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Original Article

Overexpression of FAM3C protein as a novel biomarker for epithelial-mesenchymal transition and poor outcome in gastric cancer

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Abstract: Objective: In recent years, overexpression of FAM3C protein has been proved to contribute to epithelial to mesenchymal transition (EMT) and correlate with poor prognosis in several malignant tumors. However, the role of FAM3C in gastric cancer (GC) is still not clear. Thus, we detected the expression of FAM3C by immunohistochemistry (IHC) and determined the association of FAM3C expression with EMT, clinicopathologic characteristics, and prognosis in GC. Methods: We detected the expression of FAM3C, PDGFR-β, E-cadherin, and vimentin in 150 patients with GC by tissue chip technology and IHC methods. All statistical analyses were conducted using SPSS 22.0 software. Results: FAM3C expression in gastric carcinoma tissues was significantly higher than in matched adjacent normal tissues (P = 0.037). The expression of FAM3C positively correlated with vimentin expression and negatively correlated with E-cadherin expression (P = 0.045 and 0.029, respectively). However, there was no correlation between expression of FAM3C and PDGFR-β (P = 0.095). FAM3C overexpression was significantly associated with depth of invasion, lymph node metastasis and TNM stage (P = 0.004, 0.016 and 0.022, respectively). Multivariate analysis revealed that high expression of FAM3C is an independent prognostic factor for poor prognosis in GC patients (P = 0.007). Conclusions: Overexpression of FAM3C is a potential marker for EMT and predicts poor outcome in gastric cancer.

Keywords: FAM3C, gastric cancer, prognosis, epithelial-mesenchymal transition

Introduction

Although the incidence of gastric cancer/(GC) has been decreasing, it still remains the fifth most common cancer and the third leading cause of cancer-related death in the world [1]. In GC, metastasis is the main cause of cancer-related death [2]. Tumor metastasis is a multistep process in which malignant tumor cells move from the primary site to the other parts throughlymphatic, vascular and other pathways. In this metastatic process, epithelial-to-mesenchymal transition (EMT) has been proven to play an important role [3, 4]. Epithelial-mesenchymal transition (EMT), a developmental process in which epithelial cells acquire a mesenchymal cell phenotype, may promote tumor development, invasion or metastasis. In EMT, cells gradually lose the epithelial characteristics of intercellular adhesion, thereby acquiring migratory fibroblastoid properties, and becoming resistant to apoptosis [5]. EMT is characterized by downregulation of epithelial markers such as E-cadherin and upregulation of mesenchymal markers such as vimentin [6, 7]. Accumulating evidence suggests that loss of E-cadherin expression and positive expression of vimentin play important roles in cancer invasion and metastasis, and EMT may predict a relatively poor prognosis [8-10]. EMT may be induced by secreted factors of diverse cells which a re recruited into the tumor microenvironment by tumor cells [11].

FAM3C, also called interleukine-like EMT inducer (ILEI), is one of the important secreted factors which can induce EMT [12]. FAM3C belongs to the FAM3 cytokine family. All four members of the family (FAM3A, B, C and D) can encode a protein with a hydrophobic leader sequence,

Table 1. Correlation between FAM3C, PDGFR, E-cadherin, and vimentin expression and clinicopathological characteristics of patients with gastric carcinoma

Variables	n	FAM3C		*P* value	PDGFR		*P* value	E-cadherin		*P* value	Vimentin		*P* value
		IS High (>6)	IS Low (≤6)		IS High (>6)	IS Low (≤6)		IS High (>4)	IS Low (≤4)		Positive	Negative	
Age				0.943			0.507			0.062			0.079
<60	74	52	22		41	33		24	50		18	56	
≥60	76	53	23		38	38		36	40		10	66	
Gender				0.741			0.309			0.537			0.254
Male	93	66	27		52	41		39	54		20	73	
Female	57	39	18		27	30		21	36		8	49	
Tumor size				0.093			0.393			0.385			
<5 cm	71	45	26		40	31		31	40		16	55	0.279
≥5 cm	79	60	19		39	40		29	50		12	67	
Histological grade				0.969			0.536			0.001			0.088
Well and moderate	47	33	14		23	24		28	19		5	42	
Poor and others	103	72	31		56	47		32	71		23	80	
Tumor location				0.406			0.607			0.368			0.7
Upper	24	17	7		13	11		12	12		4	20	
Middle	51	39	12		24	27		17	34		8	43	
Lower	75	49	26		42	33		31	44		16	59	
Depth of invasion				0.004			0.002			0.029			0.12
T1+T2	45	24	21		15	30		24	21		5	40	
T3+T4	105	81	24		64	41		36	69		23	82	
Lymph node metastasis				0.016			0.004			0.047			0.038
Absent	58	34	24		22	36		29	29		6	52	
Present	92	71	21		57	35		31	61		22	70	
TNM stage				0.022			<0.0001			0.029			0.032
I-II	59	35	24		18	41		30	29		6	53	
III-IV	91	70	21		61	30		30	61		22	69	

IS, immunoreactivity score; T1, tumor invades lamina propria, muscularis mucosae, or submucosa; T2, tumor invasion of the muscularis propria; T3, tumor invasion subserosal connective tissue; T4, tumor invasion serosal or adjacent structures.

which contains 224-235 amino acids [13]. FAM3C gene is located on chromosome 7 (7q31), which was initially considered as a candidate gene for autosomal recessive non-syndromic hearing loss [14]. Moreover, FAM3C is also considered to be associated with the occurrence and development of tumor [15]. And high levels of FAM3C have been found in human esophagus, breast, colon, prostate, lung, liver, head and neck tumors [16]. Recently, Zhu et al. [17] showed that FAM3C expression is upregulated in esophageal squamous cell carcinoma and is associated with aggressive tumor behavior, metastasis and poor clinical outcome. Moreover, Gao et al. [18] demonstrated that overexpression of FAM3C is significantly associated with tumor metastasis and poor prognosis in colorectal cancer. However, the expression and clinical significance of FAM3C in GC patients remains not yet well-documented. Moreover, several works have shown that FAM3C was a key regulator of EMT and contributed to metastatic progression in both human and animal models [16, 19]. Moreover, downregulation of FAM3C could reduce the induction of EMT [19, 20]. Furthermore, Lahsnig et al. [21] revealed that FAM3C could stimulate the upregulation of PDGFR-β and govern hepatocellular EMT through mechanisms involving PDGFR-β signaling in liver carcinoma. Guo et al. [22] have revealed that PDGFR-β was found to be highly expressed in gastric cancer and PDGFR-β signaling might induce EMT to promote metastasis of gastric carcinoma. However, whether FAM3C expression has a connection with PDGFR-β and EMT in gastric cancer remains unclear.

In the present study, immunohistochemistry (IHC) was used to evaluate the expression of FAM3C, PDGFR-β, E-cadherin and vimentin. We used downregulation of E-cadherin and upregulation of vimentin to evaluate the occurrence of EMT. Ultimately, we aim to investigate the cor-

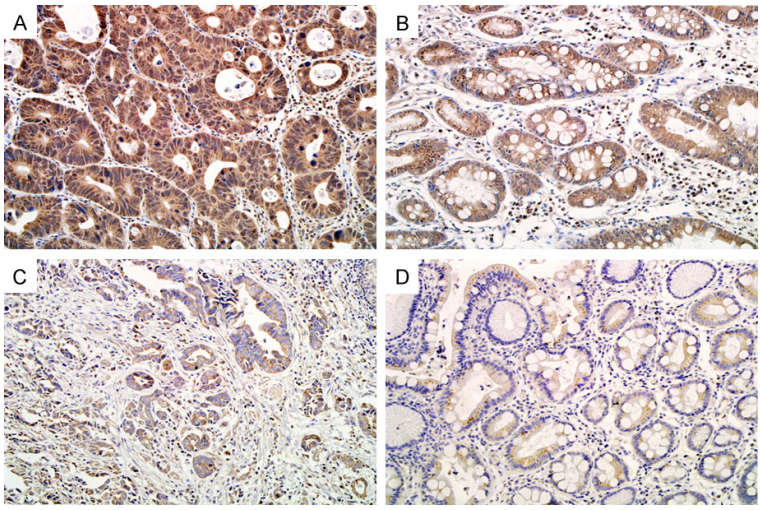

Figure 1. IHC staining of FAM3C in GC and corresponding paracancerous tissue. The staining of FAM3C protein (brown) was mainly located in the cytoplasm of GC tumor cells (×200): (A) High FAM3Cexpression in GC, (B) High FAM3C expression in non-neoplastic tissue, (C) Low FAM3C expression in GC, and (D) Low FAM3C expression in non-neoplastic tissue. GC, gastric cancer; IHC, immunohistochemical.

Table 2. Expression of FAM3C in gastric cancer and normal gastric mucosa

	FAM3C expression		*P* value
	High (%)	Low (%)	
Gastric cancerous tissue (n = 150)	105 (70%)	45 (30%)	0.037
Paracancerous tissue (n = 40)	21 (52.5%)	19 (47.5%)	

relation of FAM3C expression with PDGFR-β, EMT, clinicopathological characteristics, and prognosis in a number of GC cases.

Materials and methods

Patients and tissue samples

GC tissues and matched adjacent normal tissues were obtained from 150 GC patients who underwent surgical resection at Zhongnan Hospital of Wuhan University from 2010 to 2012. No patient had received preoperative chemotherapy or radiotherapy. The clinicopathological information of all the GC patients, including age, gender, tumor size, tumor location, histological grade, depth of invasion, lymph node metastasis status, and pathological TNM stage (pTNM stage), was collected from the Department of Oncology, Zhongnan Hospital of Wuhan University (**Table 1**). Pathological diagnoses were performed independently by two experienced pathologists. TNM staging of GC was conducted based on American Joint Committee on Cancer [23]. The study was approved by the ethics committee of Zhongnan Hospital of Wuhan University. Written informed consent for the use of tumor tissues was obtained from all the GC patients before the surgical resections. All the patients were followed up by telephone enquiry or mail communications. The final follow-up was completed in December 2017. The follow-up time ranged from 3 to 96 months (mean 57 months).

Preparation of tissue chips

150 cases of GC paraffin tissue blocks and 40 cases of matched adjacent normal paraffin tissue blocks were sent to Shanghai Outdo Biotech Company and made into tissue microarray. Through HE stained tumor sections, the donor wax blocks were selected and marked. Two different sampling sites were selected for each sample, and the donor tissue cores were placed in the receptor wax block through punching needle. Then, a dot matrix was arranged on the receptor wax block in the order of prior design, and then they were routinely sliced and stained with HE, which was confirmed by the senior pathologist.

Antibodies and reagents

The primary antibodies used were rabbit polyclonal anti-human FAM3C (14247-1-AP, Proteintech, Wuhan, China), rabbit polyclonal anti-human PDGFR-β (3169T, Cell Signaling Technology, Danvers, USA), rabbit polyclonal anti-human E-cadherin (20874-1-AP, Proteintech, Wuhan, China) and rabbit polyclonal anti-human vimentin (10366-1-AP, Proteintech, Wuhan, China).

Immunohistochemistry

We used immunohistochemistry (IHC) to analyze the expression of FAM3C, PDGFR-β, E-ca-

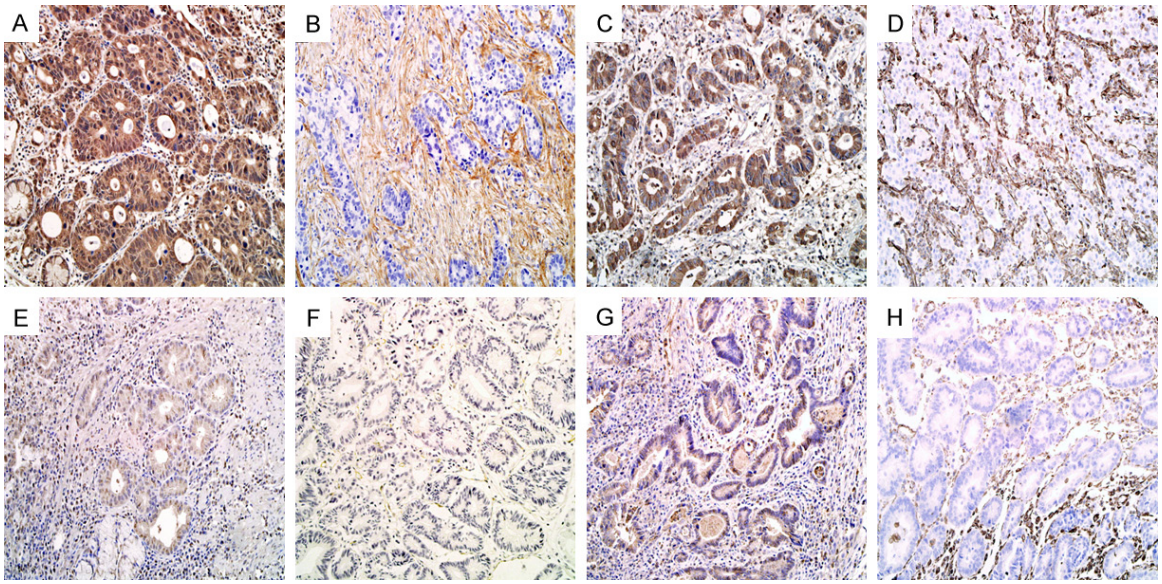

Figure 2. Immunohistochemical staining for FAM3C, PDGFR-β, E-cadherin and vimentin in gastric cancer(×200): (A) High FAM3C expression, (B) High PDGFR-β expression, (C) High E-cadherin expression and (D) Positive vimentin expression; (E) Low FAM3C expression, (F) Low PDGFR-β expression, (G) Low E-cadherin expression and (H) Negative vimentin expression.

dherin and vimentin in human GC. Briefly, the sections were dewaxed in xylene and then dehydrated in ascending concentrations of ethanol. Next, the slices were heated for 15 min to repair the antigen by using electric pottery furnace and then cooled down to room temperature. Subsequently, the sections were rinsed with phosphate-buffered saline (PBS) (3×3 min) and then blocked with 3% peroxide for 15 min at room temperature for endogenous peroxidase ablation. After being blocked with normal goat serum for 30 min at room temperature, sections were incubated overnight at 4°C with primary antibodies at the following dilutions: anti-FAM3C, 1:50; anti-PDGFR-β, 1:100; anti-E-cadherin, 1:100; and anti-vimentin, 1:100. After being washed with PBS (3×3 min), sections were incubated with horseradish peroxidase-labeled secondary antibodies (goat anti-rabbit for FAM3C, PDGFR-β, E-cadherin and vimentin) for 20 min at 37°C. Then, the sections were immersed in 3,3-diaminobenzidin (DAB) for 10 min at room temperature without light. Finally, all the sections were counterstained with hematoxylin and mounted. PBS was used instead of the primary antibody as a negative control.

Evaluation of immunostaining

All the sections were evaluated repetitively and independently by two experienced pathologists who were blind to both clinical and pathological data. All of the disagreements were resolved by the third investigator. According to those established methods, both of the stain intensity and proportion of the positive cells were evaluated. The staining intensity was scored on a 0-3 scale: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. The percentage of positive cells was scored on a 0-4 scale: 0, ≤5% positive tumor cells; 1, 6-25% positive tumor cells; 2, 26-50% positive tumor cells; 3, 51-75% positive tumor cells; 4, 76-100% positive tumor cells. We assessed the percentage of positive cells through calculating the percentage of positive tumor cells among the entire carcinoma-involved area. All the slides were examined by light microscope and five random fields (400× magnification) were selected. The immunoreactivity score (IS; 0-12) was generated by multiplying the intensity and reactivity rates [24, 25]. For FAM3C and PDGFR-β, an IS score of 06 was considered low expression, and scores above 6 were high expression [18, 26]. For E-cadherin, an overall staining score of 4 or less was considered as down-regulated, and scores above 4 were high [18]. However, expression of vimentin was considered positive when more than 10% of tumor cells showed positive vimentin staining and negative when 10% or less of tumor cells showed positive vimentin staining according to previously published methods [27, 28].

Table 3. Correlation between FAM3C, PDGFR, E-cadherin, and vimentin expression in gastric cancer

Variable	FAM3C expression		r	P
	High	Low		
PDGFR expression			0.137	0.095
High	60	19		
Low	45	26		
E-cadherin expression			-0.178	0.029
High	36	24		
Low	69	21		
Vimentin expression			0.164	0.045
Positive	24	4		
Negative	81	41		

Statistical analysis

All statistical analyses were conducted using SPSS 22.0 software. The differences in the protein expression patterns between gastric cancerous and paracancerous tissues were analyzed by the chi-square test. The chi-square test was also adopted for analysis of associations between FAM3C expression and clinicopathological parameters of GCs. The associations of FAM3C expression with PDGFR-β, E-cadherin and vimentin expression were analyzed with the nonparametric Spearman rank correlation coefficient. Survival curves were assessed by the Kaplan-Meier method and intergroup differences were analyzed with the log-rank test. Univariate and multivariate analysis were performed to evaluate the independence of significant prognostic factors by Cox proportional hazards regression model which was used to calculate hazard ratios (HR) of mortality for multiple clinicopathological variables and protein markers. Two-tailed *p*-value <0.05 was considered statistically significant.

Result

Expression of FAM3C, PDGFR-β, E-cadherin and vimentin in GC

IHC was conducted to analyze expression of FAM3C in 150 cases of GC tissues and 40 cases of matched adjacent normal tissues. As shown in **Figure 1**, positive staining of the FAM3C protein was most obviously detected in the cytoplasm of tumor cells. The proportion of high FAM3C expression was 70% (105/150) in GC and 52.5% (21/40) in matched adjacent normal tissues (**Table 2**). The chi-square test indicated that FAM3C expression in GC tissues was significantly higher than that in matched adjacent normal tissues (P<0.05).

Immunohistochemical staining for PDGFR-β was mainly detected in the cytoplasm of cancer cells and surrounding stromal cells (**Figure 2**). PDGFR expression in GC tissues was significantly upregulated. Among the GC cases, 79 of 150 (52.7%) showed high PDGFR expression.

As shown in **Figure 2**, immunoreactivity for E-cadherin was present predominantly at the cell membrane. Expression of E-cadherin in GC tissues was significantly downregulated. The proportion of E-cadherin low expression was 60% (90/150) in GC tissues.

Vimentin was predominantly observed in the cytoplasm of GC cells. The percentage of vimentin positive expression was 18.7% (28/150) in GC tissues. Representative images are shown in **Figure 2**.

Associations of FAM3C expression with PDGFR-β, E-cadherin and vimentin expression in GC

As shown in **Table 3**, the Spearman rank test showed that high expression of FAM3C had obviously positive correlation with vimentin expression (r = 0.164, P = 0.045) and negative correlation with E-cadherin expression (r = -0.178, P = 0.029). However, there is no correlation between expression of FAM3C and PDGFR-β (r = 0.137, P = 0.095).

Correlations of clinicopathologic characteristics with FAM3C, PDGFR-β, E-cadherin, and vimentin expression

The relationships between FAM3C, PDGFR-β, E-cadherin, and vimentin expression and clinicopathologic characteristics are shown in **Table 1**. High expression of FAM3C and PDGFR-β was significantly associated with depth of invasion (P = 0.004 and 0.002, respectively), lymph node metastasis (P = 0.016 and 0.004, respectively), and TNM stage (P = 0.022 and P< 0.0001, respectively), but not with tumor size, tumor location or histologic grade (P>0.05). The down-regulated expression of E-cadherin was significantly associated with histological

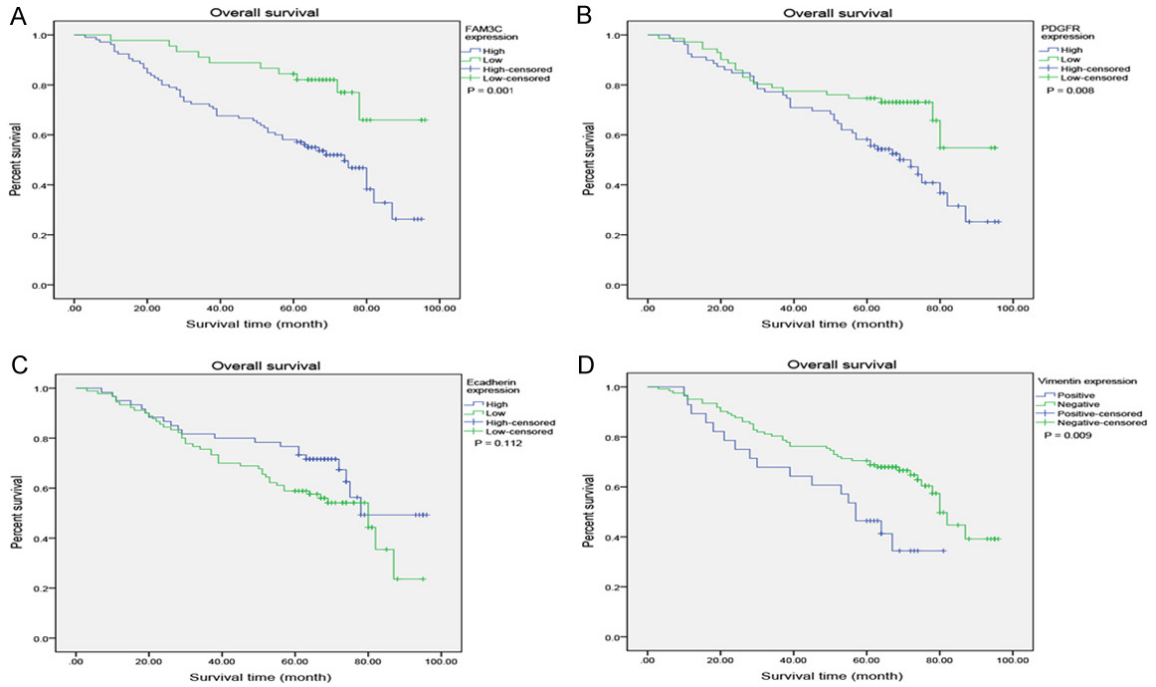

Figure 3. Analysis of OS relative to FAM3C, PDGFR-β, E-cadherin and vimentin expression levels: A. FAM3C expression: low versus high expression (P = 0.001). B. PDGFR-β expression: low versus high (P = 0.008). C. E-cadherin expression: low versus high (P = 0.112). D. Vimentin expression: negative versus positive (P = 0.009).

grade (P = 0.001), depth of invasion (P = 0.029), lymph node metastasis, (P = 0.047) and TNM stage (P = 0.029), but not with tumor size or location (P>0.05). The positive expression of vimentin was significantly associated with lymph node metastasis (P = 0.038) and TNM stage (P = 0.032), but not correlated with tumor size, tumor location, histological grade, or depth of invasion (P>0.05).

Survival analysis

Patients with high expression of FAM3C had significantly lower overall 1, 3, 5-year survival rates (92.4%, 72.4% and 58.1%, respectively) than those expressing low levels of FAM3C (97.8%, 91.1% and 84.4%, respectively). As shown in **Figure 3**, Kaplan-Meier analysis indicated that overall survival (OS) of patients with high FAM3C expression was significantly shorter than those with low FAM3C expression (log-rank, P = 0.001). Patients displaying high PDGFR-β expression had a shorter survival duration compared to those with low expression (log-rank, P = 0.008). Similarly, the OS of patients with positive vimentin expression was significantly lower than those with negative expression (log-rank, P = 0.009). However, low E-cadherin expression had no significant impact on OS of GC patients (log-rank, P = 0.112).

Univariate analysis indicated that FAM3C expression (P = 0.002), PDGFR-β expression (P = 0.009) and vimentin expression (P = 0.011) could serve as prognostic factors for GC patients (**Table 4**).

Multivariate analysis by using forward stepwise Cox regression analysis revealed that high expression of FAM3C (P = 0.007) and TNM stage (P<0.0001) were independent prognostic factors in GC patients (**Table 4**).

Discussion

In the current study, it was observed that overexpression of FAM3C was closely correlated with poor outcomes for GC patients and could serve as a novel marker for EMT. This is the first research investigating the relationship between FAM3C and EMT for its clinical significance in GC.

Recently, several works have shown that high levels of FAM3C were detected in many epithelial tumors and may be involved in tumor initia-

Table 4. Univariate and multivariate analyses of survival in gastric cancer

Variable	Patients (n)	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P	HR (95% CI)	P
Age		0.806 (0.493-1.317)	0.389		
<60	74				
≥60	76				
Gender		0.614 (0.359-1.049)	0.074		
Male	93				
Female	57				
Tumor size		1.455 (0.886-2.389)	0.138		
<5 cm	71				
≥5 cm	79				
Histological grade		1.701 (0.964-3.002)	0.067		
Well and moderate	47				
Poor and others	103				
Tumor location		0.786 (0.575-1.075)	0.132		
Upper	24				
Middle	51				
Lower	75				
Depth of invasion		3.807 (1.87-7.747)	<0.0001		
T1+T2	45				
T3+T4	105				
Lymph node metastasis		2.395 (1.372-4.183)	0.002		
Absent	58				
Present	92				
TNM stage		3.139 (1.748-5.635)	<0.0001	0.35 (0.194-0.63)	<0.0001
I-II	59				
III-IV	91				
FAM3C expression		0.346 (0.176-0.678)	0.002	0.393 (0.199-0.773)	0.007
High	105				
Low	45				
PDGFR expression		0.5 (0.297-0.842)	0.009		
High	79				
Low	71				
E-cadherin expression		1.52 (0.902-2.56)	0.116		
High	60				
Low	90				
Vimentin expression		0.481 (0.274-0.843)	0.011		
Positive	28				
Negative	122				

CI, Confidence interval; HR, Hazard ratio.

tion, progression, and metastasis, which indicated that overexpression of FAM3C might be closely associated with the genesis and development of cancer [17, 29]. Importantly, FAM3C overexpression has been proven to be an independent predictive factor for poor survival in various malignant tumors, such as esophageal squamous cell carcinoma [17] and colorectal cancer [18]. However, there was no prior research concerning the expression pattern and clinical significance of FAM3C in GC. In our study, we found that FAM3C expression in GC tissues was significantly higher than that in matched adjacent normal tissues. Statistical

analysis showed that higher levels of FAM3C expression were detected in tumors with aggressive clinicopathological features including deeper invasion, lymph node metastasis and advanced TNM stage, indicating an underlying promotion of FAM3C in GC formation, progression, and metastasis. Furthermore, our study showed that high expression of FAM3C was significantly associated with relatively poor outcome for GC patients. Patients with high expression of FAM3C displayed a relatively lower rate of 5-year OS than those expressing low levels of FAM3C. Using multivariate analyses, our data showed that high FAM3C expression is an independent factor of poor prognosis for GC. Therefore, these findings suggested that FAM3C expression could act as a predictive factor for poor outcomes or a potential therapeutic target for the clinical management of GC.

Metastasis is one of the decisive factors that affect outcomes of cancer patients [30]. EMT has been found to be involved in many aspects of tumor metastasis [31]. Moreover, an increasing number of articles have demonstrated that FAM3C overexpression can induce EMT, thereby promoting tumor growth and metastasis in vitro and in vivo [16, 21]. Previous research found that only covalent FAM3C self-assembly is essential for EMT induction, elevated tumor growth and metastasis [32]. Therefore, we have also investigated the expression of EMT-related proteins (E-cadherin and vimentin) in GC tissues by immunohistochemistry and have analyzed the relationship between FAM3C and these proteins. E-cadherin is a member of the epithelial cadherin family of glycoproteins, which play important roles in maintaining cell adhesion and intercellular connections [33]. The down-regulated expression of E-cadherin could result in an advanced histologic grade, tumor invasion, and metastasis [34]. Vimentin is a major component of intermediate-sized filaments present in the cytoskeleton, which is essentially type III intermediate filament protein [35]. Vimentin plays a significant role in maintaining cell shape and the integrity of the cytoplasm and stabilizing cytoskeletal interactions [36]. Several studies have demonstrated that positive expression of vimentin could be correlated with poor prognosis and high frequency of metastasis in patients with GC [37, 38]. In this study, we found that increased vimentin and reduced E-cadherin were closely linked to metastasis, advanced TNM stage and poor outcomes in GC. Moreover, for the first time, we found that high expression of FAM3C had obviously positive correlation with vimentin expression and negative correlation with E-cadherin expression, which indicated that FAM3C could be a novel marker for epithelial-mesenchymal transition for GC. The result is consistent with some other studies of colorectal cancer [18], breast cancer [16] and liver carcinoma [21]. Howeverthe precise mechanisms of FAM3C involved in EMT are still not completely clear.

In recent investigations, several studies have demonstrated that FAM3C could mediate the occurrence of EMT through TGF-β signaling in murine epithelial cells [19, 20]. FAM3C was required but not sufficient to induce TGF-β-mediated EMT [19]. However, Lahsnig et al. [21] demonstrated that FAM3C required cooperation with oncogenic Ras to induce and maintain EMT of hepatocytes in a TGF-β-independent fashion in human hepatocytes. Moreover, Waerner et al. [16] revealed that overexpression of FAM3C alone was enough to induce EMT and promote tumor growth and metastasis by TGF-β-independent signaling in murine mammary epithelial cell EpH4. These results indicated that overexpression of FAM3C could induce tumor cells to undergo EMT and the molecular mechanisms of FAM3C involved in the process of EMT might vary with the type of epithelial cell. Interestingly, upregulation of PDGF/PDGFR induced by FAM3C may be involved in both murine mammary epithelial EMT and hepatocellular EMT [16, 21]. Therefore, we also detected the expression of PDGFR in GC tissues and assessed their potential relationship with FAM3C. The results showed that PDGFR-β was highly expressed in GC, and the high expression was significantly associated with depth of invasion, lymph node metastasis and TNM stage. However, we found that there was no correlation between expression of FAM3C and PDGFR-β in GC. Similarly, differing associations between FAM3C and PDGFR-β expression have been shown in different kinds of cancer, suggesting that FAM3C might induce and maintain EMT by PDGFR-β-independent signaling in GC. Further work regarding the association between FAM3C and PDGFR-β in GC isstill needed.

In conclusion, FAM3C is a potential marker of EMT in GC. Moreover, high expression of FAM3C may promote GC invasion and metastasis through the EMT, and is an independent predictive factor for poor prognosis in GC patients.

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Disclosure of conflict of interest

None.

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