Overexpression of lncRNA-422 inhibits proliferation and invasion in colorectal cancer

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Abstract: Long non-coding RNA-422 (lncRNA-422) has been recently reported to be involved in the progression of colorectal cancer (CRC). But its effects on CRC cell phenotypes remain elusive. In the present study, we assessed the function of lncRNA-422 on the aggressive phenotypes of CRC cells in vitro. SW1116 cell was transfected with specific recombinant plasmid vector to enhance lncRNA-422 expression. The cell proliferation, invasion and migration were investigated and compared between overexpression group and control group. The apoptosis related protein levels in transfected CRC cells were also detected by western blot analysis. We found that up-regulation of lncRNA-422 inhibited the proliferation and invasion of CRC cells, and activated the apoptosis-related proteins, including JNK, p38, Erik, P-p53, PARP, caspase-3. In conclusion, our findings suggested that lncRNA-422 was implicated in the progression of CRC, which might provide the potential therapeutic target for this fetal cancer.

Keywords: lncRNA-422, proliferation, invasion, apoptosis, colorectal cancer

Introduction

Colorectal cancer is the third most prevailing cancer in the world with high mortality [1]. According to recent statistical data, the number of new patients with CRC is more than one million annually, and it remains the second leading cause of cancer-related death in the USA [2]. The occurrence rate of CRC in China has also obviously increased in recent years [3]. Therefore, it is critically important to elucidate the molecular mechanisms involved in the development of CRC [4, 5].

Long non-coding RNAs (IncRNAs) are described to be longer than 200 nt [6], and a rising number of studies have reported their biological roles as regulatory RNAs in a variety of human diseases including oncogenesis [7-9], and deregulation of IncRNAs is also implicated in the pathogenesis of CRC [10, 11]. Kogo reported that high expression of HOTAIR is associated with a poor prognosis of CRC [12]. Ling revealed that a novel IncRNA CCAT2 (colon cancer associated transcript 2) is significantly overexpressed in microsatellite-stable CRC and underlies metastatic progression and chromosomal instability in colon cancer [13]. Yan and Madamanchi demonstrated decreased expression of IncRNA ncRuPAR can down-regulate protease-activated receptor-1 (PAR-1), thus inhibits CRC progression [14, 15]. Accordingly, IncRNAs may function as oncogenes or tumor suppressors, add a novel layer to our understanding of the complexity of CRC development [16]. It has already been reported that IncRNA-422 was decreased in CRC [17], while detailed cellular functions and mechanisms of IncRNA-422 in CRC still remain largely elusive.

Therefore, in the present study, we investigated the role of IncRNA-422 in CRC progression. We found that elevated expression of IncRNA-422 could induce CRC cell apoptosis, and inhibit CRC cell migration and invasion capacities. Furthermore, the underlying mechanisms of IncRNA-422 in regulating CRC cell apoptosis were also determined.

Materials and methods

Cell culture and reagents

The human CRC cell lines (SW1116) were obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China), and cultured...
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Construction of lncRNA-422 plasmid and transfection

To generate plasmid particles with LncRNA-422 overexpression, ligation reactions of the LncRNA-422 gene fragments and pcDNA3.1 vectors were conducted using T4 DNA ligase Kit (TaKaRa), and the products were transformed into E. coli JM109 competent bacteria. Monoclonal colonies were picked, and positive recombinants were identified. Subsequently, SW-1116 cells were transfected with pcDNA3.1-LncRNA-422 and empty vectors, respectively. Successful transfections were confirmed by counting the number of green fluorescent protein (GFP) positive cells under a fluorescence microscope.

Reverse transcription-quantitative polymerase chain reaction analysis

After 5-day and 6-day infection, SW1116 cells were lysed to extract total RNA. Complementary DNA (cDNA) was then synthesized from 1 μg RNA, and the primers were synthesized by TaKaRa. The sequences are as follows: forward: 5'-CC AAG CTT GTC TTT TTC AGT CTG AAG TCT TTA TTT GG-3', and reverse: 5'-CTC TCT CTC GAG TAA ACC ACC AAA TTT TTA TTA AGA T-3'. GAPDH (forward primer: 5'-GGGAAGCCAAAAGGTTTGGT-3'; and reverse primer: 5'-GAGTCCTTCCAGATACCAA-3') was used as an internal control. Then RT-qPCR was performed on BioRad C1000 Real-Time PCR platform with prepared 20 μl PCR reaction mixture (10 μl 2×SYBR premix ex taq, 0.8 μl primers, 4.2 μl ddH₂O and 5.0 μl cDNA) using the following amplified procedure: pre-denaturation at 95°C for 1 min, followed by 40 cycles consisting of 5 s denaturation at 95°C and 20 s annealing extension at 60°C. The relative gene expression was analyzed using 2^ΔΔCt method.

MTT assay

SW1116 Cells (4×10^3/well) were plated onto 96-well plates after infection for 3 days. MTT assay was used to determine cell viability. Briefly, total 20 μl MTT solution was added to
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each well at day 1, 2, 3, 4 and 5 and incubated at 37°C for 4 h. Then the samples were dissolved in 100 µl acidic isopropanol containing 5% isopropanol, 10% SDS and 0.01 mol/L HCl and the optical density of each well was measured by a microplate reader at 595 nm. Each test was conducted in triplicate.

**Annexin V/7-AAD staining**

After 24 h, the transfected cells were harvested by trypsinization and Annexin V-APC/7-AAD Apoptosis Kit (KA3808, Abnova) was used to evaluate SW1116 cells apoptosis according to the manufacturer’s instruction. Percentages of cells stained with Annexin V-APC and/or 7-AAD relative to total cell numbers were measured with Becton-Dickinson FACsalibur system.

**Transwell invasion assays**

Transwell assays were performed using Boyden’s chambers. Cells were plated in the upper chamber consisting of 8-mm membrane filter inserts coated with (for invasion assay) or without (for migration assay) Matrigel (BD Biosciences). The chemoattractant in the lower chamber was supplemented with medium containing 10% FBS. Cells on the upper surface were removed after 24 h, and those attached on the lower side of the membrane were fixed and stained with crystal violet before counting under a microscope in five randomly selected fields. At least three chambers from three different experiments were analyzed.

**Wound healing assay**

Cultured plates were seeded on the back of the line before the label, cell digestion after access to 12-well plate, perpendicular to the orifice to create cell scratches. Absorb the cell culture medium, rinse the orifice plate three times with PBS, and wash away the scratches generated cell debris. Add serum-free medium, the culture plate into the incubator culture, every 4-6 hours to take pictures. Analyze the experimental results based on the collected image data.

**Western blot assay**

SW1116 cells were lysed with RIPA protein extraction reagent (Beyotime, Beijing, China) supplemented with a protease inhibitor cocktail (Roche, CA, USA). Protein extracts (40 µg) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to 0.22-µm nitrocellulose membranes (Sigma), following incubation with appropriated primary and secondary antibodies. GAPDH was

![Figure 3. Overexpression of lncRNA-422 induced apoptosis in SW1116 cells. A: Cell apoptosis of SW1116 cells was determined by flow cytometry in transfecting with pcDNA3.1-RNA-422 and empty vector. B: Quantification of apoptotic cells in SW1116 cells by flow cytometry. Annexin V+/7-ADD+, early apoptotic cells; annexin V+/7-ADD−, late apoptotic cells. *P<0.05, ***P<0.001.](image-url)
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used as a control. ECL chromogenic substrate was used, and signals were quantified by densitometry (Quantity One software, BioRad).

Statistical analysis

The data were presented as mean ± standard deviation (SD). Statistical analysis was performed using the SPSS version 15.0 software (SPSS, Chicago, IL, USA). Statistical analysis was conducted using a Student’s t-test. Differences at the level of P<0.05 were considered to be statistically significant.

Results

LncRNA-422 was upregulated after transfection in SW1116

To assess the role of IncRNA-422 in CRC, we established IncRNA-422 overexpressed SW1116 cells by infecting with pcDNA3.1-IncRNA-422. Controls were transfected with empty vectors. As depicted in Figure 1A, there were more than 80% cells infected with plasmid vectors as indicated by GFP positive cells under green fluorescence, indicating a successful infection. To further confirm the transfection efficiency, IncRNA-422 expression levels were examined using qRT-PCR. As shown in Figure 1B, IncRNA-422 expression levels were increased after transfection with pcDNA3.1-IncRNA-422. These results suggested that IncRNA-422 was up-regulated stably in CRC cells after transfection.

Overexpression of IncRNA-422 inhibited SW1116 cell proliferation

For further confirm whether overexpression IncRNA-422 caused SW1116 cell apoptosis, the quantitative analysis of SW1116 cell apoptosis was monitored by using Annexin V-APC/7-AAD Apoptosis Kit. As depicted in Figure 3, the apoptotic rates, including early apoptosis ( Annexin V+/7-AAD-) and late apoptosis ( Annexin V+/7-AAD+), in SW1116 cells with IncRNA-422 overexpression were all significantly increased (P<0.05).

Overexpression IncRNA-422 suppresses SW1116 cell invasion

The above findings indicated that overexpressed IncRNA-422 inhibited the proliferation of SW1116 cells. Thus, we detected the migration
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and invasion of SW1116 cells following pcDNA3.1-lncRNA-422 transfection. Wound healing assay was demonstrated that overexpression of lncRNA-422 inhibited the migration of SW1116 cells by comparing with the control empty vector-transfected cells (Figure 4A). In analogical results observed in transwell assay, the SW1116 cells with lncRNA-422 overexpression showed decreased invasion capacities in comparison with control cells (Figure 4B).

**Overexpression of lncRNA-422 induces SW1116 apoptosis**

To explore the mechanisms of lncRNA-422 in regulating tumorigenesis, we then investigated whether lncRNA-422 regulates cell apoptosis-related signals. The expression of apoptosis-related proteins was detected by western blot analysis. As shown in Figure 5A, up-regulated lncRNA-422 resulted in an increase in the phosphorylation levels of p-38, SAPK/JNK (p54 and p46) and MEK. Furthermore, cell apoptosis-related proteins, including p-p53, PARP, caspase-3 were also activated in the SW1116 cells with lncRNA-422 overexpression by comparing with control cells (Figure 5B).

**Discussion**

As research on human cancer was developing rapidly, IncRNAs had been regarded as crucial regulators in various biological processes [18, 19]. IncRNAs played important roles in carcinogenesis and cancer metastasis in CRC, a serious disease that was complex and heterogeneous [19, 20]. We found that IncRNAs might function as oncogenes or tumor suppressors in...
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the cancer initiatome [21]. In 2003, MALAT1 was first found to be highly expressed in non-small cell lung cancer by Ji [22]. Ji's study showed that the high expression of MALAT1 was associated with metastasis and poor prognosis. Finally in 2011, Xu identified the functional motif of MALAT-1 in CRC. They found a motif of the 3′ end MALAT-1 gene layered an important role in the biological processes of human colorectal malignancies [23].

In addition, Zheng conducted an important experiment to assess MALAT-1 expression in 146 CRC patients (stage II/III) and 23 paired normal colonic mucosa samples [24]. Results presented that the expression of MALAT-1 was up-regulated in CRC tissues, and the over-expression of MALAT-1 might serve as a negative prognostic marker in stage II/III CRC patients. In recent years, HOTAIR was found significantly overexpressed in many cancers, such as breast cancer, hepatocellular cancer and laryngeal squamous cell carcinoma [25, 26]. Kogo’s group found that the expression levels of HOTAIR were higher in cancerous tissues than in noncancerous tissues, they also indicated that expression of HOTAIR and members of the PRC2 complex (SUZ12, EZH2, and H3K27me3) had a close correlation [12].

LncRNA-422 might be implicated in carcinogenesis through regulating protein coding genes involved in special biological process relevant to cancer [17]. In the present study, we demonstrated that IncRNA-422 could inhibit CRC cell proliferation and invasiveness. Overexpression IncRNA-422 SW1116 cell line was generated, and functional experiments showed that up-regulated IncRNA422 expression could induce SW1116 cell apoptosis, and the ability of migration and invasion were also obviously decreased after transfection with pcDNA3.1-IncRNA-422. We further investigated the markers of cell apoptosis pathway in IncRNA-422-overexpressed cells by western blot. Results indicated that IncRNA-422 could promote the activities of JNK, p38, Erk. And up-regulated the expression of IncRNA-422 could induce the SW1116 cells apoptosis through caspase pathway.

In conclusion, our results suggested that IncRNA-422 was involved in the progression of CRC and might provide evidence for IncRNA-422 being as a potential target for therapy of this disease in the near future.

Disclosure of conflict of interest

None.

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