The effect and clinical significance of FN1 expression on biological functions of gastric cancer cells

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Abstract: Fibronectin 1 (FN1) is a glycoprotein molecule widely distributed in cell structures such as smooth muscle cell layer, vascular cell membrane and nerve cell layer. It participates in cell adhesion, migration and movement of various cells. In recent years, FN1 has been shown to play an important role in the regulation of various malignant tumors such as lung cancer, colorectal cancer, and ovarian cancer. However, its regulation and mechanism of action in gastric cancer have been rarely reported, and these are also associated with some controversy. The aim of this study was to investigate the clinical significance of FN1 in gastric cancer, to study the effects of FN1 on proliferation, apoptosis, migration and invasion of GC cells, and the mechanisms involved. The expression of FN1 in gastric cancer tissues was determined using immunohistochemistry staining. The comparative expression levels of FN1 were assayed with RT-PCR and Western blotting. The correlation amongst FN1 expression, clinicopathological parameters and prognosis of gastric cancer patients was determined. Cell transfection was used to silenceFN1 expression in gastric cancer cells. Plate cloning and CCK-8 assays were used to determine cell proliferation, while apoptosis was assayed with flow cytometry. Cell migration and invasion was measured with transwell assay. The expressions of EMT-related proteins were assayed using western blotting. The results showed that FN1 was upregulated in GC tissues and cell lines, and its expression level was closely related to tumor invasion, TNM stage, lymph node metastasis and survival. Inhibition of FN1 expression significantly reduced proliferation, migration, invasion and EMT processes of GC cells, and enhanced cell apoptosis. These results confirm that FN1 is up-regulated in GC, thereby functioning as an oncogenic gene. The high expression of FN1 might affect the clinicopathological parameters and prognosis of gastric cancer patients.

Key words: Gastric cancer; FN1; Clinical pathological parameters; EMT.

Introduction

Gastric cancer (GC), one of the most common malignant gastrointestinal tumors, is associated with high morbidity and mortality worldwide, thereby seriously affecting human health. With advancements in medical technology, the incidence of GC in developed countries has declined, but in developing countries, the annual growth of new cases of GC is still increasing annually. The prognosis of GC patients is poor (1). At present, the preferred treatment for GC is surgical resection, followed by chemotherapy. Improvements in endoscopic and bio-diagnostic techniques have enhanced early diagnosis of GC. However, the symptoms of GC are not obvious at the early stage. Thus, most patients are diagnosed at an advanced stage of metastasis, thereby missing the best period for surgical intervention. In effect, the prognosis of GC patients is poor. Therefore, studies on the pathogenesis of GC and the search for new early diagnostic markers and therapeutic targets, are of great significance for improving not only the 5-year survival of GC patients, but also their quality of life. The pathogenesis, infiltration and metastasis of GC are processes involving dysregulation of multiple genes. In recent years, developments in molecular biology and gene technology have provided new ideas and opportunities for the study of the molecular mechanisms involved in GC. Studies on GC have focused mainly on gene expressions, inhibition of tumor growth, and targeted therapy.

Fibronectin 1 (FN1) is an extracellular matrix glycoprotein with a molecular weight of 440 kDa. It is widely distributed in smooth muscle cell layer, vascular cell membrane and nerve cell layer, and is involved in cell adhesion, cell migration, and cell movement (2, 3). It is expressed in plasma and in a variety of cells. While FN1 produced by hepatocytes is plasma-soluble, cellular FN1 is insoluble in plasma, but soluble in cells (4). Since all FN1s are involved in opsonization, purified FN1 has been used clinically as an alternative treatment for sepsis, trauma, shock and other fields (5, 6). A number of studies have confirmed that FN1 is involved in the pathogenesis of a variety of malignant tumors. However, the regulatory role and clinical significance of FN1 in GC are still unclear and controversial. Therefore, this study was aimed at investigating the clinical significance and regulatory effects of FN1 expression.
on GC, and underlying mechanism(s).

Materials and Methods

General information on patients
A total of 56 GC patients who were treated in our hospital from 2012 to 2014 were chosen. They comprised 34 males and 22 females, aged 38-72 years, with an average age of 56.19±10.24 years. All patients underwent radical resection. Fresh tumor tissue and adjacent normal tissue specimens (greater than 5 cm) were obtained for analysis. The specimens were immediately preserved in liquid nitrogen. The patients did not undergo any anti-tumor treatment before surgery, and the GC specimens were confirmed by two senior pathologists. The patients and their families signed informed consent. The study was approved by the Medical Ethics Committee of our hospital. All patients were followed up through their regular return visits to the hospital.

Cell line and cell culture
Four gastric cancer cell lines (AGS, SGC-7901, MGC-803 and BGC-823), and human normal gastric mucosal cells (GES-1) were purchased from the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences. All cells were cultured in DMEM medium containing streptomycin (100 mg/mL), penicillin (100 U/mL) and 10% FBS at 37 °C in a cell incubator containing 5% CO₂. An FN1 silencing expression vector was constructed. The cells were stably transfected with the vector when the cells were grown to 90% confluence, and cells that stably expressed FN1 were established.

Immunohistochemistry staining
Paraffin tissue sections were prepared, routinely dewaxed, washed with distilled water and PBS buffer; placed in antigen repair solution for antigen retrieval, and heated for 10 min. The sections were cooled at room temperature, and 3% hydrogen peroxide solution was added dropwise, followed by incubation for 15 min to block endogenous peroxidase. Then, goat serum was added and incubated for 15 min. After the treatment, the serum was removed, and the sections were incubated overnight with FN1 primary antibody (1:1000 dilution) at 4 °C overnight. Then, secondary antibody was added, followed by incubation at 37 °C for 30 min. Color development was done using DAB, followed by counters. Staining with hematoxylin, dehydration and transparentizing, and sealing with neutral gum. The sections were observed under a x400 microscope, and recorded. Positive staining was judged and scored independently by two pathologists. The FN1 positive cells appeared as light yellow, brownish yellow or brown particles in the cytoplasm. The positive cells accounted for tumor cells. They were divided into 4 grades, based on the degree of positive staining: <5%, 5-30%, 31-50%, 51-70% and >71%, which were scored 0, 1, 2, 3 and 4 points, respectively. For staining intensity of the positive cells, light yellow, brown or tan were scored 1, 2 and 3, respectively. The products of these two scoring results were used as the final evaluation result, and was defined as negative by 0 ≤ IS < 3, and 3 < IS < 12 was defined as positive. For each slice, three sites were observed for analysis, and the scores were averaged.

RT-PCRs
Total RNA was extracted from various tissues and cells with total RNA extraction kit, and cDNA was obtained through reverse transcription using reverse transcription kit. The cDNA was used as a template for RT-PCR, with GAPDH as an internal reference gene. The FN1 mRNA level was determined with RT-PCR, and the corresponding primers were designed using Primer Premier 5.0 software. The upstream primer sequence of FN1 was 5’-TAC CAT CAG GTA GGC GTC ATG CAT A-3’, while the downstream primer sequence was 5’-AAG AAC TGC AAG CTG GTC TGC TGC G-3’. The upstream of primer sequence of GAPDH was 5’-GTC ACC CTG AGC GGT CCA TGT CTG TA-3’, while the downstream primer sequence was 5’-GTC CAG CCA CCT GTA TTG CTG CAC GT-3’. The expression of the target gene mRNA was calculated using the 2⁻ΔΔCt method.

Cell Proliferation assay
Cells at logarithmic growth phase in each transfection group were digested with trypsin so as to prepare a cell suspension. The cells were seeded at a density of 5×10⁵ cells/well in a 96-well plate (100μl/well), and 10μl CCK-8 reagent was added to each well. The cells were placed in a carbon dioxide incubator for 2 hours. The absorbance of each well was read at 450 nm at different times, in a microplate reader, and three replicates were set for each sample. Cells in good growth state were inoculated into a 6-well plate at a density of 1 × 10⁵ cells/well, and the cells were evenly distributed by gently shaking the 6-well plate. After 14 days of culture in a carbon dioxide incubator, the cells were washed with pre-cooled PBS buffer, and fixed with appropriate amount of 4% paraformaldehyde for 30 min, and washed 3 times with PBS buffer. Then, 2 ml of 0.1% crystal violet was added. After 2 hours of staining, cell images were obtained through photographing, and the number of clones was calculated and statistically analyzed.

Apoptosis assay
The transfected cells were made into single cell suspensions, and the cell density was adjusted to 1×10⁶/ml. After centrifugation, the supernatant was discarded, 1 ml of pre-cooled 75% ethanol was added, mixed, and then fixed on ice overnight. The fixed cells were washed with PBS buffer, centrifuged at 1000 °C for 5 min at 4 °C, and the supernatant was discarded. The cells were resuspended in 100μl of PBS buffer, and 5μl of Annexin-V-FITC and 10μl of PI were added. After careful mixing and incubation for 30 min in the dark, the cells were subjected to flow cytometric analysis. There were 3 replicates in each group.

Migration and invasion assay
Cell migration assay
The cells were starved for 24 h, trypsinized and seeded into a single cell suspension, and the cell concentration was adjusted to 5×10⁴ cells/ml. Then, 600μl of RPMI-1640 medium containing 10% FBS was added to a 24-well plate, and the Transwell chamber was placed directly into a 24-well plate. Then, 200μl of the cell sus-
expression was added to the upper chamber, and placed in a 5% CO₂, incubator at 37 °C for 18 h. After the incubation, formaldehyde was added, followed by fixing at room temperature for 30 min. The cells were washed thrice with PBS buffer, and cells that did not pass through the surface of the upper chamber were wiped off. After drying, 5 fields of view were randomly selected, photographed and counted under a microscope.

Cell invasion assay

Matrigel was pre-cooled with serum-free RPMI-1640 medium at a ratio of 1:8. Then, 100μl of Matrigel was evenly added to the upper Transwell chamber, and incubated at 37 °C for 1 h to achieve a solid state. The cells were seeded at a density of 5 x 10⁵ cells/ml. The other steps were consistent with those in the cell migration experiments. The number of cells that passed through the Transwell Matrigel and the membrane into the lower chamber reflected the invasive ability of the cells.

Western blotting

The total protein in each tissue sample and cell sample was extracted using RIPA lysis solution, and the protein concentration was determined with BCA method. Then, 40 μg of the protein sample was subjected to SDS-polyacrylamide gel electrophoresis, and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk powder solution, after which it was incubated with FN1 primary antibody (1:500) overnight at 4°C. After rinsing, the membrane was incubated with secondary antibody (goat anti-rabbit, 1:10000) for 1 h at room temperature. Thereafter, the membrane was washed three times with PBST (10 min for each wash), and chemiluminescent substance and chromogenic reagent were added. The membrane was then subjected to DAB color development in the dark. Image acquisition was performed using a gel imaging system, while grayscale analysis was done with Quantity One software.

Statistical analysis

This was done using SPSS19.0 software. All data are presented as mean ± standard deviation (SD). The results were statistically compared between 2 groups using t-test, while analysis of variance was used for comparison of multiple groups. Values of p <0.05 were considered as indicative of statistical significance of differences.

Results

Immunohistochemical assay of FN1 expression in gastric cancer

The expression levels of FN1 in GC tissues and their matched normal tissues were assayed with immunohistochemical staining. The results showed that 34 out of 56 GC tissue samples were positive for FN1 expression, while 9 cases were positive in adjacent normal tissues. Therefore, the degree of positive expression of FN1 in tumor tissues (60.7%) was significantly higher than that in adjacent tissues (16.1%) (p<0.05). These results are shown in Fig. 1.

Expression of FN1 mRNA and protein in gastric cancer

The expression of FN1 mRNA in 56 cases of GC tissues and adjacent normal tissues was determined with RT-PCR. The results showed that the average comparative expression level of FN1 mRNA in GC tissues was 1.68±0.11, which was significantly higher than that in adjacent normal tissues (1.00±0.03) (p<0.05). Western blotting results showed that the protein expression level of FN1 in tumor tissues was significantly lower than that in normal tissues, which was consistent with RT-PCR results, indicating that the expression level of FN1 was up-regulated in GC. These results are shown in Fig. 2.

Correlation between FN1 expression and clinicopathological parameters in patients with GC

Results showed that FN1 expression was closely related to TNM stage, depth of invasion, and lymph node metastasis (p<0.05). However, FN1 expression was not significantly correlated with gender, age, degree of differentiation and tumor size (p >0.05), as shown in Table 1. These results indicate that the expression of FN1 affects the degree of tumor progression in GC patients.

Correlation between FN1 expression and prognosis in GC patients

As shown in Fig. 3, FN1 expression was significantly correlated with patient's overall survival (OS) and disease-free survival (DFS). The mean OS and DFS...
in low FN1 expression were significantly higher than those of FN1 ($p<0.05$), implying that patients with low expression of FN1 had longer OS and DFS. Thus, high FN1 expression of FN1 affects the prognosis of GC patients.

**Expression level of FN1 in GC cell lines**

The expression of FN1 mRNA in cells was determined with RT-PCR. The results showed significantly increased expression of FN1 mRNA in four GC cell lines ($p<0.05$). Furthermore, results from Western blotting showed that the expression level of FN1 protein in each GC cell line was consistent with the mRNA expression level. Therefore, FN1 was highly expressed in the various cell lines. There were significant differences in GES-1 among normal gastric mucosal cells ($p<0.05$), with FN1 having the highest degree of up-regulation in SGC-7901. Thus, SGC-7901 was selected for use in subsequent functional studies. These results are presented in Fig. 4.

**Establishment of a stable silencing FN1 SGC-7901 cell line**

In a previous study, siRNAs that stably inhibited FN1 expression were successfully designed and screened, and siRNA-FN1 and its negative control siRNA-con were transfected into GC cell SGC-7901. Blank control was set as NC group. To verify the transfection efficiency, the comparative expression levels of FN1 mRNA and protein in the three groups were determined with RT-PCR and Western blotting after 48 h of transfection. The results showed that the comparative FN1 mRNA and protein expression levels in siRNA-FN1 cells were significantly lower than those in siRNA-con group ($p<0.05$), while the expression levels of FN1 mRNA and protein in siRNA-con group were comparable with those in NC group, indicating successful transfection.

Moreover, the expression vector did not cause cytotoxicity to GC cell SGC-7901, as shown in Fig. 5.

**Effect of FN1 on proliferation of GC cells**

After transfection for 48 hours in GC cell line SGC-7901, cell proliferation in each group was determined with CCK-8 assay. The results showed that the proliferation capacity of siRNA-FN1 group was significantly lower than that of siRNA-con group and NC group ($p<0.05$). The results of plate cloning experiments showed that the number of cloning cells in the siRNA-FN1 group was $194.5\pm6.8$, which was significantly lower than that in the negative control siRNA-con group ($399.7\pm5$) and the blank control NC group ($411.6\pm15.3$).

### Table 1. Correlation between FN1 expression and clinicopathological parameters in patients with GC.

<table>
<thead>
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<th>Clinical features</th>
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Figure 3. Correlation of FN1 expression with overall survival (OS) and disease-free survival (DFS) in GC patients. A. Relationship between FN1 expression and patient OS; B. Relationship between FN1 expression and patient DFS.

Figure 4. Expression of FN1 mRNA and protein in gastric cancer cell lines.
FN1 is associated with tumorigenesis and progression in gastric cancers.

**Effect of FN1 on apoptosis of GC cells**

As shown in Fig. 7, GC cell apoptosis in siRNA-FN1 group was significantly higher than that in siRNA-con group and NC group ($p<0.05$). Thus, inhibition of FN1 expression promoted apoptosis of GC cells.

**Effect of FN1 on cell migration and invasion of GC cells**

The results of Transwell migration assay showed that the migration capacity of SGC-7901 cells was significantly decreased after silencing FN1 ($p<0.05$), indicating that FN1 promoted the migration capacity of GC cells. Similar results were seen in the Transwell invasion assay. The average number of invasive cells in the siRNA-FN1 group was significantly lower than that in the siRNA-con and NC groups ($p<0.05$), indicating that FN1 promoted the cell migration and invasion in GC cells.

**FN1 inhibited gastric cancer EMT**

To determine whether FN1 is involved in the EMT of GC, Western blotting was used to determine the expression of EMT-related markers in GC cells. The results showed that the expression levels of N-cad and Vimentin proteins were significantly decreased after overexpression of FN1. The expression level of E-cad was significantly increased (Fig 9), indicating that FN1 promoted the EMT of GC.

**Discussion**

The optimal treatment for GC is radical surgery. However, early symptoms of GC are not usually obvious. Moreover, there are certain shortcomings in early clinical screening methods, which result in majority of GC patients missing the best operation period at the time of diagnosis. For GC patients unable to undergo surgical resection, drug therapy is the main treatment.
Conventional chemotherapy regimens such as cisplatin, fluorouracil combination or combination with paclitaxel have improved GC patient survival in recent years. However, multi-drug resistance, tumor metastasis and other factors result in poor therapeutic outcomes in some patients, thereby decreasing their overall 5-year survival (7). The occurrence of GC is the result of long-term effects of various carcinogenic factors. Activation of proto-oncogenes and inactivation of tumor suppressor genes are closely related to the pathogenesis of GC. Fibronectin 1 is an extracellular matrix with multiple functions, and it is a macromolecular glycoprotein. It acts as a ligand for the integrin receptor family, and it is involved in cell adhesion and cell migration (8).

In recent years, the regulation of FN1 in the development of malignant tumors has attracted much research attention. With respect to local infiltration and invasion of tumors, FN1 binds to integrin transmembrane receptors on the surface of various malignant tumor cells, regulates the adhesion of tumor cells to ECM, and affects the removal of tumor cells and ECM proteins. In other words, tumor cells can pass through the basement membrane and induce tumor cell migration under the induction of matrix degradation products and insulin-like growth factors (9, 10). Studies have reported that FN1 expression is significantly up-regulated in colorectal cancer tissues and cell lines. Through gene knockout of FN1, the inhibition of cell proliferation, migration and invasion of colorectal cancer cells SW116 and LOVO cells were observed (11). Wang et al. used RT-PCR to assay the expression of FN1 mRNA in 109 renal cell carcinoma tissue samples. The results showed that the comparative expression of FN1 in tumor tissues was nearly 7 times that of kidney tissue, and its expression level was significantly correlated with clinical stage (12). Another study reported that high expression of FN1 was seen in invasive human bladder cancer T24 cells, and that inhibition of FN1 expression reduce the migration capacity of T24 cells through a mechanism related to targeted of PKC signaling pathway (13). In cervical cancer, high expression of FN1 activates the expression of pro-MMP-9, resulting in a signaling cascade (14). Than et al. reported that FN1 activated MMP-2 promoter, thereby increasing the expression of MMP-2 in ovarian cancer cell OVCA, while high expression of MMP-2 further enhanced the combination of OVCA cells and fibronectin which is broken down into small segments and cleavage receptor integrin. Thus, it promotes peritoneal adhesion in ovarian cancer cells (15).

In this study, immunohistochemistry was first used to measure the expression level of FN1 in 56 GC tissues and corresponding adjacent normal tissues. It was found that the degree of positive expression of FN1 in gastric cancer tissues was significantly higher than that in adjacent normal tissues. Furthermore, the level of FN1 mRNA in tumor tissues was determined with RT-PCR. The results were consistent with those of immunohistochemical staining. Therefore, FN1 was up-regulated in GC tissues. Furthermore, FN1 expression was closely related to TNM stage, depth of invasion and lymph node metastasis. Therefore, patients with high FN1 expression had higher TNM stage and depth of invasion than those with low expression of FN1, and those with high FN1 expression were more likely to have lymph node metastasis, suggesting that FN1 may promote local invasion and distant metastasis of GC cells through certain molecular mechanisms. Some clinical studies have found that some gastric cancers that grow into the cavity have large tumors but only infiltrate to the submucosa, while some GCs that grow out of the cavity have small tumors but can infiltrate into the serosa layer (15). These are consistent with the findings in this study, in that FN1 expression was significantly associated with invasive depth, and had no significant correlation with tumor size. Kaplan-Meier survival curve was used to analyze the prognosis of GC patients. It was found that the overall survival and disease-free survival of patients with low FN1 expression were significantly higher than those with high FN1 expression. Therefore, the patients with high FN1 expression had shorter survival cycle, suggesting that FN1 expression affects the prognosis of GC patients. Our results were consistent with previous report that the expression of FN1 in GC tissues is up-regulated compared with adjacent normal tissues, and it is a potential biomarker of poor prognosis in patients with GC [16].

Furthermore, the effect of FN1 on the biological function of GC cells was studied at the cellular level. It was found that the expression levels of FN1 mRNA and protein in GC cell lines were significantly higher than those in normal gastric mucosal cells, and the comparative expression levels of FN1 mRNA differed amongst different GC cell lines. The SGC-7901 with the highest comparative expression level was chosen as research target. Various molecular technologies such as small RNA interference (siRNA) and gene cloning have played important roles in the study of the mechanisms involved in the pathogenesis of GC. It is known that siRNA is a chemically modified double-stranded RNA which can inhibit the expression of specific genes after induction of transcription, and has high interference efficiency. It can be recognized by dicer enzyme after entering the cells through transient transfection. It also binds to intracellular RISC and degrades homologous mRNA, thereby silencing the expression of specific genes (18, 19). Therefore, in order to study the effect of FN1 expression on GC, the expression of FN1 in SGC-7901 cells was silenced with transfection, and the transfection efficiency was determined with RT-PCR. After successfully establishing silenced expression of FN1 in GC cell lines, cell proliferation of each group was determined using CCK-8 method and plate cloning assay. It was found that the proliferation of SGC-7901 cells was significantly lower than those of the negative control group and NC group after silencing FN1 expression, indicating that FN1 promoted the proliferation of GC cells. Results from flow cytometry showed that apoptosis of SGC-7901 cells was significantly increased after silencing FN1 expression. Therefore, FN1 promoted the apoptosis of GC cells.

Clinically, tumor invasion and metastasis are major difficulties in the treatment of GC, and they are also important factors in tumor recurrence. A variety of enzymes and protein factors are involved in the regulation of tumor invasion and metastasis. These include matrix metalloproteinase which promote the formation of blood vessels around the tumor by degrading the extracellular matrix, thereby enhancing the ability of the tumor to in-
vade the surrounding tissues (20). Therefore, cell matrix degradation and cancer cell detachment are among the major factors involved in tumor invasion and metastasis. Generally, tumor cells undergo secondary invasion through endothelial target and basement membrane. To determine the effect of FN1 on migration and invasion of gastric cancer cells, Transwell assay was used to analyze GC cell migration and invasion. The results of cell migration assay showed that after silencing FN1 expression, the number of cells passing through the membrane of SGC-7901 cells was significantly lower than that of the control group, indicating marked weakening of the migration ability of the cells. Similar results were obtained in the Transwell invasion assay: cell invasion in the siRNA-FN1 group was significantly lower than that in the siRNA-con and NC groups, indicating that FN1 promoted the migration and invasion capacity of GC cells. Epithelial-mesenchymal transition (EMT) is generally considered a hallmark of tumor migration and invasion. It refers essentially the transformation of epithelial-derived tumor cells from an epithelial phenotype to a mesenchymal phenotype (21-23). Results from western blotting showed that after overexpression of FN1, the expression levels of N-cad and Vimentin proteins in GC cells were significantly decreased, while the expression levels of E-cad were significantly increased, indicating that FN1 promoted EMT in GC.

In summary, this study confirmed that FN1 is up-regulated in GC, and that high expression of FN1 might affect the degree of progression and prognosis of GC patients. These findings indicate that FN1 has important clinical significance. It promotes cell proliferation, migration, invasion and EMT of GC cells, and inhibits their apoptosis. Thus, FN1 is a cancer-promoting gene.

Acknowledgments
None.

Conflicts of interest
There are no conflicts of interest in this study.

Author’s contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Xinguo Zhu; Yuan Zhou, Guangxin Cao, Hongyu Cai, Haijing Huang, Xinguo Zhu collected and analysed the data; Yuan Zhou and Guangxin Cao wrote the text and all authors have read and approved the text prior to publication. Yuan Zhou and Guangxin Cao contributed equally to this work as co-first author.

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