Abnormal expression of FOSB correlates with tumor progression and poor survival in patients with gastric cancer

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Abstract. FOSB protein is encoded by the FOSB gene in humans, which shares structural similarities with the prototype of the Fos family. FOSB plays a role by AP-1 complex which is composed of heterodimers of Jun and Fos members. Our experiment aimed to evaluate the effect of FOSB in gastric cancer (GC) patients and then probe its significance in prognosis. We detected the expression of FOSB in GC and adjacent non-cancerous tissues by western blot analysis and real-time quantitative PCR (qRT-PCR). Moreover, we analyzed FOSB expression in patients who underwent resection procedures using immunohistochemistry. The relationship between the expression of FOSB, the clinicopathological characteristics and the patients survival were also investigated. Furthermore, in vitro, we evaluated the effects of FOSB gene on gastric cancer cell viability, proliferation and migration by MTT, clone formation and Transwell assays. Finally, the Kaplan-Meier method and log-rank test were used to compare the overall survival between high FOSB expression group and low FOSB expression group. Immunohistochemical staining data showed that FOSB expression was significantly decreased in gastric cancer cases. In addition, we confirmed FOSB downregulation in both mRNA and protein levels in GC tissues compared with matched adjacent non-cancerous tissues. Downregulated expression of FOSB was correlated with poor differentiation, lymph node metastasis and advanced TNM stage. Moreover, we found that low FOSB expression exhibited a significant correlation with poor prognosis for GC patients by Kaplan-Meier survival analysis. Overexpression of FOSB significantly suppressed cell proliferation, clone formation and migration in GC cell lines. In contrast, silencing of FOSB expression in GC cells promoted proliferation, clone formation and migration. Our results showed that FOSB plays a crucial role in the suppression of GC, and that it may be a useful biomarker in diagnosis and prognosis for GC patients.

Introduction

According to WHO, gastric cancer (GC) is the fourth most common cancer and the second most common cause of cancer-related death worldwide (1). In China, gastric cancers are considered to be the second most frequently diagnosed cause of cancer death (2). Recently, owing to the global environment worsening and change in people's habits, incidence of gastric cancer has increased year by year. Despite some advances in diagnosis, staging and treatment of GC, especially surgery, chemotherapy and radiotherapy, the long-term survival rate remains quite low because of local invasion and distant metastasis. The incidence, development, infiltration and metastasis of GC are a multistep and multifactor process. It may be regulated by many genes and involves a variety of gene activation, regulated disorder or inactivation (3). Recently, many characteristic biological markers are being studied, which may be very helpful to clinicians to predict metastatic progression and prognosis of GC patients.

FBJ murine osteosarcoma viral oncogene homolog B, also known as FOSB (in humans) or FosB (in other species), is a protein that is encoded by the FOSB gene that is localized on chromosome 19q13 and is composed of four exons in humans (4,5). FosB is an acidic protein of 338 amino acids which shares structural similarities with the prototype of the Fos family, c-Fos, namely, a prolin-rich basic DNA-binding region, a leucine zipper required for dimer formation, and a C-terminal transactivation domain (6). FOSB gene encodes leucine zipper proteins that dimerize with proteins of the JUN family, thereby forming the transcription factor complex

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AP-1. AP-1 regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress and bacterial and viral infections (7). It was shown in various experimental systems that stronger proliferation, malignant transformation and enhanced aggressiveness are accompanied by a change in AP-1 complex composition (8-10). Similarly, the inhibition of proliferation and induction of differentiation processes leads to a shift of expression of the individual AP-1 proteins (11-13). In the past several years, some studies identified that downregulated expression of FOSB is frequent in breast carcinomas (14) and pancreatic cancer (15). Up to now, there has not been research reporting the role of FOSB in GC. Thus, the purpose of the present study was to determine FOSB expression in human GC patient specimens and then to evaluate the clinicopathological implications of FOSB expression in GC. Furthermore, we investigated the potential influence of FOSB in the development of GC.

Materials and methods

Tissue collection. We retrospectively analyzed clinicopathological data from 116 gastric cancer patients who underwent surgical resection at the Second Affiliated Hospital of Nantong University from January 2008 to December 2010, and obtained fresh gastric cancer and surrounding non-cancerous tissue samples randomly from 40 GC patients. After surgical resection, fresh samples were frozen in liquid nitrogen immediately and divided into two parts, and one was maintained at -80˚C until use for real-time PCR, the other used for western blot analysis. Paraaffin-embedded samples were obtained from 116 GC patients for immunohistochemistry. The clinical information related to the 116 GC patients, including gender, age, tumor size, TNM stage and lymph node metastasis was also collected. Staging and grading were referred to the classification of the International Union against Cancer (UICC) criteria. None of the patients were administered preoperative radiotherapy or chemotherapy. All human tissues were collected using protocols approved by the Ethics Committee of The Second Affiliated Hospital of Nantong University.

Western blot analysis. Paired cancer and their paracancerous tissue specimens were used for western blot analysis. We used lysis buffer which contain protease inhibitors (Promega, Madison, WI, USA) to extract total protein. Then, equal amounts (30 µg) of protein separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After that, they were transferred to a polyvinylidene fluoride (PVDF) membrane, and we used 5% non-fat milk in TBST (Tris-buffered saline containing 0.1% Tween-20) to block non-specific binding for 2 h. After the incubation with the primary antibodies overnight at 4˚C a rabbit polyclonal anti-human FOSB antibody (1:100, sc-48; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or a rabbit anti-β-actin as internal reference, at 1:2,000 dilution (Sigma-Aldrich, St. Louis, MO, USA), membranes were washed three times in TBST for 5 min and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1,000 dilution; Sigma-Aldrich) for 2 h at room temperature. Finally signals were scanned with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA), and analyzed with PDQuest 7.2.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

Quantitative real-time PCR. The mRNA expression of FOSB was analyzed by the quantitative real-time PCR. Total RNAs were isolated from tumor tissue samples (100-200 mg) adjacent to the region which was used for protein extraction. Quantitative real-time PCR was performed using HotStart-IT SYBR-Green qPCR Master Mix (2X; USB Corp., Cleveland, OH, USA). On the basis of the HotStart-IT protocol, 25 µl reactions were run with 2 µl of cDNA. In addition, RT-PCR experiments were performed in a LightCycler 480 system (Roche Applied Science). PCR procedures: first, hot start at 95˚C for 10 min; 40 cycles of amplification/quantification at 95˚C for 10 sec, then 60˚C for 30 sec, and 72˚C for 30 sec when fluorescence was measured. Melting curve analysis was performed using continuous fluorescence acquisition from 65 to 97˚C. These cycling parameters generated single amplicons for both primer sets used according to the presence of a single melt peak. The sequences of the primers for FOSB were: FOSB forward, 5’-TGACAGTTTATCCCAAGCCC-3’ and FOSB reverse, 5’-CCAGGAGGCCGCAATC-3’. The β-actin was selected as the internal reference. All quantitative real-time PCRs were repeated three times for each gene and each sample was done in triplicate.

Immunohistochemistry. The tissue sections were deparaffinized with dimethylbenzene and rehydrated through 100, 95, 90, 80 and 70% ethanol. After three washes in phosphate-buffered saline (PBS), the slides were boiled in antigen retrieval buffer containing 0.01 M sodium citrate-hydrochloric acid for 10 min in a microwave oven. After rinsing with PBS, the tissue sections were incubated with a rabbit polyclonal anti-human FOSB antibody (1:100; Santa Cruz Biotechnology) and the slides were then rinsed in 3% hydrogen peroxide to block endogenous peroxidase. The sections were incubated with a donkey anti-rabbit second antibody conjugated horseradish peroxidase (1:5,000; Abcam, Cambridge, UK) at 4˚C overnight and then incubated with horseradish peroxidase (HRP) (at room temperature for 30 min. After washing in PBS, the visualization signal was developed with 3,3’-diaminobenzidine (DAB) solution, and all of the slides were counterstained with hematoxylin.

The total FOSB immunohistochemistry staining score was calculated as the sum of the percentage of positively stained cells and the staining intensity. Briefly, the percentage of positive staining was scored as 0 (0-9%), 1 (10-25%), 2 (26-50%) or 3 (51-100%), and the intensity was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining) or 3 (strong staining). The expression level of FOSB was defined as follows: ‘-’ (negative, score of 0), ‘+’ (weak positive, score of 1-3), ‘++’ (positive, score of 4-6) and ‘+++’ (strong positive, score of 7-9). We defined high FOSB expression as a total score of >3, and low FOSB expression as a total score of <3.

Cell culture, plasmid construction and transfection. The human GC cells lines, AGS, SGC7901, BGC823 and MKN45 and the human normal gastric epithelial mucosa cell lineGES-1 were all obtained from the Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of
USA) according to the manufacturer's instructions. In addition, RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) was transfected into GC cells, respectively, using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In addition, siRNA3 could effectively reduce endogenous FOSB expression by western blotting assay. Therefore, siRNA3 was chosen for downstream experiment.

FOSB overexpression vector pcDNA 3.1(+)–FOSB was constructed using the PCR method. The PCR products were confirmed by direct DNA sequencing and cloned into the mammalian expression vector pcDNA 3.1(+). We obtained stably transfected clones by G418 selection (Promega). A stable transfectant of the pcDNA 3.1(+) vector was used as a control. For transfection, pcDNA 3.1(+)–FOSB expression plasmids were transfected into GC cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The level of FOSB expression after transfection was analyzed by western blot analysis.

Cell viability assay. Cell viability was tested by using MTT assay. All cells were plated in 96-well plates at 5x10^3 cells/well in complete medium and cultured for 24 h, and replaced with medium which contained 10% FBS, then 10 µl MTT was added to each corresponding test well and the plates were further incubated for 4 h. All samples were measured at 490 nm spectrophotometric absorbance. All experiments were performed in triplicate.

Colonies formation assay. After transfection, cells (5x10^4/well) were separately plated in a 24-well plate. After 24 h, the cells were collected and seeded (1,000-1,500/well) in a fresh 6-well plate for 12 days. Surviving colonies (>50 cells per colony) were counted after fixed with methanol/aceton (1:1) and stained with 5% Gentian violet (ICM Pharma Pte. Ltd., Singapore, Singapore), after that rinsed three times with PBS to remove excess dye, photographed and counted. The experiment was carried out in triplicate wells three times.

Cell migration assay. According to the manufacturer's instructions, the cell migratory capacity was determined by Transwell chambers (BD Biosciences, San Jose, CA, USA). After transfection, cells were harvested at 24 h and then 3.0x10^5 transfected cells or untreated cells in serum-free medium were added to each upper surface. The chemotactic factor, DMEM medium (Gibco) which contained 10% FBS (500 µl) was added to the lower chamber. After incubation for 48 h, non-migrated cells on the upper surface were removed gently with a cotton swab and the migrated cells on the lower membrane surface were fixed in methanol. Cells were stained with 0.1% crystal violet, photographed and counted. The experiment was performed in triplicate and repeated three times.

Statistical analysis. The SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The relationship between the FOSB expression level and the clinicopathological characteristics was subjected to the χ² test. Survival curves were calculated by the Kaplan-Meier method and the differences were analyzed with the log-rank test. A multivariate analysis of several prognostic factors was carried out using the Cox proportional hazards regression model. The results were expressed as the mean ± SD of at least three independent experiments, for all statistical analyses. P-value <0.05 was considered to be statistically significant.

Results

FOSB expression analyzed in gastric cancer tissue samples by western blot analysis and qRT-PCR. Twenty-four gastric cancer specimens as well as their adjacent non-cancerous tissues were selected from 40 gastric cancer patients randomly and used to evaluate FOSB protein expression by western blot analysis. The representative results of western blot analysis in five cases are shown in Fig. 1A. The results show that the expression of FOSB protein level was significantly downregulated in most of GC tissues (79.17%, 19 of 24) compared with adjacent non-cancerous tissues (P=0.004). We also performed qRT-PCR analysis in 40 GC tissues and matched adjacent non-cancerous tissues. As shown in Fig. 1C, the average relative expression of FOSB mRNA level was significantly lower in GC tissues compared with paired adjacent non-cancerous tissues (P=0.004).

To further investigate FOSB expression level, a total of 116 paraffin-embedded GC blocks were evaluated by IHC analysis. We found that FOSB was expressed at various levels in the GC tissues and the adjacent non-cancerous tissue samples (Fig. 2). Overall, 64 of 116 (55.17%) cases showed low FOSB expression (FOSB - or FOSB +) in GC tissues, while the remaining
52 (44.83%) cases displayed high SPOP expression (FOSB ++ or FOSB ++++) (Table I). The adjacent non-cancerous tissues showed the strongest FOSB-positive staining (Fig. 2A).

**Overexpression of FOSB inhibits gastric cancer cell viability, proliferation and migration in vitro.** FOSB was significantly decreased in GC tissues and acted as a tumor suppressor gene. MKN45 cell line with the lowest expression of FOSB of the five tested GC cell lines (Fig. 3) was chosen to examine whether overexpression of FOSB affected cell viability, proliferation and migration in GC. The effects of FOSB on cell viability and proliferation were evaluated by MTT and colony formation assay, and the results showed that overexpression of FOSB suppressed the viability and the number of colonies of MKN45 cells (Fig. 4B and C; P<0.05). In addition, these results were further confirmed by assay, overexpression of FOSB could decrease the transformation phenotype of GC cells in vitro. Besides, we investigated the potential role of FOSB on cellular migration by Transwell assays. MKN45 cells were transfected with FOSB overexpressing plasmid or control plasmid and seeded in the chamber, and their migratory abilities were determined 24 h later. The results showed that the overexpression of FOSB inhibited the migratory capacity of MKN45 cells (Fig. 4C; P<0.05).

**Knockdown of FOSB expression promotes gastric cancer cell viability, proliferation and migration in vitro.** To further study the effects of FOSB on the viability, proliferation and migra-
tion of gastric cancer cells, SGC7901 cells that possessed the highest FOSB expression in the four GC cells (Fig. 3) were transfected with FOSB siRNA1, FOSB siRNA2 and FOSB siRNA3. Western blot analysis showed that siRNA3 reduced the level of endogenous FOSB expression more significantly than siRNA1 and siRNA2 (Fig. 5A). The effects of FOSB on cell viability and proliferation was assessed by MTT and colony formation assays, and the results indicated that downregulation of FOSB expression promoted the viability of SGC7901 cells and increased the number of colonies of Table I. Correlation between the FOSB expression and the clinicopathological characteristics in patients with gastric cancer.

<table>
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<th>High (n=52)</th>
<th>P-value</th>
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*P-value <0.05 was considered statistically significant.

Figure 4. Overexpression of FOSB inhibited the viability, proliferation and migration of GC cells *in vitro*. (A) Western blot analysis of FOSB protein expression levels. The FOSB overexpression transfectant (FOSB) and the empty vector transfectant (vector) are shown. (B) Cell viability was evaluated with MTT assay using absorbance readings at 490 nm. MKN45 cells were transfected with pcDNA3.1 (+)-FOSB or empty vector for 1, 2, 3 and 4 days, respectively. The values are indicated by the mean of three determinations. (C) Colony numbers of the MKN45/FOSB and control cells in plate colony formation assays. The data represent the means ± SEM of three independent experiments. (D) Transwell assays demonstrated that overexpression of FOSB in MKN45 cells transfected with pcDNA3.1 (+)-FOSB plasmid suppressed cell migration compared with empty vector (*P<0.05). Representative photos of stained cells are shown with the original magnification of x100.
SGC7901 cells compared with the control cells (Fig. 5B and C; P<0.05). In addition, knockdown of FOSB expression dramatically reduced the migrated cell number of SGC7901 cells by Transwell assay (Fig. 5D; P<0.05).

FOSB expression is correlated to clinicopathological factors in GC. Chi-square test was used to analyze the correlation between the FOSB expression in GC tissues and various clinicopathological characteristics. Table I, indicates that we found...
that decreased expression of FOSB was significantly correlated to poor differentiation (P<0.001), lymph node metastasis (P=0.001) and high TNM stage (P=0.021). However, there was no statistically significant correlation between FOSB expression and gender, age, tumor size, location, invasive depth, *H. pylori* infection and distant metastasis (P>0.05). To further estimate the correlation between the FOSB expression and the prognosis of GC patients, we used Kaplan-Meier survival curves and the log-rank test to investigate the prognostic effect of FOSB on the overall survival rate of GC patients by comparing the 5-year survival rate of patients with high or low levels of FOSB expression. The patients with the low levels of FOSB expression had a poorer prognosis than the patients with high levels of FOSB expression ($\chi^2$, 13.421, P<0.001; Fig. 6).

Univariate and multivariate analysis was performed to evaluate the independent prognostic roles of FOSB (Table II). Univariate Cox regression analysis showed that histological grade, invasive depth, TNM stage, lymph node metastasis and FOSB expression were significantly associated with overall survival in GC patients. Moreover, multivariate Cox regression analysis confirmed FOSB expression and TNM stage as independent predictors of the overall survival of GC patients.

### Discussion

Gastric cancer has one of the highest incidence rates in the world, and the 5-year survival rate of is less than 20-25% in China because of frequent relapse and high metastasis rate postoperatively (16), and most patients present advanced disease at diagnosis making its treatment very intricate (17). It is widely accepted that early diagnosis and treatment are keys for better clinical outcome in patients with gastric cancer (18). There have been many advances in diagnostics and therapeutics in GC, however, dismal prognosis, despite these improvements, still persist (19). Many cancer-related molecules have been characterized with the goal of developing novel anticancer therapies, including targeted drugs or antibodies and cancer vaccines (20). FosB is known as a kind of the biological markers, but there is no related report on FOSB in GC.

FOSB is a member of the Fos gene family. It is activated by growth factors and is a nuclear protein of 338-amino acids that shows 70% homology with Fos. Furthermore, like Fos, it forms complexes with the Jun-family of transcription factors 8, suggesting that it is involved in regulating gene expression (6). Together with the Jun family members, the Fos family of transcription factors form the group of AP-1 proteins which, after dimerization, bind to so-called TPA-responsive elements in the promoter and enhancer regions of target genes (21). In contrast to Jun proteins, Fos family members are not able to form homodimers, but heterodimerize with Jun partners, giving rise to various trans-activating or trans-repressing complexes with different biochemical properties (22). AP-1 in turn controls a number of cellular processes including differentiation, proliferation and apoptosis (7). To further analyze the role of the FOSB transcription factor in GC and to explore whether the level of FOSB expression is involved in GC cell viability, proliferation and migration, we used immunohistochemistry on paraffin sections of 116 gastric carcinomas. According to our new measurements, we demonstrated that FOSB was expressed higher in adjacent non-cancerous tissues than in GC tissues. In addition, we also found that a downregulated expression of FOSB was markedly connected with advanced TNM stage, poor differentiation and lymph node metastasis. Thus, the abnormal expression of FOSB might be involved in GC tumor progression and metastasis, and we speculated that FOSB may play a tumor suppressor role in GC. Subsequently, qPCR and western blot analysis were used to find that FOSB expression was decreased at the mRNA and protein levels in most cancer tissues compared to their adjacent non-cancerous tissues. Moreover, we showed that the viability, proliferation and migration in vitro of MKN45 cells were observably inhibited by overexpression of FOSB, whereas knockdown of FOSB expression promoted the viability, proliferation and migration of SGC7901 cells in vitro. Furthermore, it is well known that a high prevalence of *H. pylori* is always accompanied by a high incidence of gastric cancer (23). One study demonstrates that *H. pylori* positivity is a beneficial prognostic indicator in patients with gastric cancer (24). However, in the present study, there was no significant discrepancy in the expression of FOSB in the patients with and without *H. pylori* infection.

In accordance with the Kaplan-Meier survival, it showed that low FOSB expression had significant relationship with shorter survival time of GC patients. Multivariate analysis suggested that in patient overall survival, FOSB expression was an independent prognostic indicator. Moreover, in a study on human keratinocytes, the calcium-induced differentiation and expression of the differentiation marker involucrin was accompanied by an increase of FOSB and a decrease of Fra-1 expression (13). Regarding the functional differences between Fos family members, FOSB is the only AP-1 protein which cannot bind and inactivate glucocorticoid receptors (25). Vice versa, unlike other AP-1 family members, FOSB proteins cannot be blocked by GR proteins which are expressed in the epithelial cells of terminal lobular units and which play a role in the differentiation of mammary epithelial cells (26,27).
The knowledge on FOSB is still not complete, thus, further research on the effect of FOSB will be necessary. The mechanisms which lead to the downregulated expression of FOSB in GC patients still require further investigation.

The above data show that the downregulated expression of FOSB is involved in tumor progression of GC patients and might represent a prognostic indicator. Thus, extra experiments will be necessary to prove this hypothesis and that FOSB may play a useful role in new therapeutic interventions in GC patients.

References


