

High tumor-associated macrophages infiltration is associated with poor prognosis and may contribute to the phenomenon of epithelial–mesenchymal transition in gastric cancer

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Background: Recent studies show that epithelial–mesenchymal transition (EMT) and tumor-associated macrophages (TAMs) contribute to the progression and poor prognosis of carcinoma through multiple mechanisms. Both inflammation and changing of epithelium have a close relationship with tumorigenesis of gastric cancer. However, the relevance between EMT and TAMs is still unclear in gastric cancer and needs more scientific research. This study is designed to explore the relationship between EMT and TAMs in gastric cancer.

Materials and methods: Immunohistochemistry was used to detect the expression of EMT-related proteins and TAM markers in cancer tissues and normal gastric tissues.

Results: High levels of EMT and TAMs infiltration are related to aggressive features and independent prognostic factors in gastric cancer, respectively. In addition, expression of the two indicators is associated with expression of transforming growth factor- β 1 (TGF- β 1). Infiltration of TAMs is also associated with EMT-related marker in gastric cancer.

Conclusion: Our results suggest that high levels of EMT and TAMs infiltration are related to aggressive features and independent prognostic factors in gastric cancer, respectively. A correlation was found between EMT- and TAM-related indicators, which may be associated with TGF- β signaling pathway. The level of TAMs infiltration plays an important role in gastric cancer, the markers of which can be used as prognostic indicators.

Keywords: gastric cancer, prognosis, tumor-associated macrophages, E-cadherin, TGF- β 1

Introduction

There are approximately one million patients diagnosed with gastric cancer every year worldwide.¹ Although the incidence rate of gastric cancer is continually decreasing in recent years, it is still the second leading cause of cancer-related deaths.^{1,2} So, it is necessary for researchers to figure out new targets and therapies for gastric cancer. Since this is a typical inflammation-associated epithelial cancer,³ cancer-related inflammation and the change of normal epithelium are two crucial points that need to be focused on. Both invasiveness and metastasis are the characteristic features of malignant cancer and are the main cause of cancer-related deaths. Recent researches suggest that tumorigenesis has a close relationship with cancer-related inflammation and epithelial–mesenchymal transition (EMT), both of which contribute to invasiveness and metastasis of various cancers, especially in gastric cancer.^{4–7} Macrophages appear in almost 50% of the solid tumors, and tumor-associated macrophages (TAMs),

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no doubt, act as one of the vital components of cancer-related inflammation in gastric cancer.⁸

The process of epithelial cells transforming into mesenchymal cells is defined as EMT, and the reverse process is known as mesenchymal–epithelial transition.⁹ Both of them, especially the progress of EMT, hold an important position in embryonic development, fibrosis, pathogenesis of cancer, and progression of other diseases.^{10–12} EMT has been proved to be one of the crucial processes of tumorigenesis in various cancers, and high expression of EMT-related proteins indicates a poor prognosis in cancer patients.^{13–15} It has been confirmed that in the process of EMT, deconstruction of cell polarity, reorganization of cytoskeleton, and changes of the signaling programs improve motility and invasiveness of cancer cells through multiple mechanisms.^{16,17} Extracellular cues stimulate the correlative signaling pathways (including Hedgehog, integrin, transforming growth factor- β [TGF- β], Wnt, platelet-derived growth factor, Notch, Akt, PI3K, NF- κ B, Ras, and so on), which may reprogram the gene expression in EMT.^{18–25} Then, the transcription factors that mediate the whole switch of EMT are finely adjusted at different levels.²⁶ Though the combined action of all the related signaling pathways is necessary, TGF- β family signaling pathway plays a primary role in EMT.^{26,27}

TAM is one of the dominating inflammatory components of immune cell infiltration in cancer stroma, which is discovered in tumor microenvironment of many cancers, including gastric cancer.²⁸ Furthermore, a high-level infiltration of TAMs is always related to poor prognosis of cancer.^{29,30} Considerable evidences indicate that cancer cell is implicated in many critical respects of tumor progression by changing the phenotype of TAMs and remodeling TAM activities.^{31,32} Cytotoxic activity of TAMs is enhanced or suppressed by molecular products derived from tumor. TAMs facilitate growth, migration, and metastasis of tumor cells under the influence of these released products, including TGF- β 1.^{33–35} In addition, previous studies have shown that TGF- β 1 can promote cancer invasiveness and metastasis through effects on the tumor microenvironment, ie, by immune suppression, angiogenesis, and so on.^{36,37} However, the relationship between TGF- β signaling pathways and TAMs in the tumor is not clear.³⁸

TGF- β family is a huge cloud of extracellular growth factors including TGF- β s, activins, bone morphogenetic proteins, and so on.³⁹ TGF- β 1 is the key cytokine with dual and inverse functions in cancer biology that regulates growth, migration, angiogenesis, and immune response to cancer.⁴⁰ It has an inhibiting effect in the early stage

of oncogenesis, while in the later stage acts as a tumor promoter.⁴¹ Accumulating evidences have shown that high expression of TGF- β 1 is associated with a poor prognosis in patients with cancers, including gastric cancer.^{42–44} Furthermore, TGF- β 1 expressed during immune response contributes to the transformation of TAMs and tumor microenvironment.^{45,46} Moreover, TGF- β signaling pathway has been reported to be a dominating signaling pathway of EMT, and TGF- β 1 can activate some EMT-related pathways, such as IL-6/STAT3 and PI-3/Akt.²⁶ As TGF- β 1 plays an important role both in EMT and TAM, it may have a close relationship with tumorigenesis of gastric cancer. In this study, we demonstrate a connection between EMT and TAM related to TGF- β 1. High expression of TGF- β 1 induced by TAM in tumor microenvironment may facilitate the progression of EMT, which is associated with the poor prognosis of gastric carcinoma.

Materials and methods

Clinical and tissue samples

A total of 178 gastric cancer tumor samples were collected from the First Affiliated Hospital, Medical School, Xi'an Jiaotong University and the 215th Hospital in Shaanxi Province from 2004 to 2009. The patients included 125 males and 53 females, with age ranging from 25 to 81 years. All the gastric patients were diagnosed with gastric adenocarcinoma, with a complete clinical history and detailed follow-up information. Clinicopathological parameters of all the patients were assessed in detail according to the tumor node metastasis (TNM) staging mentioned in the *AJCC Cancer Staging Manual*.⁴⁷ The clinicopathological data of 178 patients are documented in Table 1. None of the patients accepted chemotherapy or radiation therapy before surgery.

Immunohistochemistry and scoring

Formalin-fixed and paraffin-embedded tissue specimens (4 μ m in thickness) of cancer and pericarcinous tissues from each patient were used. Tissue sections were placed on charged glass slides, and then were subjected to

Table 1 Expression of CD163 and TGF- β 1 in gastric cancer and normal tissues

Group	CD163			TGF- β 1		
	High	Low	P-value	High	Low	P-value
Gastric cancer tissue	84	94	<0.001	111	67	<0.001
Normal tissue	38	140		37	141	

Abbreviation: TGF- β 1, transforming growth factor- β 1.

deparaffinization and rehydration. Then, antigen retrieval was performed using citrate buffer at pH 6.0. Antibodies against E-cadherin (ZA-0565-3), CD163 (ZM-0428), and TGF- β 1 (bs-0086R), purchased from Beijing Zhongshan Biotechnology (Beijing, People's Republic of China), were used for blocking. Streptavidin–peroxidase technique was used for the purpose of staining. The sections were stained with 0.02% diaminobenzidine solution followed by counterstaining with hematoxylin for 2 minutes. Irrelevant rabbit antiserum was used as a first antibody for the duplicated section as a negative control.

The indicator expression was evaluated, respectively, by two pathologists who were blinded to the clinical data. The staining results of three indicators were scored semi-quantitatively on the basis of immunostaining intensity and percentage of positive tumor cells. Sections were observed with a light microscope at high power (200 \times) in at least five areas. The mean staining results of E-cadherin and TGF- β 1 were scored and placed into five categories: 0 point for <5%, 1 point for 5%–25%, 2 points for 26%–50%, 3 points for 51%–75%, and 4 points for >75%. The staining intensity was scored as 0, 1, 2, and 3, corresponding to no coloring, slightly yellow, brown yellow, and tan, respectively. Sum of the two aforementioned scores was calculated as the final staining score which was defined as follows: 0–5, low expression and ≥ 6 , high expression. However, for the positivity of TAM markers (CD163), staining density was estimated by the mean count of the stained cells in five areas. If the average number in each case was less than the mean, the section was allocated to the group of low expression; otherwise, it was allocated to the group of high expression.

Ethics statement

The Protection of Human Subjects Committee of The First Affiliated Hospital, Medical School, Xi'an Jiaotong University, approved this study which complies with the Declaration of Helsinki. Informed consent was obtained from the patients.

Statistical analysis

Associations between clinicopathologic variables and expressions of CD163, TGF- β 1, and E-cadherin were examined by the chi-square test. The Spearman's rank correlation coefficient was used to confirm the association among E-cadherin, CD163, and TGF- β 1. Kaplan–Meier test was used to calculate survival curves, which were compared using the log-rank test. Factors showing prognostic significance were evaluated with the multivariate Cox regression model. The normally distributed variables were checked by Student's

t-test or one-way analysis of variance. *P*-values <0.05 and 0.01 were considered to be statistically significant. Data were analyzed by SPSS software package (Version 16.0; SPSS Inc., Chicago, IL, USA).

Results

Differential expression of CD163 and TGF- β 1 markers in cancer tissues and normal mucosae and their association with clinicopathologic features in gastric cancer

Expression of CD163, TGF- β 1, and E-cadherin was significantly different in gastric cancer tissues and normal gastric tissues (Table 1). The clinicopathologic features of gastric cancer patients and their correlation with the expression of CD163, TGF- β 1, and E-cadherin are described in Table 2. High and low expressions of CD163, TGF- β 1, and E-cadherin, according to typical immunohistochemical staining patterns, in gastric cancer and paracancer normal tissues are shown in Figure 1. High expression of TGF- β 1 was observed in the cytoplasm of tumor cells in 111 cases (62.4%) and in the cytoplasm of normal cells in 37 cases (20.8%), which shows a statistical significance ($P < 0.001$). TGF- β 1 expression was found to be higher in low- and advanced-grade tumors, and statistical analysis revealed that high expression of TGF- β 1 was related to tumor size, differentiation, Borrmann type, TNM stage, nodal involvement, and metastasis ($P < 0.05$). High expression of CD163 was detected in 94/178 (52.8%) cases of gastric cancers and in 38 (21.3%) cases with normal tissues. The data show statistical significance ($P < 0.05$). Significant associations were also seen with respect to tumor size, differentiation, Borrmann type, TNM stage, nodal involvement, and metastasis.

Association among expression of EMT, TGF- β 1 markers, and TAMs infiltration

Significant associations among the expression of CD163, TGF- β 1, and E-cadherin are described in Tables 3 and 4. High expression of CD163 was related to low expression of E-cadherin ($r = -0.421$, $P < 0.001$) and high expression of TGF- β 1 ($r = -0.427$, $P < 0.001$) in gastric cancer tissues. High expression of TGF- β 1 was correlated with a loss of E-cadherin expression ($r = -0.452$, $P < 0.001$) in the same samples. In order to have a better illustration, the relationships between expression of TGF- β 1 and E-cadherin were calculated by different CD163 infiltration group in gastric cancer tissues, and we found almost exactly the same result (Table 5).

Table 2 Correlations of TGF- β 1 and CD163 expressions with clinicopathologic characteristics of gastric cancer patients

Variable	n	TGF- β 1		P-value	CD163		P-value
		High	Low		High	Low	
Sex				0.738			0.623
Male	125	79	46		68	57	
Female	53	32	21		26	27	
Age (years)				0.536			0.548
≤ 60	94	61	33		42	42	
> 60	84	50	34		52	42	
Tumor size (cm)				0.015			0.020
< 3	44	22	22		17	27	
3–5	54	30	24		26	28	
> 5	80	59	21		51	29	
Borrmann				0.032			0.029
I	22	11	11		10	12	
II	55	32	23		21	34	
III	62	36	26		38	24	
IV	39	32	7		25	14	
Differentiation				< 0.001			< 0.001
High	12	3	9		3	9	
Moderate	57	24	33		20	37	
Poor	109	84	25		71	38	
Depth of invasion				0.033			0.002
T1	16	5	11		5	11	
T2	18	10	8		3	15	
T3	67	47	20		39	28	
T4	77	49	28		47	30	
Lymph node metastasis				< 0.001			< 0.001
N0	49	18	31		13	36	
N1	36	17	19		13	23	
N2	35	26	9		11	13	
N3	58	50	8		46	12	
Distant metastasis				0.003			0.019
M0	138	78	60		66	72	
M1	40	33	7		28	12	
TNM stage				0.002			< 0.001
I	21	8	13		5	16	
II	34	17	17		10	24	
III	83	53	30		51	32	
IV	40	33	7		28	12	

Abbreviations: TGF, transforming growth factor; TNM, tumor node metastasis.

Association of TGF- β 1 and CD163 expression with survival times of gastric cancer patients

Survival curves were calculated by the Kaplan–Meier method and log-rank test. Patients with high expression of CD163 have an unfavorable overall survival (28.00 ± 2.20 months, $P < 0.001$) compared to those with low CD163 expression (46.00 ± 3.05 months, $P < 0.001$) (Table 6; Figure 2A). The same is true for the expression of TGF- β 1 in the groups exhibiting differential expression of CD163 ($P < 0.001$) (Table 6; Figure 2B and C). Instead, the low expression of E-cadherin has a statistically significant correlation with

poor overall survival ($P < 0.001$) (Figure 2D and E) and with poor prognosis ($P < 0.001$) in CD163 differential expression groups. Multivariate Cox proportional hazard analysis of overall survival reveals that low expression of E-cadherin and high expression of CD163 are independent prognostic factors in patients with gastric cancer ($P < 0.05$) (Table 7).

Discussion

Being the second leading cause of cancer-related deaths, gastric cancer is still one of the major public health problems worldwide.^{1,48} Because tumorigenesis of gastric cancer has a close relationship with inflammation and changing of epithelium, it is necessary to figure out whether there is

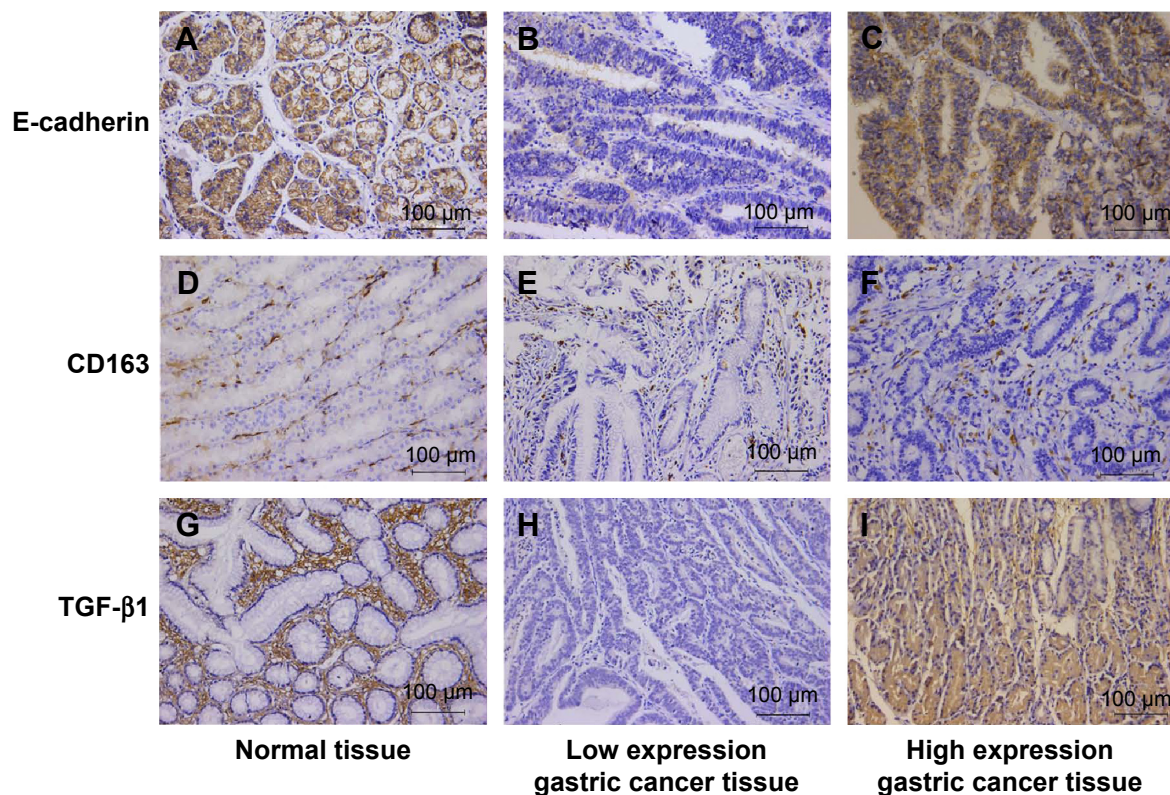


Figure 1 Immunohistochemical results of E-cadherin, TGF- β 1, and CD163 in the gastric cancer and paracancer tissue.

Notes: (A) Typical high expression of E-cadherin in paracancer tissue of gastric cancer. Staining was localized predominantly in the cytomembrane. (B) Typical low expression of E-cadherin in gastric cancer tissue. (C) Infrequent high expression of E-cadherin in gastric cancer tissue. (D) Low expression of CD163 in normal tissue. Staining was localized predominantly in the cytosol. (E) Low expression of CD163 in gastric cancer tissue. (F) High expression of CD163 in gastric cancer tissue. (G) Low expression of TGF- β 1 in paracancer tissue. Staining was localized predominantly in the cytosol. (H) Low expression of TGF- β 1 in gastric cancer. (I) High expression of TGF- β 1 in gastric cancer. Magnification, 200 \times .

Abbreviation: TGF- β 1, transforming growth factor- β 1.

a correlation between these two aspects in gastric cancer. Recent studies showed that both EMT and TAMs contribute to progress of gastric cancer, which is related to epithelial change and inflammation, respectively.^{20,49} However, the relevance between EMT and TAMs in gastric cancer still needs more attention. This study was designed to explore the relationship between EMT and TAMs in gastric cancer.

The present study showed a high expression of TGF- β 1 (62.36%) and CD163 (52.8%), but a low expression of

E-cadherin (33.71%) in gastric cancer tissues. We demonstrated a significant relationship between TAMs and EMT based on the expression of typical indicators CD163 and E-cadherin. Furthermore, both high expression of CD163 and low expression of E-cadherin were correlated to high expression of TGF- β 1 in gastric cancer. High expression of TGF- β 1 and CD163 was related to tumor size, differentiation, Borrmann type, TNM stage, nodal involvement, and distant metastasis of gastric cancer. Significant association of low expression of E-cadherin was also seen with tumor size, differentiation, Borrmann type, TNM stage, nodal involvement, distant metastasis, and

Table 3 Association between CD163 infiltration with expression of E-cadherin and TGF- β 1 in gastric cancer tissues

Group	CD163		<i>r</i>	<i>P</i> -value
	High	Low		
E-cadherin			-0.421	<0.001
High	14	46		
Low	80	38		
TGF- β 1			0.427	<0.001
High	77	34		
Low	17	50		

Abbreviation: TGF- β 1, transforming growth factor- β 1.

Table 4 Expression correlation of TGF- β 1 with E-cadherin in gastric cancer tissues

Group	TGF- β 1		<i>r</i>	<i>P</i> -value
	High	Low		
E-cadherin			-0.452	<0.001
High	19	41		
Low	92	26		

Abbreviation: TGF- β 1, transforming growth factor- β 1.

Table 5 Association between expression of E-cadherin and TGF- β 1 of differential CD136 infiltration in gastric cancer tissues

Group	TGF- β 1	CD136 high		<i>r</i>	<i>P</i> -value	CD136 low		<i>r</i>	<i>P</i> -value
		High	Low			High	Low		
E-cadherin				-0.385	<0.001			-0.440	<0.001
	High	12	13			8	28		
	Low	59	10			32	16		

Abbreviation: TGF- β 1, transforming growth factor- β 1.

Table 6 Univariate analysis of overall survival

Variable	N	Overall survival		
		Median \pm SE	95% CI	<i>P</i> -value
Sex				0.973
Male	125	34.00 \pm 2.97	28.16–39.83	0.223
Female	53	38.00 \pm 3.63	30.86–45.13	
Age (years)				0.019
≤ 60	94	40.00 \pm 3.87	32.40–47.59	
> 60	84	32.00 \pm 1.13	29.77–34.22	0.502
Size (cm)				
< 3	44	46.00 \pm 5.20	35.79–56.20	0.107
3–5	54	34.00 \pm 1.83	30.40–37.51	
> 5	80	32.00 \pm 0.98	30.06–33.93	0.005
Borrmann				
I	22	39.00 \pm 7.62	24.06–53.93	<0.001
II	55	40.00 \pm 3.69	32.76–47.23	
III	62	34.00 \pm 3.50	27.14–40.85	<0.001
IV	39	31.00 \pm 1.77	27.51–34.48	
Differentiation				<0.001
High	12	40.00 \pm 6.06	28.11–51.88	
Moderate	57	39.00 \pm 6.11	26.97–51.02	<0.001
Poor	109	32.00 \pm 1.74	28.59–35.41	
Depth of invasion				<0.001
T1	16	46.00 \pm 8.66	29.01–62.98	
T2	18	52.00 \pm 15.91	20.81–83.18	<0.001
T3	67	35.00 \pm 4.60	25.98–44.01	
T4	77	31.00 \pm 1.45	28.14–33.86	<0.001
Lymph node metastasis				
N0	49	55.00 \pm 9.79	35.79–74.20	<0.001
N1	36	43.00 \pm 7.80	27.22–58.78	
N2	35	36.00 \pm 3.54	29.04–42.95	<0.001
N3	58	29.00 \pm 2.53	24.03–33.96	
Distant metastasis				<0.001
M0	138	40.00 \pm 3.91	32.32–47.67	
M1	40	29.00 \pm 1.57	25.91–32.08	<0.001
TNM stage				
I	21	49.00 \pm 3.21	42.69–66.71	<0.001
II	34	45.00 \pm 5.83	33.57–56.42	
III	83	34.00 \pm 2.27	29.53–38.41	<0.001
IV	40	29.00 \pm 1.57	25.91–32.08	
E-cadherin				<0.001
High	60	54.00 \pm 2.08	48.52–59.48	
Low	118	29.00 \pm 1.45	26.15–31.85	<0.001
TGF- β 1				
High	111	31.00 \pm 1.31	28.41–33.58	<0.001
Low	67	50.00 \pm 5.26	39.68–60.31	
CD136				<0.001
High	94	28.00 \pm 2.20	23.68–32.31	
Low	84	46.00 \pm 3.05	40.01–51.98	

Abbreviations: SE, standard error; CI, confidence interval; TNM, tumor node metastasis; TGF- β 1, transforming growth factor- β 1.

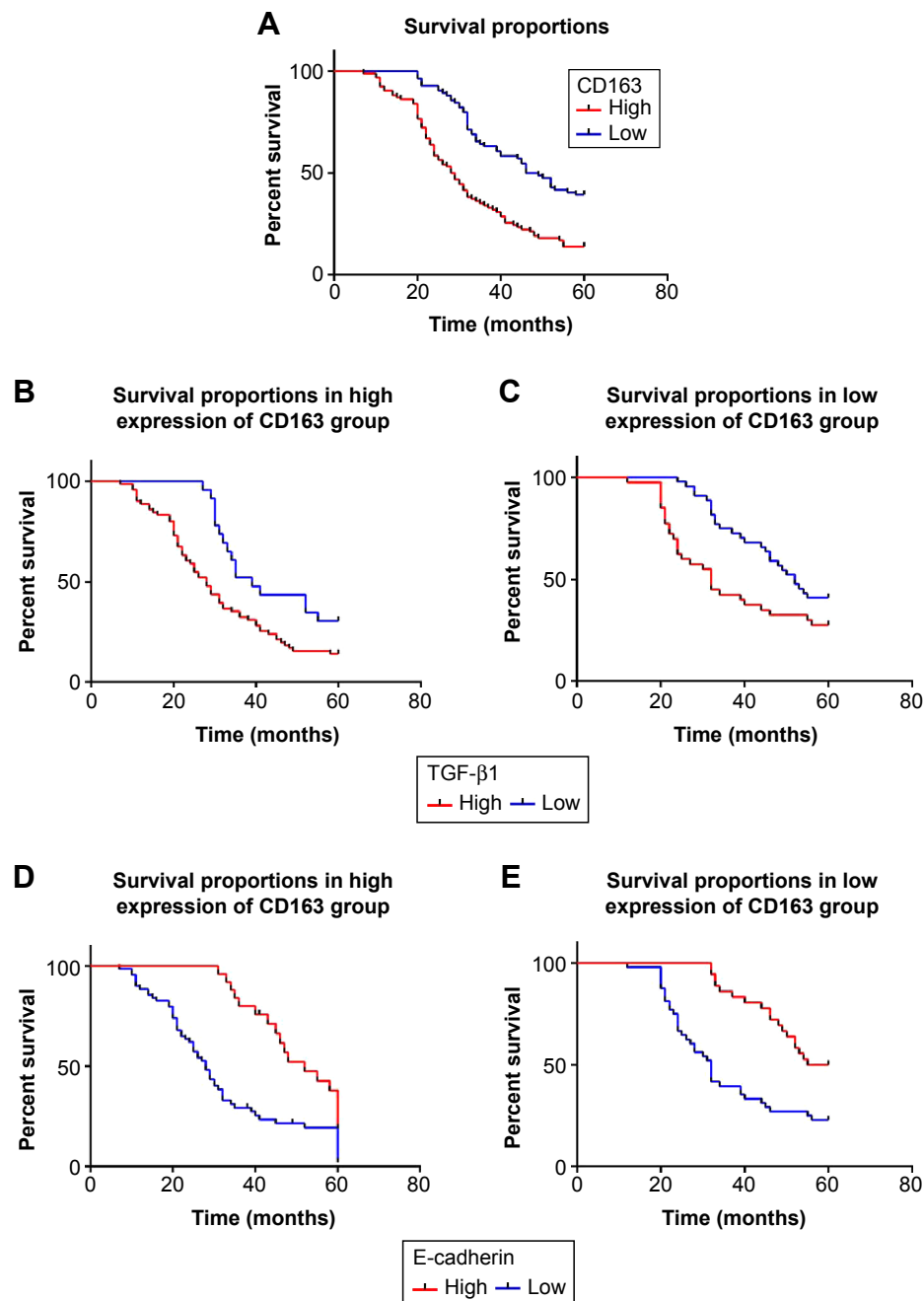


Figure 2 Kaplan–Meier curves for differential expression of E-cadherin, TGF-β1, and CD163 in gastric cancer.

Notes: Differential expression of three indicators showed a significant difference in cumulative overall survival. (A) Patients with high expression of CD163 showed a poor overall survival ($P < 0.001$). (B, C) Patients with high expression of TGF-β1 had a poor overall survival in high/low expression of CD163 group ($P < 0.001$). (D, E) Patients with low expression of E-cadherin had a poor overall survival in high/low expression of CD163 group ($P < 0.001$).

Abbreviation: TGF-β1, transforming growth factor-β1.

Table 7 Multivariate Cox proportional hazard analysis of overall survival

Variable	Overall survival		
	HR	95% CI	P-value
Lymph node metastasis	1.238	1.050–1.460	0.011
E-cadherin	2.559	1.711–3.829	<0.001
TGF-β1	0.651	0.430–0.985	0.042
CD163	0.597	0.406–0.878	0.009

Abbreviations: HR, hazards ratio; CI, confidence interval; TGF-β1, transforming growth factor-β1.

poor prognosis. High expression of both TGF-β1 and CD163 indicates a poor prognosis. Our findings manifest a connection between TAMs and EMT in the tissues of gastric cancer, both of which are associated with TGF-β1.

The mechanism by which EMT and TAMs interact with each other in gastric cancer tissues has not been clear yet. Accumulating evidence shows, in lung cancer and hepatocellular carcinoma, TGF-β1 is a major inducer of EMT, which

can activate other EMT-related signaling pathways.^{50,51} So, TGF- β 1 originated from TAMs and tumor-related microenvironment, and TGF- β signaling pathway may be one of the key ways by which TAMs may cause an effect on the progress of EMT in gastric cancer. It has been reported that TAMs promote progress of EMT through activation of Gas6/Axl-NF- κ B17 in oral cancer.⁵² Moreover, a recent study demonstrated that CCL18 secreted by TAMs downregulates miR98 and miR27b to promote EMT in breast cancer.⁵³ More intensive studies are needed to figure out the mechanism by which EMT and TAMs interact with each other.

In general, our study demonstrated a relationship between EMT and TAMs, associated with TGF- β 1. High infiltration of TAMs and expression of TGF- β 1 are related to aggressive features and poor prognosis of gastric cancer, respectively. The expression levels of E-cadherin, TGF- β 1, and CD163 may serve as prognostic factors for gastric cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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