Original Article

CTHRC1 promotes human colorectal cancer cell proliferation and invasiveness by activating Wnt/PCP signaling

Xiao-Mei Yang*, Hai-Yan You*, Qing Li, Hong Ma, Ya-Hui Wang, Yan-Li Zhang, Lei Zhu, Hui-Zhen Nie, Wen-Xin Qin, Zhi-Gang Zhang, Jun Li

State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200032, Peoples Republic of China. *Equal contributors.

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Abstract: Collagen triple helix repeats containing 1 (CTHRC1) participates in vascular remodeling, bone formation, and developmental morphogenesis. Recently, CTHRC1 has been found up-regulated in many solid tumors and contributes to tumorigenesis, but its role in the progression of human colorectal cancer (CRC), remains unclear. In this study, CTHRC1 expression in human CRC cell lines was evaluated by quantitative real-time PCR and immunoblot analyses. The role of CTHRC1 in CRC cell proliferation and extracellular matrix invasion in vitro was analyzed by gene over-expression and recombinant protein. Reporter luciferase assay was used to reveal key relevant signaling pathways involved in CRC cells. The results show that CTHRC1 is secreted both by colorectal epithelia cells and stromal fibroblasts. Recombinant CTHRC1 promotes CRC cell migration and invasion dose-dependently. CTHRC1 overexpression promotes CRC cell migration, invasion and proliferation in vitro. Wnt/PCP signaling but not Wnt/catenin signaling was activates by CTHRC1 in CRC cells. Together, CTHRC1 promotes CRC cell proliferation, migration and invasion in vitro, which is possibly mediated by activating Wnt/PCP pathway.

Keywords: CTHRC1, colorectal cancer, metastasis, extracellular matrix

Introduction

Colorectal cancer (CRC), one of the most common malignant tumors, ranks the third and second among all commonly encountered malignancies in terms of incidence and mortality, respectively [1]. The high mortality rate of advanced CRC is attributable to limited treatment options. Metastasis is the major cause of recurrence and death in CRC patients which is a complex interplay between malignant cancer cells and surrounding tumor microenvironments.

Besides genetic changes that are intrinsic to cancer cells, alterations within the tumor microenvironment also play important role in tumor cell proliferation, migration, metastasis and other important molecular and cellular processes [2]. The role of the tumor microenvironment in CRC invasiveness and metastasis had been a focus recently [3, 4]. Molecules involved in the interaction between tumor cell and extracellular matrix (ECM) have been illustrated to promote cancer progression by increasing cancer cell growth, migration, invasion, and metastasis [5, 6].

Collagen triple helix repeat containing 1 (CTHRC1), an ECM-related protein, was first identified in a screen for differentially expressed sequences in balloon-injured versus normal rat arteries [7]. It may contribute to tissue repair by limiting collagen matrix deposition and promoting cell migration [8]. Elevated expressions of CTHRC1 were found in various cancer types including breast cancer [9, 10] and invasive melanoma [11]. CTHRC1 high expression was also found to be of prognostic value in multiple cancers including hepatocellular cancer, gastric cancer, non-small cell lung cancer and gastrointestinal stromal cancer [12-15]. Overexpression of CTHRC1 was recently reported to promote invasion of CRC cells [16]. However, the precise
role of CTHRC1 in human CRC cell viability, motility, invasion and in vivo carcinogenesis has not been addressed.

In the present study, by gene over-expression and recombinant protein, we have demonstrated that CTHRC1 promotes CRC cell migration, invasion and proliferation. Wnt/PCP signaling but not Wnt/catenin signaling was activated by CTHRC1 in CRC cells. Moreover, we have revealed that both colon epithelial cells and stromal fibroblasts are sources of CTHRC1 in CRC microenvironment. Our data indicate that CTHRC1 is an important player that promotes CRC cell proliferation, migration and invasion in vitro, which is possibly mediated by activating wnt/PCP pathway.

Materials and methods

Cell lines

CRC cell lines Lovo, HCT-8, HT-29, Colo205, LS174t and HCT116 were purchased from American Type Culture Collection (Rockville, MD). SW480 and SW620 were purchased from Cell Bank of the Chinese Academy of Sciences. Cells were maintained in DMEM (Dulbecco’s modified Eagle medium) supplemented with 10% (v/v) fetal calf serum at 37°C in a humidified incubator under 5% CO₂ condition.

Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from CRC cell lines using Trizol reagent (Takara, Dalian, China) followed the manufacturer instructions. The reverse-transcription reactions were carried out with random primers and M-MLV Reverse Transcriptase (Takara, Dalian, China). The cDNAs were used for templates of quantitative real-time PCR reaction in SYBR-Green method. All the qPCR reactions were performed on a StepOne™ real-time PCR System (Applied Biosystems, Foster City, CA, USA). The forward and reverse CTHRC1 primer sequences were: 5’-TGGTATTTCACATTCAATGGAGCTG-3’ and 5’-TGGGTAATCTGAACAAGTGCCAAC-3’, respectively. Beta-actin was used as an internal control. The 2-ΔΔCt method was used to quantify the relative CTHRC1 expression levels.

Western blot

Cells were lysed in Western and IP lysis buffer (P0013, Beyotime, Jiangsu, China) supplemented with 1 mM PMSF (Adamas beta, Shanghai, China). The lysis buffer includes, 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na₃VO₄, leupeptin. Proteins were separated by 10% SDS-PAGE under reducing condition, followed by blocking in phosphate-buffered saline/Tween-20 containing 1% BSA (Bovine Serum Albumin). The NC (Nitrocellulose filter membrane) membrane was incubated with antibodies for CTHRC1 (1:1000, mouse, Huaan, Hangzhou, China) and species-specific secondary antibodies. Bound the IRDye 680 antimouse (LI-COR, 1:20000) secondary antibodies were revealed by Odyssey imaging system (LI-COR).

Immunohistochemical staining

Human CRC tissues were bought from Shanghai Superchip Company. Immunohistochemistry was performed using a two-step standard protocol. After microwave antigen retrieval, tissues were incubated with rabbit anti-CTHRC1 antibody (1:200) for 60 min at room temperature. Following 30 min incubation with secondary antibody (Novolink Polymer RE7112), sections were developed in DAB solution under microscopic observation and counterstained with hematoxylin.

Protein expression, purification and characterization

Human full length CTHRC1 was cloned into the episomal expression vector pCEP-Pu-Strep II-tag (C-terminal) in-frame with the sequence of the BM-40 (SPARC/osteonectin) signal peptide downstream of the CMV promoter. Cthrc1 was recombinant expressed in EBNA-293 cells after transfecting reconstructed plasmid by using X-treme GENE 9 DNA Transfecting Reagent (Roche, Mannheim, Germany). The transfected cells were cultured with 5 μg/ml puromycin initially for screening and were subsequently cultured in large scale in DMEM supplemented with 10% (v/v) FBS and 1 μg/ml puromycin. The culture media was collected and centrifuged and the supernatants were applied to the StrepTactin sepharose column (IBA). The column was washed with binding buffer and eluted by elution buffer containing 2.5 mM desthiobiotin. The collected fractions were further quantified and identified by Coomassie Brilliant Blue (CBB) staining and Western blot.
Migration and Matrigel invasion assay

CRC cell lines were detached and resuspended in serum-free DMEM. Approximately $5 \times 10^4$ cells in 0.1 ml were placed in Matrigel (BD biosciences, Bedford, MA)-coated inserts (Millipore) seated on the 24-well plate. DMEM containing 5% (v/v) FBS and recombinant CTHRC1 was added to the bottom chamber. Cells were incubated at 37°C and allowed to migrate or invade through Matrigel for 48 h. After incubation, filters were fixed and stained with 0.1% (w/v) Crystal Violet. Non-migrated or Non-invading cells were removed using a cotton swab while invading cells on the underside of the filter were counted under a microscope at a magnification of 200×. At least five grids per filter were counted and the experiments were repeated twice. To rule out the effects of different cell proliferation rates that might alter the results, cells were treated with 10 μg/mL of mitomycin C before the assay.

Cell proliferation assay

Cells were seeded into a 96-well plate at $3 \times 10^3$ cells per well with 100 μl complete medium and cultured at 37°C. 10 μl Cell Counting Kit-8 (CCK-8, WST-8, Dojindo, Japan) solution was added to each well after 24 h, 48 h, 72 h, 96 h and 120 h, respectively. In viable cells, WST-8 was metabolized to produce a colorimetric dye that is detected at 450 nm using a microplate reader. The experiment was performed in triplicate and repeated twice.

Lentivirus production and cell transduction

cDNAs encoding human CTHRC1 were amplified and cloned into pEZ-lv105 vector. Virus packaging was performed in 293T cells after cotransfection of pEZ-lv105 vector (GeneCopoeia) using Lipofectamine 2000...
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Figure 2. Recombinant CTHRC1 promotes CRC cell migration. A: Characterization of affinity purified CTHRC1 by silver staining, CBB staining and western blot. B: Representative images of migratory cells (left) and statistical analysis of cell migration (right) stimulated with CTHRC1 or vehicle control. C: Analysis of migratory cells in response to different doses of CTHRC1 or vehicle control. D: Representative images of invaded cells in response to different doses of CTHRC1 or vehicle control and relative cell count analysis. Results shown are means ± SD of migratory or invaded cells photographed at 200× magnification per field. *, P < .05 and **, P < .01, Student’s t test.

(Vivitrogen). Viruses were harvested at 48 hour and 72 hour after transfection, and virus titers were determined. Target cells (1×10⁵), including Lovo, HCT-8 cells, were infected with 1×10⁶
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recombinant lentivirus-transducing units in the presence of 6 μg/ml polybrene (Sigma).

**Luciferase reporter assay**

CRC cells were seeded in 96-well plates and transfected with mixture of 100 ng TOPFLASH and FOPFLASH, or 100 ng pFR and 10 ng ATF2, and 10 ng Renilla following the recommended protocol for the Lipofectamine 2000 transfection system. One group of CRC cells were treated with rCthrc1 protein at a concentration of 20 nm. After 48 hours of incubation, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI) from the cell lysates.

**Statistical analysis**

Statistical analyses were done using SPSS 12.0 for windows (SPSS, Chicago, IL). All statistical tests were two-sided. \( P < 0.05 \) was considered statistically significant.

**Results**

**Expression of CTHRC1 in colorectal cancer cells**

We detected CTHRC1 expression in several colorectal cancer cell lines both by quantitative RT-PCR (Figure 1A) and western blot (Figure 1B). The results showed that CTHRC1 was weakly expressed in HCT-8, SW620, LoVo, Colo205 and Ls174t cells, while SW480 and HCT116 exhibit relative higher CTHRC1 expression.

**Human colon fibroblasts are also a major source of CTHRC1**

By immunohistochemical staining of human colorectal cancer samples, we found that CTHRC1 was highly expressed in CRCs in comparison of adjacent normal tissues, which was consistent with previously reported [16]. Furthermore, we found that CTHRC1 was expressed not only by colon epithelial cells but also stromal fibroblasts (Figure 1C). This result suggests that both autocrine and paracrine CTHRC1 contribute to CRC carcinogenesis.

**Recombinant CTHRC1 promotes migration and invasion of colorectal cancer cells**

Recombinant CTHRC1 was expressed and purified by silver staining, Coomassie Brilliant Blue (CBB) staining and Western blot (Figure 2A). To investigate whether CTHRC1 directly promote colorectal cancer cell migration, 10 nM of recombinant CTHRC1 protein was applied in a migration assay. Compared to vehicle control, CTHRC1 significantly promoted the migration of colorectal cancer cells including HCT-8, HT-29 and LoVo (Figure 2B). We further demonstrated that CTHRC1 can promote Lovo cell migration in a dose-dependent manner (Figure 2C).

Next, we investigated whether CTHRC1 promote colorectal cell invasion through matrigel. The results showed that recombinant CTHRC1 significantly increased the number of invasive colorectal cancer cells, including HT-29 and LoVo cells, compared with those treated with vehicle control. The effect of CTHRC1 on colorectal cancer cell invasion was also demonstrated to be dose-dependent (Figure 2D).

We also detected whether cell proliferation was affected by CTHRC1 treatment, and the results showed that colorectal cancer cell proliferation was not significantly changed by CTHRC1 treatment (data not shown). In addition, CTHRC1 neither affect colorectal cancer cell differentiation as detected by the sphere formation assay (data not shown).

**Over-expression of CTHRC1 promotes proliferation, migration and invasion of colorectal cancer cells**

To examine the functional consequence of elevated CTHRC1 expression on colorectal cancer cell migration and invasion, we established stable cell lines transduced by the lentivirus carrying the CTHRC1 gene, designated as Lenti-CTHRC1, in Lovo and HCT-8 cells, which exhibited a low endogenous level of CTHRC1. CTHRC1 was truly overexpressed in lenti-CTHRC1 infected cells as characterized both by quantitative RT-PCR (Figure 3A) and western blot (Figure 3B). Invasion assay showed that the number of invasive cells in CTHRC1 overexpressed group was clearly increased compared with those in the control group (Figure 3C). In addition, cell proliferation assay revealed slightly but statistically significant increase of cell proliferation resulted from CTHRC1 overexpression in both LoVo and HCT-8 cells (Figure 3D).
Figure 3. Overexpression of CTHRC1 promotes CRC cell invasion and proliferation. A: Identification of CTHRC1 overexpression in mRNA level. B: Identification of CTHRC1 overexpression in protein level. GAPDH was detected as a loading control. C: Overexpression of CTHRC1 promotes cell invasion in HCT-8 and LoVo cells. D: Cell proliferation of CRC cells overexpressed with Lenti-CTHRC1. *, $P < .05$ and **, $P < .01$, Student’s t test.
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**CTHRC1 activates Wnt/PCP signaling in colorectal cancer cells**

To understand the underlying mechanism by which CTHRC1 promotes CRC cell migration and invasion, we examined the activation of the canonical Wnt pathway and the non-canonical Wnt pathway. CRC cells were transfected with a Wnt/β-catenin reporter plasmid (TCF/catenin plasmid) and negative control counterpart plasmid or non-canonical Wnt/PCP pathway reporter plasmid (ATF2 plasmid). Recombinant CTHRC1 or vehicle control was added 24 hours after transfection, and luciferase activity was determined. The results showed that Wnt/β-catenin signaling was not altered while the non-canonical Wnt/PCP signaling was clearly activated by recombinant CTHRC1 protein in both HCT-8 and LoVo cells (Figure 4). This result suggests a possible mechanism that CTHRC1 promote CRC cell migration and invasion by activating Wnt/PCP pathway.

**Discussion**

By secreted factors, cancer cells can modify their microenvironment to facilitate their own survival, growth, invasion and metastasis. CTHRC1, a secreted protein, has been reported to be up-regulated in many solid tumors, including melanoma, breast cancer, gastrointestinal, and HCC [11]. Tang et al has shown that CTHRC1 protein was weak or undetectable in benign nevi and in non-invasive melanoma tumors, but highly expressed in invasive melanoma [11]. Over-expression of CTHRC1 in melanoma cell lines enhances cell migration and adhesion, and protects melanoma cells from serum deprivation induced apoptosis [17]. In breast cancer, the stromal expression of CTHRC1 is enhanced in patients with bone metastasis [18]. These data suggest that CTHRC1 is an important regulator for tumor invasion and metastasis in tumor microenvironment. In accord with previous reports, we have found that CTHRC1 is a cancer growth- and invasion-promoting protein in tumor microenvironment. CTHRC1 is not only secreted by colon epithelial cells but also by stromal fibroblasts, suggesting that CTHRC1 acts autocrine and paracriney to promote CRC cell growth and metastasis.

The Wnt pathways are important participants in the microenvironment of CRC carcinogenesis [19, 20]. The best-characterized Wnt canonical...
pathway is involved in determining cell fate and the regulation of growth, including the formation of the body axis, patterning of the neuroectoderm and amplification of neural progenitors [21]. The Wnt/planar cell polarity (PCP) pathway, the non-canonical Wnt pathway, controls tissue polarity and cell movement through the activation of RHOA, c-Jun N-terminal kinase (JNK) and nemo-like kinase (NLK) signaling cascades [20]. CTHRC1 can interact with multiple extracellular components of Wnt signaling, Fzd proteins and the Wnt/PCP co-receptor Ror2. These components form a Cthrc1-Wnt-Fzd/Ror2 complex to selectively activate the Wnt/PCP pathway and suppress the canonical Wnt pathway [22]. In this study, we illustrated for the first time that CTHRC1 can active Wnt/PCP pathways in CRC cells.

The data presented by us showed that recombinant CTHRC1 can not but overexpression of CTHRC1 can significantly promote CRC cells viability. The reason for this inconsistence might be that the effect of recombinant CTHRC1 on CRC cell proliferation was transient in contrast to the persistent effect by overexpression. The in vivo role of CTHRC1 in CRC carcinogenesis was totally unknown up to now. Further investigations with CRC mouse models spontaneously induced by genetic alterations (e.g. APC^min/+ mice) or induced by chemical carcinogens are needed to reveal the pathological role of CTHRC1 on colonic carcinogenesis.

In summary, our study provide evidences that CTHRC1 is an important player that promotes CRC cell proliferation, migration and invasion in vitro, which is possibly mediated by activating wnt/PCP pathway. Besides colonic epithelial cells, colon fibroblasts are also a major source of CTHRC1. Our data indicate that extracellular protein CTHRC1 produced in the tumor microenvironment promote CRC carcinogenesis autocrinely and paracrinely. Future further functional experiments with CRC mouse models induced by genetic alterations or chemical carcinogens are needed to clarify the role of CTHRC1 in CRC progression and metastasis.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jun Li, State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, 5/2200 Xietu Road, Shanghai 200032, Peoples Republic of China. E-mail: junli@shsci.org

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