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Original Article

Cancer-associated fibroblasts are associated with poor prognosis in esophageal squamous cell carcinoma after surgery

Yufeng Cheng[1,2*], Kai Wang[3*], Wei Ma[4], Xiaomei Zhang[2], Yipeng Song[5], Jianbo Wang[2], Nana Wang[2], Qingxu Song[2], Fangli Cao[2], Bingxu Tan[2], Jinming Yu[1]

[1]Tianjin Medical University, Tianjin 300000, People's Republic of China; [2]Oncology Center, Qilu Hospital of Shandong University, 107 West Wenhua Road, Jinan 250012, People's Republic of China; [3]Oncology Center, Qilu Hospital of Shandong University (Qingdao), 758 Hefei Road, Qingdao 266035, People's Republic of China; [4]General Hospital of Ningxia Medical University, Yinchuan 750004, People's Republic of China; [5]Department of Oncology, Yantai Yuhuangding Hospital, Yantai 264000, People's Republic of China. [*]Equal contributors.

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Abstract: Objective: Cancer-associated fibroblasts (CAFs; α-SMA positivity), as a representative of the tumor microenvironment, play an important role in influencing the proliferation, invasion and metastasis of cancer cells. The objective is to investigate the prognostic value of CAFs density in esophageal squamous cell carcinoma (ESCC) after surgery. Method: A total of 95 patients who underwent esophagectomy for ESCC in 2007 were included in this study. These specimens were immunostained with α-smooth muscle actin (α-SMA) antibodies to quantify CAFs. Antibodies D2-40 and CD34 were used to evaluate the lymphatic vessel density (LVD) and microvessel density (MVD) of the lesions. The Cox proportional hazards model was used to determine the hazard ratio of CAFs density on 3-year overall survival and 3-year disease-free survival. The correlation between CAFs density and lymphatic vessel density (LVD) or microvessel density (MVD) were analyzed. Results: 3-year overall survival rate in the CAF-poor group (63%) was significantly better than those in the CAF-rich group (42%) ($P < 0.01$). In the Cox univariate and multivariate analysis of 3-year overall survival, the hazard ratio (HR) of CAFs density was 1.870 (95% CI 1.033-3.385; $P = 0.039$) and 2.196 (95% CI 1.150-4.193; $P = 0.017$), respectively. CAFs density was proved to be an independent prognostic factor for 3-year overall survival. CAFs density correlated significantly with increased LVD and MVD in ESCC. Conclusion: CAFs density may be a marker for predicting prognosis and guiding therapeutic management of ESCC.

Keywords: Esophageal squamous cell carcinoma, survival, cancer-associated fibroblasts, lymphatic vessel density, microvessel density

Introduction

Esophageal carcinoma is the eighth most common cancer, and the sixth cause of cancer mortality in the world [1]. The incidence of esophageal cancer varies in different genders and areas. In China and other East Asia countries, more than 90% of cases are esophageal squamous cell carcinoma (ESCC). Esophageal cancer is 3 to 4 times more common among males than females in worldwide. In north China, the area with the highest incidence of ESCC, male patients is more susceptible than female to the ESCC due to tobacco use and alcohol consumption [2]. As a highly lethal disease, the overall survival rate of ESCC is less than 20%. It is important to find new and reliable markers for predicting prognosis and guiding therapeutic management of ESCC.

Carcinoma was long viewed as a disease of transformed epithelial cells. However, tumor progression is not achieved solely by the evolving cancer cells. Stromal components-the microenvironment of the tumor-also play a key role in this process [3]. Fibroblasts are an important component of the tumor stroma. As reactive fibroblasts, carcinoma-associated fibroblasts play an essential role in some aspects of cancer biology including transformation, progression, tumor growth, and drug resistance [4], and their presence in large numbers is often

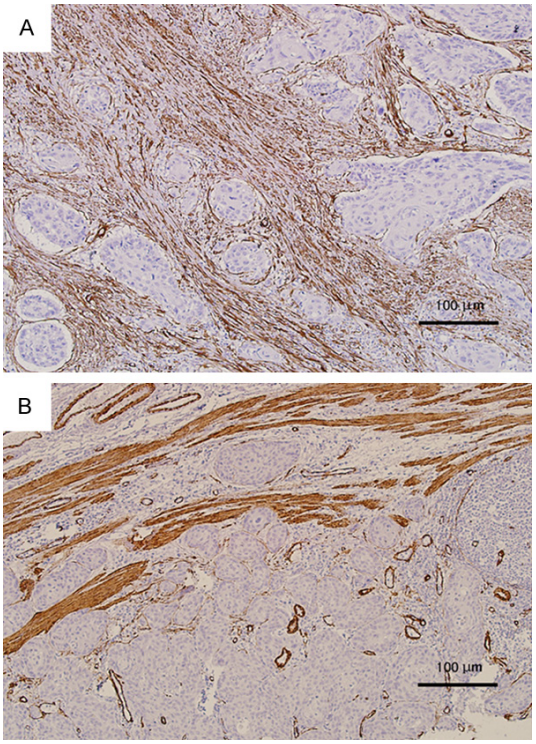

Figure 1. α-SMA-stained 5 μm sections of esophageal squamous cell carcinoma; 100× magnification (10× objective). A. example of CAF-rich (dense overlapping of CAFs distributed throughout the tumor predominantly of epithelioid morphology). B. example of CAF-poor (CAFs not distributed throughout the entire tumor).

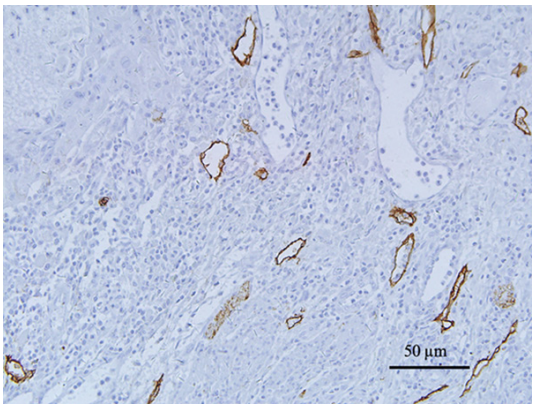

Figure 2. Lymphatic vessel density (LVD) D2-40-stained 5 μm sections of esophageal squamous cell carcinoma; 200× magnification (20× objective). The vessels exhibiting a typical morphology (lumen) were considered lymphatic vessels.

associated with the development of high-grade malignancies and poor prognosis [5]. Recently, CAFs density was proved to be associated with poor prognosis in rectal cancer after chemoradiotherapy, mobile tongue cancer [6, 7]. To our knowledge, the prognostic value of CAFs density remained to be explored for ESCC.

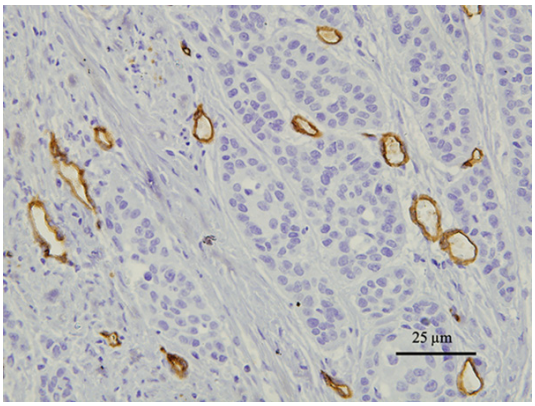

Figure 3. microvessel density (MVD) CD34-stained 5 μm sections of esophageal squamous cell carcinoma; 400× magnification (40× objective). The vessels exhibiting a typical morphology were considered microvessel.

The objective of this study was to evaluate the prognostic value of CAFs density in ESCC, and its relationship with lymphatic vessel density (LVD) and microvessel density (MVD).

Materials and methods

Patients and tissue samples

From the database of the Qilu Hospital of Shandong University, we selected patients with ESCC who underwent resection with curative intent at the Department of Thoracic Surgery of the Qilu Hospital from 1 January to 31 December 2007. Patients treated with neoadjuvant therapy which could interfere with the evaluation of CAFs density were excluded, as were patients who died within 30 days after surgery. Patient, tumor and treatment characteristics were retrieved from the Medical Records Room. Both original pathological reports and immunohistochemistry stained sections from the primary tumor were retrieved from the Department of Pathology. All patients signed informed consent to this study, and the protocol was approved by the Ethics Committee of Qilu Hospital of Shandong University.

Histopathological protocol

The available 132 blocks were cut into 5-μm-thick sections and stained with α-SMA monoclonal antibody (clone 1A4, 1:100, Dako A/S, Denmark), D2-40 monoclonal antibody

Table 1. Patient, tumor and treatment characteristics for patients who underwent esophagectomy for esophageal squamous cell carcinoma, grouped by CAFs density

Characteristics	Total (n = 95)		CAF-poor (n = 46)		CAF-rich (n = 49)		*P*-value
	No. of patients	%	No. of patients	%	No. of patients	%	
Gender							0.674
Male	82	86.3	39	84.8	43	87.8	
Female	13	13.7	7	15.2	6	12.2	
Median age at surgery (range)	60 (42-77)		62 (42-74)		60 (42-77)		0.214
Tumor location							0.898
Upper	10	10.5	5	10.9	5	10.2	
Middle	56	59.0	28	60.9	28	57.1	
Low	29	30.5	13	18.3	16	32.7	
pN status							0.302
pN0	56	58.9	26	56.5	30	61.2	
pN1	25	26.3	11	24.0	14	28.6	
pN2	11	11.6	6	13.0	5	10.2	
PN3	3	3.2	3	6.5	0	0	
pTNM stage							0.270
I	13	13.7	9	19.6	4	8.2	
II	47	49.5	21	45.7	26	53.1	
III	35	36.8	16	34.8	19	38.8	
Differentiation grade							0.406
Well	28	29.5	12	26.1	16	32.7	
Moderate	40	42.1	18	34.1	22	44.9	
Poor	27	28.4	16	34.8	11	22.4	
Radicality							0.214
R0	92	96.8	44	95.7	48	98.0	
R1	2	2.1	2	2	0	0	
R2	1	1.1	0	0	1	2.0	
Adjuvant therapy							0.470
No	47	49.5	21	45.7	26	53.1	
Yes	48	50.5	25	54.3	23	46.9	

Abbreviations: TNM, tumor node metastasis; CAFs, Cancer-associated fibroblasts. *P*-value < 0.05 was considered significant.

(1:50 Dako A/S, Denmark) and CD34 monoclonal antibody (prediluted, Dako A/S, Denmark). All analyses were performed by at least two of the authors who were unaware of the clinical outcome at the time of analysis.

The α-SMA cases were graded as CAF-rich and CAF-poor group according to CAFs density, similar to a previous description of this technique in tongue and oral squamous cell cancer [8, 9]. CAF-rich pattern means dense overlapping of CAFs distributed throughout the tumor predominantly of epithelioid morphology, with essentially no distinct border with the ESCC. And CAF-poor pattern means somewhat less dense, or CAFs not distributed throughout the entire tumor. Each tumor was scored according to the most severe grade (**Figure 1**).

LVD was assessed as follows [10]. Briefly, the most vascularized intratumoral and peritumoral areas (hot spot areas) were identified under low magnification (40× and 100×). The number of immunostained lymphatic vessels found in 5 hot spot areas was counted at 200× magnification. Only the vessels exhibiting a typical morphology (lumen) were considered lymphatic vessels (**Figure 2**). The LVD in each case was expressed as means (total number of vessels in 5 hot spot areas/5).

For evaluation of microvessel density, the immunostained sections were scanned at low

Table 2. Cox univariate analysis for 3-year survival and 3-year disease-free survival in 95 patients who underwent esophageal resection for squamous cell carcinoma

	Univariate analysis					
	3-year overall survival			3-year disease-free survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
CAFs density			0.039			0.405
CAF-poor	1.000	Ref.	-	1.000	Ref.	-
CAF-rich	1.870	1.033-3.385	0.039	1.258	0.733-2.160	0.405
Differentiation grade			0.558			0.470
Well	1.000	Ref.	-	1.000	Ref.	-
Moderate	1.450	0.699-3.009	0.318	1.303	0.660-2.575	0.446
Poor	1.456	0.658-3.207	0.351	1.573	0.763-3.243	0.219
Tumor location			0.359			0.426
Upper	1.000	Ref.	-	1.000	Ref.	-
Middle	2.046	0.767-5.460	0.153	0.780	0.327-1.866	0.577
Low	1.296	0.659-2.550	0.452	0.558	0.213-1.464	0.236
pN status			0.041			0.100
pN0	1.000	Ref.	-	1.000	Ref.	-
pN1	1.772	0.900-3.491	0.098	1.317	0.693-2.504	0.401
pN2	2.534	1.121-5.729	0.025	2.034	0.922-4.486	0.078
PN3	3.557	1.060-11.938	0.040	3.450	1.042-11.427	0.043
pTNM stage			0.012			0.072
I	1.000	Ref.	-	1.000	Ref.	-
II	7.830	1.053-58.244	0.044	1.442	0.586-3.550	0.426
III	13.730	1.854-101.702	0.010	2.441	0.989-6.025	0.053
Radicality			0.048			0.070
R0	1.000	Ref.	-	1.000	Ref.	-
R1	3.424	0.813-14.432	0.094	2.363	0.568-9.837	0.237
R2	7.154	0.933-54.842	0.058	8.296	1.069-64.351	0.043
Adjuvant therapy			0.449			0.215
No	1.000	Ref.	-	1.000	Ref.	-
Yes	1.252	0.700-2.236	0.449	1.412	0.818-2.438	0.215

Abbreviations: N, pathological node stage; TNM, tumor node metastasis; CAFs, Cancer-associated fibroblasts; HR, hazard ratio; CI, confidence interval. Analysis was performed using the Cox proportion hazard model. *P*-value < 0.05 was considered significant.

magnification (×40). Three areas of carcinoma with the greatest number of distinctly highlighted intratumoral microvessels, hot spots, were selected at the same time. Then the slides were evaluated for microvessel counting using ×400 magnification (**Figure 3**). Depending on the size of the hot spot, 1 to 3 readings were taken. The MVD in each case was expressed as me-ans (total number of vessels in 3 hot spot areas/3).

Follow-up

Follow-up data were collected until death or March 2011. All patients had a regular follow-up schedule including a complete history and physical examination every 3 months during the first 2 years after surgery and every 6 months thereafter. Routine radiological examinations were performed.

Statistical analysis

Differences in patient, tumor and treatment characteristics were assessed using the Mann-Whitney test for continuous variables and the chi-square test for categorical variables. The Cohen's kappa coefficient was used to analyze the variability between the two investigators. The Kaplan-Meier method and Log-Rank test were used for analysis and comparison of survival curves. For the analysis of 3-year disease-

Table 3. Multivariate Cox analysis for 3 year survival in 95 patients who underwent esophageal resection for squamous cell carcinoma

	Multivariate analysis		
	3-year overall survival		
	HR	95% CI	*P*-value
pN status (N3)	2.557	0.708-9.234	0.020
CAFs density (CAF-rich)	2.196	1.150-4.193	0.017
pTNM stage (III)	2.168	1.316-3.572	0.002
Radicality (R1-2)	5.720	1.652-19.812	0.006

Abbreviations: pN, pathological node stage; TNM, tumor node metastasis; CAFs, Cancer-associated fibroblasts; HR, hazard ratio; CI, confidence interval. Analysis was performed using the Cox proportion hazard model. *P*-value < 0.05 was considered significant.

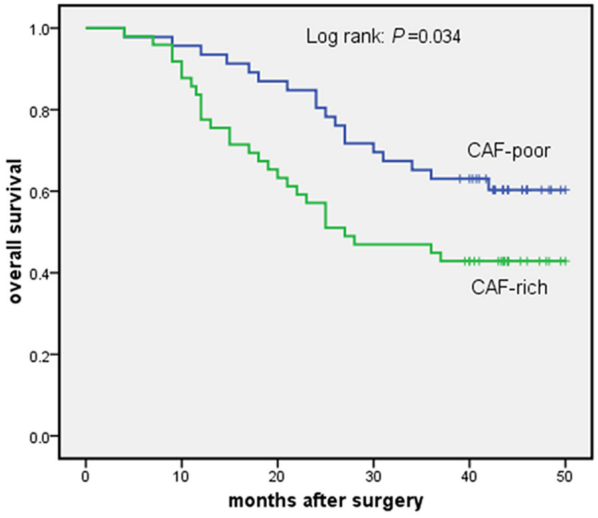

Figure 4. 3-year survival curves for all 95 patients who underwent esophagectomy for esophageal squamous cell carcinoma. CAF-poor group versus CAF-rich group.

free survival, events were defined as first locoregional or distant tumor relapse or death from any cause. For the analysis of 3-year overall survival, events were defined as death from any cause. The Cox proportional hazards model was used to determine the hazard ratio of variables on 3-year disease-free survival and 3-year overall survival in univariate and multivariate analysis. The Chi-square test was used to compare the relationship between CAFs density and increased LVD or MVD in ESCC.

The results are given as hazard ratios with their 95% confidence interval (CI). *P*-values < 0.05 were considered statistically significant. Data were analyzed using statistical package SPSS version 17.0 (SPSS Inc, Chicago, IL, USA).

Result

Clinicopathological features

Ninety-five patients (82 men and 13 women) were included in this study. The median age of the patients were 60 (range 42-77) years at the date of surgery. The median follow-up time was 40 (4-50) months of patients. Clinicopathological and treatment characteristics of patients are shown in **Table 1**.

CAFs density in esophageal squamous cell carcinoma

Using a 4× and 10× objective, α-SMA stained sections from the primary tumors were analyzed for the presence of CAFs. Estimation of the CAFs density was performed successfully in all tumors. Assessed by two independent researchers, 46 cases were CAF-poor and 49 were CAF-rich. Cohen's kappa coefficient revealed an almost perfect agreement in two researchers (kappa = 0.81).

Correlation of CAFs density with other prognostic factors

Table 1 shows patient, tumor and treatment characteristics for the CAF-rich and the CAF-poor groups. There were no significant differences between two groups. Follow-up was complete. In the Cox univariate model, CAFs density, lymph node status, pTNM stage and radicality of resection were significantly related to 3-year overall survival. Lymph node status (N3) and pTNM stage (III) were significantly related to 3-year disease-free survival (**Table 2**). In the Cox multivariate model, CAFs density, pTNM stage and radicality of resection remained independent prognostic factors for 3-year overall survival (**Table 3**). The hazard ratio of CAFs density was 2.196 (95% CI 1.150-4.193; *P* = 0.017). 3-year overall survival curves are shown in **Figure 4**. The difference of survival curves between two groups remained statistically significant (*P* = 0.034). In univariate analysis, our results suggested that adjuvant therapy was not significantly associated with the 3-year overall survival (**Table 2**). According to receiving adjuvant therapy or not, the stratified analysis was used for analysis and comparison of survival curves between CAF-poor and CAF-rich group. For patients received no therapy, significant difference was observed between the two groups (*P* = 0.034) (**Figure 5**). While for

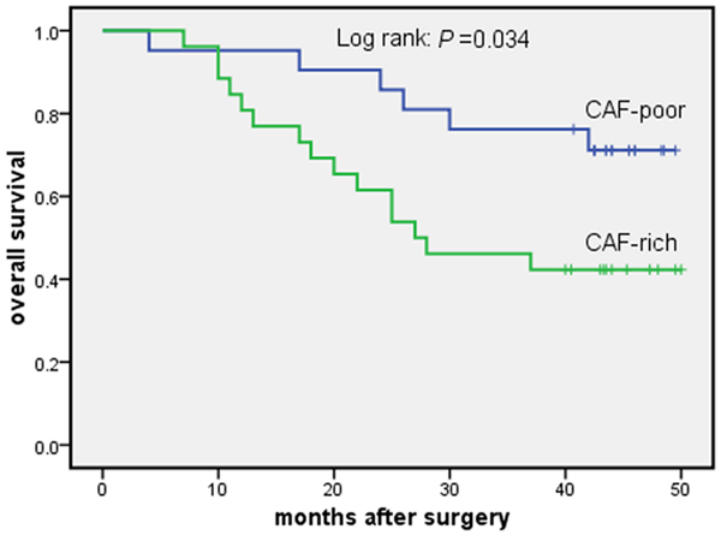

Figure 5. 3-year survival curves for patients who received no adjuvant therapy after esophagectomy for esophageal squamous cell carcinoma. CAF-poor group versus CAF-rich group.

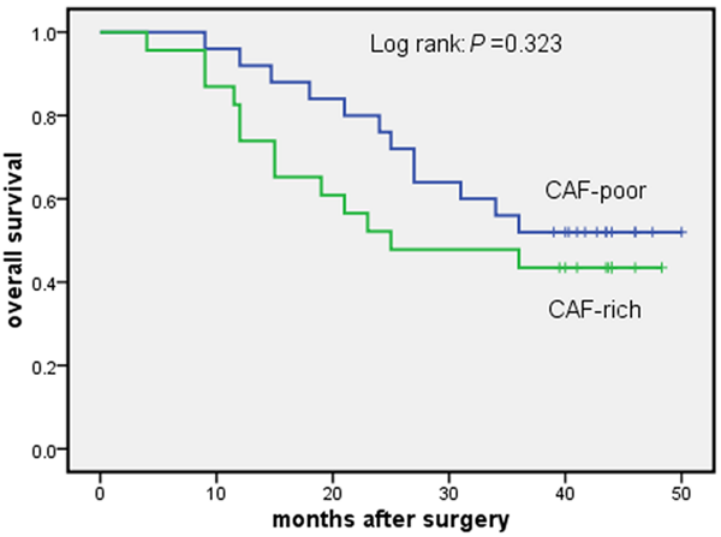

Figure 6. 3-year survival curves for patients who received adjuvant therapy after esophagectomy for esophageal squamous cell carcinoma. CAF-poor group versus CAF-rich group.

patients received adjuvant therapy, no significant difference was observed between the two groups (P = 0.323) (**Figure 6**). For 3-year disease-free survival, pTNM stage (III) was proved to be an independent prognostic factor (**Table 4**). The hazard ratio of pTNM stage (III) was 1.843 (95% CI 1.067-3.184; P = 0.031). CAFs density wasn't significantly associated with the 3-year disease-free survival (P = 0.405). **Tables 5** and **6** show CAFs density in ESCC correlated significantly with increased LVD and MVD.

Discussion

The present investigation showed that CAFs density correlated significantly with increased LVD and MVD, and was associated with poor prognosis of ESCC patients.

The use of CAFs density as a prognostic factor has been introduced by previous studies. After analysis of 128 patients with Mobile tongue squamous cell carcinoma (MTSCC), Ibrahim O. Bello and colleagues found that CAFs density was independently and relatively strongly associated with elevated mortality from MTSCC [7]. Our previous study showed stroma-rich tumors were associated with poor prognosis and an increased risk of relapse in ESCC [11]. Our hypothesis was that CAFs density might be an important prognostic parameter for ESCC.

Ninety-five patients (82 men and 13 women) were included in this study. From the constituent ratio of gender, the male patients are more than female patients. Considering that CAFs density isn't gender dependent, gender bias didn't affect analytic results in this study. For 3-year overall survival, we found that CAFs density, lymph node status, pTNM stage and radicality of resection were significantly related to 3-year overall survival in multivariate analysis. And CAFs density may be an independent prognostic factor of ESCC. For patients received no therapy, significant difference was observed between CAF-rich and CAF-poor group. The present study suggested that adjuvant therapy may be beneficial to CAF-rich group. CAFs density may be a marker for guiding therapeutic management of ESCC.

Different molecular mechanisms underlying the tumor-promoting capabilities of CAFs have

Table 4. Multivariate Cox analysis for 3-year disease-free survival in 95 patients who underwent esophageal resection for squamous cell carcinoma

	Multivariate analysis		
	3-year disease-free survival		
	HR	95% CI	*P*-value
pN status (N3)	2.193	0.650-7.400	0.206
pTNM stage (III)	1.843	1.067-3.184	0.031
Radicality (R1-2)	3.156	0.964-10.328	0.057

Abbreviations: pN, pathological node stage; TNM, tumor node metastasis; HR, hazard ratio; CI, confidence interval. Analysis was performed using the Cox proportion hazard model. *P*-value < 0.05 was considered significant.

Table 5. Correlation between CAFs density and LVD

	LVD			
CAFs density	low	high	r	*P*-value
poor	29	17		
high	20	29	0.222	0.030

Abbreviations: r, correlation coefficient; CAFs, Cancer-associated fibroblasts; LVD, Lymphatic vessel density. *P*-value < 0.05 was considered significant.

Table 6. Correlation between CAFs density and MVD

	MVD			
CAFs density	low	high	r	*P*-value
poor	23	23		
high	14	35	0.220	0.032

Abbreviations: r, correlation coefficient; CAFs, Cancer-associated fibroblasts; MVD, microvessel density. *P*-value < 0.05 was considered significant.

been emerging to explain why CAFs are associated with poor prognosis of esophageal squamous cell carcinoma.

First, the role of tumor stromal fibroblasts is in promoting tumor progression. Neoangiogenesis is an important pathophysiological process in the growth and progression of cancer. MVD is a quantitative indicator of angiogenesis. And it is regarded as the mark of angiogenesis activity effect. Our results demonstrated that CAFs density in ESCC correlated significantly with increased tumoral MVD. Vascular endothelial growth factor (VEGF) is a potent pro-angiogenic factor that stimulates endothelial cell migration and proliferation [12]. It is believed to play an important role in angiogenesis. As the principal source of host-derived VEGF, CAFs drive vascular network formation through the stimulation of VEGF release [13].

Second, the role of CAFs is in promoting tumor cell metastasis. Tumor lymphangiogenesis is a prognostic indicator for lymph node Metastasis. Lymphatic vessel density (LVD) at tumor sites might be a sensitive prognostic indicator of metastasis to lymph nodes and disease progression. An increase in tumor-associated LVD correlates with lymph node metastasis and unfavorable prognosis in esophageal squamous cell carcinoma [14]. Our results demonstrated that CAFs density in ESCC correlated significantly with increased tumoral LVD. As lymphangiogenic growth factors, expression of VEGF-C by tumor-associated stroma correlated with lymph node metastasis in ESCC [15].

Although our study population was homogenous, the study has its shortcomings. This was a retrospective study with a relatively small sample size and the follow-up time is short. It is of greater value to conduct a prospective study with a larger sample size in the future. And we will investigate the mechanism behind the association between CAFs and prognosis of ESCC at the next period.

The present results clearly showed that tumor stromal fibroblasts could boost neoangiogenesis and lymphangiogenesis, and the CAFs density of the tumor microenviroment had the significant impact on patient survival. CAFs density may be a marker for predicting prognosis and guiding therapeutic management of ESCC. These novel findings may have major consequences on ESCC management and prognosis.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jinming Yu, Tianjin Medical University, Tianjin 300000, People's Republic of China. Tel: 86-531-82169830; Fax: 86531-86927544; E-mail: jinmingshandong@163.com

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