

**UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
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PHD THESIS

***PROGNOSTIC FACTORS STUDY IN
LARYNGEAL SQUAMOUS CELL CARCINOMAS***

ABSTRACT

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INTRODUCTION

Head and neck cancers are ranked eighth in the world as frequency, and laryngeal carcinomas represent 30-40% [2,3]. Laryngeal carcinoma accounts for 2.4% of all neoplasms diagnosed annually [4]. Laryngeal squamous cell carcinoma is considered to be a male affection, commonly diagnosed between 40-70 years of age, accompanied by increased mortality among them, clearly superior to women [7,9]. In recent years, an increase in this pathology has also been identified among female patients most likely due to the habit of drinking or consuming alcohol [5-6]. Regarding the localization of the tumor at the various stages of the larynx, glottic localization is the most frequent localization encountered, the rarest being diagnosed localization in the infraglottic stage [9]. A variety of risk factors that influence the appearance of cancer such as tobacco, alcohol, diet, occupation, various external agents, socio-economic status, but also the presence of precursor lesions, such as leukoplasia or chronic laryngitis, are described [6,11,13,21].

Cancer occurs because of repeated changes and between which there are various types of association, being influenced by both genetic predisposition and various external factors [95]. It was found that suppression of suppressor genes and activation of oncogenes allow the appearance of tumor cells that begin to proliferate to ultimately form primitive tumor [117]. It is necessary to identify the numerous phenomena that are involved in the occurrence and progression of cancer, because they provide useful information for both the carcinogenesis process and the possibility of identifying prognostic factors.

This study aimed to evaluate clinical, epidemiological, histopathological and immunohistochemical markers for the identification of possible prognostic targets, as well as an accurate diagnosis for using the most useful treatment from the earliest stages.

Key words: laryngeal squamous carcinoma, laryngeal carcinogenesis, prognostic factors, immunohistochemical markers.

CHAPTER I. EPIDEMIOLOGICAL CONSIDERATIONS AND RISK FACTORS IN LARYNGEAL SQUAMOUS CELL CARCINOMAS- This chapter is dedicated to recent data from the literature on epidemiology and risk factors involved in laryngeal squamous carcinomas.

CHAPTER II. RECENT DATA ABOUT LARYNGEAL CARCINOGENESIS- is a chapter that describes the stages of a complex process, involving the disruption of many genetic and molecular phenomena.

CHAPTER III. PROGNOSTIC FACTORS IN LARINGIAN SCULPTAIN CARCINOUSES - presents data on prognostic factors associated with the occurrence of laryngeal squamous carcinomas: age, sex, tumor localization, presence of lymph node metastases, degree of differentiation or mutations of p16 genes, p53.

CHAPTER IV. CLASSIFICATION OF LARYNGEAL TUMORS - The latest classification proposed by the World Health Organization was made in 2017 and brings new information on laryngeal cancer. These recent data show changes in the enteropathogenesis, especially related to the HPV virus.

CHAPTER V. MATERIAL AND METHODS - The present study was conducted for a period of 3 years and includes 293 cases of laryngeal squamous carcinomas. The material used for the research was human material from patients admitted to the Otolaryngology Clinics of the Emergency County Hospital of Craiova and diagnosed in the Pathology Department. The pieces were processed by the usual technique with paraffin embedding and Hematoxylin-Eosin stain. The immunohistochemical technique comprised a total of 38 cases and we used LSAB2-HRP (Label Streptavidin-Biotin 2 System; DAKO, Redox, code K0675) as a working method. The antibodies used are: EGFR, Ki67, p53, bcl-2, E-cadherin, β -cadherin, β -catenin, cyclin D1, Snail.

CHAPTER VI AND VII. RESULTS AND DISCUSSIONS - these chapters contain the data obtained from the present study and later they were compared with the data from the recent specialized literature.

We have noticed that the incidence of laryngeal squamous carcinomas showed a progressive increase from the fifth decade to the seventh decade, and regarding the

incidence of lesions on sex, we found a net predominance of male affections. The literature confirms the same issues [136,155].

In 91.8% of cases, the presence of smoking habits was identified. Smoking is the main risk factor in the incidence of carcinomas, and various studies report high values for this habit, ranging from 86.8 to 95% [6,156].

In our cases, we found that all cases of laryngeal cancer corresponded to squamous carcinoma, and as variations were non-keratinized forms in 82.3% of cases, 16.4% of cases corresponded to the keratinized appearance, basaloid form 0.7% of cases and one case of warts carcinoma and papillary carcinoma respectively, representing 0.3%. Non-keratinized aspect corresponds to poorly differentiated forms that are usually associated with a poor prognosis of patient progression [4]. Regarding the histological degree, the most cases belonged to moderate-differentiated carcinomas that were diagnosed in 50.5% of the cases, followed by the poorly-differentiated forms with 31.8% and well-differentiated in 17.7% of the cases.

Vascular invasion was identified in 82 cases and the perineural one in 169 cases. The analysis of lymph node involvement indicated the presence of metastatic adenopathy in 25.4% of cases and its absence in 74.6% of cases. The evaluation of tumor stage included 3 cases that belonged to stage I, 10 cases that belonged to stage II, 37 cases belonged to stage III and 17 cases belonged to stage IV of the disease.

For the immunohistochemical study we used the following marker categories: growth and proliferation factors (EGFR, Ki67), apoptosis markers (p53, bcl-2), adhesion molecules (E-cadherin, β - cadherin, β - of the cell cycle (cyclin D1) and transcription factors (Snail).

The analysis of EGFR immunoeexpression in the laryngeal squamous carcinomas of the present study showed membranous and cytoplasmic positivity in 34 of the 38 immunohistochemically investigated cases. We found an incidence of 54.7%, with a high expression in moderate and poorly differentiated forms of laryngeal squamous carcinomas compared to well-differentiated forms. Using the chi-square test, we obtained

significant ($p = 0.015$) values between EGFR marker expression and degree of differentiation, the expression being high in poorly differentiated cases.

Ki-67 immunoassay analysis indicated a nuclear positivity in 42.6% of the 38 analyzed cases. Also, in our study, we obtained statistically significant association ($p = 0.000$) between the Ki-67 marker and the degree of differentiation using the Anova test.

Immunohistochemical analysis of the p53 marker revealed a 52.5% positivity in our study of the 38 immunohistochemically investigated cases. Both the percentage and the intensity of the positive reactions varied with the degree of differentiation and the pTNM stage. The highest values of 75.7% were obtained in poorly differentiated forms. In our study, we obtained a significant association between p53 and the pTNM stage ($p = 0.031$, chi-square test).

The positivity of the bcl-2 marker was observed in 33 cases of the 38 immunohistochemically investigated in our study. In advanced stages, III and IV, as well as poorly differentiated carcinomas we obtained the highest values and the average percentage was 42.7%. The statistical analysis of our study demonstrated significant associations between the marker and the degree of differentiation ($p = 0.018$, Anova test) but also the pTNM stage (0.015, Anova test).

In our study, E-cadherin was identified at both membrane and cytoplasmic levels in a superior number of 53.6% in well-differentiated cases, 19.8% in moderately differentiated and 17.4% in poorly differentiated cases. Statistical analysis showed a significant association between the degree of tumor differentiation and E-cadherin expression ($p = 0.000$, chi-square test).

Immunoreaction of P-cadherin we identified in 46.09% of immunohistochemical investigations. Advanced stages and poorly differentiated forms showed the highest values. Moreover, we also obtained a significant association between the marker and the degree of differentiation ($p = 0.000$, chi-square test).

In the present study β -catenin was identified in all locations, membrane, cytoplasmic and nuclear, in 37.2% of cases. In moderate and poorly differentiated forms as well as in advanced disease states, we have found the presence of cytoplasmic and

nuclear marker localization in comparison to well-differentiated forms. The statistical analysis for this marker demonstrated significant association between the marker and the degree of differentiation ($p = 0.043$, chi-square test).

Following the investigation of cyclin D1 marker expression, we achieved a 29.3% positivity in the 36 investigated cases. The cyclin D1 expression increased with the degree of differentiation, the highest values being obtained in poorly differentiated forms. In our study, we obtained a significant association between cyclin D1 and the degree of differentiation ($p = 0.010$, chi-square test).

Snail immunoexpression was evident at the nuclear level in 61.5% of the 38 analyzed cases. The reaction was intense and very well expressed in poorly differentiated cases and in advanced stages. We obtained significant associations between Snail expression and N category ($p = 0.020$, chi-square test), tumor stage ($p = 0.010$, chi-square test) and degree of differentiation ($p = 0.004$, chi-square test).

CHAPTER VIII. CONCLUSIONS

Among the most important conclusions, we mention:

Most of the analyzed cases, namely 118 cases (40.2%), corresponded to the interval between 60-69 years.

We found a net predominance of male affections in 98.3% of cases.

The risk factors study showed that in most of cases smoking and alcohol consumption were the main risk factors in 269 and 102 cases, respectively.

Regarding the histopathological varieties we noticed the following forms: nonkeratinized forms in 241 cases, 48 cases corresponded to the keratinized form, the basaloid form in 2 cases and the verrucous carcinoma in the same number with the papillary carcinoma, each with one case (0.3%).

Another histopathological aspect of our study was the degree of differentiation, which allowed us to identify a majority of 148 moderately differentiated cases, followed by 93 cases of poorly differentiated squamous carcinomas and 52 well differentiated laryngeal squamous carcinomas.

Lymphatic ganglion status was an important parameter and most cases of laryngeal squamous carcinomas (50 cases) did not associate the presence of metastatic adenopathy. The presence of lymphatic invasion was observed in 17 cases: N1 category corresponded to 7 cases and N2 category 10 cases.

Regarding the tumor stage, we found that most cases corresponded to stages III and IV, with 37 cases belonging to the third stage and 17 cases to fourth stage. Followed stage II with 10 cases and stage I with 3 cases.

Statistical analysis of the histopathological study identified statistically significant differences ($p < 0.05$, chi-square test) between the degree of differentiation and localization but also the histopathological varieties. Associations were also obtained between tumor stage and T category, N category and vascular and perineural invasion.

A high expression of EGFR was obtained in moderate and poorly differentiated forms of laryngeal squamous carcinomas compared to well-differentiated forms. Using the chi-square test, we obtained significant ($p = 0.015$) values between EGFR marker expression and degree of differentiation, with high expression in poorly differentiated forms.

High values of the Ki-67 index corresponded to moderate and poorly differentiated forms while literature mentions high marker expression is associated with poorly differentiated carcinoma cases with the presence of lymph node metastases and rapid recurrence.

The immunohistochemical expression of the markers of apoptosis, p53 and bcl-2, was similar, the highest values were associated with poorly differentiated forms and in stage IV. Studies discovered p53 and bcl-2 coexpression is associated with a poor prognosis and a lower survival time.

For E-cadherin, the highest values have been identified for well-differentiated laryngeal squamous carcinomas in the early stages of the disease, stage I and II.

Cases with the most unfavorable progression, advanced stages and poorly differentiated forms, showed the highest values for the P-cadherin expression. Moreover,

we also achieved significant association between the marker and the degree of differentiation.

Immunoexpression of β -catenin in moderately and poorly differentiated forms as well as in advanced disease states corresponded to the cytoplasmic and nuclear localization of the marker versus well-differentiated forms. Nuclear localization is commonly found in cases of head and neck cancers with a reserved prognosis.

We found in the present study that the expression of cyclin D1 increased with the degree of differentiation, the highest values being obtained in poorly differentiated forms.

The Snail reaction was intense and very well expressed in poorly differentiated cases and in advanced stages. We obtained significant associations between Snail expression and N category, tumor stage and degree of differentiation.

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