UNIVERSITY OF MEDICINE AND PHARMACY
CRAIOVA

DOCTORAL THESIS

SUMMARY

ANGIOGENESIS ROLE IN LIPS SQUAMOUS CELL CARCINOMA PROGRESSION - CLINICAL, HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

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CRAIOVA 2014

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INTRODUCTION

Squamous cell carcinomas are the most common epithelial tumors of the oral area and they are currently estimated by various statistics at 95% of all oral cancers, ranking sixth in the world most frequent cancers. The report published in 2012 by the EU oral health research comitteee members considers oral cancer the 8th most common form of cancer worldwide and lip cancer ranks as the 12th most common type of cancer in men in the EU countries.

Increased prevalence of the disease was recorded in Spain and Hungary and it continues to rise in Eastern Europe countries.

In 2007, there were about 8 million deaths worldwide from cancer and its complications, and there are 12 million deaths projected for 2030.

More than 300,000 new cases of squamous cell carcinomas are diagnosed every year worldwide, representing about 90% of the oral cavity neoplasms and lip cancer reaches a rate of 25-30% of the oral cavity cancers. Lips squamous cell carcinoma shows increased incidence in tropical countries, influenced by local socio-demographic and culture factors and sums up 40% of head and neck carcinomas.

The 107 patients included in this study were first clinico - epidemiological analyzed, which led to the following conclusions : most cases of lips squamous cell carcinomas were recorded in male patients , seventh decade of life , predominantly from rural areas and the lesions were over 90 % located on the lower lip .

The microscopic study of the 107 investigated lip squamous cell carcinomas had been analyzed regarding the tumor's growth pattern, tumor type and histological quantification of the inflammatory infiltrate , presence / absence of the lymph-vascular, perineural and muscle invasion, tumor's invasion pattern, surgical resection limits and associated lesions, presence / absence of metastatic adenopathy .

Immunohistochemical analysis was performed for 40 lip squamous cell carcinomas and associated dysplasia and tracked CD105 immunoexpression and MVD CD105 + evaluation, quantified the maturity degree for CD105 and α -SMA tumor vessels, the vascular and tumoral proliferation degree by CD105 and Ki67 double immunostaining, quantified VEGF and its receptors VEGFR1 , VEGFR2 and angiopoietin 2 immunoexpression. Carcinoma markers expression had been compared to the associated dysplastic lesions and with the corresponding metastases .

Keywords: squamous cell carcinoma, lip, angiogenesis, immunohistochemical factors, microvascular density, prognosis.

Chapter I. Epidemiological aspects and risk factors involved in oral squamous cell carcinoma occurrence- provides data on the oral cancer world incidence and information on the main risk factors and on the influence they have on the carcinogenesis process: chronic alcohol consumption, smoking, nutritional deficiencies, prolonged exposure to solar radiation, immunodeficiency, potential malignant lesions.

Chapter II. Tumor angiogenesis involved in oral carcinogenesis - presents information about the research on the angiogenesis process that had been done since the last century, about tumor angiogenesis stages, description of the proangiogenic and antiangiogenic factors and their functions, whose understanding is essential for tumor therapy and clinical application of the results.

Chapter III. Lip oral squamous cell carcinoma prognosis- provides information on clinical factors (sex, age, race, socioeconomic conditions, anatomical location of the tumor), histopathological factors (metastasis, TNM staging) and immunohistochemical factors (VEGF, Hox genes) that have a role in assessing the prognosis of lips squamous carcinomas.

RESEARCH PURPOSE AND OBJECTIVES
This study proposes a comprehensive research of the angiogenesis process of the lips squamous cell carcinoma, using traditional investigation methods and also modern techniques such as morphometry and immunohistochemistry.
The specific objectives of this study are:
1. the evaluation of the clinical and epidemiological, histopathological and immunohistochemistry characteristics of the lips squamous carcinomas, for three years, by quantifying the following parameters:
- c*linical:* patients' gender and age, origin, risk factors, time since the onset of illness, symptoms, tumor location, presence / absence of metastatic adenopathy.
 - *histopathological*: the development pattern and tumor invasion pattern, differentiation degree, inflammatory infiltrate, presence / absence of metastatic adenopathy, perineural, vascular and muscular invasion, presence / absence of residual malignant cells in the surgical safety margins, presence of associated lesions.
 - *immunohistochemical*: quantification of CD105, VEGF, VEGFR1, VEGFR2, ANG2 expression.
- *morphometric*: assessing MVD.
2. the quantification of the lip squamous cell carcinoma blood vessels in order to highlight the angiogenesis process involvement on the development and progression of the lesions;
3. determining the vessel neoformation proliferative activity degree;
4. identification and quantification of the angiogenic proteins and their receptors role in identifying proangiogenic sources and establishing their role in each stage of the lip squamous cell carcinogenesis;
5. identifying synergistic or antagonistic molecular mechanisms involved in the

angiogenesis modulation.
6. selection of the statistically significant data, from the analysis of the parameters that have a role in carcinogenesis and may constitute possible therapeutic targets.

Chapter IV. Material and methods- provides data on the studied material and on the research used methods.

THE STUDIED MATERIAL
   The study was conducted over a period of three years (2010-2012), comprising a total of 107 cases of squamous cell carcinoma located on the lips. The investigated material was surgical excision samples with variable resection limits, and in 25 cases in which lymphadenectomy was performed, the samples were accompanied by lymph nodes. The biologycal material came from patients hospitalized in the Oral-Maxillofacial Surgery Clinic of the Emergency County Hospital Craiova.

RESEARCH USED METHODS
   I started by obtaining epidemiological and clinical data from the 107 patients included in the study group, regarding sex, age, origin, risk factors, time since the onset of illness, symptoms, tumor location, presence / absence of metastatic adenopathy.
   The histopathological analysis was performed on 107 cases of lip squamous cell carcinomas and included the following assessment criteria: tumor development pattern, differentiation degree, inflammatory extent, tumoral invasion pattern, presence / absence of metastatic adenopathy, perineural, sanguine and lymphatic invasion, presence / absence of muscle invasion, presence / absence of residual malignant cells on the surgical safety margins, presence of associated lesions: dysplasia, actinic cheilitis, solar elastosis, ulcers, etc..
   The immunohistochemical study included 40 selected cases that have been analyzed regarding vascular microdensity (CD105) , blood vessels maturity (CD105/α-SMA), vascular and tumoral proliferation degree(Ki67/CD105) and the growth factors and their receptors (VEGF , VEGFR1 , VEGFR2 , ANG2) expression.
   We have used single and double reactions; for the simple reactions and for the some of the double reactions we used LSAB2 -HRP amplification system and CSAII (Biotin -Free Catalyzed Amplification System- CD105 monoclonal) with DAB chromogen development (brown). For the second part of the double rections (α -SMA , CD105 - polyclonal), after blocking with avidin- biotin , we used LSAB2 -AP System and Vulcan Fast Red chromogen (red) .
   Morphometric analysis examined CD105 and α -SMA stained microdensity vessels by the " hot spot " method, which consisted in manually quantifying the vessels . Vascular microdensity was analyzed both intratumoral and at the invasion front . If SMA There have been analyzed only vessels with double staining, CD105 + and SMA + .

The following antibodies panel has been used:

ANTIBODY	CLONE/ MANUFACTURER	DILUTION	ANTIGENIC RETRIEVAL	POSITIVE CONTROL
CD105 (endoglin)	SN6h/ Dako	1:1000	-	Kidney
CD105 (endoglin)	Policlonal/ Thermo Scientific	1:50	Citrate buffer, pH 6	Kidney
α-SMA (smooth muscle actin)	1A4/ Dako	1:50	Citrate buffer, pH 6	Colon
Ki67	MIB 1/ Dako	1:200	Citrate buffer, pH 6	Breast Carcinoma
VEGF	C1/ Dako	1:100	Citrate buffer, pH 6	Kidney
VEGFR1 (Flt-1)	C17/ Dako	1:150	Citrate buffer, pH 6	Skin
VEGFR2 (KDR/Flk-1)	Policlonal/ Abcam	1:300	Citrate buffer, pH 6	Kidney
ANG 2	F1/ SantaCruz Biotechnology	1:50	Citrate buffer, pH 6	Placenta

The statistical analysis has used t -Student tests, ANOVA, chi square and Pearson using SPSS 10 software; the images were acquired using Nikon Eclipse E600 microscope and Lucia 5 software.

CHAPTER V- Results and **CHAPTER VI - Debates** render the study results, which are related to the recent and classical literature data.

The clinic- epidemiological analysis revealed that the highest percentage of lips squamous cell carcinomas was recorded in male patients (78.5 %) in the seventh decade of life (34.6 %), predominantly from rural area (63.5 %) . The lesions location was 96.2 % for the lower lip and only 2.8 % of tumors have been located in the upper lip. The results are consistent with data from other publications, which reveal a much higher incidence among males, with a ratio of 6:1 to the cases diagnosed in women, affecting primarily men over 50 years [273] . The study of the incriminated risk factors showed that they were often associated; on the top there has been the smoking and chronic alcohol consumption association. Most of the studied patients, 71 % were smokers and only 28.9 % said they didn't smoke. The data are consistent with the literature: alcohol consumption and smoking are factors with certain role in the etiopathogenesis of [119 , 206, 309]. Regarding the rate of metastases , lips squamous cell carcinomas, especially small ones , are associated with the lowest incidence of cervical metastasis of all oral cancers [336, 364] . The survival rate of patients with LSCC is longer than 5 years [204] and this neoplasia has a better prognosis compared to other aero - digestive tract cancers [153, 6]; no significant differences in the survival of men and women has been found [204].

The histopathological study revealed that most tumors were well differentiated , representing 48.5 % of the analyzed cases, followed in order of frequency of moderately differentiated forms (37.3 %) and poorly differentiated carcinomas (13.9 %). A multivariate analysis study showed that tumor grade was significantly related to the presence of lymph node metastases when diagnosed [185] , most studies reporting that this classification system is a good indicator of disease progression and response to treatment [264, 352, 265] . We have observed a different amplitude inflammatory infiltrate, predominantly at high intensity. Lympho- plasmatic type peritumoral

inflammatory response was directly correlated with survival and inversely proportional to the metastasis frequency [316] . Muscle invasion is described as fairly common in lips squamous cell carcinoma, most likely due to superficial location of the orbicularis muscle . In our study , this aspect has been identified in 56 cases out of the 107 investigated, regardless of tumors histological degree. Vascular- lymphatic invasion analysis revealed its presence in 36 cases (33.6 %), in poorly and moderately differentiated squamous cell carcinomas. In some studies , the incidence of perineural invasion was reported in 3.7% of cases, statistically significantly associated with the metastasis risk [284] . Perineural invasion was identified in 33 cases , representing 30.8 % of the cases . In 92 cases , there has been some associated modification on the uninvaded tumor resection limits. Thus, 16 cases showed aspects of dysplasia, actinic cheilitis in 7 cases and solar elastosis in 59 cases . In 10 cases , the lesions were associated dysplasia- actinic cheilitis and solar elastosis -actinic cheilitis. Solar elastosis and actinic cheilitis accompanying tumors are significant arguments of the relationship between these diseases [232] . Framing the tumors in pTNM system indicates that most cases in this study were classified into stage I of the disease (60.7 %) , 9.3 % of cases at the stage II, 19.4 % in stage III and 10.2 % into stage IV. The tumors predominance in early clinical stages is invariably accompanied by high rates of overall survival at 5 years, regardless of therapeutic modality.

Immunohistochemical analysis was performed in relation to interest clinicopathological parameters: age , sex, risk factors, macroscopic appearance , tumor differentiation degree, associated inflammation degree, the invasion pattern, vascular, perineural and muscle invasion, tumor size and extension (T category) , the presence of lymph node metastasis (N category), tumor stage. The markers expression was analyzed compared to the associated dysplastic lesions and with the corresponding metastases. CD105 immunostaining was found in the endothelial cells cytoplasm, both in carcinomas and associated dysplasia. In relation to the differentiation degree, this study showed poorly differentiated carcinomas with higher average level CD105 intratumoral MVD (p = 0.030) and at the front of invasion (p = 0.045), compared to the moderately or well-differentiated carcinomas. The number of α -SMA + stained vessels was higher at the front of invasion compared to the intratumoral area, showing statistical relationships with various clinico - pathological parameters of the analyzed categories (p < 0.05). Intratumoral, the number of vessels had a 3.7 ± 2.0 average score of MVD α -SMA + versus invasion front, where it was 6.1 ± 2.4 MVD α -SMA +, statistically significant difference (p < 0.01) . Compared to primitive tumors , metastases showed α -SMA MVD averages significantly higher in comparison to the invasion front (p = 0.003) . Ki67 staining showed significant differences regarding the carcinomas' differentiation degree; the highest mean values were observed in poorly differentiated carcinomas , followed by moderate and well differentiated forms; there were no significant differences between the intratumoral and the invasion front compartments. Regarding IP Ki67 correlation to the tumor stage, our study showed superior IP Ki67 signicant differences in discohesive invasion pattern carcinomas (p = 0.040) , with vascular invasion (p < 0.001) , perineural (p < 0.001) and muscle invasion (p < 0.001), in T3 -T4 category (p < 0.001) and III - IV tumor stage (p < 0.001) . These results indicate the presence of a reduced vascular proliferative activity in dysplastic lesions , which becomes consistent in invasive lesions . In the study , we found VEGF positivity in 80 % of LSCC in different tumor stages and

with various differentiation degrees. Although VEGF average score was higher in carcinomas compared to associated dysplastic lesions, we found no significant statistic differences; there were no significant staining differences related to the dysplasia degree. Literature data on VEGF correlation with clinical and histopathological parameters are not homogeneous . While some authors have demonstrated a significant correlation between VEGF expression and clinical parameters for squamous cell carcinomas with different locations in the oral mucosa [151 , 169, 301, 332] , others have found no such correlation [196, 213 , 178, 179 , 9, 330]. ANG -2 immunostaining has been seen in the cytoplasm of 62.5 % of the analyzed lesions; negative cases belong to variable different differentiation degree squamous carcinomas, in different tumor stages . Similar data were communicated by other studies: Ang -2 positivity rate was significantly higher in tumor area compared to non-tumor adjacent tissue or normal oral mucosa (51.22 % vs 26.67 % and 0 , $P < 0.05$) [195]. ANG - 2 mean scores analysis in relation to the analyzed clinicopathological parameters showed statistically significant differences in the differentiation degree (p = 0.014) , T category (P = 0.018) and tumor stage (p = 0.037) . Regarding the Ang-2, VEGF and its receptors R1 and R2 expression, we found no significant statistical relationship between ANG -2 immunoexpression and other analyzed markers.

Chapter VII. - Conclusions presents the study's conclusions :

- The highest level of lips squamous cell carcinomas was recorded in male patients (78.5 %), in the seventh decade of life (34.6 %), predominantly in rural areas (63.5%) .
- 96.2 % of the lesions were located on the lower lip and only 2.8 % of tumors have been located on the upper lip.
- Most patients , 71 % were smokers; alcohol consumption was also frequent; 83 % of the participants were both smokers and alcohol drinkers.
 - Macroscopic aspect of neoplasia determined their classification into the following clinical forms: ulcerated (44.8 %) , vegetative (33.6 %), ulcerated- vegetative (18.6 %) and infiltrated, in 2.8% of cases .
- Most cases were well differentiated forms of squamous carcinomas: 46.7 % had a conventional appearance and only in 1.8 % of cases we have diagnosed warty carcinomas; 34.5 % of the moderately differentiated carcinomas corresponded to conventional forms and in 2.8% of cases to acantolitic squamous cell carcinomas; 13 % of poorly differentiated squamous cell carcinomas corresponded to conventional aspects and only 0.9 % to basaloid carcinoma.
- Vascular- lymphatic analysis invasion revealed its presence in 33.6 % of cases and perineural invasion was identified in 30.8% of the studied cases .
- Surgical resection limits showed tumor invasion in 13.9 % of cases : 11.1 % in only one surgical safety border and 2.8 % at both surgical safety borders.
- Associated lesions to the surgical resection borders have been seen in 85.9 % cases , of which 16 cases of dysplasia , 7 cases of actinic cheilitis and 59 cases of solar elastosis. In 10 cases , the lesions were associated: dysplasia - actinic cheilitis and solar elastosis - actinic cheilitis.
- pTNM system indicated that most of the tumors were classified as stage I of disease

(60.7 %); 9.3 % stage II; 19 4% stage III and 10.2% in disease stage IV .
- The tumors that have been immunohistochemistry analyzed showed a vegetative aspect (35 %) , most of the lesions were well differentiated (40 %) , with various degrees of associated inflammation and invasive patterns. In 9 cases (22.5 %), there have been identified high-grade dysplastic lesions (3 cases) or low grade dysplastic lesions (6 cases), associated to the adjacent carcinomas mucosa.
- CD105 MVD was higher at the front of invasion compared to the intratumoral area , regardless of the clinical and pathological analyzed parameters.
- At the front of the invasion we found a negative linear correlation CD105 MVD / α - SMA MVD, which shows, at least for the invasive tumor growth compartment, increased immaturity of the neoformation vessels.
- IP Ki67 showed significantly higher values in poorly differentiated carcinomas, with discohesiv invasion pattern, vascular, perineural and muscle invasion, in T3- T4 category, with nodal metastasis and advanced tumor stages .
- VEGFR1 and VEGFR2 immunoreactivity was present in the cytoplasm in 67.5 % and 75 % of analyzed lesions; poorly differentiated tumors , in T3/T4 category, in advanced stages and having high IP Ki67 showed significantly higher scores; there has been a positive linear correlation between the two markers and a negative linear VEGF correlation.
- ANG -2 immunoreaction has been seen in the cytoplasm of 67.5 % of the analyzed lesions; moderately differentiated tumors , in T3 category and in advanced stages had the highest scores.
- CD105 , α -SMA and Ki67 staining analysis indicates the invasion front as the tumor area with a exacerbated immaturity degree and a significant proliferative activity. The existence of medium-caliber vessels with proliferative activity in the analyzed carcinomas, rather pleads for the existence of two types of neoformation vessels , some with a proliferative activity and a variable maturity degree (α -SMA±) and others immature, with no proliferative activity (α-SMA-).
- The analysis of VEGF , VEGFR1 and VEGFR2 imunoexpression results indicates a VEGF-/VEGFR1 + / VEGFR2 + immunophenotype in aggressive tumors in advanced stages, and relationships of R1 and / or R2 VEGF receptors to MVD CD105 and MVD α -SMA. Aggressive tumors, with VEGFR1 and VEGFR2 high scores showed a consistent vascular neoformation process and an emphasized vascular immaturity degree.

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