RINT1 is a new suppression target to reduce colon cancer cell growth, migration and invasion through regulating ZW10/NAG-1 expression

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Abstract
This study explored the function and mechanism of RAD50 interactor 1 (RINT1), Zeste white 10 (ZW10) or nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) in cell growth of colon cancer. The mRNA expression of RINT1 was suppressed in patients with colon cancer. In addition, disease free survival (DFS) in colon cancer patients with high expression of RINT1, ZW10 and NAG-1 were promoted compared with colon cancer patients with low expression of RINT1, ZW10 and NAG-1. Over-expression of RINT1, ZW10 or NAG-1 reduced colon cancer cell growth, migration and invasion in vitro model. In contrast, down-regulation of RINT1, ZW10 or NAG-1 promoted colon cancer cell growth, migration and invasion in vitro model. Over-expression of RINT1 promoted while down-regulation of RINT1 suppressed ZW10 and NAG-1 expression. Following knockdown of ZW10 and NAG-1 protein expressions, RINT1 induced changes of colon cancer cell growth, migration and invasion were inhibited. Taken together, it is indicated that RINT1 is a new suppression target to reduce cell growth, migration and invasion of colon cancer by targeting ZW10/NAG-1.

Keywords RINT1 · ZW10 · NAG-1 · Cell growth · Colon cancer

Introduction
Colorectal cancer (CRC) is one of the common malignancies [1]. The incidence rate ranks to third in males and the second in females worldwide, and the morbidity and mortality are gradually increasing, posing serious threat to human health [1]. The incidence is relatively higher in populations over 50 years old and relatively lower in those under 50 years old [2]. According to reports from developed countries such as the United States and Australia, due to the popularity of CRC screening in recent years, the incidence of CRC in the United States has declined [3]. The incidence of CRC in males and females has decreased by 2.8% and 2.2% annually on average, respectively. However, the incidence of CRC in young adults is increasing. The incidence of CRC among young males and females in the 20–49 age group is increased by 1.5% and 1.6%, respectively per year in the United States [3].

Zeste white 10 (ZW10) is a highly evolutionarily-conserved protein which was originally found in Drosophila cells [4]. Subsequent studies have demonstrated the wide distribution of ZW10 in multicellular eukaryotes [4, 5]. DNA damage repair is a protective mechanism of cells and also an important pathway of tumor apoptosis [6]. Neuroblastoma-amplified gene-1 (NAG-1), a member of the TGF-β superfamily, is also known as macrophage inhibitory cytokine-1, placental transforming growth factor-β, prostate-derived factor, placental bone morphogenetic protein, or growth differentiation factor-15 (GDF-15) [7, 8]. NAG-1 is involved in the carcinogenesis and tumor progression [9]. Changes in NAG-1 gene expression can be detected in multiple types of tumors [10]. Although NAG-1 has been reported as a component of anti-tumor complex to...
participate in tumor apoptosis, the mechanism involved is extremely complicated and might change according to tumor microenvironment [9, 11].

DNA damage repair process is an important research field in tumor biology. Multiple classical genes involved in DNA damage response, such as P53 and breast cancer susceptibility gene-1 (BRCA1), play roles as tumor suppressor genes [12, 13]. RINT1 is critically involved in the repair of double-strand breaks, which also participates in the proliferation and apoptosis of various types of tumors [12]. This study explored the function and mechanism of RINT1, ZW10 or NAG-1 in cell growth, migration and invasion of colon cancer.

**Materials and methods**

**Clinical samples**

From March 2013 to September 2014, colon cancer tissue and normal colon tissue samples were obtained from 42 patients who were diagnosed with colon cancer at Tangshan Gongren Hospital, China. Tissues were immersed in liquid nitrogen and were stored at − 80 °C until use. Approval for this study was provided by the Ethics Committee of Tangshan Gongren Hospital, China. Informed consent was obtained from patients.

**RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from colon cancer tissue samples using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using an ABI 7900 PCR Thermal Cycler with a SYBR Green kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The mRNA expression levels of genes were measured using the $2^{-\Delta\Delta C_t}$ method.

**Cell Culture and transfection**

HCT 116 cell line was obtained from the Cell Bank (Shanghai, China). Cells were cultured in Dulbecco’s modified eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in an incubator at 37 °C with 5% CO2. Following 24 of culture, cells were transfected with RINT1, ZW10 and NAG-1 mimics, and si-RINT1, si-ZW10, si-NAG-1 plasmids using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA).

**Cell viability and cytotoxicity**

Cell viability was analyzed using methythiazolyldiphenyl-tetrazolium bromide (MTT) assays (Beyotime, Nantong, China) at 0, 24, 48 and 72 h after transfection. A total of 20 μL of MTT solution was added to each well and incubated for 4 h at 37 °C. Supernatants were removed and formazan crystals were dissolved in 150 μL dimethylsulfoxide (DMSO). Absorbance was measured using a Multiskan Spectrum Microplate Spectrophotometer (Thermo Scientific™, Waltham, MA, USA) at a wavelength of 492 nm.

Cytotoxicity for analyzing Lactate dehydrogenase (LDH) activity was measured using LDH activity kits (Beyotime, Nantong, China). Absorbance was measured using a Multiskan Spectrum Microplate Spectrophotometer (Thermo Scientific™, Waltham, MA, USA) at a wavelength of 450 nm.

**Scratch test**

A total of 100 μL of transfection cells (1 × 10⁶/mL) were resuspended in serum-free medium and seeded onto each well of 6 well plates. Then, 200 μL tips were used to scratch and cells were observed using microscope at 24 h after scratch.

**Western blot analysis**

Total proteins of cells were collected using Radio-immunoprecipitation assay (RIPA) lysis buffer and protease inhibitor cocktail (PMCF; 1:100, Beyotime, Nantong, China), and the concentration of these protein was determined by using BCA Kit (Beyotime, Nantong, China). Then, 50 μg protein samples were loaded to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinyl difluoride (PVDF; Thermo Scientific™, Waltham, MA, USA) membranes. Membranes were incubated with RINT1, ZW10, NAG-1, TGF-β and GAPDH antibodies at 4 °C overnight after blocking with 5% non-fat milk in Tris-buffered saline with 0.1% tween 20 (TBST). The membranes were washed with TBST for three times and then incubated with anti-rabbit secondary antibody (1:5000) for 2 h at room temperature. All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Immunoreactive bands were visualized using the ECL kit (Thermo Scientific™, Waltham, MA, USA) and integrated density of the bands was quantified by Quantity One software 3.0 (Bio-Rad, CA, USA).
Statistical analysis

Results are expressed as means ± standard deviation (SD). Statistical analysis was performed with the Student unpaired t test or One-way analysis of variance (ANOVA) and Tukey’s post test in Statistical Product and Service Solutions (SPSS) version 19.0 (SPSS Inc., Chicago, IL, USA). p values < 0.05 were considered to indicate statistical significance.

Results

RINT1, ZW10 and NAG-1 expression observed in colon cancer patients

The mRNA expression of RINT1, ZW10 and NAG-1 in normal tissues and cancer tissues of colon cancer patients was determined by qRT-PCR. It was found that mRNA expression of RINT1 (Fig. 1a, b), ZW10 (Fig. 1d, e) and and NAG-1 (Fig. 1g, h) was reduced in colon cancer tissues of patients, compared with normal tissues of patients (p < 0.01). mRNA expression of RINT1 (Fig. 1c), ZW10 (Fig. 1f) and NAG-1 (Fig. 1i) in tissues of patients with stage IV colon cancer was lower than that of patients with stage III colon cancer (p < 0.01). In addition, tissue mRNA expression of RINT1 (Fig. 1c), ZW10 (Fig. 1f) and NAG-1 (Fig. 1i) in patients with stage III colon cancer was lower than that of patients with stage I-II colon cancer (p < 0.01).

Effects of RINT1, ZW10 and NAG-1 on the survival rate of colon cancer patients

The effects of RINT1, ZW10 and NAG-1 on the survival rate of colon cancer patients were investigated. It was suggested that overall survival (OS) in colon cancer patients with high expression of RINT1 (Fig. 2a), ZW10 (Fig. 2c) and NAG-1 (Fig. 2e) was similar to patients with low expression of RINT1, ZW10 and NAG-1. However, DFS in colon cancer patients with high expression of RINT1, ZW10 and NAG-1...
were higher than colon cancer patients with low expression of RINT1 (Fig. 2b), ZW10 (Fig. 2d) and NAG-1 (Fig. 2f).

**RINT1 regulated growth, migration and invasion of colon cancer cells**

To confirm the effects of RINT1 on colon cancer growth, migration and invasion, RINT1-mimic plasmids were transfected into HCT 116 cells. The expression of RINT1 was increased in cultured cancer cells, compared with the negative group (p < 0.01; Fig. 3a). It was found that, compared with the negative control, over-expression of RINT1 reduced cell growth (p < 0.01; Fig. 3b), increased cytotoxicity (p < 0.01; Fig. 3c), suppressed migration (p < 0.01; Fig. 3d, e) and invasion rate (p < 0.01; Fig. 3f, g) and increased caspase-3/9 activity levels (p < 0.01; Fig. 3h, i) in vitro model of colon cancer.

To further prove the results of RINT1, si-RINT1 was used to treat with HCT 116 cells. RINT1 expression was heavily reduced in si-RINT1 transfected colon cancer cells, in comparison with the negative group (p < 0.01; Fig. 3j). The down-regulation of RINT1 promoted cell growth (p < 0.01; Fig. 3k), accelerated migration (p < 0.01; Fig. 3l, m) and invasion rate (p < 0.01; Fig. 3n, o), reduced cytotoxicity (p < 0.01; Fig. 3p), and decreased caspase-3/9 activity levels (p < 0.01; Fig. 3q, r) in vitro model of colon cancer, compared with the negative group.
ZW10 regulated growth, migration and invasion of colon cancer cells

This study further confirmed the function of ZW10 in cell growth, migration and invasion of colon cancer. As shown in Fig. 4a, ZW10 mimic plasmid significantly promoted ZW10 expression in vitro cultured colon cancer cells ($p < 0.01$). Furthermore, compared to the negative control, over-expression of ZW10 reduced cell growth ($p < 0.01$; Fig. 4b), increased cytotoxicity ($p < 0.01$; Fig. 4c), reduced migration ($p < 0.01$; Fig. 4d, e) and invasion rate ($p < 0.01$; Fig. 4f, g) and increased caspase-3/9 activity levels ($p < 0.01$; Fig. 4h, i) in vitro model of colon cancer, compared with the negative group ($p < 0.01$; Fig. 4j). ZW10 down-regulation promoted cell growth ($p < 0.01$; Fig. 4k), accelerated migration ($p < 0.01$; Fig. 4l, m) and invasion rate ($p < 0.01$; Fig. 4n, o), reduced cytotoxicity ($p < 0.01$; Fig. 4p), and decreased caspase-3/9 activity levels ($p < 0.01$; Fig. 4q, r) in colon cancer cells, compared with the negative group.

NAG-1 regulated growth, migration and invasion of colon cancer cells

This study confirmed that the anti-effects of NAG-1 in cell growth, migration and invasion of colon cancer. Compared to the negative control, NAG-1 mimic plasmid induced higher NAG-1 expression in vitro cultured colon cancer cells ($p < 0.01$; Fig. 5a). Over-expression of NAG-1 reduced cell growth ($p < 0.01$; Fig. 5b), increased cytotoxicity ($p < 0.01$; Fig. 5c), reduced migration ($p < 0.01$; Fig. 5d, e) and invasion ($p < 0.01$; Fig. 5f, g) rate and increased caspase-3/9 activity levels ($p < 0.01$; Fig. 5h, i) in vitro model of colon cancer, compared with negative group. Moreover, si-NAG-1 decreased NAG-1 expression significantly in vitro model of colon cancer, indicating the good efficiency of si-NAG-1 ($p < 0.01$; Fig. 5j). NAG-1 down-regulation induced cell growth ($p < 0.01$; Fig. 5k), accelerated migration ($p < 0.01$; Fig. 5l, m) and invasion ($p < 0.01$; Fig. 5n, o), reduced cytotoxicity ($p < 0.01$; Fig. 5p), and decreased caspase-3/9 activity levels ($p < 0.01$; Fig. 5q, r) in colon cancer cells, compared with the negative group.
To determine the mechanism of RINT1 in colon cancer, we used gene chip to analyze the expression gene regulated by RINT1. As showed in Fig. 6a, RINT1 might regulate ZW10/NAG-1 to reduce reactive oxygen species (ROS) production. Over-expression of RINT1 promoted ZW10 (Fig. 6b, d) and NAG-1 (Fig. 6b, c) protein expressions in colon cancer cell line, compared with the negative group (p < 0.01). Conversely, compared with the negative control, down-regulation of RINT1 obviously suppressed ZW10 (Fig. 6e, f) and NAG-1 (Figs. 6e, g) protein expressions in colon cancer cell line (p < 0.01).

The inhibition of ZW10/ NAG-1 suppressed the effects of RINT1 on growth of colon cancer cells

To further analyze the role of ZW10 in the effects of RINT1 on growth of colon cancer cells, the expression of ZW10 was inhibited using si-ZW10. As shown in Fig. 7a–c, compared with the RINT1 over-expression group, si-ZW10 suppressed ZW10 and NAG-1 protein expressions induced by over-expression of RINT1 in cultured colon cancer cells (p < 0.01). Furthermore, RINT1 over-expression induced changes of cell viability (Fig. 7d), migration (Fig. 7e, f), invasion (Fig. 7g, h), cytotoxicity (Fig. 7i), and caspase-3/9 activity (Fig. 7g, k) of colon cancer cells were significantly inhibited following the inhibition of ZW10 (p < 0.01).

It was indicated that RINT1 changed growth of colon cancer cells by the inhibition of ZW10.

Next, the role of NAG-1 in the effects of RINT1 on growth of colon cancer cells was further determined by si-NAG-1. It was shown that si-NAG-1 suppressed NAG-1 protein expressions induced by over-expression of RINT1 in colon cancer cells (Fig. 8a, b). The inhibition of NAG-1 promoted cell growth (Fig. 8c), migration (Fig. 8d, e) and invasion (Fig. 8g, h), and inhibited cytotoxicity (Fig. 8i) and caspase-3/9 activity (Fig. 8j) in vitro model of colon cancer induced by over-expression of RINT1 (p < 0.01).
Discussion

Compared with CRC in old population, the incidence of CRC in young population is relatively lower. In general, CRC is not common in population under 40 years old [14, 15]. The risk of CRC under 40 years old is 1:1200, while it rises to 1:25 in population over 70 years old [15]. In recent years, due to the popularity of CRC screening in the elderly, the incidence of CRC in the old population in the United States, Europe, and other developed Western countries has gradually decreased [16]. However, the incidence and mortality of CRC in young people has been increasing, and CRC has become the third leading cause of death following leukemia and neurological diseases among population with 20–39 years old [14]. This study proved that down-regulation of RINT1, ZW10 and NAG-1 expression was observed in colon cancer patients. RINT1 reduced colon cancer cell growth, migration and invasion by targeting ZW10 or NAG-1.

RINT1 was first identified as a RAD50-interacting protein of 792 amino acids. An N-terminally truncated RINT1 protein over-expression results in a defect in the radiation-induced G2/M checkpoint, indicating that RINT1 plays an important role in cell cycle progression [17]. Lin et al. reported that RINT1 serves as a tumor suppressor and maintains Golgi dynamics for cell survival [18]. This study also suggested that RINT1 expression is decreased in patients with colon cancer, and RINT1 reduces colon cancer cell growth, migration and invasion.

NAG-1 is associated with multiple diseases including cancer. The expression of NAG-1 is lower in glioblastoma cell lines than that in benign glioma cell lines and normal human astrocytes [19]. Existing in vitro studies have shown suggest that NAG-1 expression inhibits tumor cell growth [19–21]. A study provided the evidence that over-expression...
of NAG-1 can inhibit the proliferation of breast cancer cells, epithelial tumor cells and prostate cancer cells [22]. In addition, the tumor formation ability of human colon cancer and glioma cells with NAG-1 overexpression is significantly decreased in nude mice [23]. Further studies have demonstrated that overexpression of NAG-1 can cause caspase-dependent apoptosis in prostate cancer cells [24]. Above results suggest that NAG-1 is closely associated with tumorigenesis. In this study, we also found that NAG-1 can suppress colon cancer cell viability, migration and invasion, and its protein expression can be regulated by RINT1.

ZW10 plays an extremely important role in both mitosis and meiosis [24]. ZW10 mutations exert similar effects on mitosis and meiosis, causing delayed chromosome and chromosomal unequal separation, ultimately regulating tumor proliferation and apoptosis [25]. We also identified...
the inhibition of ZW10 or NAG-1 reduced the effects of RINT1 on cell growth of colon cancer. Arasaki et al. showed that RINT1 coordinates the localization and function of ZW10 at the ER and the Golgi apparatus throughout the cell cycle [26]. This investigation suggests that RINT1 activation may be associated with ZW10/NAG-1 and subsequent improvement of colon cancer.

**Conclusions**

In summary, our data indicate that RINT1, ZW10 and NAG-1 expression were down-regulated in colon cancer patients. Over-expression of RINT1, ZW10 or NAG-1 reduced cell viability, migration and invasion in vitro model. In addition, RINT1 induced ZW10 and NAG-1 protein expressions to control colon cancer cell growth, migration and invasion. RINT1,
ZW10 and NAG-1 may help lead to the development of new drugs to cure colon cancer.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that there are no potential conflicts of interest to disclose.

**Ethical approval** The study was approved by the Tangshan Gongren Hospital, China.

**Informed consent** Informed consent was obtained from patients.

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