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Prognostic factors in squamous cell carcinoma of the lower lip – an immunohistochemical study

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Abstract

Squamous cell carcinoma (SCC) of the lip represents 15–30% of SCC of cephalic extremity, located on the lower lip in about 90% of cases. The present paper aimed to define the profile of SCC of the lip with major risk factors. The study included 20 selected cases diagnosed with lower lip SCC, using a panel of antibodies which addressed cell proliferation (Ki67), perturbation of the cell cycle (p53), angiogenesis (VEGF – vascular endothelial growth factor), factors related to tumor cell interaction with the extracellular matrix (CD44). Ki67 immunoexpression was identified in all the cases. Poorly differentiated (PD) SCC presented a mean value of Ki67 positivity index (PI) significantly higher compared to well-differentiated (WD) and moderately differentiated (MD) SCC. We found significantly higher mean values of Ki67 PI in pT3 lesion, compared with pT2 and pT lesions, and with no statistically significant differences in lip SCC with associated lymph node metastasis (pN1), compared to those with no lymph node metastasis (pN0). PD SCC presented a higher mean value of p53 PI compared to WD and MD SCC, but without significant differences. Analysis indicated significantly higher values in pT3 lesions and in pT2 and pT1 and in pN1 SCC. In WD SCC, CD44 immunoexpression had a higher intensity. For PD and MD SCC the immunolabelings presented low/moderate heterogeneous intensity. WD lip SCC presented a statistically significant higher mean value for CD44 PI compared to MD and PD SCC, and not statistically significant higher in pT1, pT2 then in pT3 and in pN0 cases then in pN1. WD lip SCC presented statistically significant higher mean value of VEGF PI related to those with MD and PD SCC. VEGF PI values were higher in pT1, pT2 then in pT3 and in the pN0 SCC, but without statistically significant differences. We found a positive linear correlation for Ki67/p53, although statistically not significant and for CD44/VEGF statistically significant (p=0.001). Also, the analysis identified negative a linear statistically significant correlation for Ki67/CD44 and for Ki67/VEGF statistically significant as well (p=0.001). Immunohistochemical investigations in lip SCC, regarding the expression of p53, Ki67, CD44 and VEGF, revealed results that suggest their ability to assess the prognosis and progression of tumor evolution.

Keywords: lower lip squamous cell carcinoma, prognosis, immunohistochemistry.

Introduction

Squamous cell carcinoma (SCC) of the lip is an epithelial malignant tumor, infiltrating and destructive, with lymphatic and/or blood metastatic potential. It represents 15–30% of SCC of cephalic extremity and 1/5 of the upper aerodigestive tract cancers. Lip SCC is located on the lower lip in about 90% of cases [1, 2]. It is estimated that annually occur in the US approximately 40 000 new cases of oral SCC and about 500 000 new cases worldwide. In the US, there are diagnosed each year 3500–4000 new cases of lip cancer, and the incidence is of 2% [3, 4]. Extension is the tendency that characterizes SCC of the lip and it manifests itself, either towards the surface, either in depth, or in both directions. This process is connected to many factors, some dependent on the tumor, some on the patient's history. In the first phase, the evolution is strictly localized in SCC of the lip, afterwards we can discover metastasis in the regional lymphatic nodes, and bone affliction in SCC of the lip represents a localization with a higher risk of metastasis then SCC of the skin, for which there have been described more prognostic factors (clinical and histopathological) [1].

Mean survival rate of patients with SCC of the lip is 90% at two years, and 83% at five years. Patients with T3 or T4 and those with metastasis have unfavorable prognostic. After treatment, patients must be examined periodically for at least five years for finding possible recurrence and eventual regional metastasis. Almost 75% of metastasis appears in the first year after surgery, a period in which examinations must be accurate [2].

The paper aimed as a prime objective and also as an element of innovation, to define the profile of SCC of the lip with major risk. With this cause, we have evaluated and correlated the signification of immunohistochemical parameters (p53, Ki67, CD44, VEGF – vascular endothelial growth factor) counted as being important for establishing the prognosis of patients with this type of lesion.

Materials and Methods

The immunohistochemical study had as an objective finding the correlation between the expression of p53, Ki67, CD44 var. v6, VEGF and the prognostic of SCC of the lip, on o number of 20 selected cases of SCC with localization on the lip, found in different levels of tumor progression. The biological material was represented by samples of surgical excision, which came from the Clinic of Dermatology, Emergency County Hospital of Craiova, Romania, and diagnosed between 2014 and 2015, in the Laboratory of Pathology of the same Hospital. Tumor fragments were fixed in 10% buffered neutral formalin, processed using the classical method of paraffin embedding and Hematoxylin–Eosin (HE) staining.

The primary parameters of prognostic of the lesion, represented by differentiation degree, size/invasion (pT), metastasis in the lymph nodes (pN) and tumor stage were analyzed. In this study, the distribution for the category T and tumor stage were the same.

Immunohistochemical (IHC) analysis was performed by using a panel of antibodies, which addressed cell proliferation (Ki67), perturbation of the cell cycle (p53), angiogenesis (VEGF) and tumor cell interaction with extracellular matrix (CD44), the latter being an indicator of squamous epithelial tumor differentiation. The antibody panel used is described in the following table (Table 1).

Table 1 – ***Antibodies panel***

Antibody	Clone / Manufacturer	Dilution	Pretreatment	External control
Ki67	MIB1 / Dako	1:50	Citrate buffer microwaving, 15 minutes, pH 6	Tongue tonsil
p53	DO-7 / Dako	1:50	Citrate buffer microwaving, 15 minutes, pH 6	Breast carcinoma
CD44	DF1485 / Dako	1:50	Citrate buffer microwaving, 20 minutes, pH 6	Spleen
VEGF	Polyclonal / Santa Cruz Biotechnology	1:200	Citrate buffer microwaving, 20 minutes, pH 6	Kidney

The amplification system used was LSAB™+ Kit Universal (code K0679), and developing the reactions was realized with 3,3'-diaminobenzidine (DAB). The immunohistochemical analysis followed the distribution of the immunolabelings and the intensity of the reactions at the site of SCC. Also, for quantification, an average positivity index (PI) was calculated by reporting the number of positive cells to the total number of cells, on a microscopic objective of 40×. For each case, there were counted 2000 cells in areas with maximum immunohistochemical staining. Image acquisition was performed with a Nikon Eclipse E600 microscope, a color CCD camera, and the Lucia 5 software.

The statistical analysis followed the association of IHC expression of the analyzed markers and in relation to prognostic histopathological parameters. Data were reported as average ± standard deviation (SD), compared using Student's *t*-test, and ANOVA (analysis of variance), while correlations were sought using Pearson's test, in SPSS 10, with *p*-values <0.05 being considered significant.

Results

Histopathological analysis of the investigated cases indicated the predominance of well-differentiated SCC (40%), with a diameter less than 2 cm (65%), in tumoral stage 1 (65%) (Table 2). Regional lymph node metastases were present in only two cases of SCC, poorly differentiated and in advanced stages of evolution.

Table 2 – ***Distribution of analyzed cases***

T category	Differentiation degree		
	Well differentiated (WD)	*Moderately differentiated (MD)*	*Poorly differentiated (PD)*
T1	7	5	1
T2	1	2	1
T3	0	1	2

Ki67 immunoexpression

Ki67 immunoexpression was identified in all the analyzed cases, in the nucleus of the tumor cells and on some stromal elements (Figures 1–3).

The mean value of Ki67 PI for the analyzed group was of 42.4±19.6. In this stage, we found differences in expression of Ki67 in related to differentiation degree and size/stage of the lip SCC (Table 3).

Table 3 – ***Ki67 PI values in rapport with analyzed histological parameters***

Parameter	Variable	Mean Ki67 PI (*p*-value)
Differentiation degree	Well differentiated	30.6±10.1
	Moderately differentiated	37.8±11.3
	Poorly differentiated	75±10.8
		*p=0.000
Size (pT)/ Stage	T1	35.2±14.2
	T2	46.2±16.5
	T3	68.3±25.6
		*p=0.001
Lymph node metastasis (pN)	N0	37.9±14.6
	N1	82.5
		**p=0.061

*p-value for ANOVA test; **p-value for Student's t-test.

Therefore, in relation to differentiation degree of the tumors, lip SCC with low differentiation presented a mean value of Ki67 PI of 75±10.8. Compared to cases with well and moderately differentiated carcinomas, the values were of 30.6±9.1 and 37.8±11.3, statistical analysis indicating significant differences (p=0.000, ANOVA) (Figure 4).

For the category pT of analyzed lip SCC, we found significantly higher mean values of Ki67 PI in pT3 lesion, respectively of 68.3±25.6, in comparison with pT2 and pT1, the values being of 46.2±16.5 and 35.2±14.2 (p=0.001, ANOVA) (Figure 5).

Although the values of Ki67 PI were superior in cases of lip SCC with associated lymph node metastasis (pN1), 82.5, compared to those who did not have lymph node metastasis, being of 37.9±14.6, statistically these aspects being not significant (p=0.061, Student's t-test).

The nuclear antigen Ki67 is associated with cell

proliferation and is used to determine the rate of tumor proliferation and at the same time, it is useful in identifying some types of aggressive cancer and their metastatic potential.

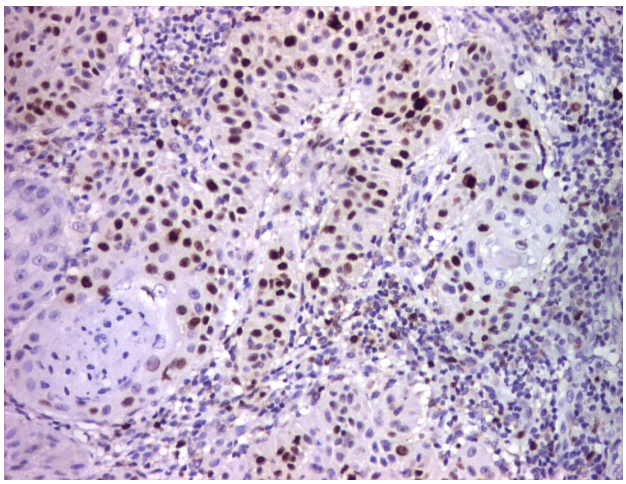

Figure 1 – ***Well differentiated squamous cell carcinoma (Ki67 immunostaining, ×100).***

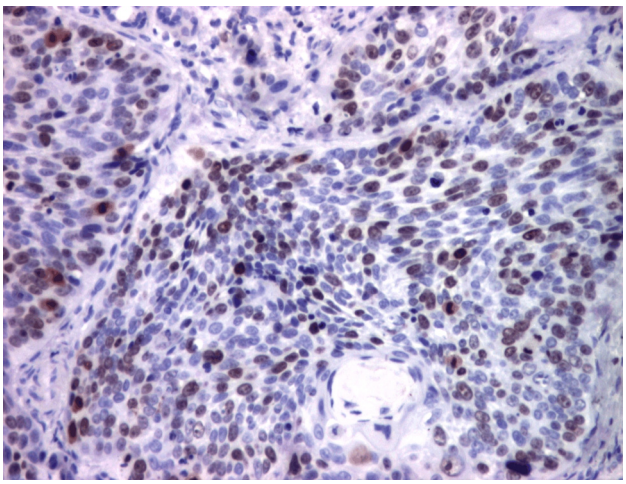

Figure 2 – ***Moderately differentiated squamous cell carcinoma (Ki67 immunostaining, ×100).***

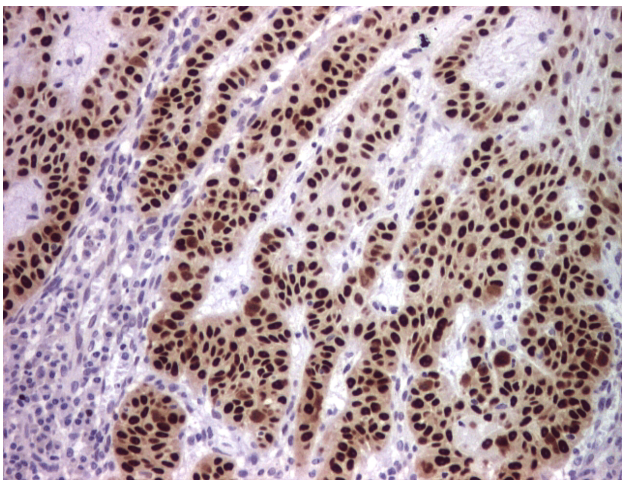

Figure 3 – ***Poorly differentiated squamous cell carcinoma (Ki67 immunostaining, ×100).***

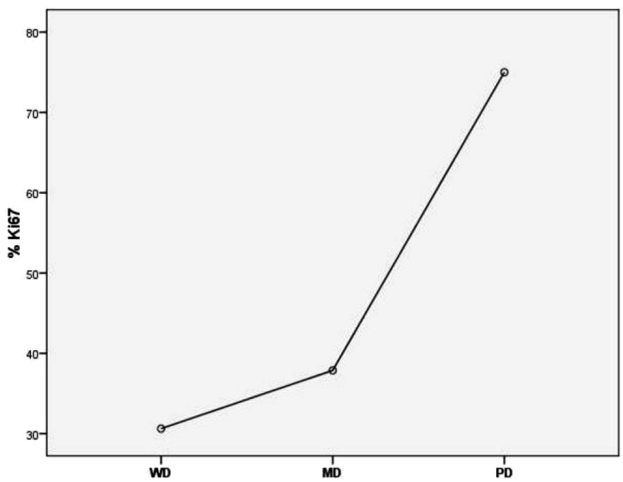

Figure 4 – ***Ki67 PI values in rapport with differentiation degree. WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated.***

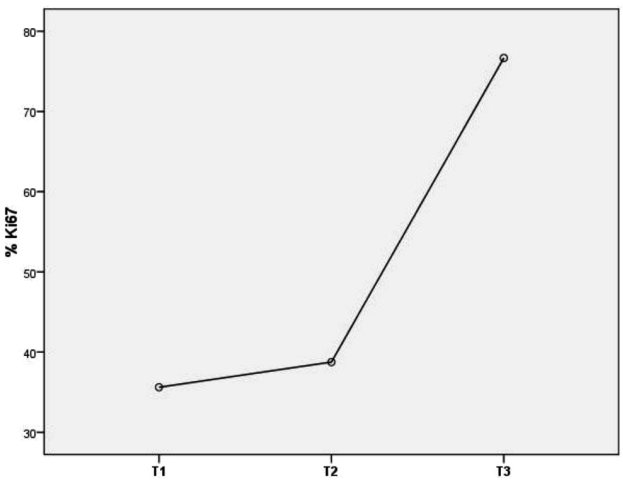

Figure 5 – ***Ki67 PI values in rapport with pT category.***

p53 immunoexpresion

p53 immunoexpresion was identified in the tumoral cell's nucleus, the mean value of p53 PI for the analyzed group being of 31.5±12.9. In this stage, we found significant differences in the expression of p53 in relation to the pT and pN categories of lip SCC (Table 4).

In the case of well and moderately differentiated carcinomas, the stainings were with low/moderate intensity, predominantly in the periphery of the tumor islands, while for poorly differentiated carcinomas the markings represented a heterogeneous distribution with a moderate/high intensity (Figures 6–8).

In relation to the tumor differentiation degree, poorly differentiated SCC presented a mean value of p53 PI of 33.7±16.5, in comparison with well and moderately differentiated SCC, in which the values were of 30.8±8.6 and respectively 32±16, the aspect being of no statistical significance (p=0.898, ANOVA) (Figure 9).

Analysis of size/tumoral stage indicated significantly higher values of p53 PI in pT3 lesions, 53.3±10.4, in comparison with those found in pT2 and pT1, those being 36.2±8.5 and respectively 25±7.6 (p=0.009, ANOVA) (Figure 10).

Also, p53 staining values were significantly higher in cases of SCC who were associated with lymph node metastasis (pN1), 45.5, compared to those who did not have associated metastasis, 29.7±12.3 (p=0.005, Student's t-test).

Table 4 – ***p53 PI values in rapport to analyzed histological parameters***

Parameter	Variable	Mean p53 PI (*p*-value)
Differentiation degree	Well differentiated	30.8±8.6
	Moderately differentiated	32±16
	Poorly differentiated	33.7±16.5
		*p=0.898
Size (pT)/ Stage	T1	25±7.6
	T2	36.2±8.5
	T3	53.3±10.4
		*p=0.009
Lymph node metastasis (pN)	N0	29.7±12.3
	N1	47.5
		**p=0.005

*p-value for ANOVA test; **p-value for Student's t-test.

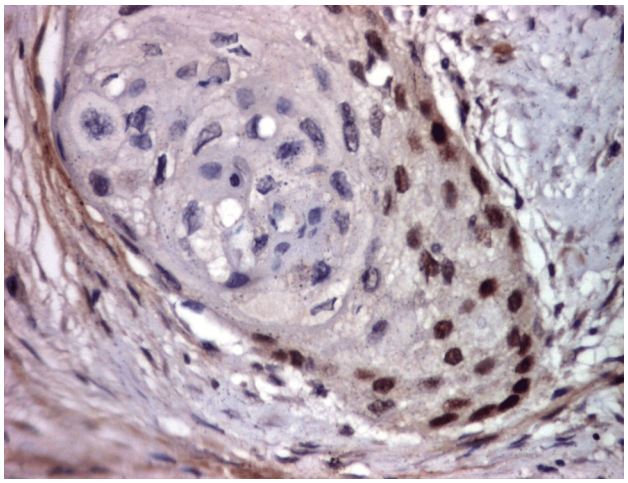

Figure 6 – ***Well differentiated squamous cell carcinoma (p53 immunostaining, ×200).***

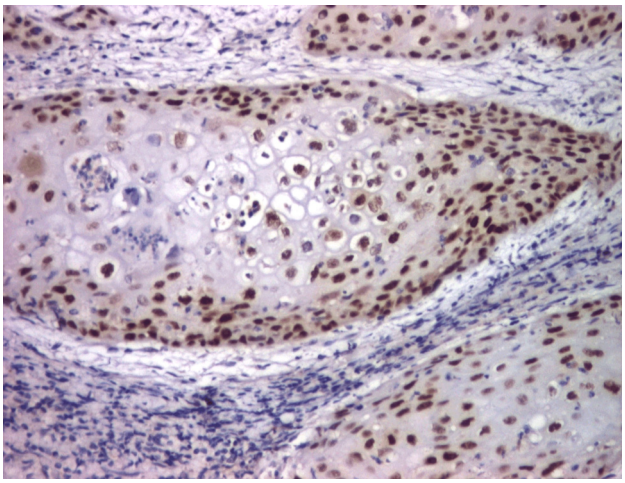

Figure 7 – ***Moderately differentiated squamous cell carcinoma (p53 immunostaining, ×100).***

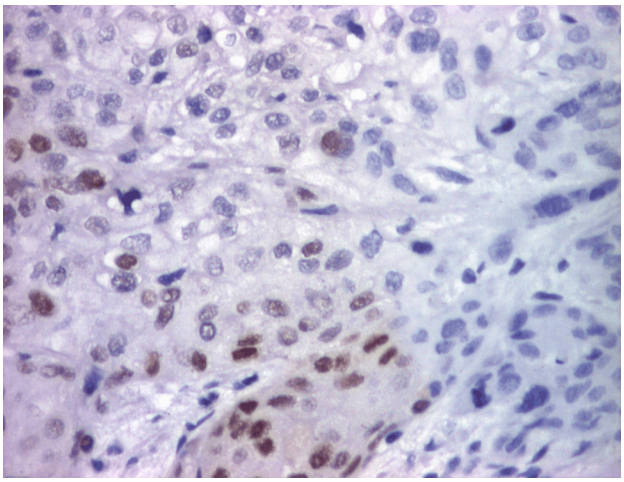

Figure 8 – ***Poorly differentiated squamous cell carcinoma (p53 immunostaining, ×100).***

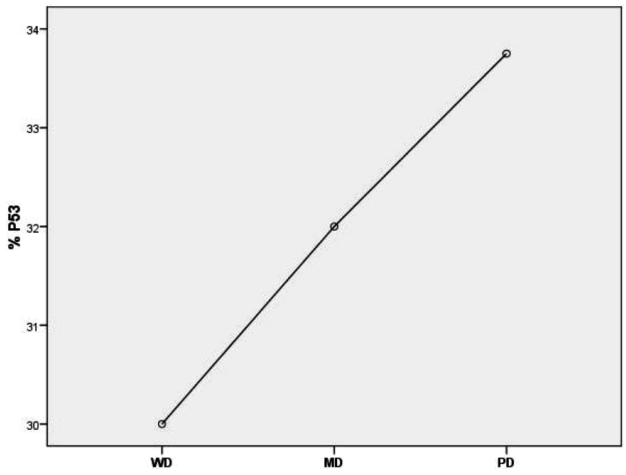

Figure 9 – ***p53 PI values in rapport with differentiation degree. WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated.***

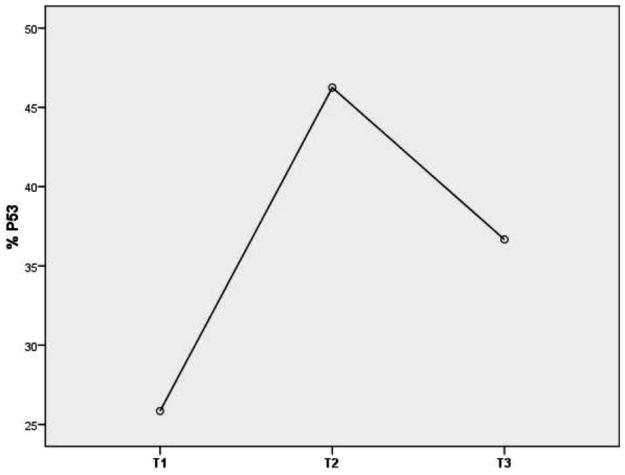

Figure 10 – ***p53 PI values in rapport with pT category.***

CD44 immunoexpression

CD44 immunoexpression was identified in tumor cells in 90% of the cases, with a membrane and apical cytoplasmic localization. The reaction was evident also in stromal elements, such as lymphocytes, plasma cells, macrophages and fibroblasts. In the case of well-differentiated carcinomas, the markings had a higher intensity; diffuse in the tumoral islands, the intensity being superior in the peripheral area. For low and moderately differentiated carcinomas, the markings presented low/moderate heterogeneous intensity (Figures 11 and 12).

The mean value of CD44 PI for the entire group was of 51±16.2, in this study we found significant differences in the expression of CD44 related to tumoral differentiation degree (Table 5).

Well differentiated lip SCC presents a mean value for CD44 PI of 64.3±12.6, in comparison with moderate and low differentiated carcinomas, in which the values were of 44±8.5 and 31.6±2.8, the aspect being statistically significant (p=0.000, ANOVA) (Figure 13).

In relation to pT, CD44 PI values in cases of lesions pT1, pT2, pT3 were of 53.5±14.1, 56.6±25.1 and 35±5, but the differences were not statistically significant (p=0.169, ANOVA) (Figure 14).

Table 5 – ***CD44 PI values in rapport with analyzed histological parameters***

Parameter	Variable	Mean CD44 PI (p-value)
Differentiation degree	Well differentiated	64.3±12.6
	Moderately differentiated	44±8.5
	Poorly differentiated	31.6±2.8
		*p=0.000
Size (pT)/ Stage	T1	53.5±14.1
	T2	56.6±25.1
	T3	35±5
		*p=0.169
Lymph node metastasis (pN)	N0	53.3±15.7
	N1	39.5
		**p=0.086

*p-value for ANOVA test; **p-value for Student's t-test.

In relation to the presence of metastasis, CD44 staining values were superior in cases of SCC which had no lymph node metastasis (pN0), 53.3±15.7, compared with those who presented lymph node metastasis, respectively 39.5, but the aspect was not statistically significant (p=0.086, Student's t-test).

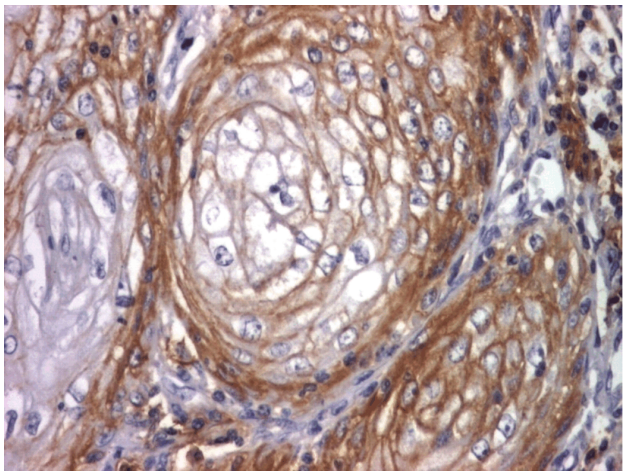

Figure 11 – ***CD44+ well differentiated squamous cell carcinoma (CD44 immunostaining, ×200).***

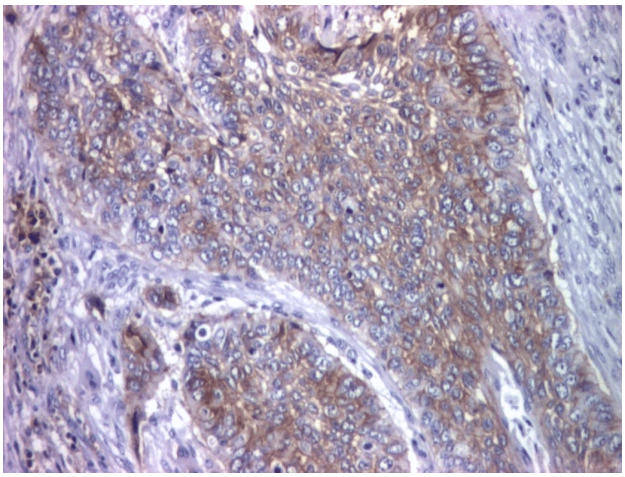

Figure 12 – ***CD44+ poorly differentiated squamous cell carcinoma (CD44 immunostaining, ×100).***

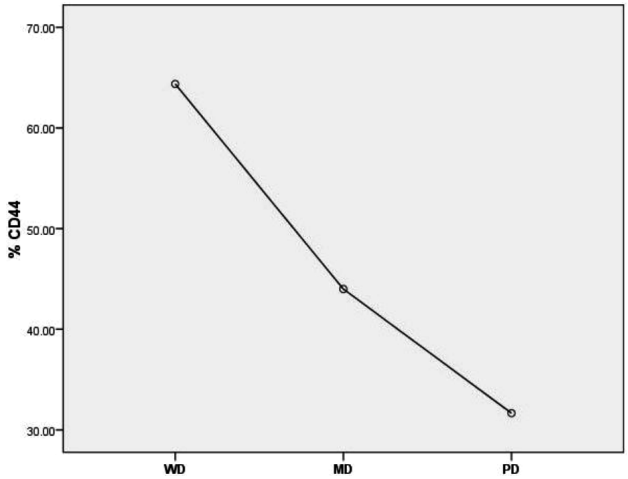

Figure 13 – ***CD44 PI values in rapport with differentiation degree. WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated.***

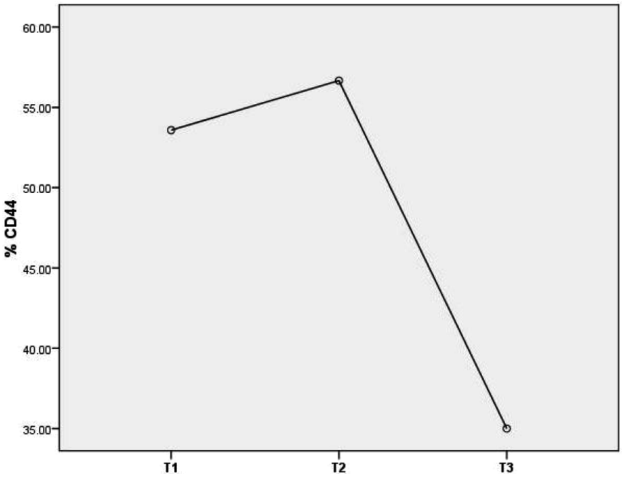

Figure 14 – ***CD44 PI values in rapport with pT category.***

VEGF immunoexpression

VEGF immunoexpression was observed in the cytoplasm of tumoral cells in 85% of the analyzed cases, and also in some stromal elements such as lymphocytes, plasma cells, fibroblasts, endothelial cells (Figures 15–17).

The mean value of VEGF PI for the analyzed group was of 43.8±14. In this study, we found significant differences of VEGF expression related to tumoral differentiation degree (Table 6).

Therefore, well differentiated lip SCC presented a mean value of VEGF PI of 55.5±8.1, related to those with moderate and poorly differentiated degree in which values were of 44.6±8.7 and 20±5, the aspect being statistically significant (p=0.000, ANOVA) (Figure 18). In the case of well and moderately differentiated carcinomas, staining levels were moderate/increased, diffuse in the tumoral islands, while for the poorly differentiated carcinomas the staining had low intensity.

In relation to pT, VEGF PI values in cases of lesions type pT1, pT2 and pT3 were of 42.2±13, 49 and 22.5, but the aspect was not statistically significant (p=0.059, ANOVA) (Figure 19).

In relation to the presence of metastasis, VEGF markings values were superior in the case of oral squamous cell carcinoma (OSCC) with associated lack of lymphatic node metastasis (pN0), respectively 46.7±12.3, in comparison to those with associated metastasis, respectively 32.5, but these differences were not statistically significant (p=0.108, Student's t-test) (Table 6).

Table 6 – ***VEGF PI values in rapport with analyzed histopathological parameters***

Parameter	Variable	Mean VEGF PI (p-value)
Differentiation degree	Well differentiated	52.2±8.1
	Moderately differentiated	44.6±8.7
	Poorly differentiated	20±5
		*p=0.000
Size (pT)/ Stage	T1	46.2±13
	T2	49
	T3	22.5
		*p=0.059
Lymph node metastasis (pN)	N0	46.7±12.3
	N1	32.5
		**p=0.108

*p-value for ANOVA test; **p-value for Student's t-test.

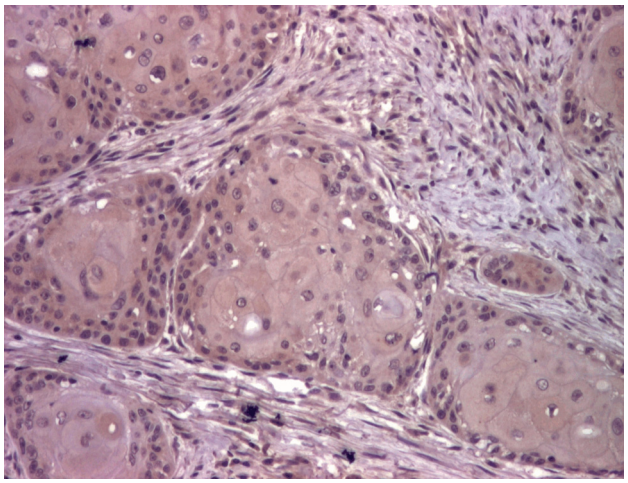

Figure 15 – ***VEGF+ well differentiated squamous cell carcinoma (VEGF immunostaining, ×100).***

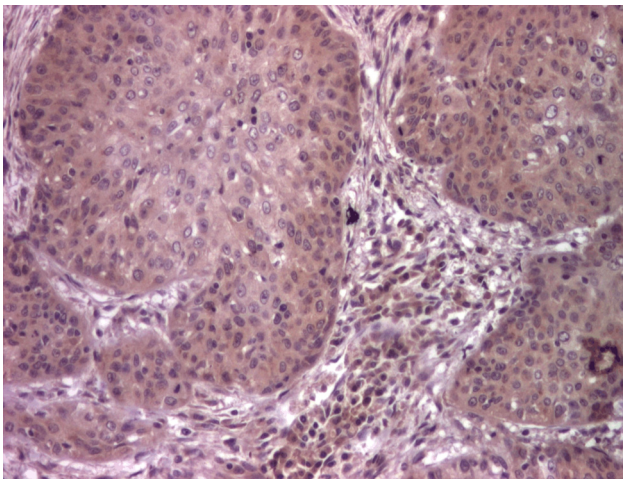

Figure 16 – ***VEGF+ moderately differentiated squamous cell carcinoma (VEGF immunostaining, ×100).***

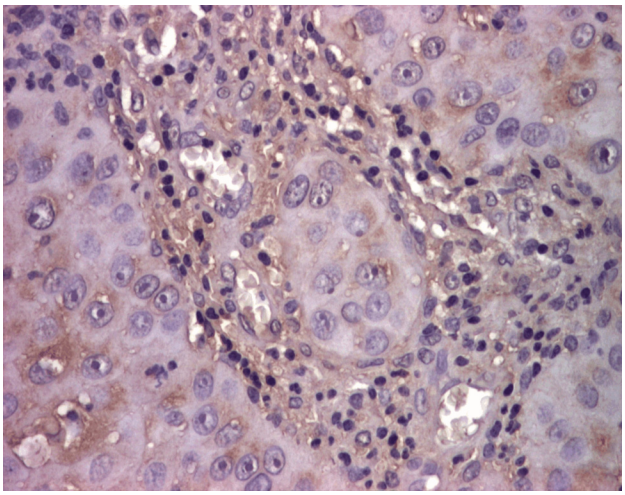

Figure 17 – ***VEGF+ poorly differentiated squamous cell carcinoma (VEGF immunostaining, ×200).***

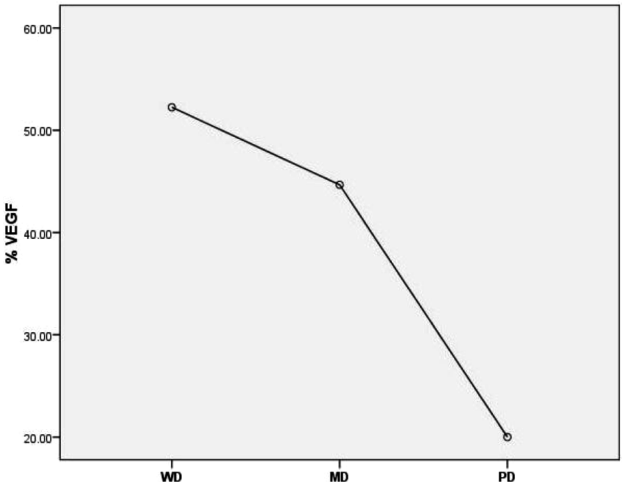

Figure 18 – ***VEGF PI values in rapport to differentiation degree. WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated.***

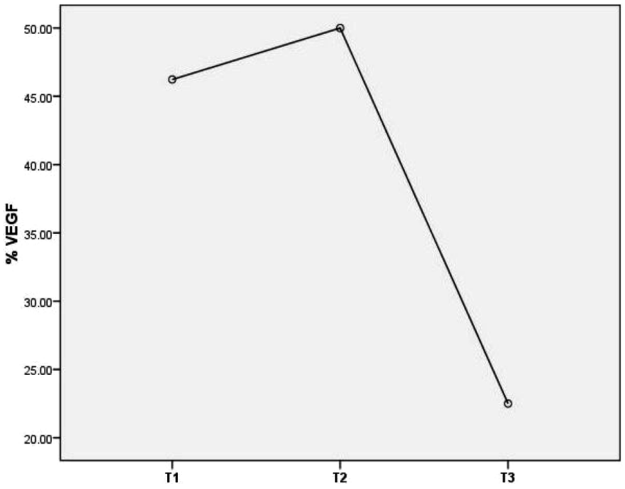

Figure 19 – ***VEGF values in rapport with pT category.***

The analysis of the markers value distribution with Pearson's test identified positive linear relations between Ki67/p53, statistically not significant (p=0.332), and between CD44/VEGF statistically significant (p=0.001) (Figure 20).

Also, the analysis of the markers value distribution in the Pearson's test identified negative linear relation statistically significant for Ki67/CD44 (p=0.035), and between Ki67/VEGF, statistically significant (p=0.001) (Figure 21).

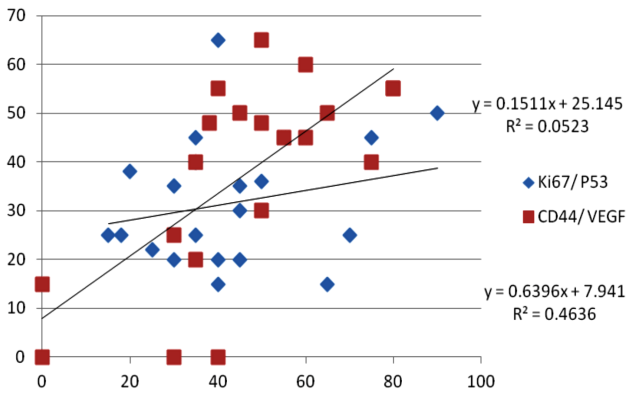

Figure 20 – ***Distribution of percentage values for Ki67/p53 and CD44/VEGF.***

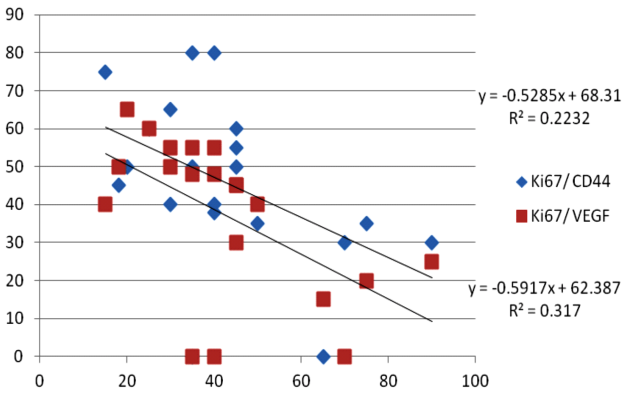

Figure 21 – ***Distribution of percentage values for Ki67/CD44 and Ki67/VEGF.***

Discussion

Lip SCC affects with predilection the inferior lip of men over the age of 60, with prolonged exposure to sun and who smoke. SCC of the lip starts frequently on lesions with malignant potential, especially on chronic keratosic cheilitis, thus revealing the importance of early diagnosis and correct treatment of preblastomatous cheilitis [3]. In the first phase, the evolution is strictly local of SCC of the lip; afterwards, we can find metastasis in the regional lymphatic nodes, affliction of the nearby bones and rarer far metastasis. In 2000, the *American Society for Cancer*, reported that on a global level 389 650 of such new cases, of which 266 672 were SCC of the oral cavity and 122 978 of the pharynx. These represented 5% of cancer in men and 2% of cancer in woman [4]. Lately, there are mentions of a continuous increase in the incidence of lesions in woman and younger people, especially of the lingual localization [5]. Data regarding evaluation of lesions for both sexes indicate the fact that this type of cancer represents the 4th most common type of cancer for men and 6th for woman [6, 7].

In SCC of the lip, regional lymph node metastases are the most common secondary lesions. The frequency of these, reported to data in specialty literature, differs in large limits from 2.7% (Breuninger) to 37% (Stoll) [8, 9]. The mean is close to 8–10%. Higher numbers are offered by studies made on patients from the services of the oro-maxilo-facial-surgery departments [10]. After the centralization of many studies, totaling in 11 094 cases of lip cancer, a study has found metastasis after five years of surveillance in 13.7% of patients. Most of lymph node metastases appear in the first two years of evolution of lip SCC. These are more frequent in cases with T2 and T3, in comparison with patients in T1 [1].

Extraganglionary metastasis are very rare in lip SCC (<1% of cases) and bone affliction in the course of the evolution of the tumor, is because late diagnosis and afterwards tumor aggressively. As far as recurrences are concerned, after the treatment of the primary tumor, these were correlated with cancer of great size and with low histological differentiation of the primary lesion. The frequency of these is of 11.3% (Korenvs *et al*. – study on 189 patients), close values (10.8%) being found in another study on 223 patients [11].

In a large study published by Sousa *et al.*, in 2009, 24 cases of varying degrees of dysplasia there were not identified differences regarding p53 expression, while in 2008, Angiero *et al.* noticed in a study of biopsies from 58 specimens of the same type of injuries, an increased expression of p53 and Ki76 in higher degrees of dysplasia. Authors conclude that the p53 is potentially predictive for evolution of a dysplasia to invasive carcinoma. Both p53, Ki67 and can be used as prognostic markers in assessing of oral SCC [12, 13].

Apparently, Ki67 overexpression is associated with the idea of uncontrolled growth and proliferation of tumor cells, thus it would be associated with prognostic factors and requires prioritization of approaching these patients [14]. A retrospective Canadian study showed that overexpression of Ki67 is correlated with an increased risk of recurrent squamous cell carcinoma of the cervix after radiotherapy and therefore such cases should be subject to a more targeted treatment. In early stages in squamous carcinomas of the head and neck in particular has demonstrated that overexpression of markers such as p53 and Ki67 is a high risk factor and a factor of worse prognosis [13, 15].

An important factor implicated in the process of malignancy of the squamous epithelium and in the progression of carcinomas of the head and neck is represented by the adhesion molecules, especially CD44, respectively the v6 variant of it (CD44 var. v6). This molecule has the tendency to disappear in premalignant and malignant lesions, but is relatively conserved in basal cell carcinoma [16], a tumor in which individual tumor areas have been showed to keep contact with the basement layer of the epidermis or of the hair follicle appendage [17]. Also, it has been established the existence of a good correlation between the level of expression of this molecule and the rate of survival of patients with SCC of the lip. Therefore, these patients with a lower expression of CD44 have a more reserved prognostic [18].

Thirty studies with 2102 patients met the inclusion criteria for the meta-analysis. Fifteen studies used anti-pan-CD44 antibody, nine used anti-CD44-v6 antibody, two used anti-CD44-v3 and two used anti-CD44s antibody, one used anti-CD44-v9, and one used anti-CD44-v6, -v3 and -v4/5 simultaneously. CD44 has been reported to be involved in tumor growth and metastasis and has also been implicated as a SCC marker in head and neck squamous cell cancer (HNSCC) and suggested that CD44 is related to worse T category, N category, tumor degree and prognosis, in pharyngeal and laryngeal cancer, but no clear association was revealed between CD44 expression and oral cancer [19]. Also, some data suggest CD44 variant isoforms may be important molecular markers and possible therapeutic targets in HNSCC treatment [20].

Another factor, which can influence the aggressiveness of the tumor, is VEGF, a protein whose production is stimulated in conditions of hypoxia and which promotes increases in vascular permeability and endothelial cell proliferation. In squamous cell carcinoma, a high level of VEGF is associated with a higher rate of local recurrence, far metastasis and a lower rate of survival. These findings open up new therapeutic perspectives, along with the classical ones, in an attempt to increase the rate of survival of patients with oral cancer including those of the lip [21, 22].

Many other studies have found that the level of expression of VEGF in positive oral SCC increases with the invasion depth during the evolution of the tumor. Also, it has been shown that a higher expression of VEGF occurs in tumoral cells located in the vicinity of necrosis areas. It was suggested that hypoxia was responsible for this, thus regulating the expression of VEGF but also HIF-1α (hypoxia-inducible factor 1-alpha). The majority of studies on oral SCC have shown that there is no association between the level of VEGF expression and the histological degree of the tumor. In contradiction with this fact, other studies, including this one have found a significant correlation between VEGF expression and histological tumor degree; therefore, VEGF expression was lower in poorly differentiated carcinomas [23].

Conclusions

Immunohistochemical investigations in SCC of the lip, regarding the expression of p53, Ki67, CD44 and VEGF suggest their ability to assess the prognosis and progression of tumor evolution. By corroborating clinical, histopathological and immunohistochemical data, we will have the possibility of creating the profile of lip SCC with major risk (increased metastasis potential, bone affliction, recurrences). Knowing the prediction factors of the evolution of this cancer will be decisive in choosing the methods for optimum treatment, which will lower the number of recurrences and will increase the rate of survival of patients with SCC of the lip.

Conflict of interests

The authors declare that they have no conflict of interests.

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