Abstract

**Aim**—To investigate the expression of p53 protein in invasive squamous cell carcinoma (SCC) of the larynx and dysplasia in relation to histological grade and tobacco smoking.

**Method**—Paraffin wax embedded tissue sections from 41 cases of invasive SCC of the larynx, 28 cases of dysplasia and 14 control laryngeal biopsy specimens were studied immunohistochemically using two anti-p53 monoclonal antibodies (DO7 and 801). The Streptavidin/horseradish peroxidase method was used after microwave antigen retrieval and a semiquantitative method was applied to assess the extent of p53 expression.

**Results**—Of the cases of invasive SCC of the larynx, 78% (32/41), regardless of histological grade, overexpressed p53 compared with only 30% (eight of 28) of cases of mild dysplasia. A gradual increase in p53 expression from mild to severe dysplasia (60%) was observed, and only three of 14 control biopsy specimens of laryngeal nodules showed occasional weakly positive basal cells.

**Conclusion**—The gradual increase in p53 expression from mild to severe dysplasia to invasive SCC indicates that p53 overexpression is an early event in laryngeal carcinogenesis which may lead to invasive malignancy. p53 overexpression may be related to environmental factors as most of the patients smoked tobacco. Microwave postfixation may be essential for the reliable detection of p53.

Keywords: p53, squamous cell carcinoma, dysplasia, larynx.

Wild-type p53 is a short-lived nuclear phosphoprotein coded by the p53 tumour suppressor gene located on chromosome 17p13.1

Its main function is to downregulate the cell cycle reducing the mutagenic effects of DNA damage hence preventing carcinogenesis.2,3

Mutational change of the p53 gene is the commonest anomaly detected in human malignant neoplasia resulting in loss of action of the p53 gene. The p53 protein product of the altered gene becomes stable and easily detectable immunohistochemically. Mechanisms of p53 overexpression without p53 gene mutations have also been reported.4

Invasive squamous cell carcinoma (SCC) of the larynx is a common malignancy and to-bacco, alcohol, human papilloma viruses (HPV), and malnutrition have all been implicated as aetiological factors in laryngeal carcinogenesis although their mode of action is not clear.7 There is evidence that alterations in the p53 gene are important for tumour progression as has been shown in malignancies with distinct sequential morphological features such as those which occur in the development of cancer in the oral cavity,5 colon6 or uterine cervix.10 In laryngeal carcinogenesis dysplasia usually precedes the development of in situ and invasive SCC.11 Several studies indicate significant overexpression of stable p53 protein in invasive SCC of the larynx12-14 and this phenomenon has been associated with heavy smoking15-17 closely resembling that observed in lung carcinomas.18-20 The latter are characterised by several p53 mutations in exons 4 to 9 with a “hot spot” in the 238 to 248 region.21 It seems that the mutated p53 gene plays a significant role in the early stages of carcinogenesis and may determine progression of the disease during the late stages of tumour development.11,22 Greek patients with laryngeal SCC are usually heavy smokers and the present study was undertaken to assess p53 expression during the sequential steps of laryngeal carcinogenesis as very few studies refer to this phenomenon.17

**Methods**

Archival paraffin wax embedded sections of laryngeal biopsy or laryngectomy specimens fixed in neutral buffer formol were studied retrospectively, using immunohistochemical methods. The length of fixation was unknown for the laryngectomy specimens but the laryngeal biopsy specimens were fixed for up to 24 hours. The study population comprised 83 patients in total, including 41 with laryngeal invasive SCC, 28 cases of mild (n=8), moderate (n=8) or severe (n=10) dysplasia without associated invasive malignancy, and a control group of laryngeal nodules (14 cases) without evidence of malignancy despite a history of heavy smoking (10 of 14 cases). Heavy smokers (group A) were patients who smoked more than 20 cigarettes daily for at least five years, patients in group B smoked less than 20 cigarettes daily and those in group C were non-smokers. The clinicopathological features of all patients are indicated in table 1.

The microwave postfixation Streptavidin/ horseradish peroxidase method was applied to paraffin wax sections (5-6 μm)23,24 which were then incubated with the two monoclonal anti-
Table 1  Clinicopathological features of patients with invasive SCC of the larynx and dysplasia

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>Grade</th>
<th>No. of patients</th>
<th>Sex (M/F)</th>
<th>Mean age (years)</th>
<th>Tobacco Smoking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>SCC (n=41)</td>
<td>1</td>
<td>14</td>
<td>14/0</td>
<td>63-7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18</td>
<td>18/0</td>
<td>65-1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9</td>
<td>8/1</td>
<td>60-4</td>
<td>1</td>
</tr>
<tr>
<td>Dysplasia (n=28)</td>
<td>1</td>
<td>10</td>
<td>8/2</td>
<td>53-3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8</td>
<td>8/0</td>
<td>53-3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10</td>
<td>8/2</td>
<td>63-5</td>
<td>4</td>
</tr>
<tr>
<td>Controls (n=14)</td>
<td>1</td>
<td>14</td>
<td>10/4</td>
<td>57-6</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>83</td>
<td>74/9</td>
<td></td>
<td></td>
<td>19</td>
</tr>
</tbody>
</table>

A = >20 cigarettes daily (heavy smokers); B = <20 cigarettes daily (moderate smokers); C = non-smokers; D = not known.

Table 2  p53 expression in laryngeal SCC and dysplasia

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>Grade</th>
<th>No. of patients</th>
<th>p53 expression*</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>SCC</td>
<td>1</td>
<td>14</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>1</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Controls</td>
<td>14</td>
<td></td>
<td>Scanty, weakly positive basal cells in three cases</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>83</td>
<td></td>
<td>17</td>
<td>16</td>
</tr>
</tbody>
</table>

* See text for scoring system used.

p53 antibodies (DO7 and 1801).25,26 p53 expression was assessed semiquantitatively using the following scoring system: less than 10% positive cells (+), 10–50% positive cells (+ +), more than 50% positive cells (+ + +). As the intensity of the nuclear staining varied, all positive nuclei were considered equally.

Results

In table 2 the extent of p53 overexpression has been correlated with histological diagnosis, grade and smoking habits. Of the SCC cases, 78-5% (11/14) of grade 1 and 77-8% of grades 2 (14/18) and 3 (seven of nine) overexpressed p53 (fig 1). Only in three control biopsy specimens were scanty, weakly positive basal cell nuclei identified. Of the 17 p53 negative cases, four were from group A and 11 from group B. The extent and intensity of staining was comparable for both monoclonal antibodies but there was a slight increase in sensitivity with DO7 (three cases negative with 1801 were positive with DO7).

The staining intensity within individual cases varied from area to area, particularly in the laryngectomy specimen sections. In addition, there was no significant difference in p53 overexpression between in situ and invasive areas of laryngeal carcinoma, thus no distinction has been made between these in recording the results. Dysplasia adjacent to invasive SCC (18 cases) also showed positive immunoreactivity. A significant difference in p53 overexpression was observed between grade 1 dysplasia where only 30% (three of 10) of cases were positive and grades 2 and 3 where 75% (six of eight) and 60% (six of 10) positivity was observed (fig 2). Although more cases were positive in grade 2 compared with grade 3 (75% v 60%) most of them (five of eight) showed positive immunoreactivity in less than 10% of cells. In grade 3 dysplasia four of eight cases showed diffuse immunoreactivity.

Although there was no linear correlation between the percentage of positive cells and the degree of smoking, all but three of the patients overexpressing p53 were smokers. All of those patients with grades 2 and 3 dysplasia with varying degrees of p53 immunoreactivity smoked.

Discussion

In this retrospective study of 83 Greek patients with laryngeal premalignant changes and invasive SCC of the larynx marked p53 overexpression (78%) was observed regardless of histological grade. As follow up was available for three years only and no reported deaths or lymph node metastases were reported in these patients during that time, the results have not been correlated with stage or survival but with histological grade and smoking habit.

To our knowledge, this is the first study to address the phenomenon of p53 overexpression in laryngeal dysplasia without adjacent invasive malignancy and our results indicate a distinct difference between mild dysplasia where only 30% positivity was observed compared with grade 2 and 3 dysplasia (75% and 60%, respectively). We did not observe the same pattern of homogeneity in p53 expression in staining of normal epithelial nuclei adjacent to invasive SCC, as reported by Dolcetti et al,22 and this may be because of the different immunohistochemical methods used to detect p53 overexpression, or the variable length of laryngectomy specimen fixation. In the same study frequent expression of p53 protein was observed in dysplasia adjacent to invasive SCC but the extent of p53 expression in relation to the histological grade of dysplasia was not reported. We confirmed previous reports that p53 overexpression is an early phenomenon in laryngeal carcinogenesis22 with clonal expansion of p53 positive cells during the sequential stages of laryngeal carcinogenesis, a phenomenon that may be advantageous to the malignant cells for tumour progression. We did
Figure 2 This figure illustrates the difference in p53 immunostaining between grade 2 (A) and grade 3 dysplasia (B) with monoclonal antibody DO7.

not observe any significant increase in p53 protein expression in benign squamous epithelium (normal or hyperplastic) despite a number of occasional weakly positive basal cell nuclei in the squamous epithelium of laryngeal nodules. Follow up of the cases with dysplasia was short at three years hence we could not correlate the significance of p53 overexpression with future invasive potential of the malignant cells, as has been determined in oral malignancies.9

It was impossible to demonstrate p53 protein expression reliably using the Streptavidin/ horseradish peroxidase method without pretreatment in a microwave oven.24 Intensity of staining varied in the same section, particularly in the laryngectomy specimens, possibly due to the length of fixation which is important for the detection of p53 overexpression.25 Both antibodies gave comparable results with DO7 being slightly more sensitive.26 Most of the patients included in this study were heavy smokers, confirming an association between p53 overexpression and heavy smoking although three non-smokers with invasive SCC of the larynx overexpressed the p53 protein. We did not find any association between the histological grade of carcinomas and confirmed other reports that p53 overexpression is common in SCC of the larynx.

Other studies indicate that high frequency p53 gene alterations are associated with p53 overexpression, a finding supporting the hypothesis that tobacco carcinogens may contribute to the inactivation of the p53 tumour suppressor gene and although this phenomenon may be an early event in carcinogenesis it is not necessarily related to aggressive biological behaviour as has been shown in breast adenocarcinoma.27 28 Apart from tobacco smoking, several aetiological factors have been implicated in laryngeal carcinogenesis. The role of HPVs in laryngeal carcinogenesis has not been clarified and it will be interesting to assess HPV positivity in laryngeal SCC as the virus binds the p53 protein, through the E6 region, with subsequent protein degradation.29 30 Mutations in the p53 gene have also been observed in animals with formaldehyde induced malignant tumours of the upper respiratory tract.31 Further studies are therefore required to assess the significance of early p53 overexpression in laryngeal carcinogenesis, and its premalignant role and invasive potential.

17 Field JK, Pavelic ZP, Stambrook PJ, Jones AS, Gluckman...