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ASOCIJACIJA FIBROBLASTA POVEZANIH SA KARCINOMOM SA KLINIČKO-PATOLOŠKIM PARAMETRIMA ORALNOG KARCINOMA SKVAMOZNIH ĆELIJA: IMUNOHISTOHEMIJSKA STUDIJA

ASSOCIATION OF CARCINOMA-ASSOCIATED FIBROBLASTS WITH CLINICO-PATHOLOGICAL PARAMETERS OF ORAL SQUAMOUS CELL CARCINOMA: AN IMMUNOHISTOCHEMICAL STUDY

Sanela S. Idoska[1], Danica A. Popovic-Monevska[1], Senada S. Pepić[2], Antonio LJ. Kirkov[1], Goran T. Pančevski[1], Suzana D. Božovic[1], Aleksandar T. Ilijev[1], Jana N. Trambulova[1], Aleksandar C. Stamatovski[1], Filip LJ. Koneski[1]

[1]UNIVERZITET „SVETI ĆIRILO I METODIJE", UNIVERZITETSKA KLINIKA ZA MAKSILOFACIJALNU HIRURGIJU, SKOPLJE, SEVERNA MAKEDONIJA
[2]PORODIČNA KLINIKA ZA ALERGIJE I ASTMU, ČARLSTON, ZAPADNA VIRDŽINIJA, SAD

[1]SS. CYRIL AND METHODIUS UNIVERSITY, UNIVERSITY CLINIC FOR MAXILLOFACIAL SURGERY, SKOPJE, NORTH MACEDONIA
[2]FAMILY ALLERGY AND ASTHMA CLINIC, CHARLESTON, WEST VIRGINIA, USA

Sažetak

Uvod: *Fibroblasti povezani sa karcinomom (CAFs) iz tumorske mikrosredine (TMS) imaju važnu ulogu u progresiji, recidivu i lošoj prognozi oralnog karcinoma skvamoznih ćelija (OKSĆ).*
Cilj: *Utvrditi gustinu CAFs-a u tumorskoj stromi (TS) i utvrditi povezanost gustine CAFs-a sa kliničko-patološkim parametrima i relapsom bolesti kod pacijenata sa OKSĆ-om.*
Materijal i metode: *Imunohistohemijsko bojenje uzoraka tkiva iz primarnog OKSĆ-a obavljeno je kod 45 pacijenata operisanih na Univerzitetskoj klinici za maksilofacijalnu hirurgiju u Skoplju, u Severnoj Makedoniji. Za vizuelizaciju CAFs-a korišćeno je mišje primarno monoklonsko antitelo anti-α-SMA. Gustina CAFs-a α-SMA+ grupisana je u četiri stepena, a dobijeni podaci statistički su analizirani.*
Rezultati: *Prisustvo CAFs-a nije utvrđeno u uzorcima tkiva kod svih pacijenata sa OKSĆ. Postoji signifikantna povezanost gustine CAFs-a u primarnom tumoru sa T, N i TNM statusom, (p = 0,0006; p = 0,0255; p = 0,0164). Difference testom utvrđeno je da je samo u slučaju relapsa bolesti u vidu lokalnog recidiva procentualna zastupljenost pacijenata sa prisutnim CAFs-om značajno veća u poređenju sa pacijentima koji nisu imali CAFs (p = 0,0001).*
Zaključak: *Ovi nalazi sugerišu ucešće CAFs-a u agresivnosti tumora i progresiji bolesti, ali nisi dovoljni da bi bili uključeni kao parametar u standardizovani histopatološki nalaz kod OSCC-a.*

Ključne reči: *tumorska mikrosredina (TMS), α-SMA pozitivni CAFs (CAFs α-SMA+), planocelularni karcinom usne šupljine (PKUD)*

Corresponding author:
Assistant Professor Sanela Pepić, DMD, Phd
University Clinic of Maxillofacial Surgery, Skopje,
North Macedonia
Email: sanela@dr.com
Phone:+38970289525

Abstract

Introduction: *Cancer-associated fibroblasts (CAFs) from the tumor microenvironment (TME) play an important role in the progression, recurrence and poor prognosis of OSCC.*
The aim: *To determine the density of CAFs in the tumorstroma (TS) of OSCC, and to determine the association of the density of CAFs with clinico-pathological parameters and disease relapse in patients with OSCC.*
Materials and methods: *Immunohistochemical staining of tissue samples from primary OSCC was performed in 45 patients operated at the University Clinic for Maxillofacial Surgery, in Skopje, North Macedonia. A mouse primary monoclonal antibody: anti-α-SMA was used to visualize CAFs. The density of CAFs α-SMA+ was grouped into 4 grades, and the obtained data were statistically analyzed.*
Results: *The presence of CAFs was not determined in tissue samples from all patients with OSCC. There is a significant association of the density of CAFs in the primary tumor with T, N and TNM-status, respectively (p=0.0006, p=0.0255, p=0.0164).*
The Difference test determined that only in case of disease relapse in the form of local recurrence, the percentage representation of patients with CAFs present was significantly higher compared to patients who did not have CAFs (p=0.0001).
Conclusions: *These findings are suggestive of the role of CAFs in disease progression, but are insufficient to be included as a parameter in a standardized histopathological finding.*

Key words: *tumor microenvironment (TME), α-SMA-positive carcinoma associated fibroblasts (CAFs α-SMA+), oral squamous cell carcinoma (OSCC)*

Uvod

Oralni karcinom skvamoznih ćelija (OKSĆ) je malignitet sa veoma visokim mortalitetom[1] i niskom stopom preživljavanja (manje od 50%), prvenstveno zbog relapsa bolesti, lokalnih recidiva i metastaza u udaljenim organima. Biološko ponašanje oralnog OKSĆ-a je nepredvidivo, a varijabilnost progresije tumora motiviše istraživače da se fokusiraju na faktore koji utiču na prognozu ovog malignog oboljenja[2].

Prethodno prihvaćeni model u kojem se progresija oralnog karcinoma zasniva na kumulativnim genetskim promenama oralnog epitela prolazi kroz promene; predlaže se nov model u kojem tumorsko mikrookruženje (TMs) ima značajan doprinos u progresiji karcinoma. Novi model ima prognostički i terapeutski značaj[3].

OKSĆ se sastoji od dveju međusobno zavisnih komponenenti: tumorskih epitelnih ćelija i strome, u kojoj su ove ćelije dispergovane. TME ili tzv. stroma (TS) predstavlja složen sistem sastavljen od ćelijskih i nećelijskih komponenata. Ćelijska komponenta ne sadrži samo maligne ćelije već i ćelije mezenhimskog i hematopoetskog porekla, dok je nećelijska komponenta TMS-a ekstracelularni matriks (ECM). U takvom okruženju, tumorske ćelije reprogramiraju okolne stromalne ćelije za tumorigenezu, progresiju karcinoma i invaziju u okolno tkivo[4].

Podskup fibroblasta pod imenom fibroblasti povezani sa karcinomom (CAF) ima kritičnu ulogu u tumorigenezi, invaziji i progresiji OKSĆ-a, a kroz stimulaciju angiogeneze i rekonstrukciju ECM-a vrši pripreme za metastaze u ranoj fazi bolesti; takođe, promoviše recidiv tumora predviđajući lošu prognozu bolesti[5,6,7].

Unakrsna komunikacija između neoplastičnih i stromalnih ćelija modifikuje diferencijaciju, proliferativni i invazivni kapacitet tumorskih ćelija, stvarajući svojstva povezana sa malignitetom, kao što su otpornost na apoptozu, invazija kroz brže i bolje kretanje kroz ECM i sposobnost metastaza. Interakcije između invazivnih tumorskih ćelija i ECM-a rezultiraju novostvorenim okruženjem, koje je pogodno za rast i metastaze neoplastičnih ćelija[8].

Smatra se da CAF u TS-u potiče od normalnih fibroblasta (NF), ali mehanizam tranzicije NF u CAF u OKSĆ-u još uvek nije potpuno razjašnjen.

Introduction

Oral squamous cell carcinoma (OSCC) is a malignancy with a very high mortality[1] and low survival (less than 50%) due primarily to disease relapse, local recurrences, and distant organ metastasis. The biological behavior of oral SCC is unpredictable, and the variability of tumor progression is a motivation for researchers to focus on factors influencing the prognosis of this malignancy[2].

The previously accepted model in which oral cancer progression is based on cumulative genetic alterations of the oral epithelium is undergoing changes, and a new model is proposed in which the tumor microenvironment (TME) has a significant contribution to cancer progression. The new model has prognostic as well as therapeutic importance[3].

SCC consists of two interdependent components: tumor epithelial cells and stroma, in which they are dispersed. The TME or so-called "stroma" (TS) is a complex system composed of cellular and non-cellular components. The cellular component not only contains the malignant cells, but also contains cells of mesenchymal and hematopoietic origin, while the non-cellular component of the TME is the extracellular matrix (ECM). In such an environment, tumor cells reprogram the surrounding stromal cells for tumorigenesis, cancer progression, and invasion of the surrounding tissue[4].

A subset of fibroblasts called cancer-associated fibroblasts (CAFs) has a critical role in tumorigenesis, invasion and progression of OSCC, and through stimulation of angiogenesis and reconstruction of the ECM makes preparations for metastasis in the early stage of the disease. It also promotes tumor recurrence anticipating a poor disease prognosis[5,6,7].

Cross-communication between neoplastic and stromal cells modifies the differentiation, proliferative and invasive capacity of tumor cells, generating properties associated with malignancy such as resistance to apoptosis, invasion through faster and better movement through the ECM, and the ability to metastasize. Interactions between invading tumor cells and the ECM result in a newly created environment that is convenient to the growth and metastasis of neoplastic cells[8].

CAFs in TS are thought to originate from normal fibroblasts (NFs), but the mechanism of transition of NFs to CAFs in OSCC is still poorly elucidated. Many consider them to arise from NFs in which tumor cells induce epigenetic changes with

Mnogi smatraju da nastaju iz NF-a u kojima tumorske ćelije indukuju epigenetske promene sa posledičnom mutacijom NF u CAF. Međutim, CAF mogu nastati i epitelno-mezenhimskom tranzicijom (EMT) epitelnih ćelija iz matičnih ćelija koštane srži koje su podvrgnute EMT-u ili iz transdiferenciranih ćelija, kao što su adipociti, periciti ili ćelije glatkih mišića, citokini dobijeni iz karcionoma[9]. CAF proizvode nekoliko proteina specifičnih za mezenhim, uključujući alfa-aktin glatkih mišića (α-SMA). α-SMA odražava ekspresiju CAF-a u mezenhimu tumora i najčešći je marker CAF-a[10]. CAF koji su pretežno α-SMA pozitivni fibroblasti (CAFS α-SMA+), koji se nazivaju MF, jedan su od glavnih ćelijskih sastojaka TS-a.

Utvrđivanje stadijuma bolesti kod bolesnika sa OKSĆ-om preduslov je za definisanje hirurškog i onkološkog lečenja, ali i za procenu rizika od relapsa bolesti i procenu ukupnog preživljavanja (UP), i ima ključnu prognostičku vrednost. TNM sistem za stadijum karcinoma zasniva se na proceni veličine primarnog tumora (T), zahvatanju lokalno regionalnih limfnih čvorova sa metastazama (N) i udaljenim metastazama (M), ali TNM klasifikacija uzima u obzir samo anatomsko proširenje bolesti. Ugrađivanjem drugih parametara, kakvi su stepen diferencijacije malignih ćelija (G), limfo-vaskularna invazija (LI/VI) i perineuralna invazija (PnI), u postojeće patološke sisteme stadijuma, poboljšana je stratifikacija rizika za nepovoljne ishode, a samim tim i utvrđivanje optimalnog tretmana. Višestruke karakteristike u histopatološkom izveštaju imaju prognostički značaj[11-15]. Uspostavljanje preciznog sistema koji bi definisao anatomski stadijum uz uključivanje bioloških prognostičkih informacija predstavlja izazov za AJCC/UICC. Objavljivanje osmog izdanja AJCC/UICC 2017. godine dovelo je do velike prekretnice u određivanju patološkog stadijuma OKSĆ-a[16,19]. Preporučuju se dalje modifikacije poslednjeg, osmog izdanja, jer u određivanje stadijuma, za razliku od histopatološke analize, biomarkeri i novine iz molekularnih studija za OKSĆ još nisu ugrađeni[20].

Tradicionalni pristupi u histopatološkoj proceni i dalje se fokusiraju na sam tumor, a ne na interakcije između strome i domaćina. Iako su predloženi multifaktorski sistemi pokušali da naprave poboljšanja, trenutno su istraživanja raka fokusirana na ispitivanje uloge TME u progresiji tumora. Thode i saradnici[21] su na osnovu svojih rezultata predložili ažuriranje ovih sistema uvođenjem karakteristika CAF α-SMA+ u standardizovani histopatološki izveštaj.

consequent mutation of NFs in CAFs. But CAFs can also arise by epithelial-mesenchymal transition (EMT) of epithelial cells, from bone marrow stem cells that have undergone EMT, or from trans-differentiated cells such as adipocytes, pericytes or smooth muscle cells Transdifferentiation of NFs into CAFs is driven by cancer-derived cytokines[9]. CAFs produce several mesenchyme-specific proteins including alpha-smooth muscle actin (α-SMA). α-SMA reflects the expression of CAFs in the tumor mesenchyme and is the most common marker of CAFs[10]. CAFs which are predominantly α-SMA-positive fibroblasts (CAFS α-SMA+) called MFs, are one of the main cellular constituents of TS.

Determining the disease stage of a patient with OSCC is a prerequisite for defining surgical and oncological treatment, but also for assessing the risk of disease relapse and estimating overall survival (OS), and has a key prognostic value. The TNM system for cancer staging is based on an assessment of the size of the primary tumor (T), involvement of locoregional lymph nodes with metastasis (N) and distant metastases (M), but TNM classification only takes into account the anatomic extension of the disease. By incorporating other parameters, including the degree of differentiation of malignant cells (G), lymphovascular invasion (LI/VI) and perineural invasion (PnI) into existing pathological staging systems, the stratification of risks for unfavorable outcomes has been improved and consequently, determining the optimal treatment. Multiple features in the histopathological report have prognostic significance[11-15]. Establishing a precise system that would define anatomic staging while incorporating biological prognostic information is a challenge for the AJCC/UICC. The publication of the 8th edition of the AJCC/UICC in 2017 led to a major milestone in determining the pathologic staging of OSCC[16-19]. Further modifications of the last 8th edition are recommended because in determining the stage, apart from histopathological analysis, biomarkers and novelties from molecular studies for OSCC have not yet been incorporated[20].

Traditional approaches in histo-pathological evaluation still focus on the tumor itself, rather than on the interactions between the stroma and the host. Although proposed multifactorial systems have attempted to make improvements, currently cancer research is focused on examining the role of the TME in tumor progression. Thode et al.[21] based on their results proposed updating these systems by introducing the characteristics of CAFs α-SMA+ in the standardized histopathological report.

Materijal i metode

Uzorci tkiva

Ovo je retrospektivno-prospektivna kontrolisana studija, koja je obuhvatila ukupno 45 pacijenata sa patohistološki potvrđenim primarnim oralnim karcinomom skvamoznih ćelija (OKSĆ) na šest lokacija u usnoj duplji (jezik, dno usta, gingiva mandibularnog i maksilarnog alveolarnog grebena, bukalna sluzokoža, nepčana sluzokoža i retromolarno područje). Pacijenti su operisani na Univerzitetskoj klinici za maksilofacijalnu hirurgiju u Skoplju, u Severnoj Makedoniji, u periodu od 2016. do 2021. godine. Pacijenti koji su primali hemoterapiju i radioterapiju pre operacije nisu bili uključeni u studiju.

Uzorci tkiva primarnog karcinoma podvrgnuti su IHC-u i analizirani su na Institutu za patologiju u okviru Univerzitetskog kliničkog centra „Majka Tereza" u Skoplju. Imunohistohemija je izvedena korišćenjem *Dako EnVision flek* sistema. Primarno mišje monoklonsko antitelo anti-α-SMA korišćeno je za vizuelizaciju CAF-a.

Sprovođenje studije odobrila je Etička komisija Stomatološkog fakulteta „Sv. Kirilo i Metodije" Univerziteta u Skoplju, u Severnoj Makedoniji.

Bodovanje rezultata imunološkog bojenja

Analiza slajdova obavljena je digitalnim svetlosnim mikroskopom Nikon 80.

CAF su viđeni kao veliki fibroblasti u obliku vretena sa izduženim jezgrom, koje izražava α-SMA. Za određivanje gustine CAFs-a koristili smo modifikaciju sistema klasifikacije koji su dali Kellerman i saradnici[6,22] i Fujii i saradnici[23]. Nivoi gustine kategorisani su u četiri grupe:

Ocena 0 (negativno);
Ocena 1 (retko);
Ocena 2 (fokalna);
Ocena 3 (obilno).

Uzorci u kojima nisu identifikovani CAF ili oni sa manje od 1% MF obojenih α-SMA klasifikovani su kao negativni, uzorci koji su pokazali sporadično prisustvo rasutih CAF-a po celoj stromi klasifikovani su kao retki, oni sa žarišnim rasporedom koji se vidi kao nepravilno koncentrisano bojenje kao fokalni[23], dok su uzorci koji su pokazali brojne i gusto raspoređene CAF klasifikovani kao obilni (Figura1).

Material and methods

Tissue samples

This is a retrospective-prospective controlled study, which included a total of 45 patients with pathohistologically confirmed primary oral squamous cell carcinoma (OSCC) at six locations in the oral cavity (tongue, floor of mouth, gingiva of the mandibular and maxillary alveolar ridge, buccal mucosa, palatal mucosa and retromolar area). The patients were operated at the University Clinic for Maxillofacial Surgery in Skopje, North Macedonia in the period of 2016-2021. Patients who received chemotherapy and radiotherapy preoperatively were not included in the study.

Tissue samples from primary cancer 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. Primary mouse monoclonal antibody: anti-α-SMA was used for visualization of CAFs.

The conduct of the study was approved by the Ethics Committee at the Faculty of Dentistry, Ss. Cyril and Methodius University in Skopje, North Macedonia.

Scoring of immunostaining results

Analysis of the slides was performed with a Nikon 80 digital light microscope.

CAFs were seen as large spindle-shaped fibroblasts with an elongated nucleus expressing α-SMA. To determine the density of CAFs we used the modification of the classification system of Kellerman et al.[6,22] and Fujii et al.[23]. Density levels were categorized into 4 categories:

- Grade 0 (Negative)
- Grade 1 (Rare)
- Grade 2 (Focal)
- Grade 3 (Abundant)

Samples where no CAFs were identified or less than 1% of MFs stained with α-SMA were classified as negative; samples that showed the sporadic presence of scattered CAFs throughout the stroma were classified as rare; those with a focal arrangement seen as irregular concentrated staining were classified as focal[23]; while samples that showed numerous and densely arranged CAFs were classified as abundant (see Figure 1).

α-SMA immunoreactivity observed in endothelial cells in vessel walls was used as an internal positive control[24], and was not included in the calculation[23].

α-SMA imunoreaktivnost primećena u endotelnim ćelijama u zidovima krvnih sudova korišćena je kao interna pozitivna kontrola[24] i nije uključena u analizu[25].

Patološki stadijum bolesti (pTNM) određen je u skladu sa kriterijumima sedmog izdanja UICC/AJCC sistema za gradaciju tumora iz 2010. godine za oralni skvamocelularni karcinom[25-27]. Diferencijacija tumora klasifikovana je u četiri stepena prema Broderovoj histološkoj klasifikaciji diferencijacije tumorskih ćelija (Broderov stepen deskriptivnog sistema)[12,28] – dobro, umereno, loše, nediferencirani (anaplastični)−karcinomi.

Statistička analiza

Podaci dobijeni tokom istraživanja statistički su obrađeni pomoću softverskog paketa SPSS, verzija 20.0 za *Windows* (SPSS, Čikago, IL, SAD).

Fisherov egzaktni test i Fisher–Freeman–Halton egzaktni test korišćeni su kako bi se utvrdila povezanost između određenih atributivnih dihotomnih podataka. Za poređenje proporcija korišćen je test razlike. Za određivanje statističke značajnosti korišćena je dvostrana analiza sa nivoom značajnosti $p < 0,05$.

Pathological stage of disease (pTNM) was determined according to the 2010 UICC/AJCC Cancer Staging System 7th edition criteria for oral squamous cell carcinoma[25,26,27]. Tumor differentiation was classified into 4 grades: well, moderate, poor, undifferentiated (anaplastic)−carcinomas according to Broder's histological classification of differentiation of tumor cells (Broder's grading descriptive system)[12,28].

Statistical analysis

The data obtained during the research were statistically processed using the SPSS software package, version 20.0 for Windows (SPSS, Chicago, IL, USA).

Fisher exact test and Fisher Freeman Halton Exact test were used to determine the association between certain attributive-dichotomous data. Difference test was used to compare the proportions. A two-sided analysis with a significance level of $p<0.05$ was used to determine statistical significance.

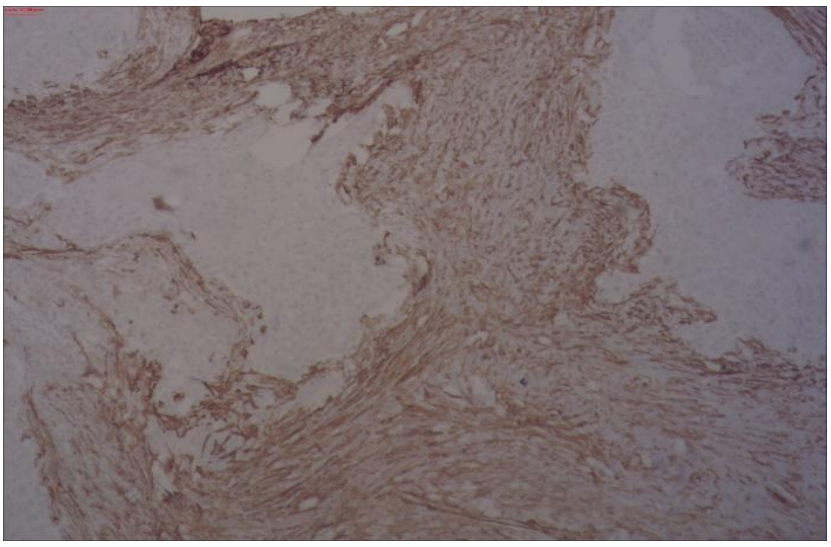

Figura 1. Obilna (Stepen 3) ekspresija α-SMA u tumorskoj stromi OKSĆ-a lokalizovanoj na podu usne duplje pTNM = pT3 pN2b Mk L1 Stadijum IVA

Figure 1. Abundant (Grade 3) expression of α-SMA in tumor stroma of OSCC localized in the floor of the mouth. pTNM=pT3 pN2b Mx L1 Stage IVA

Rezultati

α-SMA pozitivni CAFs (CAFs α-SMA+) utvrđeni su samo u tumorskoj stromi (TS) u 75,5% tumora, dok u 24,4% nisu pronađeni (Tabela 1).

U celom uzorku od 45 pacijenata (100%) *Difference* testom utvrđena je ocena 3 (obilno) kao najzastupljenija; postojala je statistički značajna razlika u zastupljenosti stepena 3 (obilno) u odnosu na ostale stepene gustine CAF-a (Tabela 1).

Nijedan od 45 pacijenata nije imao N3 i G4 stepen diferencijacije malignih epitelnih ćelija.

Fisher–Freeman–Halton egzaktnim testom utvrđena je značajna povezanost T, N, TNM stadijuma sa gustinom CAF-a, odnosno višim T, N, TNM stadijumima (veća tumorska lezija, pozitivni limfni čvorovi na vratu i uznapredovali stadijum bolesti) značajno povezani sa većom gustinom CAF-a, što je potvrđeno i dodatnim grupisanjem T, N i TNM stadijuma u dve kategorije (T1 → T2/ T3 →T4; N0 / N1 → N2 i TNM1 → TNM2/ TNM3 → TNM4), to su mali i veliki tumori, nemaju/imaju metastaze u limfnim čvorovima na vratu i rani/uznapredovali stadijum bolesti (Tabela 2).

Difference testom pokazano je da je procenat pacijenata koji imaju OKSĆ lokalizovan na jeziku sa CAF-om značajno veći od onih kod kojih CAF-a nije bilo. Kod svih pacijenata sa karcinomom dna usne šupljine utvrđeno je prisustvo CAF-a; ta ocena bila je „fokalna" u 12,5% i „obilna" u 87,5% slučajeva. Ni kod jednog od pacijenata sa ovom lokacijom nije primećeno odsustvo CAF-a (Tabela 2).

Nije utvrđena značajna povezanost stepena diferencijacije tumora (G), limfne invazije (LI), perineuralne invazije (PnI) i relapsa bolesti (R) sa gustinom CAFs-a (Fisher–Freeman–Halton egzaktni test) (Tabela 3).

Difference testom utvrđeno je da je samo u slučaju relapsa bolesti u vidu lokalnog recidiva (LR) procentualna zastupljenost pacijenata sa prisutnim CAF-om značajno veća u poređenju sa onima kod kojih CAF nisu pronađeni (Tabela 3).

Results

α-SMA positive CAFs (CAFs α-SMA+) were determined only in the tumor stroma (TS) in 75.5% of tumors, while in 24.4% they were not found (see Table 1).

In the entire sample of 45 patients (100%), the *Difference* test determined the grade 3 (abundant) as most represented percentage, and there was a statistically significant difference in the representation of grade 3 (abundant) in relation to the other degrees of density of CAFs (see Table 1).

None of the 45 patients had N3 and G4-grade of differentiation of the malignant epithelial cells.

Fisher Freeman Halton Exact test determined a significant association of T, N, TNM stage with the density of CAFs, i.e. higher T, N, TNM stages (bigger tumor lesion, positive neck lymph nodes and more advanced stage of the disease) significantly associated with a higher density of CAFs, which was also confirmed by additional grouping of T, N and TNM stage into two categories (T1→T2/ T3→T4; N0 / N1→N2 and TNM1→TNM2/ TNM3→ TNM4), that is small and large tumors, no/have neck lymph node metastases, and early/advanced disease stage (see Table 2).

The *Difference* test showed that the percentage of patients with OSCC localized on the tongue who had CAFs was significantly higher compared to those where CAFs were absent. In all patients with carcinoma of the floor of the mouth, the presence of CAFs was determined, and that grade was "focal" in 12.5% and "abundant" in 87.5%. In none of the patients with this location, the absence of CAFs was determined (see Table 2).

No significant association of the degree of tumor differentiation (G), lymphatic invasion (LI), perineural invasion (PnI) and disease relapse (R) with the density of CAFs was determined (Fisher Freeman Halton Exact test) (seeTable 3).

The *Difference* test determined that only in case of relapse of the disease in the form of local recurrence (LR), the percentage representation of patients with CAFs present was significantly higher compared to those where CAFs were not found (see Table 3).

Tabela 1. Analiza odabranih kliničko-patoloških parametara prema stepenu gustine CAF α-SMA+

Table 1. Analysis of selected clinical-pathological parameters according to degrees of density of CAFs α-SMA+

Parameters	CAFs α-SMA+ grade				p
	CAFs 0 N=11	CAFs 1 N=8	CAFs 2 N=6	CAFs 3 N=20	
T - stage					
T1 (N=12)	9 (75%)	2 (16.67%)	0 (0%)	1 (8.33%)	T1→T4 / N+R→F+A †p=0.0006*
T2 (N=16)	1 (6.25%)	4 (25%)	2 (12.5%)	9 (56.25%)	
T3 (N=8)	0 (0%)	2 (25%)	2 (25%)	4 (50%)	
T4 (N=9)	1 (11.11%)	0 (0%)	2 (22.22%)	6 (66.67%)	
T – stage (groups)					
T1→T2 (N=28)	10 (35.71%)	6 (21.43%)	2 (7.15%)	10 (35.71%)	†p=0.0491*
T3→T4 (N=17)	1 (5.88%)	2 (11.76%)	4 (23.53%)	10 (58.82%)	
N - stage					
N0 (N=31)	10 (32.26%)	6 (19.35%)	1 (3.23%)	14 (45.16%)	N0→N1 / N→A †p=0.0255*
N1 (N=11)	1 (9.09%)	2 (18.18%)	4 (36.36%)	4 (36.36%)	
N2 (N=3)	0 (0%)	0 (0%)	1 (33.33%)	2 (66.67%)	-
N – stage (groups)					
N0 (N=31)	10 (32.26%)	6 (19.35%)	1 (3.23%)	14 (45.16%)	†p=0.0164*
N1→N2 (N=14)	1 (7.14%)	2 (14.29%)	5 (35.71%)	6 (42.86%)	
TNM - stage					
TNM1 (N=10)	8 (80%)	2 (20%)	0 (0%)	0 (0%)	TNM1→TNM4 / N→R+F+A †p=0.00001*
TNM2 (N=12)	1 (8.33%)	4 (33.33%)	0 (0%)	7 (58.33%)	
TNM3 (N=11)	1 (9.09%)	2 (18.18%)	3 (27.27%)	5 (45.45%)	
TNM4 (N=12)	1 (8.33%)	0 (0%)	3 (25%)	8 (66.67%)	
TNM – stage (groups)					
TNM 1→TNM2 (N=22)	9 (40,91%)	6 (27,27%)	0 (0%)	7 (31,82%)	††p=0,0165*
TNM 3→TNM4 (N=23)	2 (8,70%)	2 (8,70%)	6 (26,09%)	13 (56,52%)	
Localisation					
Floor of the mouth (N=8)	0 (0%)	0 (0%)	1 (12.50%)	7 (87.50%)	-
Tongue (N=20)	6 (30%)	5 (25%)	1 (5.00%)	8 (40%)	†††p=0.0125*
Buccal mucosa (N=7)	2 (28.57%)	2 (28.57%)	3 (42.86%)	0 (0%)	†††p=0.1224
Gingival mucosa (N=8)	3 (37.50%)	0 (0%)	1 (12.50)	4 (50%)	†††p=0.3329
Retromolar area (N=1)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	-
Palate (N=1)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	-

T stadijum (veličina tumora); N stadijum (metastaze u cervikalnim limfnim čvorovima); TNM – patološki staging tumor–čvor–metastaza
Negativno + Retko/Fokalno + Obilno = N + R → F + A
† Fisher–Freeman–Halton egzaktni test; † † CAFs: nema/ima – Fisherov egzaktni test; ††† Test razlike (CAF nema/ima), *nivo značajnosti $p < 0,05$
CAFs0 = Negative; CAFs1 =Rare; CAFs2 = Focal; CAFs3 = Abundant
T- stage (tumor size); N- stage (cervical lymph node metastasis);TNM – pathological tumor-node-metastasis staging
Negative+Rare/Focal+Abundant = N+R→F+A
†Fisher Freeman Halton Exact test;† † CAFs : no/have – Fisher exact test ;†††Difference test (CAFs no/have),*significant for $p<0.05$

Tabela 2. Analiza odabranih kliničko-patoloških parametara prema stepenu gustine CAFs α-SMA+

Table 2. Analysis of selected clinical-pathological parameters according to the degree of density of CAFs α-SMA+

Parameters	CAFs α-SMA grade				p
	CAFs 0 N=11	CAFs 1 N=8	CAFs 2 N=6	CAFs 3 N=20	
G - grade					
G1 (N=6)	2 (33.33%)	2 (33.33%)	0 (0%)	2 (33.33%)	G1→G3 / N+R→F+A †p=0.3573
G2 (N=28)	9 (32.14%)	3 (10.71%)	4 (14.29%)	12 (42.86%)	
G3 (N=10)	0 (0%)	3 (30%)	2 (20%)	5 (50%)	
G – grade (groups)					
G1→ G2 (N=34)	11 (32.35%)	5(14.71%)	4 (11.76%)	14 (41.18%)	††p=0.4744
G3 (N=10)	0 (0%)	3 (30%)	2 (20%)	5 (50%)	
LI– Lymphatic invasion					
No invasion (N=28)	9 (32.14%)	6 (21.43%)	3 (10.71%)	10 (35.71%)	†p=0.2609
Have invasion (N=17)	2 (11.76%)	2 (11.76%)	3 (17.65%)	10 (58.82%)	
PnI – Perineural invasion					
No invasion (N=37)	11 (29.73%)	6 (16.22%)	3 (8.11%)	17 (45.95%)	No→Have / N+R→F+A ††p=0.4355
Haveinvasion (N=8)	0 (0%)	2 (25%)	3 (37.50%)	3 (37.50%)	
R - Recurrence					
No (N=17)	3 (17.65%)	3 (17.65%)	3 (17.65%)	8 (47.06%)	†p=0.7948
Have (N=27)	8 (29.63%)	5 (18.52%)	3 (11.11%)	11 (40.74%)	
TR–Type of reccurence					
LNM (N=5)	3 (60%)	0 (0%)	1 (20%)	1 (20%)	†††p=0.5485
LR (N=18)	3 (16.67%)	5 (27.78%)	1 (5.56%)	9 (50%)	†††p=0.0001*
LR+LNM (N=4)	2 (50%)	0 (0%)	1 (25%)	1 (25%)	†††p=1.000

CAFs0 = Negativni; CAFs1 = Retko; CAFs2 = Fokalno; CAFs3 = Obilno
G – stepen diferencijacije; G1 – dobro diferentovan; G2 – umeren; G3 – slab; G4 – nediferentovan (anaplastični karcinom)
R – rekurentan; TR – tip rekurencije; LNM – metastaze cervikalnih limfnih čvorova; LR – lokalni recidiv; LR + LNM – lokalni recidiv i metastaze u cervikalnim limfnim čvorovima
† Fisher–Freeman–Halton egzaktni test; † † CAFs stepen: negativan + retko/fokalno + obilan – Fisherov egzaktni test; ††† Test razlike (CAF nema/ima)
*nivo značajnosti $p < 0,05$CAFs0 = Negative; CAFs1 =Rare; CAFs2 = Focal; CAFs3 = Abundant
G-grade of differentiation; G1-well differentiated, G2-moderate; G3-poor; G4-undifferentiated (anaplastic carcinoma)
R-reccurence, TR- type of reccurence; LNM – cervical lymph node metastasis; LR – local recurrence;LR+LNM - local recurrence and cervical lymph node metastases
†Fisher Freeman Halton Exact test ;† † CAFs degree: Negative+Rare/Focal+Abundant – Fisher exact test; †††Difference test (CAFs No/Have)
*significant for $p<0,05$

Tabela 3. Distribucija OKSĆ pacijenata prema stepenu gustine CAFs a-SMA+

Table 3. Distribution of OSCC patients according to degrees of density of CAFs α-SMA+

OSCC (N=45)		
CAFs α-SMA grade / stepen	N (%)	[1]p
CAFs 0 – Negative / Negativno	11 (24.44%)	N/R – p=0.4421 N / F - p=0.1806 N/ A - p=0.0471* R / F - p=0.5617 R / A - p=0.0066* F / A - p=0.0012*
CAFs 1 – Rare / Retko	8 (17.79%)	
CAFs 2 - Focal / Fokalno	6 (13.33%)	
CAFs 3 - Abundant / Obilno	20 (44.44%)	

[1]Difference test *nivo značajnosti / [1]Difference test *significantp<0,05

Diskusija

U tumorskoj stromi OKSĆ-a, gustina CAF-a u interakciji sa kancerskim ćelijama je promenljiva. U in vitro studiji Kellermann i sar.[22] uočeno je povećanje ekspresije α-SMA u OSCC-u tokom transdiferencijacije NF u CAF posredovane stimulacijom TGF-b1[22]. Intrigantni su podaci o visoko-polimorfnom duktalnom karcinomu (dojke in situ) i neinvazivnom urotelnom karcinomu mokraćne bešike kod kojih su CAF identifikovani u stromi koja okružuje kancerske ćelije; pokazujući da faktori koji potiču iz agresivnih malignih epitelnih tumorskih ćelija mogu prelaziti bazalnu membranu koja stimuliše transformaciju MFs-a[29].

Chaudhari i sar.[30], Kapse i sar.[31] i Chauhan i sar.[32] istraživali su ulogu CAF-a u progresiji premalignih lezija u oralnom skvamoznom karcinomu i utvrdili da je povećana distribucija CAF-a u korelaciji sa progresijom potencijalno malignih lezija oralne sluzokože u invazivni fenotip, tj. kod karcinoma skvamoznih ćelija usne šupljine, zaključivši da se mogu koristiti kao stromalni markeri za maligne lezije oralne sluzokože.

U ovoj studiji klasifikovali smo gustinu CAF-a u četiri stepena, koristeći novu definiciju „fokalne" distribucije (Ocena 2)[23] i vodeći se nalazima određenih studija o raku dojke i kolorektalnom karcinomu koje su pokazale da su stepeni 2 i 3 koncentrisani na invazivnom prednjem delu tumora i sugerisale da se interakcija CAF-a sa ćelijama raka dešava upravo na invazivnom prednjem delu tumora[23,33].

Analizom T stadijuma u našoj studiji utvrđeno je da najveći procenat (75%) pacijenata sa malim tumorima (T1) nije imao CAF, kao i da je najveći procenat (66,7%) pacijenata sa velikim tumorima (T4) imao obilnu (Ocena 3) gustinu CAF-a; dakle, što je tumorska lezija veća, to je veća gustina CAF-a. Ne samo da se gustina CAF-a kvantitativno povećava u većim tumorima, u primarnim tumorima sa pozitivnim limfnim čvorovima na vratu za metastatske naslage (N+) i u uznapredovalim stadijumima bolesti, već su i veliki tumori i N+ vrata takođe značajno povezani sa „fokalnim" obrascem (Stepen 2) distribucije CAF-a. Kaplan–Mejerova analiza preživljavanja u studiji OKSĆ-a otkrila je značajno nižu stopu preživljavanja i najveći mortalitet kod pacijenata sa „fokalnom distribucijom" CAF-a (Stepen 2) u poređenju sa drugim stepenom gustine.

Discussion

In the tumor stroma of OSCC, the density of CAFs interacting with cancer cells is variable. Kellermann et al.[22] in vitro study reported an increase in α-SMA expression in OSCC during transdifferentiation of NFs into CAFs mediated by stimulation of carcinoma-derived TGF-β1[22]. Intriguing is the data on highly polymorphic ductal carcinoma in situ of the breast and non-invasive urothelial carcinoma of the bladder in which CAFs were identified in the stroma surrounding the cancer cells, demonstrating that factors derived from aggressive malignant epithelial tumor cells are able to cross the basement membrane stimulating transformation of MFs[29].

Chaudhary et al.[30], Kapse et al.[31] and Chauhan et al.[32] investigated the role of CAFs in the progression of premalignant lesions in oral squamous cell carcinoma, and determined that an increased distribution of CAFs correlated with the progression of potentially malignant lesions of the oral mucosa to an invasive phenotype ie. in oral squamous cell carcinoma, concluding that they can be used as stromal markers for malignant lesions of the oral mucosa.

In our study, we classified the density of CAFs into 4 grades, using a new definition of "focal" distribution (grade 2)[23], guided by findings of some studies of breast cancer and colorectal cancer, which demonstrated that grades 2 and 3 are concentrated at the invasive front of the tumor and suggested that the interaction of CAFs with cancer cells occurs precisely at the invasive front of the tumor[23,33].

Analysis of T-stage in our study determined that the highest percentage (75%) of patients with small tumors (T1) had no CAFs, while the highest percentage (66.7%) of patients with large tumors (T4) had abundant (Grade 3) density of CAFs, that is, the larger the tumor lesion, the higher the density of CAFs. But not only does the density of CAFs increase quantitatively in larger tumors, in primary tumors with positive neck lymph nodes for metastatic deposits (N+) and in advanced disease stages, but large tumors and N+ neck are also significantly associated with the "focal" pattern (Grade 2) of CAFs distribution. Kaplan–Meyer survival analysis in a study of OSCC revealed a significantly lower survival rate and the highest mortality in patients with a "focal distribution" of CAFs (Grade 2) compared to other density grades. They explained this by the scattered distribution of CAFs in Stage 2 which allows cancer cells to migrate between CAFs, and the interactions between these two types of cells contribute to tumor growth and disease

To je objašnjeno raštrkanom distribucijom CAF-a u fazi 2, koja omogućava kancerskim ćelijama da migriraju između CAF-a; interakcije između ovih dvaju tipova ćelija doprinose rastu tumora i progresiji bolesti[23]. Nasuprot našim rezultatima, neki istraživači nisu uspeli da pronađu povezanost gustine CAF-a sa veličinom primarnog tumora[22, 23], ali su opsežne studije ustanovile značajnu povezanost između visoke gustine CAF-a i velikih karcinoma jezika[34] i nazofarinksa[35].

Marilin Vered i sar.[36] su u studiju CAFs-a i EMT-a u metastatskom OKSĆ-u jezika uključili 19 podudarnih parova uzoraka primarnog tumora i cervikalnih limfnih čvorova sa metastazama. Korišćenjem panela imunohistohemijskih markera za identifikaciju CAF-a i EMT-a utvrđeno je da se sa EMT-om gubi epitelni fenotip karcinoma i stiču mezenhimske karakteristike neophodne za invaziju tumora i metastaze. Ulogu stromalnih miofibroblasta (MF) kao prediktivnih markera za metastaze u limfnim čvorovima potvrdili su Smitha i sar.[37]. Rezultati njihove studije pokazali su značajno veći broj CAF α-SMA+ u N+ statusu u poređenju sa N0 statusom, potvrđujući pozitivnu korelaciju između visokih gustina CAF-a i N stadijuma kod pacijenata sa OKSĆ-om, a to potvrđuje i rezultat dobijen u našoj studiji.

Određene kliničko-patološke studije pokazale su da gustina CAF-a u OKSĆ-u značajno korelira sa N statusom, VI i LI, što sugeriše da CAF pomažu u metastazama, dok obilno prisustvo CAF-a u prednjem delu invazivnog tumora korelira sa PnI i kraćim vremenom preživljavanja, tj. obilno prisustvo CAF-a u njihovoj studiji indikativno je za postojanje biološki agresivnijeg krcinoma[6].

Analiza odsustva/prisustva CAF-a u primarnim tumorima u odnosu na relaps bolesti pokazala je da nema značajne razlike u procentualnoj zastupljenosti pacijenata sa CAF-om i bez njih. Stepen 3 je preovladavao skoro podjednako kod pacijenata sa relapsom i bez recidiva. Kod pojave lokalnog recidiva, procentualna zastupljenost pacijenata koji su imali CAF bila je značajno veća u odnosu na pacijente koji nisu imali CAF, što možda predviđa da će kod pacijenata koji imaju CAF doći do eventualnog relapsa bolesti u vidu lokalnog recidiva. U studiji Kellermana i sar.[22] obilno prisustvo CAF-a u primarnom OKSĆ-u značajno je povezano sa N+ na početnoj prezentaciji i sa pojavom LNM-a na vratu nakon tretmana, odnosno visoka gustina CAF-a ukazuje na postojanje izrazito agresivnih tumora koji pokazuju tendenciju recidiva, u vidu metastaza na vratu.

progression[23]. In contrast to our results, some researchers have failed to find an association of CAFs density with the size of the primary tumor[22,23], but extensive studies have established a significant association between a high density of CAFs and large cancers of the tongue[34] and nasopharynx[35].

Marilyn Vered et al.[36] in a study of CAFs and EMT in metastatic OSCC of the tongue included 19 matched pairs of primary tumor and cervical lymph node specimens with metastatic deposits. Through the use of a panel of immunohistochemical markers for the identification of CAFs and EMT, she determined that with EMT, the epithelial phenotype of cancer is lost, and mesenchymal characteristics necessary for tumor invasion and metastasis are acquired. The role of stromal myofibroblasts (MFs) as predictive markers for lymph node metastasis was confirmed by Smitha et al.[37]. Their results demonstrated a significantly higher number of CAFs α-SMA+ in N+ status compared to N0 status, confirming a positive correlation between a high density of CAFs and N-stage in OSCC patients, which is also confirmed by the result obtained in our study.

Some clinicopathological studies have demonstrated that the density of CAFs in OSCC significantly correlates with N-status, VI, and LI, suggesting that CAFs assist in metastasis, while the abundant presence of CAFs in the invasive tumor front correlates with PnI and shorter survival time i.e. the abundant presence of CAFs in their study was indicative of the existence of a biologically more aggressive cancer[6].

The analysis of the absence/presence of CAFs in primary tumors in relation to disease relapse showed that there was no significant difference in the percentage representation of patients who did not have/have CAFs. Grade 3 predominated almost equally in relapsed and non-relapsed patients. At the occurrence of local recurrence, the percentage representation of patients who had CAFs was significantly higher compared to patients who did not have CAFs, which perhaps anticipates that in patients who have CAFs, eventual relapse of the disease would occur in the form of local recurrence.

In the study by Kellerman et al.[22] the abundant presence of CAFs in primary OSCC was significantly associated with the N+ at the initial presentation and with the appearance of neck LNMs after treatment, i.e. the high density of CAFs indicated the existence of distinctly aggressive tumors that

Ova studija je, prema našim saznanjima, prvi zapis do sada o tome da visoka gustina CAF-a može biti korisna u predviđanju prognoze pacijenata sa oralnim karcinomom, pošto su cervikalni LNM jedan od najvažnijih prognostičkih faktora za bolest. Suprotno našim rezultatima, obilno prisustvo CAF-a u njihovoj studiji nije bilo povezano sa lokalnim recidivom bolesti.

Analiza šest lokalizacija oralnog karcinoma pokazala je da je najčešći karcinom jezika. Ovaj karcinom se razlikuje od karcinoma drugih lokalizacija i prema rezultatu koji je pokazao da je broj pacijenata sa karcinomom jezika, koji imaju CAF (70%) značajno veći od onih koji nemaju CAF. Za razliku od ovog karcinoma, svi pacijenti sa karcinomom dna usne šupljine imali su CAF, a čak 87,5% njih imalo je „obilnu" (Stepen 3) gustinu. Imajući u vidu dobijene rezultate, možda su CAF ključni akteri odgovorni za to što je karcinom ovih dveju lokalizacija najteže kontrolisati. Prema analizi baze podataka *Surveillance, Epidemiology, and End Results* (SEER), vredi napomenuti da je jezik najčešća lokalizacija i da je povezan sa većom smrtnošću oralnih karcinoma drugih lokalizacija[20].

Opšte je prihvaćeno da CAF ispoljavaju protumorski efekat stimulišući rast i progresiju tumora. Međutim, nedavne studije pokazuju efekat CAF-a na inhibiciju tumora, što sugeriše da oni pokazuju sličan stepen plastičnosti kao i druge stromalne ćelije. Recipročne interakcije sa okruženjem tumora i različitim izvorima porekla pojavljuju se kao dva važna faktora koja podržavaju heterogenost CAF-a. Ova studija naglašava nedavni napredak u razumevanju biologije CAF-a i predlaže da se proširi pojam ćelijske „polarizacije", koji je prethodno uveden kako bi se opisala različita stanja aktivacije CAF-a, čime se potvrđuje njihova fenotipska raznolikost[38].

Sa idejom da se razjasni uloga CAF-a u OSCC-u, zaključci studije Vereda i sar.[39] i Lima i sar.[40] ukazuju na to da njihova gustina odražava biološka svojstva tumora. Shodno tome, veća gustina ukazuje na agresivniji tumor, sa većom moći za recidiv i lošijom prognozom.

show a tendency to relapse, in the form of neck metastases. This study, to our knowledge, is the first record so far that a high density of CAFs can be useful in predicting the prognosis of patients with oral cancer, since cervical LNMs are one of the most important prognostic factors for the disease. Contrary to our results, the abundant presence of CAFs in their study was not associated with local disease recurrence.

The analysis of the 6 localizations of oral cancer showed that the most common was tongue cancer. Tongue carcinoma differs from carcinomas of other localizations and according to the result that indicated that the number of tongue carcinoma patients who have CAFs (70%) is significantly higher compared to those who do not have CAFs. In contrast to this carcinoma, all patients with carcinoma of the floor of the mouth had CAFs, and even 87.5% of them had "abundant" (Grade 3) density. Considering the obtained results, perhaps CAFs are the key players responsible for the fact that cancers of these two localizations are the most difficult to control. According to the analysis of the *Surveillance, Epidemiology, and End Results* (SEER) database, it is noteworthy that the tongue is the most common localization and is associated with higher mortality than oral cancers of other localizations[20].

It is generally accepted that CAFs exhibit a pro-tumor effect by stimulating tumor growth and progression. But recent studies demonstrate a tumor-inhibitory effect of CAFs suggesting that they exhibit a similar degree of plasticity as other stromal cells. Reciprocal interactions with the tumor environment and different sources of origin appear as two important factors underpinning the heterogeneity of CAFs. This study highlights recent advances in understanding of the biology of CAFs and proposes to extend the term cell "polarization" previously introduced to describe different activation states of CAFs thus confirming their phenotypic diversity[38].

With the idea of elucidating the role of CAFs in OSCC, the conclusions of the study by Vered et al.[39] and Lim et al.[40] indicated that their density reflects the biological properties of the tumor. Accordingly, a higher density indicates a more aggressive tumor, with a greater power for recurrence and a worse prognosis.

Zaključak

Nekoliko studija ukazalo je na mogućnost upotrebe CAF-a kao važnog prognostičkog faktora kod različitih tumora, iako je njihov klinički značaj u prognostičke svrhe za oralni karcinom skvamoznih ćelija retko prijavljivan. Napredak istraživanja biomarkera specifičnih za oralni karcinom skvamoznih ćelija još uvek je nezadovoljavajući; otuda postoji potreba za prospektivnim analizama za njihovu identifikaciju i upotrebu u skriningu i identifikaciji pojedinaca u riziku od razvoja primarnog karcinoma ili identifikacije agresivnih tumora sa rekurentnim karakteristikama koji imaju lošu prognozu.

Zahvalnica: Nema

Sukob interesa: Nema

Conclusion

Several studies have indicated the possibility of CAFs being used as an important prognostic factor in a variety of tumors although their clinical significance in prognostic purposes for oral squamous cell carcinoma has rarely been reported. Research progress on biomarkers specific to oral squamous cell carcinoma is still unsatisfactory. Hence, there is a need for prospective analyses for their identification and use in screening and identification of individuals at risk of developing primary cancer or identification of aggressive tumors with recurrent features that have a poor prognosis.

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