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Overexpression of Spleen Tyrosine Kinase Inhibits Proliferation and Promotes Apoptosis of Colorectal Cancer Cells by Regulating Fra-1 (Postprint)

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Date: 2018-06-15T00:00:00+00:00

Abstract

Objective: To investigate the effects of spleen tyrosine kinase (SYK) overexpression on the proliferation and apoptosis of colorectal cancer cells and the potential underlying mechanisms. Methods: The recombinant plasmid pcDNA.3.1-SYK was constructed using the pcDNA.3.1 vector and transfected into colorectal cancer cells to overexpress SYK. The experimental groups were as follows: (1) pcDNA.3.1-SYK (HCT116): HCT116 cells transfected with pcDNA.3.1-SYK; (2) pcDNA.3.1 (HCT116): HCT116 cells transfected with pcDNA.3.1 empty vector; (3) Normal (HCT116): Normal HCT116 cells. (1) pcDNA.3.1-SYK (Sw480): Sw480 cells transfected with pcDNA.3.1-SYK; (2) pcDNA.3.1 (Sw480): Sw480 cells transfected with pcDNA.3.1 empty vector; (3) Normal (Sw480): Normal Sw480 cells. qRT-PCR was used to detect the mRNA expression levels of SYK and Fra-1 in colorectal cancer tissues and adjacent normal tissues. Western blot was performed to determine the protein expression levels of SYK and Fra-1. MTT assay was used to assess cell viability. BrdU assay was employed to measure cell proliferation activity. Caspase-3 activity was measured using a commercial kit. Cell apoptosis was detected by Annexin-V FITC/PI staining. Results: SYK expression was downregulated in both colorectal cancer tissues and colorectal cancer cell lines ($P<0.01$). Transfection of colorectal cancer cells with pcDNA.3.1-SYK significantly increased both SYK mRNA ($P<0.01$) and protein expression levels ($P<0.01$), indicating successful SYK overexpression. Following SYK overexpression, the viability and proliferative activity of colorectal cancer cells were significantly decreased ($P<0.01$), while cell apoptosis was increased ($P<0.01$). Additionally, Fra-1 expression was significantly suppressed after SYK overexpression ($P<0.01$). Conclusion: SYK overexpression inhibits the proliferation of colorectal cancer cells and promotes their apoptosis, and the underlying mechanism may be related to the regulation of Fra-1 by SYK, pro-

viding a theoretical basis and reference value for the prevention and treatment of colorectal cancer.

Full Text

Preamble

Spleen Tyrosine Kinase Inhibits Proliferation and Promotes Apoptosis of Colorectal Cancer Cells In Vitro via Regulating Fra-1

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Abstract

Objective: To investigate the effects of spleen tyrosine kinase (SYK) overexpression on proliferation and apoptosis of colorectal cancer cells and explore the possible underlying mechanisms.

Methods: Recombinant pcDNA.3.1-SYK plasmid was constructed and transfected into colorectal cancer cells to achieve SYK overexpression. The experimental groups were as follows: (1) pcDNA.3.1-SYK (HCT116): HCT116 cells transfected with pcDNA.3.1-SYK; (2) pcDNA.3.1 (HCT116): HCT116 cells transfected with empty pcDNA.3.1 vector; (3) Normal (HCT116): untreated HCT116 cells. Similarly for Sw480 cells: (1) pcDNA.3.1-SYK (Sw480); (2) pcDNA.3.1 (Sw480); and (3) Normal (Sw480). qRT-PCR was used to detect mRNA expression levels of SYK and Fra-1 in colorectal cancer and adjacent tissues, while Western blotting measured their protein expression. Cell viability was assessed by MTT assay, proliferation activity by BrdU incorporation, Caspase-3 activity by commercial kit, and apoptosis by Annexin-V FITC/PI staining.

Results: SYK expression was significantly reduced in both colorectal cancer tissues and cell lines ($P<0.01$). Transfection with pcDNA.3.1-SYK markedly increased SYK mRNA and protein expression ($P<0.01$), confirming successful overexpression. SYK overexpression significantly decreased colorectal cancer cell growth and proliferation ($P<0.01$) while increasing apoptosis ($P<0.01$). Additionally, Fra-1 expression was significantly suppressed following SYK overexpression ($P<0.01$).

Conclusion: Overexpression of SYK inhibits proliferation and promotes apoptosis of colorectal cancer cells, possibly through regulation of Fra-1 expression. These findings provide a theoretical basis and reference value for colorectal cancer prevention and treatment.

Keywords: colorectal cancer; spleen tyrosine kinase; overexpression; Fra-1

Colorectal cancer is one of the most common gastrointestinal malignancies. In addition to genetic factors, dietary factors and various precancerous lesions such as colorectal adenomas, non-adenomatous polyposis, and inflammatory bowel disease can all contribute to colorectal carcinogenesis [1-4]. Its incidence and mortality rates continue to rise, with increasing incidence among women, making the diagnosis and treatment of colorectal cancer a formidable challenge [5-6]. Spleen tyrosine kinase (SYK) is a non-receptor protein tyrosine kinase with tumor suppressor functions that has been extensively studied in breast cancer, where reduced or absent SYK expression correlates with enhanced invasiveness [7-9]. In myeloma, SYK similarly inhibits cell proliferation and migration [10]. Furthermore, gastric cancer progression and lymph node metastasis are associated with decreased SYK expression [11]. However, the relationship between SYK expression and proliferation and apoptosis in colorectal cancer cells remains unreported in the literature. Therefore, this study aimed to investigate the impact of SYK overexpression on colorectal cancer cell proliferation and apoptosis to provide valuable insights for colorectal cancer prevention and therapy.

Materials

HCT116, Sw480, and FHC cells were purchased from the American Type Culture Collection (ATCC). Fetal bovine serum and DMEM medium were obtained from Gibco (USA). SYBR Premix Ex Taq II, TRIZOL reagent, and plasmid mini-prep kits were purchased from Takara Bio (Dalian, China). XhoI, Turbofect transfection reagent, EcoRI, and reverse transcription kits were from Thermo Fisher Scientific. SYK mouse monoclonal antibody and Fra-1 mouse monoclonal antibody were from Abcam (UK). GAPDH mouse monoclonal antibody and HRP-conjugated goat anti-mouse secondary antibody were from Beijing Biosynthesis Biotechnology. DH5 competent cells were from Tiangen Biotech (Beijing). MTT reagent was from Shanghai Sangon Biotech, and BCA protein assay kit was from Pierce (USA).

1.2 Cell Culture and Tissue Collection

Colorectal cancer cell lines HCT116 and Sw480, along with normal colon epithelial cells FHC, were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO . Cells were passaged upon reaching 80% confluence.

Twenty newly diagnosed colorectal cancer patients provided tumor tissues (n=10) and adjacent normal tissues (n=10) through Henan Provincial Hospital of Traditional Chinese Medicine, with approval from the Ethics Review Committee. The mean patient age was 65 years (range 50-78). Adjacent tissues were collected at least 1 cm away from the tumor margin and immediately snap-frozen in liquid nitrogen.

1.3 qRT-PCR Analysis

Tissues were pulverized in liquid nitrogen and homogenized for lysis. Total RNA was extracted using TRIzol reagent and reverse-transcribed into cDNA using a reverse transcription kit. qRT-PCR was performed with GAPDH as the internal reference. The reaction protocol included an initial denaturation at 94°C for 1 min, followed by 40 cycles of amplification, and a final extension at 72°C for 10 min. Each 20 L reaction mixture contained 10 L SYBR Premix Ex Taq II and 1 L cDNA. The primer sequences were as follows: SYK sense primer 5' -TGTCAAGGATAAGAACATCATAG-3' and antisense primer 5' -CACCACGTCATAGTAGTAATTG-3' ; GAPDH sense primer 5' -CGTCTTCACCACCATGGAGA-3' and antisense primer 5' -CGGCCATCACGCCACAGTTT-3'; Fra-1 sense primer 5'-GAGTAAGGCGCGAGCGGAACAA-3'and antisense primer 5'-TGGAACATAGAGGGAAAGGGGTCC-3'. Relative gene expression was calculated using the 2^(-ΔΔCT) method, with each sample run in triplicate.

1.4 Western Blot Analysis

Cells were harvested and lysed to extract total protein, with concentrations determined using a BCA assay kit. Protein samples (25 g) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes using semi-dry transfer. Membranes were blocked with 5% non-fat milk for 2.5 h, then incubated overnight with primary antibodies: SYK mouse monoclonal antibody (1:500), Fra-1 mouse monoclonal antibody (1:500), and GAPDH mouse monoclonal antibody (1:1000). After washing with PBS, membranes were incubated with HRP-conjugated goat anti-mouse secondary antibody (1:1000) for 1 h at room temperature. Protein bands were visualized using a gel imaging system, with GAPDH as the loading control. Experiments were repeated three times and averaged.

1.5 Construction of Recombinant pcDNA.3.1-SYK Plasmid and Cell Transfection

Total RNA was extracted from 9×10 FHC cells using TRIzol reagent and reverse-transcribed into cDNA. Using this cDNA as template, full-length SYK was amplified by PCR with the following primers: sense primer 5' -CGTACCTCGAGGCGGAATTCGCCACCATGGCCAG-3' (XhoI site underlined) and antisense primer 5'-GGAATTCGGCCTCGAGTTAGTTCACCACGTCATAGTAG-3' (EcoRI site underlined). PCR conditions were: initial denaturation at 94°C for 1 min, 35 cycles of amplification, and final extension at 72°C for 6 min. The 25 L PCR product was gel-purified, double-digested with XhoI and EcoRI, and ligated into similarly digested pcDNA.3.1 vector. The ligation product was transformed into DH5 competent cells, and positive clones were selected for plasmid amplification. Recombinant plasmids were verified by double digestion and sequencing (Shanghai Invitrogen).

For transfection, HCT116 and Sw480 cells cultured in 6-well plates at 70% confluence were transfected with 6 g of either pcDNA.3.1-SYK or empty pcDNA.3.1 vector using 7 L Turbofect according to the manufacturer' s protocol. After 48 h, transfection efficiency was verified by qRT-PCR and Western blot. The experimental groups were: (1) pcDNA.3.1-SYK (HCT116): HCT116 cells transfected with pcDNA.3.1-SYK for SYK overexpression; (2) pcDNA.3.1 (HCT116): HCT116 cells transfected with empty vector as negative control; (3) Normal (HCT116): untreated HCT116 cells. The same grouping was applied for Sw480 cells.

1.6 Annexin-V FITC/PI Apoptosis Detection

Apoptosis was assessed using the Annexin-V FITC/PI kit according to the manufacturer' s instructions. Transfected cells were pre-chilled with PBS, then incubated with 12 L Annexin-V FITC (1 g/mL) at 4°C for 35 min. After washing with PBS, cells were incubated with 8 L propidium iodide (PI) for 8 min and analyzed by flow cytometry.

1.7 Caspase-3 Activity Assay

Cells were harvested and washed with PBS. Caspase-3 activity was measured using the BD ApoAlert Caspase-3 Fluorescent Assay Kit following the manufacturer' s protocol.

1.8 Cell Viability and BrdU Proliferation Assays

For MTT assay, cells were seeded at 5×10 cells per well in 96-well plates and cultured for 24 h. Then, 0.2 g of recombinant plasmid or empty vector was mixed with 0.4 L Turbofect in 200 L serum-free DMEM and added to each well. After 48 h, 20 L MTT (5 mg/mL) was added and incubated for 4 h. The supernatant was replaced with 150 L DMSO to dissolve formazan crystals, and absorbance at 450 nm was measured.

For BrdU assay, after cell seeding and transfection in 96-well plates, the supernatant was removed and 200 L fixation/denaturation solution was added for 30 min at room temperature. After removal, 95 L anti-BrdU-POD working solution was added for 90 min at room temperature, followed by 100 L substrate for 15 min. The reaction was stopped with 2 M sulfuric acid and absorbance at 450 nm was measured. All experiments were performed in triplicate.

1.9 Statistical Analysis

Data were analyzed using SPSS 16.0 software and expressed as mean ± standard deviation. One-way ANOVA was used for comparisons among multiple groups, with SNK-Q test for pairwise comparisons. $P<0.05$ was considered statistically significant.

2.1 Reduced SYK Expression in Colorectal Cancer Tissues and Cells

qRT-PCR and Western blot analyses revealed significantly downregulated SYK mRNA expression in colorectal cancer tissues compared to adjacent normal tissues. Similarly, SYK protein expression was markedly reduced in HCT116 and Sw480 cells compared to normal FHC colon epithelial cells ($P<0.01$, [Figure 1: see original paper]).

2.2 Upregulated SYK Expression After Cell Transfection

To verify transfection efficiency, SYK mRNA and protein levels were measured after transfection. Both mRNA and protein expression of SYK were significantly higher in pcDNA.3.1-SYK transfected cells compared to empty vector controls (mRNA: $P=0.001$ for HCT116, $P=0.004$ for Sw480; protein: $P=0.001$ for HCT116, $P=0.005$ for Sw480), confirming successful SYK overexpression ([Figure 2: see original paper]).

2.3 SYK Overexpression Inhibits Colorectal Cancer Cell Proliferation

MTT and BrdU assays demonstrated that SYK overexpression significantly reduced cell viability and proliferation activity compared to negative control groups ([Figure 3: see original paper]).

2.4 SYK Overexpression Promotes Colorectal Cancer Cell Apoptosis

To evaluate the effect of SYK on apoptosis, caspase-3 activity and Annexin-V FITC/PI staining were performed. Both caspase-3 activity and apoptotic rates were significantly elevated in SYK-overexpressing cells compared to controls (caspase-3 activity: $P=0.002$ for HCT116, $P=0.017$ for Sw480; apoptosis: $P=0.011$ for HCT116, $P=0.004$ for Sw480, [Figure 4: see original paper]).

2.5 SYK Overexpression Decreases Fra-1 Expression

To determine whether SYK overexpression affects Fra-1 levels, we measured Fra-1 mRNA and protein expression. Both were significantly reduced in SYK-overexpressing cells (mRNA: $P=0.003$ for both HCT116 and Sw480; protein: $P=0.006$ for HCT116, $P=0.007$ for Sw480, [Figure 5: see original paper]).

Colorectal cancer represents a major threat to human health, ranking second in cancer-related mortality and accounting for 10% of malignant tumor incidence and 8% of cancer deaths, with rising incidence and mortality rates [12]. The protein tyrosine kinase family comprises enzymes that catalyze tyrosine phosphorylation of substrate proteins, regulating cellular invasion and proliferation

[13-15]. As a non-receptor protein tyrosine kinase, SYK is associated with cell proliferation and is considered a tumor suppressor [16-17].

SYK is widely expressed in hematopoietic cells and functions as a B-cell receptor effector, regulating B-cell clonal expansion, proliferation, and apoptosis [18]. Studies have linked SYK to cell migration in squamous cell carcinoma [19] and shown that reduced SYK expression inhibits breast cancer cell growth and metastasis [7]. SYK represents a potential therapeutic target for prostate cancer [20] and can reduce invasion in lung and pancreatic cancers [13]. Shin et al. [21] demonstrated that SYK acts as a tumor suppressor in hepatocellular carcinoma by inhibiting proliferation and invasion. Conversely, SYK inhibition can enhance therapeutic efficacy in certain cancers, such as potentiating paclitaxel-induced cytotoxicity in ovarian cancer [22] and treating high-risk precursor B-cell acute lymphoblastic leukemia [23]. However, the role of SYK expression in colorectal cancer cell proliferation and apoptosis had not been previously reported.

Our study revealed significantly decreased SYK expression in colorectal cancer tissues and cells. SYK overexpression reduced colorectal cancer cell viability while increasing apoptosis, indicating that upregulated SYK inhibits proliferation and promotes apoptosis in colorectal cancer cells.

Caspase-3, a key cysteine protease in mammalian apoptosis known as the “death protease,” triggers downstream events that affect multiple proteins and promote cell death upon activation [24-28]. In our study, SYK overexpression significantly increased caspase-3 activity, thereby promoting colorectal cancer cell apoptosis. The proto-oncogene Fra-1 is a member of the Fos subfamily of nuclear transcription factor AP-1 [29]. While SYK can activate PI3K, the precise mechanism remains unclear [30-31]. Cao et al. [32] found that PI3K regulates Fra-1 expression through AKT in vascular smooth muscle cells, suggesting SYK may modulate Fra-1 levels. Fra-1 is closely associated with cell proliferation, differentiation, apoptosis, and tumor transformation [33-34]. Fra-1 protects tumor cells from apoptosis, and its downregulation promotes cancer cell death [35]. Studies show that reduced Fra-1 expression inhibits colon cancer cell migration, invasion, and proliferation [36], while Fra-1 upregulation suppresses apoptosis in lung cancer [34]. In our study, SYK overexpression inhibited Fra-1 expression, correlating with decreased proliferation and increased apoptosis. Collectively, these findings suggest that SYK overexpression affects colorectal cancer cell proliferation and apoptosis through regulation of Fra-1.

In summary, SYK expression is significantly reduced in colorectal cancer tissues and cells. SYK overexpression inhibits colorectal cancer cell proliferation and promotes apoptosis while suppressing the oncogene Fra-1. These results identify SYK as a tumor suppressor in colorectal cancer and suggest it as a potential therapeutic target.

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