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CLINICAL RELEVANCE OF TUMOR-ASSOCIATED MACROPHAGES (TAMs) AND CARCINOMA-ASSOCIATED FIBROBLASTS (CAFs) IN ORAL SQUAMOUS CELL CARCINOMA (OSCC): IMMUNOHISTOCHEMICAL STUDY

КЛИНИЧКА РЕЛЕВАНТНОСТ НА МАКРОФАГИ-АСОЦИРАНИ СО ТУМОР (TAMs) И ФИБРОБЛАСТИ-АСОЦИРАНИ СО КАРЦИНОМ (CAFs) КАЈ ОРАЛЕН ПЛАНОЦЕЛУЛАРЕН КАРЦИНОМ (OSCC): ИМУНОХИСТОХЕМИСКА СТУДИЈА

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Abstract

Introduction: The factors, which influence or play a key role in tumor invasion and metastasis of the squamous cell carcinoma of the oral mucosa (OSCC), are subject of many scientific researches. **Objectives:** The aim of this study was to investigate the clinical relevance of OSCC infiltration with TAMs and CAFs. **Materials and methods:** Immunohistochemical analysis of both stromal/tumoral CD68+ TAMs and α-SMA positive CAFs was performed in paraffin–embedded tissue specimens from 23 OSCC patients and correlated with the clinical stage and degree of malignant cell differentiation. **Results and conclusions:** The presence of CAFs was not detected in the specimens of all 23 patients, but TAMs were found in all of them. Stromal myofibroblasts are heterogeneously detected in the OSCC, and the lowest density of CAFs is in the first clinical stage. There is a statistically significant correlation between clinical stage 1 and the degree of density of CAFs compared to other clinical stages **(p=0.006474).** There is a statistically significant difference between clinical stage 1 and other stages 2, 3 and 4, and the presence of TAMs in the tumor stroma **(p<0.033179)** and in the tumor nests also **(p<0.033179)**. There is no statistically significant difference between the density of CAFs and the degree of differentiation of tumor cells, and also the degree of differentiation of tumor cells does not correlate with the expression of CD68+ TAMs neither in the tumor stroma **(p=0.438807),** nor in the tumor nest **(p=0.9488644)**. **Key words:** Tumor microenvironment, carcinoma-associated fibroblasts, tumor-associated macrophages, oral squamous cell carcinoma.

Апстракт

Вовед: Кои чинители влијаат или имаат клучна улога во туморската инвазија и метастазирање на планоцелуларниот карцином на оралната мукоза (OSCC) се сеуште предмет на многу научни истражувања. **Цели:** Оваа студија има за цел истражување на клиничката релевантност на инфилтрација на OSCC со TAMs и CAFs, евалуирајќи ги туморските маркери CD68 и α-SMA во туморската строма. **Материјали и методи:** Направена е имунохистохемиска анализа на стромални/интратуморски CD68-позитивни TAMs и α-SMA-позитивни CAFs на ткивни примероци од орален планоцелуларен карцином кај 23 пациенти, а потоа резултатите се корелирани со клиничкиот стадиум на болеста и со степенот на диференцијација на малигните клетки. **Резултати и заклучоци:** Не е утврдено присуство на CAFs кај сите 23 пациенти, но кај сите 23 пациенти се пронајдени TAMs. Стромалните миофибробласти се хетерогено детектирани во OSCC, а најмала густина на CAFs има во првиот клинички стадиум. Постои значајна статистичка корелација помеѓу густината на CAFs во првиот клинички стадиум на болеста споредено со останати клинички стадиуми **(p=0.006474)**. Исто така, постои статистички значајна разлика помеѓу клинички стадиум 1 и останатите стадиуми 2, 3 и 4 и присуство на CD68+ TAMs во туморска строма **(p<0.033179)** и туморското гнездо **(p<0.033179)**. Не постои статистички значајна разлика помеѓу густината на CAFs и степенот на диференцијација на туморските клетки за **p=0.72158,** и исто така степенот на диференцијација на туморските клетки не корелира со експресија на CD68+ TAMs ниту во туморската строма **(p=0.438807)**, ниту во туморското гнездо **(p=0.9488644)**. **Клучни зборови:** Туморска микросредина, фибробласти-асоцирани со карцином, макрофаги-асоцирани со тумор, орален планоцелуларен карцином.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the malignancies with a very high incidence and mortality and poses a significant health problem all around the world[1]. Despite advances in understanding the molecular biology of this cancer, diagnosis and treatment including multimodal tumor therapy, mortality remains below 50% as 30 years ago, due to locoregional relapses and metastasis.

Staging and grading of OSCC is necessary for determining the following surgical and oncologic treatment. The TNM system of cancer staging is based on assessment of the size of the primary tumor (T), involvement of locoregional lymph nodes (N), and the distant metastases (M). This classification is important not only for treatment planning, but for estimating the risk of tumour recurrence and assessment of the overall survival. However, this classification only considers the anatomic extension of the disease. OSCC staging and grading criteria are periodically changed and improved, introducing modifications that influence the risk stratification as well as the treatment of this type of malignancy[2].

Histopathological grading for squamous cell carcinoma of the lip was first introduced by Broders, and was based on the differences in differentiation between tumors. Later, more complex grading systems were suggested. These multifactorial systems consider the differentiation of the tumor cells, the tumor invasion patterns and host reactions e.g. inflammatory response[3]. These systems should be updated by introducing the feature of cancer-associated fibroblasts (CAFs) assessed with the use of immunohistochemistry for α-SMA[4]. Until now, the cause of cancer progression has been attributed to cumulative genetic changes in the oral epithelium, but this model, based only on alterations in the oral epithelium alone, has changed, and a new model is proposed, in which the tumor microenvironment (TME) has a significant contribution to cancer progression. In 1889, Paget proposed the "seed and soil" hypothesis, in which invasion of neoplastic tissue was explained by the fact that cancers induced changes in adjacent stromal cells[5].

TME is a complex system composed of various stromal cells such as fibroblasts, endothelial cells, immune cells, and various components of the extracellular matrix (ECM). In such environment, the tumor cells reprograms the surrounding stromal cells for tumorigenesis, cancer progression and invasion of the surrounding tissue. The stromal cells in TME, unlike cancer cells, are not prone to mutations and their behavior is modulated by several cytokines. The most numerous and important immune cells in TME are tumor-associated macrophages (TAMs); in solid tumors these cells make up 5-40% of the tumor mass[6].

TAMs are a mixed population of macrophages constituting both M1 and M2, but mainly composed by M2 macrophages which are recruited and educated by cancer cells[7]. TAMs are identified by immunohistochemistry. The antibody against CD68 is a pan-macrophage marker that is widely used to identify all macrophages regardless of their phenotype[8] (Macrophages in the microenvironment). Under physiological conditions, macrophages are polarized into proinflammatory and anti-tumor M1 phenotype; however, tumor cells may influence redirection of the macrophage polarization to an alternatively activated M2 phenotype. The M2 phenotype, in turn, secretes cytokines, chemokines, enzymes, growth factors, and several matrix metalloproteinases, thereby intensifying inflammation as well as promoting tumor progression, immunosuppression, angiogenesis, as well as resistance to the treatment also[6]. In vitro studies have shown that, depending on the chemokine stimuli, the polarization from M1 to M2 can be reversed[9]. The clinical relevance of macrophage subpopulations in cancer is not clear, yet. Recent studies show that a high degree of TAM infiltration in tumor tissues has been correlated with poor prognosis in many cancers such as lymphoma, cervical cancer, bladder cancer, and breast cancer. In that manner, many studies have also confirmed that a high degree of TAM infiltration is correlated with tumor metastasis. The results of some studies on animal models show that low TAM infiltration can inhibit tumor growth and metastasis[10].

Local residents i.e. normal fibroblasts (NFs) are the major source of cancer-associated fibroblasts (CAFs). CAFs are distinguishable from NFs by their tumor-supportive properties. However, the mechanism underlying the transition of NFs to CAFs in OSCC remains still unclear. α-SMA reflects the expression of CAFs in the tumor mesenchyme and is the most common marker of CAFs. Normal mesenchymal fibroblasts do not express α-SMA. Activated fibroblasts, which are mostly α-smooth muscle actin-positive fibroblasts termed myofibroblasts, are one of the major cellular constituens of the tumor stroma. Stromal fibroblasts are not only activated in injured tissue, but are activated by cancer cells also. Myofibroblasts synthesize ECM components, several proteins, growth factors, and cytokines, thus creating a permissive environment that supports tumor growth. Unlike wound healing changes, tumor fibroblasts that are adjacent to the tumor remain activated, as in chronic inflammation. The results of many studies define CAF as very important element of the TME in OSCC due to their significant role in tumor invasion and development, suggesting a poor disease outcome[11].

Objectives

This study aims to determine the presence of TAMs and CAFs in OSCC and also investigates the clinical relevance of tumor infiltration with these two cell types. TAMs and CAFs are immunohistochemicaly analyzed with two tumor markers: CD68 (tumor marker for both M1 and M2 macrophages) and α -SMA (tumor marker for CAFs). Stromal/tumoral expression of CD68 and stromal expression of α-SMA were evaluated, as well as possible relation with the clinical stage of the disease and the degree of tumor cell differentiation.

Materials and methods

Patients and tissue samples

Tissue samples from 23 patients confirmed for OSCC were obtained and stored at the Institut for Pathology. The patients underwent surgery at the University Clinic for Maxillofacial surgery in Skopje between 2016 and 2018. None of the patients received preoperative chemotherapy or radiotherapy. Patient and tumor characteristics, including clinical stage of the disease and the histologic grade, were determined from the patient's medical record and pathology reports. The stage of the disease was classified according to the criteria of the 7th Edition of AJCC/UICC TNM classification system of the OSCC. Tumor differentiation was classified into 4 levels: good, moderate, poorly differentiated, and undifferentiated (anaplastic) according to Broder's histological classification of tumor cell differentiation in OSCC (Broder's grading descriptive system)[12].

Immunohistochemistry (IHC) and IHC evaluation

Tissue samples underwent IHC and were analyzed at the Institute of Pathology, within the University Clinical Center "Mother Teresa" in Skopje. Immunohistochemistry was performed using Dako EnVision flex system. The following primary mouse monoclonal antibodies were used: anti-α-SMA and CD68. Nikon 80 digital microscope was used.

CAFs and TAMs Assessment

CAFs were evaluated by immunohistochemistry. Positive or negative fibroblasts were identified on the basis of α-SMA expression. CAFs were defined as large spindle-shaped fibroblasts that express α-SMA. A modification of the classification system of Kellerman et al. was used to determine the density of CAFs[13]. Density levels were categorized into 4 categories: Negative (0), Rare (1), Focal (2) and Abundant (3). Samples in which no stromal myofibroblasts were detected were classified as negative. Samples showing sporadic stromal myofibroblasts were classified as rare. Samples showing focal arrangement were classified as focal. Samples showing numerous and densely arranged stromal myofibroblasts were classified as abundant.

CD68 expression was determined by counting the number of CD68-positive macrophages (TAMs) in the invasive areas of the OSCC specimens. Macrophages were defined as stromal cells larger than 10μm in diameter, which express CD68. Each section was displayed on a low power magnification (low-power field with 100x magnification) to identify the areas with the highest macrophage density, then the macrophages were counted in three fields on high power magnification (high-power field on 400x magnification) and the mean number of macrophages per HPF was obtained[14]. Additionally, we assessed the number of TAMs in TS (tumor stroma) and TN (tumor nest).

Statistical analysis

All collected statistical data are processed using the following statistical methods:

- All statistical series are tabulated
- Analysis of relationships between attributive statistical series is done with $\chi 2$ – Test
- Testing the significance of differences between two arithmetic means in independent samples (between groups) was done with Student's t-test
- Samples are divided into groups with low and high number of CD68+ TAMs in tumor stroma (TS) and tumor nest (TN) based on their number, using cut-off values of 55 for TAMs in tumor stroma and 6 for TAMs in the tumor nest.

Results

The presence of α-SMA + CAFs was not detected in all patients with OSCC. Out of 23 patients, in 5 of them CAFs were not detected, and in the remaining 18 CAFs were present. Out of 23 patients, 7 patients are in the first clinical stage (Stage 1), of which 4 have no CAFs, and the other three patients have CAFs but scanty CAFs (grade 1). Of the remaining 16 patients, which are in more advanced clinical stages 2, 3, and 4, just one patient had no CAFs, and that patient was in the second clinical stage (Stage 2); CAFs were found in the other 15 patients. Therefore, there is a statistically significant correlation between clinical stage 1 and the degree of density of α-SMA-positive CAFs, compared to other clinical stages. There is a statistically significant difference between the density of CAFs in stage 1 and other stages (2, 3 and 4) ($p = 0.006474$). χ^2 e **7.4134**. (see Table 1).

Table 1. Correlation of α-SMA-positive CAFs degree with the pathological stages of the patients with OSCC.

	α-SMA+ CAFs (degrees)		
Pathological stage (pTNM)	0	1, 2, 3	**p-value** <0.05
pTNM 1	4	3 (degree 1)	**0.006474**
pTNM 2, 3, 4	1	15	

Pathological stages of OSCC: Pathological stage 1, 2, 3 and 4 (pTNM 1, 2, 3, 4). Density of CAFs in TS (degrees): 0 - no CAFs, 1.) scanty, 2.) focal, 3). abundant.

Table 2. Correlation of α-SMA-positive CAFs degree with the degrees of malignant cell differentiation in patients with OSCC.

	α-SMA+ CAFs (degrees)		
Degree of malignant cell differentiation	**0**	**1,2,3**	**p-value** <0.05
G1/G2	**5**	**16**	0.72158
G3/G4	**0**	**2** (both patients have degree 1 CAFs (scanty))	

Density of CAFs in TS (degrees): 0 - no CAFs, 1 - scanty, 2 - focal, 3 - abundant. Degrees of malignant cell differentiation: G1 - well differentiated, G2 - moderately, G3 - poor, G4 - undifferentiated (anaplastic).

Table 3. Correlation of number of CD68-positive TAMs with the pathological stages in patients with OSCC.

	CD 68+ TAMs (TS)			CD68+ TAMs (TN)		
Pathological stage (pTNM)	Low	High	p-value	Low	High	p-value
pTNM 1	6	1	**< 0.033179**	6	1	**< 0.033179**
pTNM 2, 3, 4	6	10		6	10	

As stated above in the text, out of 23 patients, CAFs were not found in 5 patients. All 5 patients had a G1/G2degree of cell differentiation. CAFs were found in the other 18 patients, 16 of whom had G1/G2, and 2 had G3/G4 degree of differentiation. Both patients with G3/G4 degree of differentiation have little CAFs present, i.e. in oral carcinomas with poor cell differentiation and in anaplastic carcinomas there is a lower number of CAFs, thus worse differentiated tumors have fewer CAFs. **χ^2 is 0.127** and for p = 0.72158 there is no statistically significant difference between the density of CAFs and the degree of differentiation of tumor cells (see Table 2).

The presence of CD68-positive TAMs was found in both the tumor stroma (TS) and the nests (TN) in all 23 patients with OSCC. There is a statistically significant difference between clinical stage 1 and other stages (2, 3 and 4), and the presence of TAMs in the tumor stroma **(p<0.033179)**.I In the same manner, there is a statistically significant difference in the presence of TAMs in tumor nests between clinical stage 1 and the other clini-

Table 4. Correlation between the number of CD68-positive TAMs and the degree of malignant cell differentiation. Degrees of malignant cell differentiation: G1 - well differentiated, G2 - moderately, G3 - poor, G4 - undifferentaited (anaplastic).

	CD 68+ TAMs (TS)			CD68+ TAMs (TN)		
Degree of malignant cell differentiation	Low	High	p-value	Low	High	p-value
G1/G2	12	9	**0.438807**	1	1	**0.9488644**
pTNM 2, 3, 4	0	2		11	10	

Degrees of malignant cell differentiation in OSCC: G1 - well differentiated, G2 - moderately, G3 - poor, G4 - undifferentiated (anaplastic).

Table 5. Expression of CD68 + TAMs in tumor stroma (TS) and tumor nest (TN). Student's t-test (t=-5.41148).

Groups	Mean	SD	min	max	p-value
CD68+ TAMs (TS)	65.22	41.44762	9	156	< 0.00001
CD68+ TAMs (TN)	14.35	17.73443	0	55	

cal stages (2, 3 and 4) **(p <0.033179)**. Namely, it is observed that out of 7 patients, who were in the first clinical stage, 6 patients have a low number of TAMs, and only one patient had a high number of TAMs. We got the same results in tumor nests. $\chi^2 = 4.5365$. (see Table 3).

The degree of differentiation of tumor cells does not correlate with the expression of TAMs neither in the stroma nor in the nests. There is no statistical significance between TAMs infiltration of tumor stroma **(p=0.438807)** and tumor nests **(p=0.9488644)** with the degree of malignant cell differentiation. χ^2**=4.5365**. However, although there is no statistical significance, it is noted that out of 2 (two) patients with poorly differentiated/ anaplastic cancer, both patients have a high number of TAMs that infiltrate the tumor stroma (TS). (see Table 4).

There is a statistically significant difference in the expression of TAMs between stroma and nest for p<0.00001. Testing of the significance of the differences between two arithmetic means between the tumor stroma and the tumor nest was performed with the Student's t-test **(t=-5.41148)**. (see Table 5).

Discussion

It is widely accepted that tumor development of OSCC depends on a complex interaction between malignant cells and the tumor microenvironment (TME)[15]. In our group of 23 patients, among other, we investigated the presence of CD68-positive TAMs and α-SMA-positive CAFs in the tumor stroma, and found that stromal myofibroblasts are not detected in all 23 patients, so the question arises whether CAFs play any role in oral carcinogenesis? A study by Eliene-Magda de-assis et al.[16], which evaluates the presence of stromal myofibroblasts in oral leukoplakia (OL), normal mucosa and OSCC, found no presence of stromal myofibroblasts in all 30 patients with OL, and in 10 patients with normal oral mucosa as a control group. This indicates that these cells have no relevance during oral carcinogenesis. In the rest of the 41 patients with OSCC, approximately one-third (26.8%) had no presence of stromal myofibroblasts, while two-thirds (73.2%) had them in the tumor stroma, so this result is identical to our finding where 21.8% of tumor stromas do not have stromal fibroblasts, and the remaining 78.2%, in which stromal fibroblasts have been identified, have different densities. However, this heterogeneous presence of stromal fibroblasts in OSCC suggests that myofiboblasts are associated with the creation of a conducive environment for tumor invasion in OSCC[16]. The presence of myofibroblasts in OSCC stroma in our study, but also their absence in epithelial dysplasias in the studies of numerous other authors, suggests the need for further investigation to elucidate the role of myofibroblasts in the carcinogenesis of OSCC.

The results of Etemad-Moghadam et al.[17] of the 40 OSCC study samples, showed positive immunostaining in myofibroblasts in all oral squamous cell carcinomas, i.e. the presence of myofibroblasts in the stroma of all OSCCs was confirmed, but not in the samples with oral epithelial dysplasia or in normal oral mucosa. In addition, the results of the study showed that there is no significant difference between the different degrees of tumor differentiation and the number of myofibroblasts[17]. Our results are consistent with Ethemad's.

If we compare the clinical stages of the patients in our study and the degree of differentiation of cancer cells with the number of CAFs, we come to the conclusion that the number of CAFs is lowest in the lowest clinical stage, and vice versa, the worse the cell differentiation is (G3/G4), the less CAFs.

Patients in lower clinical stages have a better prognosis and live longer and, according to our research, these patients have a lower number of CAFs; according to that, in order to came to the exact conclusion about the prognosis of our patients, we need to do further investigations. This difference in number and distribution of CAFs is confirmed by the difference between patients in the first clinical stage and patients in higher stages. Therefore, from the obtained results we can conclude that patients in the first clinical stage either do not have or have a very small number of CAFs which is consistent with the findings of Ibrahim O'Bello[18] and Kellerman et al.[19]. Ibrahim O. Bello's research on tongue cancer has shown that the density of CAFs is associated with the degree of tumor malignancy, tumor growth and progression. Kellerman's findings are similar, confirming that the higher degree of infiltration with CAFs is associated with more advanced TNM stage and lymph node metastasis.

Our results contradict the findings of Fujii N. et al.[20], in his study of 108 respondents with OSCC they found

that there is no significant correlation between the distribution of CAFs and the clinical stage of the disease. In a study conducted by Yahui Yu et al.[21], the authors included 152 patients with nasopharyngeal carcinomas and examined the association between α-SMA expression levels and clinical features of the patients[21]. The summarized results show that there is no significant correlation between the level of α-SMA expression and the clinical stage of the disease.

The presence of CD68 + TAMs macrophages in tumor stroma was observed in all 23 patients with OSCC in our study. The results showed the presence of a small number of TAMs in the first clinical stage, in both TS and TN, i.e. a strong association of early-stage disease with the presence of small number of TAMs has been established. In addition, the results of some studies on animal models show that low TAM infiltration can inhibit tumor growth and metastasis, thus corresponding with our findings[10].

Our results do not match the results of many authors such as Fujii N.[20] and Yahui Yu[21]. The results of Fujii N. et al., obtained from 108 patients with OSCC, testify that there is no significant correlation between the level of expression of CD68 with clinicopathological parameters such as the clinical stage and the degree of differentiation of cancer cells in oral squamous cell carcinomas[20]. In a study by Yahui Yu et al., the authors included 152 patients with nasopharyngeal cancers and examined the association between CD68 expression levels and clinical features of patients; the summarized results show that there is no significant correlation between CD68 expression levels and clinical stages of the disease[21].

Faustino J. Suarez-Sanchez[6] investigated the clinical relevance of OSCC infiltration with TAMS. He and his colleagues evaluated the expression of CD68 as a panmacrophage marker of both M1 and M2 macrophages, and CD163 as a tumor marker of M2 macrophages in tumor nests and the surrounding tumor stroma, and found that tumor infiltration with CD68-positive TAMs was not associated with none of the examined clinicopathological parameters, i.e.. it is neither associated with the clinical stage of the disease nor with the degree of malignant cell differentiation. However, a large number of CD68-positive cells have been reported to infiltrate the stroma in larger tumors (T3 – T4), in more advanced stages (Stage 3 and 4), and in moderately/poorly differentiated tumors, although the differences are not statistically significant6. In our study, although there is no statistical significance, it is observed that patients with poorly differentiated/anaplastic cancer have a high number of stromal CD68-positive TAMs (out of two patients with G3/G4, both of them have a high number of CD68-positive TAMs in the tumor stroma (TS)).

Studying tumor infiltration with TAMs by assessing CD68-positive TAMS with immunohistochemistry, Lin et al.[22] in 84 patients with laringeal carcinoma found that the infiltration of carcinomas with these cell types significantly correlated with poor prognosis. In contrast, Troiano et al.[23], in the HNSCC meta-analysis, reports that there is no association between CD68-positive macrophage expression in tumor nests or stroma and survival. Therefore, these findings raise the question about the use of the panmacrophage marker CD68 in IHC analysis as it has no prognostic utility in patients with HNSCC.

The association of infiltration with TAMs and patient prognosis differs among tumors. As we find in literature, TAM infiltration predicts poor prognosis in breast cancer, uterine cervix cancer, and bladder cancer, but it predicts good prognosis in other tumors i.e. prostate cancer, lung cancer, and brain tumors, and these differences may be related to various factors which refer to the tumors, but perhaps also to the manner in which the research was conducted[22].

Some results in literature suggest that TAM infiltration can be used to evaluate patients' condition and predict the prognosis of the carcinomas. Therefore, if we hypothesize that TAM infiltration may be an early, sensitive prognostic indicator, then expanded radical surgery or appropriate expansion of surgical range should be considered even for patients with early stage and well-differentiated OSCC once high TAM infiltration is identified. However, there is a general conclusion among investigators that further investigations are necessary to determine how to apply this potentially predictive indicator into everyday clinical work.

In addition, we expand the investigation for TAMs in our research as we found statements in literature for possible influence of TAMs infiltration of the tumor nests on the prognosis of the OSCC patients. According to our results, there is a statistically significant difference in the number of CD68-positive TAMs in tumor stroma and tumor nest in favor of tumor stroma. Searching for data in literature, we found that in endometrial cancer, infiltration with TAMs in TN shows a positive correlation with reduced recurrence, while in invasive breast cancer it suggests an unfavorable prognosis. If these results are taken into account, there are strong recommendations to examine TAMs localization, both in TN and TS[24].

Conclusion

More recent researches focused on the TME in OSCC aims to overcome the poor success of conventional treatment of this malignancy. Predicting the aggressiveness of the tumor and the prognosis of

patients with OSCC is still a weakness of the standard pathological report, and the introduction of new pathological parameters is a big step towards personalized treatment of patients with OSCC.

Our results for CAFs and TAMs are within the numerous limitations of this research, among others, and the small group of patients with OSCC. Therefore, to obtain more precise and relevant answers to the question of the clinical relevance of these two types of cells, we consider that it will be necessary to conduct further, far more extensive and better-designed studies in the future.

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