Study of CK18 and GDF5 immunoexpression in oral squamous cell carcinoma and their prognostic value

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Abstract
Oral cavity cancer remains one of the most common cancers worldwide, with an increased incidence in young adults, although there has been lately a decrease tendency in the incidence of this form of cancer. Lingual localization has a very high mortality and tends to be more aggressive becoming frequently metastatic at the regional lymph nodes. The purpose of this study is to investigate the immunohistochemical expression of cytokeratin 18 (CK18) and the reactivity to GDF5 (CDMP-1), called the morphogenetic protein-1, cartilage-derived, in lingual squamous cell carcinoma and the correlation between the immunoreactivity of this panel of antibodies, and the clinical stage, the degree of differentiation and the invasion pattern. In this regard, we studied the immunohistochemical behavior of these markers in 15 cases of lingual squamous cell carcinoma. In our study, we observed the correlation of CK18 and GDF5 expression with the clinical stage, differentiation degree and invasion pattern, the highest levels of immunoreactivity being recorded in poorly differentiated forms, in high-level invasion patterns and in the most advanced stages. The markers used can become therapeutic targets, which could help increase the quality of life and life expectancy for these patients.

Keywords: lingual squamous cell carcinoma, prognostic value, immunoexpression, invasive front.

Introduction
Oral cavity and oropharyngeal cancers are among the most common cancers worldwide, although there has been lately a decrease tendency in the incidence of oral cavity cancers in most parts of the world, most likely due to the reduction of tobacco consumption [1]. In Romania, according to the data provided by the International Agency for Research on Cancer (IARC) from 2012, there was an incidence of lip and oral cavity cancer in 1518 new cases per year (3.5 cases per 100 000 inhabitants) and a mortality rate of 878 new cases every year (3% cases per 100 000 inhabitants), this form of cancer occupying the 8th position in incidence of all cancers at various sites. Moreover, over the past 20 years, the epidemiological studies have shown a steady increase in the incidence of these cancers in young adults aged between 18 and 45 years [2, 3].

Lingual localization represents one of the sites with a very high mortality rate of oral squamous cell carcinoma. In addition, the tumors developed on the mobile part of the tongue tend to be more aggressive, becoming frequently metastatic at the regional lymph nodes. Therefore, there is a need for comprehensive studies, which would better assess the prognosis of these tumors. The purpose of this study is to investigate the immunohistochemical expression of cytokeratin 18 (CK18) and the reactivity to GDF5 (CDMP-1), called the morphogenetic protein-1, cartilage-derived, in lingual squamous cell carcinoma and the correlation between the immunoreactivity of this panel of antibodies and the clinical stage, differentiation degree and invasion pattern.

Materials and Methods
The studied material consisted of 15 cases of lingual squamous cell carcinoma from the casuistic of the Laboratory of Pathological Anatomy within the Emergency County Hospital of Craiova, Romania. Of the 15 cases, eight were moderately differentiated, four were well differentiated and three cases were poorly differentiated.

The 4-μm sections were applied to slides treated with poly-L-lysine, deparaffined with benzene and hydrated by passing through four alcohol baths with decreasing concentrations. The immunohistochemical (IHC) study used as working method the LSAB (Labeled Streptavidin–Biotin2 System) technique. The kit used was manufactured by Dako, Redox, Romania (code K0675). The result of these immunohistochemical reactions was to visualize the antigens investigated using the 3,3’-diaminobenzidine (DAB) chromogen, by coloring them in brown.

In the immunohistochemical study of the 15 cases of lingual squamous cell carcinoma, we used concentrated anti-human directed antibodies developed in mice or rabbits, whose main characteristics are shown below (Table 1).
For each antibody used, it was performed a successive positive external control–negative external control, using the same LSAB technique. The external positive control was performed on normal tissues containing the investigated target antigen (positive sections). They were processed under the same conditions as the investigated tumor. This test was first performed to verify the effectiveness of the reagents and the accuracy of the technique used in the process.

The markers expression assessment was qualitatively performed, the reactions intensity being measured according to the score: 0 – negative, 1 – weakly positive, 2 – moderately positive, 3 – strongly positive. The images were captured using the Nikon Eclipse 55i microscope (Nikon, Apidrag, Bucharest, Romania), equipped with a video camera with 5-megapixel cooling, while the processing and the interpretation were performed with the imaging software AMS7 Image ProPlus (Media Cybernetics Inc., Buckinghamshire, UK).

The semiquantitative analysis assessed the number of positive cells at a magnification ×400 on five random fields. The results were grouped as it follows: 0 – absence of reactivity, +1 (weak) – less than 10% of positivity in tumor cells, +2 (moderate) – homogeneous or intense in 10–75% of tumor cells and +3 (intense) – intense homogeneous in more than 75% of tumor cells.

Table 1 – Antibodies used in the study of lingual squamous carcinomas

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Antigen unmasking</th>
<th>Dilution</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin 18 (CK18)</td>
<td>Mouse monoclonal, DC 10</td>
<td>Proteinase K</td>
<td>1:30</td>
<td>Gastric mucosa</td>
</tr>
<tr>
<td>GDF5</td>
<td>Rabbit polyclonal</td>
<td>Citrate, pH 6</td>
<td>1:100</td>
<td>Gastric mucosa</td>
</tr>
</tbody>
</table>

Results

CK18 reactivity

The reactivity was detected in 10 of the 15 investigated cases. The maximum semiquantitative score (score 3) was recorded in only three cases. The reaction pattern was a cytoplasmic one and the reaction was more intense in the cells at the carcinoma proliferation periphery, particularly in the invasive front (Figure 1, A and B). We did not notice any CK18 immunoreactivity in any of the well-differentiated tumors. It was present in seven of the eight moderately differentiated cases and in all three poorly differentiated cases of lingual squamous cell carcinoma.

The correlations of the semiquantitative score of CK18 immunoreactivity with the morphoclinical main parameters investigated are presented in Table 2. Thus, the CK18 immunoreactivity was correlated with the clinical stage, the degree of differentiation and the invasion pattern. The more advanced was the clinical stage the greater the number of immunoreactive cells. Meanwhile, high scores of CK18 reactivity were obtained in poorly differentiated forms compared to the well-differentiated ones and in the invasion patterns of higher degree compared to those of lower degree (the significant difference being between the 1st and 4th degrees).

Table 2 – Case distribution according to the number of cells with CK18 marking reported in major morphological parameters (clinical stage, differentiation degree and invasion pattern)

<table>
<thead>
<tr>
<th>Morphological parameter</th>
<th>Subcategory</th>
<th>CK18 reactivity (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical stage</td>
<td>I (n=5)</td>
<td>9.43±13.35</td>
</tr>
<tr>
<td></td>
<td>II (n=3)</td>
<td>17.75±25.13</td>
</tr>
<tr>
<td></td>
<td>III (n=4)</td>
<td>21.34±19.57</td>
</tr>
<tr>
<td></td>
<td>IV (n=3)</td>
<td>31.56±24.63</td>
</tr>
<tr>
<td>Differentiation degree</td>
<td>Well differentiated (n=4)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated (n=8)</td>
<td>11.53±15.46</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated (n=3)</td>
<td>34.73±25.73</td>
</tr>
<tr>
<td>Invasion pattern</td>
<td>Grade 1 (n=3)</td>
<td>3.51±9.73</td>
</tr>
<tr>
<td></td>
<td>Grade 2 (n=4)</td>
<td>15.38±21.33</td>
</tr>
<tr>
<td></td>
<td>Grade 3 (n=5)</td>
<td>22.36±19.65</td>
</tr>
<tr>
<td></td>
<td>Grade 4 (n=3)</td>
<td>31.67±27.43</td>
</tr>
</tbody>
</table>

GDF5 reactivity

The reactivity was a granular cytoplasmic one, with the highest intensity in the acantholysis areas, in the oval and fusiform morphology cells (Figure 2A). Overall, the reactivity of the carcinoma islets was greater to the oral epithelium adjacent, while topographically, the tumor reactivity appears to be higher in the deep areas of the tumor, at the invasive front compared to the superficial tumor areas (Figure 2, B and C). In the well-differentiated forms, reactivity prevailed in the dyskeratotic cells adjacent to keratin pearls inside the proliferation carcinoma (Figure 2D). In poorly differentiated forms, the pattern was a consistent one in the islets or predominantly at the periphery (Figure 2E). We also noticed a GDF5 reactivity in the stroma, where some inflammatory cells and the endothelial cells of blood vessels became positive (Figure 2F).

In addition, we noticed reactivity in the skeletal muscle fibers and in the adipocytes.

In Table 3 there is presented the correlations of the semiquantitative score of GDF5 immunoreactivity with the morphoclinical main investigated parameters.

Table 3 – Case distribution according to the number of tumor cells with GDF5 reactivity reported to the main morphological parameters (TNM stage, tumor differentiation degree and type of invasion pattern)

<table>
<thead>
<tr>
<th>Morphological parameter</th>
<th>Subcategories</th>
<th>Tumor parenchyma Reactivity to GDF5 (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical stage</td>
<td>I (n=5)</td>
<td>31.56±24.63</td>
</tr>
<tr>
<td></td>
<td>II (n=3)</td>
<td>43.75±35.13</td>
</tr>
<tr>
<td></td>
<td>III (n=4)</td>
<td>61.34±31.57</td>
</tr>
<tr>
<td></td>
<td>IV (n=3)</td>
<td>64.36±43.63</td>
</tr>
<tr>
<td>Differentiation degree</td>
<td>Well differentiated (n=4)</td>
<td>43.57±27.63</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated (n=8)</td>
<td>37.53±29.46</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated (n=3)</td>
<td>62.13±55.23</td>
</tr>
<tr>
<td>Type of invasion pattern</td>
<td>Degree 1 (n=3)</td>
<td>23.51±11.73</td>
</tr>
<tr>
<td></td>
<td>Degree 2 (n=4)</td>
<td>35.38±31.33</td>
</tr>
<tr>
<td></td>
<td>Degree 3 (n=5)</td>
<td>51.36±49.35</td>
</tr>
<tr>
<td></td>
<td>Degree 4 (n=3)</td>
<td>61.67±57.73</td>
</tr>
</tbody>
</table>
Figure 1 – (A) Lingual squamous cell carcinoma – moderately differentiated form, cytoplasmic expression of CK18 in the carcinoma cells in the invasive front (invasion in the minor salivary glands with increased reactivity to CK18) [IHC staining for CK18 (brown)/GDF5 (red), ×40]. (B) Lingual squamous cell carcinoma – moderately differentiated form, cytoplasmic expression of CK18 in the cells of the carcinoma proliferations periphery, in the invasive front [IHC staining for CK18 (brown)/GDF5 (red), ×100].

Figure 2 – (A) Lingual squamous cell carcinoma – moderately differentiated form, acantholytic area, cytoplasmic expression of GDF5 in the carcinoma cells with oval and fusiform morphology [IHC staining for CK18 (brown)/GDF5 (red), ×100]. (B) Lingual squamous cell carcinoma – moderately differentiated form, cytoplasmic expression of GDF5 in the tumoral dysplastic epithelium cells suprajacent and a weakly reactivity from the superficial carcinoma cells [IHC staining for CK18 (brown)/GDF5 (red), ×40]. (C) Lingual squamous cell carcinoma – moderately differentiated form, cytoplasmic expression of GDF5 in the carcinoma cells in the invasive front [IHC staining for CK18 (brown)/GDF5 (red), ×40]. (D) Lingual squamous cell carcinoma – well-differentiated form, cytoplasmic expression of a different intensity to GDF5 in the dyskeratosis carcinoma cells adjacent to keratin pearls [IHC staining for CK18 (brown)/GDF5 (red), ×100].
Figure 2 (continued) – (E) Lingual squamous cell carcinoma – poorly differentiated form, cytoplasmic homogenous expression of GDF5 in the carcinoma cells in the invasive front [IHC staining for CK18 (brown)/GDF5 (red), ×100]. (F) Lingual squamous cell carcinoma – poorly differentiated form, cytoplasmic expression of GDF5 in the inflammatory cells and in the vascular endothelium cells in the invasive front [IHC staining for CK18 (brown)/GDF5 (red), ×100].

The semiquantitative investigation of tumor immunoreactivity highlights the prevalence of score 2 (66.66%). In terms of correlations with the morphological parameters investigated, we noticed significant differences between stage I and stages III/IV, the reactivity being higher in the more advanced stages. Of the degrees of differentiation, we recorded the highest reactivity in poorly differentiated forms, and related to the pattern of invasion, the immunoreactivity was marked in the 3rd and 4th degree invasion patterns.

Regarding this protein co-localization with CK18, we observed their co-expression, especially in the invasion front, for the poorly differentiated forms of lingual carcinoma and in highly invasive degree patterns.

Discussion

CK18 reactivity

CK18 belongs to the 1st group of the cytokeratin acids (CK9–CK20) and it is mainly present in the simple epithelia, with a cytoplasmic and perinuclear subcellular localization [4, 5]. It is co-expressed with CK8 in a number of adult epithelial tissues: liver, lung, kidney, pancreas, gastrointestinal tract and in the mammary glands, being also expressed in the cancers developed in these tissues [6]. CK18 participates in the cell cytoskeleton build-up, thus providing cell resistance to external aggressions [7, 8] and maintaining in normal parameters the mitochondrial structures [9], the cell apoptosis [10, 11], the mitosis [12], the cell cycle progression [13] and cell signaling [14].

Pathologically, CK18 is involved in epithelial cell motility and in cancer progression [15], its expression being considered a prognostic factor in patients with such cancers [16, 17]. In oral squamous cell carcinoma, the levels of mRNA expression and of the protein CK18 expression were significantly raised together with the stage and grade of the tumors [16]. In addition, CK18 expression was used to differentiate the origin of the various primary squamous cell carcinomas of head and neck, this marker being more frequently expressed in the squamous cell carcinoma of the larynx and hypopharynx, in comparison to the oral squamous cell carcinoma [18].

Regarding oral cavity cancers, literature shows an aberrant CK18 expression in the localizations in oral mucosa and the tongue [19, 20]. Later, it was shown that the aberrant CK8/18 expression and the lack of CK10 expression represented a common feature of the oral mucosa and tongue during fetal development, which is why it was speculated that the expression of such a phenotype, also present in the correspondent squamous cell carcinomas, could emphasize the ability of these tumors to repeat the existing phenotype during fetal development [21]. It was recorded an increase of the CK8/18 expression with the stage and degree of the tumors, representing an independent prognostic factor that may indicate a decline in the overall survival of such patients [16, 22]. The percentage of oral squamous cell carcinoma cases, which express aberrant CK18 seems to be quite high, at least 50% of them expressing such a marker [23].

Our study revealed the presence of an aberrant CK18 expression in 66.66% of the investigated cases, with an immunoreactivity value that varied significantly in almost equal percentages between scores 1–3. We also noticed the correlation of this cytokeratin expression with the clinical stage, the degree of differentiation and the pattern of invasion, the highest levels of its immunoreactivity being recorded in the poorly differentiated forms, in the high degrees of invasion patterns and in the most advanced stages.

GDF5 reactivity

GDF5 (CDMP-1), also known as the morphogenetic cartilage-derived protein 1, is a member of the bone morphogenetic proteins (BMPs) family and of the transforming growth factor beta (TGF-β) superfamily, which is expressed in the central nervous system development [24] and has a role in the skeletal and joints development [25, 26].

Studies on this protein expression in human cancers are relatively few and those concerning the squamous cell
carcinomas do not exist in the literature. Some authors reported the CDMP-1 expression in the grooved and interleafing ducts of the normal salivary gland, suggesting the involvement of this marker in morphogenesis by branching the salivary ductal system rather than in preserving the myoepithelial cells [27]. However, the same authors observed the CDMP-1 expression in myoepithelial cancer cells with cuboidal morphology, in the hypocellular areas that seem to have a pre-chondroprogenitor morphological phenotype [28]. It was suggested that CDMP-1 would play a key role in accelerating the process of trans-differentiation from the myoepithelial cuboid cancer cells into chondroid incomplete cells in an autocrine manner. The authors suggested the involvement of such a protein in the EMT and transdifferentiation processes, which underline this tumor tumorigenesis [29].

Data from the literature also indicate a weak reactivity to GDF5 from lung carcinomas without small cells, which, along with the other members of the bone morphogenetic proteins family, contribute to the progression and growth of these tumors [30]. It has been shown that TGF-β produced by breast cancer cells induces the GDF5 expression in the endothelial cells, which in its turn stimulates the angiogenesis both in vivo and in vitro [31].

In our study, we recorded a GDF5 reactivity in all the investigated cases, with reactivity differences from one case to another, but in the vast majority (66.66%) it was recorded a semi-quantitative score 2 (the number of immunoreactive cells ranging between 10–75%). This reactivity was more highlighted in the advanced clinical stages, in the invasive front, in the poorly differentiated forms and in the higher degree invasion patterns.

Regarding the collocalization of this protein with CK18, we observed their co-expression, particularly in the invasive front, especially for the poorly differentiated lingual squamous cell carcinoma forms and for the cases with a higher degree of the invasive pattern.

### Conclusions

CK18 immunoreactivity was noticed in 66.66% of the investigated cases, their scores being correlated with the clinical stage, the grade of differentiation and the invasion pattern. The highest levels of immunoreactivity were recorded in poorly differentiated forms, in higher degree of the invasion patterns and in the most advanced stages. GDF5 was expressed in all the investigated cases and in general at quite high level, the highest scores of immunoreactivity occurring in the advanced clinical stages, in the invasive front, in the poorly differentiated forms and in those with a high degree of invasion pattern. The CK18/GDF5 co-expression was mainly present in the invasive front, especially in the poorly differentiated forms of lingual squamous cell carcinoma and also in the cases of higher degree invasion patterns. The investigated markers may represent therapeutic targets, which could help increase the quality of life and life expectancy for these patients.

### Conflict of interests

The authors declare that they have no conflict of interests.

### References


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