UNIVERSITY OF MEDICINE AND PHARMACY FROM CRAIOVA
DOCTORAL SCHOOL

DOCTORAL DISSERTATION

STUDY OF THE PROANGIOGENIC MARKERS INVOLVED IN THE PROGRESSION OF THE TONGUE SQUAMOUS CARCINOMA

- abstract -

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INTRODUCTION

Nowadays, on the entire globe, oral cancer is the 8th most frequent malignant neoplasia, and the twelve cause of decease due to cancer among men. In the last decade it has been noted an increasing tendency amongst women and youth, the mortality rate from cancer evolving in a series of state member in the Est Europe. It has been observed an increase among youth females and the white breed for his specific lingual localization.

Moreover, its localization on the mobile part of the tongue is associated with a higher rate of mortality due to the fact that these tumors, at an early stage have a predilection to metastasis in the lymph node situated in the near area.

Our study has investigated a number of 54 pacients diagnosed with lingual squamous carcinoma from the epidemiological, histopatological, immunohistochemistral and statistical point of view, for about 3 years, beetwen 2012-2014.

The epidemiological study identified the prevalence of the squamous carcinoma localized on the 2/3 anterior tongue for the male gender, living on the countryside, chronically exposed to smoking and alcohol, in the 6th decade of life.

The histopatological study outlined an aggressive phenotype among the non-keratinized forms, with a moderate grade of differentiation, a discohesive pattern of invasion, with muscular invasion in more than 2/3 of the cases, vascular invasion and lymph-nodes dissemination in 1/3 from the cases.

A third of these cases have been divided in stage II and III pTNM.

The immunohistochemistral analysis outlined the particularities of the angiogenesis process and it showed:

The existence of an intense angiogenesis process in the invasion front, certified on high values of MVD-CD105, most of the blood-vessels being immature and intermediary type and with a high index of proliferation.

The proliferation index and the vascular maturity degree was even higher as the tumors were undivided, with an invasion pattern discohesive type and with the advance of the tumoral stage.

The existence of a particular angiogenic profile in the aggressive forms of lingual squamous carcinoma (barely divided, in an advanced stage and with a high invasive pattern) VEGF-/VEGFR1+/VEGFR2+ ,

The immunoreactivity scores for the 2 markers being correlated to MVD-CD105, to the maturity index and proliferation index.

The involvement in the progression of the oral squamous carcinoma of the growth factors VEGF, TGFβ1 and angiopoietin-2,given to the tumoral immunoreactivity of the tumors for these markers and their receptors, the highest scores of immunoreactivity being signalised in those tumors with a specific histological aggresivity.

CHAPTER I. THE ETIOPATHOGENESIS OF ORAL SQUAMOUS CARCINOMA- it presents the newest informations about the etiological factors of squamous carcinoma and how are they involved in the carcinogenesis .

CHAPTER II. PRO AND ANTIANGIOGENIC FACTORS INVOLVED IN THE ORAL CARCINOGENESIS – offers informations about the tumoral angiogenesis process and its implications in the human carcinogenesis, then it describes the main proangiogenic and antiangiogenic factors and their functions during the carcinogenesis of the mucous membrane of the oral cavity.

CHAPTER III. ANGIOGENESIS AS A FACTOR PREDICT IN THE SQUAMOUS CARCINOMA OF THE ORAL CAVITY – it presents the implications in the angiogenesis process such as the initiation, progression, and dissemination of the cancerous processes, adding information about the influence of the angiogenesis in the squamous carcinoma of the oral cavity.

STUDY GOALS

1.The evaluation of the epidemiological and histopathological characteristics of the lip cancer, evaluated for 3 years on these parameters:

• epidemiological : sex and age of the patients, origin, risk factors, localization of the tumors.

• histopathological : the degree of differentiation, the quantification of the inflammatory infiltrate, tumoral invasion pattern, presence/absence of the vascular, perineural,muscular invasion,presence/absence of the microscopical invasion at the bounds serving to the surgical precision, presence of other associated injuries.

II. Establishing the angiogenic immunoprofile of these tumors through the following investigations:

• the maturity degree of the tumoral vessels with the help of the double immunohistochimical CD105/KI-67;

• tumoral reactivity, of the vascular endothelium and of the other stromal cellular partners (fibroblasts and inflammatory cells) to a series of known factors with proangiogenic effect (VEGF, TGFβ1 and angiopoietin-2) and their receptors (VEGFR1, VEGFR2 and TGFβ1R1);

• the significant statistical data selection, as an outcome of the analysis of the reminded parameters, with the realisation of some statistical significant correlations concerning the emphasis on the predict factors.

CHAPTER IV-Methods and Material- this offers data of the studied material and the methods used in research

THE STUDIED MATERIAL-from the Laboratory of Pathological Anatomy of the Clinical Hospital of Emergency Number 1 Craiova's cases and has been represented by the paraffin blocks archieved. The study has been extended on a 3 year period, the cases been selected in 2012-2014, a number of 54 cases of lingual tumors that have been the main objective of the histopathological study.

UTILISED METHODS

The epidemiological study's purpose was to collect the following data about the included sick people: sex, age, environment of provenience, risk factors and location of tumors.

THE HISPATOLOGICAL ANALYSIS has been made according to the following morphological variables: the hispatological options, the differentiation grade, the proportions of the inflammatory infiltrate, the tumoral invasion pattern, the presence/absence of the sanchine and limfatic vascular invasion, the presence/absence of the perineural invasion, the presence/absence of the muscular invasion, the presence/absence of the metastatic adenopathy, the presence/ absence of the residual malign cells in the edge of the chirurgical safety; the presence of associate injuries: displacements.

IMUNOHISTOCHIMICAL STUDY had a number of 20 selected case studies, for which the vascular microdensity has been analysed (CD105), the maturity grade of the veins (CD 105/ α-SMA), the proliferation grade of vascular and tumoral (Ki67/CD105), as well as the expression of some growing factors and their receptors(VEGF şiVEGFR1/ VEGFR2, TGFβ1 şi TGFβ1R1, ANG2).

I have used simple and double reactions; for the simple reactions and for the first part of the double reactions I have used the amplification system LSAB2-HRP şi CSAII (for- CD105 monoclonal), the development being made with the DAB cromogen(brown color). The second part of the double reaction (α-SMA; CD105-policlonal), after being blocked with biotin avidin, i have used the visualisation LSAB2-AP and the cromogen Vulcan Fast Red (red color).

Using the morphometric method I have analysed the microdensity of the veins marked with CD 105/ α-SMA using the „hot spot" technique , which meant the manual cuantification of the 3 aries veins with a maximum mark on the obiective x20. The vascular microdensity has been analysed intratomural as well as in the invasion front. In the SMA case I have only analysed the double marked vessels CD105+ and SMA+.

In the imunohistochimical study I have used only concentrated antibodies,that have the principal characteristics shown in the following table:

Antibody	Clone/ Producer	Diluation	Antigenical Demask	Positive Control
CD105 (endoglin)	SN6h/ Dako	1:1000	-	Kidneys
CD105 (endoglin)	Policlonal/ Thermo Scientific	1:50	Citrate, pH 6	Kidneys
α-SMA (smooth muscle actin)	1A4/ Dako	1:50	Citrate, pH 6	Colon
Ki67	MIB 1/ Dako	1:200	Citrate, pH 6	Mamary Carcinom

VEGF	C1/ Dako	1:100	Citrate, pH 6	Kidneys
VEGFR1 (Flt-1)	CL0344 / Sigma-Aldrich	1:150	Citrate, pH 6	Tegument
VEGFR2 (KDR/Flk-1)	Policlonal/ Abcam	1:300	Citrate, pH 6	Kidneys
ANG 2	F1/ SantaCruz Biotechnology	1:50	Citrate, pH 6	Placenta
TGFβ1	3C11/ SantaCruz Biotechnology	1:250	Citrat, pH 6	Kidneys
TGFβ RI	H-100/ SantaCruz Biotechnology	1:50	Citrat, pH 6	Kidneys

Antibodies used in the lingual squamous carcinomas study.

For the statistical analysis the Student, Anova,Chi Square and Pearson tests have been used with the SPSS 10 soft, and the acquired images were done with the help of the NIKON NIKON ECLIPSE 55i microscope and the Image-Pro Plus soft.

CHAPTER V- Results and **CHAPTER VI- Discussions**, give the result of our study, then they are reported to the classic and recent data from the special literature.

The epidemiologic analysis has shown that most of the cases included male gender with 64,81%, the statement on sexes being around 1:1,2 for male gender. The injury topographic has shown that most of the time squamous carcinoma of the tongue has developed in the 2/3 anterior tongue (33,33%),most of the people coming from the countryside(57,41%), and the major factor was the permanent exposure to alcohol and smoking(76%).

According to the data coming from the Surveillance Program, to the epidemiologic analysis and to the Final results between the years 1975-2007, it has been registered a growth in the oral squamous carcinoma especially among young females, and more specifically to the tongue.

According to the special literature data frequently lingual tumors develop on the tongue's edges, in the 2/3 anterior tongue.

In literature the most incriminated factors in the etiology of the oral cancer are tobacco(chewing and smoking) and alcohol, and occasionally can be blamed the nutritional deficiencies , bad dentition and viruses [381,384,385].

Histopathological study has shown that most of the tumors corresponded to the non-keratinized (44,44%), followed closely by the keratinized forms (37%) and then the acantholytic form(13%).

The latest tendency is to group all the oral squamous carcinoma in 3 categories: I.non-keratinized, in which etiopatogeny is blamed the HPV infection , localized in the oropharynx, II. Keratinized, no etiopatological connection to the HPV , III. Keratinized, with squamous differentiation ,the localization of the keratin not going beyond 10% of the tumor and not necessary connected to HPV infection.

From the point of view of the differentiate grade most of the cases were diagnosed in the category of the moderate differentiate (57,4%), most of the cases are acantolythic tumors, nonkeratinized carcinoma and only 5 keratinized squamous carcinoma with rare keratin pearls.

This histological system of gradation of the malignancy even if it's frequently used in the Clinic it still has a reduced utility in the Squamous Carcinoma and those localized on the tongue particularly.[401-403]

According to the pattern of the tumoral invasion most of the cases had a prevalence in the type 3 invasive(42,59%), the invasion was formed in small cell groups or cords of infiltrative cells.

Most of the studies have confirmed that the squamous oral with a high invasive pattern is also associated with a bad prognosis.[409-410]. In comparison with the presence of the invasion in the lymph-nodes we have noticed that this aspect was present in only 27,77% of the total cases. Literature notes indicated that the lymph-node invasion is not a favorable prognosis in the lingual squamous carcinoma, associated with the metastases in the lymph-nodes or the loco-regional reccurence.[414,415] According to the invasion of the tumors in the own tongue muscular , it has been recorded the fact that this aspect has been present in more than half of the cases (62,96%).

Date in literature indicated that the muscular invasion is a morphological appearance frequently encountered , more than 85% of these invasive tumors in the muscular tongue, and in 91% of the reccurences this appearance was present.[419]

In our casuistry we recorded the presence of positive edges in 1/3 cases (31,48%)frequently only one of the edges was invaded (24,7% from the investigated cases, and 76,47% from the cases with the invasion of the limits). General consense is that the presence of the edges with a clear resection it's an important prognostic factor, but it is still controversial what a clear resection is. Some of the researchers have recommended that a clear resection are those of no more than 2 cm, whilst others believed that it should not pass 3 mm.[424],but the tendency nowadays is to consider 1 tridimensional cm, as an adequate edge.[425]

The analysis of our casuistry in relation to the staging pTNM has shown that 2/3 from the cases (70,38%) have been diagnosed in stage II and III in equal percentage (35,18%).Most of the data in literature indicates the diagnose of the tongue cancer in stage I and II, and less in more advanced stages.[413]

Immunohistochemistry analysis. Using the CD105 marker we mostly observed tumoral vessels with aberrant morphology, with ramifications, and small dimensions (<15μm in diameter), or we observed the presence of a vascular architecture more complex with numerous points of blossom and isolated endothelial cells CD105 reactive , especially in the invasion tumoral front. The cuantification of MVD-CD105 has proofed the existence of an angiogenesis process more active at the level of the invasion front in comparison with the intratumoral areas, the process is even more obvious in the forms the are weakly differentiates, especially in stages II-IV pTNM. Studies which used the CD105 as endothelial marker and have quantified MVD in squamous carcinoma of head and neck and shown its prognostic value,

tumors with high values of MVD-CD105 usually being associated with a bad prognostic.[349,445,446]

On the investigated casuistry the evaluation of MVD- αSMA has shown the prevalence of αSMA+ vessels at the side of the invasion front in comparison with the intratumoral areas, with the existence of significant static differences. It has also been observed the existence of significant static corelations of the MVD- αSMA and the grade of differentiation, intratumoral but also in the invasion front. Literature studies indicate the existence of various grades of covering of the new tumoral vessels with pericites (CD34+/αSMA+) and so presenting different grades of vascular maturity in diverse human tumors.[455]

In the study that we developed we observed a correlation of the tumoral proliferation index with the grade of tumoral differentiation, highest values being registered on the tongue tumor poorly differentiated.

In addition to this we observed: (1) variations of this index with the grade of the invasive pattern, great values being registered on the 3rd and 4th pattern; (2) IP-Ki67 had greater values in the tumors with muscular and vascular invasion in comparison to the non-invasive tumors; (3) tumors in an evolved stadium presented higher values of the tumoral proliferation index.

Studies made on the proliferation index of Ki-67 in oral squamous carcinoma, including lingual carcinoma have shown that the higher values of this index are significantly higher for the people with cervical lymph-nodes metastasis, which is an important prognostic independent in the evaluation of the survival of these people.[456]

The analysis of our casuistry has shown the existence of correlation of the immune markers for VEGF and the grade of tumoral differentiation , immunoreactivity scores for VEGF were higher in the well differentiated forms in comparison to the poor ones. Literature notes say that the reactivity for VEGF indicates the existence of significant static differences of reactivity in the invasion front vs. periphery the biggest expression being registered in the invasion front of the oral squamous carcinoma.[213]

When it comes to the tumoral reactivity to the receptors of the growth factor VEGF we observed the fact that VEGFR1 was expressed in 60% of the investigated cases, whilst VEGFR2 reacted in 55% of the cases. Both immunoreactivity scores of the two receptors correlated significantly with the tumoral differentiation grade and the moment of evolution of the disease.

Literature notes indicates the expression of the proangiogenic VEGF factor receptivity both at the tumoral cells and the endothelial vascular tumoral cells from the oral squamous carcinoma.[470-472]

This indicates the involvement of the VEGF-VEGFR1,2 axis in the tumoral progression directly and through some sort of mechanisms, but also indirectly by stimulating the tumoral angiogenesis.[473]

Investigation of our casuistry outlined the fact that the tumoral immune reactivity for TGFβ1 was the highest in the squamous carcinomas moderately differentiated. In addition we observed the correlation of the IHS score for TGFβ1 with the tumoral proliferation index. In a series of studies developed on squamous

carcinomas of the head and neck we outlined the fact that in more than 80% of the tumors the TGFβ1 factor was expressed ,and its expression is correlated with the advanced stages of the disease and the lower rate of survival [481-482].

In our study cca.80% of the investigated cases expressed the TGFβr1 factor. Highest immunoreactivity has been observed in the moderate differentiated forms, cases in which it also existed a correlation with the growth factor TGFβ1.

The investigation of the TGFb expression in squamous carcinomas of the head and neck showed a lower expression of the TGFbR2 and TGFbR3 receptors,35% of the oral tumors specimens presenting a weak reactivity or the absence of the expression TGFbR2 and 53% the absence or a small reactivity such for TGFrB3[486].

Ang2 on our casuistry came positive in 65% of the tongue tumors. Tumoral immunoreactivity presented variations with the differentiation grade and the tumoral stage. In a recent study developed on oral squamous carcinomas it has been found a high percentage 54% of tumors that expressed Ang2 and VEGF, and the angiogenic parameters investigated have significantly correlated to the expression of this proangiogenic factor.

CHAPTER VII Conclusions- shows the most important findings of our study.

-The general epidemiological profile of these patients shows the apparition casuistry at males (64,81%), in the VI'th decade of the life(33,33%) with a higher frequency at the 2/3 previous level of the tongue (33,33%).

-The histopathological study has shown the apparition of the nonkeratinized forms of squamous carcinoma(44,44%), of the moderate differentiate forms(57,4%) with an invasion pattern, most common type being the type 3(42,59%), showing a vascular invasion in 27,77%, perineural in 33,33% of the cases, muscular invasion in 62,96%, and 31,48% of the cases and the microscopic invasion of the resection's edge. At the same time 27,78% have presented lymph-nodes dissemination at the time of the diagnosis, and 71% of the cases have been diagnosed in the stage II and III pTNM.

-MVD-CD105 outlined an angiogenesis process more active in the invasion front in comparison to intratumoral areas, especially in the poor differentiated forms and in the advanced stages of disease.

- In the invasion front we observed the accentuation of the vascular immaturity whilst the invasion pattern became more discohesive, in the situation of vascular invasion, perineural and muscular invasion and also for the advanced tumors.

- The proliferation index for Ki-67 presented significant superior values for the poorly differentiated carcinomas, with discohesive invasion pattern, vascular and muscular invasion with metastasis in the lymph-nodes and in advanced stages .

- Tumoral immunoreactivity for VEGF had significantly higher values in the well differentiated forms with a growth tendency at the periphery of the tumoral islands , in comparison with their centre. Also we observed a correlation with the vascular microdensity.

- Even if the expression of the two receptors didn't correlate with the VEGF factor, they had numerous correlations but weak,and they both correlated with the

proliferation index Ki-67, highest rate of immunoreactivity being obtained by the tumors with the highest rate of proliferation.

-Reactivity of the tumoral vessels for TGFβ1, especially thos from the invasion front are suggesting a direct involvement of this factor in the angiogenesis process.

- The active involvement of Ang2 in the angiogenesis tumoral process is proved through the expression of this factor in the endothelial cells and also because of the fact that his tumoral reactivity correlated with the one of VEGF.

- The prognostic value of investigating the VEGF,MVD-CD105 and IP-Ki67 markers in the tongue carcinomas, allowing the selection of the aggressive tumors.

- The participation at the angiogenesis tumoral process from the oral squamous carcinomas as an expression of the investigated markers on the level of the endothelial cells with the participation of their stromal cellular partners.

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