UNIVERSITY OF MEDICINE AND PHARMACY IN CRAIOVA

IMMUNOHISTOCHEMICAL, MORPHOLOGICAL AND CLINICAL STUDY OF PREMALIGNANT LESIONS OF THE LARYNX

Abstract of Ph. D. Thesis

PhD student

Dr. FLORIN ANGHELINA

Scientific coordinator

Prof. Dr. ELENA IONIȚĂ

SUMMARY

I. Background and the Reasons for Choosing the Subject of the Thesis……………………2
II. Aims and Objectives………………………………………………………………………3
III. Material and methods …………………………………………………………………3
IV. Results and discussions …………………………………………………………………3
    IV.1. Results of clinical study……………………………………………………………3
    IV.2. Results of immunohistochemical study………………………………………5
V. Conclusions………………………………………………………………………………7
VI. Curriculum vitae………………………………………………………………………..9

KEY WORDS: Larynx, immunohistochemistry, premalignant lesions of the larynx, chronic laryngitis, dysplasia, p53, PCNA, Ki-67, CD44, AE1/AE3, VEGF
I. Background and the Reasons for Choosing the Subject of the Thesis

The concept of precancer, clinical, biological and histological entity, has generated extensive controversy, a long time the possibility of the existence of a premalignant condition to reach the stage of malignancy was denied. Currently most authors recognize the possibility of a precancerous condition. In recent decades, various aspects of laryngeal carcinogenesis have been extensively studied, including etiology, histological classification, treatment, neoplastic transformation frequency and predictive factors. A particular interest in benign laryngeal disorders has been focused on the lesions with the highest rate of malignant transformation. These lesions are described, variously, as a potential malignant precursor risk preneoplastic or precancerous lesions. The correct assessment of precancerous states imposes checking the speed and frequency of their malignity; witch in terms allows the assessment of their severity. Current knowledge does not allow for objective assessment of future development of precancerous lesions to invasive cancer. One of the premises of this study is to identify and propose criteria for selection of larynx cancer screening.

II. Aims and Objectives

The main goal is to investigate premalignant laryngeal disease (precancerous condition) with malignant risk in terms of etiology, epidemiology, clinical and therapeutic. This involved the study of immunohistochemical markers in precursor lesions of malignancy larynx with the highlight significant changes and their correlation with clinical and morphological aspects.

III. Material and Methods

Own research are divided into two main parts:

1. Clinical study - evaluation and monitoring of patients with laryngeal pathology (laryngeal precancerous lesions) hospitalized in ENT Craiova from 01/01/1998 to 12/31/2007. The clinical study was done by examining retro and prospective pre-and postoperative data of a batch of 485 hospitalized patients with laryngeal precancerous lesions in ENT Craiova. All patients enrolled underwent suspended microlaryngoscopy biopsy and surgical treatment of lesions and histological confirmation of diagnosis. I watched malignancy in the group of patients over 12 years and have highlighted possible correlations. The results were analyzed statistically using Microsoft Excel 2007 software.

2. Histological and immunohistochemical study on precancerous laryngeal lesions.
Between November 2004 and November 2006 in the research project "Clinical, morphological and immunohistochemical study of precursor lesions of laryngeal malignancy" project of the Academy of Medical Sciences, Program Viasan contract 335/2004 have been investigated 54 patients with laryngeal hypertrophic lesions. In the research protocol proceeded to biopsy sampling suspended microlaryngoscopy. For immunohistochemical study of specific markers was used for unmasking technique called Steptavidin-Biotin Complex method (sABC) / Horse Radish Peroxide (HRP). Following immunohistochemical markers were used: p53 (clone DO-7 diluted 1 / 25), AE1/AE3 (clone AE1-AE3, dilution 1 / 150), CD44 (clone DF 1485, dilution 1 / 50), VEGF (clone VG 1, dilution 1 / 50), Ki67 (clone MIB-1, dilution 1 / 10), PCNA (clone PC 10, dilution 1 / 100). We analyzed the expression of these markers in accordance with the degree of injury.

IV. Results and Discussions

IV.1. Results of clinical study

Distribution by years of study of benign hypertrophic laryngeal diseases (BHLD). We had a yearly average of 48.5 with a standard deviation of 13.14. Compared to the number of new cases admitted with laryngeal cancer in the same period we found a correlation between the increase or decrease in tandem both benign disease and malignant in certain years of the existence of correlated peaks in both positive and negative sense. It is alarming that the incidence of cancer within 10 years with newly ENT diagnosed doubled (204 cases in 2007 to 102 in 1998), similarly to the phenomenon leading to duplication of laryngeal cancers (111 in 2007 to 55 in 1998).

The distribution by sex in BHLD lot. Given the clear predominance of male gender disorder in cancer of the larynx is not surprising that there is a much higher percentage of damage to men (77.73%) in BHLD. Regarding the correlation between histological type and sex of patient diagnosis, high proportions affecting male sex occurs in advanced dysplasia and carcinoma in situ values of 96% and 100% respectively.

The distribution by age. Lot of 485 patients followed ALCHR had a mean age of 49.3 ± 13.7 significantly lower than the average age of occurrence of laryngeal cancer (between 55 and 65 after various authors) and reported and the limit of what other authors. There was a significant difference in age between mild forms of dysplasia and advanced, and the apparent evolution, like increasing, the average age of male sex with the degree of dysplasia.

The division according to risk factors. We determined a high prevalence of risk factors,
with 42.88% (208 patients), alcohol consumption and 64.94% (315 patients) active smokers or former smokers.

**Distribution by type of BHLD.** Chronic hypertrophic lesions of the larynx have been grouped under the following conditions, in order of frequency were: polyp 225 cases (46.39%), papillomas - 131 (27.01%), red hypertrophic laryngitis -57 (11.75%), white hypertrophic laryngitis - 37 (7.63%), pseudo-myxomatous laryngitis - 24 (4.95%), cyst - 9 (1.86%), laryngocele -2 (0.41%).

**Distribution by histopathological type of precancerous lesion.** Of the 485 cases were histopathologically differentiated 3 carcinoma in situ representing 0.62%, 25 severe dysplasia (5.15%), 72 moderate dysplasia (14.85%), 290 mild dysplasia (59.79%) 90 papillomas (18.56%), a capillary hemangioma (0.2%), a osteochondroma (0.2%) and a histyocitoma (0.2%), two inflammatory infiltrates seen in the two laryngocele.

**Treatment and postoperative monitoring.** Treatment in the study group has been adapted both clinical diagnosis and histological lesions associated. Surgical treatment provided in all cases the removal of the pathological process, without affecting or with minimal damage healthy tissue. In 476 cases (98.14%) underwent endoscopic microsurgery with cold instruments. In 8 cases open surgery was necessary - cordectomy (1.64%) and one case (0.2%) received frontolateral laryngectomy extended to third past the opposite vocal cord for massive recurrence of hypertrophic laryngitis.

**Characteristics of malignant subgroup.** The group of 485 patients followed over 12 years, 13 representing 2.68% showed malignant transformation. Compared to literature data rate of malignancy was relatively low from 2.3 to 21.4% as reported from other authors. The average age of patients with progression to malignancy surprised at the time of registration was 62.23 ± 9.6 differ significantly (p <0.005) average age of hypertrophic lesions in the study: 49.3 with a standard deviation of 13.7. This requires higher attention to patients diagnosed with precancerous lesions close to the age of risk. Of the 13 patients 12 were male (92.30%) and one female (7.70%). In the malignant group we noted a higher frequency of chronic hypertrophic laryngitis (69%). In group 9 showed malignant histopathology at baseline representing a mild dysplasia percentage 69.23%, 3 severe dysplasia (23.07%) and a patient I encountered an moderate dysplasia (7.69%). Malignancy frequency and relative risk of malignant transformation of each histopathological varieties are given in Table 1.
Table No. 1. Statistical correlations of transformed malignant cases

<table>
<thead>
<tr>
<th>Dg. HP</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignancy(no.)</td>
<td>9</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Cases</td>
<td>300</td>
<td>72</td>
<td>25</td>
</tr>
<tr>
<td>Malign Freq.</td>
<td>3.00%</td>
<td>1.39%</td>
<td>12.00%</td>
</tr>
<tr>
<td>P0</td>
<td>0.021621622</td>
<td>0.029056</td>
<td>0.021739</td>
</tr>
<tr>
<td>RR</td>
<td>1.3875</td>
<td>0.478009</td>
<td>5.52</td>
</tr>
</tbody>
</table>

Severe dysplasia had a malignancy rate of 12%. RR relative risk of malignant transformation is RR = 5.52. Compared to literature data with malignancy between 9.3% and 57.1% in this study falls within the minimum reporting.

Moderate dysplasia had a malignancy rate of 1.39%. Compared to literature data with malignancy between 4% and 24% reporting current study falls below the minimum.

Mild dysplasia had a malignant rate of 3%. Compared to literature data with malignancy from 0% to 11.5% this study falls within the minimum reporting.

Time elapsed until the malignancy. The criterion for inclusion in the group of malignant transformation was that it must have happened at least 3 months after diagnosis and harvesting the first evidence that the treatment of baseline lesion and biopsy. The average malignant transformation of the study group was 25.38 months (4-84 months), malignancy occurred earlier reports from the literature.

IV.2. Results and discussion of immunohistochemical study group (group IHC)

2.1. IHC analysis of nuclear markers PCNA, Ki-67, p53

2.1.a) PCNA analysis. In the IHC study, positive PCNA immunostaining was directly correlated with the degree of dysplasia: mild dysplasia (43%), moderate dysplasia (90%), severe dysplasia (100%), CIS (100%). The coefficient of determination R2 equal to 73% indicates that there is good correlation between actual data and the model of linear increase in the frequency of positive immunostaining PCNA with the transition from one stage to another more advanced dysplasia (R² = 0.7356).

2.1.b) Ki67 analysis. In the IHC study, Ki-67 positive immunostaining was directly correlated with the degree of dysplasia: mild dysplasia (21%), moderate dysplasia (80%), severe dysplasia (100%), CIS (100%). The coefficient of determination R2, equal to 78% indicates that there is good correlation between actual data and the model of linear increase in the frequency of positive immunostaining Ki-67 with the transition from one stage to another.
more advanced dysplasia \( (R^2 = 0.788) \).

**2.1.c) Analysis of p53.** p53 is a nuclear phosphoprotein encoded by p53 gene (wild type - wild-type) located on the short arm of chromosome 17 \((17p13)\). P53 expression in this study was directly correlated with the severity of dysplastic lesions: mild dysplasia \((17\%)\), dysplasia moderate \((46\%)\), severe dysplasia \((67\%)\), CIS \((100\%)\). The coefficient of determination \(R^2\), equal to 99\% indicates that there is a very good correlation between actual data and the model of linear increase in the frequency of positive immunostaining p53 with the passage from one stage to another more advanced dysplasia \( (R^2 = 0.9935) \). The results suggest that p53 over expression may play a role in the pathogenesis and development of laryngeal cancer.

**2.2. IHC membranar markers**

**2.2.a.) Analysis of CD 44.** CD44 is a transmembranar glycoprotein. In this study on 54 patients, 9 of mild dysplasia cases \((45\%)\) was observed membrane immunostaining positive basal layer cells of squamous epithelium, and in one of these instances, parabazal cells. Its intensity was variable from one case to another, showing intense positive reaction areas and areas with lower intensity. Also, in the latter case, immunostaining was focal and in cells in the interlayer. On moderate dysplasia cases, CD44 expression was intensely positive in 38\% of cases, diffuse in the interlayer by squamous cells. A very weak positivation have registered a case with severe dysplasia. A high level of CD44 expression was more frequently found in moderate dysplastic lesions, the marker expression level was weaker or absent in other lesions.

**2.2.b.) AE1/AE3 analysis.** Anti-AE1/AE3 monoclonal antibody \( (\text{clone AE1/AE3}) \) is represented by a cocktail of two monoclonal antibodies AE1 and AE3, that interacts with several types of cytokeratin present both in normal epithelial tissues and in the cancer. In this study we found a weak positive for all mild dysplasia and positive in almost all moderate and severe dysplasia especially for papilloma, increasing the intensity of immunostaining with grade dysplastic lesion.

**2.3. IHC stromal markers**

**2.3.1. VEGF expression.** VEGF-A is mainly known as vascular permeability factor. The study performed on 30 cases, 20 with mild dysplasia, 8 moderate dysplasia, 1 severe dysplasia and 1 Cis, showed a variable expression of VEGF in the cases studied. In two cases \((10\%)\) with moderate dysplasia positive immunoreactivity was observed in simple, low-intensity diffusely distributed in the stromal cells. Immunostaining was slightly positive in rare cases with stromal cells from 4 medium dysplasia \((50\%)\) and average negative for
dysplasia and Cis. According to available data a positive VEGF expression was not correlated with the degree of dysplasia.

V. General Conclusions

1. The progression from dysphasic epithelium to squamous cell carcinoma of the larynx is a lengthy, complex and stages related to the progressive accumulation in the cascade of genetic changes that lead to the selection of a transformed epithelial cell clones.

2. The incidence of newly diagnosed cancers ENT localization doubled (204 cases in 2007 to 102 in 1998), similarly phenomenon leading to duplication of laryngeal cancer (111 in 2007 to 55 in 1998), precancerous lesions following a course of these increases graphic distribution symmetrical with the annual linear laryngeal carcinomas.

3. There is a much higher percentage of damage to men (77.73%) for benign hypertrophic laryngeal diseases, especially encountered in severe dysplasia and carcinoma in situ, with values of 96% and 100% respectively.

4. In the patients with benign hypertrophic laryngeal affections we have determined a high prevalence of risk factors, with 42.88 % alcohol consumers and 64.94 % smokers or former smokers.

5. For the evaluation of benign hypertrophic larynx lesions it is necessary to use complex investigation methods such as direct laryngoscopy fibrolaryngoscopy, suspended microlaryngoscopy, contact videoendoscopy, videolaryngostroboscopy and absolutely the anatomopathological exam.

6. The localization of the hypertrophic lesion at the vocal folds dominated (81.65%) with the form of laryngeal polyps (54.04 %) and hypertrophic laryngitis (28.72%).

7. Dysphonia is the dominant and major symptom of most benign hypertrophic laryngeal lesions.

8. Chronic hypertrophic lesions of the larynx were seen mostly as polyps (46.39%) and papillomas (27.01%).

9. Malignant transformation was seen in 2.68% of cases, similar data from the medical literature, the percentage of malignancy was the lowest limit of reporting.

10. The average age of the patients with progression to malignancy at the date of registration was 62.23 ± 9.6 years, statistically different ( p <0.005) from the median age of hypertrophic lesions in the study : 49.3 ± 13.7 years. This requires higher attention to patients diagnosed with precancerous lesions close to the age of risk.
11. The average malignant transformation of the study group was 25.38 months, the
degree of dysplasia being a risk factor for neoplastic transformation; severe dysplasia
exhibited the highest frequency of malignancy: 12% (Fisher's exact test, two-tailed: \( P = 0.0246 \) which is considered a statistically significant association) located within the minimum
reporting in medical literature.

12. VEGF is expressed with different intensities in the dysplastic lesions but no one
correlation with the degree of dysplasia.

13. AE1-AE3 cytokines are found in intense positive expression in all the cases with
laryngeal papillomas and also positive in the other epithelial lesions.

14. The positivity of the CD44 expression is decreased with the severity of the dysplastic
laryngeal lesions, being inversely correlated with them.

15. Immunostaining with PCNA, Ki-67 and p53 can be utilized as a marker of the cellular
proliferative activity in the hypertrophic cell larynx lesions, their positive expression being
correlated with the severity of premalignant lesions.

16. The histological classification of laryngeal epithelial lesions is subjective, and for this,
an improvement of the alterations of the cell cycle would increase the objectivity of the
histological evaluation. Involvement of tumor markers detected immunohistochemically is
undisputed due to the presence and persistence of their altered expression in the evolution
from premalignant to malign.

17. Establishing a correct diagnostic, applying an adequate treatment and the dynamic
supervising of the pathological process in the lesions with malignant degeneration risk, will
contribute to the decrease in the number of patients with irreversible, recurrence or
progression to malignancy.
CURRICULUM VITAE

PERSONAL DATA:
Surname: ANGHELINA, First name: FLORIN,
Date of birth: 1972-06-11. Place of birth: Balș. Address: Clinic Emergency Hospital, str. Tabaci no. 1, Craiova, Dolj, Tel. 0742031003. Marital status: married, 1 children

SECONDARY AND HIGH SCHOOL
1978-1984 Primary School: Iancu Jianu, Olt
1984-1986 Primary School No. 30, Craiova
1986-1990 High School No.1, Craiova- Baccalaureate Exam

ACADEMIC STUDIES
1991-1997 The Faculty of Medicine of Craiova, University of Craiova

OTHER TITLES
Master degree in Management of Hospitals

TEACHING ACTIVITY
01.10.1999-01.04. 2003 ENT junior assistant, UMF Craiova
01.04.2003 to present: ENT assistant professor, UMF Craiova

PROFESSIONAL ACTIVITY
1998-1999 Trainee Physicians - Clinical Emergency Hospital no. 1, Craiova
1999 Admitted at the National Contest for Residents; between 1999-2004 Resident, Specialty ENT - Clinical Emergency Hospital no. 1, Craiova
2004 Specialist in ENT Clinical Emergency Hospital, Craiova
2009 Attending Physician, Specialty ENT Clinical Emergency Hospital, Craiova

FOREIGN LANGUAGE: English, French

Scientific activity:
I. Articles/Abstracts published in ISI indexed journals:3 (citation:2)
II. Articles published in CNCSIS indexed journals: 16
III. Books/Book Chapters: 4
IV. National and international scientific presentations: 102

International Scientific Awards: 3
Member of Scientific Societies: Romanian ENT Society
Accumulated experience with projects/programs/grants: member in 3 national grants