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The value of RACK1 and peripheral blood M2/M1 monocyte ratio on the prognosis of patients with oral squamous cell carcinoma

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Abstract: This study is to evaluate the effect of receptor for activated c kinase 1 (RACK1) and peripheral blood M2/M1 monocytes ratio on the prognosis of patients with oral squamous cell carcinoma (OSCC). A total of 115 OSCC patients who underwent radical surgery in North China University of Science and Technology Affiliated Hospital from January 2015 to December 2015 were included in the experimental group and 34 healthy individuals after a physical examination during the same period were included in the control group. Cancer and para-cancerous tissues were collected, and the relationship between RACK1 and M2/M1 ratio and the prognosis of OSCC patients and its predictive value were analyzed. RACK1, M2/M1 ratio, clinical stages, lymphatic metastasis, recurrence and metastasis were considered independent factors for the prognosis of OSCC patients ($p<0.05$); In addition, RACK1 and the M2/M1 ratio were proven to be of significant predictive values for the prognosis of OSCC patients ($p<0.05$). RACK1 and peripheral blood M2/M1 monocytes ratio demonstrate great potential as prognostic predictors of OSCC patients.

Keywords: OSCC; RACK1; M2/M1 ratio; prognosis

INTRODUCTION

OSCC, as the most malignant and harmful tumor of the head and neck, is mostly found in the tongue, gingiva, and palate. (Mukdad *et al.*, 2019). Surgery is the mainstay for the treatment of OSCC in the current stage (Shanti and O'Malley, 2018); however, it is criticized for a poor five-year postoperative survival down to 50% and a high incidence of relapse and metastasis (Weckx *et al.*, 2020). A clear understanding of the mechanisms underlying the occurrence and development of OSCC would benefit the development of novel treatment options. RACK1, a free cytoplasmic scaffold protein located on human chromosome 5, has been confirmed to take part in multiple biological events, including cell migration. It has also been found by prior research that RACK1 is strongly associated with the progression of OSCC, which is closely related to the prognosis of patients (Liu *et al.*, 2018). Differentiated from monocytes, tumor-associated macrophages (TAM) are the most abundant immune cells in tumor tissues. Among all TAMs, M1 and M2 are the two polarization states, in which M1 kills tumor cells and amplifies inflammation, and M2 facilitates tumor growth and metastasis (Yunna *et al.*, 2020). Nonetheless, whether RACK1 is related to the recruitment and differentiation of TAM and the underlying mechanisms remains obscure. In this study, the cumulative postoperative five-year survival rate was used as an indicator to analyze the relationship between the RACK1 and peripheral blood M2/M1 monocytes ratio and the prognosis of OSCC patients, aiming to provide a theoretical basis for clinical treatment and prognostic judgment.

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MATERIALS AND METHODS

Clinical data

A total of 115 OSCC patients who underwent radical surgery in North China University of Science and Technology Affiliated Hospital from January 2015 to December 2015 were identified as the research objects.

Inclusion criteria: (1) Patients aged 18-80 years; (2) Patients who were pathologically diagnosed as primary OSCC (Zhong, 2020); (3) Patients who had no contraindications to radical surgery, and received no anti-tumor treatment such as radiotherapy and chemotherapy before surgery; (4) Patients who received the same adjuvant treatment scheme after surgery.

Exclusion criteria: (1) Patients with other malignant tumors or a history of previous malignancies; (2) Patients with vital organ dysfunction, infectious diseases, autoimmune diseases; (3) Patients with incomplete medical records; (4) Patients with poor compliance.

Cancer and adjacent tissues of the OSCC patients were collected (at least 5cm away from cancer tissue). Of all OSCC patients, 70 were males and 45 were females, aged 38 to 76 years, with a mean age of (57.59±10.25) years. Another 34 healthy individuals after physical examination in the hospital during the same period were included in the control group, including 21 males and 13 females, aged 39-80 years old, with an average age of (57.63±10.21) years. No evidence of significant difference regarding the general information such as gender and age between the two groups was found ($p>0.05$). The study was approved by the North China

University of Science and Technology Affiliated Hospital ethics committee and all the enrolled subjects signed an informed consent form.

Main reagents and instruments

The Rabbit anti-RACK1 monoclonal antibody (No. ab129084), HRP labeled secondary antibody (No. ab6721) were purchased from Abcam Co. Ltd., America. The SABC reagent (No. SA1024) was purchased from BOSTER Biological Technology Co. Ltd., Wuhan. The FITC-CD14 antibody, APC-CD163 antibody, and PE-CD86 antibody were purchased from Biolegend Co. Ltd., America, the PE-CD 204 antibody and red blood cell lysate were purchased from RD Co. Ltd., America and the flow cytometer (Specifications: FACS Calibur Ⅱ) was purchased from RD Co. Ltd.

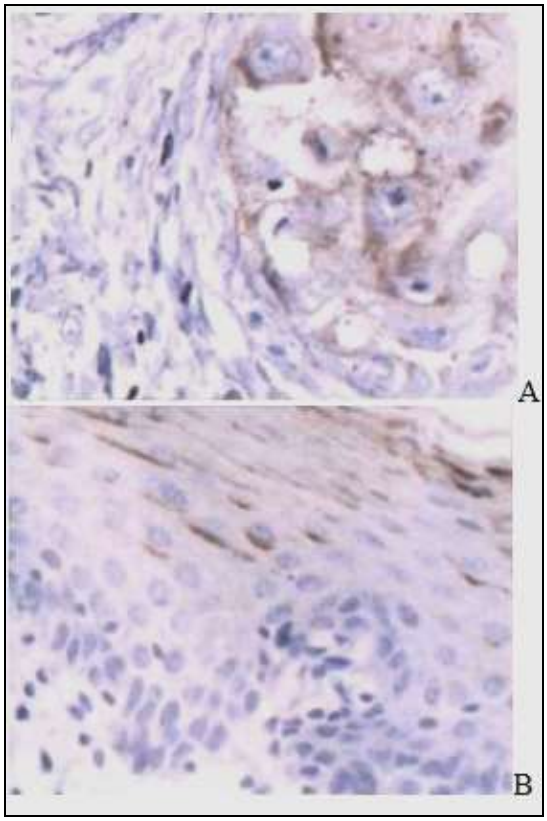
Method

Immunohistochemistry SABC method

A paraffin section with a thickness of 5μm was prepared and placed in a 37°C incubator for drying. Xylene and alcohol were used for dewaxing and the section was rinsed with clean water. 3% H2O2 solution was used to remove endogenous catalase. The section was then rinsed with clean water, added with Citrate buffer and microwaved for 3 minutes at medium heat. The section was cooled to room temperature, rinsed with clean water twice for 5 minutes and phosphate buffer saline (PBS) twice for 5 minutes. Rabbit anti-RACK1 monoclonal antibody was added to the experimental group, PBS of the equivalent volume was added to the control group and sections of the two groups were both incubated at 4℃ overnight. The next day, the section was rinsed in PBS three times, 5 minutes each and added with HRP labeled secondary antibody, followed by a 30-min incubation at 37℃. Then the section was rinsed with PBS three times, 5 minutes each and added with strept avidin-biotin complex (SABC), prior to another 30-min incubation at 37℃. The section was then rinsed with PBS three times, 5 minutes each and was dropwise added color developing agent to observed the coloration through a microscope. Subsequently, the section was rinsed in clean water, soaked in hematoxylin-eosin for 30s for a counterstain rinsed in water, dehydrated by ethyl alcohol and xylene, and mounted by neutral balsam. The double-blind method was used to examine the section with 3 epithelial or cancerous fields randomly selected under the microscope at 400 times. Positive staining indicates a brown color of cytoplasm or cell membrane, with the number of positive cells below 30% being negative and above 30% being positive.

Flow Cytometry

200μL of peripheral blood was collected from both OSCC patients and healthy individuals and equally divided into 2 flow tubes. One tube was added with 7.5μL of FITC-CD14 antibody, 5.0μL of APC-CD163 antibody and 7.5μL of PE-CD204 antibody to determine the proportion of M1 monocytes. The other tube was added with 7.5μL of FITC-CD14 antibody, 5.0μL of APC-CD163 antibody, and 7.5μL of PE-CD86 antibody to determine the proportion of M2 monocytes. After uniform oscillation, the tubes were rested at room temperature and allowed to stand for 25 minutes away from light. Then red blood cell lysate and 1% paraformaldehyde were added dropwise, oscillated evenly and rested at room temperature for 10 minutes. The gating was set as the intermediate cell group for forwarding scattered light and lateral scattered light. Flow cytometry was employed to analyze the proportion of M1 monocytes and M2 monocytes in CD14+ monocytes and the M2/M1 ratio was calculated.



Note: A: OSCC tissue; B: adjacent tissue

Fig. 1: The expression of RACK1 in OSCC and its adjacent tissues (SABC, ×400)

Follow-up visit

All patients will be followed up immediately after the radical operation through outpatient follow-up and telephone follow-up and the follow-up contents include postoperative conditions such as recurrence, metastasis, and death. The follow-up started on the day of surgery and ends on the day of death or December 31, 2020, with a frequency of every 3 months for the first 2 years and every 6 months for the following 3 to 5 years. The cumulative survival, recurrence and metastasis of the patients one, three, and five years after the surgery were calculated.

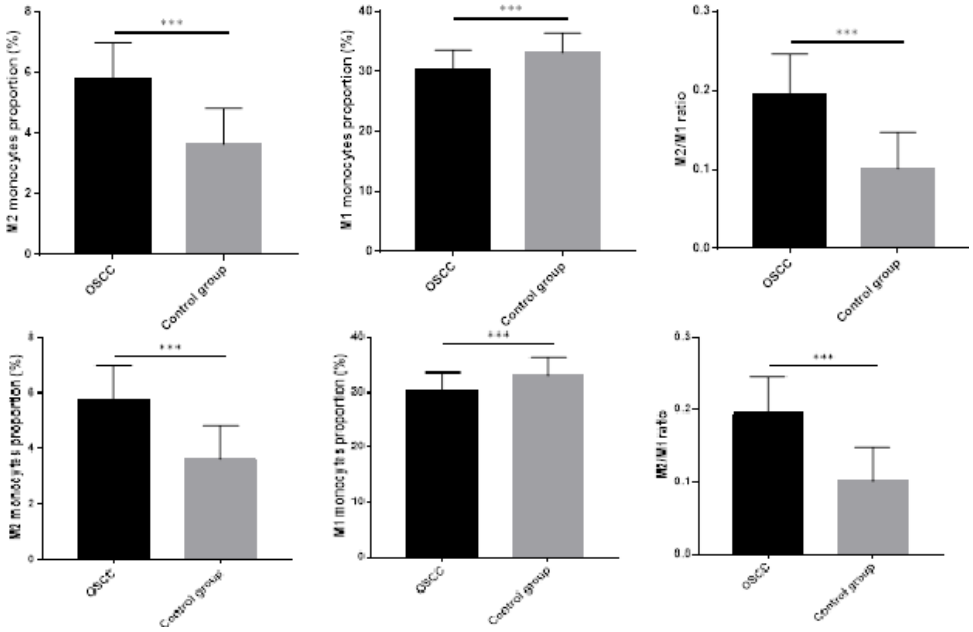


Fig. 2: Comparison of M1 and M2 monocytes between the two groups, *** indicated p < 0.001

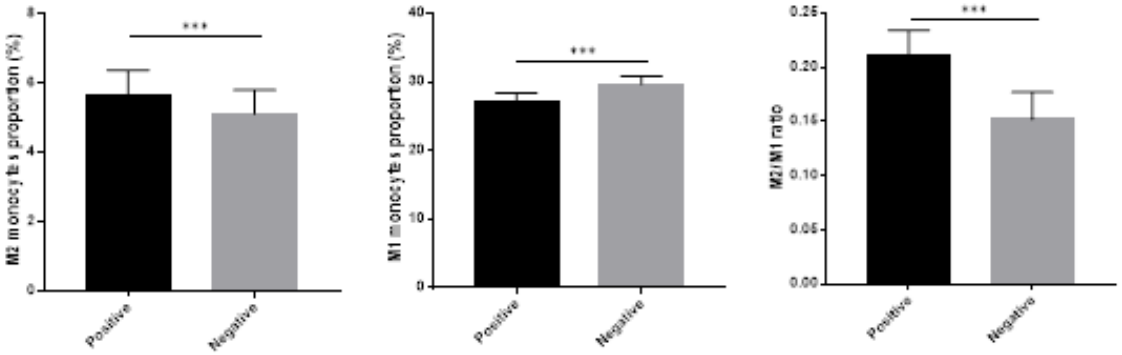


Fig. 3: Correlation analysis of RACK1 and M2/M1 ratio in OSCC patients, ***indicated p<0.001

STATISTICAL ANALYSIS

Data analysis was conducted using SPSS 20.0. The measurement data conforming to the normal distribution were expressed as (x ±s) using t-test for analysis and the count data were expressed by frequency or composition ratio using the chi-square non-correction method, with the total number of cases ≥40 and the minimum theoretical frequency >5. The correlation analysis was conducted using Pearson analysis. The relationship between RACK1 and M2/M1 ratio and the 5-year cumulative survival of OSCC patients were analyzed using the Kaplan-Meier curve analysis. The relationship between RACK1 and M2/M1 ratio and the prognosis of OSCC patients were analyzed using Univariate and multivariate COX regression analysis. The receiver operating characteristic (ROC) curve was drawn to calculate the area under the curve (AUC) and analyze the prognostic value of the RACK1 and M2/M1 ratio. The difference was considered statistically significant when a p-value was less than 0.05.

RESULTS

The general conditions of OSCC patients

A follow-up rate of 100% was obtained in a total of 115 OSCC patients after receiving radical surgery, with the median follow-up time of 62 (7-82) months as of December 31, 2020. The 1-year, 3-year and 5-year cumulative survival rates and recurrence and metastasis rates of OSCC patients were 88.70% (102/115) and 26.96% (31/115), 73.91% (85/115) and 33.91% (39/115), and 61.74% (71/115) and 41.74% (48/115), respectively (table 1).

Results of immunohistochemical staining

Positive cells of OSCC tissue were mainly distributed on the edge of cancer nests with cytoplasm or cell membrane in brown (fig. 1A). A small amount of RACK1 staining could be seen in the adjacent tissues, mainly distributed in the basal layer (fig. 1B). The positive expression rates of RACK1 in OSCC and its adjacent tissues were 70.43%

(81/115) and 11.30% (13/115), respectively. The higher positive rate of RACK 1 in OSCC tissue was significantly higher than that in adjacent tissues. See fig. 1 ($\chi 2$=83.192, p<0.01).

Comparison of RACK1 in patients with different clinicopathological characteristics

Strong evidence of a significant difference in the comparison of RACK1 expression in patients with different clinical stages, degree of differentiation, lymphatic metastasis, nerve invasion, recurrence and metastasis and the prognosis was found in table 2 (p<0.05).

Table 1: Clinicopathological features of 115 patients

Features	Cases	Proportion %
Age		
<60	59	51.30
≥60	56	48.70
Gender		
Male	70	60.87
Female	45	39.13
Drinking		
Yes	47	40.87
No	68	59.13
Smoking		
Yes	62	53.91
No	53	46.09
History of hypertension		
Yes	29	25.22
No	86	74.78
History of diabetes		
Yes	18	15.65
No	97	84.35
Tumor location		
Tongue	40	34.78
Cheek	33	28.70
Gingiva	16	13.91
Palate	9	7.83
Mouth floor	17	14.78
Clinical stages		
Ⅰ~Ⅱ	55	47.83
Ⅲ~Ⅳ	60	52.17
T stages		
T1~T2	74	64.35
T3~T4	41	35.65
Differentiation		
Low/medium	35	30.43
High	80	69.57
Depth of invasion		
<5mm	92	80.00
≥5mm	23	20.00
Lymphatic metastasis		
Yes	43	37.39
No	72	62.61
Nerve invasion		
Yes	32	27.83
No	83	72.17
Recurrence and metastasis		
Yes	56	48.70
No	59	51.30

Comparison of M1 and M2 monocytes between the two groups

In comparison with the control group, the proportion of M2 monocytes and M2/M1 ratio in OSCC patients has shown a sharp increase and the ratio of M1 monocytes presented a significant decline (p<0.05). See fig. 2.

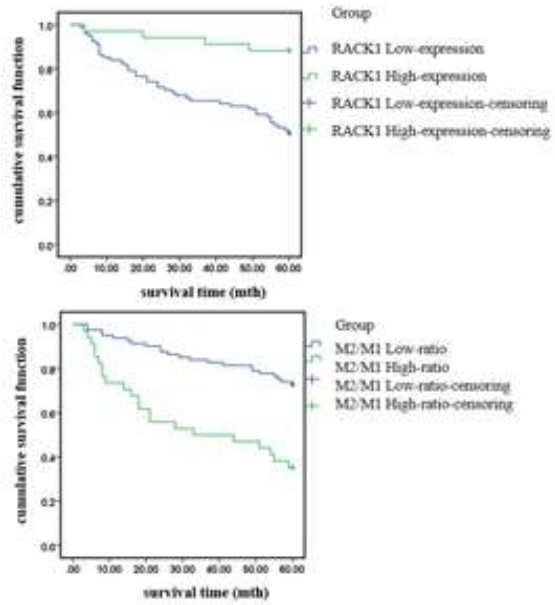


Fig. 4: The relationship between RACK1 and M2/M1 ratio and the 5-year cumulative survival rate of OSCC patients (Kaplan-Meier curve)

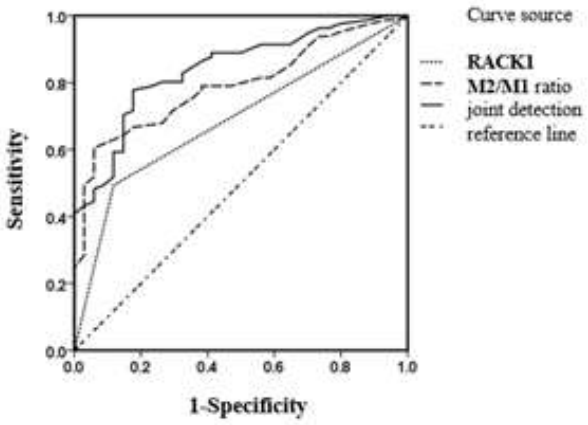


Fig. 5: The predictive value of RACK1 and M2/M1 ratio on the prognosis of OSCC patients (ROC curve)

Table 2: Comparison of RACK1 in patients with different clinicopathological characteristics

Features	Cases	RACK1 expression		χ^2	*p*
		Positive (n=81)	Negative (n=81)		
Age				1.092	0.296
<60	59	39	20		
≥60	56	42	14		
Gender				2.394	0.122
Male	70	53	17		
Female	45	28	17		
Drinking				2.622	0.105
Yes	47	37	10		
No	68	44	24		
Smoking				1.864	0.172
Yes	62	47	15		
No	53	34	19		
History of hypertension				1.467	0.226
Yes	29	23	6		
No	86	58	28		
History of diabetes				1.705	0.192
Yes	18	15	3		
No	97	66	31		
Tumor location				4.712	0.318
Tongue	40	33	7		
Cheek	33	21	12		
Gingiva	16	11	5		
Palate	9	6	3		
Mouth floor	17	10	7		
Clinical stages				23.062	<0.01
Ⅰ~Ⅱ	55	27	28		
Ⅲ~Ⅳ	60	54	6		
T stages				3.092	0.079
T1~T2	74	48	26		
T3~T4	41	33	8		
Differentiation				14.765	<0.01
Low/medium	35	16	19		
High	80	65	15		
Depth of invasion				3.769	0.052
<5mm	92	61	31		
≥5mm	23	20	3		
Lymphatic metastasis				8.038	0.005
Yes	43	37	6		
No	72	44	28		
Nerve invasion				6.201	0.013
Yes	32	28	4		
No	83	53	30		
Recurrence and metastasis				22.323	<0.01
Yes	56	51	5		
No	59	30	29		
Prognosis				14.346	<0.01
Survival	71	41	30		
Death	44	40	4		

Comparison of M2/M1 monocytes ratio in patients with different clinicopathological characteristics

Significant differences were found in the comparison of M2/M1 monocytes ratio in patients with different clinical stages, degree of differentiation, lymphatic metastasis, nerve invasion, recurrence and metastasis and prognosis in table 3 ($p<0.05$).

Correlation analysis of RACK1 and M2/M1 ratio in OSCC patients

Results from fig. 3 presented a positive correlation between the RACK 1 expression and M2 monocytes proportion (r=0.629，p=0.023) and a remarkably higher M2 monocytes proportion of RACK 1-positive than RACK 1-negative patients ($p<0.05$).

Table 3: Comparison of M2/M1 monocytes ratio in patients with different clinicopathological characteristics (x±s)

Features	Cases	M2/M1 ratio	*t*	*p*
Age			0.363	0.717
<60	59	0.159±0.028		
≥60	56	0.161±0.031		
Gender			0.440	0.661
Male	70	0.178±0.023		
Female	45	0.176±0.025		
Drinking			0.216	0.829
Yes	47	0.164±0.025		
No	68	0.163±0.024		
Smoking			0.155	0.877
Yes	62	0.183±0.034		
No	53	0.182±0.035		
History of hypertension			0.351	0.726
Yes	29	0.167±0.028		
No	86	0.165±0.026		
History of diabetes			0.140	0.889
Yes	18	0.175±0.027		
No	97	0.174±0.028		
Tumor location			0.124	0.976
Tongue	40	0.195±0.016		
Cheek	33	0.197±0.015		
Gingiva	16	0.195±0.014		
Palate	9	0.194±0.015		
Mouth floor	17	0.196±0.016		
Clinical stages			2.458	0.016
Ⅰ~Ⅱ	55	0.187±0.025		
Ⅲ~Ⅳ	60	0.198±0.023		
T stages			0.339	0.735
T1~T2	74	0.186±0.031		
T3~T4	41	0.188±0.029		
Differentiation			1.988	0.049
Low/medium	35	0.177±0.028		
High	80	0.188±0.027		
Depth of invasion			176.296	<0.01
<5mm	92	0.169±0.025		
≥5mm	23	0.181±0.023		
Lymphatic metastasis			17.325	<0.01
Yes	43	1.745±0.018		
No	72	1.689±0.016		
Nerve invasion			12.525	<0.01
Yes	32	1.718±0.021		
No	83	1.667±0.019		
Recurrence and metastasis			26.525	<0.01
Yes	56	1.891±0.023		
No	59	1.772±0.025		
Prognosis			14.645	<0.01
Survival	71	1.692±0.026		
Death	44	1.764±0.025		

The relationship between RACK1 and M2/M1 ratio and the 5-year cumulative survival rate of OSCC patients

The cumulative survival rate of OSCC patients with higher RACK 1 expression and M2/M1 ratio presented a greater decrease than patients with lower data results during the five-year follow-up visit ($p<0.05$). (table 4)

Univariate and multivariate COX regression analysis on the prognosis of OSCC patients

Univariate analysis showed that RACK1, M2/M1 ratio, clinical stage, degree of differentiation, depth of invasion, lymphatic metastasis, nerve invasion, recurrence and metastasis are involved in the prognosis of OSCC patients ($p<0.05$). Further multivariate COX regression analysis demonstrated that all aforementioned factors apart from the degree of differentiation, depth of invasion and nerve invasion were considered as independent factors in the prognosis of OSCC patients ($p<0.05$) (table5).

The predictive value of RACK1 and M2/M1 ratio on the prognosis of OSCC patients

The RACK1 and M2/M1 ratios were proved to be of high prediction values for the prognosis of OSCC patients, with a higher AUC of the combined assay than the single assay ($p<0.05$), as shown in table 6 and fig. 5.

DISCUSSION

Relevant data have revealed a stark rise in the morbidity and mortality of OSCC in China (Xu *et al.*, 2018). Treatment strategies that rely primarily on surgery, followed by radiotherapy and chemotherapy to enhance the quality of life of OSCC patients, are critically hampered by the low postoperative five-year survival rate and high recurrence and metastasis rate due to severe local invasion and the high metastatic latency of OSCC (Cheraghlou *et al.*, 2018; Qin *et al.*, 2018). Therefore, the improvement of the accuracy of OSCC prognostic factors serves to optimize the quality of life of patients (Wang *et al.*, 2020).

It has been reported that RACK1 widely expressed in the brain, liver, spleen, and other organs of mammals can be used as a ribosomal scaffold protein, to bind tightly with ribosomes through the interaction between its high

Table 4: Correlation analysis of RACK1 and M2/M1 ratio in OSCC patients

RACK1 expression	Cases	M2 monocytes proportion (%)	M1 monocytes proportion (%)	M2/M1 ratio
Positive	81	5.64±0.73	27.21±1.14	0.211±0.023
Negative	34	5.11±0.69	29.69±1.16	0.152±0.025
t		3.610	10.591	12.233
p		0.001	<0.01	<0.01

Table 5: Univariate and multivariate COX regression analysis on the prognosis of OSCC patients

Variable	Univariate analysis			Multivariate analysis		
	HR	95%CI	*p*	*HR*	95%CI	*p*
RACK1	3.809	2.647~12.517	0.001	2.647	1.497~5.891	0.005
M2/M1 ratio	4.295	2.274~16.184	0.004	3.025	1.808~9.706	0.010
Clinical stages	2.603	1.803~5.085	<0.001	2.215	1.219~4.479	0.016
Degree of differentiation	3.081	2.110~9.364	0.003	-	-	-
Depth of invasion	2.929	1.732~4.997	<0.001	-	-	-
Lymphatic metastasis	2.448	1.320~3.445	<0.001	1.751	1.022~2.763	0.027
Nerve invasion	1.976	1.206~3.217	0.007	-	-	-
Recurrence and metastasis	2.235	1.411~3.524	0.001	1.928	1.328~3.136	0.003

Table 6: The predictive value of RACK1 and M2/M1 ratio on the prognosis of OSCC patients

Indexes	Sensitivity /%	Specificity /%	*p*	AUC	OR (95%CI)
RACK1	49.4	88.2	0.001	0.688	0.588~0.788
M2/M1 ratio	60.5	94.1	0.000	0.792	0.710~0.873
Combined assay	77.8	82.4	0.000	0.841	0.766~0.915

conservativeness and specification in the protein translation process (Nielsen *et al.*, 2017). Recent studies have found that RACK1 induces apoptosis by activating downstream factors such as JNK (Zhang *et al.*, 2019) and inhibits MTK1-mediated apoptosis by binding to stress granules (Zou *et al.*, 2018). In addition, the up-regulation of the expression of RACK1 in a variety of malignant tumors (Hu *et al.*, 2019) is demonstrated by previous studies to be involved in the poor prognosis of cancer patients (Han *et al.*, 2018). Liu S *et al.* (Liu *et al.*, 2018) found that the positive expression rate of RACK1, an independent organ-specific indicator for predicting the risk of OSCC death in OSCC tissues, ranges from 54.6% to 68.2%. In this study, the positive expression rate of RACK1 in OSCC tissue was 70.43%, which was in line with the results of the previous research that revealed a markedly higher positive expression rate of RACK1 in OSCC tissue than that in adjacent tissues, and found a correlation between prognosis and the different clinical stages, degree of differentiation, lymphatic metastasis, nerve invasion, recurrence and metastasis. TAM is the main immune cell of tumor tissue infiltrating immune cells and is closely related to tumor growth, angiogenesis, and metastasis (Li *et al.*, 2020). Under the influence of the local microenvironment, activated TAM differentiates into M1 and M2 monocytes, among which M1 macrophage markers mainly include CD14, CD163 and CD204 and M2 macrophage markers mainly include CD14, CD163, and CD86 (de Groot and Pienta, 2018). Clinical experiments have shown that the polarization of TAM into M2 monocytes was indicative of a poor prognosis and treatment resistance in cancer patients (Cai *et al.*, 2019). The results of this study presented that, compared with healthy individuals, OSCC patients observed a higher proportion of M2 monocytes, an M2/M1 ratio and a decrease of M1 monocytes proportion, which was similar to the research results given by Li Shumei *et al.* (Li *et al.*, 2017) who proposed that M2 macrophages could promote lymphatic metastasis in patients with OSCC. Moreover, significant differences were found in the M2/M1 ratio of patients with different clinical stages, degree of differentiation, depth of invasion, lymphatic metastasis, nerve invasion, recurrence and metastasis, and prognosis, suggesting the involvement of TAM polarization to M2 in the occurrence and development of OSCC. Herein, a higher proportion of M2 monocytes in patients with positive expression of RACK1 than negative RACK 1 and a corresponding positive interaction were found. Consequently, the relevancy between high expression of RACK1 and the increase of M2 macrophage infiltration further indicates that the suppression of tumor growth and metastasis can be achieved by restraining M2 monocyte polarization in the down-regulation of RACK1 expression, to further ameliorate the prognosis of patients. Furthermore, a lower 5-year cumulative survival rate of OSCC patients with high RACK1 expression and high

M2/M1 ratio through Kapla-Meier curve analysis implied their impact on the prognosis of OSCC. It was also obtained that both RACK1 and the M2/M1 ratio were of predictive values for the prognosis of OSCC, and the hybrid detection reaped huge fruits in mitigating the prediction accuracy of the poor prognosis of OSCC.

CONCLUSION

RACK1 and peripheral blood M2/M1 monocytes ratio demonstrate great potential as prognostic predictors of OSCC patients. The limitation of this study lies in the small sample size, which will be expanded in future studies.

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