There was no correlation between p53 positivity and HPV 16 DNA status.

### Discussion
Intraepithelial neoplastic lesions of the anus (AIN) were first described a decade ago. The natural history of these lesions is unknown, although possible parallels between AIN and cervical intraepithelial neoplasia (CIN) have been suggested with regard to progression to invasive squamous cell carcinoma. This hypothesis is supported by the fact that high grade AIN 3 lesions have been detected in resection specimens for anal squamous cell cancer. The lesions have been found adjacent to the invasive tumours as well as in areas separated from the tumours by normal mucosa. AIN seems to be fairly common in certain risk groups: homosexual men who practise receptive anal intercourse; immunosuppressed organ transplant recipients; and group IV HIV seropositive individuals, and as part of multifocal anogenital squamous cell neoplasia. In view of the rarity of anal squamous neoplasia (3% of large bowel cancers), most of the AIN lesions seem to regress or remain static. This poses problems for their management as treatment of all lesions may result in avoidable morbidity in some patients. On the other hand, the possibility that a small number of patients with untreated AIN may progress to invasive cancer has to be considered. How, then, do we determine which AIN lesions are likely to progress to invasive cancer? Ideally, a marker identifying abnormal anal cells would aid in the management of such patients.

Mutations of the p53 tumour suppressor gene are the most common genetic abnormality in human cancers. These mutations seem to alter the structure of the p53 protein and also to stabilise it, leading to increased concentrations of mutant p53 protein in cells. Furthermore, certain mutant p53 proteins act as dominant transforming oncogenes. Recent studies have shown that in cervical tumours and cell lines, as well as in a small series of anal tumours, there seems to be an inverse relation between the presence of p53 mutations and oncogenic HPV sequences: only HPV negative tumours seem to contain mutations of the p53 gene. This suggests that an alteration in the function of p53, either by mutation or binding to HPV encoded proteins, is implicated in the pathogenesis of anogenital neoplasia.

### Table 3: HPV 16 DNA status in anal squamous cell neoplasia

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Total No (n=126)</th>
<th>HPV 16 positive</th>
<th>HPV 16 negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal anal epithelium</td>
<td>39</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>AIN 1/HPV</td>
<td>14</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>AIN 2</td>
<td>7</td>
<td>3</td>
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<tr>
<td>AIN 3—no invasion</td>
<td>20</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>AIN 3—invasion</td>
<td>12</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Anal cancer</td>
<td>34</td>
<td>26</td>
<td>8</td>
</tr>
</tbody>
</table>

### Table 4: HPV 16 status in p53 positively staining specimens (n = 55)

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Total No p53 positive</th>
<th>HPV 16 positive</th>
<th>HPV 16 negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal anal epithelium</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AIN 1/HPV</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>AIN 2</td>
<td>6</td>
<td>2</td>
<td>4</td>
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<tr>
<td>AIN 3—no invasion</td>
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<td>3</td>
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<tr>
<td>AIN 3—invasion</td>
<td>9</td>
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<td>2</td>
</tr>
<tr>
<td>Anal cancer</td>
<td>23</td>
<td>16</td>
<td>7</td>
</tr>
</tbody>
</table>
The results of our study show that increased p53 protein concentrations occur in high grade AIN and invasive anal squamous cell carcinomas. The similarity in the pattern of distribution of p53 concentrations in invasive tumours and adjacent AIN 3 lesions suggest that a field effect phenomenon may be operating. Furthermore, in two specimens of AIN 3 obtained from patients without demonstrable invasive disease, which stained strongly for the p53 protein, the patients subsequently developed invasive anal squamous cell carcinomas which had a similar staining pattern to that of the AIN 3 specimens. These results suggest that mutations of the p53 gene (leading to expression of high concentration of mutant p53 protein) may have a role in the pathogenesis of anal squamous neoplasia. The accumulation of mutant p53 protein may also possibly serve as a marker of likely disease progression from AIN 3 to invasive lesions. Long term follow up of the AIN 3 lesions, however, are required to confirm these results.

These results are similar to those of studies which have examined p53 expression in squamous neoplasia at other sites. These studies have shown increased concentrations of the p53 protein in oesophageal, skin, and head and neck squamous cell carcinomas. Furthermore, some of these studies have shown a close correlation between increased concentrations of p53 protein and mutations of the p53 gene locus. Increased p53 concentrations have also been shown in dysplastic and normal epithelium obtained from patients with p53 positive invasive squamous cell carcinomas.

The expression of increased p53 protein concentrations in HPV positive AIN and invasive tumours (table 4) is surprising, as current evidence suggests that there is an inverse relation between the presence of p53 mutations and oncogenic HPV sequences. The E6 oncoprotein of HPV 16 and 18 has been shown to complex with wild type p53 protein, accelerating its degradation, and thereby nullifying the tumour suppressor effects of the wild type p53 protein. In HPV negative tumours it is thought that mutations of the p53 gene leads to loss of function of the wild type allele. The results from recent studies, however, suggest that the relation between HPV infection and p53 mutation in anogenital neoplasia may not be quite so clear cut. These studies suggest that there is no obvious association between the presence or absence of HPV sequences and mutations or allelic loss at the p53 gene locus.

We do not think that the results of the present study are due to non-specific cross reaction as antibodies to different epitopes on the p53 molecule were used. The results with these antibodies correlated strongly with each other (table 2) and were consistently repeatable. Further investigation into the status of the p53 gene in anal squamous neoplasia, however, is required. We are currently sequencing the p53 gene locus in the specimens examined in the present study.


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doi: 10.1136/jcp.46.6.507

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