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Original Articles

Thioredoxin-like protein 2b facilitates colon cancer cell proliferation and inhibits apoptosis via NF-κB pathway

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ABSTRACT

Our previous work identified thioredoxin-like protein 2 (Txl-2), a novel thioredoxin family member, as the target of the monoclonal antibody MC3 which can detect colon cancer with high sensitivity and specificity. In the present study, the function of the most abundant isoform Txl-2b in cell proliferation and apoptosis was investigated. Txl-2 overexpression correlated with increased clinical stages. Inhibition of Txl-2b suppressed cell proliferation, induced cell cycle arrest at the G1/S phase, and led to responsiveness to the vincristine-induced apoptosis in SW620 cells. Txl-2b overexpression in LoVo cells had the opposite effect, which was dependent on Trx domain function. *In vivo* studies validated that Txl-2b expression promoted colon cancer tumorigenesis in nude mice. Further studies revealed that nuclear factor-κB (NF-κB) signaling was activated by Txl-2b. Inhibition of NF-κB activation partly abrogated the pro-proliferation and anti-apoptotic phenotypes mediated by Txl-2b via reduced Cyclin D1, Bcl-2, Bcl-xL and Survivin expression and increased Caspase-3 activation. Overall, our results indicate that Txl-2b expression stimulates cancer cell proliferation, accelerates the cell cycle and contributes to apoptosis resistance in colon cancer and provides a potential therapeutic target for colon cancer treatment.

Introduction

Colorectal cancer is a major cause of cancer mortality worldwide with approximately 694,000 recorded deaths from the disease in 2012 [1]. In the last 30 years, the major expansion of genomic and proteomic data has greatly contributed to the discovery of novel cancer biomarkers and targeted molecules for its treatment [2,3]. Using a homogenate preparation of colon cancer tissues, we developed a colon cancer-specific monoclonal antibody named MC3-Ab. Previous immunohistochemical studies revealed that MC3-Ag overexpression was strongly correlated with the histological grade and prognosis of colon cancer patients. Through proteomics techniques, we identified thioredoxin-like protein 2 (Txl-2) as the target of the MC3 monoclonal antibody [4].

Txl-2, also named TXNDC6, is a member of the thioredoxin (Trx) family, which was cloned and characterized in 2003 [5]. Txl-2 is mainly expressed in normal testis, and deregulated Txl-2 expression presented in colon cancer, thus categorizing Txl-2 as a putative novel cancer-testis (CT) antigen. CT antigens are normally only expressed in germ cells and yet are aberrantly activated in a variety of human cancers. They are potential targets for cancer immunotherapy because of their restricted expression in immune-privileged germ cells and various malignancies [6].

Although Txl-2 function in cancer remains largely unknown, Trx family members were widely reported to play pivotal roles in tumor development and progression. Trx, NADPH and thioredoxin reductase (TrxR) comprise a thioredoxin system that exists in nearly all living cells. It functions in thiol-dependent thiol-disulfide exchange reactions, which play critical roles in the regulation of cellular redox homeostasis [7]. The Trx system has multi-faceted roles in mammalian cells, including implications in cancer, where cells exist in a stressed environment and rely on Trx for protection against stress-induced deregulated redox signaling. Mounting data indicate that the Trx system contributes to many cancer hallmarks, such as growth signal self-sufficiency, insensitivity to antigrowth signals, apoptosis evasion and sustained angiogenesis [8]. For instance, Trx-1 overexpression reportedly induces vascular endothelial growth factor production and enhances tumor angiogenesis [9]. Elevated Trx-1 expression is associated with poor prognosis in colon cancer patients with liver metastasis [10]. TXNDC5 is significantly up-regulated in colorectal and gastric cancers and promotes gastric cancer cell proliferation and invasion [11,12]. TXNDC9, another Trx family member, is overexpressed in colorectal cancer and facilitates cancer growth and metastasis [13].

Abbreviations: AP-1, activator protein 1; ASK1, apoptosis signal-regulating kinase 1; CT antigen, cancer-testis antigen; NF-κB, nuclear factor-κB; Txl-2, thioredoxin-like protein 2; Trx, thioredoxin; VCR, vincristine.

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In total, three different Txl-2 isoforms were identified [4], including full length Txl-2 (Txl-2a), Txl-2b (splice variant lacking exons 1 and 5), and Txl-2c (splice variant lacking exons 1, 4 and 5). Of these, Txl-2b was the most abundant isoform in colon cancer, with an expression rate of 88% compared with Txl-2a (8%) and Txl-2c (20%) [14]. In the present study, we investigated the role of Txl-2b on colon cancer cell proliferation and apoptosis. Txl-2 overexpression correlated with increased clinical stages. Txl-2b inhibition led to the suppression of cell proliferation and induced apoptosis, whereas Txl-2b overexpression resulted in the opposite effects in a Trx-domain-dependent fashion. Furthermore, we determined that NF-κB is activated upon Txl-2b induction, and the expression of NF-κB regulated gene products changed accordingly. The current study provides a novel biomarker and target molecule for the diagnosis and treatment of colon cancer.

Materials and methods

Cell culture

Human colon adenocarcinoma cell lines SW480, SW620, LoVo, HT-29, Caco-2 cells and the non-malignant human intestinal epithelial cell line HIEC were preserved by our institute. Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 μg/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO) at 37 °C with a humidified atmosphere of 95% air and 5% CO_2.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tumor tissue and corresponding normal tissue blocks were collected from 86 colon cancer patients who underwent surgical resection during 2009 in the Department of Pathology of Xijing Hospital of Digestive Diseases, China. Txl-2b immunostaining was performed by an avidin–biotin method as described previously [4] with anti-Txl-2 antibody (Abcam, Cambridge, MA). All sections were examined and scored independently by two experienced pathologists.

Plasmid construction and stable transfection

pSilencer3.1-H1 (Life Technologies, Carlsbad, CA) was used for the construction of human Txl-2b siRNA expression vectors pSilencer Txl-2b#1 and #2 according to the manufacturer's protocol. pEGFP-N3-Txl-2b and pEGFP-N3-Txl-2b^{CS} were constructed as previously described [14]. For pEGFP-N3-Txl-2b^{CS} generation, cysteine residues in the Trx domain active site were replaced with serine residues (Cys- > Ser). Transfection was done using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described in the manufacturer's protocol. After 24 hours, G418 (400–600 μg/ml) was added into the culture medium for establishing stable clones. The mixed clones were expanded for an additional 6 weeks and used for subsequent assays within 10 passages.

Cell proliferation assay

The monolayer culture growth rate was determined by the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis, MO) into water-insoluble formazan crystals by viable cells. 2×10^4 cells/well were plated in 48 well plates. At each time point, the cells were incubated with MTT (5 mg/ml) for 3 hours at 37 °C. The formazan crystals were dissolved by adding 150 μl DMSO. The absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 650 nm with a multimode microplate reader (Thermo Fisher, Waltham, MA). All measurements were performed in quadruplicate, and each experiment was repeated at least three times.

Colony formation assay

Log phase cells were trypsinized into a single cell suspension and seeded at approximately 800 cells/well in 6-well plates in triplicate. After 12 days of incubation, plates were gently washed with PBS and stained with 0.1% crystal violet. Colonies from 3 replicate wells were counted per group. Experiments were performed in triplicate.

Cell cycle and apoptosis assays

For cell cycle analysis, target cells in log phase were fixed in 70% ethanol and stained with propidium iodide supplemented with RNaseA (Roche, Mannheim, Germany) for 30 min. For apoptosis assays, vincristine (VCR) was added to cells at a concentration of 0.3 μg/ml. After 18 hrs, cells were harvested and washed twice with PBS. After incubation with a mixture containing APC-conjugated Annexin V and propidium iodide (BD Pharmingen, San Diego, CA) in a binding buffer for 15 min, cellular fluorescence was measured by a fluorescence-activated cell sorter FACSCanto (BD Biosciences, San Jose, CA).

Immunofluorescence staining

Immunofluorescence staining was done as previously described [14]. Anti-Caspase 3 antibody (Abcam, Cambridge, MA) or anti-NF-κB p65 antibody (Cell Signaling Technology, Beverly, MA) were applied at 4 °C overnight. Cy3-conjugated secondary antibodies were loaded and incubated for 2 h at room temperature. Immunostaining signal and Hoechst 33342-stained nuclei were visualized by a FluoView confocal scanning laser microscopy (Olympus, Tokyo, Japan).

Western blot analysis

Western blot analysis was done as previously described [14]. The following primary antibodies were used: antibody that specifically recognizes Txl-2b (Abcam, Cambridge, MA); NF-κB Pathway Sampler Kit, anti-CyclinD1, Bcl-2, Bcl-xL, Survivin and Lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA); β-actin (Sigma-Aldrich, St. Louis, MO). The NF-κB-specific inhibitor Bay 11–7082 was from Beyotime (Beijing, China).

In vivo *tumorigenicity assay*

Every group had five 4–6-week-old female BALB/C nude mice that were obtained from the Shanghai Laboratory Animal Center of China. Approximately 3×10^6 cells in log phase were collected and injected subcutaneously into the dorsal flank of the nude mice. The tumor diameter was measured every 3 days before harvest. The mice were sacrificed 4 weeks later, and the tumor weight was evaluated. The experimental procedures were approved by the Animal Ethics Committee of the Fourth Military Medical University.

Statistical analysis

All of the statistical analyses were performed using SPSS 16.0 software (Chicago, IL). The Mann–Whitney U-test for two groups and the Kruskal–Wallis H-test for multiple groups were used to analyze immunohistochemical results. Numerical data are presented as mean ± SEM. Differences among groups were analyzed using Student's t-test or one-way analysis of variance. $P < 0.05$ was considered to be statistically significant.

Results

Txl-2 expression is significantly increased in colon cancer tissue and cell lines

Txl-2 expression was examined by immunohistochemistry in 86 colon cancer patients. Txl-2 expression was mainly located in the cytoplasm of colon cancer cells (Fig. 1A). Txl-2 staining was positive (++ ~ +++) in 66 (76.7%) colon cancer samples, while negative or weak staining (− ~ +) was observed in 20 cases (23.3%). Txl-2 expression was significantly correlated with the degree of cancer cell differentiation and tumor TNM stages (Table 1). However, there was no correlation among Txl-2 expression, patient age or gender. These results were consistent with our previous finding that Txl-2 mRNA was present in approximately 80% of colon cancer tissues [14]. Then, we examined Txl-2b expression in 5 colon cancer cell lines (HT-29, LoVo, SW480, SW620 and Caco-2) and the adherent non-malignant human intestinal epithelial cell line HIEC. Western blot analysis showed that Txl-2b was absent in HIEC cells, whereas its expression was higher in SW620 and Caco-2 cells and lower in SW480, LoVo and HT-29 cells (Fig. 1B). Next, we selected SW620 and LoVo cells to perform the knockdown and overexpression of Txl-2b respectively based on their highest and lowest Txl-2b expression among the 5 colon cancer cell lines that we have analyzed.

Txl-2b expression was knocked down in SW620 cells using Txl-2b-specific siRNA. The pEGFP-N3-Txl-2b vector was used to upregulate Txl-2b-EGFP expression in LoVo cells. A redox inactive Txl-2b variant (Txl-2b^{CS}) was also generated in which the cysteine residues at the active site of the thioredoxin domain were replaced by serine residues [14]. Densitometric analysis indicated that Txl-2b expression was decreased 51.3% in SW620-siTxl-2b#2 cells and 78.2% in SW620-siTxl-2b#1 cells compared with the