FRAT1 expression regulates proliferation in colon cancer cells

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Abstract. Colorectal cancer is one of the most common gastric malignancies worldwide. However, the underlying mechanism of colon cancer development and valuable indicators of the disease remain unclear. In this study, the expression of frequently rearranged in advanced T-cell lymphomas 1 (FRAT1) in colon cancer was investigated and the association between FRAT1 expression and biological properties of tumors was analyzed. A total of 147 colon cancer tissue samples and adjacent normal tissues were collected between January 2013 and June 2014. The FRAT1 gene and protein expression levels were analyzed in tissues with different TNM and pathological stages. Small hairpin RNAs (shRNAs) containing the human FRAT1 gene were constructed and transfected into colon cancer HT-29 cells. The proliferation and migration of the cells was also analyzed in relation to a reduction in FRAT1 expression. In colon cancer tissues, the expression of FRAT1 was significantly higher when compared with adjacent tissues. In addition, FRAT1 expression was found to positively correlate with the degree of tumor malignancy, and this difference was determined to be statistically significant ($P<0.05$). Following shRNA transfection in HT-29 cells to decrease the expression of FRAT1, the proliferation and migration of the HT-29 cells decreased (due to conversion of the shRNA into small interfering RNA). These results indicate that in colon cancer, FRAT1 may present a novel tool for analyzing the tumor progression and may be a novel therapeutic target for the treatment of colon cancer.

Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies worldwide. It is ranked fifth in terms of cancer-associated mortality in China (1). The incidence of colon cancer has been increasing in a large number of countries over the previous 20 years (2,3). The 5-year relative survival rate for colon cancer patients is 64.2% (4). Approximately 694,000 patients succumb to CRC globally every year (5). The CRC incidence and mortality both increased prior to the onset of organized screening in middle-income countries, such as China and Thailand (6), which inflicts a high socio-economic burden.

The majority of cases of colorectal cancer develop from polyps, however, patients with colon polyps and precancerous tumors typically have no obvious symptoms. For diagnosis of CRC, sigmoidoscopy or colonoscopy with biopsy can be used to confirm the presence of cancer tissues. Surgery is the most common method of treatment for CRC. Due to advances in surgical techniques and neoadjuvant chemotherapy, the median survival of patients with early-stage colon cancer continues to improve (7), however, prognosis remains poor for patients with advanced colorectal cancer. Previous studies indicated that 10-30% of patients with CRC eventually develop recurrent disease, despite receiving radical treatment (8,9).

The pathology and clinical stage of colon cancer tumors has been shown to be closely associated with oncogene activation and suppression (10). The Wnt/β-catenin pathway is thought to be central to the process of carcinogenesis in CRC (11-13). Recently, a novel proto-oncogene, frequently rearranged in advanced T-cell lymphomas 1 (FRAT1), has increasingly received attention. The FRAT1 gene has been demonstrated to be involved in the regulation of β-catenin expression and secretion (14). Previous studies have demonstrated that in non-small cell lung cancer and gastric cancer tissues, overexpression of FRAT1 activates the Wnt signaling pathway and promotes tumor malignancy (15,16). However, few studies have investigated the effect of FRAT1 expression in colon cancer. In the present study, colon cancer specimens were obtained from colon cancer patients undergoing gastrointestinal surgery. The aim of the present study was to investigate the expression of FRAT1 in colon cancer, and to analyze the association between FRAT1 expression and proliferation and migration of tumor cells.

Materials and methods

Patients and tissue samples. A total of 147 colon cancer tissue samples and adjacent normal control samples were obtained from patients diagnosed with colon cancer who underwent radical surgery at The Second Hospital of Shandong University (Jinan, China) between January 2013 and June 2014. All patients exhibited primary tumors and none of the patients had received chemotherapy or radiation therapy prior to tumor excision. The patient cohort included 93 males and

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54 females, with a median age of 62 years (range, 37-72 years). Patient clinicopathological factors are shown in Table I. The present study was approved by the Ethics Committee of the Second Hospital of Shandong University (Jinan, China) and additionally received institutional approval. The experiments were performed in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research.

Western blot analysis. Resected tissues were washed using phosphate-buffered saline (PBS), cut into sections and lysed with RIPA lysis and extraction buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The lysates were centrifuged in an ice bath to obtain the supernatant and total protein was calculated using the bicinchoninic acid assay method (Pierce BCA Protein Assay kit; Thermo Fisher Scientific, Inc.). A total of 50 μg protein was used for each sample. Following electrophoresis with 12% gel, samples were transferred to polyvinylidene fluoride membranes and incubated with FRAT1 rabbit monoclonal antibody (1:500; catalog no., ab108405; Abcam, Cambridge, UK) overnight at 4˚C. Next, the membranes were incubated with goat anti-rabbit IgG-horseradish peroxidase (1:2,000; catalog no., sc-2005; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37˚C for 2 h and visualized using a chemiluminescence detection reagent (Applygen Technologies, Inc., Beijing, China). The western blot gray values were quantified using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNA was isolated from the tissue samples using mechanical homogenization and TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China). cDNA was synthesized from the total RNA using SuperScript III RNase H-Reverse Transcriptase (Takara Biotechnology Co., Ltd.). Gene expression was quantified using a One-Step SYBR PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd.). The primers were designed as described previously (17). The sequences of the primers were as follows: Forward, 5'-GCC CTGTCTAAAGTGTATTTTCAG-3' and reverse, 5'-CGC TTGAGTAGGACTGCAGAG-3' for FRAT1 (Invitrogen; Thermo Fisher Scientific, Inc.). GADPH was used as the internal reference gene and its primers were as follows: forward, 5'-GAAGTGAAGGTCGGAGTCA-3' and reverse, 5'-TTCACACCCATGACGAACAT-3'. The gene expression was analyzed on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR was performed in a 10 μl reaction volume, which consisted of 5 μl One-Step SYBR buffer III, 0.2 μl Takara Ex *Taq* HS, 0.2 μl PrimerScript RT enzyme mix II, 0.2 μl forward primer, 0.2 μl reverse primer, 1 μl RNA and 3.2 μl RNase-free dH_20. PCR was performed under the following conditions: Initial denaturation at 90˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 45 sec, annealing at 55˚C for 40 sec and extension at 72˚C for 50 sec (18).

Cell transfection with FRAT1. The specific sequence of the shRNA targeting FRAT1 was designed as previously described (19-22) using the following primer sequences: Forward, 5'-GCAGTTACGTGCAAAGCTTTTCAAGAG AAAGCTTTGCACGTACTGC-3' and reverse, 3'-CGTCAA TGCACGTTTCGAAAAGTTCTCTTTCGAAACGTGCAT TGACG-5'. The shRNA template was then added to two endonucleases, *Hind*III and *BamH*1, for digestion. The synthetic template was annealed to form a double-stranded oligo and then cloned into the vector pLV-H1-EF1a-puro (Biosettia Inc., San Diego, CA, USA). Finally, recombinant pshRNA-FRAT1 was successfully constructed and verified by Sanger dideoxy DNA sequencing. HT-29 cells were transfected with the recombinant plasmid to screen the stable cell line for FRAT1 inhibition. In addition, HT-29 cells were tranfected with empty vector pLV-H1-EF1a-puro and used as the control. To ensure efficient transfection, reverse transcription (RT)-PCR and western blot analysis were used to analyze FRAT1 gene and protein expression.

Table I. Clinicopathological factors of 147 colon cancer patients.

Parameter	Patients, n
Gender	
Male	93
Female	54
Age (median, range) (years)	62 (37-72)
TNM stage	
I	26
II	45
III	51
IV	25
Tumor differentiation	
Well-differentiated	41
Moderately differentiated	82
Poorly differentiated	24

MTT assay. Cell proliferation was analyzed using the MTT colorimetric method. HT-29 cells ($1x10^4$ cells/well) were subcultured to 80% confluence and seeded in 96-well plates. The cells were incubated for 48 h, and were cultured for 4 h with 5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA). The optical density (OD) was then measured (ELx800 UV universal microplate reader; Bio-Tek Instruments, Inc., Winooski, VT, USA) and the inhibition rate was calculated according to the following formula: Inhibition rate = 1 - experimental OD / control OD (23).

Cell migration was performed using Transwell inserts (8-μm pore size; Merck Millipore, Darmstadt, Germany). The upper chamber was coated with 1 mg/ml Matrigel (Corning, Inc., Corning, NY, USA). The cells were incubated without serum for 12 h and seeded at a density of $2x10^5$/ml into the upper chamber, while 600 μl 10% serum medium was placed into the lower chamber. The cells were cultured for 36 h and subsequently the cells on the upper surface were removed gently with a cotton swab. The Transwell chambers were fixed in 95% ethanol for 15 min and washed with PBS 3 times. Eosin was used to stain the chambers for 10 min, followed by washing with PBS. Finally, the chambers were viewed under a high-powered inverted microscope (CKX41; Olympus

Table II. FRAT1 expression in 147 colon cancer tissue samples.

Clinical characteristics	Patients, n	FRAT1 expression (±SEM)	FRAT1 WB gray (±SEM)	P-value
Histological grade				
Well differentiated	41	7.39±2.65	2.42±2.21	0.012
Moderately differentiated	82	4.39±1.98	4.92±1.77	
Poor differentiated	24	3.12±1.66	5.78±1.98	
TNM stage				0.015
I	26	2.13±0.79	1.79±0.32	
II	45	3.96±1.46	2.12±0.51	
III	51	5.77±1.82	3.79±0.98	
IV	25	8.02±2.04	5.13±1.22	

Data are expressed at the mean ± SEM. FRAT1, frequently rearranged in advanced T-cell lymphomas 1; PCR, polymerase chain reaction; WB, western blot; SEM, standard error of the mean; gray, gray value.

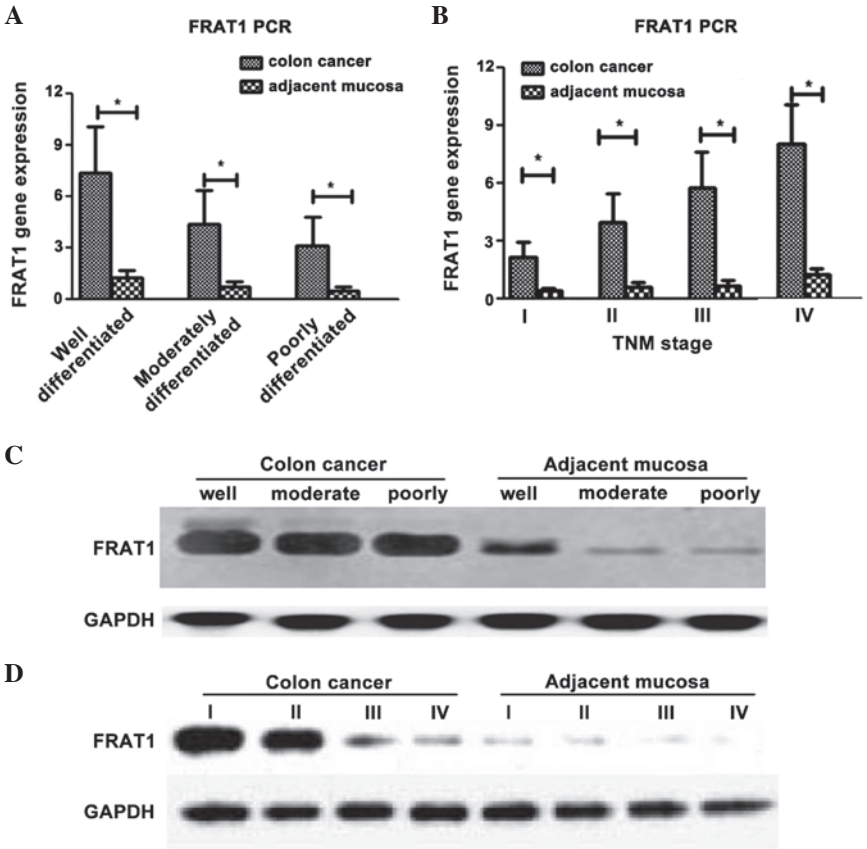

Figure 1. PCR and western blot analysis of FRAT1 gene and protein expression. PCR revealed that (A) FRAT1 mRNA expression was significantly higher in colon cancer tissues than in adjacent tissues at all pathological stages (*P<0.05) and (B) at different TNM stages (*P<0.05). Western blot analysis revealed that FRAT1 protein expression was significantly higher in colon cancer tissues of (C) all pathological stages and (D) TNM stages, when compared with the adjacent tissues. PCR, polymerase chain reaction; WB, western blot; FRAT1, frequently rearranged in advanced T-cell lymphomas 1.

Corp., Tokyo, Japan). Cells were counted in 6 random fields at magnification, x200.

Statistical analysis. All data analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of the mean. Differences between groups were determined using Student's *t*-test and the correlation between FRAT1 expression, TNM stage and pathological stage was determined using χ^2 and Spearman's rank correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

FRAT1 protein and gene expression in colon cancer and normal tissues. As shown in Fig. 1, mRNA expression of

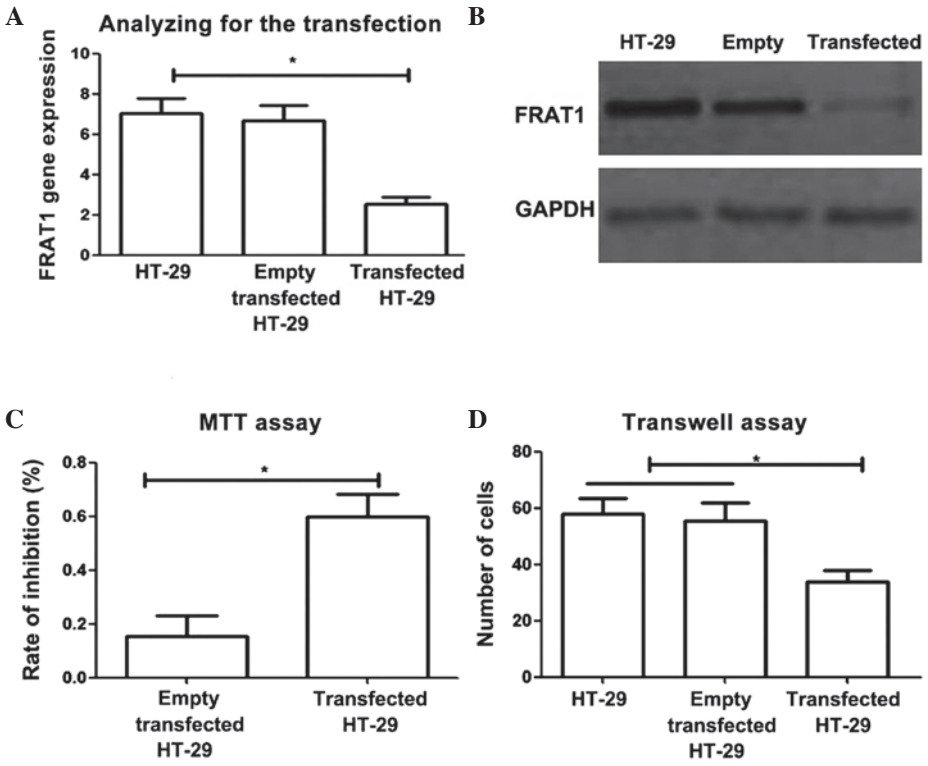

Figure 2. Analysis of HT-29 colon cancer cell FRAT1 gene and protein expression following transfection with empty vector and pshRHA-FRAT1. (A) FRAT1 gene and (B) protein expression in HT-29, empty vector cells and transfected HT-29 cells. GAPDH was used as the internal reference gene. FRAT1 gene expression was significantly higher in HT-29 and empty transfected HT-29 cells when compared with transfected HT-29 cells. (C) MTT assay revealed that inhibition of proliferation was significantly higher in transfected HT-29 cells when compared with empty transfected HT-29 cells (*P<0.05). (D) Transwell assay revealed that migration was significantly decreased in the transfected HT-29 cells compared with the control group (*P<0.05 vs. control).

FRAT1 was significantly increased in colon cancer tissues compared with adjacent tissues at different pathological stages (P<0.05; Fig. 1A) and various Tumor-Node-Metastasis (TNM) stages (P<0.05; Fig. 1B) (24). Western blot results revealed that the expression of FRAT1 significantly increased in colon cancer tissues of various pathological (P<0.05; Fig. 1C) and TNM stages (P<0.05; Fig. 1D) compared with adjacent tissues.

Correlation between clinicopathological stage and FRAT1 expression. In the colon cancer tissues, increased FRAT1 gene and protein expression was found to significantly correlate with increased tumor malignancy and a higher TNM stage (P<0.05) (Table II).

Effects of regulating FRAT1 expression on colon cancer cell proliferation and apoptosis. Small hairpin RNA (shRNA)-transfected HT-29 cells were analyzed using PCR and western blotting to determine the effects of reducing FRAT1 expression. The results revealed that levels of FRAT1 gene and protein expression were significantly decreased in transfected HT-29 cells (due to conversion of shRNA to small interfering RNA) compared with control cells (P<0.05).

To investigate the effects of FRAT1 gene regulation on the proliferation and migration of colon cancer cells, MTT and Transwell assays were performed. MTT assay revealed that reducing FRAT1 expression significantly inhibited the proliferation of colon cancer cells (Fig. 2C; P<0.05). Furthermore, Transwell assay demonstrated that in transfected HT-29 cells, the number of cells which passed through Matrigel within 12 h was significantly lower than that in normal HT-29 cells and empty vector transfected HT-29 cells (P<0.05). These results indicated that reducing FRAT1 expression significantly decreased the migration ability of tumor cells (Fig. 2D).

Discussion

FRAT1 was originally identified as a T-cell lymphoma proto-oncogene in mice (25). Recent studies have confirmed that the abnormal activation of FRAT1 increased by competing with Axin for glycogen synthase kinase 3 phosphorylation sites binding, subsequently inhibiting the degradation of Wnt/β-catenin phosphorylation and improving the cytoplasmic secretion and retention of β-catenin. Subsequently, the activation of β-catenin led to altered cell proliferation and apoptosis (26-28).

High FRAT1 expression has been demonstrated in a variety of tumors, including non-small cell lung cancer, ovarian cancer, brain glioblastoma and other tumor tissues (14,29-31). High FRAT1 expression is associated with the increased malignancy and a higher clinical stage, thus FRAT1 affects tumor biological characteristics (19). However, studies investigating FRAT1 expression in digestive tract tissue are limited. Previous studies have demonstrated an association between increased FRAT1 expression and a higher TNM stage and pathological stage in gastric cancer. However, further study is required (16,18).

Previous studies using surgical specimens and statistical analysis have been performed (1,27,28,32). In the present

study, gene and protein expression of FRAT1 was analyzed in colon cancer specimens of different TNM and pathological stages. The results showed that FRAT1 expression was significantly higher in stage III and IV patients than stage I and II patients ($P<0.05$). Furthermore, the pathological findings revealed that FRAT1 expression was significantly increased in poorly differentiated colon cancer tissues compared with that in well- and moderately-differentiated colon cancer tissues ($P<0.05$). Previous studies have also shown that in colon cancer, FRAT1 expression is positively correlated with clinical and pathological progression of tumors (33-35). Taking into consideration a previous study, which investigated the mechanism of colon cancer development (34), we hypothesize that in colon cancer, regulation of the FRAT1-Wnt/β-catenin pathway occurs. Therefore, similar to the non-small cell lung cancer, FRAT1 may present a novel therapeutic target for colon cancer.

Although statistical analysis has revealed a positive correlation between tumor progression and FRAT1 expression, such analysis does not show the direct effect of FRAT1 expression on tumor cells (36). Therefore, *in vitro* shRNA interference experiments were conducted to investigate the effects of FRAT1 expression on tumor cells. An interference expression vector was constructed and transfected into the human colon cancer cell line, HT-29, which inhibited FRAT1 expression, as confirmed by RT-PCR and western blot analysis. MTT and Transwell assays revealed that migration and proliferation of the transfected colon cancer cells were significantly decreased. Thus, regulation of FRAT1 expression may be used to inhibit cancer cell proliferation and migration, which are known to be associated with the clinicopathological stage. Thus, we hypothesize that FRAT1 exerts an important role in the development of colon cancer, and the inhibition of FRAT1 expression effectively inhibits cancer cell progression.

In conclusion, the results of the present study indicate that in colon cancer, FRAT1 gene and protein expression is positively correlated with TNM and pathological stage. In addition, a positive correlation was also identified between FRAT1 expression and the degree of tumor malignancy. Further cell transfection experiments revealed that inhibition of FRAT1 expression significantly reduced proliferation and migration in colon cancer cells. Therefore, FRAT1 may present an important tool for evaluation and an important therapeutic target for the treatment of colon cancer, however, further study is required.

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