Abstract. – OBJECTIVE: Recent studies have discovered that long noncoding RNAs (lncRNAs) play an important role in malignant tumors. In this research, IncRNA ROR1-AS1 was selected to identify how it affected the development of colorectal cancer (CRC).

PATIENTS AND METHODS: ROR1-AS1 expression was detected by Real-time quantitative polymerase chain reaction (RT-qPCR) in CRC tissue samples. ROR1-AS1 expression level and patients’ overall survival time were analyzed. Functional experiments were conducted to identify the changes of biological behaviors in CRC cells after knockdown of ROR1-AS1. Moreover, we also explored the underlying mechanism.

RESULTS: Detection of ROR1-AS1 expression level in patients’ tissues showed that ROR1-AS1 was higher in CRC tissues than that in adjacent ones. ROR1-AS1 expression was negatively associated with patients’ overall time. Cell growth ability was inhibited due to knockdown of ROR1-AS1 in vitro. Moreover, cell migration and invasion abilities were repressed after ROR1-AS1 was knockdown. Furthermore, due to the knockdown of ROR1-AS1, the targetted proteins in Wnt/β-catenin signaling pathway were suppressed.

CONCLUSIONS: These results suggested that ROR1-AS1 could enhance cell metastasis and proliferation via targeting Wnt/β-catenin signaling pathway, which might offer a potential therapeutic target in CRC.

Keywords: Long noncoding RNA, ROR1-AS1, Colorectal cancer, Wnt/β-catenin signaling pathway.

Introduction

The incidence rate of colorectal cancer (CRC) remains high both in male and in female worldwide1. 1.36 million cases were diagnosed of CRC annually globally, and almost 0.6 million cases died of CRC. Although technological advances have been made in early detection and intervention for the past decades, the prognosis for CRC patients is still dismal2. Thus, it is crucial to uncover the molecular mechanism involving the progression of CRC and find out potential targets to improve the poor prognosis of this disease.

As one subtype of noncoding RNA (ncRNA), long noncoding RNAs (lncRNAs) regulate a variety of cellular processes and pathways in the development of cancers. For instance, down-regulation of lncRNA linc-ITGB1 inhibits cell invasion, cell migration and epithelial-mesenchymal transition in non-small cell lung cancer by decreasing Snail expression3. The expression level of lncRNA-CCHE1 is positively related to the malignancy of colorectal carcinoma and it regulates ERK/COX-2 pathway4. Through regulating the stability of DNMT1 and depressing the expression of tumor suppressors, lncRNA LUCAT1 promotes esophageal squamous cell carcinoma formation and cell metastasis5. Activated by ZEB1, lncRNA HCCCL5 accelerates cell viability, cell migration, epithelial-mesenchymal transition and the malignancy of hepatocellular carcinoma6. LncRNA SCHLAP1 contributes to the development of aggressive prostate cancer by antagonizing the function of the SWI/SNF complex7. However, how lncRNA ROR1-AS1 participates in the progression of CRC remains unknown.

In this study, ROR1-AS1 was remarkably higher-expressed in CRC tissues when compared with adjacent normal tissues. Moreover, ROR1-AS1 promoted the proliferation and invasion of CRC in vitro. Our further experiments also showed that ROR1-AS1 participated in tumorigenesis of CRC through Wnt/β-catenin signaling pathway.
Patients and Methods

Clinical Samples
Tumor samples and the adjacent tissues (≥ 5 cm away from the edge of tumor) were gathered from CRC patients (n = 52) who underwent surgery at Shanxi Provincial People’s Hospital between 2014 and 2017. Written informed consent was taken before operation. All fresh tissues were preserved at -80°C. This study was approved by the Ethics Committee of Shanxi Provincial People’s Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture
Human CRC cell lines (HCT116, HT29, SW620, SW480) and normal human colonic epithelial cell line (NCM460) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA), and then cultured in Roswell Park Memorial Institute-1640 (RPIM-1640) (HyClone, South Logan, UT, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in an incubator containing 5% CO2 at 37°C.

Cell Transfection
Short hairpin RNA (shRNA) targeting ROR1-AS1 (sh-ROR1-AS1), scrambled oligonucleotides (NC) were purchased from GenePharma (Shanghai, China). The complementary DNA encoding ROR1-AS1 was PCR-amplified, which was then inserted into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). Those treated cells were used for the following experiments.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)
24 h after transfection, total RNA was extracted from CRC cells or tumor tissues from CRC patients (using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand complementary deoxyribonucleic acid (cDNA) was synthesized using a first-strand cDNA synthesis kit (Invitrogen Life Technologies Co., Ltd., Dainan, China) according to the manufacturer’s instructions. Primers used are the primers using-forward 5′-CTGACGAAACACTGGAACTC-3′ and reverse 5′-GTCTGATTTGGTAGCTTGATG-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward 5′-CCAAAATCAGATGGGGCAATGGCTGG-3′ and reverse 5′-TGATGGCATGGACTGTGGTCATTCA-3′. Thermal cycle was as follows: 30 sec at 95°C, 5 sec for 40 cycles at 95°C, 35 sec at 60°C.

MTT (3-(4,5-Dimethylthiazol-2,5-Diphenyl Tetrazolium Bromide) Assay
Before transfection, CRC cells (100 cells/well) were seeded into 96-well plates. 15 μL MTT was added to each well and incubated for 4 h when cultured for 0, 24, 48, and 72 h. To stop the reaction, 0.2 mL dimethyl sulfoxide (DMSO) was added. The absorbance at 490 nm was assessed using an enzyme-linked immunosorbent assay (ELISA) reader (Multiskan Ascent, LabSystems, Helsinki, Finland).

Colony Formation Assay
Those transfected cells were placed in a 6-well plate for 2 weeks. Cell colonies were treated with methanol for 30 min. Then 0.5% crystal violet was used for staining for 5 min. Colonies containing more than 50 cells were counted and the mean colony numbers were calculated. Analysis was performed with Image-Pro Plus 6.0 (Silver Springs, MD, USA).

Wound Healing Assay
Cells, seeded in 6-well plates, were incubated overnight. After scratched with a pipette tip, cells were cultured in serum-free DMEM. Related distance was viewed under a light microscope (Olympus Corp., Tokyo, Japan) at 48 h. Each assay was independently repeated in triplicate.

Transwell Assay
2×104 cells in serum-free Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Rockville, MD, USA) were replanted in the upper chamber and coated with 30 μL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). DMEM and FBS were added into the bottom chamber. After 24 h of incubation, the cells were immersed with 4% paraformaldehyde for 10 min and stained in 1% crystal violet for 30 min to remove any uninfected cells from the upper chamber. Next, cells were counted and photographed in randomly selected fields with a Leica DMi4000B microscope (Leica Microsystems, Heidelberg, Germany).

Western Blot Analysis
Cell samples were washed with precooled phosphate-buffered saline (PBS) and then lysed with cell lysis solution (RIPA) (Beyotime, Shanghai, China). Protein concentration was
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Samples (Figure 1A). ROR1-AS1 expression level was higher in CRC cells than that in NCM460 (Figure 1B).

The Association between ROR1-AS1 Expression Level and the Prognosis of CRC Patients

We divided 52 patients into two groups, high ROR1-AS1 level group and low ROR1-AS1 level group, via median expression. Kaplan-Meier analysis showed that patients in high ROR1-AS1 level group had shorter overall survival time compared with those in low ROR1-AS1 level group (Figure 2).

Cell Proliferation was Inhibited in CRC Cells via Knockdown of ROR1-AS1

As ROR1-AS1 expression level was the highest in SW620 among four CRC cell lines, SW620 cells were used for the transfection of ROR1-AS1 shRNA or scrambled oligonucleotides (NC). RT-qPCR was utilized for detecting the ROR1-AS1 expression (Figure 3A). Moreover, the results of MTT assay revealed that the cell growth ability of CRC cells was obviously repressed via knockdown of ROR1-AS1 (Figure 3B). The outcome of colony formation assay also revealed that the number of colonies was remarkably reduced via knockdown of ROR1-AS1 in CRC cells (Figure 3C).

Cell Migration and Invasion was Inhibited in CRC Cells via Knockdown of ROR1-AS1

Wound healing assay results revealed that the relative migrated ability of CRC cells was
obviously repressed via knockdown of ROR1-AS1 (Figure 4A). The outcome of Transwell assay also revealed that the number of invaded cells was remarkably reduced via knockdown of ROR1-AS1 in CRC cells (Figure 4B).

**The Interaction between Wnt/β-Catenin Signaling Pathway and ROR1-AS1**

To explore the underlying mechanism of ROR1-AS1 function in CRC, RT-qPCR and Western blot assay were conducted to detect the target proteins in Wnt/β-catenin signaling pathway such as Wnt3a, β-catenin, C-myc and Survivin. RT-qPCR results showed that Wnt3a, β-catenin, C-myc and Survivin could be down-regulated via knockdown of ROR1-AS1 (Figure 4C). Results of Western blot assay showed that Wnt3a, β-catenin, C-myc and Survivin could be down-regulated.

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**Figure 2.** The association between ROR1-AS1 expression level and the prognosis of CRC patients. Expression levels of ROR1-AS1 were negatively associated with patients’ overall survival time. *p<0.05.

**Figure 3.** ROR1-AS1 promoted CRC cell proliferation. **A,** ROR1-AS1 expression in CRC cells transfected with ROR1-AS1 shRNA (sh-ROR1-AS1) or scrambled oligonucleotides (NC) was detected by RT-qPCR. GAPDH was used as an internal control. **B,** MTT assay revealed that the cell growth ability of CRC cells was obviously repressed in sh-ROR1-AS1 group compared with NC group. **C,** Colony formation assay also revealed that the number of CRC cell colonies was remarkably reduced in sh-ROR1-AS1 group compared with NC group. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.
ROR1-AS1 promotes tumorigenesis of colorectal cancer via targeting Wnt/β-catenin signaling pathway. These results suggested that ROR1-AS1 participated in the regulation of Wnt/β-catenin signaling pathway and further promoted CRC growth and metastasis.

Discussion

Numerous studies have proved that ncRNAs take part in a variety of important biological processes, including tumor growth. Previously,
evidence revealed that several lncRNAs participate in the development of CRC. For instance, lncRNA TP73AS1 promotes cell apoptosis of CRC by sponging miR103\textsuperscript{10}. LncRNA RUNXI-IT1 acts as a tumor suppressor in CRC by inhibition of cell migration and cell proliferation, which suggests RUNXI-IT1 could function as a novel diagnostic biomarker\textsuperscript{11}. In addition, lncRNA H19 promotes 5-Fu resistance in CRC via sponging to miR-194-5p\textsuperscript{12}.

Located in 1p31.3, ROR1-AS1 is a newly discovered lncRNA which is firstly discovered in mantle cell lymphoma\textsuperscript{13}. In the current study, we conducted experiments to identify the role of ROR1-AS1 in CRC. Results showed that ROR1-AS1 was upregulated in CRC samples and was associated with patients’ prognosis. Besides, CRC proliferation and invasion was found to be inhibited via knockdown of ROR1-AS1. Above results indicated that ROR1-AS1 promotes tumorigenesis of CRC and might act as an oncogene.

Previous researches have suggested that aberrant activation of the Wnt/β-catenin signaling pathway plays an important role in regulating development of several human cancers. For instance, lncRNA CCAL promotes hepatocellular carcinoma progression by regulating Wnt/β-catenin pathway\textsuperscript{14}. MicroRNA-495 inhibits the progression of non-small-cell lung cancer by targeting TCF4 and inactivating Wnt/β-catenin signaling pathway\textsuperscript{15}. Wnt/β-Catenin signaling activated by c-Myb promotes proliferation and metastasis of breast cancer\textsuperscript{16}. Wnt/β-Catenin/Axin2 signaling is involved in the occurrence and development of several human cancers. For instance, lncRNA RUNX1-IT1 plays a tumour-suppressive role in colorectal cancer\textsuperscript{17}. In the current study, we conducted experiments to identify the role of ROR1-AS1 in CRC. Results showed that ROR1-AS1 promotes tumorigenesis of CRC and might act as an oncogene.

Conclusions

Above data suggested that ROR1-AS1 could promote CRC cell proliferation and invasion by activating Wnt/β-catenin signaling pathway. These findings indicate that ROR1-AS1 might promote CRC cell proliferation and invasion by activating Wnt/β-catenin signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

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