

Fra-1 is upregulated in gastric cancer tissues and affects the PI3K/Akt and p53 signaling pathway in gastric cancer

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Abstract. Gastric cancer is an aggressive disease that continues to have a daunting impact on global health. Fra-1 (FOSL1) plays important roles in oncogenesis in various malignancies. We investigated the expression of Fra-1 in gastric cancer (GC) tissues by qPCR, immunohistochemistry (IHC) and western blot technologies. The results showed that Fra-1 was overexpressed in gastric cancer tissues compared with the adjacent non-cancerous tissues. To explore the possible mechanism of Fra-1 in GC, we elucidated the effect of Fra-1 in the apoptosis and cell cycle of gastric cancer cells, AGS, and found that a considerable decrease in apoptotic cells and increase of S phase rate were observed for AGS cells with Fra-1 overexpression. We identified and confirmed that Fra-1 affected the expression level of CTTN and EZR *in vitro* through LC-MS/MS analyses and western blot technology. Furthermore, we found that Fra-1 was correlated with dysregulation PI3K/Akt and p53 signaling pathway in gastric cancer tissues *in vitro*. Moreover, we found that Fra-1 overexpression affected the expression of PI3K, Akt, MDM2 and p53 *in vivo*. In summary, our results suggest

that Fra-1 is upregulated in gastric cancer tissues and plays its function by affecting the PI3K/Akt and p53 signaling pathway in gastric cancer.

Introduction

Fra-1 is a member of the Fos transcription factor family (c-Fos, FosB, Fra-1 and Fra-2) that is highly expressed in multiple malignancy, and playing important roles in proliferation, transformation and metastasis (1,2). FRA-1 forms activator protein-1 complexes in association with members of the JUN family (c-Jun, JunB and JunD) and drives gene transcription (1,2). Fra-1 can positively regulate transcription. Fra-1 activity is regulated transcriptionally as well as post-translationally (3,4). FRA-1 has been implicated in the development of airway squamous metaplasia and is frequently overexpressed in squamous cell carcinomas of the stomach and esophagus (2). Fra-1 gene induction and the accumulation of Fra-1 protein might contribute to the neoplastic phenotype in HNSCC (head and neck squamous cell carcinoma) (3). Fra-1 is required for invasion and Fra-1 might be a good target for intervention in colorectal cancer (3,5,6). In addition to its pro-invasive and pro-migratory effect, Fra-1 might influence the metastatic potential of breast cancer cells by changing the expression of adhesion molecules, resulting in increased adherence to endothelial cells (1,7,8). Although some evidence has been reported, the mechanism of Fra-1 in malignancy is not fully understood. Moreover, at present, scarce information exists to study the relationship between Fra-1 and gastric. Thus, it is urgent to explore the effect and mechanism of Fra-1 in gastric cancer.

The PI3K (phosphoinositide 3-kinase)/Akt signaling pathway is a major driving force in a variety of cellular functions. Dysregulation of this pathway has been implicated in many human diseases including cancer (9-12). Therapy resistance is critical to tumor maintenance and severely limits

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Abbreviations: GC, gastric cancer; FOSL1 (also known as Fra-1), FOS-like antigen 1; CTTN, cortactin; EZR, ezrin; TP53, tumor protein p53; AKT1 (also known as AKT), v-akt murine thymoma viral oncogene homolog 1; IHC, immunohistochemistry; GADPH, glyceraldehyde-3-phosphate dehydrogenase

Key words: Fra-1, gastric cancer, PI3K, p53, signal pathway

Table I. Characteristics of gastric cancer patients.

Samples	Age (years)	Gender	Histological diagnosis
1	60	Female	Gastric intermediately differentiated adenocarcinoma
2	60	Male	Gastric poorly differentiated adenocarcinoma
3	46	Male	Gastric intermediately differentiated adenocarcinoma
4	49	Male	Gastric poorly differentiated adenocarcinoma
5	57	Male	Gastric poorly differentiated adenocarcinoma
6	72	Male	Gastric poorly differentiated adenocarcinoma
7	53	Male	Gastric intermediately differentiated adenocarcinoma
8	63	Female	Gastric poorly differentiated adenocarcinoma
9	54	Male	Gastric poorly differentiated adenocarcinoma
10	66	Female	Gastric poorly differentiated adenocarcinoma
11	57	Male	Gastric poorly differentiated adenocarcinoma
12	47	Male	Gastric poorly differentiated adenocarcinoma
13	70	Male	Gastric poorly differentiated adenocarcinoma
14	54	Female	Gastric intermediately differentiated adenocarcinoma
15	59	Male	Gastric poorly differentiated adenocarcinoma
16	62	Female	Gastric intermediately differentiated adenocarcinoma
17	60	Male	Gastric poorly differentiated adenocarcinoma
18	73	Male	Gastric poorly differentiated adenocarcinoma
19	47	Male	Gastric poorly differentiated adenocarcinoma
20	63	Female	Gastric poorly differentiated adenocarcinoma

cancer patient survival. The PI3K pathway has emerged as a major driver of resistance to diverse anticancer agents (10). It has been shown that p53 was a crucial transcription factor and p53 is an important sensor of cellular stress under genotoxic, chemotoxic, pathological, and even normal physiological conditions (13,14). MDM2 is a negative regulator of p53. Abnormalities in the p53 gene and overexpression of MDM2 are commonly observed in malignancy. The MDM2-p53 feedback loop plays an important role in tumor progression (15,16). Mutations activating the PI3K/Akt signalling pathway and inactivating the p53 tumor suppressor gene are common mechanisms that cancer cells require to proliferate and escape pre-programmed cell death (17).

In the present study, we examined the expression levels of Fra-1 in gastric cancer tissues. Furthermore, we studied the influence of Fra-1 to gastric cancer and explored the possible mechanism.

Materials and methods

Cell culture. AGS, a human gastric cancer cell line was cultured in HAM'S/F-12 (HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco by Life Technologies™, Grand Island, NY, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in the presence of 5% CO₂.

Patient samples. Twenty participants were recruited at the Cancer Hospital of Hunan province, Central South University (Changsha, Hunan, China). Consent forms were obtained from each individual patient, and experimental protocols

were approved by the Institutional Review Board of the Cancer Hospital of Hunan province. All subjects enrolled in the study were Chinese. All clinical and biological data were available for the samples (Table I). Gastric cancer tissue and corresponding non-tumor normal tissue were collected, and each biopsy sample was divided into two sections, one was submitted to routine histological diagnosis, and the remaining section was studied by qPCR, immunohistochemistry and western blot experiments.

Total RNA extraction and quantitative real-time PCR analysis. Total RNA was extracted from gastric cancer tissues and corresponding non-tumor normal tissues using TRIzol reagent (CWBio, Beijing, China) and cDNA synthesis was carried out using the RevertAid First Strand cDNA Synthesis kit (CWBio) according to the manufacturer's recommendations. Quantitative real-time PCR (qRT-PCR) was done with GoTaq® qPCR Master Mix (Promega Corp., Fitchburg, WI, USA). For detection of Fra-1 mRNA expression levels, GAPDH was amplified in parallel as an internal control. The sequences of the primers used for qPCR were as follows: Fra-1 forward, 5'-gcatgggctaaggatttgaa-3' and reverse, 5'-tcccaaatttagcctgttgg-3'; GAPDH forward, 5'-cgaccactttgcaagctca-3' and reverse, 5'-actgagtgtggcaggactc-3'. The expression of mRNA was assessed by evaluated threshold cycle (CT) values. The CT values were normalized with the expression levels of GAPDH and the relative amount of mRNA specific to each of the target genes was calculated using the 2^{-ΔΔCT} method (4,18-21). qPCR was done with the Bio-Rad CFK96™ Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). The data were analyzed by Bio-Rad CFK manager software (Bio-Rad Laboratories).

Expression of mRNA was assessed by evaluated threshold cycle (CT) values, and GAPDH was used as an internal control.

Immunohistochemistry (IHC) and evaluation of staining. Immunohistochemistry was done using the peroxidase anti-peroxidase technique following a microwave antigen retrieval procedure. Antibody for *Fra-1* was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Antibody against *Fra-1* (1:100) was overlaid on gastric cancer and corresponding non-tumor normal tissue sections and incubated overnight at 4°C. Secondary antibody incubation (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was performed at room temperature for 30 min.

Sections were blindly evaluated by two investigators in an effort to provide a consensus on staining patterns by light microscopy (Olympus). *Fra-1* staining was assessed according to the methods described by Hara and Okayasu (22) with minor modifications. Each case was rated according to a score that added a scale of intensity of staining to the area of staining. At least 10 high-power fields were chosen randomly, and >1,000 cells were counted for each section. The intensity of staining was graded on the following scale: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining. The area of staining was evaluated as follows: 0, no staining of cells in any microscopic fields; 1+, <30% of tissue stained positive; 2+, between 30 and 60% stained positive; 3+, >60% stained positive. The minimum score when summed (extension + intensity) was, therefore, 0, and the maximum, 6. A combined staining score (extension + intensity) of ≤ 2 was considered to be negative (low staining); a score between 3 and 4 was considered to be moderate; whereas, a score between 5 and 6 was considered to be strong.

Construction of pEGFP-N1-Fra-1 vector and cell transfection. The coding region of *Fra-1* gene was generated by PCR with the primer pair 5'-atactcgaatgaacctggccatcagcat-3' and 5'-cgcgaaattccacagggacatgaaatccg-3'. The PCR was performed under the following conditions: one cycle for 5 min at 94°C; 30 cycles for 45 sec at 94°C, 45 sec at 55°C, and 90 sec at 72°C, and extension of 10 min at 72°C. The fragments were cloned into the TA vector (Promega) and used to transform *E. coli* JM109 (Takara Bio, Dalian, China). Following selection and propagation, the pure plasmid DNA was prepared by standard methods. The DNA fragments were removed from the TA vector by restriction enzyme digestion with *Xho*I and *Eco*R1 (Promega) to subclone into the pEGFP-N1 vector. The fusion sequences were verified by DNA sequencing using ABI 3730.

Cell transfection. Cell transfection was accomplished by using Lipofectamine, according to the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). A 2×10^5 cells were planted into each well of a 6-well plate 24 h prior to the transfection. For each transfection, 2 μ g of pEGFP-N1-Fra-1 plasmid and pEGFP-N1 vector plasmid was transfected into AGS cells, respectively. The plasmids were diluted with 100 μ l of serum-free media and 4 μ l Lipofectamine was added into 100 μ l serum-free media. The two solutions were combined, mixed gently and incubated at room temperature for 30 min. Then the 200 μ l mixture and 200 μ l of serum-free media were added into each well. The cells were then incubated at 37°C

for 24 h, followed by replacing the transfection media with fresh complete culture media. After an additional 24-h culture, the cells were harvested for the following western blot experiments.

Flow cytometric analysis of cell cycle. The cell cycle was analyzed by flow cytometric analysis using a MoFlo™ XDP high-performance cell sorter (Beckman Coulter, Brea, CA, USA) propidium iodide (PI) (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Briefly, cells (2×10^5) were placed in each well of a 6-well plate 24 h prior to the transfection. For each transfection, 2 μ g of pEGFP-N1-Fra-1 plasmid and pEGFP-N1 vector plasmid was transfected into AGS cells, respectively. The plasmids were diluted with 100 μ l of serum-free media and 4 μ l Lipofectamine was added into 100 μ l serum-free media. The two solutions were combined, mixed gently and incubated at room temperature for 30 min. The 200 μ l mixture and 200 μ l of serum-free media were added into each well. The cells were then incubated at 37°C for 24 h, followed by replacing the transfection media with fresh complete culture media. After an additional 24-h culture, the cells were harvested for cell apoptosis analysis. The cells were harvested and fixed in 70% ethanol at 4°C. Subsequently, the cells were washed with cold PBS and stained with propidium iodide (PI) in working solution (0.5 mg/ml RNase, and 0.1 mg/ml propidium iodide in PBS). The cell cycle was characterized by flow cytometric analysis using a MoFlo™ XDP high-performance cell sorter (Beckman Coulter) and the data were analyzed by the summit 5.2 software (Beckman Coulter).

The effect of *Fra-1* to gastric cancer cell apoptosis. The cell apoptosis was analyzed by flow cytometric analysis using a MoFlo™ XDP high-performance cell sorter (Beckman Coulter) propidium iodide (PI) and Hoechst 33342 double staining (Nanjing KeyGen). Briefly, cells (2×10^5) were placed in each well of a 6-well plate 24 h prior to the transfection. For each transfection, 2 μ g of pEGFP-N1-Fra-1 plasmid and pEGFP-N1 vector plasmid was transfected into AGS cells, respectively. The plasmids were diluted with 100 μ l of serum-free media and 4 μ l Lipofectamine was added into 100 μ l serum-free media. The two solutions were combined, mixed gently and incubated at room temperature for 30 min. The 200 μ l mixture and 200 μ l of serum-free media were added into each well. The cells were then incubated at 37°C for 24 h, followed by replacing the transfection media with fresh complete culture media. After an additional 24-h culture, the cells were harvested for cell apoptosis analysis. Cells were collected in an Eppendorf tube for 24 h and washed twice with PBS by centrifugation. The supernatants were discarded. To detect apoptosis, 500 μ l PBS, 5 μ l Hoechst 33342 and 5 μ l PI were added to each tube, and the contents of the tube were mixed in the dark, at room temperature for 15 min, followed by FCM testing (Beckman Coulter). The data acquired were analyzed with summit v5.2 software (Beckman Coulter).

Identification of differential proteins of *Fra-1* overexpressing AGS cells by LC-MS/MS analysis. The cell lysate consisted of 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and sodium orthovanadate, sodium fluoride, EDTA, leupeptin, supplemented with 1X halt

protease inhibitor cocktail (CWBio) and 1X halt phosphatase inhibitor cocktail (BestBio). The protein concentration was estimated by bicinchoninic acid (BCA) method. The gel was run at 80 V 40 min, then 120 V 90 min. Then, 50 μg of each preparation was loaded on a 10% SDS-PAGE gel. SDS-PAGE was run at 80 V 40 min, then 120 V 90 min. Protein bands were visualized using Coomassie brilliant blue G-250 (Sigma, Carlsbad, CA, USA) and excised. The protein spots were destained using 15 mM $\text{K}_4\text{Fe}(\text{CN})_6$ and 50 mM sodium thiosulfate. 1.25 μg trypsin (1:20 enzyme/substrate ratio) was added to each band and in-gel-digestion was performed at 37°C overnight (~16 h). The generated peptides were extracted by sonication (15 min, ice cooling) of the gel pieces in ~20 μl of 50% acetonitrile in 0.1% FA, twice. After extracting from gel pieces, peptides were dried by vacuum centrifugation to ensure a complete removal of acetonitrile and reconstituted in 20 μl 0.1% FA (23-25).

LC-MS/MS analyses were performed on an Ultimate™ 3000 RSLCnano system online coupled to an LTQ Orbitrap Velos Pro mass spectrometer (both from Thermo Scientific, Bremen, Germany). Peptide diluted with 0.1% FA, was injected in each analysis of 30 μl sample. After injection, peptides were pre-concentrated with 0.1% FA, 3%ACN on a trap column (μ -Precolumn C18 PepMap 100, 300 μm x 5 mm, 5 μm , 100 Å; Thermo Fisher Scientific) at a flow rate of 300 nl/min for 5 min. Subsequently, the analyte was transferred to the analytical column (Acclaim® PepMap RSLC, 75 μm x 15 cm, nano Viper, C18, 2 μm , 100 Å; Thermo Fisher Scientific) and separated using a 120-min gradient from 5 to 40% solvent B at a flow rate of 300 nl/min (solvent A, 0.1% formic acid; solvent B, 0.08% FA 80% acetonitrile). The mass spectrometer was operated in a data-dependent mode. The general mass spectrometric parameters were: spray voltage, 2.0 kV; capillary temperature, 275°C. For data-dependent MS/MS analyses, the software Xcalibur (Thermo Fisher Scientific) was used. Full scan MS spectra were acquired at a mass resolution of 60,000 (mass range 350–2000 m/z) in the Orbitrap analyzer. For label-free analyses, tandem mass spectra of the ten most abundant peaks were acquired in the linear ion trap by peptide fragmentation using collision-induced dissociation (CID). Normalized collision energy (NCE) was set to 35% and an isolation width of 2 m/z was chosen (23-25).

Protein identifications were performed with Proteome Discoverer software. Briefly, Thermo raw-files were imported and searched against UniProtKB/Swiss-Prot database (release 2014_10). For database searches, mass tolerances were set to 10 ppm and 0.8 Da for precursor and fragment ions, respectively. Taxonomy was restricted to human and one enzymatic miscleavage was allowed. For label-free analyses, modifications of cysteine (carbamidomethyl, static) and methionine (oxidation, variable) were considered. Confidence of peptide identifications was estimated using percolator function, implemented in Proteome Discoverer. Instead of determining the peptide confidence based on a single metric such as Mascot ion score, we decided to use percolator as it discriminates correct from incorrect peptide spectrum matches based on multiple orthogonal score criteria leading to accurate and sensitive peptide identifications. Peptide identifications with false discovery rates $\leq 1\%$ (q-value ≤ 0.01) were discarded (23-25).

Western blot analysis. The gastric cancer tissues, corresponding non-tumor normal tissues, and AGS cells were lysed in RIPA buffer (CWBio) and total protein concentration was determined using Pierce® BCA protein assay kit (Thermo Scientific, Inc., Rockford, IL, USA). Extracts containing 50 μg of proteins were separated in 10% SDS-PAGE gels and electroblotted onto nitrocellulose membranes (HyClone Laboratories). The membranes were inhibited using Tris-buffered saline/Tween-20 (25 mM Tris-HCl, 150 mM NaCl, pH 7.5 and 0.05% Tween-20) containing 5% non-fat milk followed by overnight incubation at 4°C with primary antibodies (rabbit anti-PI3K polyclonal antibody, catalog no. 4292; Cell Signaling Technology, Inc., Danvers, MA, USA; dilution, 1:500; rabbit anti-Akt polyclonal antibody, catalog no. 9272, Cell Signaling Technology; dilution, 1:300; rabbit anti-MDM2 antibody, 1:200 and rabbit anti-p53 antibody, 1:200; Wuhan Boster). Following three washes, the membranes were incubated with horseradish peroxidase-conjugated second antibodies (catalog no. sc-2491; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; dilution, 1:5,000) and the specific signals were visualized using an ECL detection system. Anti-GAPDH antibody (Santa Cruz Biotechnology; 1:3,000) was used as a loading control.

Statistical analysis. Differences of non-parametric variables were analyzed by the Mann-Whitney U test. Differences of the quantitative variables between groups were analyzed by the Student's t-test using SPSS 11.0 program (SPSS, Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Fra-1 is highly expressed in gastric cancer tissue. To detect the mRNA expression levels of the Fra-1 molecular in gastric cancer and the adjacent non-cancerous tissues, 20 samples of each were selected to perform qPCR of the Fra-1 gene. The data were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method and the fold change in the expression of these genes relative to the internal control gene, GAPDH, was analyzed. The expression of the Fra-1 gene was higher in the gastric cancer samples compared with the adjacent non-cancerous tissues and the normalized Fra-1 gene expression in gastric cancer was upregulated 2.99-fold ($P=0.017$) (Fig. 1A and Table II).

To determine whether the Fra-1 gene was expressed at a higher level in gastric cancer compared with the adjacent non-cancerous tissues, the protein expression levels of Fra-1 were further examined by western blot analysis in 1-5 samples (Fig. 1B). In comparison with the adjacent non-cancerous tissues, the expression level was identified to be greater in gastric cancer tissues, which corresponded with the qPCR results.

To confirm the pattern of Fra-1 in gastric cancer, immunohistochemistry (IHC) was carried out with antibodies against Fra-1 protein in gastric cancer and the adjacent non-cancerous tissues. Fra-1 was identified as differentially expressed between gastric cancer tissues versus the adjacent non-cancerous tissues. IHC showed a similar pattern in protein expression with western blot results. There was 61.5% (16/26) high score of Fra-1 in gastric cancer tissues and 26.9% (7/26) in the adjacent non-cancerous tissues. The distribution of low score was 23.1% (6/26) and

Table II. Identification of the mRNA expression level of Fra-1 in gastric cancer and adjacent non-cancerous tissues by qPCR.

Gene	Sample	n	Fra-1 CT (mean ± SD)	GAPDH CT (mean ± SD)	ΔCT (mean ± SD)	ΔΔCT (mean ± SD)	Fold ^a
Fra-1	Gastric cancer tissues	20	26.86±1.05	16.27±0.64	10.59±0.43	-1.58±0.52	2.99
	Non-cancerous tissues	20	29.34±1.65	16.73±0.66	12.17±0.45		(2.08-4.28)

^aMean fold change in expression of the target gene, Fra-1, relative to the internal control gene, GAPDH, was calculated using the $2^{-\Delta\Delta CT}$ equation by Livak *et al* (18): $\Delta\Delta CT = (CT_{\text{Target}} - CT_{\text{GAPDH}})_{\text{cervical cancer}} - (CT_{\text{Target}} - CT_{\text{GAPDH}})_{\text{control}}$. At least three replicates of each reaction were performed. CT, threshold cycle; qPCR, quantitative polymerase chain reaction.

Table III. The difference of Fra-1 expression between gastric cancer and the adjacent non-cancerous tissues by immunohistochemistry.

	n	Score			χ^2	P-value
		Low (0-2)	Moderate (3-4)	High (5-6)		
Gastric cancer	30	6 (20.0%)	8 (26.7%)	16 (61.5%)	7.38	0.024<0.05
Non-cancerous tissues	30	15 (42.3%)	8 (30.8%)	7 (26.9%)		

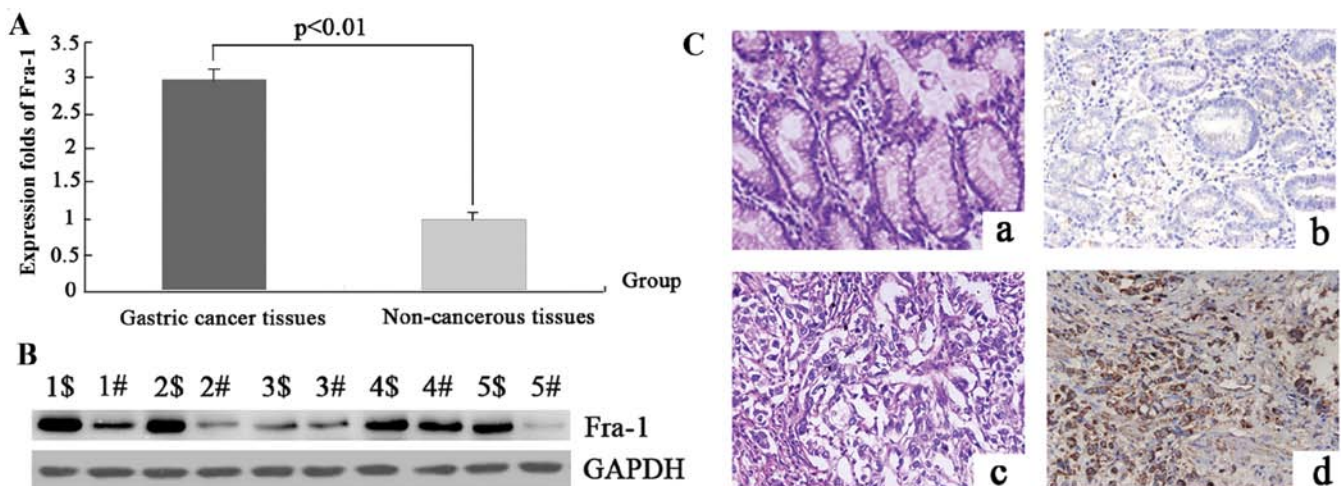


Figure 1. The detection of expression levels of FRA-1 in the gastric cancer tissues and the adjacent non-cancerous tissues by qPCR, western blot analysis and IHC. (A) qPCR was performed to validate the expression of FRA-1 in gastric cancer tissues and the adjacent non-cancerous tissues. GAPDH was used as an internal control and for normalization of the data. The mRNA expression levels of the FRA-1 was significantly upregulated in the gastric cancer tissues (n=20) compared with the adjacent non-cancerous tissues (n=20). *P<0.01. (B) 1, 2, 3, 4 and 5 tissues which were used in the detection of mRNA expression levels by qPCR were selected to detect the expression levels of FRA-1 protein by western blot analysis. \$ indicates gastric cancer specimens and # indicates adjacent non-cancerous tissues. Data are representative of three independent experiments. (C) Immunohistochemistry analysis of the expression of FRA-1 protein in the gastric cancer and the adjacent non-cancerous tissues. Antibody of FRA-1 protein was used; brown grains denote positive signal. (a) H&E staining of gastric epithelial tissue, (b) FRA-1 staining of gastric epithelial tissue, (c) H&E staining of gastric cancer tissue, (d) FRA-1 staining of gastric cancer tissue. Original magnification, x200.

42.3% (11/26) in gastric cancer and the adjacent non-cancerous tissues, respectively (P=0.024) (Fig. 1C and Table III), thus, corresponding with the qPCR results.

Analysis of cell cycle in AGS cells overexpressing Fra-1. Cell cycle progression was assessed in AGS, AGS/vector and AGS/Fra-1 cells by flow cytometry. For AGS and AGS/vector cells, the percentages of G0/G1, S and G2/M phases were 59.2, 22.4, 18.4 and 60.1, 22.0, 17.9%, respectively. Whereas, AGS cells

with Fra-1 overexpression were mostly in the proliferative phase, and showed significant increase in S phase and reduction in G0/G1 phase, the percentages of G0/G1, S and G2/M phases were 48.9, 32.4 and 18.7%, respectively (Fig. 2A). The results suggested that Fra-1 affected cell cycle distribution of gastric cancer cells.

Fra-1 inhibits gastric cancer cell apoptosis. Promotion of cell proliferation is usually mediated by inhibition of cell apop-

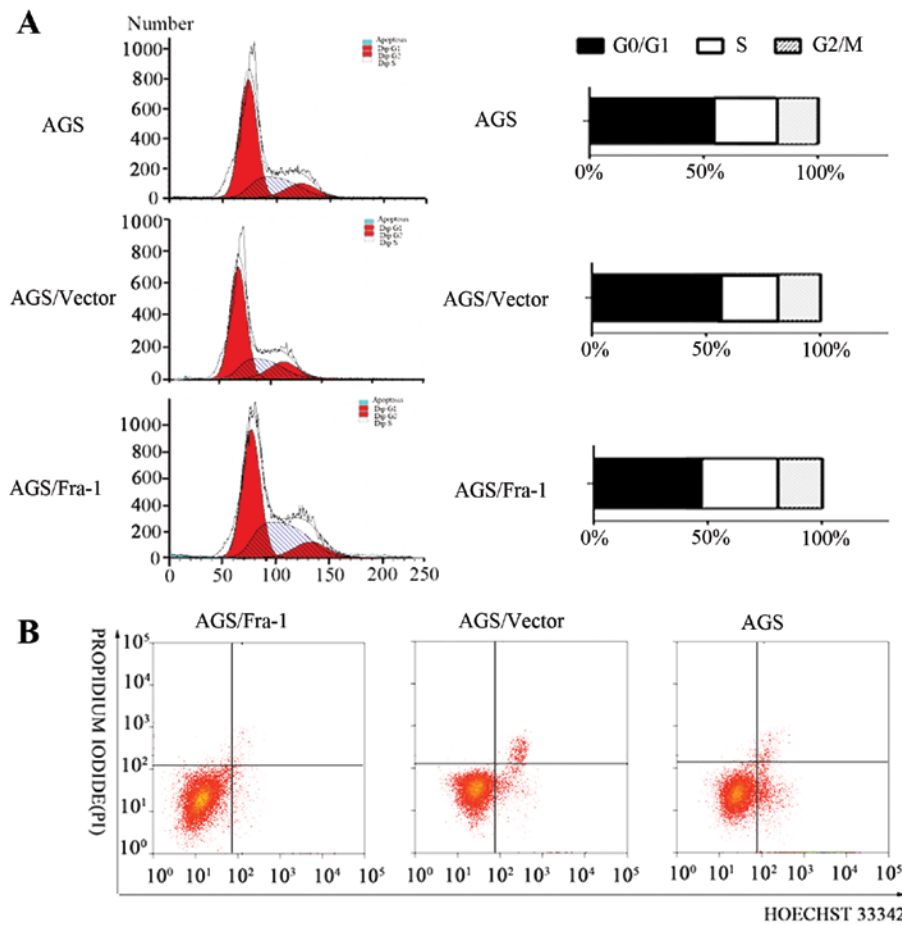


Figure 2. Fra-1 affects the distribution of cell cycle and inhibits cell apoptosis in AGS gastric cancer cells. (A) The distribution of the cell cycle in AGS gastric cancer cells. AGS/Fra-1, transfection with pEGFP-N1-Fra-1 plasmid after 48 h, AGS/vector, transfection with pEGFP-N1 plasmid after 48 h, AGS, transfection without plasmid. (B) The effect of Fra-1 to AGS gastric cancer cells. AGS/Fra-1, transfection with pEGFP-N1-Fra-1 plasmid after 48 h, AGS/vector, transfection with pEGFP-N1 plasmid after 48 h, AGS, transfection without plasmid. Data are representative of three independent experiments.

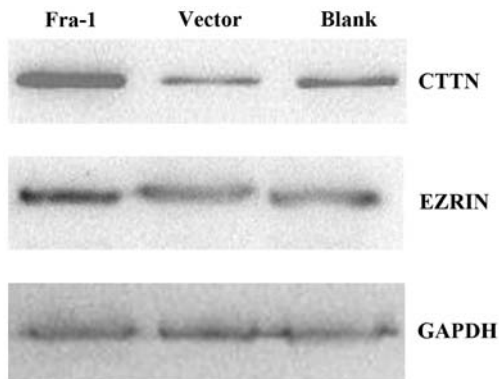


Figure 3. Expression levels of CTTN and EZR protein in AGS gastric cancer cells. Fra-1, AGS cells transfected with pEGFP-N1- Fra-1. Vector, AGS cells transfected with pEGFP-N1. Blank, AGS cells not transfected with plasmid. Data are representative of three independent experiments.

tos. To determine whether apoptosis mediated the growth in AGS, AGS/vector and AGS/Fra-1 cells, we performed a Hoechst 33342/PI double-staining experiment. A considerable decrease in apoptotic cells was observed for AGS/Fra-1

cells ($5.27 \pm 0.24\%$), AGS cells ($11.54 \pm 0.87\%$), and AGS/vector cells ($10.97 \pm 0.73\%$) (Fig. 2B). Our results suggested that Fra-1 inhibited gastric cancer cell apoptosis.

Identification of proteins affected by Fra-1 through LC-MS/MS analyses. We found that Fra-1 was highly expressed in gastric cancer tissues and inhibited apoptosis of gastric cancer cells *in vitro*. Furthermore, we identified the differential molecule affected by Fra-1 through LC-MS/MS analyses. Our results showed that there were 21 proteins which were only present in AGS/Fra-1 cells and there were 16 proteins which were only at present in AGS/vector cells. According to the frequency of unique peptides, the CARS protein was the highest in AGS/Fra-1 cells followed by CLTB, CTTN, EHD1, EPS8L2, and EZR (Table III). ACTG1 protein was the highest in AGS/vector cells (Table IV).

To confirm the reliability of the proteins identified, we tested the expression levels of CARS, CLTB, CTTN, EHD1, EPS8L2 and EZR by western blot analysis. The results showed that CTTN and EZR were upregulated in AGS/Fra-1 cells compared with AGS/vector cells and AGS cells (Fig. 3). It corresponded with the LC-MS/MS results. Further four proteins were no significantly different among AGS/Fra-1 cells, AGS/vector cells and AGS cells.

Table IV. The differential proteins affected by FRA-1 through LC-MS/MS analyses.

No.	AGS/FRA-1				AGS/vector			
	Protein	Unique peptides	Coverage (%)	Protrin score	Protein	Unique peptides	Coverage (%)	Protrin score
1	CARS	48	25.05	262.99	ACTG1	44	23.05	231.28
2	CLTB	33	21.63	171.87	ARF6	42	11.23	224.82
3	CTTN	33	19.64	132.1	CSTB	30	24.68	150.08
4	EHD1	32	9.13	114.32	DDX56	29	23.47	156.81
5	EPS8L2	31	24.33	179.56	EIF4A1	28	43.83	119.16
6	EZR	31	25.90	157.76	LAMB3	26	16.73	137.39
7	GIPC1	30	46.99	149.72	MTA2	26	16.30	72.34
8	GPRC5A	27	20.13	144.92	MYOF	25	44.80	153.84
9	HMBS	26	45.72	184.4	PPM1G	25	18.73	110.75
10	ITGA3	26	14.31	158.29	RRP9	24	28.79	121.47
11	KRT17	24	48.59	151.03	S100A16	23	47.65	168.64
12	LMO7	23	17.38	132.3	TBRG4	23	32.13	96.54
13	LRRFIP1	21	26.11	104.45	TM4SF1	21	37.00	175.58
14	MRPL17	20	36.47	158.23	TOR4A	21	51.35	167.67
15	NOP16	20	46.78	124.36	TUBA1C	21	12.18	102.87
16	NUDT5	20	29.51	105.72	UBC	20	39.47	157.66
17	RALB	20	17.95	101.27				
18	TTLL12	19	39.36	216.67				
19	TUBB4B	19	48.45	135.76				
20	TUBG1	19	35.49	117.77				
21	VPS26A	19	32.71	95.42				

Ranked by unique peptides.

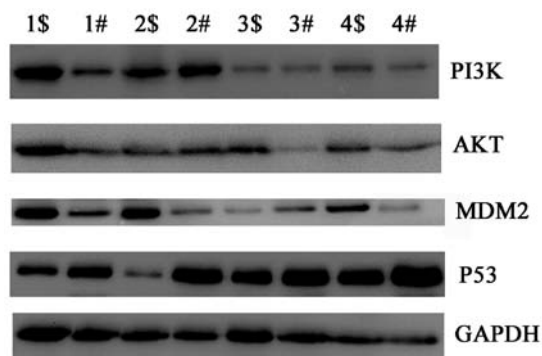


Figure 4. Expression levels of PI3K, AKT, p53 and MDM2 protein in gastric cancer and the adjacent non-cancerous tissues. In total, 1, 2, 3 and 4 tissues which were used in the detection of mRNA expression levels by qPCR were selected to detect the expression levels of PI3K, AKT, p53 and MDM2 protein by western blot analysis. \$ indicates gastric cancer specimens and # indicates adjacent non-cancerous tissues. Data are representative of three independent experiments.

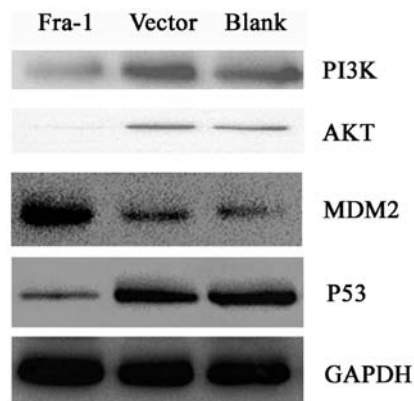


Figure 5. Expression levels of PI3K, AKT, p53 and MDM2 protein in AGS gastric cancer cell lines. Fra-1, AGS cells transfected with pEGFP-N1-Fra-1. Vector, AGS cells transfected with pEGFP-N1. Blank, AGS cells not transfected with plasmid. Data are representative of three independent experiments.

Fra-1 is correlated with dysregulation of PI3K, Akt, MDM2 and p53 in gastric cancer tissues *in vitro*. To uncover the possible mechanism of Fra-1 in gastric cancer, we tested the expression levels of key molecules in the PI3K/Akt signaling pathway by western blot technology. PI3K and Akt were upregulated in gastric cancer compared with the adjacent

non-cancerous tissues. MDM2 had the same tendency with PI3K and Akt, but p53 was downregulated in gastric cancer (Fig. 4). Combined with the above results in which Fra-1 was highly expressed in gastric cancer, we inferred that Fra-1 is correlated with dysregulation PI3K/Akt and p53 signaling pathway in gastric cancer tissues *in vitro*.

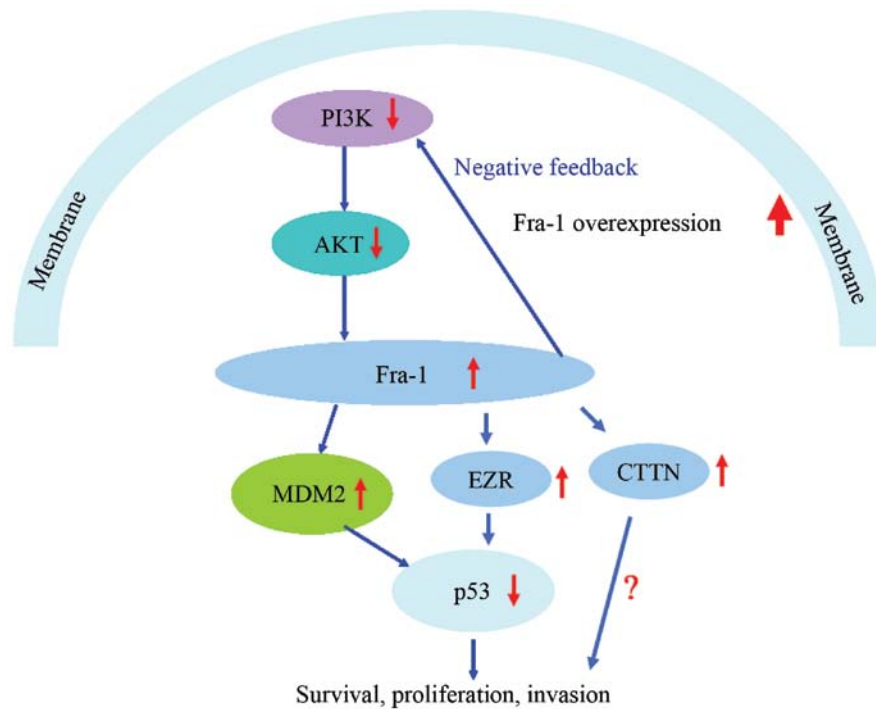


Figure 6. The proposed Fra-1 related pathway.

Fra-1 overexpression affects the expression of *PI3K*, *Akt*, *MDM2* and *p53* *in vivo*. To confirm whether *Fra-1* affects the expression of *PI3K*, *Akt*, *MDM2* and *p53* *in vivo*, we constructed the plasmid pEGFP-N1-*Fra-1*. The plasmid of pEGFP-N1-*Fra-1* and pEGFP-N1 were transfected into AGS gastric cancer cells. We harvested the cells after 48 h with transfection and tested the expression levels of *PI3K*, *Akt*, *MDM2* and *p53* proteins *in vivo*. The *PI3K* and *Akt* were downregulated in AGS cells which *Fra-1* was overexpressed. The *MDM2* was upregulated in AGS cells in which *Fra-1* was overexpressed. Furthermore, *p53* was downregulated in AGS cells in which *Fra-1* was overexpressed (Fig. 5). Our results suggested that *Fra-1* overexpression affected the expression of *PI3K*, *Akt*, *MDM2* and *p53* *in vivo* (summary in Fig. 6).

Discussion

Despite an overall decline in incidence over the last several decades, gastric cancer (GC) remains the fourth most common type of cancer and is the second leading cause of cancer-related death worldwide (26,27). Although some evidence has been reported, the mechanism of GC is not fully understood (28-30). Thus, identifying molecular aberrations in GC may improve our understanding of gastric carcinogenesis and help us subdivide patients into biologically and clinically relevant subgroups, as well as develop novel therapeutic strategies.

In the present study, we found that the expression of the *Fra-1* gene was higher in the gastric cancer samples compared with the adjacent non-cancerous tissues and the normalized *Fra-1* gene expression in gastric cancer was upregulated 2.99-fold ($P < 0.01$). Similar tendency was seen by western blot experiments. IHC showed a similar pattern in protein expression with qPCR results and western blot results.

Adisheshaiah *et al* (2) found that *FRA-1* was frequently overexpressed in squamous cell carcinomas of the stomach and esophagus. High *Fra-1* expression is associated with a more malignant cell phenotype and *Fra-1* could have a pivotal role in breast cancer progression (31). Philips *et al* (32) found that high *Fra-1* concentration was crucial for the negative regulation of AP-1 activity by estradiol and that *Fra-1* might take part in estradiol-induced inhibition of cell proliferation in ER alpha-breast cancer cells transfected with ER alpha expression construct. The results of the study by Shirsat *et al* (33) showed that overexpression of *Fra-1* could lead to proliferation inhibition of C6 glioma cells. Our results are consistent with these reports.

Our results showed that *Fra-1* was able to inhibit cell apoptosis of gastric cancer cell and increase the rate of S phase of cell cycle. Deregulation of the cell cycle underlies the aberrant cell proliferation that characterizes cancer and loss of cell cycle checkpoint control promotes genetic instability. Moreover, we identified and confirmed that *Fra-1* affected the expression level of *CTTN* and *EZR* *in vitro* by LC-MS/MS analyses. Our results suggested that *Fra-1* might function by regulating the expression of *CTTN* and *EZR*. Zhang and Qi (34) found that *MTSS1* suppressed cell migration and invasion by targeting *CTTN* in glioblastoma. Cortactin (*CTTN*) overexpression in osteosarcoma is correlated with its advanced stage and reduced survival (35). Luo *et al* (36) found that amplification and overexpression of *CTTN* (*EMS1*) contributed to the metastasis of esophageal squamous cell carcinoma by promoting cell migration and anoikis resistance. Our results also hinted that *CTTN* might play an important role in the malignancy.

The *PI3K/Akt* pathway is known to play key roles in cell apoptosis, cell proliferation, and cell survival in various kinds of cells (37). It is generally demonstrated that the *PI3K/AKT* signaling pathways regulate metastasis in a variety of cancer

cells (38,39). Our results showed that Fra-1 was correlated with dysregulation of PI3K/Akt and p53 signaling pathway in gastric cancer tissues *in vitro* and that Fra-1 overexpression affected the expression of PI3K, Akt, MDM2 and p53 *in vivo*. Degradation of p53 is regulated by its interaction with specific E3 ubiquitin ligases, the best known, being encoded by MDM2 (40). Damage to p53-dependent mechanism is often caused by overexpression of MDM2, which codes for a p53-regulating protein (41).

In summary, our results suggested that Fra-1 was upregulated in gastric cancer and affected the expression of CTTN and EZR in AGS gastric cancer cells. We found that Fra-1 was involved with dysregulation of PI3K/Akt and p53 signaling pathway *in vitro* and that Fra-1 overexpression affected the expression of PI3K, Akt, MDM2 and p53 *in vivo*. Thus, as shown (Fig. 6) we inferred that Fra-1 functions by regulating the expression of CCTN and EZR and dysactivation of the PI3K/Akt and p53 signaling pathway.

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