Prognostic Value of Tumor-stroma Ratio in Oral Squamous Cell Carcinoma: Contribution of Cancer Associated Fibroblasts

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Research

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Abstract

Background: The aim of this study is to confirm the prognostic value of the tumor–stroma ratio (TSR) in a large cohort of oral squamous cell carcinoma (OSCC) and further demonstrated the cancer associated fibroblasts (CAFs)-stroma ratio (CSR) served as a critical biomarkers contributed to the prognostic value of TSR

Results: The threshold level of TSR value is 50%, which divides patients into high (>50%) and low (<50%) stroma. We examined the TSR on hematoxylin and eosin-stained tissue samples from 581 patients with oral squamous cell carcinoma and 298 cases were included in the high-stroma group. In multivariate analysis, the TSR was identified as an independent prognostic factor for disease-free survival (DFS) (hazard ratio (HR), 2.11; 95% confidence interval (CI), 1.56–2.86; $p < 0.001$) and oral cancer-specific survival (OCSS) (HR, 2.56, 95% CI, 1.78–3.67; $p < 0.001$). The interaction term reached statistical significance for histological grade. Multivariate analysis confirmed the discriminative value of the TSR in well differentiated tumors for DFS and OCSS separately ($P=0.001$, $P=0.003$). The prognostic value of TSR was not varied by other clinically subgroups. Furthermore, the high-stroma group had a higher Fibroblast Activation Protein (FAP+) CSR and α -Smooth Muscle Actin (α -SMA+) CSR than the low-stroma group ($p < 0.001$).

Conclusion: High-stroma levels indicated a negative consequence and a higher CAFs–stroma ratio than low-stroma levels in OSCC. The TSR is not altered by other clinically elements rendering it a credible histological parameter and informing the rational design of individual cancer management.

Background

Annually, 270,000 people are affected by oral cancer worldwide, and roughly 90% of cases are oral squamous cell carcinoma (OSCC), which usually develop in the lingual, buccal, and gingival areas with a mortality rate of 40–50% [1, 2].

Currently, the paradigms of treatments for OSCC remain surgical resection with postoperative chemotherapy and/or radiotherapy [3]. Whether adopting neck lymph node dissection partly depends on the pathological diagnosis through hematoxylin and eosin stained slides (H&E) focusing on the tumor cell, while ignoring the surrounding stroma, which characterized all kinds of elements adjacent to the cancer cells, consisting of fibroblasts, endothelial cells, inflammatory cells, intertwined blood vessels, and the extracellular matrix (ECM) [4]. Unlike normal tissue stroma, the tumor micro-environment is distinctly disparate in mechanical yet physiological properties partly ascribe to the bountiful cancer associated fibroblasts (CAFs), which establish the complex signal network between cell and stroma crosstalk via various cytokines, chemokines and extracellular vesicles facilitating events such as tumor proliferation, angiogenesis, invasion, metastasis and therapy resistance [5]. In view of the importance of the tumor stroma, extensive studies were conducted to determine the prognostic elements, whereas integration into

clinical application is limited. Incorporating relevant parameters that translate microenvironment in the routine pathology diagnosis is imperative.

The tumor–stroma ratio (TSR) has a promising prospects, which represents the relative abundance of stroma in cancer cells validated in many solid tumors, including colon, breast and oesophagus cancers [6–8]. Additionally, the core competitiveness of TSR evaluation is easy, quick, reproducible, and inexpensive by visually assessing routinely retrieved H&E slides used for pathological cases.

Previously, in early-stage oral tongue cancers Almangush et al. has assessed the prognostic impact of TSR [9]. By comparison, we confirmed the preponderance of the TSR as a prognostic tool in the whole oral cancer, specifically those with buccal, tongue, and gingival squamous cell carcinoma. Furthermore, we found the prognostic value of TSR is chiefly discerning in moderately and highly differentiated tumors. More importantly, we firstly substantiated that a difference in the cancer-associated fibroblast (CAFs)–stroma ratio(CSR) exists between the high and low-stroma groups and this discrepancy has influence on the prognostic power of TSR. Our present work clarified the prognostic value of TSR and highlight the contribution of CAFs–stroma ratio (CSR).

Results

Patients and tumor characteristics

Initially n = 850 patients with oral squamous cell carcinoma at School & Hospital of Stomatology, Wuhan University were incorporated. After telephone follow-up n=632 patients with available follow-up data were included. Finally, we evaluated 581 H&E-stained slides from patients for TSR scoring because of the loss or inferior quality of the excluded slides(n=51). It is worth noting that inferior quality refers to difficulty in judging the TSR groups of tumor with little invasiveness (inplementary materias figure 5). Interestingly most of the analyzed area on the slide are in the invasive front. The mean age of the patients was 57.08 years at the time of operation. The mean follow-up period was 30.33 months (range, 1–36 months). The basic clinicopathological characteristics of the patients are shown in Table 1. Most tumors were of oral tongue cancers (56.11%), male (62.13%), no lymph node metastasis (73.67%), <4cm (81.41%), and grade I or II (87.09%).

Table 1
Overview of the baseline patient and tumor characteristics (Grouped by Tumor Stroma Ratio).

	Stroma-high			Stroma-low		
	n	n=298	%	n=283	%	p-value
Gender		298		283		
Male	361	181	60.7	180	63.6	0.477
Female	220	117	39.3	103	36.4	
Age(years)						
<40	58	26	8.7	32	11.3	0.592
40to<49	100	57	19.1	43	15.2	
50to<59	150	80	26.8	70	24.7	
≥60	273	135	45.3	138	48.8	
Tstatus						
T1	177	85	28.5	92	32.5	0.394
T2	296	150	50.3	146	51.6	
T3	66	38	12.8	28	9.9	
T4	42	25	8.4	17	6.0	
Nstatus						
N0	428	203	68.1	225	79.5	0.012
N1	80	48	16.1	32	11.3	
N2	69	44	14.8	25	8.8	
N3	4	3	1.0	1	0.4	
pTNM stage						
I	154	73	24.5	81	28.6	0.078
II	214	101	33.9	113	39.9	
III	112	63	21.1	49	17.3	
IV	101	61	20.5	40	14.1	
Grade						
I	216	95	31.9	121	42.8	0.025

	Stroma-high			Stroma-low		
II	290	162	54.4	128	45.2	
III	75	41	13.8	34	12.0	
location tumor						
buccal	156	76	25.5	80	28.3	0.307
tongue	326	178	59.7	148	52.3	
gingival	99	47	15.8	52	18.4	

We used NDP.view2 to visually assess the TSR in the most invasive part of the tumor. Cohen's Kappa coefficient of inter-observers increased from 0.762 at the beginning to a nearly perfect accordance at second evaluation, indicating a good agreement and reproducibility in the method. Ultimately, 298 patients were scored as high stroma, and 283 patients were scored as low stroma (Figure.1A). Meanwhile the excluded slides with little invasiveness were shown in supplementary material figure 6.

The Prognostic Value Of The Tsr

To assess the prognostic value of the TSR, we performed the chi-square test. No significant differences in gender, age, T status, pTNM stage, and tumor location were observed between the high and low-stroma groups (Table 1).

The Kaplan–Meier and log-rank tests of the TSR showed that the high-stroma group had a significantly better prognosis ((Figure.1B). Specifically, the DFS and OCSS of the high-stroma group were 77.74% and 55.70%, respectively, whereas those of the low-stroma group were 85.10% and 63.95%, respectively, indicating that the low-stroma group had a worse prognosis. Even after correcting for confounders in the subsequent multivariate Cox regression analysis, the hazard ratio (HRs) of the TSR was 2.11 (95% confidence interval (CI) 1.56–2.86; $p < 0.001$) for DFS and 2.56 (95% CI, 1.78–3.67; $p < 0.001$) for OCSS. Kaplan–Meier curve was also created for OS ($P < 0.001$) including the multivariate analysis (Supplementary materials Table 1, Figure.1). Additionally, the prognostic value of the TSR was significant within subgroups: buccal ($p = 0.006$), lingual ($p < 0.001$), and gingival ($p = 0.009$) (Supplementary materials Figure.2).

Then, we performed univariate Cox regression analysis and found that not only TSR (DFS, HR = 2.34, 95% CI 1.73–3.16, $p < 0.001$; OCSS, HR = 2.74, 95% CI 1.92–3.92, $p < 0.001$) but also gender, T status, N status, pTNM stage, and differential grade were statistically significant for DFS and OCSS (Table 2). Furthermore, in the multivariate Cox regression analysis (Table 2), gender remained statistically significant for OCSS ($p = 0.006$), and differential grade was statistically significant for DFS ($p < 0.001$) and OCSS ($p < 0.001$).

Table 2

Univariate and multivariate analysis of disease-free survival (DFS) and oral cancer-specific survival (OCSS) in Cox regression analysis.

univariate analysis							
	DFS				OCSS		
	n	HR	95%CI	P-value	HR	95%CI	P-value
Gender							
Male	361			0.315			0.005
Female	220	0.86	0.64-1.15		0.60	0.42-0.86	
Age(years)							
<40	58			0.252			0.101
40to<49	100	1.28	1.70-2.36		1.48	0.71-3.08	
50to<59	150	1.65	0.94-2.91		2.05	1.04-4.04	
≥60	273	1.28	0.74-2.22		1.42	0.73-2.76	
T status							
T1	177			0.013			0.017
T2	296	1.49	1.05-2.12		1.57	1.04-2.36	
T3	66	1.63	1.00-2.66		1.60	0.90-2.84	
T4	42	2.29	1.36-3.85		2.54	1.41-4.57	
N status							
N0	428			<0.001			<0.001
N1	80	1.57	1.07-2.30		1.34	0.83-2.15	
N2	69	2.32	1.61-3.34		2.97	2.01-4.38	
N3	4	2.65	0.65-10.71		3.78	0.93-15.37	
pTNM stage							
I	154			<0.001			<0.001
II	214	1.41	0.94-2.12		1.34	0.83-2.16	
III	112	1.84	1.18-2.87		1.56	0.91-2.65	
IV	101	2.53	1.64-3.90		3.14	1.94-5.08	
Grade							

univariate analysis							
I	216			<0.001			<0.001
II	153	2.15	1.51-3.06		2.59	1.69-3.97	
III	27	3.12	2.03-4.81		3.89	2.35-6.45	
tumor location							
buccal	156			0.416			0.327
tongue	326	0.91	0.66-1.27		0.99	0.67-1.46	
gingival	99	1.17	0.77-1.77		1.34	0.83-2.16	
TSR							
stroma-low	283			<0.001			<0.001
stroma-high	298	2.34	1.73-3.16		2.74	1.92-3.92	
multivariate analysis							
DFS				OCSS			
	n	HR	95%CI	P-value	HR	95%CI	P-value
Gender							
Male	361			0.373			0.006
Female	220	0.87	0.65-1.18		0.59	0.41-0.86	
T status							
T1	177			0.515			0.555
T2	296	1.22	0.62-2.39		1.48	0.66-3.30	
T3	66	0.96	0.44-2.10		0.93	0.37-2.34	
T4	42	1.75	0.71-4.32		1.33	0.48-3.70	
N status							
N0	428			0.169			0.215
N1	80	1.01	0.53-1.94		0.84	0.39-1.79	
N2	69	2.30	0.95-5.60		1.69	0.64-4.47	
N3	4	5.20	0.94-28.67		5.74	0.99-33.28	
pTNM stage							
I	154			0.692			0.873

univariate analysis					
II	214	1.04	0.48-2.25	0.79	0.32-1.98
III	112	1.29	0.53-3.17	1.10	0.39-3.12
IV	101	0.72	0.23-2.31	1.07	0.29-3.95
Grade					
I	216		<0.001		<0.001
II	153	1.85	1.28-2.66	2.36	1.52-3.67
III	27	2.56	1.63-4.02	3.17	1.88-5.34
TSR					
stroma-low	283		<0.001		<0.001
stroma-high	298	2.11	1.56-2.86	2.56	1.78-3.67

Tsr Stratified By Clinically Crucial Subgroups

To find the prognostic value of TSR in clinically essential subgroups, Cox regression analysis was performed. We introduced the interaction term to assess the clinically discriminative subgroups and found that grade was statistically significant for DFS ($p < 0.001$) and OCSS ($p = 0.003$) (Table 3). No statistical differences were observed between the groups when stratified by gender, T status, N status, and pTNM stages. In the multivariate Cox regression analysis, the prognostic value of the TSR was most discriminative in grade I carcinomas for DFS (HR, 4.38; 95% CI, 2.14–8.95; $p < 0.001$) and in grade I, II carcinomas for OSCC (HR, 4.75; 95% CI, 1.93–11.68; $p = 0.001$ and HR, 2.46; 95% CI, 1.53–3.97; $p < 0.001$, respectively). The Kaplan–Meier analysis for DFS of the TSR combined with grade displayed a statistically significant difference among the subgroups (Figure.1C).

Table 3

Results of the tumor–stroma ratio stratified by clinically important prognostic parameters in the Cox regression mode.

TSR stratified by group	Subgroups	DFS	OCSS
Gender		P=0.524	P=0.131
Tstatus		P=0.379	P=0.558
Nstatus		P=0.678	P=0.769
pTNM stage		P=0.275	P=0.793
Grade		P=0.001	P=0.003
	I	HR 4.38,95%CI 2.14-8.95,P<0.001	HR 4.75,95%CI 1.93-11.68,P=0.001
	II	HR 1.59,95%CI 1.07-2.35,P=0.021	HR 2.46,95%CI 1.53-3.97,P<0.001
	III	HR 2.25,95%CI 1.04-4.84,P=0.039	HR 1.59,95%CI 0.69-3.67,P=0.274

The CAFs–stroma ratio(CSR) essentially advanced to the prognostic value of TSR

To further examine whether the prognosis of the TSR is related to the CAFs–stroma ratio, we selected 100 patients from the low and high-stroma groups to detect C^{FAP+}SR and C^{SMA+}SR through immunohistochemistry. The baseline characteristics of the patients and tumors are shown in Supplementary materials Table 2. For negative control, we used PBS instead of antibodies against FAP, α -SMA and vimentin for immunohistochemistry and no positive staining for FAP, α -SMA and vimentin (Supplementary materials Figure 4). Then we stained OSCC slides with FAP and α -SMA, specific markers for CAFs, and vimentin a specific marker for stroma. Expression of FAP and α -SMA were separately regarded a CAF subtype to evaluate the CAFs-stroma ratio as low CAFs group, medium CAFs group and high CAFs group (Figure 2). Medium and high expression of CAFs-stroma ratio were observed for FAP (72%, 72/100) and α -SMA (73%, 73/100) respectively (Table 4). What's more, Patients with high stroma had higher C^{FAP+}SR and C^{SMA+}SR than those with low stroma ($p < 0.001$) (Table 4) via immunohistochemical analysis. The Kaplan–Meier curves are shown in Figure 2, comparing the low, medium, and high CAFs groups for SMA and FAP with statistical significance. In the Cox multivariate model, the C^{FAP+}SR was significantly correlated with DFS ($p < 0.001$) and OCSS ($p = 0.008$) after correcting for confounders (Table 4). Meanwhile the C^{SMA+}SR was significantly related to the DFS ($p < 0.001$) and OCSS ($p = 0.002$) (Table 4). In addition, it's impressive to find the high expression of FAP in the tumor cells in the stroma-low group ($n = 17$) compare to the stroma-high group ($n = 4$), which may account for the disparity of the prognosis in the stroma-high and stroma-low groups (Supplementary materials Figure 5).

Table 4

Difference of the CAF-stroma ratio in the selected patients for Immunohistochemistry and multivariate analysis of disease-free survival and oral cancer-specific survival of CAFs^{FAP}-stroma ratio and CAFs^{SMA}-stroma ratio in Cox regression analysis

	Stroma-high		Stroma-low	
	n	n=50	n=50	p-value
CAFs ^{FAP} -stroma ratio				
CAFs-low	28	3	25	0.001
CAFs-medium	32	13	19	
CAFS-high	40	34	6	
CAFs ^{SMA} -stroma ratio				
CAFs-low	27	7	20	0.001
CAFs-medium	27	5	22	
CAFS-high	46	38	8	
multivariate analysis				
	Disease-free survival		Oral cancer-specific survival	
Age	P=0.244		P=0.232	
Gender	P=0.465		p=0.052	
T status	P=0.812		P=0.422	
N status	P=0.789		P=0.571	
pTNM stage	P=0.871		P=0.167	
Grade	P=0.744		P=0.931	
CAFs ^{FAP} -stroma ratio	P<0.001		P=0.008	
CAFs-Low				
CAFs-Medium	HR 2.916,95%CI 0.692-12.282		HR=4.054,95%CI 0.665-24.697	
CAFs-High	HR 15.681,95%CI 3.769-65.251		HR=14.457,95%CI 2.371-88.155	
multivariate analysis				
	Disease-free survival		Oral cancer-specific survival	
Age	P=0.183		P=0.393	
Gender	P=0.425		P=0.035	

	Stroma-high	Stroma-low
T status	P=0.560	P=0.333
N status	P=0.489	P=0.562
pTNM stage	P=0.969	P=0.600
Grade	P=0.536	P=0.509
CAFs ^{SMA} -stroma ratio	P<0.001	P=0.002
CAFs-Low		
CAFs-Medium	HR 0.371,95%CI 0.089-1.550	HR=0.312,95%CI 0.051-1.900
CAFs-High	HR 3.707,95%CI 1.316-10.438	HR=3.712,95%CI 1.055-13.065

Discussion

This study was designed to confirm the prognostic significance of TSR in OSCC in the largest cohort so far, thus rendering us to proceed to subgroup analysis in a high degree of confidence. Not only that, we show that the prognostic value of TSR was especially sensitive in tumor with grade I and II by means of introducing interaction terms in COX proportional mode. More importantly, we further ascertained that CAFs differ between high- and low-stroma tumors, uncovering the underlying possibilities of poor outcomes in the high-stroma group in a certain extent.

First, TSR was of prognostic value validated in OSCC, specifically its independent prognostic value in different locations. The high-stroma group had worse outcomes than the low-stroma group. Second, gender, N status, and differential grade were significantly correlated with DFS and OCSS through the multivariate Cox regression analysis. Meanwhile, pTNM stage ($p = 0.792$) illustrated a poor prognostic power for DFS. Additionally, we introduced the interaction terms in the Cox regression analysis, including TSR*gender, TSR*T status, TSR*N status, TSR*pTNM stages, and TSR*differential grade. The results showed that the use of the TSR was most sensitive in high differentiated tumors for DFS and OCSS. No statistically significant differences were observed among the subgroups; however, this does not mean that the prognostic effect of TSR does not differ among the total cohort. Moreover, the concept of interaction term was first used in the clinical research of the TSR in oral cancer. Finally, immunohistochemical analysis reflected that the high-stroma group has higher C^{FAP+SR} and C^{SMA+SR} than the low-stroma group. Additionally, patients with a high C^{FAP+SR} implied a poor outcome, which may be accounted for the adverse outcomes in the high-stroma group.

Moreover, this is the first study to digitally assess the TSR in oral cancer using the Mesker method [10]. Concretely, we chose a 3.2-mm² circle to perform TSR scoring, which was exactly parallel to the *10 objection in light microscopy. A study has indicated that the diameter of a light microscope does not differ in the final score ¹¹. All slides were scored three times to avoid intra-observer variability. Finally

Kappa coefficient reached perfect agreement though in the beginning only 0.762; most initial discordance were settled. Moreover, the remaining slides from four patients were consulted to the third observer to get consensus. Kappa values will further elevate to a perfect level because of the learning curve. Thus, we should digitally examine enough H&E-stained slides to ensure the reliability of the TSR score, which is also mentioned by Vangangelt et al[12]. Through proper training, the TSR as a prognostic tool will be easy, repeatable, and inexpensive.

The tumor stroma, or other the tumor microenvironment (TME), was one of the three key unsolved issues that impede effective clinical therapy for tumors [13], which were heterogeneous and plastic for including the intracellular environment of tumor bulks and the surrounding stromal cells with abundant protumorigenic factors [14]. Though tremendous advance in elucidating the mechanisms underlying tumor-promoting effects of the TME, tackling the complicated mechanisms still has a long way to go. Over the last decade, the prognosis in OSCC has been substantially studied, including biomarkers, such as caveolin-1 [15], MAGE-A11 [16], and clinicopathological parameters, such as histological grade [17, 18] according with our current study. Although accuracy is limited, Tobias Ettl et al. (2016) have found that positive frozen section margins was associated with recurrence [19]. In contrast, the TSR was more practicable as a prognostic tool.

More interestingly, Amol Ramchandra Gadail et al. (2017) have discovered that the OSCC has a better prognosis in the background of submucous fibrosis [20], which reminded the effects of CAFs implicated in the prognosis in OSCC. The term “cancer-associated fibroblast” were the most abundant stromal component in the TME, originating from at least six cellular categories, including normal fibroblast, quiescent stellate cell, endothelial cell, epithelial cell and pericyte, smooth muscle cell, and adipocyte [4]. Besides, CAFs can secrete a plentiful growth factors, chemokines, and exosomes regulating the course of cancer and immunosuppression, along with interleukin-6 and tumor growth factor-beta [21–23]. Owing to their cellular origins and breadth of functions, CAFs can have either protumorigenic or antitumorigenic effects on different solid tumors [24]. which is the major challenge for CAF-targeting therapies. Preclinical studies have indicated that nonspecific targeting of CAFs does not achieve the desired results in cancer treatment [25]. In view of the abundance and priority of CAFs in the stroma, we set to estimate the CSR in OSCC to attempt to explain the prognostic value of TSR.

Recently, several studies have mentioned the prognostic value of CAF density in OSCC [26]. Moreover, the biological behaviors of CAF were altered by microRNAs to facilitate invasion of OSCC and CD68(+) CAFs predicted poor prognosis illustrated that CAFs is Superior in Prognosis compare to the Epithelial–Mesenchymal Transition(EMT) Score [27–29]. Although molecular studies of CAFs deepen our insight of CAF intratumor heterogeneity and breadth of function, they remain hard to specify for lacking specific markers [4]. Here we chose to use α -SMA and FAP to represent CAF subtypes and found that the high-stroma group has a higher CSR than the low-stroma group. Additionally, the C^{FAP+SR} and C^{SMA+SR} were related to the prognosis, which might be accounted for the negative consequences in the high-stroma group. However, the underlying mechanism remains to be explored. Daniel Öhlund had observed the expansion of stromal fibroblast number, the process was called “stroma-genesis” with concomitant

oncogenesis [30, 31]. A study has found fibroblasts encircling early lesions, indicating that the early phase of fibroblasts could be suppressive, and these fibroblasts evolve to tumor-promoting fibroblasts through stroma-genesis [25, 32]. However, the hypothesis was difficult to testify due to the impractical and longitudinal sample of the same lesion and the conversion of the cell states. In this study, the rich-stroma group suggested a worse prognosis than the poor-stroma group, and the rich-stroma group had higher C^{FAP+SR} and C^{SMA+SR} . Moreover, patients with higher C^{FAP+SR} and C^{SMA+SR} had a worse prognosis. Additionally, we found that the low-stroma group has higher positive staining of FAP+ in tumor cells. According to the latest study that overexpression of FAP is related to the epithelial–mesenchymal transition (EMT) in OSCC [33], we inferred that low-stroma tumors might initially be transformed into high-stroma tumors through the events where fibroblasts evolve to protumorigenic fibroblasts and tumor cells via EMT, though the detailed mechanisms remain to be explored.

First, one of the study strengths is manifesting that the prognostic value of the TSR was most prominent in moderately and highly differentiated tumors, and indicating the unique role of the C^{FAP+SR} and C^{SMA+SR} in advancing the prognostic value of TSR. Second, this study has the largest oral cancer sample, which enabled us to render the subgroup analysis in high confidence. Third, the method we used to evaluate the TSR confirmed that we can locate high-stroma areas in the entire slides, and images can be stored to solidify the authenticity of the results compared to those obtained using light microscopy, which are disturbed by uncontrollable factors when scoring the TSR. Limitations of our study are retrospective study design with a short follow-up period and all cases came from a single institution; thus, whether the application of the TSR in a prospective study can obtain reproducible results in OSCC remains to be seen.

In conclusion, the prognostic value of TSR was positively validated in oral squamous cell carcinoma particularly in tumors with moderate or high differentiation. and the prognostic effect of TSR didn't differ from the prognostic value of the whole cohort in gender, tumor size, lymph node status subgroups. Furthermore, the fact that higher C^{FAP+SR} and C^{SMA+SR} in tumors with stroma-rich simultaneously suggesting the poor outcome, illustrating C^{FAP+SR} and C^{SMA+SR} contributed to the prognostic impact of TSR in OSCC. In a Word, the advantages of TSR were convenient, practicable and lowcost in routine pathological examination and do not need extra staining, which opened a great opportunity for decision-making in individual therapy.

Conclusions

We show that tumor–stroma ratio (TSR) as a prognostic tool in the whole OSCC especially in highly differentiated tumors. There is also a difference in the cancer-associated fibroblast (CAFs)–stroma ratio(CSR) exists between the high and low-stroma groups and its influence on the prognostic power of TSR, which informed the rational design of individual cancer management especially for pathological diagnosis.

Methods

Study patients

Data of patients with OSCC were obtained from the database of School & Hospital of Stomatology, Wuhan University. We incorporated every patient with primary OSCC who had surgery for the first time from January 1, 2012 to December 31, 2016. All cases were staged based on the seventh edition of the American Joint Committee on Cancer Staging Manual [34] because the eighth edition of the manual was used from January 2018. Patients treated with chemotherapy and/or radiotherapy before the study, died within a month, and those with non-primary tumor were excluded from the study. The basic demographic data and detailed information were collected from the Medical Records Room and the Department of Oral and Maxillofacial and Head and Neck Oncology. Every pathological report and H&E section were retrieved from the Pathology Department.

Assessment Of The Tsr

We visually evaluated the TSR using 3- μ m H&E slides of primary OSCC using NDP.view2 (Hamamatsu Photonics, Hamamatsu City, Japan), a software for the digital assessment of microscopic images. The original H&E slides were scanned using NanoZoomer (S210 C13239-01, NanoZoomer) to make high-resolution digital images for subsequently analysis.

First, all H&E-stained slides were visually assessed to find areas with the most abundant stroma. Then, we used a 10 $\times$ objective and ensured that tumor cells exist in all borders. Almost all chosen fields were near the site of the invasive front. The cutoff TSR value was determined as 50% according to the study by Mesker [10], and we divided into two groups according to the cutoff TSR value: low stroma (<50% stroma) and high-stroma (>50% stroma) groups. A stroma proportion of approximately 50% was considered stromal-rich. Two blinded observers recorded the slides three times to decrease intra-observer variability. Disagreements between the two observers were resolved by a third observer to get the final consensus.

Immunohistochemistry And Evaluation Of Immunoreactivity

We chose 100 patients from the aforementioned groups (50 patients from the high and low-stroma groups, respectively). And we could not adopt completely random way due to the paraffin block available and we made good use of admission number as random as possible. Detailed information about the admission number was listed in supplementary materials figure 7. Before immunohistochemical staining, paraffin-embedded slides (3 μ m) of tissues were air-dried at 37°C overnight. The antibodies and the corresponding dilution ratio used were as follows: FAP (1:250; Abcam, Cambridge, UK), α -SMA (1:200; Abcam, Cambridge, UK), and vimentin (1:4000; ProteinTech Group, Rosemont, IL, USA).

The immunohistochemically stained slides were scanned, and images were analyzed using NDP.view2. We examined the CAFs–stroma ratio throughout the slide based on the distribution of CAFs and

separately counted the FAP + fibroblast and α -SMA + fibroblast subtypes. To determine the stroma, we referred to the expression of vimentin and original H&E-stained slides. The patients were further divided into three groups according to the CAFs–stroma ratio: low CAFs group (CSR <33%), medium CAFs group (CSR 33–66%), and high CAFs group (CSR >66%).

Statistical analysis

Statistical Package for the Social Sciences (version 22; IBM Corporation, Armonk, NY, USA) was used to perform all statistical analyses. First, the chi-square test was used for categorical variables in the low and high-stroma groups. The Fisher–Freeman–Halton exact test was performed when the table was larger than 2×2 , and the Mann–Whitney U-test was used as the patients' age was a continuous variable. Then, we employed the Kaplan–Meier method to plot the disease-free survival (DFS) and oral cancer-specific survival (OCSS) as well as overall survival (OS). and the log-rank test was used to evaluate the difference between the two groups using GraphPad Prism (version 8.0; GraphPad Software Inc., San Diego, California, USA). DFS was defined as the time from the date of primary surgery to the date of local regional recurrence, distant recurrence, or death for any cause. OCSS was defined as the time from the date of primary surgery to the date of oral cancer-specific death. OS was defined as death from any cause. Cohen's Kappa coefficient was used to examine the inter-observer variability. Finally, all relevant risk factors (i.e., age, gender, T status, N status, pTNM stage, TSR, grade, and tumor location) were absorbed into the Cox proportional-hazards models to perform univariate and multivariate analyses. The results are expressed as the means $\pm$ SD. Differences with p values of < 0.01 were considered significant.

Abbreviations

TSR, Tumor–stroma ratio; OSCC, Oral squamous cell carcinoma; CAFs, Cancer associated fibroblasts; CSR, Cancer associated fibroblasts (CAFs)-stroma ratio; DFS, Disease-free survival; OCSS, Oral cancer-specific survival; OS, Overall survival; HR, Hazard ratio; CI, Confidence interval; FAP, Fibroblast Activation Protein; α -SMA, α -Smooth Muscle Actin; H&E, Hematoxylin and eosin stained slides; ECM, Extracellular matrix; EMT, Epithelial–mesenchymal transition.

Declarations

Ethics approval and consent to participate

Our study has been accordingly approved and supervised by Ethics Committee of School and Hospital of Stomatology, Wuhan University(IRB-ID:2021A18).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors have no conflicts of interest.

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Authors' contributions

J.J Qiu, E.H Jiang, Z.J Shang, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Figures

Figure 1

Tumor with stroma-high display a relatively poor outcome especially in moderately and highly differentiated cancer. (A) Hematoxylin and eosin stained 5 μ m sections : example of stroma-high tumor and stroma-low tumor(original magnification $\times 100$). (B) Kaplan-Meier curves for disease-free survival and oral cancer- specific survival in oral squamous cell carcinoma (stroma-low versus stroma-high). (C)

Kaplan-Meier curves for disease-free survival and oral cancer-specific survival stratified by tumor-stroma ratio combined with grade.

Figure 2

immunohistochemical expression of representative samples of OSCCs of this study as well as higher cancer- associated fibroblasts(CAFs)-stroma ratio shows negative effect in prognosis. (A) OSCC sample

demonstrates reduced of CAFs^{FAP} and CAFs^{a-SMA}-stroma ratio. (B) OSCC sample with medium CAFs^{FAP} and CAFs^{a-SMA}-stroma ratio. (C) OSCC sample displays high CAFs^{FAP} and CAFs^{a-SMA}-stroma ratio (a1-i1 original magnification ×10; a2-i2 original magnification ×50; a3-i3, original magnification ×200). (D) Kaplan-Meier curves for disease-free survival and oral-cancer specific survival of CAFs^{FAP}-stroma ratio and CAFs^{SMA}-stroma ratio in 100 patients.

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