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Original Article

TGFBI protein high expression predicts poor prognosis in colorectal cancer patients

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Abstract: Transforming growth factor-beta-induced (TGFBI) serves as a linker protein and plays a role in the activation of morphogenesis, cell proliferation, adhesion, migration, differentiation and inflammation. High expression levels of the human TGFBI gene are correlated with numerous human malignancies. In order to explore the roles of TGFBI in the tumor progression of colorectal cancer, colorectal cancer specimens from 115 patients with strict follow-up were selected for the analysis of TGFBI by immunohistochemistry. The correlations between TGFBI expression and the clinicopathological features of colorectal cancers were evaluated. In the colorectal cancer tissues, TGFBI was mainly localized in the cytoplasm and stroma and scarcely in the nucleus. TGFBI expression in the cytoplasm and stroma was not found to be associated with age, gender, tumor histopathological grading, PT category and tumor location ($P > 0.05$ for each). However, high TGFBI expression in the cytoplasm and stroma correlated with lymph node metastasis, distant metastasis and Dukes stage ($P < 0.05$ for each). The survival rate was significantly lower in patients with high TGFBI expression than in those with low TGFBI expression. Furthermore, we found that tumor node metastasis (TNM) staging (HR: 2.963; 95% CI: 1.573-1.664; $P = 0.000$), differentiation (HR: 1.574; 95% CI: 1.001-2.476; $P = 0.049$) and high TGFBI cytoplasmic expression (HR: 3.332; 95% CI: 1.410-7.873; $P = 0.000$) proved to be independent prognostic factors for survival in colorectal cancer. In conclusion, TGFBI plays an important role in the progression of colorectal cancers and it is an independent poor prognostic factor for colorectal cancer patients.

Keywords: TGFBI, colorectal cancer, immunohistochemistry, prognosis

Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer deaths worldwide [1]. Despite surgical resection and advances in radiotherapy, immunotherapy and chemotherapy, CRC still shows significant morbidity and mortality and constitutes a major burden on global health [2]. This disease seems similar when evaluating clinicopathological parameters. However, the patient response to treatment is diverse because CRC is a biologically heterogeneous disease and arises because of the accumulation of multiple genetic alterations involving critical genes that control cell proliferation and survival [3, 4]. Patients with the same stage of CRC might have different molecular drivers and different prognosis. Therefore, a better understanding of the oncogenic activities and molecular markers underlying CRC as well as the identification of new therapeutic targets for the treatment of this disease, is urgently needed.

By far, at least four different pathways have been identified as being responsible for colorectal cancer progression: the WNT/Wingless, K-ras, Transforming growth factor (TGF) and P53 pathways. The transforming growth factor-β (TGF-β) growth inhibitory pathway is altered either by mutations in the signal transduction molecules SMAD2 and SMAD4 in LOH (for loss of heterozygosity)-positive tumors or by mutations of type II TGF-β receptors (TGFβ RII) in MSI + (microsatellite instability) tumors [5]. Researchers believe that TGF-β 1 switches from an inhibitor of tumor cell growth to a stimulator

of growth and invasion, a high level of TGF-β 1 correlates with tumor progression in the process of CRC tumorigenesis, leading to high serum or plasma levels, which are associated with poor prognosis [6-11]. TGF-β secrete various types of protein, among them, transforming growth factor-beta-induced (TGFBI) (initially called βig-H3 and keratoepithelin) is a secretory protein induced by TGF-β in fibroblasts, chondrocytes, smooth muscle cells, corneal epithelial cells, and a variety of cell types [12]. TGFBI was first identified in a human lung adenocarcinoma cell line (A549) which had been treated with TGF-β. This gene encodes a highly conserved 683 amino-acid protein (68 kDa) that contains an N-terminal secretory signal peptide, four internal homologous repeats (FAS1 domain), and a C-terminal Arg-Gly-Asp (RGD) motif which can serve as a ligand recognition site for integrins [13]. TGFBI serves as a linker protein which mediates integrin binding to the extracellular matrix (ECM) proteins such as collagen, laminin and fibronectin and plays a role in the activation of morphogenesis, cell proliferation, adhesion, migration, differentiation and inflammation [12, 14-17]. Zhang L, et al previously reported that TGFBI gene was significantly elevated in colorectal cells compared with normal cells and reveal this gene may prove useful as diagnostic or prognostic markers [18]. Later experiment showed that TGFBI is also expressed at high levels in colorectal adenoma as well [19]. However, to date, only limited information is available on the expression status of TGFBI in colorectal cancer and its relationship with clinicopathological features/prognosis. To determine whether TGFBI is important in the tumorigenesis of colorectal cancers and investigate its prognostic value, 115 cases of colorectal cancer were selected for the analysis of TGFBI by immunohistochemical staining. To our knowledge, this is the first complete study to correlate TGFBI levels in colorectal cancers with histological prognostic factors to understand the role of TGFBI regulation in colorectal cancer progression.

Materials and methods

Patients and tissue samples

The present study was conducted with the approval of the Ethical and Scientific Committees of Sir Run Run Shaw Hospital, Zhejiang University (Hangzhou, China). Through the surgery consent form, patients were informed that the resected specimens would be kept by our hospital and might be used for scientific research, and that their privacy would be maintained.

A total of 115 colorectal cancer patients who underwent curative resection and follow up at the Sir Run Run Shaw Hospital between February 2003 and June 2009 were enrolled in the study. All of the patients received no treatment before surgery. The subject population patients consisted of 70 men and 45 women, the patients' age ranged between 29 and 89 years with a mean age of 62 years. Two experienced pathologists reviewed the hematoxylin and eosin-stained slides of the different biopsies according to the World Health Organization (WHO) classification guidelines and chose one appropriate paraffin block. Differentiation status was divided into three types: (1) well-differentiated, including papillary adenocarcinoma and well-differentiated tubular adenocarcinoma; (2) moderately differentiated, including highly to moderately differentiated tubular adenocarcinoma; And (3) poorly differentiated, including poorly differentiated adenocarcinoma, signet-ring cell carcinoma, mucinous adenocarcinoma and undifferentiated carcinoma. Of the 115 cases, 62 were well-differentiated, 26 were moderately differentiated and 27 were poorly differentiated cancers. The tumors were classified into T1, T2, T3, and T4 stages according to the 7th edition of the International Union Against Cancer (UICC) tumor node metastasis (TNM) classification [20]. The 115 cancer patients were followed-up for survival.

Immunohistochemical staining

The ChemMate™ EnVision™ detection kit (Dako, Carpinteria, CA, USA) was used for immunohistochemistry (IHC) according to the manufacturer's instructions. Briefly, histopathological examination of the 10% formaldehyde-fixed, paraffin-embedded and 4-um section-cut specimens was performed by a pathologist at the Pathology Division. After dewaxing and hydration with dimethylbenzene and a gradient concentration of alcohol, the sections were washed with deionized water and phosphate-buffered saline (PBS), and then an antigen retrieval process was performed at high temperature and high pressure with citrate buffer (pH 6.0) before blocking the endogenous per-

oxidase with 0.3% (v/v) H_2O_2 solution. The sections were then incubated with preimmunized goat serum for 60 min to reduce nonspecific reaction. The next step was that the sections were incubated with TGFBI antibody (1:700 dilutions; HPA008612, Sigma-Aldrich Co, St. Louis, MO, USA) overnight at 4°C. After thawing, the sections were rinsed five times with PBS, and then incubated with ChemMate EnVision/HRP, Rabbit/Mouse (ENV) reagent as a secondary antibody. Subsequently, the sections were developed using ChemMate DAB+ chromogen (Dako, Carpinteria, CA, USA) and counterstained with hematoxylin. After dehydration and transparency with a gradient concentration of alcohol and dimethylbenzene, all of the specimens were finally fixed using neutral balsam.

Evaluation of staining

Slides were reviewed under a light microscope by two observers three times, and the reviewers were blinded to the identity of the specimens between evaluations. A positive expression result was indicated by brown-yellow or brown granular deposits at the corresponding antibody expression sites. The positive expression of TGFBI is located in the cytoplasm and stroma. Because of the difference between expression of TGFBI in the cytoplasm and stroma, we used different evaluation of staining. To evaluate TGFBI staining of the cytoplasm, we analyzed the percentages of positive staining in cancer cells. The colorectal carcinoma cases were divided into two groups: low (proportion of stained cells < 10%) and high (proportion of stained cells > 10%) TGFBI expression. The result of immunostaining for TGFBI in the stroma was examined and scored according to the intensity of staining and proportion of stained cells. The percentage of positive cells was evaluated and scored in the following categories: 0, less than 5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; 4, greater than 75%. The intensity of staining cells was evaluated and scored in the following categories: 0: no staining; 1: weak staining; 2: strong staining. The two scores were summed to obtain an immunoreactivity score (IRS) value ranging from 0 to 6. To evaluate the association of TGFBI expression with clinical and pathological parameters, the patients were then grouped into two categories based on IRS values: low-expression (IRS 0-5) and high-expression (IRS 6).

Statistical analysis

Statistical calculations were performed using SSPS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Pearson's Chi-squared tests and Fisher's exact test were used to analyze the association of TGFBI protein (TGFBIp) expression with clinicopathological parameters. Overall survival analyses were performed using the Kaplan-Meier method, and differences between groups were assessed using the log-rank test. Univariate analysis comparisons (gender, age, location, differentiation, lymph node metastasis, distant metastasis, tumor stage and TGFBI expression) and multivariate survival comparisons were performed using Cox proportional hazard regression models. The estimated relative risks of dying were expressed as adjusted hazard ratios (HRs) and corresponding 95% confidence intervals (CIs). A *P* value less than 0.05 was considered statistically significant.

Results

Localization of TGFBI in colorectal cancer

In normal colorectal tissues, the cells showed no or weak staining for TGFBIp. In the colorectal cancer tissues, the positively reactive substance of TGFBIp was mainly localized in the cytoplasm and stroma (both extracellular matrix and fibroblasts), and showed scarcely positive expression in the nucleus, as detected by microscopy. For the comparison of TGFBI expression between colorectal cancer tissue and normal mucosa, the positive rate of TGFBIp expression in the cytoplasm was significantly higher in colorectal cancer tissues (40%, 46/115) than in normal mucosa (0%, 0/9). Similarly, the positive rate of TGFBIp overexpression in the stroma was 53% (61/115) in colorectal cancers, a value that was significantly higher than that in normal colorectal mucosa (0%, 0/9) ($P < 0.05$, respectively).

Relationship between TGFBI expression and clinicopathological parameters in patients with colorectal cancer

Representative immunohistochemical analysis of TGFBI protein in colorectal cancer mucosa was shown in **Figure 1**. To evaluate the relationship between TGFBIp and colorectal cancer progression, we analyzed the correlation

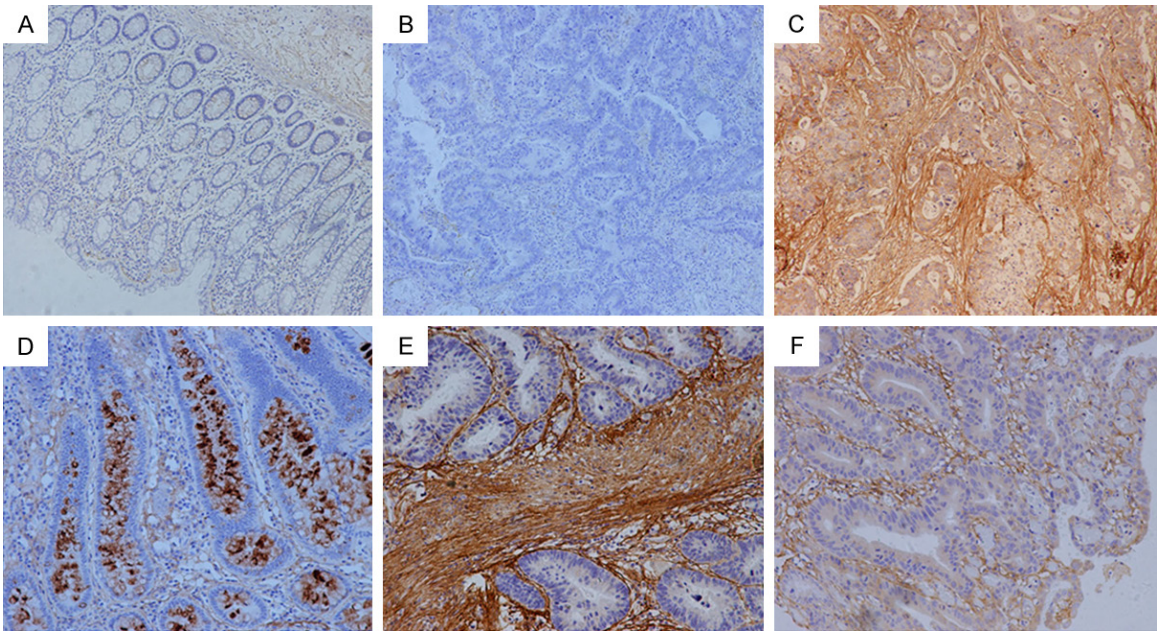

Figure 1. Representative immunohistochemical staining of TGFBI protein in normal colon mucosa and colorectal cancer. A. No or weak TGFBI expression in normal colon mucosa. B. Very weak TGFBI expression in colorectal cancer cells. C. Strong TGFBI expression in colorectal cancer cells and stroma. D. High TGFBI expression in colorectal cancer cells and weak expression in stroma. E, F. Weak TGFBI expression in colorectal cancer cells and high expression in stroma. (Original magnification, A-C × 100; D-F × 200).

between TGFBIp overexpression and clinicopathological features of colorectal cancers (**Table 1**). TGFBI expression in the cytoplasm was not found to be associated with age, gender, tumor location, tumor histopathological grading and PT category ($P > 0.05$). The strongly positive rate of TGFBIp expression was significantly higher in colorectal cancers with lymph node metastasis (51.8%, 29/59) than in cases without metastasis (28.8%, 17/56) ($P = 0.012$). The positive rate of TGFBIp expression was also higher in colorectal cancers with distant metastasis (73.3%, 11/15) than in cases without distant metastasis (35%, 35/100) ($P = 0.009$). TGFBI cytoplasmic expression also tended to correlate with Dukes stage ($P = 0.005$). The strongly positive rate of TGFBIp was significantly higher in colorectal cancers patients with Dukes C and D stage than in cases with Dukes A and B stage.

Regarding the TGFBI expression in the stroma, the overexpression of TGFBIp was not related with gender, age, tumor location, tumor histopathological grading or pT category of patients with colorectal cancer ($P > 0.05$). When patients with lymph node positivity (N1-3) were compared with patients with no lymph node involvement (N0), differences were noted in TGFBIp stromal expression in the primary tumor tissue ($P = 0.002$). The positive rate of TGFBIp stromal expression was also higher in colorectal cancers with distant metastasis (80%, 12/15) than in cases without distant metastasis (49%, 49/100) ($P = 0.025$). Patients with Dukes' C and D stage both showed significantly higher TGFBI stromal expression in tumor tissue compared with those with Dukes' A and B stage ($P = 0.012$).

In general, high-regulation of TGFBI expression was significantly associated with lymph node involvement, distant metastasis and Dukes' C and D stage ($P < 0.05$ for each).

Colorectal cancer patients with TGFBI high expression had poor prognosis

Using univariate analysis, we found that colorectal cancer patients with high TGFBI expression in the cytoplasm and stroma had significantly lower 5-year survival rates than those with low TGFBI expression tumors. Additionally, differentiation, Dukes stage, pT category, pN category, and pM category were also associated with 5-year survival rates when

Table 1. Correlation between clinicopathological background and protein of TGFBI cytoplasmic and stromal expression in 115 cases of colorectal cancer

		TGFBI cytoplasmic expression			TGFBI stromal expression		
	N	Low expression (%)	High expression (%)	*P*-value	Low expression (%)	High expression (%)	*P*-value
Total	115						
Gender							
Male	70	43 (61.4)	27 (38.6)	0.697	29 (41.4)	41 (58.6)	0.138
Female	45	26 (57.8)	19 (42.2)		25 (55.6)	20 (44.4)	
Age							
≥ 60	54	31 (57.4)	23 (42.6)	0.593	29 (53.7)	25 (46.3)	0.173
< 60	61	38 (62.3)	23 (37.7)		25 (41)	36 (59)	
Location							
Rectum	66	43 (65.2)	23 (34.8)	0.191	33 (50)	33 (50)	0.448
Colon	49	26 (53.1)	23 (46.9)		21 (42.9)	28 (57.1)	
Histopathological grading							
Well	62	38 (61.3)	24 (38.7)	0.865	31 (50)	31 (50)	0.720
Moderately	26	16 (61.5)	10 (38.5)		12 (46.2)	14 (53.8)	
Poorly	27	15 (55.6)	12 (44.4)		11 (40.7)	16 (59.3)	
Tumor invasion							
pT1	6	5 (83.3)	1 (16.7)	0.209	2 (33.3)	4 (66.7)	0.251
pT2	23	16 (69.6)	7 (30.4)		15 (65.2)	8 (34.8)	
pT3	84	46 (54.8)	38 (45.2)		36 (42.9)	48 (57.1)	
pT4	2	2 (100)	0 (0)		1 (50%)	1 (50%)	
Lymph nodal status							
pN0	59	42 (71.2)	17 (28.8)	0.012	36 (61)	23 (39)	0.002
pN1/2/3	56	27 (48.2)	29 (51.8)		18 (32.1)	38 (67.9)	
Distant metastasis							
pM0	100	65 (65)	35 (35)	0.009	51 (51)	49 (49)	0.025
pM1	15	4 (26.7)	11 (73.3)		3 (20)	12 (80)	
Dukes stage							
A	19	14 (73.7)	5 (26.3)	0.005	11 (57.9)	8 (42.1)	0.012
B	36	27 (75)	9 (25)		23 (63.9)	13 (36.1)	
C	45	24 (53.3)	21 (46.7)		17 (37.8)	28 (62.2)	
D	15	4 (26.7)	11 (73.3)		3 (20)	12 (80)	

TGFBI was expressed (**Table 2**). These data suggest that TGFBI could also be a valuable prognostic factor in colorectal cancer. Therefore, multivariate analysis was performed using the Cox proportional hazards model for all of the significant variables examined in the univariate analysis. We found that TNM category (HR: 2.963; 95% CI: 1.573-1.664; $P = 0.000$) and differentiation (HR: 1.574; 95% CI: 1.001-2.476; $P = 0.049$) proved to be independent prognostic factors for survival in colorectal cancer. Importantly, TGFBI cytoplasmic overexpression emerged as a significant independent prognostic factor in colorectal cancer (HR: 3.332; 95% CI: 1.410-7.873; $P = 0.000$) (**Table 3**).

To further substantiate the importance of high TGFBI expression in colorectal cancer progression, the prognosis between the patients with high TGFBI expression and low TGFBI expression was compared (**Figure 2**). The 5-year overall survival rates in patients with high expression and low TGFBI expression were 32.8% and 85.4%, respectively, in the cytoplasm and 55.7% and 75.3%, respectively, in the stroma. Colorectal cancer patients with high TGFBI expression in the cytoplasm had lower 5-year

Table 2. Univariate survival analyses (Cox regression model) of various factors in patients with colorectal cancer

	B	SE	Wald	HR	95% CI		*P*-value
					Lower	upper	
Gender	-.691	.440	2.464	.501	.211	1.187	.116
Age	-.095	.386	.061	.909	.427	1.936	.805
Location	-.261	.399	.429	0.770	.352	1.683	.512
Dukes stage	1.286	.272	22.216	3.610	2.120	6.177	0.000*
Differentiation	.700	.227	9.519	2.014	1.291	3.143	0.002*
PT categories	1.138	.450	6.395	3.120	1.292	7.538	0.011*
PN categories	1.657	.496	11.169	5.242	1.984	13.852	0.001*
PM categories	2.037	.397	26.376	7.668	3.524	16.684	0.000*
TGFBI cytoplasmic expression	1.591	.425	14.018	4.907	2.134	11.283	0.000*
TGFBI stromal expression	.822	.410	4.024	2.276	1.019	5.083	0.045*

B, Coefficient; SE, standard error; Wald, statistic; HR, hazard ratio; CI, confidence interval. *Significant different.

Table 3. Multivariant survival analyses (Cox regression model) of various factors in patients with colorectal cancer

	B	SE	Wald	HR	95% CI		*P*-value
					Lower	upper	
TNM categories	1.086	.294	13.607	2.963	1.573	1.664	0.000*
Differentiation	0.454	.231	3.860	1.574	1.001	2.476	0.049*
TGFBI cytoplasmic expression	1.203	.439	7.524	3.332	1.410	7.873	0.000*

B, Coefficient; SE, standard error; Wald, statistic; HR, hazard ratio; CI, confidence interval. *Significant different.

survival rates than those with low TGFBI expression in the cytoplasm (**Figure 2A**; P = 0.000). Additionally, TGFBI expression in the stroma showed a similar trend (P = 0.039, **Figure 2B**).

Furthermore, the 5-year overall survival rates in patients with high TGFBI expression (in both the cytoplasm and stroma) and low expression (cytoplasmic expression or stromal expression is low or both of them are low) were 25.0% and 79.6%, respectively. Colorectal cancer patients with high TGFBI expression in both the cytoplasm and stroma had lower 5-year survival rates than those with low TGFBI expression (P = 0.000, **Figure 2C**).

Discussion

TGFBI is a multifunctional polypeptide that widely exists in normal or cancerous tissue, there is convincing data in the literature reporting that TGFBI is overexpressed in human renal, pancreatic and colorectal cancers [21-24]. TGFBI gene was significantly elevated in colorectal cells compared with normal cells and reveal this genes may prove useful as diagnostic or prognostic markers [18]. Therefore, we did this immunohistochemistry study to determine whether the over expression of TGFBIp might serve as a biomarker for the prognostic evaluation of colorectal cancers.

In our research, TGFBI expression was not found to be associated with age, gender, tumor histopathological grading, pT category and tumor location. However, TGFBI expression tended to correlate with lymph node metastasis, distant metastasis and Dukes stage. TGFBI was upregulated in colorectal cancer patients with Dukes' C and D stage, or with lymph node metastasis or distant metastasis, indicating that tumor cells with TGFBI overexpression proliferated and attack more rapidly. Ma *et al.* also reported that TGFBI overexpression was more frequent in high-grade (Stages III and IV) tumors than in low-grade (Stages I and II) tumors, whereas no TGFBI expression was detected in the normal epithelial tissues by immunohistochemical staining of tissue microarray, in addition, ectopic expression of the TGFBIp induced the dissociation of VE-cadherin junctions between endothelial cells via activation of the

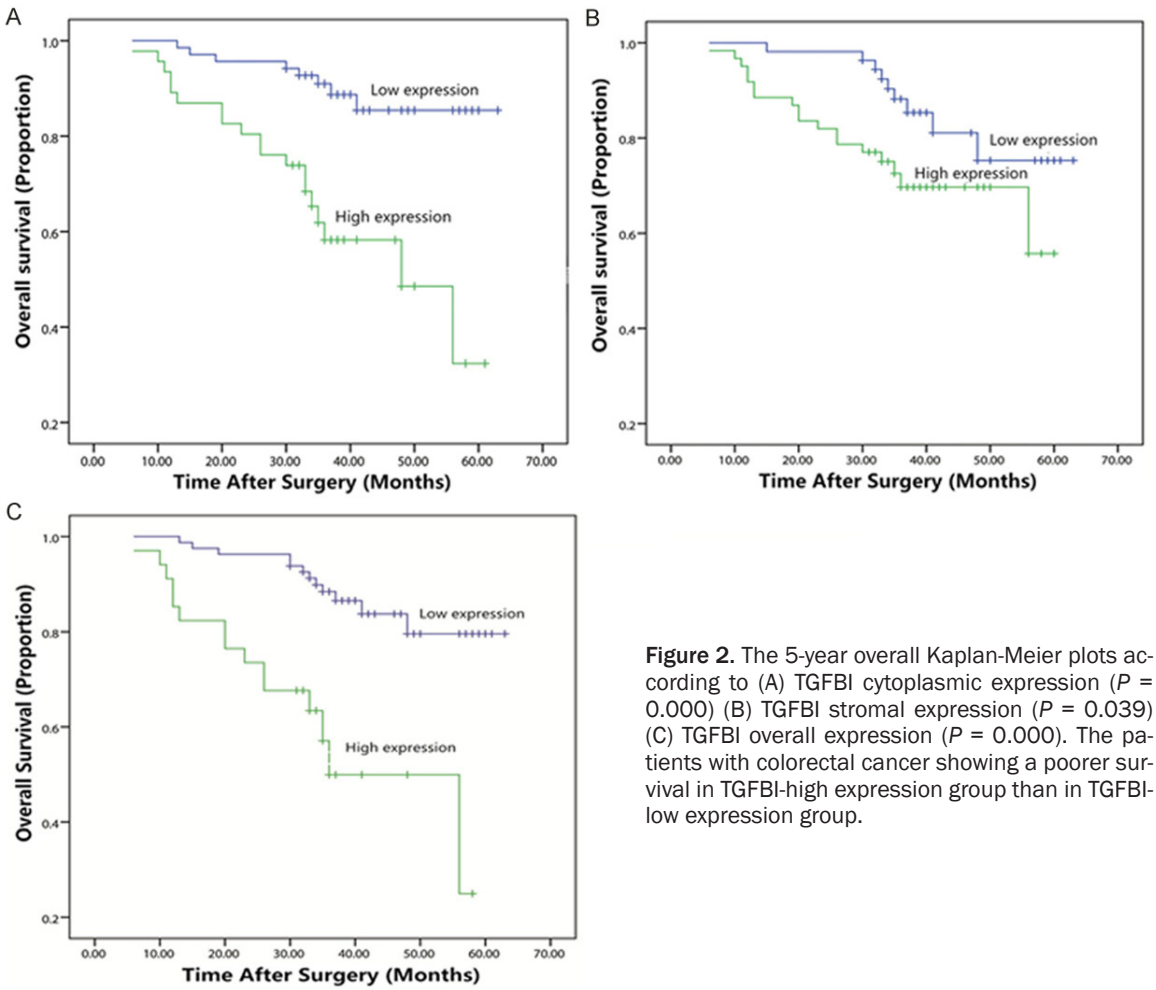

Figure 2. The 5-year overall Kaplan-Meier plots according to (A) TGFBI cytoplasmic expression (P = 0.000) (B) TGFBI stromal expression (P = 0.039) (C) TGFBI overall expression (P = 0.000). The patients with colorectal cancer showing a poorer survival in TGFBI-high expression group than in TGFBI-low expression group.

integrin $\alpha_v\beta_5$-Src signaling pathway, promoted extravasation (a critical step in the metastatic dissemination of cancer cells), enhanced the aggressiveness and altered the metastatic properties of colon cancer cells [21]. Cell migration plays important roles in cancer metastasis and angiogenesis, tumor-produced extracellular matrix (ECM) proteins can be key elements in tumor growth and metastasis. TGFBIp is a secreted ECM component that found to be a signature of high metastatic potential in melanoma recently [25]. Research reported that TGFBI promoted adhesion, migration and invasion of human renal cell carcinoma depends on inactivation of von hippel-Lindau tumor suppressor and TGFBI could be a therapeutic target against renal cell carcinoma in the future [26]. Small interfering RNAs (siRNAs) targeted against TGFBI transfected into human hepatocellular carcinoma cells also showed that TGFBI increases the invasive potential of those cells by regulating MMP-2 and -9 secretions [27]. Other research showed that Oncostatin M promotes stromal stem cell-stimulated tumor growth through a paracrine mechanism involving periostin and TGFBI [28]. Thus it can be seen that TGFBI may also be involved in the metastatic process of colorectal cancer.

Furthermore, the survival rates of the patients with high TGFBI expression were significantly lower than those of the patients with low TGFBI expression. These data uncovered that TGFBI is frequently upregulated in colorectal cancers when compared with the normal tissues counterparts, TGFBI may be an independent predictor for poor prognosis in patients with colorectal cancer, especially those with advanced stages. Furthermore, our results showed that TGFBI cytoplasmic overexpression emerged as a significant independent prognostic factor in colorectal cancer. Turtoi A, et al used matrix assisted laser desorption ionization (MALDI) image-guided proteomics and explored the het-

erogeneity of extracellular and membrane subproteome in a unique collection of eight fresh human colorectal carcinoma liver metastases, they found that novel antigens TGFBI, whose expression is a consistent feature of CRC liver metastasis, this particular hallmark can be used as part of the strategy for developing rational therapies based on multiple sets of targetable antigens [24]. These data and our results both indicate that TGFBI might be a promising therapeutic target.

We also found that TGFBI was stained not only in the cytoplasm but also in the stroma, especially the stromal fibroblasts. Our immunohistochemical study showed that TGFBI cytoplasmic expression was positively correlated with TGFBI stromal expression in patients with colorectal cancer, and tumor tissue in the cytoplasm and stroma both provide an inflammatory microenvironment with TGFBI overexpression. High TGFBIp expression was shown to be mediated by an autocrine TGF-β 1-dependent signaling pathway in several human cells [29-31]. Experiments performed in mice (knocked out of the TGF-beta type II receptor gene in mouse mammary fibroblasts) revealed that mouse fibroblasts have up-regulated expression of growth factors and increased proliferation of mammary cancer cells [32]. Therefore, we can speculate that TGFBIp and TGF-β responses mediated by stromal fibroblasts can regulate carcinoma initiation and progression of adjacent epithelium, these studies characterize a significant role for stromal TGF-beta signaling and expression of TGFBIp.

In conclusion, in colorectal cancer, TGFBI expression tended to correlate with lymph node metastasis, distant metastasis, and the Dukes stage. Furthermore, the survival rates in the patients with high-TGFBI-expression tumors were significantly lower than those in the patients with low -TGFBI-expression tumors. These results indicate that TGFBI expression could be a prognostic factor for advanced colorectal cancer with lymph node metastasis and distant metastasis. Further studies are warranted to more firmly establish this supposition.

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Disclosure of conflict of interest

None.

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