High CD38 expression in tumor-infiltrating immune cells is a favorite prognosis factor in esophageal squamous cell carcinoma with perigastric lymph node metastasis

Feng Shi (✉ cherryshifeng@163.com)

Capital Medical University Affiliated Beijing Shijitan Hospital <https://orcid.org/0000-0002-1850-7796>

Shuo Xiao

South carolina university

Yanjie Zhao

Capital Medical University Affiliated Beijing Shijitan Hospital

Yuchen Li

QIMR Berghofer Medical Research Institute

Ying Gao

Capital Medical University Affiliated Beijing Shijitan Hospital

Hong Chang

Capital Medical University Affiliated Beijing Shijitan Hospital

Qingkun Song

Capital Medical University Affiliated Beijing Shijitan Hospital

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Abstract

Background

The present study aimed to investigate the prognostic effect of CD38 in patients with esophageal squamous cell carcinoma (ESCC).

Methods

We performed a retrospective cohort study by consecutively recruiting 142 patients with ESCC. The clinicopathological features and expression of CD38, CD4, CD8, Ki-67, PD-L1 and PD-1 in tumor and immune cells were independently evaluated by two pathologists.

Results

CD38 was expressed in immune cells but not tumor cells and the median expression rate of CD38 in immune cells was 60%. Among ESCC patients with perigastric lymph node metastasis, the expression rate of CD38 was not associated with the disease-free survival ($p = 0.207$), but had a significant association with the overall survival ($p = 0.042$). The median overall survival was 13 months and not observed among patients with low and high expression rate of CD38, respectively. The crude and adjusted hazard ratio (HR) of high CD38 expression was 0.37 (95%CI 0.14, 0.95) and 0.21 (95%CI 0.06, 0.70). The expression rate of CD38 had a negative correlation with PD-L1 expressed in tumor cells and CD4 expressed in immune cells. Among patients without perigastric lymph node metastasis, the expression rate of CD38 showed a significant association with the disease-free survival ($p < 0.05$). The median disease-free survival was 45 months and not achieved for patients with high and low expression of CD38; the adjusted HR of high CD38 expression was 2.13 (95%CI 0.85, 5.33). CD38 did not have significant association with the overall survival for patients without perigastric lymph node metastasis. The expression rate of CD38 had a negative correlation with PD-L1 expressed in tumor cells and a positive correlation with PD-L1 expressed in immune cells.

Conclusion

The high expression rate of CD38 was associated with a better survival for ESCC with perigastric lymph node metastasis. The prognostic effect of CD38 on esophageal cancer should be warranted in future prospective studies.

Background

Esophageal cancer (EC) is one of the most common tumors in the world. In 2018, there were 572 million newly diagnosed EC cases and 509 million deaths worldwide, and more than half of these new cases and

deaths occurred in China [1]. An estimate of 88%-90% EC were diagnosed as the esophageal squamous cells carcinoma (ESCC) in China [2]. The 5-year survival rate of EC was about 20–30% in China, compared to 30–40% globally [3, 4]. Therefore, it is an urgent and unmet need to develop a more effectively therapeutic strategy for ESCC. The KENOTE-590 study demonstrated a promising antitumor activity of immune check-point inhibitor (ICI) treatment in advanced ESCC [5], which enables the immunotherapy of ICI as a potential management option for ESCC patients.

CD38 is a circumscribed enzyme expressed in tumor cells and active immune cells, such as the T cells, B cells, natural killer (NK) cells and Myeloid- derived suppressor cells (MDSC) [6, 7]. CD38 is involved in regulating extracellular metabolism, intracellular Ca^{2+} level, cell adhesion, activity of cyclase and hydrolytic enzyme, and signal transduction pathways, participating in the immunity regulation in tumor microenvironment (TME) [6]. CD38 has been reported to play an essential role in the primary and secondary drug resistance of ICI treatment [8]. However, the function of CD38 is complex [8]. CD38 contributes to tumor growth and immune suppression by regulating tumor immune microenvironment [6]. It was reported that CD38 drove tumor immune escape by suppressing CD8^+ T cells [9]. The expression of CD38 was associated with active immune infiltration and was consistent with the expression of FOXP3, CTLA4, PD-1, PD-L2, IDO, etc [7].

One previous study reported that CD38 reduced tumor cell senescence and increased cell metastasis in nasopharyngeal carcinoma [10]. Moreover, Zhang, et al found that the high expression of CD38 was correlated with the poor prognosis of pancreatic ductal adenocarcinoma [11]. In contrast, studies from Liu and colleagues showed a better prognosis in prostate cancer patient with high CD38 expression [12]. Given the complex function of CD38 and controversial results, the present study aimed to explore the relationship between CD38 expression, the clinicopathology features and prognosis of ESCC.

Materials And Methods

Patients and clinicopathological data. This retrospective cohort study consecutively recruited 142 patients from Beijing Shijitan Hospital, Capital Medical University, in Beijing, China between 2013 and 2016. The recruited patients received surgical treatment of esophagectomy and were diagnosed as ESCC. All patients received neither neoadjuvant therapy nor immunotherapy prior to surgical operation. The collected esophageal tissues were fixed in 4% neutral formaldehyde after operation, embedded in paraffin, and stained with hematoxylin and eosin (HE) or used for immunohistochemistry (IHC). The obtained clinical and pathological data included age, gender, tumor location, tumor size, tumor differentiation, nerve invasion, vascular invasion and lymph node metastasis. The pathological data were independently evaluated by two pathologists. The data of relapse and overall survival were followed up every six months till March 2018.

Reagents. CD38: Mouse anti-human monoclonal antibody, Clone number: SPC32, expresses in cytomembrane. PD-1: Mouse anti-human monoclonal antibody, Clone number: MRQ-22 expresses in cytoplasm. PD-L1: Rabbit anti-human monoclonal antibody, Clone number: SP142 expresses in

cytomembrane. CD4—Rabbit anti-human monoclonal antibody, Clone number: EP204—expresses in cytomembrane. CD8—Rabbit anti-human monoclonal antibody, Clone number: SP16—expresses in cytomembrane. All antibodies were purchased from Beijing Zhongshan Jinqiao Biotechnology co., LTD.

Immunohistochemistry. The paraffin-embedded samples were cut into 4 µm sections, placed on polylysine-coated slides, deparaffinized with xylene and dehydrated in alcohol series. The slides were washed in 3% hydrogen peroxide solution at room temperature for 10 min, and then in 1x phosphate-buffered solution (PBS) for 5 min for three times. Slides were repaired in citrate buffer (pH6.0) by microwave for 20 min, cooled to room temperature, and washed in PBS for 5 min for three times. Slides were incubated with the primary antibody in a moist chamber at 4 °C overnight, and then washed with PBS for 5 min for three times. The slides were incubated with the corresponding secondary antibody for 20 min at room temperature and then washed with PBS for 5 min for three times. Sav-HRP conjugates were added to the sections, incubated in a humidified chamber at room temperature for 30 min and then washed with PBS for 5 min for three times. Finally, 100 µl DAB substrate solution was added to the sections to reveal the color of antibody staining and washed with PBS for 5 min by three times. Slides were counterstained with hematoxylin.

Assessment of CD38. Tumor tissue sections stained with anti-CD38 antibody with at least 100 tumor cells and 100 immune cells were selected, and myeloma tissue sections were used as the positive controls. The positive cells were defined as the cells had partial or complete cytomembrane brown staining. The expression rate of CD38 was calculated by counting at least 100 cells in three high-power fields (HPFs). The immunohistochemically stained slides were examined by two pathologists independently.

Assessment of PD-L1, PD-1, CD4 and CD8. Tumor tissue sections stained with anti- PD-L1, PD-1, and CD8 antibodies with at least 100 tumor cells and 100 immune cells were selected, and tonsil tissues were used as the positive controls. Two pathologists independently examined the Immunohistochemically stained slides. The expression rate of PD-L1 on tumor cells and immune cells was evaluated. The Assessment of tumor-infiltrating immune cells (TILs) evaluation was conducted according to recommendations by an International TILs Working Group 2014 [13]. An average number of TILs were counted in 10 random HPF (400×) in IHC sections. The proportions of PD-1⁺TILs, CD4⁺TILs and CD8⁺TILs were calculated by the percentage of cells with positive PD-1, CD4 and CD8 expression, respectively, in 100 random TILs from three HPFs with number of TILs around the mean.

Statistical analyses. All analyses were processed by SPSS version 19.0. The expression rate of CD38 was measured by median and inter-quartile range (IQR). Spearman correlation test was used to analysis the correlation between age, tumor location, differentiation, stage and CD38. Lymph node metastasis, neural invasion and blood vessel invasion were analyzed with CD38 expression by Wilcoxon rank-sum test. The CD38 expression rate was categorized by median. Kaplan-Meier survival curve was used to estimate the difference of disease-free survival (DFS) and overall survival (OS). Cox-Hazard Proportion regression

model was used to estimate the hazard ratio (HR) and 95% confidence interval (95%CI), with further confounder adjustment. All analyses were two-tailed and significant level was 0.05.

Results

The IHC results showed that immune cells but not tumor cells had high expression of CD38 (Fig. 1a and 1b). The median expression rate of CD38 was 60% in tumor-infiltrating immune cells (Fig. 1c).

The expression rate of CD38 in immune cells was significantly correlated with patients' age and the correlation coefficient was 0.225 (Table 1). The expression rate of CD38 in immune cells also had a significant correlation with tumor differentiation, with the median expression rates at 70%, 60% and 50% in patients with poor, fair and well differentiation, respectively (Table 1). Patients with perigastric lymph node metastasis had higher expression rate of CD38 than patients without metastasis (70% vs. 50%, Table 1, $p < 0.05$). The expression of CD38 in immune cells did not show significant correlation with the sex, cancer location, periesophageal lymph node metastasis, mediastinal peripheral lymph node metastasis, stage, never invasion or blood vessel invasion (Table 1).

Table 1
Relationship between levels of CD38+ expression rate and pathological features

	Expression rate of CD38(%)		p-value
	Median	IQR	
Age, correlation coefficient*	0.225	—	0.007
Sex**			0.540
Male	60	30	
Female	70	20	
Location*			0.287
Upper esophagus	50	55	
Middle esophagus	60	30	
Lower esophagus	70	30	
Differentiation*			0.001
Poor	70	30	
Moderate	60	25	
Highly	50	35	
Periesophageal lymph node metastasis*			0.506
No	60	30	
Yes	70	20	
Perigastric lymph node metastasis*			0.002
No	50	30	
Yes	70	20	
Mediastinal peripheral lymph node metastasis*			0.429
No	60	30	
Yes	70	20	
Stage**			0.109
I	50	35	
II	60	40	
>II	70	20	

	Expression rate of CD38(%)		p-value
	Median	IQR	
Nerve invasion*			0.959
No	60	30	
Yes	60	25	
Blood vessel invasion*			0.653
No	60	30	
Yes	60	35	
IQR, inter-quartile range; *Spearman correlation test; ** Wilcoxon ran-sum test			

The expression of CD38 in immune cells was correlated with the expression of PD-L1 and Ki-67 in tumor cells and the expression of CD4 in TILs, with the correlation coefficients at -0.421, 0.174 and - 0.178, respectively ($p < 0.05$, Table 2). Among patients with perigastric lymph node metastasis, the expression rate of CD38 showed a negative correlation with the expression rates of PD-L1 in tumor cells and CD4 in TILs, with the corresponding correlation coefficients at -0.299 and - 0.337 ($p < 0.05$, Table 2). Among patients without perigastric lymph node metastasis, the expression rate of CD38 showed a negative correlation with the expression rate of PD-L1 in tumor cells and a positive correlation with PD-L1 expression in immune cells, with the correlation coefficients at -0.520 and 0.233, respectively ($p < 0.05$, Table 2). The expression of CD8 and PD-1 in TILs did not exhibit correlation with the expression rate of CD38 (Table 2).

Table 2
The correlation of CD38 with other biomarkers*

	Expression rate of CD38 in IC (n = 141)	
	Correlation coefficient	p-value
Expression of PD-L1 in tumor cells	-0.421	< 0.001
Expression of PD-L1 in IC	0.094	0.268
Ki-67 index	0.174	0.04
Expression proportion of CD4 ⁺ TILs	-0.178	0.035
Expression proportion of CD8 ⁺ TILs	0.002	0.982
Expression proportion of PD-1 ⁺ TILs	0.010	0.902
IC, immune cells; TILs, tumor-infiltrating lymphocytes, * Spearman correlation test		

The median DFS was 48 months and 19 months in patients with high and low CD38 expression, respectively ($p > 0.05$, Fig. 2a). Patients with the expression rate of CD38 higher than 60% had the median OS of 52 months but patients with lower expression rate of CD38 ($< 60\%$) did not reach the median OS ($p > 0.05$, Fig. 2b). Among patients with the perigastric lymph node metastasis, the expression of CD38 did not show any association with DFS ($p = 0.207$, Fig. 3a), but had a significant correlation with OS ($p = 0.042$, Fig. 3b). Patients with low expression of CD38 had the median OS of 13 months but the patients with high expression of CD38 did reach median OS (Fig. 3b). The crude and adjusted HR of high expression rate of CD38 was 0.37 (95%CI 0.14, 0.95) and 0.21 (95%CI 0.06, 0.70), respectively (Table 3). Among patients without perigastric lymph node metastasis, the expression of CD38 showed a significant association with DFS, and the patients with high expression of CD38 had the median DFS of 45 months and the patients with low expression of CD38 did not reach median DFS (Fig. 3c); the adjusted HR of high expression rate was 2.13 (95%CI 0.85, 5.33) (Table 3). CD38 did not have association with OS among patients without perigastric lymph node metastasis (Fig. 3d).

Table 3

Cox regression of CD38 expression on prognosis stratified by perigastric lymph node metastasis

		Disease-free survival		p-value	Overall survival		p-value
		HR	95%CI		HR	95%CI	
Patients with perigastric lymph node metastasis	Expression proportion $\leq$ 60%	1.00		0.223	1.00		0.040
	Expression proportion > 60%*	0.59	0.25, 1.39		0.37	0.14, 0.95	
	Expression proportion $\leq$ 60%	1.00		0.449	1.00		0.011
	Expression proportion > 60%**	0.67	0.24, 1.89		0.21	0.06, 0.70	
Patients without perigastric lymph node metastasis	Expression proportion $\leq$ 60%	1.00		0.049	1.00		0.162
	Expression proportion > 60%*	2.17	1.01, 4.70		1.83	0.79, 4.25	
	Expression proportion $\leq$ 60%	1.00		0.105	1.00		0.371
	Expression proportion > 60%**	2.13	0.85, 5.33		1.64	0.56, 4.84	

* univariate analysis, ** further adjusted age, sex and differentiation

Discussion

Our study found that the expression of CD38 had a significant association with OS among patients with perigastric lymph node metastasis, and patients with the expression rate of CD38 > 60% had longer survival. It has been previously reported that CD38 promoted ESCC cell growth by suppressing MDSCs and the monoclonal antibody Daratumumab against CD38 reduced the growth of esophageal tumors *in vivo* [14]. However, results from Li's study showed that the expression of CD38 was a favorable predictor for ESCC patients [15], and the study pooled all ESCC patients together including both early stage patients and patients with lymph node metastasis. In our study, the significantly prognostic effect of CD38 expression was only observed in ESCC patients with perigastric lymph node metastasis.

Compare to normal cells, though the mRNA and protein expression of CD38 was higher in malignant cells of breast cancer, lung cancer, hepatic cancer and esophageal cancer, and CD38 has been linked to the tumor differentiation, invasion and metastasis, CD38 might exhibit both stimulating and inhibiting effects to tumor development [8, 16–19]. The function of CD38 was dependent on the cancer type, tumor cell type and interaction with immune cells in the microenvironment [6, 8]. CD38 converts NAD^+ to ADP^- ribose (ADPR) and cADPR, which are essential for regulating extracellular metabolites, intracellular Ca^{2+} level, cell adhesion, and signal transduction pathways [20]. CD38 inhibited the nasopharyngeal carcinoma (NPC) cells apoptosis by regulating intracellular Ca^{2+} concentration, ATP concentration and reactive oxygen species (ROS) signals [10]. CD38 inhibited CD8^+ T-cell proliferation through producing adenosine [21]. The lack of CD38 was associated with the reduced expression of metalloproteinase-12 and inducing tumor cells apoptosis [22].

The high expression of CD38 has been reported to be a favorable prognosis factor in ESCC [15], prostate cancer [12], hepatic cancer [17] and triple negative breast cancer (TNBC) [23]. CD38 participates in the germinal center reaction with inducible co stimulator Ligand competitively and stimulated the anti-tumor immunity [24, 25]. Chen et al found no association between CD38 expression and OS in patients with early stage of lung cancer [21]. Our study showed that the high expression of CD38 was a favorable prognosis factor in patients with perigastric lymph node metastasis. The differential prognosis effects of CD 38 in patients with different degrees of ESCC may be caused by the fact that CD38 stimulates lymph node reaction and body immune system after ESCC cells metastasize into perigastric lymph node.

CD38 is a multifunctional transmembrane protein and is widely expressed in immune cells and tumor cells of prostate cancer, breast cancer, hepatocellular carcinoma [17, 23, 26]. ESCC tumor cells had CD38 mRNA expression, but immunostaining results showed that only immune cells expressed CD38 [15]. Similarly, our study also revealed that only immune cell but not tumorous cells expressed CD38.

Some immunosuppressive cells showed high CD38 expression. High expression of CD38 on $\text{CD4}^+\text{CD25}^{\text{hi}}\text{FOXP3}^+$ regulatory T cells (Tregs) was essential in maintaining tissue homeostasis and preventing immune response [27, 28]. $\text{Foxp3}^+\text{CD25}^+\text{CD4}^+$ Tregs with high CD38 expression showed a greater immunosuppressive activity than Tregs with low CD38 expression [29]. High CD38 expression in $\text{FOXP3}^+\text{CD4}^+$ T-cell was correlated to the function of CD4^+ Tregs [30]. The expression rate of $\text{CD38}^+\text{CD101}^+\text{PD-1}^+\text{CD8}^+$ T cells can be used to discriminate TNM and clinical stages [11]. The expression of CD4 and CD8 had a positive correlation with CD38 [15], but a negative correlation was observed between CD4 and CD38 in our study. In our study, the high expression of CD38 was observed in patients with poor differentiation who always had a high Ki-67 index. Therefore, the CD38 expression has a positive correlation with Ki-67 index. However, in patients without perigastric lymph node metastasis, the expression rate of CD38 had a negative correlation with PD-L1 expression in tumor cells and a positive correlation with PD-L1 expression in immune cells. PD-L1 expressed in immune cells interacts with PD-1 in immune cells and exhibits a suppressed immune function[31].

CD38 was a major molecule inducing acquired resistance to PD-1/PD-L1 blockade, and CD38 might be an independent factor in treatment-induced resistance of ICI [9, 32]. Blocking PD-1/PD-L1 could up-regulate ATRA and IFN β -mediated CD38 expression by changing inflammatory statue and an infiltration of activated T cells [21]. Chen and his colleagues concluded that the expression of PD1 and PD-L1 was related to the differentiation, invasiveness and worse prognosis of ESCC [33]. Moreover, the anti-CD38 therapy approached its therapeutic goals with acceptable adverse effects in patients with multiple myeloma [34–36]. The co-inhibition of CD38 and PD-L1 was supposed to treat solid tumor [6]. Our study found that the expression of CD38 was negatively correlated with PD-L1 expression in tumor cells and positively correlated with the PD-L1 expression in immune cells in patients without perigastric lymph node metastasis. High expression of PD-L1 in immune cells indicated a suppressed immune microenvironment. Though the expression rate of CD38 had a negative correlation with PD-L1 expression in tumor cells, the positive correlation with PD-L1 expression in immune cells introduced an inhibitive anti-tumor immunity and a non-significant prognosis effect in patients without perigastric lymph node metastasis. In Li's study, a positive correlation between PD-L1 and CD38 was observed, but the location of PD-L1 expression was not presented [15]. The background mechanism needs to be elucidated further.

Conclusion

Among patients with perigastric lymph node metastasis, the expression of CD38 was associated with a favorable prognosis of ESCC. The integration of perigastric lymph node metastasis and CD38 expression should be used to predict the survival of ESCC in future prospective studies.

Abbreviations

ESCC

esophageal squamous cell carcinoma

TME

tumor microenvironment

TILs

tumor-infiltrating immune cells

HR

hazard ratio

DFS

disease-free survival

OS

overall survival

CI

confidence interval

HE

hematoxylin and eosin

IHC

immunohistochemistry

TNBC

triple negative breast cancer

HPF

high-power field

IQR

inter-quartile range

ICI

immune check-point inhibitor

PBS

phosphate-buffered solution

Tregs

regulatory T cells

Declarations

Ethical approval and consent to participate

All procedures performed in this study involving human participants were approved by the ethical committee of Beijing Shijitan Hospital, Capital Medical University, in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments.

This study was under a retrospective study and the informed consent was waived.

Consent for publication

All authors wrote and revised the manuscript. All authors read and approved the submission.

Availability of data and material

All data and materials were evaluated by two pathologists, independently. The data analysis had done by specialist.

Competing interests

The authors declare that they have no conflict of interest.

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Author contribution

Shi Feng, Qingkun Song and Hong Chang contributed to the conception and design of the work. Shuo Xiao, Yuchen Li, Ying Gao and Yanjie Zhao contributed to acquisition, analysis or interpretation of the data.

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Figures

Figure 1

The expression of CD38 in tumor microenvironment of esophageal squamous cell carcinoma examined by immunohistochemistry (IHC). a. CD38 expression in tumor microenvironment with ×100 magnification. b. CD38 expression in tumor cells with x400 magnification. c. CD38 expression in immune cells with ×400 magnification.

Figure 2

The association between CD38 expression and ESCC prognosis of disease-free survival (a) and overall survival (b).

Figure 3

The association between CD38 expression and ESCC prognosis stratified using perigastric lymph node metastasis. a. Disease-free survival among patients with perigastric lymph node metastasis, b. Overall survival among patients with perigastric lymph node metastasis, c. Disease-free survival among patients without perigastric lymph node metastasis, d. Overall survival among patients without perigastric lymph node metastasis.