UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
DOCTORAL SCHOOL

THESIS
ANATOMOCLINICAL, MORPHOLOGICAL AND
GENETICAL CONSIDERATIONS IN
LARYNGEAL CANCER

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INTRODUCTION

The present doctoral thesis represents an anatomoclinical, morphological and genetical study of laryngeal cancer, based upon clinical and statistical data gathered from within the ENT Clinic of Craiova’s Clinical County Emergency Hospital. The study spread over 9 years, between 2010 and 2018, during which time several rare laryngeal carcinoma cases, which will be shown in this thesis in the form of clinical cases, stood out.

CURRENT STATE OF KNOWLEDGE

The current state of knowledge is divided into larynx anatomy, larynx physiology, positive diagnosis of laryngeal cancer, laryngeal cancer staging and treatment.

Larynx anatomy describes in detail the three parts of the larynx: the supraglottis, the glottis and the subglottis, as well as the laryngeal skeleton which is made up of a series of cartilages interconnected through ligaments and membranes. Further, the laryngeal extrinsic and intrinsic musculature are approached in great detail, along with the laryngeal vascularization and innervation, as well as the paraglottic and preepiglottic spaces, both of which are of great importance in tumor extension. The vocal fold’s structure is discussed in both macro- and micro-anatomical layers, through Hirano’s „cover body” model.
**Larynx physiology** explains the functions of the larynx: it’s role in phonation, starting with the principles of voice formation at a laryngeal level and the Bernoulli effect upon the vocal cords; it’s protective role over the larynx, through deglutition or coughing; it’s respiratory function.

The positive **diagnosis of laryngeal cancer** goes through an algorithm based on several stages, comprising of: medical history, clinical diagnosis (examination and palpation of the cervical region, indirect laryngoscopy and contact videolaryngoscopy), imaging (radiology, ultrasonography, CT scan, MRI, PET-CT).

**Laryngeal cancer staging** is of primordial importance in establishing a treatment plan, and is based on the TNM system of the World Health Organisation, taking into consideration the size and extension of the primary tumor (T), metastasis in the regional lymph nodes (N), and distant metastasis (M).

**Laryngeal cancer treatment** consists of transoral surgical techniques, external surgical techniques, determined in accordance with TNM staging. Transoral surgical techniques in laryngeal cancer address tumors in early or intermediary stages of glottic and supraglottic cancer, through endoscopic CO2 Laser cordectomy. External surgical techniques comprise of partial laryngectomies, total laryngectomies, salvage laryngectomies, as well as tracheostomy and lymph node dissection. Also, oncological treatment and radio-chemo-therapy tend to complete the treatment.
PERSONAL CONTRIBUTIONS

This part of the study consists of the working hypothesis, the general objectives, research methodology and concludes with the results of the study.

Working hypothesis and general objectives

The first documentation phase was applied to laryngeal carcinoma cases that have passed through the ENT Clinic of Craiova’s Clinical County Emergency Hospital – a retrospective study, documenting a period of five years (2010-2015), during which 387 patients were registered. Within the retrospective study, pre-existent data has been analysed, consisting of patients diagnosed in the clinic with laryngeal carcinoma, that have already been introduced in the database and whose biological samples have already been processed and are registered in the Pathological Anatomy Department archive.

The prospective study comprised of 153 patients diagnosed with laryngeal neoplasm, between 2015 and 2018, within the ENT Clinic. Once the patients of the prospective study have met the inclusion criteria for the research, biological samples (blood sample) have been collected in order to isolate and purify the DNA, as well as tumor samples (biopsy or resected tumor) alongside matching healthy tissue, for comparison.

By bringing together the results belonging to the retrospective study, along those of the prospective research, a database comprising of 540 patients was formed.
Results for the two studies were compiled into an electronic database, having put in specific data for each patient: identification data, background information, behaviour, work environment, comorbidities, current treatments, as well as the chief complaints (signs and symptoms) for which the patient sought medical help (dysphonia, dyspnea, dysphagia, cervical tumefaction), symptoms emergence history and the progress of the disease, as well as staging upon presentation and tumor extension.

The electronic database that contains clinical, morphological and genetic information was created through statistical analysis, using Microsoft Excel and SPSS.

The purpose of the surgery, whether it is diagnostic, curative or palliative, radical or partial, was documented along with the morphological and genetical markers, observing the correlations.

Benefitting from the experience of the Center of Excellence in Morphology from the University of Medicine and Pharmacy of Craiova, a histologic and immunohistochemical research of tumor samples (biopsy or resected tumor) from patients included in the study has been performed.

With help from the Cellular and Molecular Biology Laboratory of the University of Medicine and Pharmacy of Craiova, the identification of genetic polymorphisms involved in laryngeal carcinogenesis has been initiated. The work protocol for identifying genetic polymorphisms comprised of the following steps: isolating genomic DNA out of the blood sample, followed by isolating genomic DNA from the tissue samples, performing the spectrophotometric
analysis, identifying allelic variants through Real-Time Polymerase Chain Reaction and interpreting the results.

![Pie chart showing study group percentages]

Fig. 1. Study group percentages out of the total number of patients

**Research methodology**

The material for the study is represented by the tumor samples, whether they are laryngeal biopsies or the resected tumoral mass, collected within the ENT Clinic and processed by the Anatomical Pathology Laboratory and the Human Genetics Laboratory of the Clinical County Emergency Hospital and the University of Medicine and Pharmacy of Craiova.

The symptomatology of laryngeal cancer patients is dominated by three essential symptoms, of variable intensity, in relation to the affected laryngeal floor: dysphonia, dysphagia and dyspnea.

The patients medical history yields information regarding the patients age, gender, professional environment, home environment, alcohol and tobacco consumption, current and former treatment, allergies, case history.
The patients **clinical exam** comprised of:

- Head and neck inspection with an emphasis on the cervical region (laterocervical, prelaryngeal), taking into account any sort of alteration in colour or vascularization of the skin, or even changes in shape caused by underlying structures.

- Facial and cervical palpation, with an emphasis on lymph nodes.

- Oral cavity examination

- Laryngoscopy
  - Indirect laryngoscopy
  - Laryngeal endoscopy (70° rigid endoscope)
  - Flexible nasopharyngolaryngoscopy
    - In white light
    - NBI (Narrow Band Imaging)
  - Suspension laryngoscopy
  - Panendoscopy – whenever synchronous tumors are suspected or in case of a carcinoma with unknown primary (CUP Syndrome)

- Medical imaging
  - Cervical ecography
  - Radiography (Front and Side view)
  - Barium swallow test
  - CT scan
  - MRI
  - In order to rule out distant metastasis: Cranial CT, abdominal ecography, chest radiography, bone scintigraphy

- Laboratory tests (full blood count, ESR, etc)
Work protocols for the anatomopathological technique are established subsequent to a microscopic analysis through histopathological methods, and topped off, at times, by immunohistochemistry methods. The immunohistochemistry markers used in the study comprise of: p53, S100 protein, Ki67, PCNA, EGFR, CD44, EMA.

Genetic evaluation follows rs2245214 (c.574-12777G>C) polymorphism identification, genomic DNA isolation from peripheral blood, spectrophotometric evaluation (DNA purity and concentration evaluation) and Real Time PCR technique, in order to indentify mononucleotide polymorphisms. The first requisite to verify consists of testing the Hardy-Weinberg equilibrium (HWE). In order to exemplify the analysis, it was necessary to compare frequencies obtained in the studied population (laryngeal cancer) with a healthy lot (control group). For demonstration purposes, already published genotyping data for the studied polymorphism have been used. The statistical analysis consists in calculating the odds-ratio (OR) and the confidence intervals. Evaluation was performed using the CHI\(^2\) test (\(\chi^2\) test).

**Study results**

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<tbody>
<tr>
<td>Number of cases</td>
<td>80</td>
<td>85</td>
<td>71</td>
<td>61</td>
<td>63</td>
<td>49</td>
<td>68</td>
<td>28</td>
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Table 1. Distribution by year

As far as the distribution by gender is concerned, males were the most encountered among patients included in the study, representing 96.85% out of the patients, compared to 3.15% when it comes to women.
In regard to distribution by age groups, most cases of laryngeal carcinoma were encountered in patients of advanced age, mostly between 50 and 70 years old, followed closely by the 40-49 and the 70-79 age groups.

![Distribution by age groups graphic](image)

**Fig. 2. Distribution by age groups graphic**

It’s been noticed that following patient distribution by urban or rural area, that most patients come from a rural background – 331 cases encountered (61.3%), whereas urban cases encountered numbered 209 (38.7%).

Taking into consideration the placement of neoplastic lesions within the larynx, most carcinomas encountered incorporated all three floors of the larynx, most often starting from the supraglottis, followed by lesions starting from the glottis (according to number of cases encountered) – these two anatomoclinical spots representing the overwhelming majority of malign endolaryngeal lesions.

![Distribution of cases in regard to the involved laryngeal floor](image)

**Fig 3. Distribution of cases in regard to the involved laryngeal floor**
Concerning the clinical stage, it has been noticed that stage III and stage IV patients represented the majority, making up over 90% of documented cases between them, a situation best explained by patients presenting late for medical consultation. The first clinical case – Advanced laryngeal cancer with exteriorization - a clinical case demonstrating the extended therapeutic possibilities through radical surgery, by using a musculocutaneous flap consisting of pectoralis major.

Fig 4. Distribution of cases in regard to the stage of the disease at presentation

Out of the 540 patients, 233 (43.15%) benefitted from curative surgery, out of which 171 were total laryngectomies, 28 were cordectomies, 17 consisted of horizontal supraglottic laryngectomies, 11 were fronto-lateral laryngectomies, and 6 were bicordocomisural laryngectomies.

For most of the patients included in the study - 307 individuals (over half of the total number included in the study – 56.85%), only biopsies or palliative surgery, represented by tracheostomies, were performed. These patients have subsequently been directed towards oncological treatment.
Histological types have been represented as follows: 39 “in situ” carcinomas, 21 microinvasive carcinomas, 76 well differentiated carcinomas, 159 moderately differentiated carcinomas, 224 poorly differentiated carcinomas, 9 papillary carcinomas, 8 basaloid carcinomas, 2 verrucous carcinomas, 1 chondrosarcoma, 1 malignant melanoma. Most cases have been epidermoid carcinomas, however other histological types with rare laryngeal localization were encountered. Some of the rare histological types have been presented through clinical cases, as follows:

- Clinical case No. 2 – Laryngeal cancer with double localization and two different histological types
- Clinical case No. 3 – Basaloid squamous cell carcinoma
- Clinical case No. 4 – Chondrosarcoma of the hyoid bone
- Clinical case No.5 – Malignant melanoma with laryngotracheobronchial metastases. This patient went through a genetic molecular analysis, with detection of p.V600E mutation concerning the BRAF gene. Following the genetic analysis, the patient benefited from specific life-prolonging treatment.
- Clinical case No. 6 – Laryngeal Kaposi sarcoma
- Clinical case No. 7 – Laryngeal synovial sarcoma
Within the histological study, a number of patients required immunohistochemistry using a series of specific markers. The immunohistochemistry study has been performed on 45 selected cases, diagnosed with laryngeal cancer, in order to complement the histopathological study.

Out of the 45 patients included in the immunohistochemistry study, 36 tested positive for EGFR. PCNA was positive within the dysplastic epithelium, as well as within carcinomatous islands, its presence (20 to 80%) correlating to the degree of tumor aggressiveness. The expression of p53 has been encountered less (51% of cases) than that of PCNA, however it is good to take note of the correlation between these marker expressions – both p53 and PCNA are strongly expressed in cases of tumors with a high malignity rate.
CD44 was encountered in 11 out of the 45 cases, and is associated with a poorly differentiated pattern, a high mitotic index, a high lymph node invasion and metastasis. Ki-67 has been encountered in a considerable number of immunohistochemistry studied cases.

**ATG 5 polymorphism genotyping rs2245214 (c.574-12777G>C)**

Based on a fluorescent signal emitted by a TaqMan probe, two allele have been identified: the G allele – FAM signal and C allele – VIC signal; also, accordingly, the three genotypes, as well: homozygous CC, heterozygous CG, as well as homozygous GG, as seen in Fig. 7.

![Fig. 7. Identifying the three genotypes of ATG5 rs2245214 (c.574-12777G>C): CC, CG and GG based on the fluorescent signal emitted through VIC and FAM dyes (VIC-Yellow, FAM – Green)](image-url)
CONCLUSIONS

► Laryngeal cancer represents slightly under 2% of all cancers. Carcinomas sum up 95 to 98% of laryngeal malignancies.

► The treatment addresses both the laryngeal tumor, as well as the lymph nodes belonging to the cervical region. Currently, the treatment is complex, consisting of surgery, as well as radiotherapy, chemotherapy and immunotherapy.

► Current surgical options comprise of endoscopic laser surgery, conservative surgery, partial and total laryngectomies. Surgical techniques vary depending on the placement and extension of the primary tumor, as well as the presence or absence of lymph node metastasis.

► In the studied cases, the therapeutic conduct regarding cervical lymph nodes comprised of radical lymph node dissection modified for N3 patients, at times with internal jugular vein and sternocleidomastoid muscle „en-bloc” resection. N1 and N2 patients underwent selective jugulo-carotid lymphadenectomy, paratracheal, prelaryngeal. Cases that benefited from total laryngectomy or supraglottic horizontal laryngectomy, had all the lymph node areas verified.

► The postoperative results of patients included in the study are superposable to those forwarded by authors of expert literature, with a 5 year survival of nearly 80%.

► The histopathological study of laryngeal carcinoma has highlighted an increased number of epidermoid carcinomas (over 95%), as well as a series of special histopathological types: chondrosarcoma, biphasic synovial sarcoma, Kaposi sarcoma, malignant melanoma.
► According to their degree of differentiation, it has been observed that the G3 poorly-differentiated carcinoma was the most encountered, noting that it tends to be associated with a high rate of metastasis and an unfavourable prognosis with a high recurrence rate.

► Out of the group of patients studied within the immunohistochemistry research, 80% tested positive for EGFR, having found it significantly high in the tumor specimen, as opposed to normal mucosa – promising results that promote EGFR usefulness in the study of laryngeal carcinomas. Cases assessed through PCNA have shown a considerably higher index in lesions that have eventually went through malignant transformation. Therefore, PCNA assessment may be considered a useful marker for malignant transformation potential. In addition to PCNA, p53 has also been well expressed in tumors with high malignancy. The implication of CD44 in the mechanism of development and progression of laryngeal tumors can assist in predicting the transformation of benign or precancerous lesions into cancer. Ki-67 is a suitable marker in detecting malignant proliferation for laryngeal cancer.

► During the genetic study, a clinical case of malignant melanoma metastasis within the larynx/trachea/bronchi was encountered. It benefited from the study of V600E mutation on the BRAF gene, allowing the use of a treatment plan using Vemurafenib, a BRAF gene inhibitor, that led to a survival period of over one year.

► Molecular approach can emphasize genetical markers, which together with histological and immunohistochemical markers can represent targets for complex diagnostic, prognostic or screening protocols for laryngeal cancer.