Basic Research Paper

Effects of FRZB on growth and metasasis of gastric cancer cell line SGC-7901

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Abstract

Background and Objective: FRZB (frizzled motif associated with bone development), a member of the secreted frizzled related protein (sFRP) family, plays an important role in embryonic development. The expression of FRZB can suppress the invasion ability of prostate cancer by inhibiting the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9. This study was to investigate the effects of FRZB on tumorigenicity and metastasis of gastric cancer cell line SGC-7901. Methods: FRZB expression vector and empty vector were constructed and transfected into SGC-7901 cells to obtain stable clones (SGC-7901/FRZB and SGC-7901/VECTOR). Cell proliferation was detected by MTT assay. The tumorigenicity was investigated by soft agar colony formation test (in vitro) and xenograft mouse model (in vivo). The adhesive and invasive abilities of SGC-7901/FRZB cells were determined by Boyden chambers. The expression of MMPs in SGC-7901 cells was tested by immunocytochemistry. Results: The expression of FRZB was increased in SGC-7901/FRZB cells. Growth rate and colony formation rate of SGC-7901/FRZB cells were decreased to about 60% and 15% as compared with those of SGC-7901/vector cells, respectively. The positive dyeing of MMP-2, MMP-7 and MMP-9 in SGC-7901/FRZB cells was reduced. The numbers of invasive SGC-7901, SGC-7901/vector and SGC-7901/FRZB cells were 55.90 ± 5.68, 54.80 ± 6.97, 6.60 ± 2.63, respectively. Conclusion: FRZB exhibits anti-tumor ability in gastric cancer cell line SGC-7901 in vitro and in vivo, and decreases the expression of MMP-2, MMP-7 and MMP-9.

Gastric cancer is the second cause of cancer death in the world and ranks as the most common gastrointestinal malignancies in China.1 According to the study on cancer patients in USA, 23.24% of cancer deaths are caused by gastric cancer. Although the 5-year survival rate of early stage gastric cancer patients may reach up to 78% after gastrectomy, the 5-year survival rate of advanced gastric cancer patients is only 8–58%.2 The main cause of gastric cancer caused death is recurrence and metastasis. Thus, investigating the mechanisms involved in pathogenesis, progression and metastasis of gastric cancer will help to improve diagnosis and therapy.

Recent studies have found that the Wnt/β-catenin pathway plays an important role in the pathogenesis and metastasis of gastric cancer. It regulates cell differentiation through a series of signalling pathways and is involved in tumorigenesis. The downstream target genes of Wnt/β-catenin pathway include c-myc, Cyclin D1, Cox-2, matrix metalloproteinase-7 (MMP-7) and other proto-oncogenes as well as metastasis-associated genes. FRZB is a negative regulator of Wnt signaling. Its over-expression could inhibit tumorigenesis, and reduce the amount and activity of MMPs, thus may have the function of a tumor suppressor gene.3 In this study, we have explored the effects of FRZB on tumorgenesis and progression of gastric cancer.

SGC-7901, which was derived from gastric cancer in Chinese patients, is one of the most commonly used gastric cancer cell line in the researches on epiology and treatment of gastric cancer in China.3,4 In this study, we used SGC-7901 to explore the effects of FRZB gene on gastric cancer cells.

Materials and Methods

Cell culture. Human gastric cancer cell line SGC-7901 was purchased from the cell line bank of Shanghai Institutes of Biological Sciences, Chinese Academy of Science, and preserved by our institute. Cells were cultured in RPMI-1640 culture medium (product of Gibco Company) containing 10% calf serum (product of Hangzhou Sijiqing Co.) at 37°C in an atmosphere of 5% CO₂.
**Construction and transfection of eukaryotic expression vectors.** The full-length FRZB (GenBank Accession No. NM_001463) cDNA was amplified by polymerase chain reaction (PCR) using human gastric cancer tissue cDNA as templates. Upstream and downstream primer sequences were 5'-ATC TCG AGA GCT CCC AGA TCC TTG TGT C-3' and 5'-ATG GAT CCC TTT TTG TAT TTC GGG ATT TAG TT-3', respectively. Amplified products were cloned into pcDNA3.1 vector (product of Invitrogen Co.), and its sequence was confirmed by DNA sequencing. pcDNA3.1/FRZB and pcDNA3.1 were separately transfected into SGC-7901 cells according to the manual instruction of Lipofectamine2000 (product of Life Technology Co.). Culture medium containing 1 mg/mL of G418 was used to screen cell clones with stable FRZB gene expression, while 400 μg/mL of G418 was used for maintain culture during expansion.

**Real-time quantitative RT-pCR.** Primer sequences of FRZB were as follows: upstream, 5'-GAG GAG GTG CCT TAC GAC-3'; downstream, 5'-GAA AAT CAG CTC GTG CCG C-3'. Primer sequences of GAPDH, as an internal control, were as follows: upstream, 5'-GAA CCT GAC CTG CCG TCT AG-3'; downstream, 5'-GTA GCC CAG GTT CCT GA-3'. Total RNA (2 μg) was used to synthesize cDNA using reverse transcription system of Promega Co. A total of 20 μL PCR reaction system included 1 μL of ten times diluted cDNA as a template, upstream and downstream primers at final concentration of 200 nmol/L, and 2 × SYBRGreen PCR Master Mix (product of Applied Biosystem Co.). PCR reaction was performed on a real-time PCR apparatus, a product of MJ Research Co. PCR conditions were set as 50°C for 2 min and 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 60 s. After the last cycle, a melting curve of 55–95°C was obtained. Three wells were used for each sample. In any PCR cycle, when the fluorescence intensity was greater than the threshold value (CT) calculated by the apparatus' software, the sample was then considered as positive. The quantity of FRZB mRNA was presented as 2^ACT (ACT is the value of FRZB-CT value subtracting GAPDH-CT value). The above experiment was repeated for three times and the average value was obtained as the final expression level of FRZB.

**Detecting cell proliferation by MTT assay.** SGC-7901/FRZB, SGC-7901/vector and SGC-7901 cells at exponential growth phase were collected and seeded into a 96-well plate with a density of 1 × 10^5 cells/well; each kind of cell was seeded into three wells. The plate was cultured in an incubator with 5% CO2 at 37°C. Cell proliferation was detected by MTT assay on days 0, 3, 5, 7 and 9 of culture, while 400 μg/mL of G418 was used for maintain culture during expansion.

**Cell adhesion experiment.** Cell adhesion experiment was performed using adhesion kit (Chemicon Company) according to the manual instruction. PBS was added to a 96-well plate containing various components of extracellular matrix to hydrate for 1 h. The cells were digested and 100 μL of cells (1 × 10^6 cells/mL) were seeded into each well (each kind of cells were seeded into three wells), and then cultured at 37°C for 1 h. The plate was washed with PBS gently for three times to remove unadhered cells, and stained with 0.2% crystal violet for 5 min. After discarding suspension, the plate was washed with PBS gently for three times and dried. Extra crystal violet was dissolved by 100 μL of NaH2PO4, A value at 570 nm was measured. Cell adhesion rate = A1/A2 × 100% (A1 means A570 value at 1 h in each group, and A2 means A570 value of 1 × 10^5 cells in each group).

**Cell invasion experiment in vitro.** Chambers were purchased from Becton Dickinson Co. Matrigel was diluted with pre-chilled RPMI-1640 at 4°C, and 50 μg of Matrigel were added into each chamber by three fractions to cover up all micropores on the membrane. Prepared chambers were then exposed to ultraviolet radiation for 2 h for sterilization, and added with small amount of serum-free culture medium for hydration. After adjusting the concentration of SGC-7901, SGC-7901/vector and SGC-7901/FRZB cells to 2.5 × 10^5 cells/mL with serum-free RPMI-1640 medium, 200 μL (containing 5 × 10^4 cells) of them were added into invasion chambers; each kind of cells were tested in triplicate. Chambers were then transferred onto a 24-well plate, which contained RPMI-1640 culture solution with 10% calf serum, and cultured for 20 h. Matrigel and cells on the upper chamber surface were wiped away. After fixed with methanol and stained with crystal violet, cells invaded through Matrigel membrane were counted to obtain Invasion Index (cells in 10 visual fields of each well under high-power magnification were counted to get the average value).

**Detecting MMP-2, MMP-7 and MMP-9 expression by Sp immunocytochemistry.** Slides were pre-coated with poly-L-Lysine and then exposed to UV light for 1 h for sterilization. Subsequently, cell suspension was then dripped directly onto the coated slides, and cultured routinely. When adhered and grew on slides, cells were stained by SP immunocytochemistry. SP kit is a product of Dako Co., USA. Cells were fixed by 4% formalin for 30 min, then treated with 0.1% Triton-100 for 15 min. The expression of MMP-2, MMP-7 and MMP-9 was detected according to SP kit manual instruction. In negative control group, PBS (pH 7.2) was used instead of primary antibodies. Positive cells presented small brown-yellowish granules, and the staining intensity was higher than that of blank control cells.

**Subcutaneous tumor formation in nude mice.** Twelve male BALB/c nu/nu nude mice were provided by the Department of Laboratory Animal Science, Shanghai Jiaotong University School of Medicine, with an animal quality certification number of SCXK (Shanghai) 2004-0001. The cells were randomly divided into three groups (each group contained four mice), and received subcutaneous injection of 0.2 mL (containing 1 × 10^6 cells) of SGC-7901, SGC-7901/vector and SGC-7901/FRZB cells into the dorsal site of the hind legs, respectively. After injection, the length (L) and width (W) of tumors were recorded in every four days, and the tumor sizes (volume, V) were calculated: V = 4/3π × L/2 × (W/2)^2. Growth curves were plotted. The mice were killed 27 days later.
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Colony formation ability of cells after FRZB transfection. When cultured routinely for 14 days, the colony formation rate was significantly lower in SGC-7901/FRZB cells than in SGC-7901 and SGC-7901/vector cells [(1.5 ± 1.1)% vs. (10.4 ± 2.4)% and (11.1 ± 3.9)%, p < 0.001].

Cell adhesion and invasion ability after FRZB transfection. The adhesion ability of SGC-7901/FRZB cells to Fibronectin, Collagen IV, Laminin, Vitronectin and Collagen I was significantly increased when compared with those of SGC-7901/vector and SGC-7901 cells (p < 0.05), however, no significant difference between SGC-7901/vector and SGC-7901 cells was detected (Table 1, Fig. 3).

The number of invaded SGC-7901/FRZB cells was significantly lower than those of invaded SGC-7901/vector and SGC-7901 cells [6.60 ± 2.63 vs. 54.80 ± 6.97 and 55.90 ± 5.68, p < 0.05], however, no significant difference between SGC-7901 and SGC-7901/vector cells was detected (Fig. 4).

Changes of MMps expression in cells after FRZB transfection. The expression of MMP-2, MMP-7 and MMP-9 was obviously higher in SGC-7901 and SGC-7901/vector cells than in SGC-7901/FRZB cells (Figure 5). After FRZB transfection, the expression of MMP-2, MMP-7 and MMP-9 were all inhibited.

Cell proliferation after FRZB transfection. The proliferation rate of SGC-7901/FRZB cells was significantly slower than those of SGC-7901/vector and SGC-7901 cells (p < 0.05), but the difference between SGC-7901/vector and SGC-7901 cells was not significant (Fig. 2).

Tumors were isolated and weighed, then fixed, sliced, and stained with HE. Structural changes of tumors were observed under a light microscope.

Statistical analysis. All data were analyzed using SPSS11.0 statistic software, and presented in mean ± standard deviation. The data between groups were compared using t test. p < 0.05 indicates significant difference.

Results

Construction of FRZB eukaryotic expression vector and expression of FRZB in transfected cells. Full-length cDNA of FRZB was amplified and cloned into pcDNA3.1 vector. DNA fragments were verified by digestion of restrictive enzymes (Fig. 1), and DNA sequencing. The mRNA level of FRZB was higher in SGC-7901/FRZB cells than in SGC-7901 and SGC-7901/vector cells (2ΔCT value: 0.66 vs. 1.022E-05 and 1.048E-05), indicating the increase of FRZB expression after FRZB gene transfection. The protein level of FRZB was also higher in SGC-7901/FRZB cells than in SGC-7901 and SGC-7901/vector cells (Fig. 1).

Cell proliferation after FRZB transfection. The proliferation rate of SGC-7901/FRZB cells was significantly slower than those of SGC-7901/vector and SGC-7901 cells (p <0.05), but the difference between SGC-7901/vector and SGC-7901 cells was not significant (Fig. 2).
Effects of FRZB on growth and metastasis of gastric cancer cell line SGC-7901

The secreted glycoprotein family of Wnts is a group of signal molecules that regulate many developmental processes, including cell proliferation, cell polarity, cell specialization and cell migration. Abnormal Wnt signal transduction may lead to developmental abnormalities, and plays an important role in human tumorigenesis. The most well-studied Wnt signaling is the Canonical/β-catenin pathway, in which, the stabilization of β-catenin is the key step involved in carcinogenesis. Once activated, β-catenin may interact with one or multiple target genes, and activate a series of signal transduction. The well-known β-catenin binding proteins are cadherin, β-catenin, axin, epidermal growth factor receptor, APC, fascin (an actin-bundling protein), LEF/TCF transcription factor, Mucin-1 (a breast cancer antigen), presenilin-protein phosphatase, and IQGAP (a GTPase mediator). Among them, LEF/TCF-mediated transcription activation is involved in oncogenesis of multiple tumors. Its downstream target genes include c-myc, cyclin D1, Cox-2, MMP-7, MMP-8, and so on.

Wnt signaling is regulated by many modulators, among which secreted extracellular modulators are important, including secreted frizzled-related proteins (sFRPs), Dickkopf (DKK) protein family and Wnt inhibitory factor-1 (WIF-1). The sFRP family contains a cysteine-rich domain (CRD), which is homologous to the extracellular structure of Frizzled receptor. The proteins of sFRP family can down-regulate Wnt signaling through binding with Wnt molecules and binding with Frizzled receptor, therefore, it plays important roles in both embryonic development and tumor formation. Previous studies have revealed that the deletion of sFRP-1 and sFRP-4 occurred in the oncogenesis of colorectal cancer. Recent studies have shown that sFRPs down-regulation is caused by promoter hypermethylation, gene deletion, or other mechanisms.

FRZB/sFRP-3 is the first identified gene of the sFRP family, and it is located on chromosome 2q31-33. Embryological researches showed that FRZB is expressed in Spemann’s organizers, and can inhibit Wnt-1 and Wnt-8 signaling. Person et al. revealed that FRZB can regulate the development of atrioventricular cushion of the heart by down-regulating Wnt-9a pathway-mediated β-catenin signaling. Recently, researches on FRZB have processed into disease fields. Lories et al. found that the Arg200Trp mononucleotide polymorphism of FRZB is closely related to bony osteoarthritis and osteoporosis. Byun et al. detected the expression of FRZB protein and RNA in normal mucosa and tumor tissues of the stomach and colon using immunohistochemistry and in situ hybridization techniques, and found that FRZB possesses the function of a tumor suppressor gene. Our previous study revealed that FRZB expression in gastric cancer cells is associated with the histological type and differentiation of gastric cancers (manuscript in press).

Observing in vitro cell proliferation, colony formation and subcutaneous tumor formation in nude mice, we found that FRZB...
gelatinase A, plays an important role in the invasion and metastasis of gastric mucosa epithelial cells. MMP-9 is mainly responsible for the degradation of type IV collagen, a main component in basement membrane and ECM. MMP-9 to damage the integrity of basement membrane, and can self-secrete or induce other cells to secrete large amounts of proteases. Zi et al. reported that increased FRZB expression inhibited both the oncogenesis and growth of prostate cancer PC-3 cells.

The invasion and metastasis of tumor is a multi-step process caused by multiple factors, where extracellular matrix (ECM) plays an important role. The first step of invasion is the adhesion of tumor cells towards the basement membrane and ECM components, including collagen, gelatin, elastin, laminin, fibronectin, and so on. The second step is the degradation of matrix and basement membrane. Matrix is the natural barrier against tumor invasion and metastasis. Matrix liquefaction may eventually promote tumor invasion and metastasis. MMPs is a group of zinc-binding proteases responsible mainly for the degradation of ECM. Its overexpression or elevated activity is closely related to the growth, invasion and metastasis of tumors. MMP-7, also known as matrix dissolution factor, is an important member of the MMPs family, which can degrade the essential components of the basement membrane and ECM. The process of tumor metastasis requires basement membrane damage and ECM degradation. MMP-7 poses a wide degradative effect on ECM and is the only enzyme specifically expressed by epithelial tumor cells. Studies revealed that MMP-7 is associated with the progression of gastric cancer, colorectal cancer, breast cancer, renal cell carcinoma, pancreatic cancer and bladder cancer. The expression of MMP-7 is positively correlated to pathologic conditions, MMP-2 can disrupt the skeletal structure of cell membrane through degradating type IV collagen of ECM, and thus accelerate tumor spreading and metastasis.

Our results showed that the expression of MMP-2, MMP-7 and MMP-9 in SGC-7901 cells was significantly decreased after FRZB transfection, and the invasion ability of tumor cells was also decreased, indicating that one of the mechanisms of FRZB inhibited cell invasion is through negative regulation toward MMPs, which is possibly associated with Wnt/TCF signalling pathway. Evidences showed that there is a LEF/TCF transcription factor-binding site present at the promoter region of MMPs. Another possibility is that FRZB itself has an antagonistic effect towards MMP-7, which is due to the presence of a netrin-like domain (NTR) in FRZB protein. This domain is homologous to those in tissue inhibitors of metalloproteases (TIMP), with some inhibitory effect on metalloproteases. Zi et al. also reported that increased expression of FRZB could inhibit the activities of MMP-2 and MMP-9 in the culture supernate of prostate cancer cells, along with reduction of MMP-2 and MMP-9 expression. Our results also showed that FRZB transfection could promote the adhesive ability of SGC-7901 cells towards ECM components, but decrease cell invasive ability. The metastasis of gastric cancer cells is a very complicated process, which involves not only the ability of cell adhesion and expression of metalloproteinas but also the participant of many other kinds of cells and protein networks. According to our results, FRZB possesses inhibitory effects on the invasion of gastric cancer cells towards basement membrane, and positive regulatory effects on the adhesion of cancer cells towards matrix. We speculate that FRZB plays various roles in the metastasis and invasion of gastric cancer. The mechanisms involved still require further studies.

FRZB was initially studied in the fields of embryonic development, particularly in the research on the development of long bones. However, few studies have been conducted on the roles of FRZB in tumors. As for the same protein, why does it have different functions in different human life stages? The function of FRZB in the embryonic development is totally different from that in tumor. What exactly is the cause? Other than the Wnt/β-catenin pathway, which signaling pathway is involved in? What are the detail mechanisms? Further studies are required to explain these questions.

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References

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Figure 6. Tumor formation ability of SGC-7901, SGC-7901/vector, SGC-7901/FRZB cells. *p < 0.05, vs. SGC-7901 and SGC-7901/vector cells.