Original Article

TRAF4 promotes the growth and invasion of colon cancer through the Wnt/β-catenin pathway

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Abstract: The tumor necrosis factor receptor-associated factor 4 (TRAF4) has been linked to carcinogenesis. However, the role of TRAF4 in colon cancer is still unclear. Therefore, we investigated the role of TRAF4 in colon cancer and the underlying mechanism. In the present study, we found that TRAF4 was overexpressed in colon cancer tissues and cells, and small interfering RNA (siRNA)-mediated gene knockdown of TRAF4 significantly inhibited cell proliferation, invasion and tumorigenesis, both in vitro and in vivo, but induced apoptosis in colon cancer cells. Furthermore, siRNA-TRAF4 significantly inhibited the expression levels of β-catenin, cyclinD1, and c-myc proteins in colon cancer cells. Taken together, these results suggest that TRAF4 promoted colon cancer cell growth and invasion by potentiating the Wnt/β-catenin pathway, suggesting that TRAF4 may be a potential molecular target for colon cancer prevention and therapy.

Keywords: Tumor necrosis factor receptor-associated factor 4 (TRAF4), colon cancer, cell growth, Wnt/β-catenin pathway

Introduction

Colon cancer is one of the most commonly diagnosed cancers and the fourth leading cause of cancer mortality worldwide. The incidence is increasing rapidly in developing countries including China [1, 2]. Despite surgical resection coupled with systemic chemotherapy, about half of newly diagnosed colorectal cancer patients will still die of this disease due to tumor recurrence and metastasis [3]. Therefore, identifying the role of new molecules of diagnostic and therapeutic significance remains a major focus of current cancer research.

The tumor necrosis factor receptor (TNF-R)-associated factor (TRAF) family of intracellular proteins were originally identified as signaling adaptors that bind directly to the cytoplasmic regions of receptors of the TNF-R superfamily. The TRAF family consists of six members, and TRAF4 is a member of TRAF protein family, which mainly function in the immune system, where they mediate signaling through tumor necrosis factor receptors (TNFRs) and interleukin-1/Toll-like receptors (IL-1/TLRs) [4, 5]. TRAF4 also plays a critical role in nervous system. It is expressed in oligodendrocytes from early progenitors to mature myelinating cells, and knockdown of TRAF4 resulted in a dramatic alteration of the myelin ultrastructure and a degeneration of a high number of Purkinje cells [6]. Recently, TRAF4 has been demonstrated to play roles in carcinogenesis. In lung cancer cells and primary lung tumors, TRAF4 is overexpressed, and RNA interference (RNAi)-mediated gene knockdown blunted the malignant phenotype, exerting inhibitory effects on cell proliferation, anchorage-independent growth, and tumor development in a xenograft mouse model [7]. In addition, TRAF4 was also overexpressed in breast cancer cells, and knockdown of TRAF4 decreased cell growth, cell migration and invasion [8]. However, the role of TRAF4 in colon cancer remains unclear. In this study, we investigated the effect of manipulated TRAF4 expression on proliferation, apoptosis and invasion in colon cancer cells. Our findings showed that TRAF4 gene silencing...
TRAF4 promotes the growth and invasion of colon cancer by siRNA can inhibit proliferation and invasion, induce apoptosis of colon cancer cells, suggesting the potential of targeting TRAF4 to improve the therapeutic outcome of colon cancer.

Materials and methods

Tissue specimens

Colon cancer specimens were obtained from the surgical specimens of patients with informed consent between October, 2011 and October, 2013. This study was performed under a protocol approved by the Institutional Review Boards of Nan Yang Central Hospital and all examinations were performed after obtaining written informed consents.

Cell culture and siRNA transfection

Human colon cancer cell lines (HT29, SW480 and LS174T) and normal colonic mucosa epithelial cell (NCM460) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All of the cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in humidified atmosphere of 5% CO₂ in air. For transfection, cells were transfected with siRNA targeting TRAF4 and non-targeting siRNA (GenePharma, Shanghai, China) using LipofectamineTM2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Results were checked by real time PCR (RT-PCR) and western blot at 48 h after transfection.

RT-PCR

Total RNA was extracted from frozen tissue samples or cultured cells using the Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. 2 µg of total RNA was then subjected to TaqMan one-step reverse transcription (Applied Biosystems, Foster City, CA), followed by RT-PCR (ABI PRISM...
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7700 sequence detection system, Applied Biosystems) according to the manufacturer’s instructions. The primers for RT-PCR were the following. TRAF4: forward 5’-CTGGCTAAACCA-CAGCAGGCTC-3’, and reverse 5’-TCGTTTTCGAGTCTCTCGG-3’; GAPDH Sense 5’-CAAGCTCAT-TTTCCTGGTATGAC-3’, Antisense, 5’-CAGTGAGGTCTCTCTCTTCCT-3’; GAPDH was used as an internal control. The expression levels of the relative genes were calculated using control GAPDH mRNA and the 2^{ΔΔCT} method.

Western blot

Total protein extracts were prepared using RIPA lysis buffer (Beyotime, Nantong, China) according to the manufacturer’s instructions. The protein concentration of the lysates was evaluated using a BCA protein assay kit (Beyotime, Nantong, China). 20 μg of proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Boston, MA, USA). The membrane was blocked for 20 min at room temperature with SuperBlock T20 TBS Blocking Buffer (Santa Cruz Biotechnology, Inc., CA, USA). Blots were then probed with appropriate primary antibodies (anti-TRAF4, anti-β-catenin, anti-cyclinD1, anti-c-myc and anti-β-actin) (Invitrogen, Carlsbad, CA, USA) overnight at 4°C. The membranes were washed with TBST, and then incubated in horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature. After the membranes were washed with TBST, the proteins were finally visualized by fluorography using an enhanced chemiluminescence system.

Cell viability assay

Cell viability was measured by a cell counting kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan). In brief, HT29 cells (5 × 10^3 per well) were seeded in 96-well plates and transfected with siR-
NA-TRAF4 or siRNA-mock, respectively. After transfection for 24, 48 and 72 h, the CCK-8 reagents were added and incubated with the cells for 1 h. Then, the absorbance was detected at 450 nm according to the manufacturer’s instruction. The experiments were performed in triplicate [9].

Cell apoptosis assay

Apoptosis was assayed using the Annexin V-FITC Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the cells were harvested, washed twice with PBS, centrifuged at 1,000 × g for 5 min and resuspended in 195 μl Annexin V-FITC binding buffer. Then, 5 μl Annexin V-FITC was added gently at room temperature. After staining for 10 min in dark, the cells were centrifuged at 1000 × g for 5 min, and then were gently resuspended in 190 μl of Annexin V-FITC binding buffer and then 10 μl of PI staining solution was added to the cells, gently mixed and kept on ice in the dark. The cells were analyzed by flow cytometry [10].

Cell invasion assay

Cell invasion was assessed by matrigel precoated Transwell inserts (8.0 mm pore size with polyethylene tetraphthalate membrane) according to the manufacturer’s protocol. To assess invasion, filters were precoated with 10 mg of matrigel (BD Biosciences, NJ, USA). 1 × 10^5 cells were seeded in serum-free medium in the upper chamber. The number of cells that invaded the lower side of the membrane after 24 h was determined by counting cells in a minimum of four randomly selected areas [11].

Tumor xenograft growth assay in vivo

Animal experiment was carried out with the approval of the ethics committee of Nan Yang Central Hospital in accordance with the Guide for the Care and Use of Laboratory Animals. Nude mice were from SLAC Laboratory Animal Corp (Shanghai, China). Cells were digested and resuspended with cold phosphate buffer saline (PBS) at a density of 1 × 10^7 cells/ml. 100 μl cell suspension was subcutaneously injected into 5-6-wk-old female Balb/C nude mice in the right and left abdomen. The treatment time was 28 days. Tumor growth was monitored by tumor volume which was measured with calipers and calculated as described, $V (\text{mm}^3) = \text{width}^2 (\text{mm}^2) \times \text{length (mm)}/2$. Finally, tumors were harvested; tumor volume and weight were measured.

Statistical analysis

Results were expressed as mean ± SD. All statistical analyses were carried out using SPSS 16.0. Differences between treatment conditions were assessed for statistical significance using one-way ANOVA, followed by the LSD or Dunnett’s t test method. $P < 0.05$ was considered to be statistically significant.

Results

Expression of TRAF4 in colon cancer tissues and cell lines

To understand whether TRAF4 is involved in the development and progression of colon cancer, endogenous TRAF4 expression was examined in human normal colon, colon cancer and colon cancer cell lines. As compared with the normal colon samples, TRAF4 mRNA levels in cancerous tissue samples were significantly upregulated (Figure 1A). Moreover, we found that the expression levels of TRAF4 mRNA and protein were significantly increased in colon cancer cell lines (Figure 1B and 1C).

Knockdown of TRAF4 inhibits colon cancer cell growth in vitro

To determine the biological effect of upregulated TRAF4 expression on colon cancer development, a series of functional studies combined with a loss-of-function approach were employed to assess the role of TRAF4 in colon cancer cell growth. Cells were transfected with siRNA-TRAF4 or siRNA-mock and stably transfected cells were established. The efficacy of TRAF4 knockdown was confirmed by western blot (Figure 2A). MTT assay results show that knockdown of TRAF4 significantly inhibited cell proliferation with prolonged culture time, as compared with the control group (Figure 2B). In addition, the apoptosis rate was dramatically increased from 9.41% in mock cells to 28.30% in TRAF4 knockdown cells (Figure 2C and 2D). Together, these results suggest that TRAF4 knockdown suppresses colon cancer cell proliferation and induces cell apoptosis.
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To further explore the role of TRAF4 on colon tumor growth in vivo, the xenografted tumor in nude mouse was employed. As shown in Figure 3A, TRAF4 knockdown significantly suppressed tumor growth of Balb/C nude mice compared with the control group. The tumor volumes were calculated in each group every 7 days from day 0 to day 28; C. The tumor weights were measured at day 28. Data is expressed as mean ± SD; *P < 0.05 compared with the siRNA-mock group.

Figure 3. Knockdown of TRAF4 inhibits tumorigenicity in nude mice. A. siRNA-TRAF4 significantly inhibited tumor growth of HT29 cells implanted subcutaneously in Balb/C nude mice compared with the control group; B. The tumor volumes were calculated in each group every 7 days from day 0 to day 28; C. The tumor weights were measured at day 28. Data is expressed as mean ± SD; *P < 0.05 compared with the siRNA-mock group.

TRAF4 knockdown reduced xenografted tumor growth in vivo

To further explore the role of TRAF4 on colon tumor growth in vivo, the xenografted tumor in nude mouse was employed. As shown in Figure 3A, TRAF4 knockdown significantly suppressed tumor growth of Balb/C nude mice compared with the control group. In addition, TRAF4 knockdown significantly reduced the size and weight of the xenografted tumor (Figure 3B and 3C). These results demonstrated that TRAF4 knockdown could decrease tumor xenograft growth in vivo.

Knockdown of TRAF4 inhibits colon cancer cell invasion in vitro

Cell invasion is a basic characteristic of tumor metastasis. To examine the effect of TRAF4 on cell invasion, transwell assay was performed using TRAF4 downregulated cells. As shown in Figure 4, the invasion ability of TRAF4-downregulated cells decreased significantly compared with control cells. Thus TRAF4 likely plays a role in the invasion of colon cancer cells.

TRAF4 potentiates the Wnt/β-catenin pathway in colon cancer

β-catenin is a crucial signaling molecule, and cyclinD1 and c-myc are important target genes of the Wnt/β-catenin pathway. Therefore, the expression of β-catenin, cyclinD1, and c-myc proteins was measured by a western blot assay in TRAF4-down-regulated HT29 cells. As shown in Figure 5, the expression of β-catenin, cyclinD1, and c-myc proteins in TRAF4-down-regulated HT29 cells was significantly decreased compared with the control cells. All these results suggest that the TRAF4 promoted cell growth and invasion by potentiating the Wnt/β-catenin pathway.

Discussion

It has been known that the initiation, development, invasion and metastasis for colon cancer are controlled by many different genes and various signal transduction pathways and involved in many important biological processes. TRAF4 is an adapter protein overexpressed in certain cancers, such as breast cancer and lung cancer. However, the role of TRAF4 in colon cancer is still unclear. In this study, we found that the expression of TRAF4 mRNA and protein was upregulated in colon cancer tissues and cell lines in comparison to that in normal tissues. Furthermore, knockdown of TRAF4 inhibited cell growth and invasion by suppressing the Wnt/β-catenin pathway.

Sun et al. reported that TRAF6 is upregulated in colon cancers, which is associated with tumor grades, and it promotes proliferation of colon cancer cells via induction of cyclin D1 [12]. Consistent with the role of TRAF6 in colon cancer, in the present study, we found that TRAF4 was upregulated in colon cancer tissues and cell lines, which indicated that TRAF4 may function as an oncogene involved in the development and progression of colon cancer.
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In order to investigate the role of TRAF4 in carcinogenesis of colon cancer, our data showed that TRAF4 knockdown dramatically inhibited cellular proliferation, invasion and tumorigenesis, both in vitro and in vivo, but induced apoptosis in colon cancer cells. These results demonstrated that TRAF4 promotes the carcinogenesis of colon cancer.

Transforming growth factor-β (TGF-β)/Smad activation and induction of Smad-dependent target genes involved in metastasis were downregulated upon TRAF4 depletion in breast cancer cells. In addition, mice that were injected with TRAF4-depleted breast cancer cells did not develop any detectable bone metastases after 35 days [13]. In this study, we found that siRNA-TRAF4 significantly decreased the invasion ability of colon cancer cells, which suggested that TRAF4 promotes human colon cancer invasion.

Accumulating evidence has indicated that the Wnt signaling pathway not only plays an important role in normal mammary development, but also plays a critical role in tumor progression [14-17]. The Wnt-signaling pathway regulates gene expression by stabilizing β-catenin, which translocates to the nucleus and forms complexes with T-cell factor transcription factors [18]. β-catenin induces the transcription of several target genes, which are involved in cell survival, proliferation, and metastasis [19]. Zhen et al. reported that β-catenin expression were significantly decreased in human colon cancer cells treated with siRNA-TRAF4.

![Figure 4](image-url) Figure 4. Knockdown of TRAF4 inhibits invasion of colon cancer cells. A. Transwell invasion assay. The stable siRNA-TRAF4-transfected HT29 cells or the control cells were seeded in the upper chamber of pre-coated Matrigel 0.8 μm transwell and grown for 48 h; B. Number of the invaded cells were counted and summarized. *P < 0.05 compared with the siRNA-mock group.

![Figure 5](image-url) Figure 5. Effects of TRAF4 knockdown on regulation of Wnt/β-catenin pathway gene expression. A. Western blot analysis of β-catenin, cyclinD1 and c-myc; B. The image of proteins quantitative data. β-actin was used as an internal control. All experiments were repeated at least three times. *P < 0.05 compared with the siRNA-mock group.
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dramatically increased in eight samples of fresh colorectal cancer tissues compared with their respective adjacent non-tumor colorectal mucosa tissues by Western blot analysis [20]. Moreover, one study reported that TRAF4 plays role in enhancing transcription of β-catenin and may protect it from p53-mediated degradation [21]. Therefore, we hypothesized that TRAF4 might influence the biological function of colon cancer cells through Wnt signaling pathway. In this study, we found that siRNA-TRAF4 significantly inhibited the expression levels of β-catenin, cyclinD1, and c-myc proteins in colon cancer cells. All these results suggest that TRAF4 promoted cell growth and invasion by potentiating the Wnt/β-catenin pathway.

In summary, this study demonstrated that TRAF4 promoted cell growth and invasion by potentiating the Wnt/β-catenin pathway. Therefore, it is reasonable to speculate that TRAF4 plays a critical role in colon cancer and serves as a potential target for cancer prevention and treatment.

Disclosure of conflict of interest

None.

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