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Original Article

Increased expression of Fibulin-5 in gastric cancer associated with poor prognosis

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Abstract: The expression of Fibulin-5 and Ki67 proteins has been demonstrated to play important roles in tumor development and progression. However, the detailed clinical implications of them have not been well understood, especially in gastric cancer. In this study, immunohistochemistry was applied to investigate the expression levels of Fibulin-5 and Ki67 in a tissue microarray with following-up information, including 90 gastric cancer cases and paired para-carcinoma tissues. The results showed that the expression levels of Fibulin-5 and Ki67 in gastric cancer tissues were significantly higher than those in para-carcinoma tissues ($P<0.05$), and the expression of Fibulin-5 was significantly positive correlated with Ki67's expression ($r>0$, $P<0.05$). Clinical correlation analysis showed that the expression of Fibulin-5 in tumor cytoplasm and Ki67 in tumor cell nucleus was positively correlated with patients' age ($r=0.213$, $P=0.044$ and $r=0.241$, $P=0.023$, respectively). Expression of Ki67 also positively correlated with the tumor size ($r=0.249$, $P=0.021$). Survival analysis showed that Fibulin-5 expression in the cytoplasm of cancer tissue was significantly associated with poor prognosis of gastric cancer patients (25.0% vs. 55.6%, $P=0.027$), as an independent prognostic factor ($P=0.037$). Patients with higher Fibulin-5 expression in cancer nucleus also associated with poor survival, but their association was not significant (27.8% vs. 54.5%, $P=0.141$). In conclusion, the present study suggested that Fibulin-5 was overexpressed in gastric cancer and associated with a poor prognosis, which might serve as a novel biomarker for prognosis prediction in gastric cancer, as well as a potential therapeutic target.

Keywords: Fibulin-5, Ki67, gastric cancer, immunohistochemistry, tissue microarray, prognosis

Introduction

Gastric cancer is one of the most common malignant tumors, which ranks the fourth in incidence and second in mortality rate related to tumors [1, 2]. Therefore, exploring the expression pattern of genes associated with gastric cancer and finding the target proteins related to prognosis of gastric cancer will be essential to studying the molecular mechanism and providing solid theoretical basis for cancer treatment.

As an integrin-binding extracellular matrix protein, Fibulin family participates in the stabilization of extracellular matrix structures through the interaction with the basement member and a variety of extracellular matrix components, including laminin, elastin, aggrecan, endostatin and fibronectin [3, 4], which play an important role in regulating organogenesis, vasculogenesis, fibrogenesis, and tumorigenesis [4, 5]. As a member of Fibulin family, Fibulin-5 has been found to be involved in multiple cancers' progression and its biological functions in different cancers are in consistent. It functions as a tumor suppressor in prostate cancer, lung cancer, bladder cancer and liver cancer [6-9], while it serves as a tumor promoter in breast cancer and nasopharyngeal carcinoma [10, 11].

There are only a few studies on the function of Fibulin-5 in gastric cancer, which show that both mRNA and protein expression level of Fibulin-5 are higher in gastric cancer tissues than those in adjacent tissues. Fibulin-5 expression in cancer tissues is significantly positively correlated with the differentiation degree, lymph node status and TNM stage of gastric cancer. Fibulin-5 knockdown using corresponding shRNA significantly inhibits the proliferation and the invasion of the gastric cancer cell line MGC-803. All these results indicate that Fibulin-5 promotes gastric cancer progression

Table 1. Expression of Fibulin-5 and Ki67 in gastric cancer and para-carcinoma tissues ($\bar{x} \pm s$)

	Number of gastric cancer tissues	Expression in gastric cancer tissues	Number of para-carcinoma tissues	Expression in para-carcinoma tissues	*P*
Fibulin-5 (cytoplasm)	90	6.200±4.191	87	5.059±3.658	0.029
Fibulin-5 (nucleus)	90	7.211±3.844	87	4.914±2.460	0.000
Ki67 (nucleus)	89	4.301±2.794	89	1.545±0.660	0.000

Table 2. Correlation of Fibulin-5 and Ki67 expression in gastric cancer tissues by paired Wilcoxon test

			Fibulin-5 expression (cytoplasm)	Fibulin-5 expression (nucleus)	Ki67 expression
Spearman's rho	Fibulin-5 expression (cytoplasm)	Correlation Coefficient	1.000	0.242	0.304
		Sig. (2-tailed)		0.021	0.004
		N	90	90	89
	Fibulin-5 expression (nucleus)	Correlation Coefficient	0.242	1.000	0.244
		Sig. (2-tailed)	0.021		0.021
		N	90	90	89
	Ki67 expression	Correlation Coefficient	0.304	0.244	1.000
		Sig. (2-tailed)	0.004	0.021	
		N	89	89	89

[12]. However, this study only included 56 gastric cancer samples, without showing the correlation of Fibulin-5 expression with the prognosis of gastric cancer, and it lacked of further study on the molecular mechanism of Fibulin-5 involved in gastric tumor proliferation in details. Therefore, a gastric cancer tissue microarray containing 90 cases was carefully performed in our study. Immunohistochemistry (IHC) staining and statistical analysis were applied to study the expression correlation of Fibulin-5 with a tumor cell proliferation biomarker Ki67, as well as the correlation of these two proteins with the prognosis of gastric cancer.

Materials and methods

Gastric cancer tissue microarray (TMA)

Gastric cancer tissue microarray (HStm-Ade-180Sur-04) was obtained from Shanghai Outdo Biotech Co., Ltd. TMA was performed for 90 carcinoma tissues and paired para-carcinoma tissues. TMA production, donor paraffin-embedded sections were cut into sections and stained with hematoxylin-eosin (HE). Then, the typical pathological sites were labeled on the slices. 180 blocks on the blank recipient paraffin (the diameter was 1.5 mm) were drilled, and the target tissue core was set according to the position of HE staining. Continuous sections from tissues with a thickness of 4 um were cut with the slicer (Leica, Germany). Sections were attached to anti-off micro slides.

There were 70 male and 20 female patients, aged from 34 to 83 years, including 7 cases of stage I, 30 cases of stage II, 49 cases of stage III and 4 cases of stage IV. Of the 90 patients, there were 67 patients with lymph node metastasis and 4 patients with distant metastasis (**Table 2**).

Operations were performed from May 2007 to February 2008. The last follow-up time was August 2013. During this period, 62 patients died of disease, with a median follow-up time of 26 months (0-63 months); 27 patients were alive, with a median follow-up time of 72 months (66-75 months); one patient lost contact (censored case) at August 2012. All patients were histologically diagnosed as gastric adenocarcinoma and did not receive extra treatment before surgery.

Immunohistochemistry (IHC)

EDTA two-step IHC assay by DAKO Auto Stainer LinK48 was used: after antigen retrieval using citrate buffer, the tissue sections were blocked

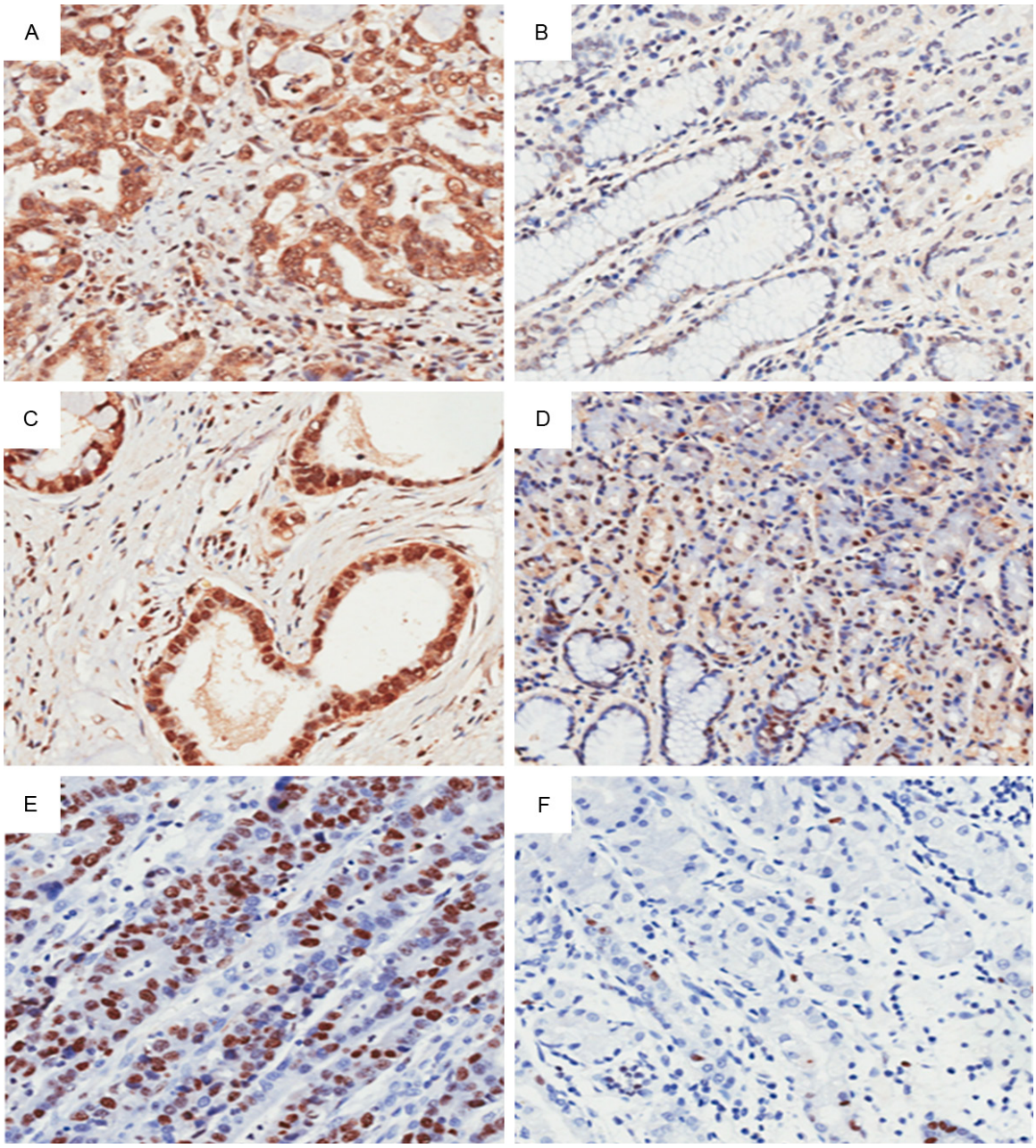

Figure 1. IHC analysis of Fibulin-5 and Ki67 in gastric cancer. A. The protein expression level of Fibulin-5 in cytoplasm of gastric cancer tissue was increased. B. The protein expression level of Fibulin-5 in cytoplasm of para-carcinoma tissue was decreased. C. The protein expression level of Fibulin-5 in nucleus of gastric cancer tissue was increased. D. The protein expression level of Fibulin-5 in nucleus of para-carcinoma tissue was decreased. E. The protein expression level of Ki67 in nucleus of gastric cancer tissue was increased. F. The protein expression level of Ki67 in nucleus of para-carcinoma tissue was decreased.

with goat serum and subsequently incubated with anti-Fibulin-5 antibody (1:100, 20097, Promab) at 4°C overnight, followed by an incubation with secondary antibody (HRP-labeled anti-mouse antibody, DAKO). After washing with PBS, the sections were visualized using diaminobenzidine (DAB) system and hematoxylin redying, observed and analyzed with microscope. Three high-magnification fields were chosen randomly under optical microscope and more than 3 × 100 cells were analyzed. Sections were scored and grouped with positive staining rate and intensity. The positive staining rate was defined according to the proportion of posi-

Table 3. Correlations between Fibulin-5/Ki67 expression and clinico-pathologic variables in gastric cancer by Spearman's correlation analysis

Clinico-pathologic Variables	Fibulin-5 high expression (cytoplasm) (n=72)	Fibulin-5 Low expression (cytoplasm) (n=18)	correlation coefficient	*P*	Fibulin-5 high expression (nucleus) (n=79)	Fibulin-5 low expression (nucleus) (n=11)	correlation coefficient	*P*	Ki67 high expression (n=73)	Ki67 low expression (n=16)	correlation coefficient	*P*
Gender			0.067	0.532			0.178	0.093			-0.012	0.914
Male	55	15			59	11			58	11		
Female	17	3			20	0			15	5		
Age			0.213	0.044			0.171	0.108			0.241	0.023
≤60	20	9			23	6			20	9		
>60	52	9			56	5			53	7		
Tumor size			0.019	0.865			0.020	0.852			0.249	0.021
≤5 cm	31	8			34	5			28	10		
>5 cm	39	9			42	6			43	5		
Lost	2	1			3	0			2	1		
Pathological grade			-0.113	0.289			0.050	0.638			-0.061	0.573
Grade I	0	0			0	0			0	0		
Grade II	21	3			20	4			22	2		
Grade III-IV	51	15			59	7			51	14		
T Stage			-0.028	0.794			-0.092	0.390			-0.024	0.821
T1	2	2			2	2			3	1		
T2	6	1			6	1			6	1		
T3	51	10			58	3			52	8		
T4	13	5			13	5			12	6		
N stage			0.019	0.860			0.046	0.668			0.151	0.157
N0	18	5			18	5			16	7		
N1	13	3			15	1			13	3		
N2	20	5			22	3			22	2		
N3	21	5			24	2			22	4		
M stage			-0.027	0.801			-0.108	0.312			-0.086	0.422
M0	69	17			76	10			70	15		
M1	3	1			3	1			3	1		
TNM stage			-0.059	0.580			-0.031	0.771			0.147	0.170
Stage I	5	2			5	2			5	2		
Stage II	26	4			27	3			24	6		
Stage III	38	11			44	5			41	7		
Stage IV	3	1			3	1			3	1		

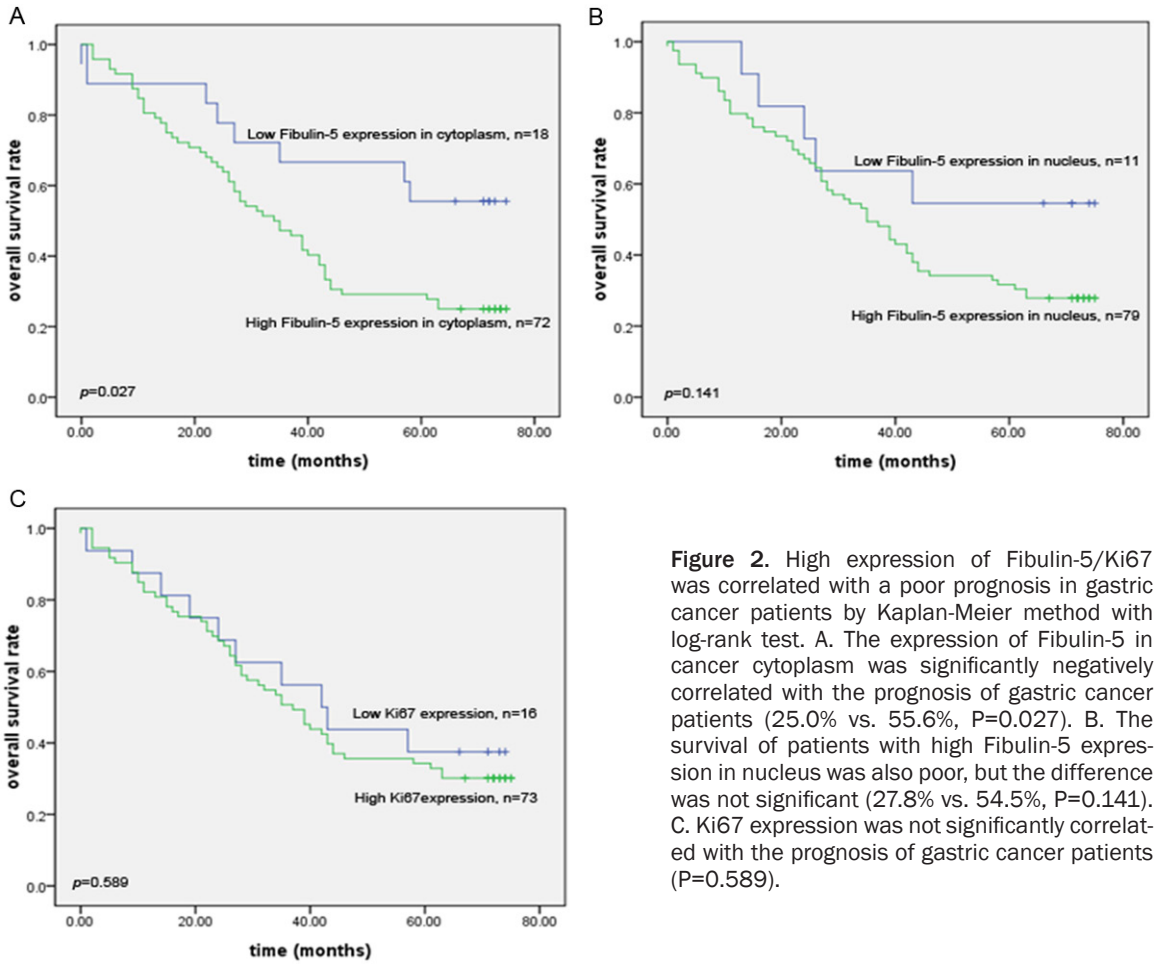

Figure 2. High expression of Fibulin-5/Ki67 was correlated with a poor prognosis in gastric cancer patients by Kaplan-Meier method with log-rank test. A. The expression of Fibulin-5 in cancer cytoplasm was significantly negatively correlated with the prognosis of gastric cancer patients (25.0% vs. 55.6%, P=0.027). B. The survival of patients with high Fibulin-5 expression in nucleus was also poor, but the difference was not significant (27.8% vs. 54.5%, P=0.141). C. Ki67 expression was not significantly correlated with the prognosis of gastric cancer patients (P=0.589).

tively stained cancer cells: "negative" was 0, "≤20%" for 1, "21%-40%" for 2, "41%-60%" for 3, "61%-80%" for 4, "81%-100%" for 5. The score of staining intensity: "negative" was 0, "1+" for 1, "2+" for 2, "3+" for 3. Patients were divided into two groups according to the scores "positive staining rate score" multiply "staining intensity score". Equal or less than 1.5 were classified into low expression, more than 1.5 were classified into high expression group.

Statistical analysis

The protein expression levels of Fibulin-5 and Ki67 in gastric cancer tissues and para-carcinoma tissues were analyzed by paired Wilcoxon test. The correlation of Fibulin-5 and Ki67 expression with clinico-pathological parameters of gastric cancer was calculated by Spearman's correlation analysis. Univariate analysis between Fibulin-5, Ki67, clinical characteristics and survival time was analyzed by Kaplan-Meier method with log-rank test. Then, statistically significant variables in univariate analysis were included in COX multivariate regression analysis of patient survival. $P<0.05$ was considered to be statistically significant.

Results

The expression levels of Fibulin-5 and Ki67 were higher in gastric cancer tissues than those in para-carcinoma tissues, respectively

IHC showed that Fibulin-5 and Ki67 proteins were expressed in gastric cancer tissues and para-carcinoma tissues. Fibulin-5 expressed in cytoplasm and nucleus, while Ki67 only expressed in nucleus (**Figure 1**). Paired Wilcoxon test showed that the expression levels of Fibulin-5 in cytoplasm and nucleus of tumor tissues were significantly higher than those in para-carcinoma tissues ($P=0.029$ and $P=0.000$, respectively). Ki67expressionintumor tissues was also significantly higher than that in the para-carcinoma tissues ($P=0.000$) (**Table 1**).

Table 4. Multivariate analysis by a cox proportional hazards regression model

						95.0% CI for Exp (B)	
	B	SE	Wald	*P*	Exp (B)	Lower	Upper
Fibulin-5 expression in cytoplasm	0.811	0.388	4.361	0.037	2.249	1.051	4.813
Tumor size	0.674	0.278	5.863	0.015	1.962	1.137	3.386
T stage	1.133	0.300	14.236	0.000	3.106	1.724	5.596
N stage	0.526	0.209	6.319	0.012	1.692	1.123	2.551
TNM stage	-0.245	0.420	0.340	0.560	0.783	0.344	1.783

The expression of Fibulin-5 and Ki67 in gastric cancer tissues positively correlated with each other

Spearman correlation analysis was used to analyze the correlation of Fibulin-5 and Ki67 expression. The results showed that cytoplasm Fibulin-5 expression, nuclear Fibulin-5 expression and Ki67 expression in gastric cancer tissues significantly positively correlated with each other (r>0, P<0.05, **Table 2**).

Correlation of Fibulin-5 expression and Ki67 expression with the clinical characteristics of gastric cancer

Spearman analysis was used to study the correlation of Fibulin-5 expression in cytoplasm, Fibulin-5 expression in nucleus, Ki67 expression with the clinical characteristics of gastric cancer patients, respectively. The results demonstrated that there were no correlations between Fibulin-5 expression and patient gender, tumor size, pathological grade, TNM stage and clinical stage, except for patients' age. Fibulin-5 expression in cancer cytoplasm significantly positively correlated with patients' age (r=0.213, P=0.044). The older patient expressed higher Fibulin-5 protein in the cytoplasm. Expression of Ki67 only positively correlated with the patient age and tumor size (r=0.241, P=0.023 and r=0.249, P=0.021, respectively) (**Table 3**).

Correlation of Fibulin-5/Ki67 expression with the clinico-pathological parameters of gastric cancer patients

Kaplan-Meier analysis with log-rank statistic test was performed to analyze the association of patient survival with Fibulin-5 expression in cytoplasm, Fibulin-5 expression in nucleus, Ki67 expression, and patient clinical characteristics. The results indicated that Fibulin-5 expression in cancer cytoplasm negatively correlated with patients' survival time. Survival time of high cytoplasm Fibulin-5 group was significantly shorter than that of the low expression group (25.0% *vs.* 55.6%, P=0.027). Survival of patients with high Fibulin-5 expression in nucleus also associated with poor prognosis, but the difference was not significant (27.8% *vs.* 54.5%, P=0.141). The expression of Ki67 didn't associate with the prognosis of gastric cancer patients (P=0.589) (**Figure 2**). Furthermore, the tumor size, T stage, N stage as well as TNM stage was significantly associated with poor overall survival of gastric cancer patients (P<0.05, respectively).

Subsequently, COX multivariate survival regression analysis was performed for the clinical characteristics related to prognosis. The results indicated that Fibulin-5 expression in cytoplasm was an independent prognostic factor of gastric cancer patients (P=0.037). In addition, tumor size, T stage and N stage were also independent prognostic factors (P<0.05) (**Table 4**).

Discussion

To explore the role of Fibulin-5 in gastric cancer, a gastric cancer tissue microarray was employed and measured by immunohistochemistry staining. The correlation of Fibulin-5 and Ki67 expression with gastric cancer occurrence, development and prognosis was further analyzed. The study demonstrated an important gene, Fibulin-5, in gastric cancer progression in the following aspects: firstly, Fibulin-5 is an oncogene for gastric cancer, especially when it was expressed in the cytoplasm. Secondly, Fibulin-5 could induce gastric cancer cell proliferation though promoting Ki67 expression to increase tumor size and then shorten the patients' survival indirectly. Thirdly, it was also possible that Fibulin-5 might dominate an unknown cancer promoting gene regulation network to reduce the patients' prognosis

directly, in view of its variety and complexity function.

There were several studies on the relationships between Fibulin-5 expression and cancers, but the conclusions were not consistent and even contradictory. On one hand, Fibulin-5 plays a definitely oncogenic role in breast cancer, nasopharyngeal carcinoma and gastric cancer [10-12]. CF Hwang *et al.* [11] showed that Fibulin-5 expression is positively correlated with T stage, M stage and TNM stage of nasopharyngeal carcinoma. Moreover, the survival of nasopharyngeal carcinoma patients with high Fibulin-5 expression is significantly decreased. Fibulin-5 could regulate nasopharyngeal carcinoma cell proliferation, migration and invasion properties, in which the cancer promoter gene FLJ10540 and AKT might be involved. However, siRNA could reverse the oncogenic ability of Fibulin-5. On the other hand, Fibulin-5 exhibited anti-tumor effects towards prostate cancer, lung cancer, bladder cancer as well as hepatocellular carcinoma (HCC) [6-9]. K Tu *et al.* [9] found that the expression level of Fibulin-5 protein was down-regulated in HCC tissues compared with that in the matched noncancerous tissues and that the tumors with high Fibulin-5 expression were associated with better overall survival and disease-free survival of HCC patients. Furthermore, Fibulin-5 was an independent prognostic factor. Further cytological studies also showed that Fibulin-5 might inhibit HCC cell invasion and metastasis through suppressing MMP-7 (matrix metalloproteinase 7) expression. All these studies had confirmed that the biological function of Fibulin-5 in cancers was various and complicated.

In conclusion, although the mechanisms of Fibulin-5 various in different cancers, its oncogenic role in gastric cancer is clear. Our study first confirmed that the expression levels of Fibulin-5 and Ki67 were significantly positively correlated with each other, and that the prognosis of gastric cancer group with high Fibulin-5 expression was significantly poorer. However, there were still some questions to be solved. For example, how is the clinical significance of the positive correlation ofFibulin-5 expression with patient's age? What genes participate in the Fibulin-5 oncogenic signal pathway? In view of the variety and complexity of Fibulin-5 function, we would plan to establish nude mouse gastric cancer xenograft model in the following study, in which gene knockout and overexpression will be applied to further explore the effect of Fibulin-5's on the prognosis and the complicated gene regulation network of gastric cancer.

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Disclosure of conflict of interest

None.

Authors' contribution

BX and JH conceived and designed the experiments. XL and CY performed the IHC experiments and data analysis. XL, CY and BX prepared a draft manuscript, and JH wrote the final version of the manuscript.

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