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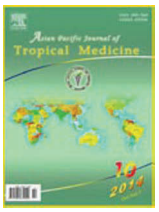

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Effect of Fibulin-5 on cell proliferation and invasion in human gastric cancer patients

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ABSTRACT

Objective: To explore the effect of Fibulin-5 expression on cell proliferation and invasion in human gastric cancer patients. **Methods:** Fibulin-5 expression was detected in 56 samples of surgically resected gastric cancer and paired noncancerous tissues using qRT-PCR and immunoblotting. Fibulin-5 was knocked down by Fibulin-5 shRNA in MGC-803 cells, then BrdU cell proliferation and transwell invasion assays were used to determine cell proliferation and invasion. **Results:** The level of Fibulin-5 mRNA in gastric cancer tissues was significantly higher as compared with that in normal tumor-adjacent tissues ($P<0.05$). Otherwise, the level of Fibulin-5 protein in cancer and noncancerous tissues was consistent with mRNA expression ($P<0.05$). Fibulin-5 protein expression in tumor tissues with poorly differentiated, lymph node metastasis and advanced TNM tumor stage was significantly higher ($P<0.05$, respectively). Fibulin-5 was obviously knocked down by Fibulin-5 shRNA ($P<0.05$), and Fibulin-5 knockdown significantly inhibited cell proliferation and invasion in MGC-803 cells ($P<0.05$, respectively). **Conclusions:** The high-expression of Fibulin-5 is associated with the malignant clinicopathologic parameters in gastric cancer and Fibulin-5 knockdown inhibits cell proliferation and invasion in MGC-803 cells, suggesting Fibulin-5 may act as a key factor in the progression of gastric cancer.

1. Introduction

Gastric cancer, one of the most common malignant cancers, results in approximately one million deaths annually around the world. The mortality of gastric cancer has been improved in the last decade, but it still remains the first cause of cancer-related death in China. The rising incidence of gastric cancer coupled with emerging evidence for major advances in diagnosis and treatment have improved the outlook for gastric cancer patients, but it has not yet achieved satisfactory curative effect. Increasing evidence suggest that many biomarkers including E-cadherin, matrix metalloproteinases (MMPs) and Human epidermal growth factor receptor-2 (HER-2) act as prognostic factors for predicting gastric cancer patients survival. Therefore, further investigating the correlation between functional change of new genes and gastric cancer development, malignant characteristics as well, play a critical role in revealing the precise molecular mechanisms of gastric cancer progression, designing reasonable molecular targeted drugs, predicting the prognosis and improving the treatment of gastric cancer in China.

The extracellular matrix (ECM) protein Fibulin-5, a TGF-β-induced glycoprotein, is a multifunctional molecule that regulates various cellular processes, including proliferation, motility and invasion, in normal and malignant cells[1-3]. Fibulin-5 overexpression triggers DNA synthesis and stimulates motility in fibrosarcoma, fibroblasts and breast cancer cells[4]. Nevertheless, the expression of Fibulin-5 is impaired in various human cancer tissues including renal cell carcinoma, bladder urothelial carcinoma, prostate cancer, lung cancer and colorectal cancer[3,5-7]. Wen *et*

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al reported that Fibulin-5 protein level in gastric cancer was obviously higher as compared with that in normal tumor-adjacent tissues[8]. But the clinical significance of Fibulin-5 and its function in human gastric cancer is poorly understood. In this study, we systemically analyzed the correlation between the expression of Fibulin-5 and clinicopathologic features and its role in cell proliferation and invasion, in order to provide a new insight for prevention and treatment of gastric cancer.

2. Materials and methods

2.1. Clinical data and cell line

A total of 56 gastric cancer patients including 41 males and 15 females were selected, who received radical operation during Jan 2009 to Dec 2012. Gastric cancer samples and paired normal tumor-adjacent samples (>3 cm distance from the margin of the resection) were collected during surgery. The demographic features and clinicopathologic data are shown in Table 1. In summary, the median age of the patients was 51 years (range, 35-72 years). Samples were collected and used after obtaining informed consent. The Zhejiang University Ethics Committee approved all protocols according to the Helsinki Declaration (as revised in Edinburgh 2000).

The human gastric cancer cell line MGC-803 was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in complete Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco) with 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma, St-Louis, MO, USA) in a humidified containing of 5% CO_2 incubator at 37 ℃.

2.2. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Fibulin-5 (forward primer: 5′-TCGCTATGGTTACTGCCAGCA-3′; reverse primer: 5′-TTGGCAAGACCTTCCATCGTC-3′) and GAPDH primers were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Total RNA was isolated from tissues and cells using TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthetized using the RevertidTM First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA). The PCR amplification for the quantification of the Fibulin-5 mRNA and the GAPDH mRNA was performed using a SYBR® Premix Ex *Taq*™ Ⅱ (Tli RNaseH Plus, Takara, Shiga, Japan). The GAPDH was used as an internal control. The amounts of Fibulin-5 mRNA were calculated with the $2^{-\Delta\Delta CT}$ method.

2.3. Western blot

Fibulin-5 (EPR4506, Epitomics, Burlingame, CA, USA) (1:1 000) and GAPDH (G8140, US Biological, Salem, MA, USA) (1:5 000) antibodies were used for immunoblotting assay. Horseradish peroxidase (HRP)-conjugated sheep anti-mouse or duck anti-rabbit secondary antibody (Bio-Rad, Hercules, CA, USA) were used at a dilution from 1:1 000-1:5 000 and detected by a Western Blotting Luminol Reagent (sc-2048, Santa Cruz, CA, USA)[9].

2.4. Plasmids transfection

Plasmids containing Fibulin-5 shRNA (5′-GAGATAAGGCTCCTCACAGCGGG-3′) or non-targeting shRNA (NT shRNA) were purchased from Invitrogen Co. The day before transfection, 1×10^6 MGC-803 cells were seeded in 60 mm dishes. 1 μg Fibulin-5 shRNA or NT shRNA plasmids were transfected into MGC-803 cells by using Effectene transfection reagent (Qiagen, Valencia, CA, USA). Further experiments were performed 48-72 h after plasmids transduction.

2.5. BrdU cell proliferation assay

For the proliferation assay, MGC-803 cells that transfected with Fibulin-5 shRNA or NT shRNA were seeded into 96-well plates at 5 000 cells/well for 24 h and assessed using a Cell Proliferation ELISA, BrdU (5-bromodeoxyuridine) (chemiluminescent) (Roche, Indianapolis, IN, USA).

2.6. Transwell cell invasion assay

Transwell assays were done in 12 well plates with Transwell inserts equipped with 5-μm pores (Corning, NY, USA) coated with 20 μg/mL Matrigel (Becton Dickinson Labware, Bedford, MA, USA) in 37 ℃ for overnight. Fibulin-5 shRNA or NT shRNA transfected MGC-803 cells (1×10^5-2×10^5) were seeded in the upper well and DMEM medium with 10% FBS, as indicated, in the lower well. After completion, membranes were removed, wiped on the side facing the upper well, and stained with crystal violet. At least 6 representative images of each well were taken and cell numbers were counted using ImageJ. The experiments were performed in triplicate.

2.7. Statistical analysis

Results are expressed as Mean±SEM. Significance was

established, with GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, CA, USA), using the student's *t*-test and Spearman rank test when appropriate. Difference were considered significant when $P<0.05$.

3. Results

3.1. Expression of Fibulin-5 in gastric cancer and paired noncancerous tissues

To determine the levels of Fibulin-5 in gastric cancer patients, the level of Fbxw7 mRNA and protein was detected by using qRT-PCR and immunoblotting in a retrospective cohort of 56 pairs of cancerous and matched noncancerous tissue samples from gastric cancer patients after surgery. It was found that Fibulin-5 mRNA level in gastric cancer tissues were significantly higher as compared with those in matched normal tumor-adjacent tissues (1.98±0.10 *vs.* 0.83 ±0.06, $P<0.001$, Figure 1). Furthermore, elevated Fibulin-5 protein level was observed in cancerous tissues as compared with matched noncancerous tissues (0.63±0.09 *vs.* 0.15±0.01, $P<0.001$, Figure 2), which was consistent with Fibulin-5 mRNA level.

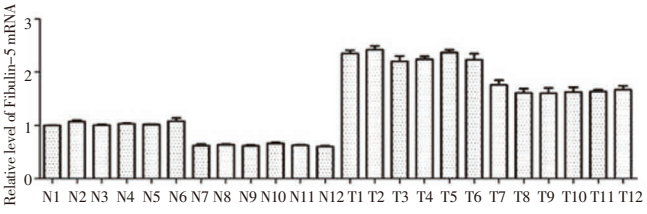

Figure 1. Expression of Fibulin-5 mRNA in gastric cancer.
Representative qRT-PCR analysis of Fibulin-5 mRNA level in cancer (T) matched noncancerous tissues (N) was shown. Quantification of the data revealed that Fibulin-5 mRNA level in gastric cancer tissues was obviously higher as compared with that in the normal tumor-adjacent tissues. $n=12$.

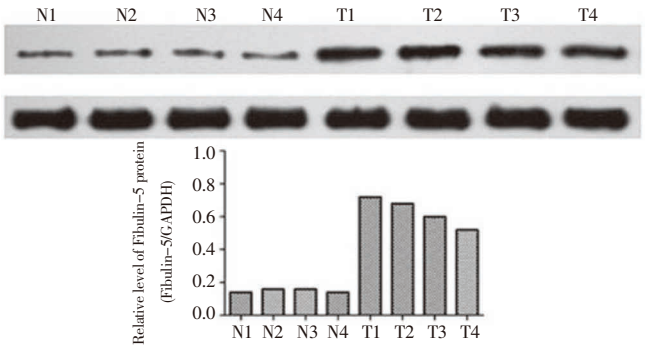

Figure 2. Level of Fibulin-5 protein in gastric cancer.
Representative western blot analysis of Fibulin-5 protein in gastric cancer (T) and matched noncancerous tissues (N) was shown. Quantification of the data revealed that Fibulin-5 protein level in gastric cancer tissues was obviously higher than that in the normal tumor-adjacent tissues. $n=4$.

3.2. Clinical significance of elevated Fibulin-5 expression in gastric cancer tissues

To analyze the correlation between Fibulin-5 expressions and clinicopathologic features in gastric cancer, Fibulin-5 protein level was quantitated by densitometry using Image J. As shown in Table 1, Clinical association analysis indicated that increased Fibulin-5 level in gastric cancer tissues was prominently related to poorly differentiated tumor ($r=0.503$, $P=0.014$), lymph node metastasis ($r=0.429$, $P=0.029$) and advanced TNM tumor stage (TNM tumor stage Ⅲ+Ⅳ; $r=0.571$, $P=0.002$). Our results indicate that the expression of Fibulin-5 was upregulated and elevated Fibulin-5 level was associated with malignant clinicopathologic parameters in gastric cancer.

Table 1
Correlation between the expression of Fibulin-5 protein and clinicopathologic characteristics in the gastric cancer patients ($n=56$).

Clinicopathologic features		*r* value	*P* value
Sex	Male	-0.035	0.809
	Female		
Age (year)	< 50	0.108	0.501
	⩾ 50		
Tumor size (cm)	< 5	0.214	0.178
	⩾ 5		
Tumor differentiation	Well and moderately differentiated	0.503	0.014*
	Poorly differentiated		
Depth of invasion	Mucosa or shallow muscularis	0.268	0.121
	Deep muscularis or whole layer		
Lymph node metastasis	No	0.429	0.029*
	Yes		
Tumor stage	Ⅰ+Ⅱ	0.571	0.002*
	Ⅲ+Ⅳ		

* $P<0.05$.

3.3. Fibulin-5 knockdown inhibits cell proliferation in MGC-803 cells

In order to explore the effect of Fibulin-5 on cell proliferation in gastric cancer, MGC-803 cells that were transfected with a specific Fibulin-5 shRNA were subjected to western blot for Fibulin-5 protein level. As measured by WB, the Fibulin-5 protein level was significantly downregulated by Fibulin-5 shRNA in MGC-803 cells (P<0.001, Figure 3). Furthermore, BrdU cell proliferation assay indicated that Fibulin-5 knockdown obviously suppressed cell proliferation in MGC-803 cells as compared with control cells (52.50±5.05 *vs.* 125.50±3.46, P=0.006, Figure 4).

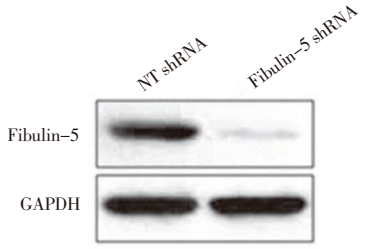

Figure 3. Fibulin-5 is knocked down by a specific shRNA MGC-803 cells.

MGC803 cells that had been transfected with Fibulin-5 shRNA and NT shRNA, respectively, were subjected to western blotting for Fibulin-5.

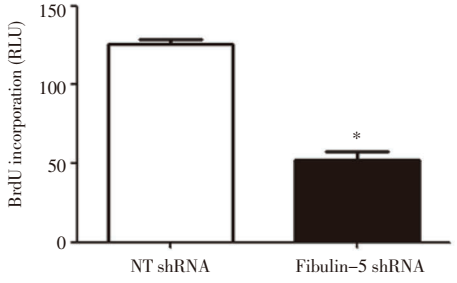

Figure 4. Fibulin-5 knockdown reduced cell proliferation in MGC-803 cells.

Cell proliferation as measured by BrdU incorporation was inhibited by Fibulin-5 knockdown in MGC-803 cells as compared with control cells. * P<0.05 by t test; n=3 repeats with similar results.

3.4. Fibulin-5 knockdown inhibits cell invasion in MGC-803 cells

To confirm whether Fibulin-5 knockdown affect cell invasion in gastric cancer, transwell cell invasion assays were performed in both Fibulin-5 shRNA and NT shRNA transfected MGC-803 cells. We found that the number of invaded MGC-803 cells in Fibulin-5 shRNA group was dramatically less than NT shRNA group (2.15±0.15 *vs.* 7.68 ±1.26, P=0.005, Figure 5). Our date indicate that Fibulin-5 may function as an oncoprotein through promoting cell proliferation and invasion in gastric cancer.

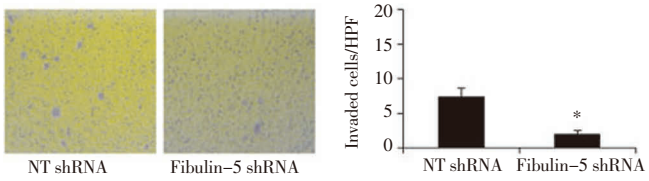

Figure 5. Fibulin-5 knockdown led to reduced cell invasion in MGC-803 cells.

Cell invasion as measured by transwell cell invasion assays was inhibited by Fibulin-5 knockdown in MGC-803 cells as compared with control cells. HPF, High power field; *P<0.05 by t test; n=3 repeats with similar results.

4. Discussion

The Fibulins is an old protein family, which are conserved in both worms and humans[4]. Fibulins are structurally consisting with a globular Fibulin-type module in C-terminal and calcium-binding epidermal growth factor-like modules[4]. In mammalian, the Fibulin family contains seven members, which were described as Fibulins 1-7. It has been reported that Fibulin gene mutations are related to various human diseases[10]. The Fibulins stabilize the structures of the ECM by interacting with many kinds of ECM components, such as laminin, elastin, aggrecan, endostatin and fibronectin[11,12]. Otherwise, Fibulins are involved in fibrogenesis, tissue organogenesis, vasculogenesis and tumorigenesis[12,13]. However, the precise mechanisms involved in these biological functions of Fibulins remain to be explored. Fibulin-5, which binds integrins through an RGD sequence, regulates endothelial cell adhesion[14]. Fibulin-5 is initially found to be expressed in the neural crest and embryonic vasculature, while is impaired in most adult vascular beds. But, the expression of Fibulin-5 is restored in injured vessels and in atherosclerotic plaques in an experimental hypercholesterolemia mouse model[7,15]. The expression of Fibulin-5 in human cancer tissues has tissue specificity. The present study revealed that Fibulin-5 was significantly up-regulated in gastric cancer tissues as compared with that in matched normal tumor-adjacent tissues, which was consistent with prior studies. Furthermore, the expression of Fiublin-5 in gastric cancer tissues with poorly differentiated, lymph node metastasis and advanced TNM tumor stage was obviously higher. Thus, these results show that the status of Fibulin-5 may be critical for prognosis determination in gastric cancer patients.

The function of Fibulin-5 is controversial duo to its tissue specificity. Lee *et al* found that Fibulin-5 initiated and enhanced TGF- β -induced epithelial-mesenchymal

transition in mammary epithelial cells[16]. Otherwise, recent study reported that Fibulin-5 promoted cell metastasis through activating the FLJ10540/AKT pathway and its overexpression was correlated with a poor 5-year overall survival in nasopharyngeal carcinoma[4]. However, Yue *et al* demonstrated that Fibulin-5 inhibited the expression of MMP-7 and subsequently suppressed lung cancer invasion[14]. Furthermore, loss of the interaction between Fibulin-5 and beta1 integrins suppresses tumor growth by up-regulating the reactive oxygen species level[17]. But low-expression of Fibulin-5 is observed in urothelial carcinoma of bladder and Fibulin-5 overexpression remarkably reduces cell growth and invasion in human bladder cancer cell line 5637[3]. The role of Fibulin-5 in gastric cancer is poorly understood. In this study, Fibulin-5 was prominently knocked down by a specific shRNA in gastric cancer cell line MGC-803. BrdU cell proliferation and transwell cell invasion assays were performed to test cell proliferation and invasion in Fibulin-5 shRNA and NT shRNA transfected MGC-803 cells. Our data demonstrated that Fibulin-5 knockdown inhibited cell proliferation and suppressed cell invasion in MGC-803 cells. Thus, Fibulin-5 may act as a carcinogenic factor through inhibiting cell proliferation and invasion in gastric cancer.

In conclusion, we find that Fibulin-5 is up-regulated in gastric cancer tissues and its high-expression is related to malignant clinicopathologic characteristics. Moreover, we demonstrate that Fibulin-5 knockdown reduces MGC-803 cell proliferation and invasion, suggesting Fibulin-5 may facilitate tumour progression by promoting tumor growth and metastasis. Altogether, we consider that Fibulin-5 may potentially act as a clinical biomarker, and may also be a therapeutic target, in gastric cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

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