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Oral squamous cell carcinomas: a histopathological review of multiple cases from Western Romania

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Abstract

Malignant tumors of the oral cavity have a growing incidence, most being squamous cell carcinomas, generally called oral cancers (OCs), clinically detected at various stages of natural evolution. The increased incidence in Romania in recent years and the lack of conclusive data have led to the development of this study. The main purpose of this study was to assess the molecular profile of tumors, the types of blood vessels associated with the tumor, and expression of tumor immunomarkers. Regarding morphological findings, focal epithelial hyperplasia, dysplastic lesions, typical mitoses, perineural invasion, parakeratosis and keratosis beads, intracytoplasmic keratinization were observed. Microvascular density was higher in the tumor area compared to the peritumoral area. Lymphovascular invasion was identified in 13% of cases, which also presented regional lymph node metastases. Podoplanin expression was identified in 79% of cases which were tested positive for the D2-40 immunomarker. All p53-positive cases co-expressed epidermal growth factor receptor (EGFR), half of the EGFR-positive cases co-expressed p53, and co-expression of CD117 and p63 was identified in 80% of EGFR-positive/cytokeratin 5 (CK5)-positive cases being proposed the basal-like subtype of OCs, defined as EGFR-positive/CK5-positive, CD117-positive and p63-positive. Results support the need for molecular classification of OCs based on of tumor immunomarker expression and gene analysis.

Keywords: squamous cell carcinoma, morphology, lymphatic features, molecular markers.

Introduction

Oral cancers (OCs) are commonly referred to as non-homogenous cancers in the form of head and neck squamous cell carcinomas (HNSCCs). OCs cause significant negative effects on patients' life quality, being of major importance in multiple studies addressing public health policies, prevention, and treatment of these diseases. According to the *International Classification of Diseases* (ICD), OCs include all malignant changes in the lips and oral cavity, salivary glands, oropharynx, nasopharynx, hypopharynx, and larynx [1]. As stated by data published by the *International Agency for Research on Cancer* (IARC), these types of malignancies caused over 240 000 deaths in 2012, increased to over 350 000 in 2018, reaching over 440 000 deaths in 2020. Regarding the number of new cases, in 2012 approximately 443 000 were reported, 710 000 in 2018 and during 2020 the number of new registered cases reached 878 348 [2–4]. Lips and oral cavity cancers are the sixth most common cancers worldwide, while larynx, pharynx and salivary glands occupies positions 21, 24 and 28 [5, 6]. The OCs group includes a very wide spectrum of malignant lesions with different tumor biologies, which implies a variable prognosis and a heterogeneous response to therapy [7].

Currently, the prognosis of patients with OCs is still very poor and reflects the fact that, although the risk factors for OCs are well known [*e.g.*, smoking, alcohol consumption, ultraviolet (UV) radiation exposure, immunodeficiency diseases, poor nutritional status, human papillomavirus (HPV) infection, blood dysfunctions: leukoplakia and/or erythroplasia], lack of data related to molecular mechanisms responsible for this malignant disease has a devastating effect [5]. The actual classification of head and neck tumors

is based on their anatomical distribution and morphology, aspects that lead to the application of a common therapy, homogeneous for a heterogeneous group of malignancies [8].

Increased incidence in Romania in recent years and lack of conclusive diagnostic data and approach led to the elaboration of the present study. Early detection of these pathologies by initiating and implementing a national screening program, to be carried out by example in dental offices, family medicine would be an important step in the prevention and early detection of various malignant and premalignant conditions located in the oral cavity. A variety of chemotherapeutic drugs used to treat cancer have limited efficacy, due to problems of release, penetration, and a moderate degree of selectivity for tumor cells, thus causing severe damage to healthy tissues.

Aim

The main purpose of this study was to assess: (*i*) the microscopic peculiarities of the carcinomas selected to study the molecular profile of these tumors, (*ii*) the types of blood vessels associated with the tumor, as a potential therapeutic target, together with the elucidation of the origin of the neoformation vessels and (*iii*) expression of tumor immunomarkers.

Materials and Methods

The current study represents a retrospective review of the cases of squamocellular injuries of the head and neck region from the Clinic of Oral and Maxillofacial Surgery and from the Ear, Nose and Throat (ENT) Clinic and analyzed at the Department of Microscopic Morphology within the Victor Babeş University of Medicine and Pharmacy, Timişoara, Romania, over three years, between 2017 and 2020. The samples included were OCs from lips, oral mucosa, tongue, pharynx, and larynx. There were 92 samples, 42 were resections and 50 were biopsies (the most relevant of the 114 biopsies evaluated), which were evaluated from the histopathological point of view. None of the patients received chemotherapy or radiation therapy before sample collection. The Human Ethics Committee of Victor Babeş University of Medicine and Pharmacy, Timişoara, approved this study, by Ethics Statement with No. 4/03.03.2017, and informed consent was obtained from all subjects involved in the study.

Biopsies were included in paraffin for morphological and molecular evaluation at the protein level. The antibodies used in this part of the study were: epidermal growth factor receptor (EGFR), cytokeratin 5 (CK5), B-cell lymphoma-2 (Bcl-2) and E-cadherin. In cases where CK5 and EGFR were positive, methods for Bcl-2 and E-cadherin were performed (Table 1). In cases where CK5 and EGFR were positive, methods for p53 protein and stem cell growth factor receptor, cluster of differentiation 117 (CD117) or c-kit, were performed to identify cells with stem element potential. The immunohistochemical (IHC) procedure used a Biotin-free working and visualization system [Bond Refine Detection System, 3,3'-Diaminobenzidine (DAB), Leica Microsystems], and the final reaction product was stained brown at nuclear level (for p53 and p63), cytoplasmic (for CK5 and CD117) and membrane (for EGFR and E-cadherin). Co-localization of p53 with EGFR, and p63 with CD117 was performed by standard double immunohistochemistry to which Bond Polymer Refine Red Detection System was added.

Table 1 – ***Antibodies, working system, and expression of the final product***

Antibody	Clone	Dilution	Antigen retrieval	Incubation	Working system, chromogen	Expression
Ki67	MIB1	RTU	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Nuclear
p53	DO7	RTU	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Nuclear
Bcl-2	124	RTU	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Nuclear, cytoplasmic
E-cadherin	NCH 38	1:100	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Membrane/ cytoplasmic or both
CK 5/6	D5/16 B4	1:80	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Cytoplasmic
EGFR	Polyclonal	RTU	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	EGFR pharmDx™ visualization reagent, DAB	Membrane, cytoplasmic
CD117	c-kit	1:300	MW, 30 minutes citrate buffer, pH 6	30 minutes, RT	LSAB+/HRP, DAB	Cytoplasmic, granular

Bcl-2: B-cell lymphoma-2; CD117: Cluster of differentiation 117; CK 5/6: Cytokeratin 5/6; DAB: 3,3'-Diaminobenzidine; EGFR: Epidermal growth factor receptor; HRP: Horseradish peroxidase; LSAB: Labeled Streptavidin–Biotin; MW: Microwave; RT: Room temperature; RTU: Ready-to-use.

IHC method was performed in a fully automated and standardized procedure for all cases and sections, with the Leica Bond-Max immunohistochemistry machine (Leica Biosystems, Newcastle upon Tyne, UK). Paraffin sections were subjected to the antigen unmasking procedure with Bond Epitope Retrieval Solution 2 for 20 minutes (Leica Biosystems). Endogenous peroxidase was blocked with 3% hydrogen peroxide for five minutes. The sections were then treated with the primary antibody. The Bond Polymer Refine Detection System was used for visualization, which includes the secondary antibody (eight minutes) and the polymer with an incubation time of eight minutes. The final reaction product was visualized with DAB dihydrochloride (10 minutes) and the nuclei were stained with modified Lillie's Hematoxylin. The stained sections were permanently mounted with Canada balsam.

The stained sections were examined with the Nikon Eclipse600 photon microscope, the images being taken with the Coolpix950 digital camera. Regarding the nuclear immunomarkers and the microvascular density, in selected cases the analysis of the microscopic image was performed with the computer program provided by Nikon, LuciaNet, on images purchased in *JPEG* format, at ×400 calibration. The image analysis was performed according to the hot-spot method, *i.e.*, the maximum density identified, choosing the microscopic fields with maximum density of positive

signals. The number of positive signals and the area were calculated.

Statistics

The frequency of distribution according to age, nodules status, tumor size, histopathological type, histological degree of differentiation was compared using the χ^2 (*chi*-squared) test with the odds ratio (OR) and 95% confidence interval (CI). We used the Statistical Package for Social Sciences (SPSS) 17.0 calculation system, the commercially available version. When we obtained a value of $p<0.05$, we considered it statistically significant. The final reporting was performed in descriptive terms for all cases and statistics for the sublots defined above.

Results

Among the clinicopathological parameters with prognostic relevance, the tumor (T) element was analyzed, most cases being T3 (45%) and T2 (38%). Only one case was T1, signaling once again the deficient early detection, and six were T4 cases. The aggressive character of these tumors also results from the distribution of cases according to the lymph node (N) element: 64% cases with N1, 26% with N2 and 9.5% with N3. Remote metastases were noted in four cases, the rest being classified as M0.

Morphological evaluation and degree of differentiation

In all cases included in the study, the resection margins showed normal tissue, including the covering epithelium and the lamina propria. Most of the usual morphological changes identified in the case of histopathological analysis of premalignant and malignant oral lesions are shown in Figure 1.

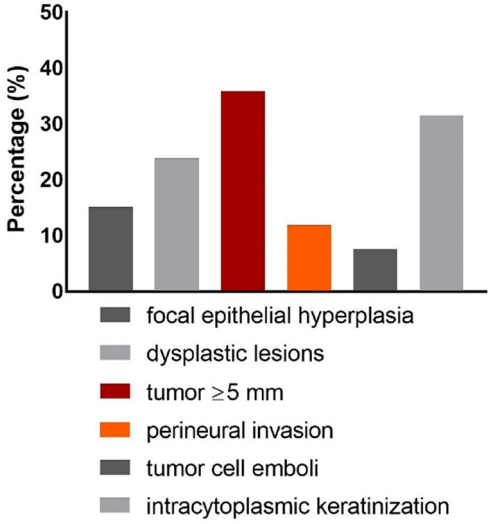

Figure 1 – ***The rate of occurrence of the usual morphological changes detected in the case of premalignant and malignant oral diseases in 92 analyzed samples.***

Focal epithelial hyperplasia was observed in 14 of the 92 (15.2%) cases in the epithelium adjacent to malignant proliferation. Microscopically, it was highlighted the numerical increase of the cells of the basal layer, and the presence of the ballooned cells, with pale or chromophobic cytoplasm in the intermediate layer. The lamina propria corresponding to focal epithelial hyperplasia showed collagen fibers arranged in thick bundles and increased density of inflammatory infiltrate compared to normal mucosa. Dysplastic lesions present in the immediate vicinity of cancer proliferation were observed in 22 of the 92 (23.9%) studied cases. Dysplastic cells, showed signs of immaturity, had the appearance of transition between basal and normal intermediates, with medium size, polygonal shape, round and relatively equal nuclei, but with the presence of prominent nucleoli and fine granular chromatin. Typical mitoses have been very rare in many cases. In the area of the inflammatory infiltrate were noticed numerous small blood vessels, with a very thin wall and content of blood cells. Tumors diagnosed with squamous cell carcinoma (SCC) showed diffuse proliferation of tumor cells located in the form of lobes, cords, nests, or compact, large beaches of different sizes, invading the stroma. All carcinomas included in this study were invasive, and of these, the tumor exceeded 5 mm in depth in 33 (35.9%) of the patients. The most common variant was tumor growth in the form of nodules of cancer cells, delimited by stroma with variable extension and high-density inflammatory infiltrate. In some cases, at the proliferation front, condensed connective tissue was observed, with the formation of dense bands of collagen fibers.

The dominant form in terms of the distribution of invasive tumor cells was in the form of islands and beaches, delimited by variable amounts of stroma, containing inflammatory cells with different densities from one case to another or even for the same case. In four cases, the tumor cells were arranged in the form of anastomosed and branched cords. Perineural invasion was identified in 11 of the 92 (11.95%) cases and tumor cell emboli in the lumen of the blood/lymph vessels were observed in seven (7.6%) of the cases.

The defining aspect of keratinization was represented by keratosis and parakeratosis beads, which have been observed in most well-differentiated cases. The parakeratosis and keratosis beads had different sizes, being formed by cells arranged concentrically, with various degrees of degeneration in the central area, which in some cases had a microcystic appearance. Sometimes the cavity was bounded by apparently viable tumor cells. In well-differentiated tumors, these areas often present as intensely acidophilic, amorphous masses, occupying large areas within the proliferation. Intracytoplasmic keratinization was highlighted in 29 of the 92 (31.5%) cases and was characterized by intense and homogeneous acidophilia which sometimes tends to mask the contour of the nucleus. Even under these conditions, at the nuclear level there are nucleoli enlarged in volume, multiple and different in size for the same nucleus, an important criterion for assessing cellular malignancy.

Lymphatic microvascular density and podoplanin expression in tumor cells

The expression of anti-podoplanin (anti-D2-40) antibody was further studied. Were monitored: (*i*) the presence, morphology, and density of lymphatic vessels, (*ii*) the

presence of lymphovascular invasion and (*iii*) D2-40 expression in tumor cells. Of the cases included in the study, 29 (31.5%) had regional lymph node metastases. Lymphatic vessels were identified in all samples as well-defined lumen structures, free of blood cells, thin-walled, with no perivascular cells around, and lymphatic endothelium was stained homogeneously with D2-40. The immunoreaction for D2-40 was positive in the lymphatic vessels, like normal tissues and did not stain the endothelium, which allows the differentiated calculation of the blood and lymphatic vessels. In the peritumoral area, lymphatic vessels were identified, which had a wide lumen, a sinuous trajectory, without surrounding peri-vascular cells (Figure 2, a and b). In the tumor area, elongated lymphatic vessels with irregular contours were identified, present both in the vicinity and in contact with tumor cells. Sporadically, the marked lymphatic vessels partially surrounded relatively large groups of tumor cells, which may give the impression of inclusion in a future lumen. Regarding the microvascular density, it was higher in the tumor area (Figure 2c) compared to the peritumoral area being also significantly higher compared to the normal mucosa. The morphology of the vessels was also different, in the peritumoral area the lumen was wide and approximately regular, while in the tumor area the dimensions were small, and the contour was irregular.

To evaluate the lymphovascular invasion were analyzed the lumen of all blood vessels in the section and were no major differences between peritumoral and intratumoral vessels. Compared to the vessels without emboli, the tumor cells were identified in both areas (peritumoral and intra-tumoral, respectively) and those in the peritumoral area showed numerous tumor cells in the lumen. Lympho-vascular invasion was identified in 12 (13%) patients who also had regional lymph node metastases.

Regarding podoplanin expression, 73 samples (79.3% of cases) were tested positive for the D2-40 marker. In the case of moderately and poorly differentiated tumors with positive immunoreaction was considered diffuse expression model, the pronounced intensity being observed at the proliferation front but also in the immediate vicinity of the lymphatic vessels, the reaction being predominant at the membrane but also random in the cytoplasm of tumor cells (Figure 2, c and d).

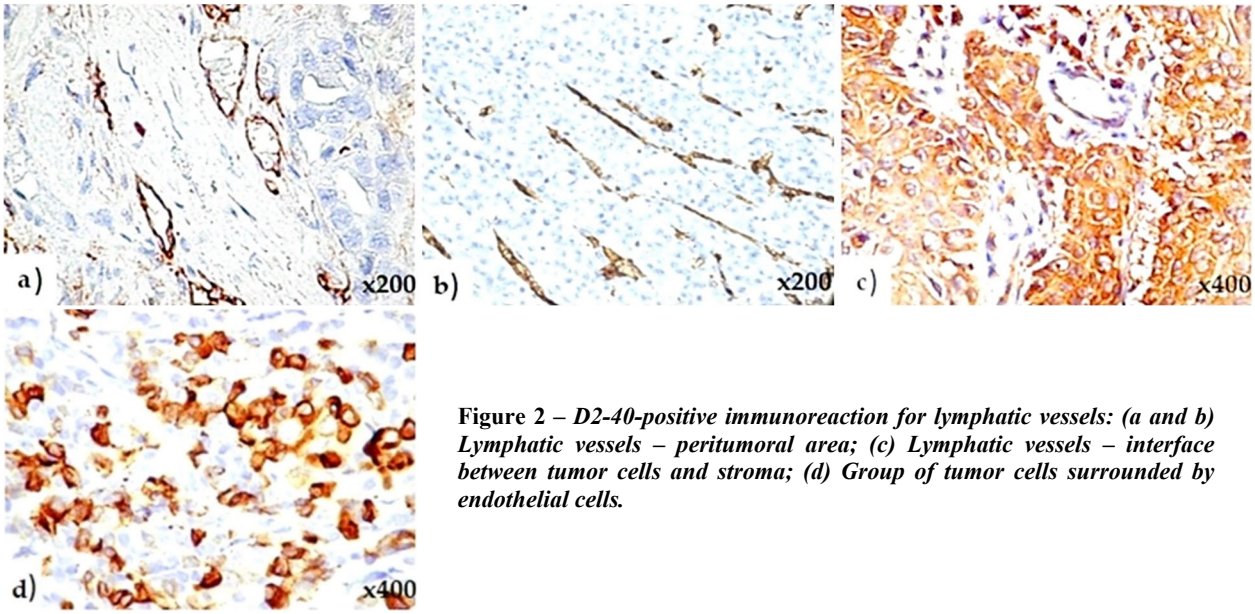

Figure 2 – ***D2-40-positive immunoreaction for lymphatic vessels: (a and b) Lymphatic vessels – peritumoral area; (c) Lymphatic vessels – interface between tumor cells and stroma; (d) Group of tumor cells surrounded by endothelial cells.***

Molecular classification

In the first stage, IHC expression of the mentioned markers (EGFR, Bcl-2, CK5, p53 and E-cadherin) was evaluated. EGFR was expressed in 85.71% cases, the final reaction product being exclusively membrane or cytoplasmic with significant membrane intensification. Given the type of immunomarker, the membrane reaction product was considered essential for interpretation (Figure 3, a and b). Most positive cases for CK5 showed a heterogeneous pattern, the final reaction product at the membrane level and the tumor cells were positive in the proportion of 20–50% (Figure 3c). A particular aspect was revealed by the immunoreaction for CD117, which in addition to the ability to visualize stem-like cells, was intensely positive in mast cells in the tumor stroma, particularly in areas rich in inflammatory infiltrate (Figure 3, d and e). Positive cells were sometimes located in the immediate vicinity of malignant plaques, while the arrangement of CD117-positive cells among negative tumor cells were observed in detail (Figure 3f). The reaction for p53 signals the final product of the strictly nuclear reaction, the results being analyzed with the semi-automatic counting system (Figure 3g).

Regarding the reporting of the results, it is worth mentioning the great variability of expression of the studied immunomarkers (Table 2). In the general evaluation of OCs cases, a statistically significant correlation was obtained between the degree of differentiation and Bcl-2 expression (p=0.033), between metastasis (M) parameter and EGFR expression (p=0.039), and between EGFR expression and p53 expression (p=0.001). p53 was expressed in a representative number of tumor cells in half of the total number of studied cases. All p53-positive cases co-expressed EGFR, but on the other hand, not all EGFR-positive cases expressed p53. No other significant differences were identified between the positive/negative p53 cases with the other investigated immunomarkers.

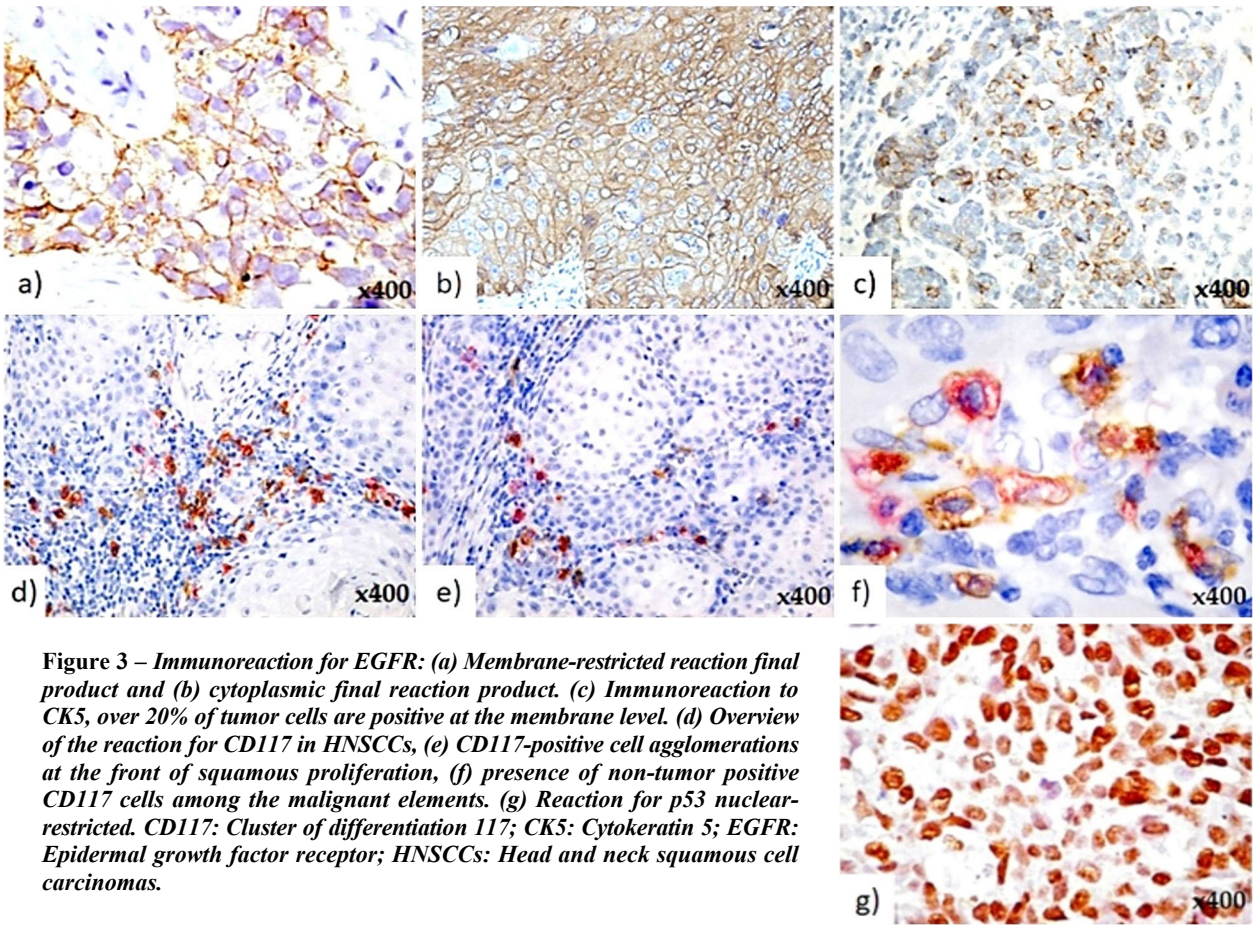

Figure 3 – ***Immunoreaction for EGFR: (a) Membrane-restricted reaction final product and (b) cytoplasmic final reaction product. (c) Immunoreaction to CK5, over 20% of tumor cells are positive at the membrane level. (d) Overview of the reaction for CD117 in HNSCCs, (e) CD117-positive cell agglomerations at the front of squamous proliferation, (f) presence of non-tumor positive CD117 cells among the malignant elements. (g) Reaction for p53 nuclear-restricted. CD117: Cluster of differentiation 117; CK5: Cytokeratin 5; EGFR: Epidermal growth factor receptor; HNSCCs: Head and neck squamous cell carcinomas.***

Table 2 – ***Heterogeneity of expression of investigated markers***

Bcl-2	p63/EGFR	p53	E-cadherin	EGFR
-	-	-	-	-
-	-	-	+	+
-	+	+	+	+
+	+	+	+	+

Bcl-2: B-cell lymphoma-2; EGFR: Epidermal growth factor receptor.

EGFR expression in cases with OCs revealed a high percentage of positive cases, respectively 89.3% had an intense or moderate reaction. More than a half of the EGFR-positive cases co-expressed p53. The highest number of CK5-positive cases was observed in EGFR-positive cases. EGFR/CK5 co-expression was noted in 72% of all cases and this combination draws attention to a particular subtype. It is well known that the normal stratified epithelium in the head and neck region expresses EGFR restricted to basal cells. In OCs, EGFR over-expression was frequently associated with CK5, suggesting that this subgroup would be a basal cell derivative by activating potential progenitor cell factors. This subgroup was further characterized by investigating the expression of p63 and c-kit (CD117). Co-expression of CD117 and p63 was identified in 80% of EGFR-positive/CK5-positive (five cases). Based on these aspects, we can try to propose the basal-like subtype of OCs, defined as EGFR-positive/CK5-positive, CD117-positive and p63-positive. For this group, a statistically significant correlation was found between degree of differentiation (G) and p63 expression (p=0.01) and also between T and CD117 expression (p=0.04). Bcl-2 and p53 immunomarkers seemed to be expressed with high frequency in EGFR-positive cases and were consistently negative in those with EGFR negativity. By correlating the data obtained based on the p53, Bcl-2 and EGFR immunoexpression, three distinct subtypes can be defined: EGFR+/p53–/Bcl-2–, EGFR+/p53+/Bcl-2– and EGFR+/p53+/Bcl-2+. From the statistical analysis of the EGFR+/p53+/Bcl-2– subtype, it appears that in this group, EGFR expression correlates with lymph node status (p=0.04) and CD117/p63 co-expression (p=0.04). CK5 was positive in 64.5% of cases, the most common co-expression being observed with EGFR and CD117/p63. For these cases, an inverse statistical relationship was noted between the T element and p53/EGFR co-expression (p=-0.03) and a positive correlation between T and CD117/p63 co-expression in tumor cells (p=0.01). Lymph node status was significantly correlated with Bcl-2 expression (p=0.04).

Discussions

Malignant tumors of the oral cavity have a growing incidence, most of them being SCCs, clinically detected in various stages of natural evolution. Oral carcinogenesis is a two-stage process characterized by an initial precancerous lesion that will develop into cancer [9]. According to the *WHO*, a precancerous lesion is defined as "modified epithelium with an increased potential for progression to squamous cell carcinoma (SCC)" [5]. During

2018, the highest rate of cancer of the lip and oral cavity was registered in Papua New Guinea (20.4/100 000), followed by Pakistan (12.2/100 000) and Bangladesh (9.5/100 000), while Romania occupied the 14th position with a rate of 5.5/100 000 [10]. Despite efforts in early diagnosis and the contributions of conventional adjuvant therapy, the long-term prognosis of these tumors remains unfavorable, and survival and disease-free survival (DFS) have not changed significantly in the last two decades. The basic treatment is represented by surgery and/or radiation therapy. The importance of histopathological analysis is recognized as being essential both at the time of diagnosis and after surgery, when additional treatment is planned. The clinician needs information about the degree of tumor and the type of resection margins, becoming decisive in detecting changes in adjacent tissue, to establish the option of personalized treatment with favorable results. Different molecular markers have been evaluated to characterize new prognostic markers and to identify new therapeutic targets. The change in the balance between prooxidants and antioxidants is manifested by oxidative stress, which leads to certain microcirculation deficits and endothelial dysfunctions with excessive growth of free radicals [11].

In recent years, both clinical and experimental studies have shown that, like normal tissue, tumors require a blood supply for growth and dissemination [12]. In the clinical observations were reported different incidence of recurrences and durations of survival for cases with the same histopathological form and degree of differentiation [13]. Most of these studies focused only on the molecular characteristics of tumor cells and study of the micro-environment, which is essential for local progression, lymph node and systemic metastasis, are scarce. On the other hand, markers that have been shown to be useful in *in vitro* and *in vivo* experiments could not be extrapolated to humans, and clinical trials based primarily on tyrosine kinase receptor inhibitors have been disappointing.

A percentage of 4% of cases from this study showed proliferation in the form of islands and beaches with tumor cells, with rich stroma, with densification of collagen fibers and reduced inflammatory infiltrate, without necrosis and extracellular keratinization. Some of these observations are described in previous studies [14]. In these cases, two subpopulations of malignant cells were morphologically identified: (*i*) those in the central area that do not differ significantly from those observed in most cases and (*ii*) those located at the periphery of invasive beaches, characterized by the presence of hyperchromic nuclei, reduced cytoplasm acidophilic. Inflammatory infiltrate was constantly observed in the tumor stroma and in all cases was polymorphic, consisting of a mixture of lymphocytes, plasma cells, macrophages and neutrophilic granulocytes and more rarely eosinophilic granulocytes. Inflammatory infiltrate in this form was observed in 90% of cases, which can be explained in part by the fact that all these tumors showed more or less extensive areas of ulceration. Particular attention has been paid to inflammatory infiltrate, mainly due to the involvement of some of these cells in angiogenesis, a condition in which they may be a source of proangiogenic factors [15]. On the other hand, mast cells are known to be involved in allergic, inflammatory, and immune reactions and exert their tumor effect through four mechanisms: immunosuppression, angiogenesis, degradation of the extracellular matrix, mitogenesis [16]. Also, there were significant correlations between vitamin deficiency indicators (vitamin D in particular) and vascular stiffness and the evaluation of certain functions, measurement of vascular stiffness indicators and biomarkers are key elements in the prevention of vascular diseases [17] and those such as inflammatory and malignant involvement, due to the link with angiogenesis.

One of the peculiarities of tumor progression and metastasis in general and oral squamous cell carcinomas (OSCCs) in particular is the formation of new blood and lymph vessels, which are the support for local and distant progression of the tumor. Neoangiogenesis is an important step in tumor progression and an important predictor of tumor behavior is intratumoral microvessel density (IMVD) [18]. Angiogenesis and lymphangiogenesis are relatively poor studied in OSCCs and precursor lesions, and there is currently much controversy about the prognostic value and the existence of real therapeutic targets in these lesions. Podoplanin was first identified in podocytes (rat glomerular epithelial cells) and is a mucin-like trans-membrane glycoprotein, expressed in the normal lymphatic endothelium, and absent from vascular endothelial cells. The binding of podoplanin is easy with murine anti-D2-40 monoclonal antibody and the immunoreactivity is intense and specific for the lymphatic endothelium. The use of D2-40 as an IHC marker to evaluate podoplanin expression is an important method of diagnosis and prognosis in various tumors [19]. More recently, micro-ribonucleic acids (miRNAs) have attracted attention as promising therapeutic markers for several conditions, including cancer, autoimmune diseases, cognitive impairment, metabolic syndrome, diabetes and others [20, 21]. OCs cells present podoplanin as a relevant functional biomarker and potential chemotherapeutic target. About half of primary tumors and over 70% of advanced tumors have increased podoplanin expression [22]. Clinical trials showed that overall 5-year survival rates are steadily declining, as follows: in approximately 90% of patients with poor podoplanin expression, in approximately 40% of patients with moderate expression, and in approximately 20% of patients with high levels of podoplanin expression [23]. Podoplanin influences the motility of OSCC cells and can lead to tumor invasion and metastasis [24].

Most malignancies developed in the oral sphere were carcinomatous tumors with varying degrees of differentiation and most of them were squamous differentiated [25]. Squamous cell neoplastic proliferation is a long-term process, with the development of malignant tumors often preceded by preneoplastic lesions, the most common of which were leukoplakia and actinic keilitis [26]. Among the microscopic prognostic elements that have demonstrated their practical applicability are the local stage of the tumor, the degree of differentiation and lymphovascular invasion. Lymphovascular invasion was recognized a prognostic factor for the development

of lymph node metastases and at a distance [27]. In many cases, research on this topic has focused on the peculiarities and molecular profile of tumor cells, and only to a small extent on the infiltrate of inflammation relatively constantly associated with tumor cell proliferation, and only a few articles have been published on the peculiarities of tumor stroma. G was calculated based on the criteria recommended by the *WHO*, namely the type of growth, the proportion of cells with nuclei with anaplasia, the rate of typical and atypical mitoses, and the intensity and extent of the keratinization process. This procedure was necessary to try to demonstrate the variations in the intensity of angiogenesis and lymph-angiogenesis in different evolutionary stages of SCC. The pattern of malignant cell disposition has been studied by many authors over time, but in terms of prognostic and predictive information for metastases, so far none of these microscopic models has met consensus in this regard.

The identification of prognostic and therapeutic markers in malignant tumors is a "gold standard" in multimodal evaluation. These markers can complete the characteristic table for prognosis and thus total survival and DFS. Various types of malignancies of heterogeneous origin, which can arise from any anatomical component of the head and neck, such as lips, tongue, larynx, pharynx, are included in a broader group known as global SCCs of the head and neck. Due to the lack of well-defined prognostic factors and the absence of molecular indicators of the therapeutic strategy, OCs is included in the group of tumors with high morbidity, high mortality, and limited therapeutic procedures except surgery, chemotherapy, and irradiation [28].

All classifications proposed so far include the EGFR expression. Our results were consistent with previous studies showing that EGFR was overestimated in over 80% of cases [29]. By comparison with previous publications were stratified EGFR-positive cases based on the expression of CK5, p53 protein and Bcl-2. When the EGFR+/CK5+ cases were analyzed, a subtype characterized by p53/Bcl-2 co-expression was identified. Such cases were not found in the group of EGFR-negative patients. The p53 protein is involved in maintaining cellular integrity after deoxyribonucleic acid (DNA) damage [30]. The *p53* gene mutation is an early event in OCs tumorigenesis [31]. P53 loss is associated with the metastatic phenotype but the appearance is shown so far only in the experimental model of head and neck cancer. The published results on the co-expression between EGFR and p53 are controversial. Some authors have shown that p53 loss stimulates the development of metastases [32], while others have shown that p53 expression can increase sensitivity to EGFR inhibitors by stopping the cell cycle and apoptosis [33]. It is proposed that in future clinical trials EGFR inhibitors be combined with irradiation for better results. Identification of EGFR/p53 co-expression in more than half of EGFR-positive cases could define a subtype of HNSCC that is more sensitive to anti-EGFR therapy and has low potential to acquire resistance to these therapeutic agents. In this group, we identified a subclass of EGFR+/p53+/Bcl-2– cases that correlates with lymphoid status and the presence of CD117-positive tumor cells. Based on these observations, it can be speculated to reduce the lymph node dissemination of tumor cells, targeting in particular EGFR.

EGFR/p53 co-expression in OCs has been shown in several studies but which included a small number of cases or in limited geographical areas [34]. Our data were consistent with those published by other authors regarding the correlation between EGFR and lymph node status and the degree of differentiation [35, 36]. Recently, Bcl-2 was investigated in OCs being considered a biomarker of importance for the clinical evolution of patients, but the results were in contradiction with those obtained in older studies. Some studies have shown that mutant overexpression of p53 decreases Bcl-2 expression so that Bcl-2 expression depends on p53 status [37, 38]. The group of EGFR-negative and CK5-negative cases were also negative for the other immunomarkers. Given the small number of cases that formed this type, we did not make statistical assessments, given that all correlations between negative immunomarkers and clinicopathological parameters have no clinical significance.

Conclusions

Our results support the need for molecular classification of OCs only based on tumor immunomarker expression and gene analysis. By IHC methods, we identified with certainty the subtype EGFR+/CK5+, subsequently sub-qualified according to the expression of p53, Bcl-2, and CD117. We consider that studies on large series of patients are needed to allow the application of a combination therapy of targeted type.

Conflict of interests

The authors declare no conflict of interests.

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Authors' contribution

Ioana Cristina Niculescu Talpoş and Ramona-Camelia Rumel contributed equally to the paper and share first authorship.

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