

Involvement of Markers D2-40, Cd10 and F-Actin in Squamous Cell Carcinoma Invasion of the Tongue

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In recent decades, an increase in the incidence of lingual squamous carcinoma, especially among young people, has been seen to be the most common form of oral cancer. In addition, this localization appears to be one of the most aggressive forms of oral squamous carcinoma, being highly locally invasive, with early metastasis, with a high rate of relapse and with a survival rate of 5 years being below 60%. To explain this phenotype, recent studies have shed light on the molecular mechanisms underlying lingual carcinogenesis, including loco-regional invasiveness with the study of markers involved in this process. From this perspective, our study proposed the investigation of the reactivity of markers D2-40, CD10 and F-actin in 57 squamous carcinomas of the tongue diagnosed in various stages, and we selected cases from the Archives of the Pathological Anatomy Laboratory of the County Emergency Hospital of Craiova (SCJUCraiova) during the years 2015-2017. The immunohistochemical technique used was that of detecting the LSAB enzyme, and the evaluation of tumor immunoreactivity was done using the semi-quantitative score described by Remmele and Stegner. Tumor reactivity was present mainly in moderately differentiated forms, usually periferic of the proliferation, in the invasion front, more intense in the acantholytic variant. On the opposite side there were cases of well differentiated squamous carcinoma, which obtained the lowest immunoreactivity scores. Reactivity was also present at the stromal level, especially in cases involving a desmoplastic reaction and inflammatory stroma cases, respectively. The pattern of predominant reactions at the periphery of the proliferation and the interface with the inflammatory or desmoplastic stroma proves the adoption by these neoplastic cells of a more aggressive phenotype with their successive involvement in local invasion and metastasis. Therefore, the markers used have prognostic value identifying the most aggressive forms of lingual squamous carcinoma thus allowing a therapeutic stratification of these patients who will benefit from a molecular targeting therapy.

Keywords: tongue, squamous cell carcinoma, invasiveness, CD10, D2-40, F-actin

Worldwide, the annual number of lingual squamous carcinomas appears to exceed 50% of all cases of oral cancer [Scully C & Bagan J, 2009]. At the same time, over the past decades, there has been a tendency towards an increase of the incidence of these cases compared to the rest of oral cancer sites [Chaturvedi AK et al., 2008; Harris SL et al., 2010; Tota JE et al., 2017], in the United States between 1975-2007, with a higher incidence among young people [Patel SC et al., 2011]. Over time, numerous studies have shown that this location of oral cancers appears to be one of the most aggressive forms of oral cancer, making it difficult to diagnose in the early stages, while the rate of a positive prognosis for this type of cancer does not appear to exceed 55-65% [Ghantous Y et al., 2015]. Unlike other oral cancers, squamous carcinomas of the tongue are noticeable much more invasive, have relatively early metastasis to the lymphatics/ganglions, and have a poor prognosis (Sano D & Myers JN, 2007; Zhou Y et al., 2015). Such an aggressive phenotype also has consequences on the quality of life of these patients, and those who survive usually have difficulty in speaking and dysfunction of mastication and swallowing [Shu HY].

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Despite advances in chemotherapy, radiotherapy and surgical treatment of these lingual cancers, the rate of relapse and death rates among these patients remain high [Chen Y et al., 2018; Shakeel Uz Zaman et al., 2016], their survival rate not exceeding 50% [Chauhan SS et al., 2015]. All these points highlight the need for more in-depth studies on lingual carcinogenesis, with the emergence of new biomarkers with both prognostic and therapeutic values that allow both longer survival and improved quality of life. In this respect, our study also aims to investigate the prognostic impact of immunohistochemical expression of markers D2-40, CD10 and F-actin on local invasiveness of 57 cases of lingual squamous carcinoma.

Experimental part

Material and method

Our study included 57 cases of squamous cell carcinoma of the tongue diagnosed and operated between 2015-2017, in the OMF Surgery Department (Oro-Maxillofacial) and the ORL Clinic (Oto-Rhino-Laryngology) of the County Emergency Clinical Hospital (SCJU) Craiova. The histopathological study was made on the basis of diagnostic slides found in the Pathological Anatomy Laboratory archive of the same hospital, diagnosed with the diagnostic criteria established by the World Health Organization (Johnson et al., 2005). For the immunohistochemical study, the corresponding histopathological samples were used, from which four-micron sections were obtained, glued to electrostatically charged microscopical slides. Subsequently, they were processed according to the LSAB (Labelled Streptavidin-Biotin2 System) enzyme detection protocol using the Dako immunohistochemical kit (Redox, Romania - K0675) and the primary antibodies of interest, the characteristics of which are reproduced in the table below (Table 1).

Tabel 1
ANTIBODIES USED IN THE STUDY AND THEIR PRINCIPLE CHARACTERISTICS

Antibody	Clone/Producer	Dilution	Antigenic exposure	External positive control
Podoplanină (D2-40)	Mouse, Monoclonal D2-40, Dako	1:100	0.1 M citrate, pH 6	Lymph nodes
CD10	Mouse, Monoclonal, 56C6, Dako Cytomation	1:50	0.1 M citrate, pH 6	Lymph nodes
F Actină	Mouse, Monoclonal, LifeSpan Biosciences	1:50	0.1 M citrate, pH 6	Prostate

Detection of antigenic targets was done using the chromogen; 3,3 diaminobenzidine (DAB, from Redox, Romania-DAKO, K3468), and the Mayer hematoxylin kit (Tunic, Romania-Bio-Optica, M06002) was used for the nuclear counter-staining. Immunohistochemical reactions were validated using positive external controls (Table 1) and negative by omitting the primary antibody.

Quantification of reactions was done using the immune-histochemical semi-quantitative score (IRS) described by W & Stegner HE Remelements, obtained by multiplying the percentage of tumor cells marked with the intensity of the reactions (Remele W & Stegner HE, 1987). We are only interested in the reactivity of tumor parenchyma and not the stromal tumor, which was only assessed qualitatively, specifying the presence of the signals and their location. Interpretation of reactions was done with the Nikon Eclipse 55i microscope, equipped with a 5-megapixel-cooled video camera and Image-Pro Plus imaging software. For statistical analysis, the SPSS 10 software and Student, Anova, square and Pearson tests were used. Contingency tables were performed with the variables of interest (age, degree of differentiation, pTNM stage and IRS scores obtained for the three markers used), then using the quadratic test for testing possible statistically significant associations if $P < 0.05$. Possible multivariable associations were tested using the ANOVA assay.

The main variables of clinico-morphologic interest of our study are shown schematically in the table below (Table 2).

Table 2
CAUSALITY DISTRIBUTION ACCORDING TO THEIR MAIN CLINICAL-MORPHOLOGICAL VARIABLES

Anatomo-clinical variables	Number of cases	(%)
Age		
<60	23	40.35
>60	34	59.65
Sex		
F	24	42.11
M	33	57.89
Topography		
Mobile portion (including edges)	38	66.67
Fixed portion	19	33.33
Degree of differentiation		
Well differentiated	18	31.58

Moderately differentiated	27	47.37
Slightly Differentiated	12	21.05
pTNM		
<i>I</i>	7	11.7
<i>II</i>	19	33.33
<i>III</i>	22	38.6
<i>IV</i>	9	16.37

Results and discussions

The clinical and morphological study highlighted an increased incidence of cases of lingual carcinomas in male subjects (57.59%), over 60 years of age (59.65%), tumors predominantly at the level of the mobile portion of the tongue (66.6 %). Histopathologically there was prevalence of the moderately differentiated cases, with almost half of the investigated cases (47.37%), and the staging of pTNM indicated stage III (38.06%) and stage II (33.33%) respectively as the most commonly diagnosed cases.

The results of the immunohistochemical study performed are shown schematically in the table below (Table 3).

Table 3
DISTRIBUTION OF MEAN IMMUNOREACTIVITY SCORES (IRS) ACCORDING
TO THEIR MAIN CLINICAL-MORPHOLOGICAL VARIABLES

Clinical and morphological variables	Average IRS scores (Average \pm STDEV*)		
	D2-40	CD10	F-actină
Age			
<60	2.83 \pm 2.38	1.33 \pm 1.40	4.79 \pm 2.88
>60	4.51 \pm 3.18	2.36 \pm 1.99	6.39 \pm 3.10
Sex			
F	4.41 \pm 3.18	2.16 \pm 1.88	6 \pm 2.99
M	3.36 \pm 2.77	1.75 \pm 1.80	5.51 \pm 3.19
Topography			
Mobile portion (including edges)	3.86 \pm 2.82	1.92 \pm 1.80	5.60 \pm 2.87
Fixed portion	3.68 \pm 3.33	1.94 \pm 1.92	5.94 \pm 3.56
Degree of differentiation			
Well differentiated	0.73 \pm 0.72	0.31 \pm 0.47	2.63 \pm 1.01
Moderately differentiated	5.96 \pm 2.44	3.34 \pm 1.64	7.69 \pm 2.70
Slightly Differentiated	4 \pm 1.80	1.41 \pm 0.99	6.33 \pm 2.05
pTNM			
<i>I</i>	0.57 \pm 0.97	0.28 \pm 0.48	2.14 \pm 1.06
<i>II</i>	3 \pm 2	1.26 \pm 0.93	1.18 \pm 1.98
<i>III</i>	4.40 \pm 2.75	2.27 \pm 1.77	6.54 \pm 2.72
<i>IV</i>	6.55 \pm 3.46	3.77 \pm 2.33	8.88 \pm 3.44

IRS = immunoreactivity score; * STDEV = standard deviation

1. Results of the immunohistochemical study with anti-D2-40 antibody

For the lingual epithelium at a distance from the neoplastic lesions, we did not report reactivity to this marker, but it was present in the epithelium adjacent to the lesions, being limited to the basal and parabasal layer with subcellular membrane and cytoplasmic localization (Figure 1A). Intensity of IRS scores and scores increased from leucoplasic to dysplastic lesions, with the highest values found in areas with aggravated dysplasia, the reactivity being both membranous and cytoplasmic, and the more superficially extensive the severity of dysplasia was bigger. Reactivity for D2-40 was also noted in the endothelial cells of the lymphatic vessels, basal cells and myoepithelial cells of the minor salivary gland component of the lingual corion (Figure 1B) and in the follicular dendritic cells of lymphoid follicles in inflammatory infiltrates associated with tumor lesions and their lymph nodes satellites (Figure 1C).

At the level of tumor specimens, the highest reactivity was recorded in cases of moderately differentiated squamous carcinoma (IRS = 5.96 \pm 2.44), the subcellular pattern of reactions being predominantly membranous and to a lesser extent cytoplasmic. In addition, in such cases the reactivity was recorded especially at the invasion front, the reactions being diffused at the level of the cells in the neoplastic proliferation component at this level (Figure 1D). The acantholytic variant showed a somewhat distinct reactivity, the reactions being more intense in the tumor areas compared to their periphery, the maximum reactivity being recorded in the cytoplasm of acantholytic neoplastic cells (Figure 1E). The weakest reactivity was recorded in cases of well differentiated squamous carcinoma (IRS = 0.73 \pm 0.72), this being exclusively present at the periphery of the proliferation cells, respectively at the stroma interface, the subcellular pattern of the reactions being predominately membranous (Fig. 1F). In the poorly differentiated variant of squamous carcinoma, the reactivity was closer to the moderately differentiated form (IRS = 4 \pm 1.80), with a diffuse and predominant cytoplasmic pattern (Figure 1G). The tumor stroma also exhibited reactivity for D2-40 in the endothelial cells of the lymphatic vessels as well as in the stromal fibroblasts / stromal myofibroblasts, the reactivity at this latter level being evident especially in the cases associated with dysplastic reaction (Fig. 1H). Reactivity was also noted in the metastasis,

the reactions being present at the periphery of the proliferations, with membranous and cytoplasmic pattern. The highest immunoreactivity scores were recorded especially in cases of lymph node metastases.

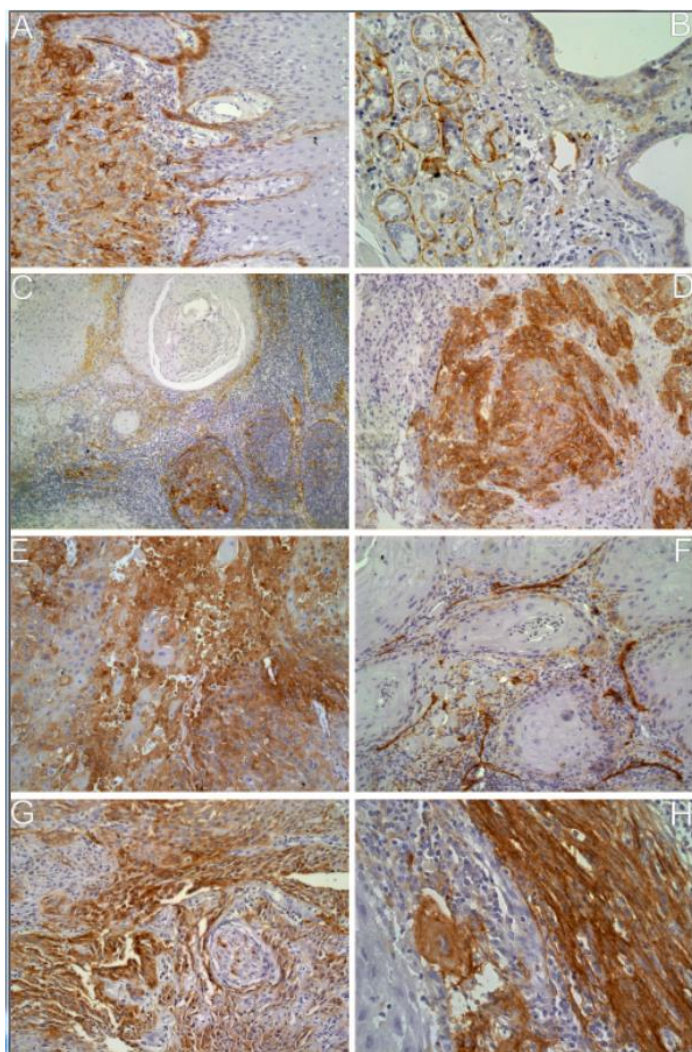


Fig.1 Lingual squamous cell carcinoma - A. Membrane and cytoplasmic reactivity of basal and parabasal layer cells for D2-40 from the lingual epithelium at a distance from the tumor. Col IHC- DAB, X200; B. D2-40 cytoplasmic reactivity of basal cells and myoepithelial cells of the minor salivary gland component of the lingual chorion, Col IHC-DAB, X400; C. Cytoplasmic reactivity to D2-40, in follicular dendritic cells, from the lymphoid follicles in lymphoganglions with well-differentiated squamous cell carcinoma metastasis. Col IHC- DAB, X100; D. Cytoplasmic reactivity of invasive frontal tumor cells at D2-40, in moderately differentiated squamous cell carcinoma, Col IHC-DAB, X200; E.

Intense cytoplasmic reactivity in acantolytic neoplastic cells, in the acantolytic form of lingual squamous cell carcinoma. Col IHC- DAB, X200; F. Lower reactivity with predominantly membrane pattern at the periphery of neoplastic proliferations from the well differentiated form of lingual foamy carcinoma. Col IHC- DAB, X200. G. Reactivity with diffuse and predominantly cytoplasmic pattern for D2-40 in the poorly differentiated form of lingual carcinoma, imaging from tumor invasion front, with perineural invasion. Col IHC- DAB, X200. H. Reactivity for D2-40 in stromal fibroblasts / myofibroblasts in cases of lingual squamous cell carcinoma with desmoplastic stroma Col IHC-DAB, X400.

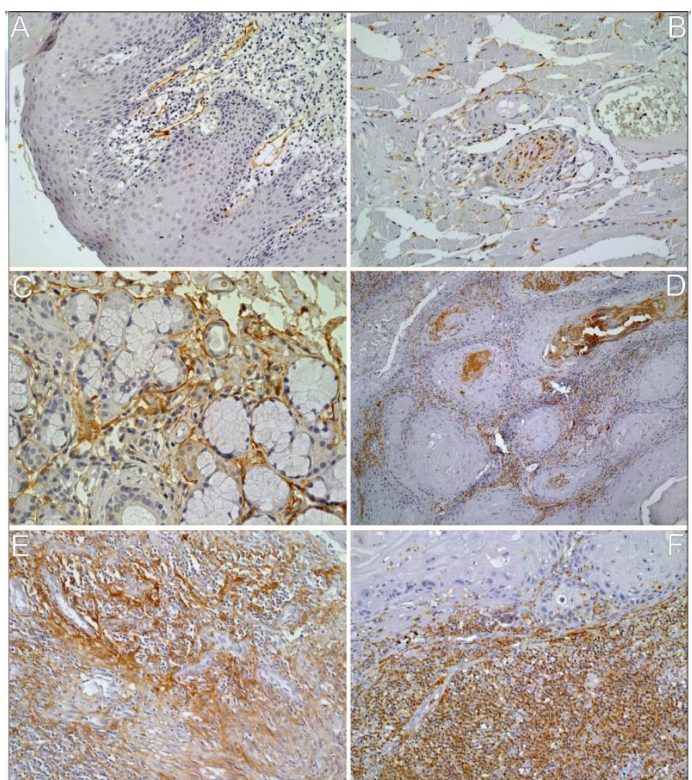


Fig. 2 Lingual squamous cell carcinoma - A. Absence of reactivity for CD10 from the lingual epithelium adjacent to the neoplastic lesions and reactivity of the vascular endothelium of the vessels in the underlying chorion, Col IHC-DAB, X200; B. Reactivity for CD10 around the threads of the peripheral nerves existing in the pieces of surgical exeresis, Col IHC-DAB, X200; C. Reactivity for CD10 in the myoepithelial cells from the minor salivary glands existing in the lingual chorion of the surgical specimens Col IHC-DAB, X400; D. Reactivity for CD10 limited to keratotic pearls from the well differentiated forms of lingual squamous cell carcinoma, Col IHC-DAB, X100; E. Reactivity of stromal fibroblasts for CD10, more evident in cases of lingual squamous cell carcinoma associated with desmoplastic reaction Col IHC-DAB reaction, X400; F. Moderate reactivity for CD10 in cases associated with an inflammatory stroma by positivity of existing lymphocytes, Col IHC-DAB, X200.

Fig. 3 Lingual squamous cell carcinoma - A. Intense reactivity for CD10 with diffuse pattern in the proliferation of moderately differentiated

2. The results of the immunohistochemical study with anti-CD10 antibody

In the normal or dysplastic lingual mucosa we did not report reactivity for CD10, this being present at the vessel endothelium level in the underlying corion (Fig. 2A). In addition, we have reported reactivity in the peripheral nerves (most likely in myelin sheaths around the nerv fascicles) (Figure 2B), at fibroblasts surrounding the striated muscle fibers, myoepithelial cells in the lower salivary glands (Figure 2C) and with a weaker response to lymphocytes and lymphocytic germination centres. I did not report reactivity in the hyperplastic and dysplastic areas of the lingual mucosa adjacent to cancerous lesions.

At the tumor specimen level, reactivity for CD10 was inferior to the D2-40 marker. In well-differentiated forms we noticed a weak reactivity ($IRS = 0.31 \pm 0.47$) present only in the keratosis pearls, the rest of the tumor cells not exhibiting reactivity for this marker (Figure 2D). A high reactivity was noted in the invasion front, namely in stromal fibroblasts, the reaction being even more evident in the cases associated with desmoplastic reaction (Figure 2E). At the same time a moderate reactivity for this marker has also been evidenced in cases associated with an inflammatory stroma due to the positive lymphocytes at this level (Figure 2F). The highest tumor reactivity was evidenced in moderately differentiated lingual squamous carcinoma ($IRS = 3.34 \pm 1.64$), the general pattern of the reaction being a diffuse one (Figure 3A), somewhat more intense at the periphery of the tumor proliferation and at the frontal invasion (Figure 3B). The subcellular pattern of reactions was predominantly membranous, but in some cases it was also cytoplasmic (Figure 3C). An intermediate reactivity to that of the well and moderately differentiated squamous carcinomas was recorded in the weakly differentiated forms ($IRS = 1.41 \pm 0.99$). The reaction pattern was diffuse, somewhat more intense towards the invasion front (Figure 3D), the reactivity being predominant cytoplasmic (Figure 3E). Regarding metastatic reactivity, the reactions were weak and somewhat more evident in the moderately differentiated proliferation and acantholytic zone (Figure 3F).

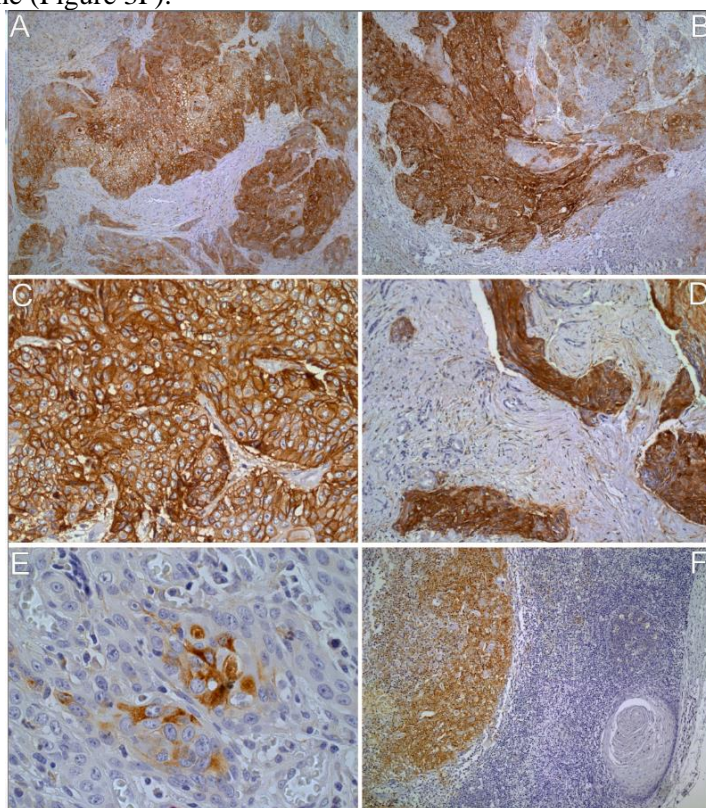


Fig. 3 Lingual squamous cell carcinoma - A. Intense reactivity for CD10 with diffuse pattern in the proliferation of moderately differentiated forms of lingual squamous carcinoma, Col IHC-DAB, X200; B. CD10 predominantly membranous reactivity of tumor cells from the invasion front of moderately differentiated squamous cell carcinomas, Col IHC-DAB, X100; C. The subcellular pattern of the reactions for CD10 was predominantly membranous, but at times it was also cytoplasmic. Col IHC-DAB, X400; D. An intermediate reactivity for CD10 in the poorly differentiated forms of poorly differentiated squamous cell carcinoma. Col IHC-DAB, X200; E. The predominantly cytoplasmic reactivity for CD10, slightly more intense towards the invasion front of the weakly differentiated lingual squamous carcinomas, Col IHC-DAB, X600; F. The lower reactivity for CD10 in the lymphoganglionic metastases, present especially in areas with moderately differentiated neoplastic proliferation, especially in the areas of tumor acantholysis, Col IHC-DAB, X100.

3. Results of the immunohistochemical study with the anti-F-actin antibody

Reactivity in the lingual epithelium adjacent to cancerous lesions was weak and present in the upper rows of the spinous layer, with a perinuclear cytoplasmic pattern in the lower ranks and one perimembranous in the upper ranks (Fig. 4A). In addition, a more intense reactivity for this marker in lymphocytes and mast cells migrating intraepithelially has been observed from place to place. Reactivity was also noted in striated muscle fibers, smooth muscle fibers in the arteriolar composition (Figure 4B), tumor infiltration associated with tumors in myoepithelial cells and even acinar cells from serous acini of the minor salivary glands (Figure 4C), as well as in lymph nodes, both in lymphoid and interfollicular follicles (Figure 4D).

At tumor tissue level, the reactivity for F-actin was by far the highest compared to the other two markers investigated. Similarly, well-differentiated forms of lingual squamous carcinoma showed the lowest reactivity ($IRS = 2.63 \pm 1.01$), the reactions for F-actin being diffused in the neoplastic proliferation (Figure 4E), somewhat more intense towards the invasion front (Fig. 4F). Reactivity was present in cells at the periphery of the proliferation, lacking at the level of the keratosis pearls. Regarding the subcellular location of the signals we recorded their diffuse and granular cytoplasmic prevalence (Figure 5A). The most intense reaction to F-actin was recorded in cases of moderately differentiated squamous carcinoma ($IRS = 7.69 \pm 2.70$), the reactivity being diffuse, present in both the tumor and the invasion front

(Fig.5B-C). Reactions seem to be more intense at the periphery of tumor proliferation with a diffuse and granular cytoplasmic pattern. The maximal reactivity was recorded in the acantholytic forms of lingual squamous carcinoma, the most intense signal being present in the cytoplasm of acantholytic neoplastic cells in the center of the proliferation (Figure 5D). A moderately differentiated form of reactivity was also noted for poorly differentiated forms of lingual squamous carcinoma ($IRS = 6.33 \pm 2.05$), with a diffuse pattern, somewhat more intense at the invasion front (Figure 5E). In cases with inflammatory stroma and those with stromal desmoplastic reaction, we also reported reactivity for F-actin and for lymphocytes and stromal fibroblasts. In contrast, in lymph node metastasis, we found the weakest reactivity in neoplastic cells at the periphery of metastatic proliferation (Figure 5f).

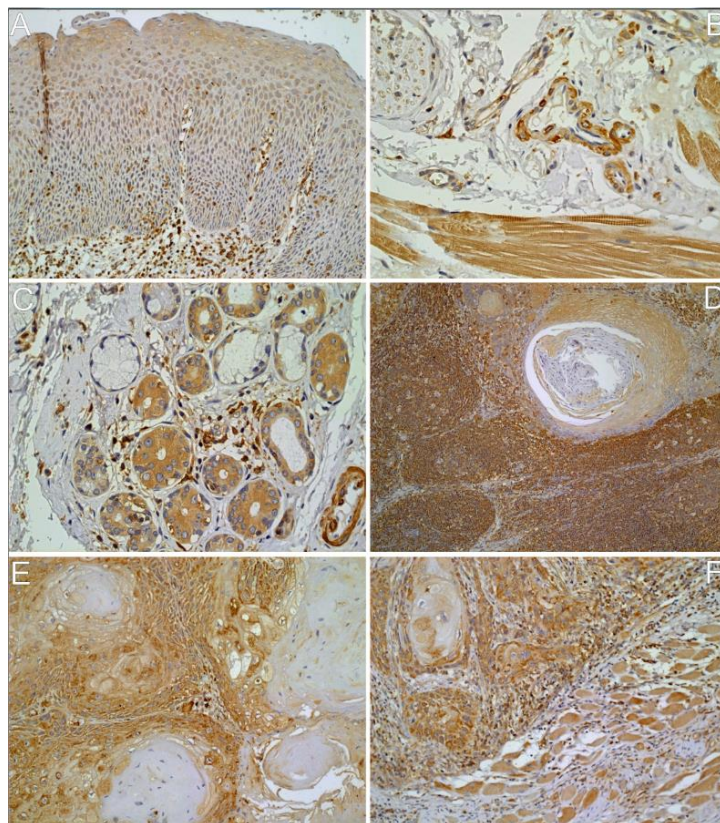
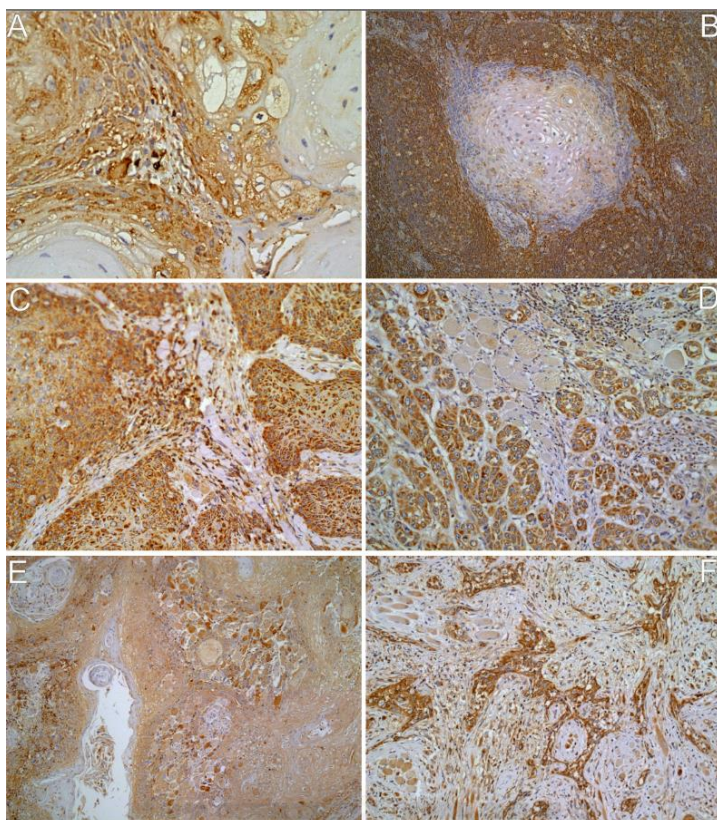


Fig. 4 Lingual squamous cell carcinoma - A. Low reactivity for F-actin in the lingual epithelium adjacent to the cancerous lesions, limited to the cell lines in the upper region of the intermediate layer, Col IHC-DAB, X200; B. Reactivity for F-actin in the striated and smooth muscle fibers from the composition of the arterioles present in the surgical specimens, Col IHC-DAB, X400; C. Reactivity for F-actin at the level of myoepithelial cells and acinar cells from serous acini of the minor chorale salivary glands present in the specimens of surgical exergy. Col IHC-DAB, X400; D. Reactivity for F-actin in the lymphoganglions, both in the lymphoid and interfollicular follicles. Col IHC- DAB, X200; E. Reactivity for F-actin predominantly at the periphery of neoplastic proliferation from well differentiated forms of lingual squamous cell carcinoma, Col IHC-DAB, X200; F. Somewhat more intense reactivity for F-actin in the invasion front of well-differentiated lingual squamous carcinomas, Col IHC-DAB, X200.



4. Results of the statistical study on tumor reactivity for these markers

The statistical analysis for the correlation between immunohistochemical reactivity for each investigated marker and the histological degree demonstrated that the lowest expression was recorded in G1 cases compared to G2 and G3 cases { $F(2,56) = 41.646$, $p < 0.001$ for D2-40; $F(2,56) = 34.134$, $p < 0.001$ for CD10 and $F(2,56) = 31.154$, $p < 0.001$ for F-actin} (Figure 6). As regards to the tumoral stage, the statistical analysis noticed that each of the three investigated markers had the lowest expression in pTNM- stage I cases compared to pTNM- stage III and in pTNM- stage IV cases { $F(3,56) = 8.568$, $p < 0.001$ for D2-40; $F(3,56) = 8.439$, $p < 0.001$ for CD10 and $F(3,56) = 12.288$, $p < 0.001$ for F-actin} (Figure 7). There was no significant difference between tumor response and patient age and lesion topography ($p > 0.05$). Finally, the Anova test demonstrated that there were very strong correlations between D2-40 / CD10 ($r = 0.903$), D2-40 / F-actin ($r = 0.829$), and CD10 / F-actin ($r = 0.819$).

Fig. 5. Lingual squamous cell carcinoma - A. The subcellular pattern of reactions for CD10 in neoplastic cells was a diffuse and granular cytoplasmic one, Col IHC-DAB, X400; B. More intense reactivity for F-actin at the level of neoplastic proliferations within the moderately differentiated forms of lingual squamous cell carcinoma, Col IHC-DAB, X200; C. Intense reactivity for F-actin at the level of neoplastic proliferation in the invasion front of the moderately differentiated forms of carcinoma lingual cuamos, Col IHC-DAB, X200; D. Intense marking for F-actin in acantholytic neoplastic cells from acantholytic forms of lingual squamous cell carcinoma, Col IHC-DAB, X100; E. Reactivity for F-actin with diffuse pattern, slightly more intense towards the invasion fundus in the weakly differentiated forms of carcinoma lingual cuamo, Col IHC-DAB, X200; F. Low reactivity for F-actin at the periphery of metastatic neoplastic proliferation in lymphoganglions diagnosed with lingual squamous cell carcinoma, Col IHC-DAB, X100.

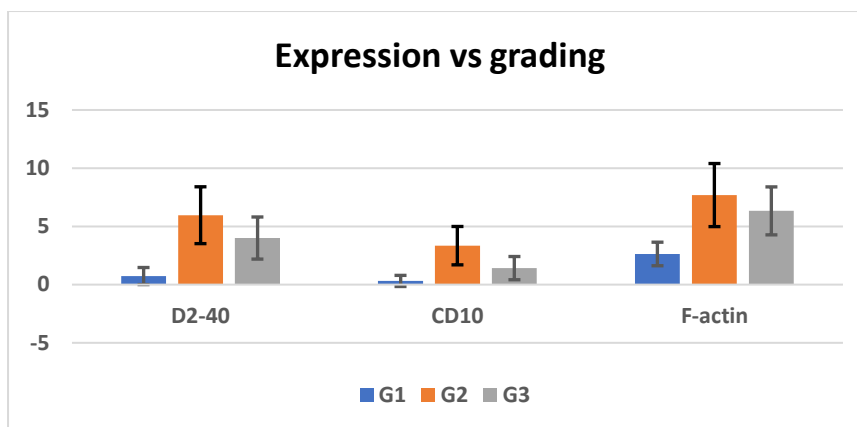


Fig. 6 Correlation between tumoral reactivity for D2-40, CD10 and F-actin versus histological degree (G1, G2, G3)

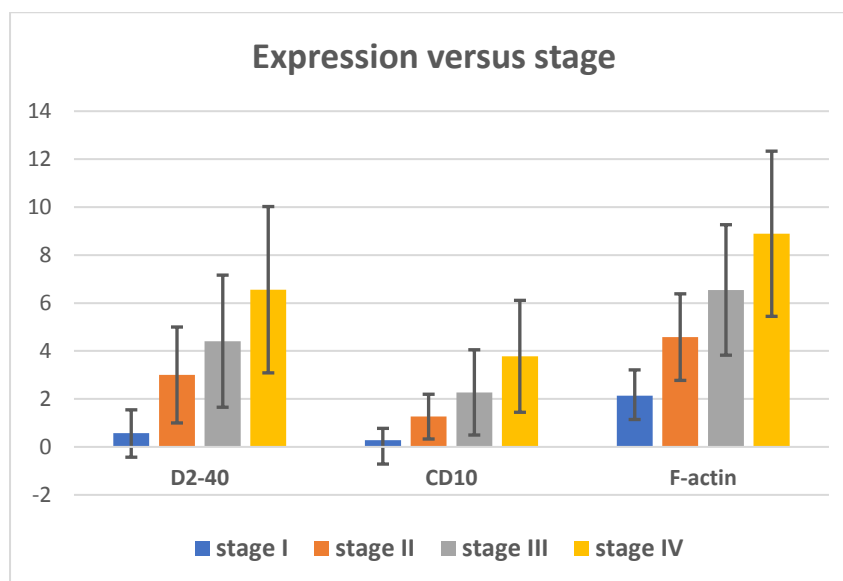


Fig. 7 Correlation between tumoral reactivity for D2-40, CD10 and F-actin versus pTNM stages (I, II, III, and IV).

Studies of recent decades have indicated that tongue cancer is one of the most common forms of oral squamous carcinoma, its incidence being increasing in civilized countries, especially among young people [Tota JE et al., 2017; Patel SC et al., 2011]. In particular, this localization of oral squamous cancers is characterized by high local aggression and early lymph node dissemination, with at least 50% of these patients presenting with metastasis at the time of diagnosis [Capote-Moreno A et al., 2010]. The most important prognostic factors of relapse rate and survival rate, especially in early forms of lingual cancer, have been found to be the depth of invasion and the pattern of high-level invasion [Almangush A, 2014; Ganly I et al., 2013; Huang SH et al., 2009; International Consortium for Outcome Research (ICOR) in Head and Neck Cancer, Ebrahimi A et al., 2014]. The depth of the invasion, defined as the distance from the surface of the reconstructed mucosa to the deepest level of invasion [Pentenero M et al., 2005], was incorporated into the 8th TNM classification in the staging T such that deep- invasion of less than 5 mm was considered less invasive, while tumors with invasion depths greater than 10 mm were considered to be deeply invasive [Amin MB et al., 2017]. In addition, for cases of lingual squamous carcinoma it is considered that tumors with invasion depths greater than 10 mm are associated with an increased risk of relapse and lymph node metastasis, selective cervical lymphadenectomy (I-III / \pm IV) being recommended in deep tumors invasion of less than 5mm [Faisal M et al., 2018]. Thus, lingual squamous carcinoma forms a particular epidemiological, histopathological and prognostic form compared to other oral cancer localizations [Bello IO et al., 2010; Jansen L et al., 2018; Li R et al., 2013], requiring additional investigations to elucidate the molecular mechanisms underlying such a tumor phenotype. In this respect, we investigated the immunohistochemical expression of markers responsible for tumor invasiveness (D2-40, CD10 and F-actin) at the level of 57 cases of lymphatic squamous carcinoma diagnosed in various stages of pTNM. The aim of the study is to establish correlations between immunohistochemical reactivity scores and a series of investigated morphoclinic parameters from the perspective of prognosis and the possibility of therapeutic stratification of such patients.

1. Discussions on immunoreactivity for D2-40

D2-40 is a type-1 transaminergic transmembrane glycoprotein found to be extensively and specifically expressed in lymphatic endothelial cells [Baluk P & McDonald DM, 2008, having a major role in the lymphangiogenesis process, since D2-40 deficient mouse has been shown to have deficient lymphangiogenesis [Schacht V et al., 2005]. This glioprotein has also been shown to be expressed in other normal human tissues and is additionally overexpressed in a series of human cancers, including squamous carcinomas, including those with development in the oral mucosa [A G D et al., 2017]. In such tumors, it appears to mediate tumor migration, invasion and metastasis by promoting the remodeling of the actinic cytoskeleton of tumor cells, increasing their motility through facilitating the formation of membrane protuberances similar to filopodia and mediating the process of epithelium-mesenchymal transition [Huber GF et al. 2011; Martin-Villar E et al., 2006; Wicki A et al., 2006].

In our study we first noted the presence of D2-40 reactivity in the basal and parabasal layer of the epithelium adjacent to tumors, the intensity and extension of the marker increasing with worsening of the eventually associated dysplasia, with maximum reactivity constituting it in the tumor tissue. At this level the highest immunoreactivity scores are observed in moderately differentiated forms, the reactivity prevailing in the peripheral neoplastic progenitor cells, especially to the invasion front. The subcellular pattern of the marker was mostly membranous, but in some cases cytoplasmic. A near-marker as well as immunoreactivity scores were recorded in poorly differentiated forms, where the signal diffused into cell proliferation, and the underlying subcellular pattern was a cytoplasmic one. In the well-differentiated forms, the immunoreactivity scores were the lowest and the reactions predominated at the stromal interface, the predominant subcellular pattern being the membrane pattern. The most intense reactivity was recorded in metastatic cases and in acantholytic forms, the reactivity being present in lymph node metastases but with a much lower intensity, close to that of the well-differentiated forms.

Among the first to show the significant prognosis of D2-40 expression in premalignant lesions oral was Kawaguchi H et al., the authors showing that extended expression in the overburden layers of these lesions would result in clonal expansion of stem cells from carcinogenesis and implicitly a higher risk of malignant transformation from these lesions [Kawaguchi H et al., 2008]. Subsequent studies also confirmed the expression of the dysplastic dysplasia in the premalignant lesion (A G D et al., 2017; Vicente JC et al., 2013; Inoue H et al., 2012). Many of these authors have shown that the intensity of expression for D2-40 increased with the severity of dysplasia, suggesting that this marker would be useful in identifying malignant lesions with high risk [Kawaguchi H et al., 2008; de Vicente JC et al., 2013; Inoue H et al., 2012; Funayama A et al., 2011; Kreppel M et al., 2012].

Numerous studies have shown overexpression of D2-40 in oral squamous carcinomas and a correlation of its expression with the degree of tumor differentiation, the maximum levels of expression being recorded in low-grade cases of tumor differentiation [A G D et al., 2017; Inoue H et al., 2012; Patil A et al., 2015; Prasad B et al., 2015]. Most authors showed that D2-40 expression in well-differentiated oral squamous carcinomas prevailed at the periphery of the proliferation, whereas tumor cells were not reactive at their center (Prasad B et al., 2015; Margaritescu C et al., 2010; Martin-Villar E et al., 2005). This would be explained by the fact that cells at the periphery of tumor proliferation have a proliferation index and a higher regeneration capacity, whereas central tumor cells would mature or undergo degenerative changes as a result of terminal-type differentiation of these cells. In addition, in moderate and poorly differentiated cases, the pattern of reactions for D2-40 was diffuse, suggesting that in these cases there were cytoskeletal alterations in tumor cells with impact on their migration and carcinogenesis [Prasad B et al., 2015; Longatto Filho et al., 2007]. In terms of subcellular tumor localization, some authors showed predominantly cytoplasmic expression in poorly differentiated forms, while moderate and well differentiated forms prevailed in membrane expression (Prasad B et al., 2015; Schacht V et al., 2005). In all degrees of differentiation, expression of D2-40 appears to be higher in stromal tumor cells [Prasad B et al., 2015; Astarita JL et al., 2012]. The vast majority of studies indicate a significant correlation between the level of D2-40 expression in oral squamous carcinomas and the cervical lymph node metastasis rate (Huber GF et al., 2011; Kreppel M et al., 2012; Chung MK et al., 2010; Yuan P et al., 2006). In the study conducted by Vicente JC et al it was shown that tumors with lingual and floor of the mouth localization developed more laterocervical lymph node metastases compared to the rest of the localizations, the highest rate being recorded in small lymph tumors (T1 and T2) and well differentiated, suggesting that tumors with these localizations would have a different carcinogenesis than the remainder of the oral tumors [Vicente JC et al., 2015]. The same authors showed that D2-40 expression was limited to the invasion front in 63% of the studied carcinomas, suggesting that this subpopulation of cells would have distinct biological properties from the rest of the tumor cells. Opinions on the prognostic role of D2-40 expression in oral cancers are shared, while some authors advocate this role [Seki S et al., 2014], others quote it would not correlate with the invasive potential and would not have independent prognostic value [Vicente JC et al., 2015].

2. Discussion on immunoreactivity for CD10:

CD10 is a zinc dependent membrane metalloproteinase, commonly known as the common lymphoblastic leukemia (CALLA) antigen. Its major role is to regulate the biological activities of a number of peptide substrates, reducing their local concentration and implicitly decreasing their availability to bind to the corresponding receptors and to trigger cell transduction mechanisms [Roques BP et al., 1993; Turner AJ & Tanzawa K, 1997]. Over time, it has been shown that this marker can be expressed by a wide variety of normal cells (kidney, liver, small intestine, placenta, coroid plexus, brain, gonads, adrenal gland cortex, leukocytes and germ cell cells) [Ordi J et al., 2003], proving to be a surface marker of stem cells from various tissues (bone marrow, adipose tissue, lung and mammary gland) [Fukusumi T et al., 2014].

Also, the expression of CD10 has been reported in a wide variety of human tumors (eg lung, stomach, liver, pancreas, kidney, urinary bladder, prostate, cervix, mammary gland, skin) [Fukusumi T et al., 2014], some of which establish a correlation between its expression level and the metastatic rate [Maguer-Satta V et al., 2011].

We recorded reactivity for CD10 only in tumor tissue, normal adjacent epithelium or with dysplastic changes not reactive for this marker. The weakest reactivity was recorded in well-differentiated forms, where it was present only at the level of keratin pearls. The most intense reactions have been observed in moderately differentiated forms, the immunodeficiency being more evident at the periphery of neoplastic proliferations and invasion, with a subcellular pattern prevalent in membranous but sometimes cytoplasmic patterns. The poorly differentiated forms had values of intermediate immunoreactivity scores in those obtained in well-differentiated and moderately differentiated forms. The pattern of reactions was diffuse in neoplastic proliferation, and the prevalence of subcellular localization was cytoplasmic. Reactivity was also noted in the tumor stroma, the immune marker being evident especially in the cases associated with the desmoplastic stroma and those with the inflammatory stroma, respectively, proving the involvement of this compartment in the process of tumor invasion of these cancers. At the level of lymph node metastases, reactivity was lower compared to primitive tumors, suggesting a change in the phenotype of cancer cells in the metastasis process.

Studies on the investigation of CD10 expression in oral squamous carcinomas are few. Thus, in the study conducted by Piatelli A et al. it was shown that between the stromal expression of CD10 and the rate of lymph node metastasis, the rate of relapse and the histological differentiation degree there would be statistically significant correlations [Piatelli A et al., 2006]. The authors suggested that CD10 would play important roles in differentiation, growth and tumor invasion, facilitating the development of metastases in oral squamous carcinomas. Subsequently, Fukusumi T et al. showed that CD10 expression was associated with chemo- and therapeutic radiosensitivity in squamous cell carcinomas of the head and neck as a result of conferring some of the tumor cells that are cancer-bearing tumor cells. This has led the authors to argue that CD10 may be a therapeutic, molecular therapeutic target, especially in cases resistant to conventional oncological treatment [Fukusumi T et al., 2014].

3. Discussion on immunoreactivity for F-actin

F-actin also known as filamentous actin or microfilaments is an important component of the cytoskeleton of eukaryotes, participating in the construction of many high-specialized structures such as stress fibers, lymphoid nodes and phallopodes [Oda T et al., 2009; Pfaendtner J et al., 2010]. F-actin plays important roles in morphogenesis and cellular stability, being involved in many major cellular processes such as division, endocytosis and cell migration [Luo BL & Low BC, 2005; Stricker J et al., 2010]. In some of the human cancers, overexpression of F-actin has been demonstrated, its level of expression correlating with the metastatic potential of these tumors [Shao J et al., 2018]. Thus, the idea was born that if we can control F-actin formation, under control and the cancer metastasis process by inhibiting the migration and invasion of cancer cells [Francis SL & Antonipillai J, 2017]. One of the mechanisms underlying the tumor invasion would be colocalization of cortactin with F-actin that would lead to the formation of invadopodia precursors [Oser M et al., 2009], which are further responsible for the extracellular matrix degradation [Clark ES et al., 2007]. In addition, a number of studies have shown overexpression of cortactin in a series of human cancers including oral squamous carcinoma [Yamada S et al., 2010].

Reactivity for F-actin was by far the highest in our study, the moderate and poorly differentiated forms with the highest immunoreactivity scores. Reactions were more evident in the periphery of the proliferation and the invasion front, the subpolar subpopulation pattern being diffuse cytoplasmic and granular. The greatest reactivity I noticed mostly in acantholytic forms of lingual squamous carcinoma and in cases associated with desmoplastic stroma and / or inflammatory tumor stroma. A weak reactivity was noted in the well-differentiated forms and in lymph node metastases, and the immune marker is usually present in the periphery of the neoplastic proliferation. Thus, neoplastic cells in lymphatic squamous carcinoma have intrinsic motile properties, which is all the more evident as the degree of histological differentiation is lower and decreases in well-differentiated forms and in metastases.

Reviewing data on F-actin expression in oral squamous carcinoma did not reveal any study that had focused on this as its objective. However, indirect data on the participation of co-authors of this cytoskeletal protein in the invasive and metastatic process of oral squamous carcinomas have been highlighted in several studies. Thus, in the study conducted by Yamada S et al, there was evidence of an overexpression of cortactin which correlated statistically high with aggressive phenotypes of oral squamous carcinomas [Yamada S et al., 2010]. The authors conclude that in this type of human cancer, cortactin may be a potential molecular target of gene therapy. In the same direction, Ramos-García P et al studied the overexpression of this protein in oral squamous carcinomas, seen as a promoter of life-threatening metastases and implicitly as a potential therapeutic target in such cancers [Ramos-García P et al., 2018]. The study by Horn D et al. has highlighted the prognostic role of monitoring the level of expression of this protein in oral squamous carcinomas, with high levels of its expression associating with poor prognosis and low survival rates, thus allowing the therapeutic stratification of these patients for individualized therapy [Horn D et al., 2018].

Our statistical analysis demonstrated the existence of correlations between tumor response for each of the three investigated markers and both histological degrees and pTNM-stages with the highest recorded reactivity in the G2 and G3 cases and respectively in the pTNM stage III and IV. We also noticed a very strong correlation between tumoral reactivity for all investigated markers.

Conclusions

Our study highlighted the reactivity of tumor tissue for all three investigated markers, moderately differentiated forms followed closely by poorly differentiated forms with the highest immunoreactivity scores, which confirmed the more aggressive nature of these forms of lingual cancer. At the level of neoplastic proliferation, the immunoassay was more evident towards their periphery at the interface with the tumor stroma, suggesting the involvement of these markers in the invasiveness and dissemination of lingual squamous carcinomas. The tumor reactivity values increased for each of the three markers investigated with the pTNM stage tumor, and there were close statistical correlations between the immunoreactivity scores of these markers. In addition, the association of a desmoplastic or inflammatory reactive stroma for the markers investigated seems to facilitate the two tumor processes, giving these cases a much more severe prognosis.

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