UNIVERSITY OF MEDICINE AND PHARMACY
CRAIOVA
FACULTY OF MEDICINE

THE MORPHOLOGICAL STUDY OF THE EPITHELIAL – MESENCHYMAL TRANSITION PROCESS IN THE SQUAMOUS CELL CARCINOMA OF THE TONGUE

SCIENTIFIC COODINATOR
PROF. UNIV. DR. CRĂIŢOIU ȘTEFANIA

PHD CANDIDATE
AFREM MIHAI-CĂTĂLIN

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INTRODUCTION

Oral cavity and oropharynx cancer are considered among the most common cancers in the world with an estimated annual incidence rate for 2008 at 400,000 new cases, of which about 223,000 would die. In Romania, according to the International Agency for Research on Cancer (IARC) in 2012, there was an incidence of cancer of the lip and oral cavity of 1,518 new cases annually (3.5 cases per 100,000 inhabitants) and a mortality rate of 878 new cases annually (3% cases per 100,000 inhabitants), this form of cancer being ranked on the 8th position as incidence of all types of cancers at various sites.

The lingual location seems to be one of the most deadly locations of oral squamous cell cancer, particularly the tumours developed in the mobile portion, which tend to be more aggressive, having metastases with greater frequency in the regional lymph nodes. During the malignant transformation there are taking place a number of cellular morphological changes, often designated as atypical changes which in fact are attributed to epithelial-mesenchymal transition process (EMT). Broadly, the term EMT designates the loss of epithelial differentiation and the gain of a mesenchymal phenotype. Relatively recent studies have shown that the presence of EMT process is both a predictor of progression of oral squamous cell carcinoma and of their prognostic factor.

Our study was in line with these latter studies investigating a series of morphological and clinical parameters and their impact on prognosis predictive carcinomas in this location. However, the histological study aimed also to highlight some of the specific morphological changes in the EMT process at the level of lingual squamous carcinomas studied. This study was continued with another immunohistochemical one in which we wanted to highlight the immunophenotypic change from the epithelial to the mesenchymal one in the lingual squamous carcinomas investigated by analyzing the most important markers in the EMT process.
CHAPTER I
EPIDEMIOLOGY OF ORAL SQUAMOUS CELL CARCINOMA

According to a report of the International Agency for Research on Cancer (IARC), the oral localized malignancies are the 11th most common form of human cancer. The most commonly, the oral cancer affects people in the developing countries (Petersen, 2003; Stewart & Kleihues, 2003). Thus, there was reported an increased incidence in the Southern-Central and Southern-Eastern Asian countries, such as India, Pakistan, Bangladesh, Taiwan and Sri Lanka (Krishna et al., 2013). In the European countries, since 1980, there was recorded an overall downward trend in the incidence and mortality of oral cancer (La Vecchia et al., 2004), but a more detailed geographical analysis showed an increase in this form of cancer mortality in some Eastern European countries, namely Bulgaria, Romania, Hungary, Slovakia and Slovenia (La Vecchia et al., 2003). The authors speculated that this trend could be related to changes related to exposure to the two major risk factors of oral cancer, namely alcohol and smoking.

Epidemiological studies over the last 20 years have shown a steady increase in the incidence of these cancers in young adults (between 18-45 years), particularly the cancers of the oral cavity and oropharyngeal cancers (Chaturvedi et al., 2008; Golas, 2007). The trend in the Western countries over the past 30 years is the decrease of oral squamous cancer incidence (Chaturvedi et al., 2008), including young people, yet for the squamous cell carcinoma of the tongue there was an increase in its incidence among white women, aged 18-44 years (von Doersten et al., 1995).

According to the data presented by WHO and the International Agency for Research on Cancer (IARC) for Romania for 2012 it was estimated for all age groups an incidence of oral and lip cancer of 1518 new cases annually in males (3.5 per 100,000 inhabitants), while in the females there were estimated 329 cases annually (0.9 per 10,000 inhabitants) (GLOBOCAN 2012). The estimated mortality rate for this cancer sites was of 878 cases annually (3 per100, 000
inhabitants) in men and 123 cases annually (0.6 per 100,000 inhabitants) among females.

According to the International Agency for Research on Cancer (IARC) the oral carcinogenicity risk factors would be represented by tobacco, alcohol, infections, radiation, occupational exposures and medication (IARC, 2014). Smoking is a dominant risk factor in squamous cancers located in the head and neck and it correlates with the intensity and the duration of smoking (Hashibe et al., 2007). The major risk factor for oral cancer among non-smokers is alcohol (Gillison, 2007; Znaor et al., 2003). The risk increases with the concentration of alcohol (there are differences in the consumption of spirits versus wine or beer), but it is unclear if ingested alcohol type may influence this risk when the total amount and the consumed alcohol concentration are adjusted (Gillison, 2007; Castellsague et al., 2004). Alcohol acts as a solvent, increasing the oral mucosal exposure to carcinogens and increases their intracellular influx. In the etiopathogenesis of oral cancers there can be criminalized also human papilloma viruses (HPV), especially serotypes 16 and 18. In Europe a meta-analytic study estimated that about 4 in 10 cases of oropharyngeal cancer (40%) would be based on HPV infections, while for oral cancers such aetiology has been estimated at 24% of the cases (Mehanna et al., 2013). The dysplastic lesions which include leukoplakia and erythroplakia preceed the oral squamous cell carcinoma. A meta-analytic study indicates their transformation rate of 12% (Mehanna et al., 2009).

**CHAPTER II
MOLECULAR MECHANISMS OF THE ORAL CARCINOGENESIS**

Oral cancer like other malignant neoplasm arises from the accumulation of a series of discrete genetic events that lead to invasive cancer. These molecular events develop consecutively to the combined effects of individual genetic predisposition and exposure to carcinogens ambient (Califano et al., 1996), such as tobacco, alcohol, chemical carcinogens, ultraviolet and ionizing radiation and microorganisms (Markopoulos et al., 2004; O'Grady & Reade, 1992; Preston et al., 2007). In the progression of oral cancers in a first phase occurs losing control of
consecutive cell cycle proliferation stimulation via the reduction of the mechanisms of apoptotic cell. Then there is the increase in cell motility as a result of which the malignant epithelial cells can penetrate the base membrane and afterwards, they metastasize. According to the model of field cancerization (Braakhuis et al., 2004) an oral epithelium stem cell accumulates a series of genetic alterations and generate daughter cells that share the same gene alterations. This "zone" (grouping) of cells expands the oral mucosa adjacent reaching sizes of a few inches, but often without being detected macroscopically.

It is believed that about 10% of all cancers have a strong hereditary component. The existence of such a component in oral cancer has been supported by a number of studies regarding the familial aggregation of such cancers (Jefferies et al., 1999).

Gene alterations may enable mutations or they can enhance those oncogenes which promote cell survival and proliferation. Mutations of this kind are the general hypomethylation of DNA, hyper- and hypomethylation of certain genes of cyclin type D and cromatinian alterations (Díez-Pérez et al., 2011; Huang et al., 2006).

Alterations in oncogenes can target (Sidransky, 1995):
• growth factors and their receptors, TGF-α, TGF-β1, EGFR / erbB, c-erbB-2 / HER-2, NGF;
• transcription factors: myc, fos, jun, c-myc;
• transducers of intracellular signals: ras, raf, State-3;
• inhibitors of apoptosis: bcl-2, bax regulators of the cell cycle: cyclin D1

Crucial events in the malignant transformation of a premalignant cell consist of the inactivation of cell-negative regulators, tumour suppressor genes, respectively. One of these suppressor genes is p53. Inactivation of p53 occurs in more than half of the cases of head and neck carcinomas. A meta-analysis study showed that oral premalignant lesions pose a much higher percentage of overexpression of p53 versus malignant lesions induced (Warnakulasuriya et al., 1998). Retinoblastoma tumour suppressor gene regulates cell cycle transition
preventing cell to G1 through hypophosphorylated pRb protein. The absence of pRb expression was observed in 66% of oral squamous cell carcinoma and in 64%
of premalignant lesions, while the expression of p16 was absent in 63% of oral squamous cell carcinoma and 59% of pre-malignant lesions (Pande et al, 1998).

One of the oncogenes viruses involved in oral carcinogenesis is HPV. Such a type of meta-analysis study showed that HPV detection probabilities is 2-3 times higher in oral precancerous lesions and it is 4-5 times higher in oral squamous cell carcinoma than in normal oral epithelium (Miller & Johnstone, 2001).

**CAPITOLUL III**

**THE IMPLICATIONS OF THE EPITHELIAL-MESENCHYMAL TRANSITION PROCESS IN HUMAN CARCINOGENESIS**

The epithelial-mezenchymal transition (EMT) is a biologically active process that allows polarized epithelial cells to undergo a series of biochemical transformations finally adopting a mesenchymal phenotype. The defining characteristics of EMT process in cultures are: (1) the deviation from the stratified and polarized epithelia morphology due to the loss of adhesion molecules; (2) the loss of epithelial specific genes; (3) the acquisition of mesenchyme specific genes; (4) the loss of sensitivity to anoikis (Kalluri & Weinberg, 2009; Scheel & Weinberg, 2011; Tiwari et al., 2012). The phenotypic plasticity of EMT process is proven by the possibility of its reversibility, respectively by the mesenchymal-epithelial transition (MET) which involves the conversion of mesenchymal cells in epithelial type derivatives. These two processes EMT and MET are closely interconnected, epithelial and mesenchymal phenotypes interconversions occurring quite frequently and providing high flexibility especially in the conduct of embryogenesis, but also facilitate the dynamic cellular remodelling during regeneration and wound healing processes.

In the EMT process it appears to be involved a series of distinct molecular processes, including the activation of transcription factors, the expression of specific cell surface proteins, reorganizations and expressions of cytoskeletal
proteins, the production of enzymes capable of degrading the extracellular matrix and alterations in the expression of specific sequences of micro RNA.

In fact many of the studies on laboratory animals and cell cultures have shown that carcinoma cells can acquire a mesenchymal phenotype, expressing mesenchymal markers such as the actinin-alpha specific to the smooth muscle (α-SMA), fibroblast specific protein-1 (FSP1), vimentin and desmin (Yang & Weinberg, 2008). Phenotypic, such cells become fusiform, lose apical basal polarity and become mobile, resistant to apoptosis and borrow features similar to stem cells (Micalizzi et al., 2010). These cells are present in the invasion front of the primary tumours and it is considered that they are the ones that come in the sequence of invasion-metastasis cascade stages (Brabletz et al., 2001; Fidler & Post, 2008; Thiery, 2002).

One of the characteristics of the EMT process is the loss of intercellular adhesion with the diminishing of E-cadherin expression. However the loss of expressiveness for E-cadherin was found to be responsible for tumour progression, metastasis and poor prognosis in various human carcinomas (Chan et al., 2000; Dorudi et al., 1993; Gould et al., 2006; Kowalski et al., 2003).

The term "change of cadherin phenotype" refers to the passage from E-cadherin expression to the expression of N-cadherin, but also includes cases where E-cadherin expression level does not change significantly, but instead begin to express or N-cadherin expresses at a higher level. This type of cadherin phenotypic change is part of the EMT process by which tumour cells "escaped" from the influence of E-cadherin-mediated intercellular interactions acquire a mobile phenotype consecutive to the N-cadherin induction, being capable of migrating and invasion, and these tumours have a poor prognosis (Gravdal et al., 2007; blasphemous et al., 2007).

The interaction between cancer cells and tumour microenvironment can induce EMT through the self-secretion and/or paracrine mediators such as growth factors, cytokines and extracellular matrix proteins (Moustakas & Heldin, 2007).
CAPITOLUL IV
THE HISTOPATHOLOGICAL STUDY OF SQUAMOUS CELL
CARCINOMA OF THE TONGUE

IV.1.1 THE MATERIAL STUDIED

The histopathological material comes from the case studies of the Anatomical Pathology laboratory of the Clinical Emergency Hospital, no. 1 Craiova and it was represented by the archived paraffin blocks. There have been selected, for the 2008-2012 time frame investigated, a total of 79 cases of oral squamous cell carcinomas that were the subject of the histopathological study.

IV.1.2 THE METHOD USED

In the morphological study I have used the classical histological technique by paraffin embedding and hematoxylin-eosin stains (H.E.) and Masson trichrome with aniline blue.

IV.2. THE RESULTS OF THE HISTOPATHOLOGICAL STUDY
OF THE SQUAMOUS CELL CARCINOMA OF THE TONGUE

The squamous cell carcinoma of the tongue represents our studied cases developed widely from the third decade of life until the ninth decade. We have found the peak incidence of the cases in the sixth decade in which fitted 28 patients, representing 34.17% of the total number of patients, followed by decades VII, VIII, and V, which included 22.78%, 16.45% and 12.65% of the patients investigated. The squamous cell carcinoma of the tongue developed especially in males, respectively 51 of the 79 cases, representing 64.55% from the cases investigated. Sex ratio of cases investigated was 1.82 for males. The most common were interested the tongue edges (35.44%), followed by the location in the 2/3 front part of the tongue (29.11%) and dorsal (21.53%)

Strictly histopathological speaking we were interested in the following aspects: histopathological variant, degree of differentiation, inflammatory infiltrate
extent, pattern of tumour invasion, metastatic adenopathy, perineural invasion, vascular invasion and surgical safety margins status.

Using the WHO diagnostic criteria, we could classify the 79 cases into the following histological varieties: keratinized squamous cell carcinoma, nonkeratinized squamous cell carcinoma, bazaloid squamous cell carcinoma, adenoid squamous cell carcinoma, spindle-cell squamous cell carcinoma, verrucous carcinoma, papillary carcinoma (Table 4.4).

Table 4.4. The distribution of the studied cases according to the histopathological variety

<table>
<thead>
<tr>
<th>HP Variety</th>
<th>Keratinized</th>
<th>Nonkeratinized</th>
<th>Acanalotitic</th>
<th>Bazaloid</th>
<th>Papilar</th>
<th>Sarcomatoid</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of cases Perc.</td>
<td>29</td>
<td>40</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>79</td>
</tr>
<tr>
<td>36.71%</td>
<td>50.63%</td>
<td>6.33%</td>
<td>1.27%</td>
<td>2.53%</td>
<td>2.53%</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

According to the data presented in the table above most frequent histopathological variety was the nonkeratinized one with more than half of the cases (50.63%), followed by the variety with keratinization (36.71%).

► The keratinized squamous cell carcinoma of the tongue were diagnosed in a total of 29 cases (36.71%). Morpho-pathologically, the tumours partially reiterated the morphology of the stratified squamous epithelium, consisting of neoplastic cells with polygonal morphology, with distinct cell borders, intercellular bridges, with abundant cytoplasm, eosinophilic, variable degree of keratinization and nuclei of varying sizes (Fig. 4.1). The growth pattern is insular and trabecular, infiltrative, with predominantly fibrous stroma (Fig. 4.2.)
Fig. 4.1 Squamous cell carcinoma of the tongue – the keratinized variety. col. H.E, X40

Fig. 4.2 Squamous cell carcinoma of the tongue – the keratinized variety, insular growth pattern and fibrous stroma. col. Trc. Masson, X40

▶ The *nonkeratinized* squamous cell carcinoma of the tongue were diagnosed in a total of 40 cases (50.63%), being made up of a relatively uniform carcinoma cell proliferation, with round-oval nuclei, vesicular, nucleoli of medium
size and frequent atypical mitoses (5-10 mitoses per 10x microscope field) (Fig. 4.5).

![Image](image.png)

Fig. 4.5 Squamous cell carcinoma of the tongue – the nonkeratinized variety. col. H.E, X40

Using the Broder classification system we could classify the 79 cases into the following degrees of differentiation: well differentiated, moderately differentiated and poorly differentiated (Table 4.5).

<table>
<thead>
<tr>
<th>Degree of differentiation</th>
<th>Well differentiated</th>
<th>Moderately differentiated</th>
<th>Poorly differentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>29</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>Percentage%</td>
<td>36.71</td>
<td>50.63%</td>
<td>12.66</td>
</tr>
</tbody>
</table>

Tabel 4.5 The distribution of squamous cell carcinoma of the tongue according to the degree of differentiation

► The moderately differentiated squamous cell carcinoma were the largest group in terms of the degree of differentiation (50.63%). In this category we have placed most of the nonkeratinized tumors (33) the papillary and adenoid squamous varieties. The tumour cells retained their polygonal morphology, but had an eosinophilic cytoplasm less abundant, intercellular limits and bridges less obvious. The growth pattern was a cordonal and insular type, variable shapes and sizes with rare diskkeratosis pearls (Fig. 4.16).
Fig. 4.16 Squamous cell carcinoma of the tongue – the moderately differentiated form. col. H.E, X100

Considering the criterion of the invasion way of tumour in the host tissue (Bryne et al., 1989) the investigated case studies presented the aspects briefly mentioned in Table 4.7

<table>
<thead>
<tr>
<th>The tumor invasion pattern</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
<th>Type 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>7</td>
<td>27</td>
<td>33</td>
<td>12</td>
</tr>
<tr>
<td>Percentage %</td>
<td>8.86%</td>
<td>34.18%</td>
<td>41.77%</td>
<td>15.19%</td>
</tr>
</tbody>
</table>

Table 4.7 The distribution of squamous cell carcinoma of the tongue according to the tumour invasion pattern

The most common type of invasive pattern identified by us on the investigated case study was the type 3, corresponding to a tumour invasion having the form of small cellular groups or cells infiltrating cords (over 15 rows of cells) (Fig. 4.21). This type of pattern being present in 33 of the investigated cases, representing 41.77% from the case studies investigated.
Fig. 4.21 Squamous cell carcinoma of the tongue – infiltrative pattern of 3rd degree. col. H.E, X40

Framing the case studies according to the pTNM system criteria of the Mixed American Joint Cancer Research (Greene et al., 2002) is shown in the table below (Table 4.12).

<table>
<thead>
<tr>
<th>pTNM stage</th>
<th>No. of Cases</th>
<th>Percentage%</th>
</tr>
</thead>
<tbody>
<tr>
<td>stage I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1N0M0</td>
<td>37</td>
<td>46.84</td>
</tr>
<tr>
<td>stage II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2N0M0</td>
<td>10</td>
<td>12.66</td>
</tr>
<tr>
<td>T1N1M0</td>
<td>6</td>
<td>7.6</td>
</tr>
<tr>
<td>T2N1M0</td>
<td>3</td>
<td>3.79</td>
</tr>
<tr>
<td>T3N0M0</td>
<td>10</td>
<td>12.66</td>
</tr>
<tr>
<td>T3N1M0</td>
<td>2</td>
<td>2.53</td>
</tr>
<tr>
<td>stage IVA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1N2M0</td>
<td>4</td>
<td>5.06</td>
</tr>
<tr>
<td>T2N2M0</td>
<td>6</td>
<td>7.6</td>
</tr>
<tr>
<td>T3N2M0</td>
<td>1</td>
<td>1.26</td>
</tr>
</tbody>
</table>

Table 4.12 The distribution of squamous cell carcinoma of the tongue according to the pTNM staging
As it can be seen, most cases were classified as stage I disease with a total of 37 cases, representing 46.84% of our case studies.

Relating to the criteria established by Brandwein-Gensler et al. (2005), reflecting the pattern of invasion, the presence of neural invasion and inflammatory infiltration degree from the tumour interface / host tissue, the cases investigated by us were classified according to the data shown in the table below (Table 4.13).

<table>
<thead>
<tr>
<th>Histological risk</th>
<th>Low degree</th>
<th>Moderate degree</th>
<th>High degree</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>12</td>
<td>44</td>
<td>23</td>
</tr>
<tr>
<td>Percentage%</td>
<td>15.2%</td>
<td>55.7%</td>
<td>29.1%</td>
</tr>
</tbody>
</table>

Table 4.13 The distribution of squamous cell carcinoma of the tongue according to the histological risk

According to the data presented in the table above, the vast majority of the investigated cases were included in carcinoma group with moderate histological risk (55.7%), followed by the high-risk category (29.1%) and the last place the patients group with low-risk (15.2%).

**SPECIFIC MORPHOLOGICAL ASPECTS OF THE EPITHELIAL-MESENCHYMAL TRANSITION PROCESS**

- The first observed aspect was the change of neoplastic cells morphology from type "thorny" neoplastic cell with polyhedral morphology to the fusiform type, elongated. This was more obvious at the tumour interface / host tissue, namely at the invasion front, especially in the deeply invasive tumours and with type 4 invasion pattern, as isolated cells, very small nests and islands of neoplastic cells.

- The loss of cohesivety, translated by the appearance of isolated neoplastic cells or groups of cells separated from the main tumour mass.
- Tumour invasiveness, property that allows neoplastic cells to penetrate deeply into the structures in which they develop: muscle, vessels and perineural (Fig. 4.34).

Fig. 4.34 Squamous cell carcinoma of the tongue – perineural invasion from the invasion front. col. tric Masson, X100

The association, especially at the invasion front with inflammatory infiltration, facilitates tumour progression and invasion, and in a more advanced stage with fibrosis (desmoplasia- Fig.4.36), which will also facilitate tumour invasion.

THE CONCLUSIONS OF THE HISTOPATHOLOGICAL STUDY OF THE PLEOMORPHIC ADENOMAS OF THE SALIVARY GLAND

- The epidemiological profile of this type of tumour in the case studies indicates squamous cell carcinoma of the tongue mainly in the course of the VI and VII decades of life (about 57%), especially in males (M: F = 1.82) and specially affecting the tongue edges (35.44%).
Histopathologically, the vast majority were nonkeratinized squamous cell carcinomas (50.63%) developed mainly on the edges of the tongue towards its base. Other histopathological varieties were also diagnosed, but they were rare (acantholytic- 6.33%, sarcomatoid- 2.53%, papilar- 2.53% and bazaloid carcinoma - 1.27%).

In terms of the degree of differentiation most cases were included in the moderately differentiated tumour group (50.63%), and at the opposite pole there were included the low differentiated forms. The latter were correlated with clinical stage; patients with these forms of squamous cell carcinoma of the tongue were detected in advanced disease stages (stages III and IV).

Vascular invasion was present in 34.18% of investigated case study, with a weak statistical correlation with the lympho-ganglionar status.

The lympho-ganglionar metastases were identified in 21.5% of the investigated cases, most of them corresponding to N1 stage (35.3%) and was directly correlated in a significant way with tumour stage and with tumour differentiation degree and poorly correlated with vascular invasion.

A weak correlation was established between the type 3/4 invasion pattern and the presence of tumour invasion with the degree of tumour differentiation, both aspects being more common in poorly differentiated forms of oral squamous cell carcinoma.

The vast majority of the cases were detected in clinical stage I (46.84%) and III (26.58%). The clinical stage on the investigated case study correlated with the degree of tumour differentiation, with vascular invasion, with lympho-ganglionar dissemination status and with histological risk of malignancy, the advanced stages corresponding to the poorly differentiated tumours, with vascular invasion, with lympho-ganglionar metastasis presence and with a histological high-risk of malignancy.

Most of the investigated squamous cell carcinomas of the tongue were included in the tumours category with histological moderate risk of malignancy (55.7%), this parameter being statistically significant correlated with the disease
stage, and the cases with high risk were presented at the time of diagnosis with the most advanced stages disease.

- The microscopic identification of some morphological aspects such as: (i) a fusiform morphology of neoplastic cells; (ii) type 4 invasive pattern with discohesive neoplastic cells infiltrating the adjacent tissues; (iii) the presence of a tumour lymphoplasmacytic infiltrate and (iii) a desmoplastic reaction next to the invasive tumour proliferations proves the involvement of an epithelial-mesenchymal transition process in the progression of the squamous cell carcinoma of the tongue.

V.

CAPITOLUL V

THE IMMUNOHISTOCHEMICAL STUDY OF THE EPITHELIAL-MESENCHIMAL TRANSITION PROCESS IN THE SQUAMOUS CELL CARCINOMA OF THE TONGUE

V.1.1 THE STUDIED MATERIAL

In the immunohistochemical study the investigated material was represented by 15 cases of squamous cell carcinomas of the tongue representative for the group of 79 cases which were the subject of the histopathological study.

V.1.2 METHOD

In the immunohistochemical study we used the paraffin blocks from which were performed the sections necessary for the classical histopathological processing, with the usual stains. These were sectioned in microtome obtaining serial sections with the thickness of 4μm which were applied to slides treated with adhesive-polylysine substance. The immunohistochemical study was a type study with enzymatic detection using LSAB technique as a working method (Labelled Streptavidin-Biotin2 System). The kit used was manufactured by Dako, Redox, Romania - Code K0675.
In the immunohistochemical study of the 15 cases of squamous cell carcinoma of the tongue we used concentrated antibodies developed in mouse or rabbit directed against man, whose main features are summarized in the table below (Table 6.1).

### Table no. 6.1 Antibodies used in the study of squamous cell carcinoma of the tongue

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Antigenic unmasking</th>
<th>Dilution</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>Monoclonal mouse-NCH-38</td>
<td>citrate pH 6</td>
<td>1:50</td>
<td>Invasive ductal breast carcinoma</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Monoclonal rabbit-EP700Y</td>
<td>citrate pH 6</td>
<td>1:50</td>
<td>Invasive ductal breast carcinoma</td>
</tr>
<tr>
<td>Citokeratina 18 (CK18)</td>
<td>Monoclonal mouse- DC 10</td>
<td>Proteinase K</td>
<td>1:30</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Monoclonal rabbit-SP20</td>
<td>citrate pH 6</td>
<td>1:200</td>
<td>Tegument</td>
</tr>
<tr>
<td>Twist1</td>
<td>Monoclonal soarece-10E4E6</td>
<td>citrate pH 6</td>
<td>1:1000</td>
<td>Colon adenocarcinoma</td>
</tr>
<tr>
<td>MMP13</td>
<td>Policlonal goat</td>
<td>citrate pH 6</td>
<td>1:40</td>
<td>Invasive ductal breast carcinoma</td>
</tr>
<tr>
<td>GDF5</td>
<td>Policlonal rabbit</td>
<td>citrate pH 6</td>
<td>1:100</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Monoclonal mouse-6G11</td>
<td>citrate pH 6</td>
<td>1:50</td>
<td>Brain</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Policlonal rabbit</td>
<td>citrate pH 6</td>
<td>1:1000</td>
<td>Tonsil</td>
</tr>
</tbody>
</table>

- The assessment of the markers expression was realised from a qualitative point of view, the reactions intensity being quantified according to the scores: 0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive.
- The semiquantitative analysis involved the assessment of the number of positive cells at a magnification of x400 for 5 fields chosen at random. The results were grouped as it follows: 0 = the absence of reactivity, +1 (weak) = the positivity
in 10% of the tumour cells, +2 (moderate) = intense or homogeneous in 10-75% of tumour cells and +3 (intense) = highly homogenous in more than 75% of the tumour cells.

The immunohistochemical diagnosis algorithm

The antibodies used in this study "targeted" especially the epithelial-mesenchymal transition process from the squamous carcinoma cells of the tongue. Thus, some addressed:

► double reactions of the type E-cadherin + vimentin; Twist1 + E-cadherin; N-cadherin + E-cadherin targeted the epithelial-mesenchymal transition process;
► double reactions of the type CK18 + GDF5; CXCR4 + MMP13 targeted the lingual squamous carcinomas invasive potential in terms of the epithelial-mesenchymal transition process;

V.2.1 THE RESULT OF THE INVESTIGATION: E-CADHERIN+ VIMENTIN

► The semiquantitative investigation of the number of positive cells with E-cadherin markage showed the predominance of score 2 in the investigated case studies, and there were not present any negative cases for this marker.

As the reactivity was present at both membrane and cytoplasmic levels, we have evaluated separately the number of positive tumour cells at the membrane level only and afterwards those with cytoplasmic marker only and we have tried to correlate these values with some of the investigated morphological parameters (the clinical stage, the degree of differentiation and the invasion pattern).

According to the data presented in the table above we have noticed a decrease in E-cadherin membrane markage in parallel with the decrease in the degree of differentiation and the increase of the invasion pattern. Thus, the membrane pattern was most evident in the most diverse forms, with invasive type 1 pattern, predominantly in the centre of the tumour islands (Fig. 5.1).
Fig. 5.1 Squamous cell carcinoma of the tongue – well differentiated form, membranary positive reactivity to E-cadherin in the keratinocytes from the center of the neoplastic island. Col IHC E-cadherin (brown)/Vimentin (red), x40.

On the other hand, the cytoplasmic pattern was clearer in the weakly differentiated forms, with type 4 pattern of invasion, being predominant at the periphery of the carcinoma proliferations and in the invasion front (Fig. 5.4).

Fig. 5.4 Squamous cell carcinoma of the tongue – moderately differentiated form, type 3 invasive pattern, positive cytoplasmic reactivity with E-cadherin. Col IHC E-cadherin (brown)/Vimentin (red), x100.
Vimentin was positive in all the investigated specimens, the cytoplasmic reactivity being mainly present at the stromal level. Strictly at the proliferations carcinoma levels, the reactivity was present in 11 of the 15 investigated cases. The vast majority of the parenchymal reactivity ranged at the semiquantitative score 1 (60% of the cases) and only in 2 cases the score was 2, none of the cases presenting score 3.

The cytoplasmic markage was more obvious in the tumour cells on the periphery of the proliferative islands, but also in the acantholytic carcinoma cells with oval or spindle morphology in the centre of neoplastic islands (Fig. 5.7).

![Fig. 5.7 Squamous cell carcinoma of the tongue – moderately differentiated form, present reactivity to Vimentin from the neoplastic keratinocytes from the invasion part, invasive pattern type 2. Col IHC E-cadherin (brown)/Vimentin (red), x100.](image)

Statistically, the significant markage E-cadherin and vimentin were correlated in reverse, so that the marking intensity was higher in E-cadherin and lower in vimentin in well-differentiated forms, with low invasion pattern and with an early clinical stage. Moreover, while E-cadherin reactivity was more evident in the centre of the proliferative areas, and at the surface of the tumours, for vimentin the markage prevailed in the periphery of the proliferative areas and in the invasion front.
**V.2.2 THE INVESTIGATION RESULT: Twist1 + E-cadherin**

The reactivity for Twist1 was present in all cases investigated at nuclear level, both at the parenchymal component level and stromal level. The stromal reactivity was more obvious than the parenchymal one.

The tumour immunoreactivity is a heterogeneous one; the nuclear marker is present in almost all tumour mass regardless of topography, both in the centre and in the periphery of proliferations, and also in the superficial and in the deep area, at the invasion front (Fig. 5.14). The reactivity appears to be higher in the invasion front, but not to prove the existence of statistically significant differences.

![Image](image.jpg)

**Fig. 5.14** Squamous cell carcinoma of the tongue - well differentiated form, highly nuclear reactivity to Twist-1 of the neoplastic keratinocytes. Col IHC E-cadherin (brown) / Twist1 (red) x40.

Regarding the co-localization of E-cadherin it was present in all the investigated cases. The simultaneous expression of the two markers is present mainly in well and moderately differentiated forms, especially in the centre of the carcinoma proliferations, where E-cadherin immunomarkage was predominantly membranary (Fig. 5.19).
Fig. 5.19 Squamous cell carcinoma of the tongue –well differentiated form, nuclear reactivity to Twist-1 from the central keratinocytes which co-express membranary and E-cadherin. Col IHC E-cadherin (brown)/Twist1 (red), x100.

**V.2.3 INVESTIGATION RESULT: N-cadherin+ E-cadherin**

The squamous carcinoma cells of the tongue reactivity investigated for the N-cadherin marker was very small; out of the 15 cases only in 7 cases it was present. The reactivity was present especially at the cytoplasmic level and rarely at the membrane level, the cells at the periphery of the carcinoma proliferations being the most reactive. However the reactivity for this marker was dominant at the invasion front and especially when it appeared as small nests of cells or as isolated cells.

Regarding the co-localization of E-cadherin, this was rarely viewed only in some of the proliferative islands from invasion front, E-cadherin being expressed at the membrane level and N-cadherin at a cytoplasmic level (Fig. 5.23).
In fact, the two expression patterns of the two cadherins were opposite to each other. Thus if the E-cadherin expression was constant in the superficial parts of the tumours, especially in the well / moderately differentiated ones, the membranary expression pattern decreasing to the invasion front and becoming one predominantly cytoplasmic, for N-cadherin the immunomarkage was predominantly cytoplasmic and it was present especially at the periphery of the proliferative islands of the invasion front, especially in weakly differentiated forms of the investigated squamous carcinoma cells of the tongue.

V.2.5 INVESTIGATION RESULT: CXCR4+ MMP13

The reactivity for CXCR4 was a cytoplasmic one, being present homogenously throughout the thickness of the tumour specimens, without variations between the superficial and deep parts of the tumours. Strictly at the level of the proliferative carcinoma islands the reactivity was more obvious inside the islands and more precisely at the level of the dyskeratotic cells bordering or not the keratin pearls (Fig. 5.36). We have noticed reactivity at the level of the
inflammatory infiltration, of the vascular endothelial cells and of the skeletal muscle fibres adjoining the tumour process.

Fig. 5.36 Squamous cell carcinoma of the tongue – moderately differentiated form, cytoplasmic expression of CXCR4 at the level of the the dyskeratotic cells in the proliferative tumoral areas. Col IHC CXCR4 (brown)/ MMP13 (red), x100.

Regarding the immunoreactivity for MMP13 we have noticed a cytoplasmic pattern of reaction, mainly present in the depth of tumour specimens, more precisely at the level of the invasion front. Strictly at the level of the proliferative islands the pattern was a homogeneous one, being present both at the level of the dyskeratotic cells in the proliferative areas as well as at the level of the cells in their periphery. The co-localizations of the two markers were more obvious in the depth of tumours, especially at the invasion front. At the level of the proliferative areas of the invasion front the reactivity to MMP-13 has been prevalent in the peripheral cells, while the reactivity to CXCR4 was predominant in the cells in the centre of these proliferations.
V.4 CONCLUSIONS OF THE IMMUNOHISTOCHEMICAL STUDY OF THE EPITHELIAL-MESENCHYMAL TRANSITION PROCESS IN THE SQUAMOUS CELL CARCINOMA OF THE TONGUE

The E-cadherin immunomark, even though it was present in all the cases, it had variations from one case to another. Thus, the membranary pattern decreased with the decreasing of the differentiation degree and with the increasing of the pattern of invasion degree, while the cytoplasmic pattern was more obvious in the weakly differentiated tumour forms, in the cases with high invasion pattern (type 4), being predominant at the periphery of the proliferation carcinoma and in the tumour invasion front.

The reactivity to vimentin was present in 73.3% of the investigated cases, but in the vast majority the semiquantitative scores were low, the number of immunoreactive cells was generally below 10%. In addition, we have noted their correlation with the degree of differentiation, the clinical stage and the pattern of invasion, the immunoreactivity was more obvious in the weakly differentiated tumours, in those with high-grade of invasion pattern and in the advanced stages of the disease.

The investigation of the coexpression E-cadherin / vimentin revealed at the invasion front of the investigated squamous carcinoma cells of the tongue, a decrease in reactivity to E-cadherin along with an increase of reactivity to vimentin in the same time with the decrease of the degree of differentiation, with the growth of the invasion pattern and with the growth of the disease stage.

The reactivity to Twist1 was present in all the investigated cases at both parenchymal and stromal levels. This marker’s immunoreactivity correlated only with the disease stage, the highest scores being recorded in the most advanced stages of disease.

The investigation of the coexpression Twist / E-cadherin revealed the existence of a direct reverse correlation, the expression E-cadherin decreasing with the overexpression Twist1 overexpression, and meantime with the decrease of the degree of differentiation and with the growth of the disease stage.
N-cadherin was positive in a few cases (33.33%), the immunoreactivity was more obvious in the weakly differentiated forms, in the tumours with invasion pattern of grade 3 and in the advanced stages III-IV of disease, but without significant correlations of the expression of this marker with these parameters.

The coexpression N-cadherin / E-cadherin was rarely shown and only in some of the proliferative islands in the invasion front, especially in the weakly differentiated forms of the investigated squamous carcinoma cells of the tongue, E-cadherin being expressed membranary and N-cadherin cytoplasmically.

Immunoreactivity to CXCR4 was found in 86.66% of the studied cases, the percentage of immunoreactive cells ranging between 10-75%. We have noticed the highest reactivity inside the proliferative areas, particularly at the level of the dyskeratotic cells and especially among patients in advanced stages of disease (III / IV).

The rate of the immunooexpression MMP-13 in the cases studied was of 66.66%, the highest scores being recorded in the tumour invasion front, in the advanced stages of disease III / IV and in the tumours with high grade invasion pattern (3 / 4).

The coexpression CXCR4/MMP-13 was more obvious evident in the depth of the tumour specimens investigated, more precisely, the maximum of colocalisation being recorded in their front of tumoral invasion.
The epidemiological study of the investigated cases has helped to shape the next epidemiologic profile of the patients with lingual carcinoma: mostly male (sex ratio being 1.82), aged 60-79 years (57%) and affecting mainly the language edges (35.44%).

The histopathological study revealed the prevalence of non-keratinizing forms of squamous cell carcinoma of the tongue (50.63%) and with a moderate histological degree of differentiation (50.63%).

The histopathological forms such as acantolitic (6.33%), sarcomatoid (2.53%), papillary (2.53%) and bazaloid (1.27%) were rare and belonged in terms of the degree of differentiation to the weakly differentiated forms. These were correlated with the clinical stage; the patients with these forms of squamous carcinoma of the tongue being detected in advanced stages of disease (stages III and IV).

The vast majority of the cases were detected in the clinical stage I (46.84%) and III (26.58%), which correlates with vascular invasion, the lymphatic dissemination status and the histologic risk of malignancy. In our study the advanced stages corresponded to the weakly differentiated tumours with vascular invasion, lymphatic metastasis presence and with high histologic risk of malignancy.

The involvement of the epithelial-mesenchymal transition process in the progression of the squamous cell carcinoma of the tongue was demonstrated by the evidence in our study, particularly at the invasion front, of the following morphological aspects: (i) fusiform morphology of neoplastic cells; (ii) type 4 invasion pattern with discohesive neoplastic cells infiltrating the adjacent tissues; (iii) the presence of a tumoral lymphoplasmacytic infiltration and (iii) a desmoplastic reaction in the vicinity of the invasive tumour proliferation.

The investigation of immunoreactivity for E-cadherin and respectively for vimentin proves the existence of an epithelial-mesenchymal transition process in
squamous cell carcinoma of the tongue, especially at the invasion front. Thus, at this level it was showed a decrease in the reactivity to E-cadherin at the same time with an increase in reactivity to vimentin with the decrease of the degree of differentination, with the increase of the pattern of invasion and with the growth of the disease stage.

- Meantime the investigation of the coexpression Twist / E-cadherin revealed the existence of a reverse direct correlation, the E-cadherin expression decreasing at the same time with the overexpression Twist1 with the decrease of the degree of differentiation and with the increasing stage of disease.

- On the other hand the presence of the cadherin "switch" (E-cadherin- N-cadherin) was reported in a few cases (33.33%) and especially in the weakly differentiated forms, in tumours with invasion pattern of grade 3 and in the advanced stages III-IV of disease. Moreover, the coexpression N-cadherin / E-cadherin was rarely shown and only in some of the proliferative islands in the invasion front, especially in the weakly differentiated forms of the investigated squamous carcinoma cell of the tongue, E-cadherin having a predominant membranary pattern, and N-cadherin a cytoplasmic one.

- The invasive potential of the investigated squamous carcinoma cells of the tongue was shown through the panel CXCR4 / MMP-13, the coexpression of the two markers was obvious especially in their invasion front, in the advanced stages III / IV and in the tumours with a high degree of invasion pattern (3/4).

- One of the general conclusions of the EMT immunohistochemical study of the investigated squamous cell carcinoma of the tongue is the prognostic utility of the immunohistochemical panels E-cadherin / vimentin and CXCR4 / MMP-13 that allow prognostic-therapeutic stratification of these patients, identifying the most aggressive forms of disease.

- On the other hand all these markers can become therapeutic targets, which could contribute to the improvement of the quality of life and of life expectancy of these patients.
SELECTIVE BIBLIOGRAPHY:


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