

## ORIGINAL PAPER

# Clinical, morphological and immunohistochemical aspects in laryngeal premalignant lesions

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### Abstract

**Objective.** We investigated the proliferative activities in premalignant laryngeal vocal chord lesions treated by epithelial stripping in microlaryngoscopy, using immunohistochemical staining with anti-p53, anti-PCNA and anti-Ki-67 monoclonal antibody and we correlated with clinic and morphologic aspects. **Material and methods.** The study was made on 32 patients hospitalized in Craiova ENT Clinic in 2005 presenting lesions precursor to vocal cord malignity. The lesion's aspect was observed using suspended microlaryngoscopy, a biopsy was performed and biological tests were examined from a pathological and immunohistochemical point of view, with the investigation of the following immunohistochemical markers: Ki-67, PCNA and p53. **Results.** 13 cases (41%) presented red hypertrophic cronic laryngitis, seven cases (22%) presented white hypertrophic cronic laryngitis, and 12 cases (37%) presented papillomas with simple, moderate, severe dysplasia and in situ carcinoma in 62.5%, 22%, 12.5% and, respectively, 3% of the cases. All the dysplastic lesions, no matter the dysplastic degree, have presented alteration of both surface eplitelium and chorion. The expression of Ki-67, PCNA and p53 was correlated with the dysplasia's degree in various proportions. **Conclusions.** In clinical practice, morphological grading of dysplasia is difficult to evaluate. The Ki-67 and PCNA markers were correlated with the dysplasia degree; the expression of p53 was present only in 28% cases with moderate dysplasia and in one case with in situ carcinoma.

**Keywords:** laryngeal premalignant lesions, immunohistochemistry, p53, PCNA, Ki-67.

### Introduction

Epithelial hyperplasic laryngeal lesions may contain preneoplastic histological changes.

Laryngeal dysplasia is a truly premalignant change and describes disorder in the usual progressive maturation of cells from the basal layer to the outer layer of the squamous epithelium, usually in association with changes in individual cells on a spectrum from benign to malignant.

The percent of malign transformations in these lesions is difficult to estimate.

Cell proliferation can be assessed through immunohistochemistry measurement of the expression of proliferation-associated antigens in tumor tissues.

### Material and methods

The study has been performed on 32 patients presenting hyperplasic laryngeal lesions treated by epithelial stripping in microlaryngoscopy, hospitalized to the Craiova ENT Clinic during November 2004–September 2005.

The histological study has been made on paraffin sections colored with usual dyes such as Hematoxylin–Eosin dye, trichromic dye with green light (Goldner–Szeckelly technique). The immunohistochemical testing was made in the Histological, Histopathological and

Immunohistochemical Laboratory from Research Center of University of Medicine and Pharmacy of Craiova, by streptavidin-biotin (sABC)/Horse Radish Peroxidase (HRP)-complex method and the reactions were developed with diaminobenzidine by a brownish color.

The following nuclear markers have been used: Ki-67 antigen (clone MIB 1, DAKO) at a 1/10 dilution, PCNA (clone PC10, DAKO) at a 1/100 dilution and p53 (clone DO-7, DAKO) at a 1/25 dilution.

For the evaluation of the proliferative activity, we estimate the proliferative index for Ki-67 and PCNA. Thus, in each case, we counted 1000 nuclei within the fields with the biggest proliferative activity, taking into account the nuclei with dark-brown color; the results of this counting were reported percentual.

The distribution of the immunostaining was considered diffuse if over 50% of the cells have had positive reaction, and focal if the percent of the positive cells was between 5–50%.

For the evaluation of the positivity for p53 protein, the results were considered positive if the nuclear immunostaining was present in over 5% of the epithelial cells.

Linear regression has been utilized for statistics analysis. Linear regression is a regression method of modeling the conditional expected value of one

variable  $y$  given the values of some other variable or variables  $x$ .

The multiple regression correlation coefficient ( $R^2$ ) is a measure of the proportion of variability explained by, or due to the regression (linear relationship) in a sample of paired data. It is a number between zero and one and a value close to zero suggests a poor model.

## Results

The study lot was formed by 21 men (66%) and 11 women (34%) aged between 33 and 77 years old with an equal urban/rural distribution.

Twenty four of these patients were smokers, 19 of them consumed alcohol frequently.

All patients presented dysphonia with beginning of semiologies contents between a month and five years.

The clinical diagnoses in patients with premalignant lesions were:

- Red chronic hypertrophic laryngitis, 13 cases (41%):
  - insular verrucose hypertrophic corditis, two cases (6%);
  - diffuse simple hypertrophic corditis, eight cases (25%);
  - contact ulcer, three cases (9%).
- White chronic hypertrophic laryngitis, seven cases (22%):
  - leucoplasia, three cases (9%);
  - exofitic pachidermia, four cases (12.5%).
- Laryngeal polyp, 12 cases (37%).

The histological study allowed us to the reflection on the varied degrees of dysplastic lesions:

- Mild dysplasia – 20 cases (62.5%), from which eight cases were associated with papilloma;
- Moderate dysplasia – seven cases (22%), from which four cases were associated with papilloma;
- Severe dysplasia, four cases (12.5%);
- *In situ* carcinoma (ISC), one case (3%).

In cases with mild dysplasia, the modifications of the epithelium were accompanied by subjacent stromal alterations. The stroma presented an inflammatory moderate infiltrate, spotty disposed, with easy vascular congestion and amyloid deposit in varied amounts.

In medium dysplasia the vascular congestion was important and the inflammatory infiltrate had a varied intensity. The immunohistochemical reactions for Ki-67 and PCNA were intense positive, focal (in about 10% of the cells), in the inferior two thirds of the epithelium.

In patients with severe dysplasia, the superficial epithelium appeared much more thickened, with cellular alterations up to the surface, but with maintaining of its normal citoarchitecture.

In the deep layers we have noticed numerous nuclear monstrosities characterized by big sized nuclei, with obviously nucleoli.

The nuclear reactions for Ki-67 and PCNA were intense positive in over 50% of the cells belonging to the deep layers even through the superficial ones.

The positive PCNA indices have had high values in 15%, 57% and 75%, 100% of the cases with mild (Figure 1), moderate (Figure 2), severe dysplasia (Figure 3) and, respectively, ISC.

The rates of Ki-67 positive cases were 20%, 42%, 75% and 100% with mild, moderate (Figure 4), severe dysplasia (Figure 5) and, respectively, ISC.

Nuclear immunostaining for p53 was present only in 28% of the cases with moderate dysplasia (Figure 6) and in the case with ISC.

In the mean time, we evaluate the distribution of Ki-67, PCNA and p53 positive immunostaining in larynx premalignant lesions by linear regression. For Ki-67 positive immunostaining, regression slope is equal with 27.2 and shows a mild increase of approximately 27% with each progression from one severity stage to another (Figure 7).

$R^2 = 99.6\%$  reveals very good correlation between the actual data and the linear progression pattern of the rate of positive results (Figure 8).

For PCNA positive immunostaining, regression slope is equal with 27.2 and shows a mild increase of approximately 27% with each progression from one severity stage to another.  $R^2 = 96.8\%$  reveals very good correlation between the actual data and the linear progression pattern of the rate of positive results (Figure 9).

After primary surgery, patients underwent close follow-up, at a six months interval, and if the laryngoscopy showed signs of epithelial hyperplasia, the therapeutic and diagnostic protocol was resumed. At four patients, treatment and investigation were repeated all over again, in two of them being encountered the same grade of dysplasia as the first time, with the same anomalies of the markers in question. The other two had simple dysplasia with no reaction of immunohistochemical markers in one case, and proliferative indices between 5–10% in the other.

## Discussions

Precancerous laryngeal lesions are pathologic conditions of the epithelium, which can undergo malignant degeneration in a higher rate than other epithelial lesions [1].

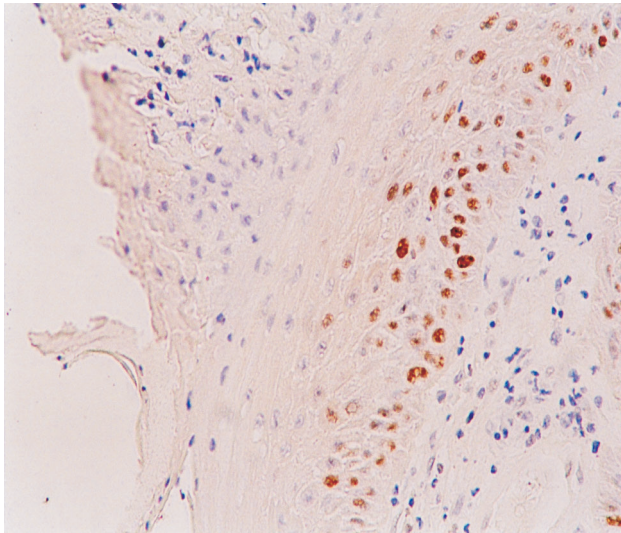
Usually the first alterations appear under the clinical aspect of a chronic laryngitis either superficial or submucous, with either circumscribed or diffuse edema.

The mucosal epithelium can also undergo thickening and abnormal keratosis, which, together with the subjacent mucosal edema produces clinical aspects of hypertrophic laryngitis.

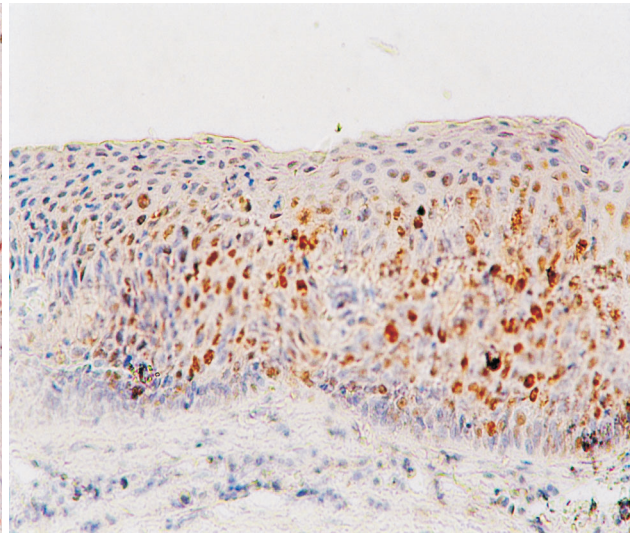
The keratinisation can be either circumscribed or diffuse, exophytic or smooth, histopathologically manifested by parakeratosis, hiperkeratosis and orthokeratosis.

The laryngoscopic aspect can be uncharacteristic, and may appear only as minor surface irregularities. In such cases only the histopathologic and immunohistochemical examination is capable of differentiating between an inflammatory process and an early tumoral process.

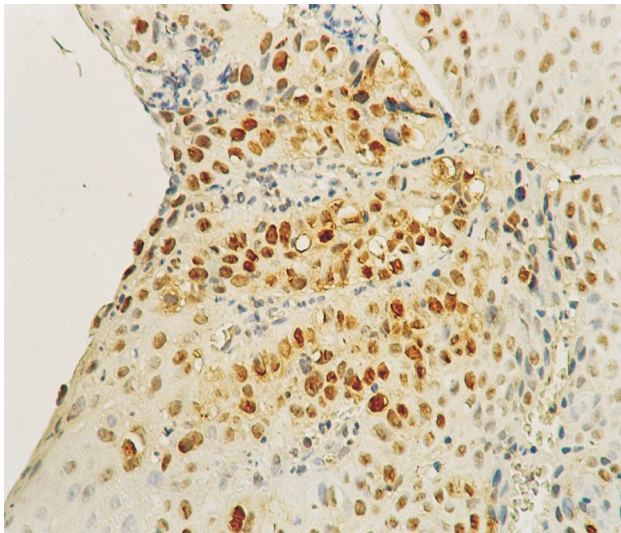




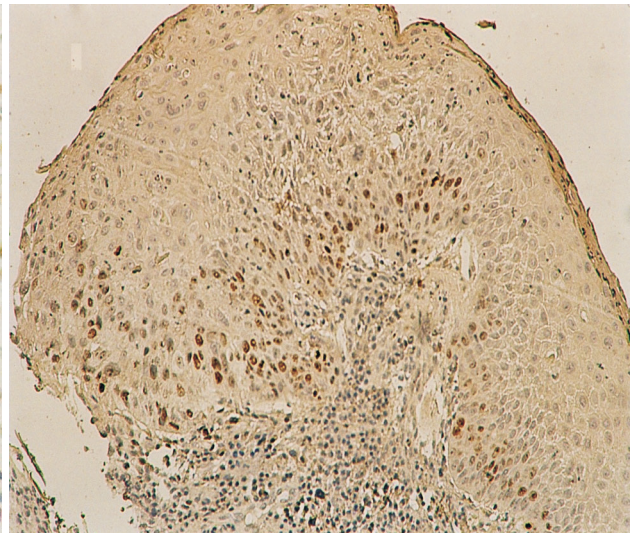
**Figure 1 – Larynx mucosa with mild dysplasia, positive nuclear immunostaining for PCNA, ×200**



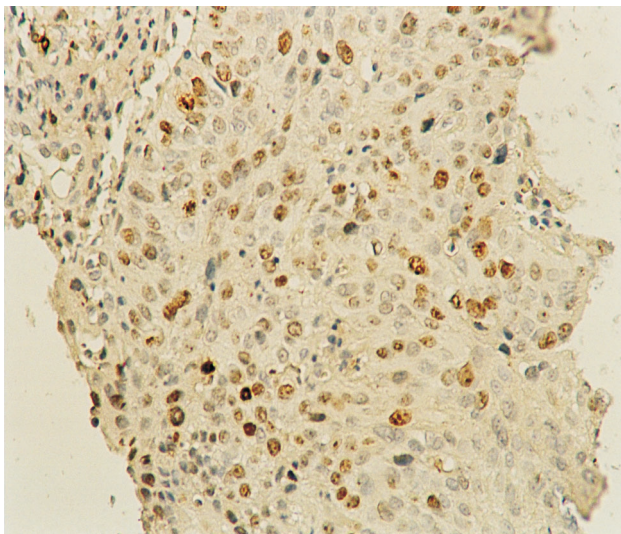
**Figure 2 – Larynx mucosa with moderate dysplasia, positive immunostaining for PCNA, ×100**



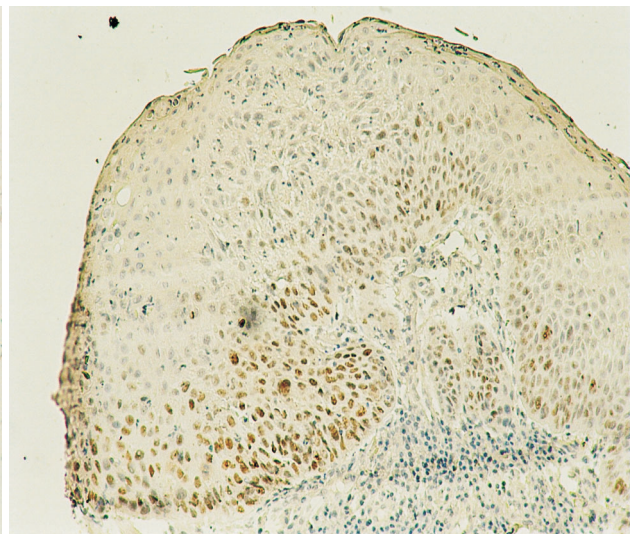
**Figure 3 – Larynx mucosa with severe dysplasia, positive immunostaining for PCNA, ×200**



**Figure 4 – Larynx mucosa with moderate dysplasia, focal positive immunostaining for Ki-67, ×100**



**Figure 5 – Larynx mucosa with severe dysplasia, diffuse positive immunostaining for Ki-67, ×100**



**Figure 6 – Larynx mucosa with moderate dysplasia, positive immunostaining for p53, ×100**

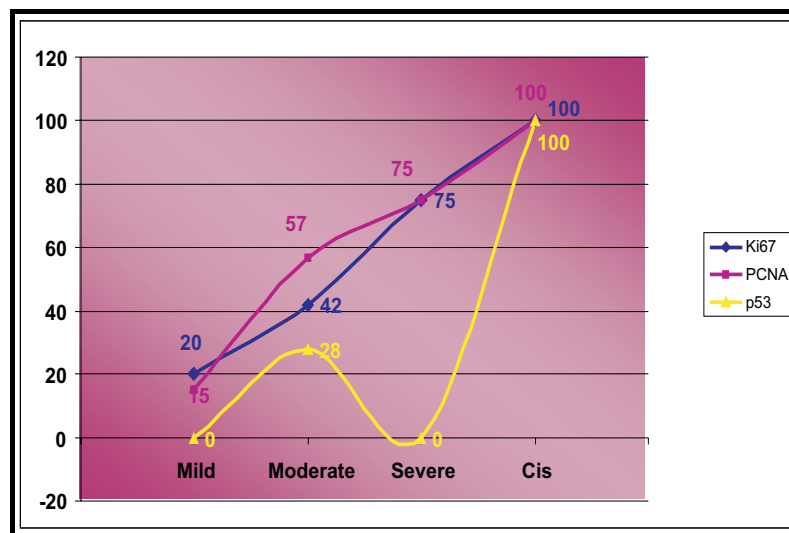


Figure 7 – Distribution of Ki-67, PCNA and p53 positive immunostaining in larynx premalignant lesions

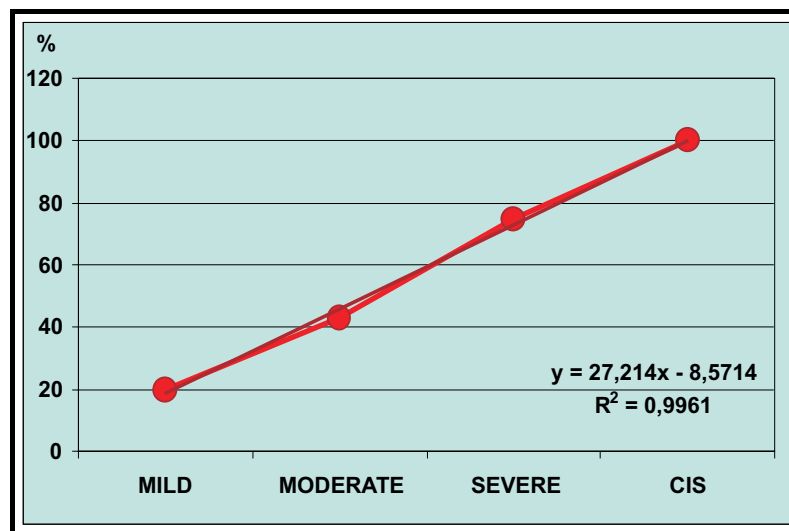


Figure 8 – Regression slope for Ki-67 positive immunostaining in laryngeal dysplasia

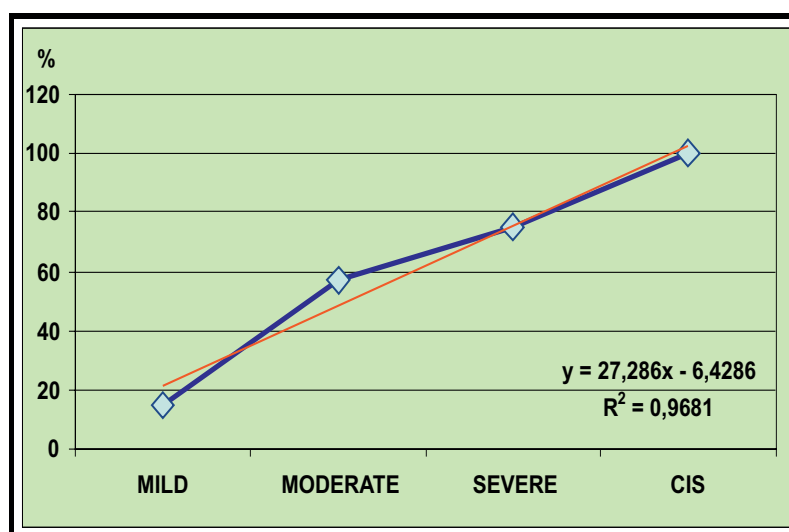


Figure 9 – Regression slope for PCNA positive immunostaining in laryngeal dysplasia



Then mucosa becomes opaque, vascular impression disappears and extravasations and fibrosis may appear; also, the mucosa may appear whitish, opaque, generating clinical aspect of leukoplakia.

Leukoplakia, exophytic keratosis, and polypoid thickening are early signs which may arouse the suspicion of malignancy. These lesions begin on the vocal cord, produce early dysphonia and can be detected early.

Proliferative markers have been broadly evaluated as prognostic and predictive factors for premalignant and malignant epithelial lesions. Several papers evaluating one or more markers have been published, often with contradictory results [2–12].

Some authors conclude that the patterns of immunoreactivity to PCNA and Ki-67 antigen correspond to the histological grade of both benign and malignant epithelial lesions of the larynx [13].

Ki-67 may play a role in the uncontrolled proliferation in papilloma and could help in estimation of the premalignant stages of the laryngeal lesions [14].

In our study, a good correlation was found between different degrees of dysplasia and the immunostaining for PCNA and Ki-67. PCNA and Ki-67 can be used as a marker of cell proliferative activity in laryngeal epithelial lesions [15].

The pattern and location of PCNA and Ki-67 positive cells in the intraepithelium had diagnostic importance. The lack of an international standardization method for antigen retrieval, staining procedures and scoring methods (semiquantitative and quantitative) is lead to a variety of results [16].

Regarding the expression of p53 protein, in our study this was positive only in some cases with moderate dysplasia.

Other studies confirms the association of p53 protein over expression with laryngeal epithelial hyperplastic lesions which have the potential to transform into malignancy but, p53 expression can not be considered a reliable prognostic factor for premalignant stages of the larynx regardless of the severity of the lesions [8].

Overexpression of p53 protein may play a role in the pathogenesis and development of premalignant lesions [17].

## ✎ Conclusions

In clinical practice morphological grading of dysplasia is difficult, mainly of clinic and microscopic diagnostic point of view between severe degree of dysplasia and carcinoma *in situ*.

PCNA and Ki-67 immunostaining can be used as a marker of cell proliferative activity in laryngeal epithelial lesions, positive expression being correlate with severity of laryngeal premalignant lesions.

We can not emphasize enough the importance of patient counseling on the matter of cancer risk,

also regarding the initiation of an appropriate follow-up planning, for the patients with PCNA and Ki-67 positive expressions.

The positive reaction for p53 protein was present only in 28% of the cases with moderate dysplasia and in one case with *in situ* carcinoma.

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