Immunohistochemically stained markers (p53, PCNA, bcl-2) in dysplastic lesions of the larynx

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Received 19 February 1999; received in revised form 12 April 1999; accepted 16 April 1999

Abstract

The percentage of malignant transformation of laryngeal dysplastic lesions is difficult to estimate. There is a need for new histological markers which could enable more objective assessment of the premalignant stages of the larynx and help in estimation of the potential of future neoplastic progression. We performed a retrospective study to determine whether immunohistochemical staining for the proliferating cell nuclear antigen (PCNA), tumour suppressor gene protein p53 and anti-apoptotic protein bcl-2 may be prognostic factors in laryngeal epithelial lesions. Staining was performed on 57 paraffin-embedded biopsies from patients with clinically detected precancerous stages of the larynx. Histopathologic examination revealed normal epithelium in six cases, mild dysplasia in 20 cases, moderate dysplasia in 18 cases, severe dysplasia in seven cases, CA in situ in four cases, papilloma in one case and CA invasivum in one case. The p53 count in mild and moderate dysplasia was 26.8 and 38.6%, respectively. This difference was statistically significant. There was significant correlation between PCNA and p53 scores. There was also a relationship between the scores of these markers and bcl-2 expression. In ten out of 45 cases of dysplastic lesions the invasive cancer developed in 4 years of follow-up. The correlation between PCNA score and malignant progression of the dysplastic lesions was on the statistical borderline. There was significant relationship between malignant transformation and age of the patients. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Larynx; Dysplasia; Prognosis; p53; PCNA; bcl-2

1. Introduction

Squamous cell carcinomas are clinically the most important malignant neoplasms in the larynx. This type of cancer often develops from precancerous lesions. The incidence of preneoplastic lesions of the larynx in Poland is difficult to estimate because there is no central register of this category of disease. Also, in the literature one can find only scant data on this problem [1].

The percentage of malignant transformation of laryngeal dysplastic lesions reveals a great difference from 3.3 up to 52.9% [2–5]. Taking these into account it is obvious that there is a need for new histological markers which could enable more objective assess-
ment of the premalignant stages of the larynx and help in estimation of the potential of future neoplastic progression.

One of the main features of malignancy is the ability of cells to proliferate. In many tumours cell kinetics have been demonstrated to correlate with malignant behaviour. The incorporation of cell kinetic data in standard pathologic diagnosis has the potential to impact on therapeutic decisions. The identification of cell cycle restricted markers which are intrinsic to the cell has simplified the evaluation of proliferating cells in tissue sections. Antibodies to these markers have been described in recent years including PCNA which is a 36-kDa, acidic, non-histone, nuclear protein whose expression is associated with the late G1 and S phases of the cell cycle [6]. PCNA was first described by Miyachi et al. [7] as a nuclear antigen found in proliferating cells that reacted with the sera of patients who had systemic lupus erythematosus. It has been proven that PCNA acts as the auxiliary protein of DNA polymerase [8]. The level of PCNA has been shown to correlate with the proliferative activity and prognosis of patients with head and neck tumours [9–11].

Tissue growth depends on both cell proliferation and the rate of cell death. Apoptosis is a specific mode of cell death by which deletion of cells occurs. One of the most important factors involved in the controlling of apoptosis is bcl-2 protooncogene which encodes a 26-kDa protein localised in intracellular membranes such as the mitochondrial membrane, endoplasmic reticulum or nuclear envelope [12]. Bcl-2 is involved in the regulation of cell death by inhibiting apoptosis. Increased expression of bcl-2 has recently been reported in different tumours [13–16].

Genetic factors play an important role in carcinogenesis. Natural genes or protooncogenes can become active oncoproteins. Mutation of p53 suppressor gene is one of the best known genetic alterations in malignant tumours. Inactivation of this gene is connected with uncontrolled cell growth. Overexpression of p53 protein has been identified in malignant and premalignant squamous epithelium [8,17–19].

The main aim of our study was to extend our knowledge of the biology of precancerous stages of the larynx and possibly to find the factors which might enable anticipation of its future progression.

2. Materials and methods

The study was performed on a group of 57 patients who underwent biopsy for endoscopically detected precancerous lesions of the larynx in the form of pachydermia, leukoplakia, hypertrophic laryngitis and papilloma. The patients have not been given any further treatment except surgical excision of the lesions. There were eight women and 49 men; the average age was 58 years, range from 34–84 years of age. The mean period of follow-up was 4 years.

The laryngeal epithelial dysplastic lesions were classified according to the WHO classification into three groups: mild, moderate and severe dysplasia.

All specimens were fixed in 10% formalin and routinely processed for paraffin embedding. Sections were cut at 3–4 μm and mounted on coated glass slides. For immunohistochemistry, tissue slides were deparaffinized in xylene and rehydrated through graded alcohols. Subsequently, the sections for PCNA and p53 were microwaved whereas those for bcl-2 were prepared in a pressure cooker. The samples were immunostained using the following antibodies: PCNA (PC-10, DAKO), p53 (DAKO) and bcl-2 (NOVOCASTRA) in a dilution 1:50, 1:50 and 1:80, respectively. Immunoperoxidase detection was employed using the standard avidin–streptavidin method. Counter-staining was performed with haematoxylin.

For PCNA and p53 all identifiable nuclear staining in the epithelial cells was recorded as positive regardless of intensity. At least 500 cells were observed under a high-power (objective magnification × 40) microscope and counted vertically from one corner to the other. The estimation was made in at least three fields. The estimations were calculated as the quotient of positive cells to the total number of cells counted, expressed as a percentage. In a few cases cytoplasmic staining was observed but nuclear staining was always stronger. The immunohistochemical results for bcl-2 were recorded as positive if there was a cytoplasmic reaction in at least 10% of cells.

2.1. Statistical methods

The significance of differences between the values of different groups was evaluated using Student’s t-test, and Wilcoxon–Mann–Whitney, Kruskal Wallis
and chi-square tests. The significance of the correlation between the values was estimated by the Pearson test. The homogeneity of variances was estimated by the Levene test. All data were processed with S-PLUS (MathSoft, Seattle, WA) statistical software. \( P < 0.05 \) was considered significant.

3. Results

Histological examination of endoscopically removed tissue specimens revealed normal epithelium in 6 cases, dysplastic lesions in 45 cases, papilloma in 1 case, carcinoma in situ in 4 cases and invasive carcinoma in 1 case (Table 1). Most of the patients had a history of chronic tobacco use. Lesions were mainly localized on the vocal cords.

The results of immunostaining in particular groups of epithelial lesions are presented in Table 1. As epithelial lesions progressed from mild to severe dysplasia the frequencies of p53 positive cells increased continuously in both the basal and suprabasal layers. The difference between the p53 count in mild and moderate dysplasia was statistically significant. In normal epithelium and mild dysplasia the p53 score was often low and staining was confined to a few scattered cells near the basal membrane (Fig. 1). In moderate and severe dysplasia the positively stained cells were detected in 2/3 of the width of the epithelium (Fig. 2). The distribution of cells stained positively for PCNA had a similar configuration as in p53 staining although more cells were positive for PCNA than for p53. There was significant correlation between PCNA and p53 scores.

Immunoreactivity of bcl-2 was detected in the cytoplasm. There was no relationship between bcl-2 positivity and the level of dysplasia (Table 1). The immunostaining of bcl-2 was rather heterogeneous although in some cases the strongest reaction was detected in basal and suprabasal layers. There was a statistically significant (Student’s \( t \)-test) relationship between bcl-2 staining and PCNA and p53 scores, e.g. cases with positive bcl-2 staining had higher PCNA and p53 scores than those with negative bcl-2 staining.

In ten of the 45 patients with dysplastic lesions of the larynx the invasive cancer developed during the 4-year follow-up period.

The age of the patients was connected with prognosis. The mean age of the patients with progressive lesions was 56.4 years whereas the mean age of the patients with non-progressive lesions was 63.8 years.

There was no correlation between the level of dysplasia and the future malignant transformation of epithelial lesions.

The correlation between a high PCNA score and malignant progression of dysplastic lesions of the larynx was on the statistical borderline (\( P \approx 0.06 \)) (Table 2).

The p53 and bcl-2 staining did not correlate with the clinical course of dysplastic lesions of the larynx (Table 2).

4. Discussion

Premalignant epithelial lesions of the larynx constitute an important clinical problem. Until now the markers that may predict the transformation of preneoplastic lesions have been poorly defined.

The bcl-2 immunostaining in epithelial hyperplastic laryngeal lesions was estimated by Hellquist [20]. More bcl-2 protein was detected in epithelia with simple hyperplasia compared to those with atypical

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Table 1
Immunohistochemical detection of p53, PCNA and bcl-2 in laryngeal epithelial lesions

<table>
<thead>
<tr>
<th>Classification</th>
<th>No. of cases</th>
<th>p53 % positive cells</th>
<th>PCNA % positive cells</th>
<th>bcl-2 % positive cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal epithelium</td>
<td>6</td>
<td>25.4</td>
<td>42.3</td>
<td>33</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>20</td>
<td>26.8</td>
<td>43.2</td>
<td>40</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>18</td>
<td>38.6</td>
<td>52.1</td>
<td>50</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>7</td>
<td>40.1</td>
<td>52.6</td>
<td>28</td>
</tr>
<tr>
<td>CA in situ</td>
<td>4</td>
<td>28.7</td>
<td>47.3</td>
<td>25</td>
</tr>
<tr>
<td>Papilloma</td>
<td>1</td>
<td>57.65</td>
<td>69.9</td>
<td>100</td>
</tr>
<tr>
<td>CA invasivum</td>
<td>1</td>
<td>37.5</td>
<td>59.1</td>
<td>100</td>
</tr>
</tbody>
</table>
hyperplasia. The author suggests that it might be a sign that an early selection for death as a self-defence mechanism is not yet needed in simple hyperplasia. The reports on bcl-2 estimation in laryngeal cancer are scant and controversial. Spafford et al. [21] found that bcl-2 is not a valuable prognostic marker in squamous cell carcinoma. Friedman et al. [22] reported that bcl-2 is a significant prognostic factor in early stages of laryngeal cancer.

Gale et al. [23] analysed p53 overexpression in laryngeal hyperplastic lesions and detected increasing positivity of staining related to the degree of abnormalities. The analysis of follow-up of the patients revealed that p53 protein may not be considered a
reliable prognostic factor in determining the risk of cancer development. Uhlman and co-workers [24] observed a close relationship between PCNA and p53 staining in all steps of carcinogenesis in the larynx. Munck-Wikland et al. [8] analysed nuclear DNA content, PCNA, and p53 immunostaining in predicting progression of laryngeal cancer in situ lesions. The authors found that the lesions, which progressed to invasive cancer, tended to have a higher grade of DNA aberration, a higher grade of intense PCNA positivity and a higher frequency of p53 immunostaining.

The main purpose of this study was to search for potential markers of future malignant transformation of laryngeal precancerous lesions. In our series of 45 cases of laryngeal dysplasia the invasive cancer developed in ten cases which is comparable to the results of Velasco et al. [25].

We observed the correlation between p53 and PCNA staining in all steps of carcinogenesis. We found the same localisation of p53 protein in the layer of proliferating cells, as shown by PCNA immunostaining. The accumulation of p53 protein in normal laryngeal epithelium exposed to carcinogens and in mild dysplasia support the hypothesis that p53 gene mutation is an early event in carcinogenesis of the larynx. The p53 immunostaining tended to increase with the degree of epithelial changes which confirms the results of other authors [23,24].

Interestingly, we found a statistically significant correlation between bcl-2 immunostaining and p53 score. This supports the theory that the p53 gene is involved in the regulation of apoptosis. Inhibition of apoptosis expressed by bcl-2 was, in our group, correlated with an increased activity of proliferation expressed by PCNA immunostaining.

The correlation of PCNA immunostaining with the severity of epithelial changes detected in our study has been reported previously [26,27].

We did not find a relationship between the level of dysplasia and prognosis. The correlation between the age of the patients and future progression of dysplastic lesions of the larynx revealed in our study may be explained by the fact that in this group the exposure for carcinogens is longer.

There was a trend towards a higher PCNA score in the precancerous lesions of the larynx that progressed to invasive cancer than in the lesions that did not. This is in accordance with the results of other authors [8,28].

Our results indicate that PCNA immunostaining may be of some value in estimating the prognosis of preneoplastic lesions of the larynx. p53 immunostaining may assist in the histological estimation of early dysplastic lesions. The estimation of bcl-2 expression in laryngeal epithelial lesion yields neither important diagnostic nor prognostic information.

References


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