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ROR1-AS1 promotes tumorigenesis of colorectal cancer *via* targeting Wnt/β-catenin

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Abstract. – OBJECTIVE: Recent studies have discovered that long noncoding RNAs (lncRNAs) play an important role in malignant tumors. In this research, lncRNA 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 analy[illegible]. Functional experiments were conducted t[illegible] tify the changes of biological behaviors [illegible] CRC cells after knockdown of ROR1-AS1. Mo[illegible]er, we also explored the underlying mechanis[illegible]

RESULTS: Detection of ROR1-AS1 expres[illegible] level in patients' tissues showed that RO[illegible] AS1 was higher in CRC tissu[illegible]hat in ad[illegible] jacent ones. ROR1-AS1 ex[illegible]ssion [illegible]s negatively associated with pa[illegible]nts' ove[illegible] survival time. Cell growth abil[illegible]s inhi[illegible] due to knockdown of ROR1-AS1 [illegible] migration and invas[illegible] abilit[illegible]e repressed after ROR1-AS1 w[illegible] knockdow[illegible]thermore, due to the knoc[illegible] of ROR1-A[illegible] targeted proteins in [illegible]enin signaling pathway were suppres[illegible]ed.

CONCLUSIONS: These [illegible]lts suggested that ROR1-A[illegible] could enhance [illegible] metastasis and prolife[illegible]on via inducing Wnt/[illegible]-catenin signaling p[illegible]way, which might offer a potential therape[illegible]rget i[illegible] CRC.

Key Wor[illegible]
[illegible]ng no[illegible], ROR1-AS1, Colorectal can- c[illegible]t/β-cate[illegible]aling pathway.

Introduction

[illegible] incidence rate of colorectal cancer (CRC) rema[illegible]s high both in male and in female worldwide[1-3]. 1.36 million cases were diagnosed of CRC ann[illegible]y [illegible]lly and almo[illegible] 0.6 million cases died of CRC[illegible]hough the technological advan[illegible] have been [illegible] early detection and int[illegible]on for the past [illegible]ades, the prognosis [illegible] patients with CRC is still dismal[4]. Thus, it [illegible] crucial to un[illegible]er the molecular mechanism [illegible]erlying the p[illegible]ression of CRC and find out p[illegible]ial targets [illegible]mprove the poor prognosis of this [illegible]nor.

As o[illegible] type of noncoding RNA (ncRNA), [illegible] noncoding RNAs (lncRNAs) regulate a [illegible]f cellular processes and pathways in [illegible] [illegible]opment 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 expression[5]. The expression level of lncRNA-CCHE1 is positively related to the malignancy of colorectal carcinoma and it regulates ERK/COX-2 pathway[6]. Through regulating the stability of DNMT1 and depressing the expression of tumor suppressors, lncRNA LUCAT1 promotes esophageal squamous cell carcinoma formation and cell metastasis[7]. Activated by ZEB1, lncRNA HCCL5 accelerates cell viability, cell migration, epithelial-mesenchymal transition and the malignancy of hepatocellular carcinoma[8]. LncRNA SChLAP1 contributes to the development of aggressive prostate cancer by antagonizing the function of the SWI/SNF complex[9]. 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.

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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% CO_2 at 37°C.

Cell Transfection

Short hairpin RNA (shRNA) targeting R[illegible]1-AS1 (sh-ROR1-AS1), scrambled oligonucleo[illegible] (NC) were purchased from GenePh[illegible]rma (Shan[illegible]hai, China). The complement[illegible] encoding ROR1-AS1 was PCR-ampli[illegible]d, whi[illegible] was then inserted into pcDNA3.1 [illegible]vitrogen, [illegible]arlsbad, CA, USA). Those treated [illegible] were [illegible] following experimen[illegible]

RNA Extracti[illegible] Real-Time Quantitativ[illegible]ol[illegible]se Chain Reaction (RT-qPCR)

24 h [illegible]r transfection, [illegible] RNA was extracted [illegible]m CRC cells or t[illegible]or tissues from CRC [illegible]ients using TRIzol reagent (Invitrogen, Ca[illegible] CA[illegible]A). First-strand complementary deoxy[illegible]ucleic a[illegible] (cDNA) was synthe[illegible]ized usi[illegible] Tra[illegible]iptor first strand cDNA [illegible]sis ki[illegible] [illegible]a Biotechnology Co., Ltd., Da[illegible] China) [illegible]ording to the manufacturer's inst[illegible]tions. Following are the primers using fo[illegible] ROR1-AS1 primers forward 5′-[illegible]GACG[illegible]ACACTGGAACTC-3′, reverse [illegible]TCTGATTTGGTAGCTTGGATG-3′; glyceral[illegible]e 3-phosphate dehydrogenase (GAPDH) prim[illegible]s forward 5′-CCAAAATCAGATGGGG-CAATGCTGG-3′ and reverse 5′-TGATGGCAT-GGACTGTGGTCATTCA-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-[illegible])-2,5-Diphenyl Tetrazolium Brom[illegible] Assay

Before transfection, CRC ce[illegible]00 cells/well) were seeded into 96-w[illegible]ll plate[illegible]2 h. 15 μL MTT was added to e[illegible] well and [illegible]ted for 4 h when cultured at [illegible]ferent times (0, 2[illegible] and 72 h). To stop th[illegible]action, [illegible]0 μL dimethyl sulfoxide (DMSO) wa[illegible]d[illegible]sorbance at 490 nm was assessed [illegible]ing a[illegible]me-link[illegible] immunosorbent ass[illegible] (ELISA) [illegible] sy[illegible]m (Multiskan Asce[illegible]Systems, He[illegible] Finland).

Colony [illegible]ormati[illegible]ssay

Those transfected c[illegible] were placed in a 6-well pl[illegible] weeks. Cell co[illegible]es were treated with [illegible]hanol for 30 min. Then 0.5% crystal violet [illegible]s used for st[illegible]ing for 5 min. Colonies con[illegible]ing more tha[illegible]0 cells were counted and the m[illegible] colony nu[illegible]ers were calculated. Analysis was [illegible]cted [illegible]ith Image-Pro Plus 6.0 (Silver Springs, [illegible], USA).

[illegible] Healing Assay

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

Transwell Assay

2×10^4 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 of 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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detected using bicinchoninic acid (BCA) (Thermo Fisher Scientific, Waltham, MA, USA). The proteins were transferred on to a polyvinylidene fluoride (PVDF) membrane, blocked in Tris buffered saline and Tween 20 (TBST) (25 mM Tris, 140 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% skimmed milk and incubated for 2 h. The proteins were incubated with the primary antibody of target proteins including Wnt3a, β-catenin, C-myc and Survivin (Abcam Inc., Cambridge, MA, USA) in Wnt/β-catenin signaling pathway and GAPDH (Abcam Inc., Cambridge, MA, USA) and incubated at 4°C overnight. After being washed (3 × 10 min) with TBST, the secondary antibody was added and incubated at room temperature for 1 h. The results were analyzed by Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

GraphPad Prism 5.0 (La Jolla, CA, USA) was adopted to conduct the statistical analysis. Data were expressed as mean ± SD (standard deviation). Student *t*-test and Kaplan-Meier m[illegible]d were utilized. It was considered of stat[illegible] significance, when p-value < 0.05.

Results

ROR1-AS1 Expression L[illegible]el in CRC Tissues

RT-qPCR was conducte[illegible] dete[illegible] AS1 expression in [illegible] patie[illegible]ues. ROR1-AS1 was significa[illegible] upregulate[illegible] CRC tissue samples (Figure 1A). ROR1-AS1 expression level was higher in CRC cells than that in NCM460 (Figure 1B).

The Association between RO[illegible]-AS1 Expression Level and the P[illegible]nosis of CRC Patients

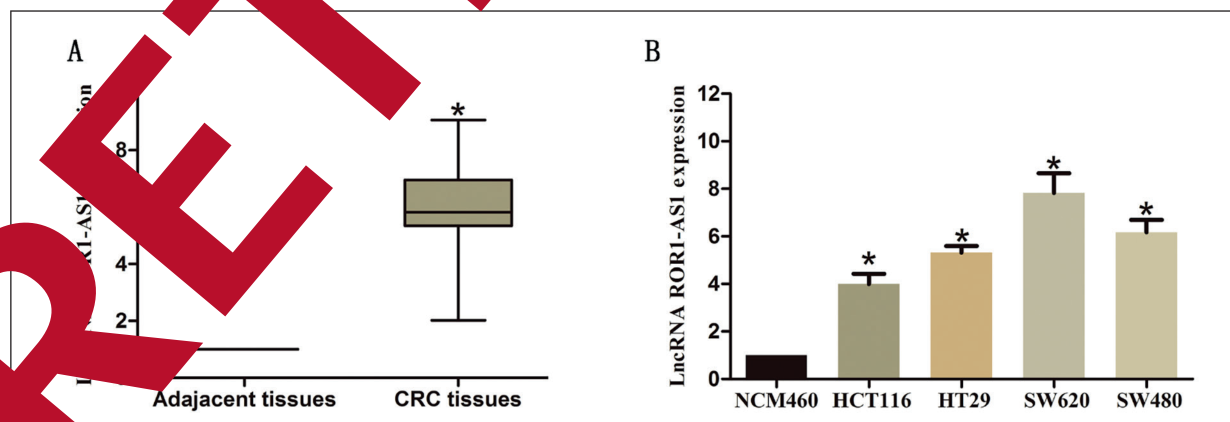
We divided 52 patients i[illegible]o two g[illegible] high ROR1-AS1 level group a[illegible] low ROR1-[illegible] level group, *via* median [illegible]ression. Kaplan-[illegible] analysis showed that [illegible]ents in [illegible]gh ROR1-AS1 level group had a po[illegible] surviv[illegible]l time compared with [illegible]ose in [illegible] ROR1-[illegible] level group (Figure [illegible]

Cell Pro[illegible] was Inhibited in CRC Cells via Knockd[illegible] of ROR1-AS1

As ROR1-AS1 expr[illegible] level was the highest in [illegible] among four C[illegible] cell lines, SW620 [illegible]s were used for the transfection of ROR1-[illegible]1 shRNA or s[illegible]mbled oligonucleotides (NC). [illegible]n, RT-qPCR [illegible]as utilized for detecting the R[illegible]AS1 expr[illegible]ion (Figure 3A). Moreover, the [illegible] of [illegible]T assay revealed that the cell growth [illegible]y of CRC cells was obviously re-[illegible]ressed *via* knockdown of ROR1-AS1 (Figure [illegible]outcome of colony formation assay also [illegible]vea[illegible] 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



Fig[illegible]1. Expression levels of ROR1-AS1 were increased in CRC patients. ***A,*** ROR1-AS1 expression was significantly increased in the CRC tissues compared with adjacent tissues. ***B,*** Expression levels of ROR1-AS1 were determined in the human CRC cell lines and normal human colonic epithelial cells (NCM460) by RT-qPCR. *$p<0.05$.

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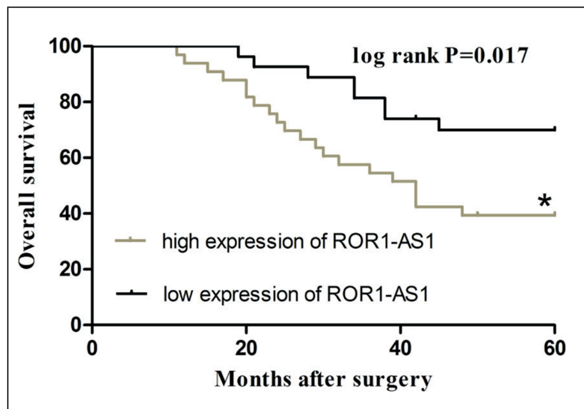
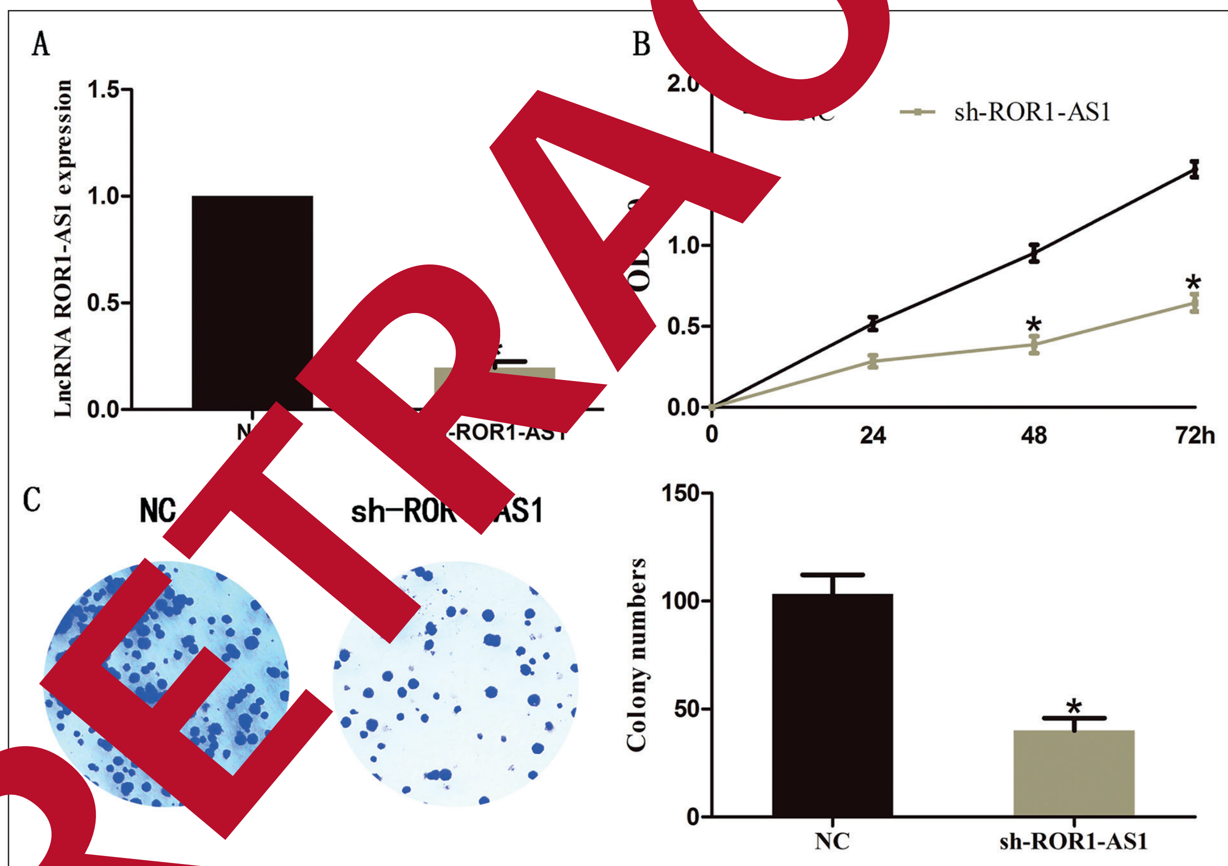


Figure 2. The association between ROR1-AS1 expression level and the prognosis of CRC patients. Expression levels of ROR1-AS1 were negatively associated with patients' overall survival time. $*p<0.05$.

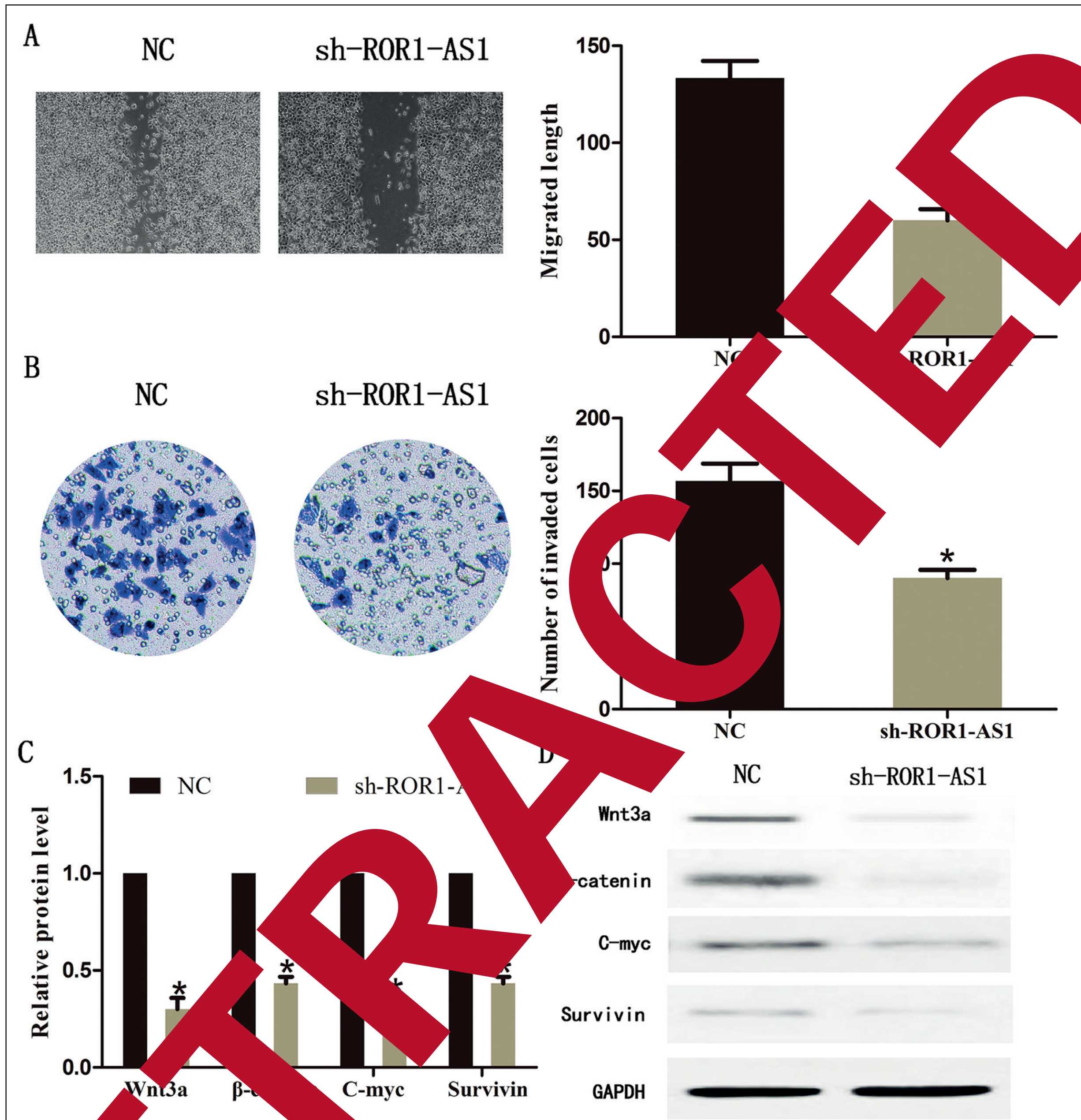
obviously repressed *via* knockdown of ROR1-AS1 (Figure 4A). The outcome of Transwell assay also revealed that the number of [illegible] cells was remarkably reduced *via* kn[illegible]down [illegible] ROR1-AS1 in CRC cells (Figure 4[illegible]).

The Interaction betwee[illegible] Wnt/[illegible]tenin Signaling Pathway a[illegible] ROR1-AS[illegible] CRC

To explore the un[illegible]lying mechanis[illegible] ROR1-AS1 function[illegible]n CRC, RT-qPCR and Western blot assay [illegible]c[illegible]cted to detect the target prote[illegible] in [illegible]atenin [illegible]naling pathway such [illegible] Wnt3a, β-[illegible]in, [illegible]myc and Survivin. R[illegible]CR results sh[illegible]hat Wnt3a, β-catenin[illegible]-m[illegible]d Survivin could be down-regulated *via* knoc[illegible]n of ROR1-AS1 (Figure 4C). Results of Weste[illegible]t assay showed that W[illegible]catenin, C-myc[illegible]d Survivin could be



[illegible]ure 3. [illegible]1-AS1 promoted CRC cell proliferation. ***A,*** ROR1-AS1 expression in CRC cells transfected with ROR1-AS1 [illegible]NA (sh-ROR1-AS1) or scrambled oligonucleotides (NC) was detected by RT-qPCR. GAPDH was used as an internal c[illegible] ***B,*** MTT assay revealed that the cell growth ability of CRC cells was obviously repressed in sh-ROR1-AS1 group co[illegible] with NC group. ***C,*** Colony formation assay also revealed that the number of CRC cell colonies was remarkably reduce[illegible] in sh-ROR1-AS1 group compared with NC group. The results represent the average of three independent experiments (mean ± standard error of the mean). $*p<0.05$.



Figur[illegible] ROR1-AS1 promoted CRC metastasis and activated Wnt/β-catenin signaling pathway. ***A,*** The migrated ability of CRC [illegible]s was sig[illegible]cantly decreased in sh-ROR1-AS1 group compared with NC group (magnification: 40×). ***B,*** Transwell assa[illegible]d th[illegible]umber of invaded CRC cells was significantly decreased in sh-ROR1-AS1 group compared with NC group ([illegible]ion: 40×)[illegible]RT-qPCR results revealed that the expression of target proteins in Wnt/β-catenin signaling pathway w[illegible]regul[illegible]in sh-ROR1-AS1 group compared with NC group. ***D,*** Western blot assay results revealed that [illegible]ressio[illegible]teins in Wnt/β-catenin signaling pathway was downregulated in sh-ROR1-AS1 group compared wi[illegible] group. [illegible]lts represent the average of three independent experiments (mean ± standard error of the mean). *$p<$[illegible].

[illegible]wnregulated *via* knockdown of ROR1-AS1 [illegible]ure 4D). These results suggested that ROR1-A[illegible]ticipated 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,

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evidence revealed that several lncRNAs participate in the development of CRC. For instance, lncRNA TP73AS1 promotes cell apoptosis of CRC by sponging miR103[10]. LncRNA RUNX1-IT1 acts as a tumor suppressor in CRC by inhibition of cell migration and cell proliferation, which suggests RUNX1-IT1 could function as a novel diagnostic biomarker[11]. In addition, lncRNA H19 promotes 5-Fu resistance in CRC *via* sponging to miR-194-5p[12].

Located in 1p31.3, ROR1-AS1 is a newly discovered lncRNA which is firstly discovered in mantle cell lymphoma[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. F[illegible] stance, lncRNA CCAL promotes hepat[illegible] carcinoma progression by regulating Wn[illegible]-catenin pathway[14]. MicroRNA-495 inhibits th[illegible]gression of non-small-cell lung cancer by ta[illegible]ing TCF4 and inactivating Wnt/β-catenin s[illegible]naling pathway[15]. Wnt/β-Cate[illegible] signaling activated by c-Myb prom[illegible] proli[illegible]ion and metastasis of breast can[illegible] Wnt10a [illegible]ts as an oncogene in CRC throug[illegible]ati[illegible]enin signaling[17]. As W[illegible]3a, β-[illegible], C-myc and Survivin were the [illegible]get proteins [illegible]t/β-catenin signaling pat[illegible]e detected t[illegible]ression of those protei[illegible]n C[illegible]lls after knockdown of ROR1-AS1. Results sho[illegible]at target proteins in Wnt/β-ca[illegible]n signaling pa[illegible] could be downregulat[illegible] *via* knockdown of [illegible]R1-AS1. All the resu[illegible]above s[illegible]ggested that ROR1-AS1 might pro[illegible]tum[illegible]genesis of CRC *via* activating Wnt/[illegible] signalin[illegible]athway.

[illegible]onclusions

[illegible] identified that ROR1-AS1 could [illegible]ance CRC cell proliferation and invasion [illegible]gh activating Wnt/β-catenin signaling pathw[illegible]ese findings indicate that ROR1-AS1 may cont[illegible]bute to therapy for CRC as a candidate target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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