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Original article

Effect of RTKN on progression and metastasis of colon cancer in vitro

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ABSTRACT

Like many epithelial-derived cancers, colon cancer results from a multistep tumorigenic process. However, the detailed mechanisms involved in colon cancer formations are poorly characterized. In the present study, we investigated the role of RTKN in colon cancer and explored underlying mechanisms. The results showed that RTKN expression was significantly increased in colon cancer tissues when compared with the adjacent tissues of patients in Shanghai People's hospital and in TCGA independent dataset. Furthermore, silencing of RTKN inhibited cell proliferation, migration, invasion, and arrested cell cycle at G1 phase in LOVO cells. Bioinformatics analysis demonstrated that DNA replication and cell cycle were involved in the regulation of RTKN. MCM2/3/5, CDK1/2 and PCNA expression had a direct relationship with the reduction of RTKN. RTKN could affect the proliferation and metastasis of colon cancer by reducing expression of MCM2/3/5, CDK1/2 and PCNA, suggesting that RTKN was a potential target for treating colon cancer.

1. Introduction

Colon cancer is the second leading cause of death from cancer in the United States [1] and generally less common in Asia, but incidence is increasing in previously low-risk areas [2]. Even though this disease is curable in early stages, frequently the tumor becomes metastatic by the time an individual presents to their physician with symptoms and thus, the mortality is very high [3].

The Rho GTPases are members of the Ras superfamily of monomeric low molecular mass (approx. 21 kDa) guanine nucleotide-binding proteins. By cycling between an active (GTP-bound) and an inactive (GDP-bound) state, Rho GTPases function as molecular switches to control signal transduction pathways in regulation of a plethora of cellular processes, including cytoskeleton reorganization, gene transcription, cell-cycle progression, and survival [4]. The diverse function of Rho GTPases is mediated through interacting with, and modulating the activity of, respective effector proteins. Rho induces the formation of focal adhesions and stress fibers, this effect being mediated through the cooperation of two downstream effector proteins, the Rho-associated kinase (ROCK/ROK/Rho kinase) and the mammalian orthologue of the Drosophila Diaphanous protein, mDia [5]. More than 30 potential effector proteins have been identified for Rho, Rac, and CDC42, including protein kinases, lipid kinases, phosphatase, lipases, and a number of scaffold proteins [4]. Much effort has now been made in characterizing the biological abilities of Rho GTPases in addition to act in organization and in identifying the individual effectors that transit the distinct signal pathways linked to these cellular responses.

We have previously shown that rhotekin (RTKN), a Rho effector protein, is overexpressed in human gastric cancer (GC) [6]. RTKN was initially isolated as a scaffold protein interacting with GTP-bound form of Rho [7]. Other than interacting with TIP-1 (human T cell lymphotrophic virus, type 1 Tax interacting protein-1) to facilitate Rho-mediated activation of serum response element [8], the role of RTKN involved in Rho-mediated signal transduction remains largely unknown.

In this study, we demonstrated the biological functions and underlying mechanisms of RTKN in colon cancer. Silencing of RTKN inhibited proliferation, cell cycle, migration and invasion of colon cancer in vitro. Bioinformatics analysis showed that DNA replication, cell cycle signaling pathways linked closely with RTKN expression. This study provides original documentation for the overrepresentation of RTKN in human cancer and its links to cancer formation.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium was obtained from Gibco BRL (Gaithersburg, MD, USA). Newborn bovine serum was supplied by Sijiqing Biotechnology (Hangzhou, China). Propidium iodide (PI) was

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purchased from Sigma Chemical Corporation (St. Louis, MO, USA). Antibodies for CDK1, CDK2, PCNA, MCM2, MCM3 and MCM5 were purchased from Abcam (Cambridge, MA, USA). Antibody for GAPDH was purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Patient and tissue samples

Paired colon cancer and adjacent non-tumor colon tissues were obtained from 30 patients who underwent primary surgical resection of colon cancer between 2006 and 2013 at Shanghai Jiao Tong University Affiliated Sixth People's Hospital, China. Colon cancer and adjacent tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C until total RNA was extracted. The study protocol was approved by the ethics committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Written informed consents were obtained from all participants in this study. All the research was carried out in accordance with the Helsinki Declaration of 1975. None of these patients had received radiotherapy or chemotherapy.

2.3. Bioinformatics analysis

Tumor RNA was harvested from fresh frozen tissue with TRIzol reagent (Invitrogen, Shanghai, China) as described [9]. TCGA RNA-Seq and corresponding clinical data were downloaded from TCGA website https://tcga-data.nci.nih.gov/tcga/ following approval of this project by the consortium. RNA-Seq analysis used data from 262 colon cancers and 41 adjacent normal tissues. To gain further insight into the biological pathways involved in colon cancer pathogenesis through RTKN pathway, a gene set enrichment analysis (GSEA) was performed. The KEGG gene sets biological process database (c2.KEGG.v4.0) from the Molecular Signatures Database-MsigDB was carried out in the analysis of enrichment.

2.4. Cell culture and transfection

Colon cancer cell lines RKO, SW620, SW480, LOVO and HCT-116 (Academia Sinica Cell Bank, Shanghai, China) were maintained in RPMI 1640 medium (Gibco BRL, Shanghai, China) supplemented with 100 mL/L heat-inactivated fetal bovine serum (Si-Ji-Qing Biotechnology, Hangzhou, China), 100 U/mL penicillin and 100 mg/mL streptomycin. Cultures were incubated in 5% CO_2 at 37 °C. Small interfering RNA (siRNA) for RTKN (20 nmol/L) was from Genesil Biotechnology (Wuhan, China). siRNA targeting position 2102–2124 (AAGAACCCUUGGAGCAAACAUTT; named RTKN-siRNA) of human RTKN mRNA was cloned into a lentiviral vector, PLKO.1-EGFP (Addgene, Beijing, China). Transfection of LOVO cells was performed with Lipofectamine 2000 (Invitrogen Life Technologies, Gaithersburg, MD, USA) following the manufacturer's protocol. Nonspecific siRNA was used as a negative control, and the selective silencing of RTKN was identified by real-time PCR analysis. The LOVO cells were analyzed 48 h after transfection.

2.5. Real-time RT-PCR

Total RNAs were extracted from adjacent tissue and colon cancer tissue and LOVO cells treated with RTKN siRNA, using TRIZOL reagent (Invitrogen Life Technologies, Gaithersburg, MD, USA) and stored at −80 °C. The DyNAmo Flash SYBR Green qPCR kit (Finnzymes Oy, Espoo, Finland) was used according to the manufacturer's instructions. The primers sequences (sense/anti-sense) used were in Table 1. Relative quantification of the signals was performed by normalizing the signals of different genes with the GAPDH signal.

Table 1
Primes sequences used in this study.

RTKN-forward	5′-GCCGCTGCTTACTATTGC-3′
RTKN-reverse	5′-GTGCTTCCCGACTTTCTG-3′
CDK1-forward	5′-ACCATACCCATTGACTAAC-3′
CDK1-reverse	5′-ATAAGCACATCCTGAAGAC-3′
CDK2-forward	5′-CCAGGAGTTACTTCTATGCCTGA-3′
CDK2-reverse	5′-TTCATCCAGGGGAGGTACAAC-3′
PCNA-forward	5′-GCCTGACAAATGCTTGCTGAC-3′
PCNA-reverse	5′-TTGAGTGCCTCCAACACCTTC-3′
MCM2-forward	5′-CTACCAGCGTATCCGAATC-3′
MCM2-reverse	5′-GTTGAGGGAGCCATCATAG-3′
MCM3-forward	5′-GAAGGCGAGGAATGTTGG-3′
MCM3-reverse	5′-TGTGGGATGGGAAGTAGG-3′
MCM5-forward	5′-TGAGCACAATGAGGAGAG-3′
MCM5-reverse	5′-CGGCAGTAGGCAATAAAC-3′
GAPDH-forward	5′-CACCCACTCCTCCACCTTTG-3′
GAPDH-reverse	5′-CCACCACCCTGTTGCTGTAG-3′
Gene	Sequences

2.6. Western blot analysis

Fifty micrograms of protein from colon cancer cell lines RKO, SW620, SW480, LOVO, HCT-116 and mutant LOVO cells treatment with RTKN siRNA were subjected to SDS-PAGE using a Bio-Rad miniature slab gel apparatus and electrophoretically transferred onto a nitrocellulose sheet. Antibody to RTKN and antibody to GAPDH were incubated with the blot overnight at 4 °C. Membranes were washed and incubated with respective secondary antibodies and were visualized by enhanced chemiluminescence (Millipore, Beijing, China) according to the manufacturer's instructions.

2.7. Cell proliferation assay

The interfering effects of the RTKN siRNA on the LOVO cells were determined by Cell Count Kit-8 (CCK-8, Dojindo, Rockville, MD, USA, http://www.dojindo.com) assay as described previously [10]. In brief, LOVO cells in logarithmic growth-phase were collected, and 5×10^4 cells/well were dispensed within 96-well culture plates in 100 μL volumes. Subsequently the cell viability was evaluated by CCK-8 following the manufacturer's instructions. The absorbance at wavelength 450 nm was measured for the supernatant of each well using the plate reader Multiskan EX (Thermo Fisher Scientific, Waltham, MA, USA, http://www.thermofisher.com).

2.8. Cell cycle and apoptosis assay by flow cytometery

LOVO cells were seeded in 12-well plates after RTKN siRNA transfection for 48 h. The percentages of cells in the different phases of cell cycle were evaluated by determining the DNA content after Annexin V-Propidium Iodine (PI) straning kit (Becton Dickinson, for apotosis) or PI staining kit (for cycle, Beyotime) [11]. Briefly, cells were washed with PBS, trypsinized and centrifuged at 1000 rpm at 4 °C for 5 min. Pellets were fixed overnight in 70% cold ethanol. After fixation, cells were washed twice with PBS and incubated in PBS containing RNase (1 mg/mL) for 10 min at room temperature. Data acquisition was done by flow cytometry (EPICSXLMCL, Beckman Coulter, US) using Cell Quest software.

2.9. Cell migration assay invasion assays

The cancer cell transwell migration assay was performed according to previous study [12]. The differences between migration and invasion were as follows: transwell membrane (Corning Costar Corporation, NY, USA) coated with Matrigel (2.5 mg/mL; BD Biosciences Discovery Labware, Beijing, China)