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Long non-coding RNA ROR1-AS1 enhances colorectal cancer metastasis by targeting miR-375

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Abstract. – OBJECTIVE: Recent research has proved that long non-coding RNAs (lncRNAs) play an important role in tumorigenesis. In this research, lncRNA ROR1-AS1 was explored to identify its role in the development of colorectal cancer (CRC).

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to measure ROR1-AS1 expression of CRC tissues. Besides, function assays including wound healing assay and transwell assay were conducted to detect the effect of ROR1-AS1 on the metastasis of CRC. Furthermore, Luciferase assays and RNA immunoprecipitation assay (RIP) were used to explore the underlying mechanism.

RESULTS: By comparison with ROR1-AS1 expression in adjacent tissues, the ROR1-AS1 expression level was significantly higher in CRC samples. Moreover, loss of ROR1-AS1 inhibited cell migration and cell invasion of CRC cells. Besides, gain of ROR1-AS1 enhanced migration and cell invasion of CRC cells. Furthermore, it was found that ROR1-AS1 acted as a competing endogenous RNA *via* sponging miR-375 in CRC.

CONCLUSIONS: The present study suggests that ROR1-AS1 could promote cell migration and invasion of CRC by sponging miR-375, which may offer a potential therapeutic target in CRC.

Key words:
Long non-coding RNA, ROR1-AS1, Colorectal cancer, MiR-375.

Introduction

Colorectal cancer (CRC) is the third most prevalent malignant tumor and the fourth leading cause of cancer-related deaths in the world[1]. The incidence rate of colorectal cancer remains high both in male and in female worldwide[2,3]. However, most patients develop resistance to chemotherapy or relapse after surgery. The prognosis of patients with CRC is still dismal with poor 5-year survival rate. Therefore, it's urgent to investigate the underlying molecular mechanisms of tumorigenesis and progression in CRC.

Genome sequencing technology has revealed that most of genome sequence transcripts are non-coding RNAs (ncRNAs). Long non-coding RNAs (lncRNAs) are a subtype of ncRNAs longer than 200 nucleotides. Research has indicated that lncRNAs are closely involved in a variety of cellular activities. LncRNA SNHG7 promotes cell proliferation in osteosarcoma by targeting miR-34a[5]. Downregulation of lncRNA linc-ITGB1 inhibits cell invasion, cell migration and epithelial-mesenchymal transition in non-small cell lung cancer by decreasing Snail expression[6]. LncRNA FENDRR suppresses cell proliferation and malignancy of non-small cell lung cancer by sponging miR-761[7]. By regulating the stability of DNMT1 and depressing the expression of tumor suppressors, lncRNA LUCAT1 promotes esophageal squamous cell carcinoma formation and cell metastasis[8]. However, the role of lncRNA ROR1-AS1 in CRC and its underlying molecular mechanism have not been studied so far.

In this work, we found out that the expression of ROR1-AS1 was remarkably higher in CRC tissues. Moreover, the migration and invasion of CRC cells were changed *via* gain or loss of ROR1-AS1 *in vitro*. We further explored the underlying mechanism of how ROR1-AS1 functioned in CRC development.

Patients and Methods

Cell Lines and Clinical Samples

52 CRC patients who received surgery at the Tianjin Union Medical Center were enrolled for

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human tissues. All fresh tissues were stored at -80°C. No radiotherapy or chemotherapy was performed before the surgery. Signed informed consents were obtained from all participants before the study. This study was approved by the Ethics Committee of the Tianjin Union Medical Center.

Cell Culture

Human CRC cell lines (HCT116, HT29, SW620 and SW480) and normal human colonic epithelial cell line (NCM460), provided by the Chinese Academy of Science (Shanghai, China), were cultured in 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), Roswell Park Memorial Institute-1640 (RPMI-1640, HyClone, South Logan, UT, USA) and penicillin. Besides, the cells were cultured in an incubator containing 5% CO_2 at 37°C.

Cell Transfection

Lentivirus expressing short-hairpin RNA (shRNA) directed against ROR1-AS1 was provided by GenePharma (Shanghai, China) and then cloned into the pGPH1/Neo vector. A viral virus targeting ROR1-AS1 was synthesized and cloned into the pLenti-EF1a-EGFP-F2A- vector (Biosettia Inc., San Diego, CA, USA). ROR1-AS1 shRNA (sh-ROR1- negative control was used for transfection of HCT116 and ROR1-AS1 lentivirus or scramble vector was used for transfection of SW480.

Wound Healing Assay

After transfection, cells were cultured in RPMI-1640 medium overnight. Then, the cells were scratched with a plastic and cultured in serum-free RPMI-1640. Wound closure was viewed at 24 h.

Transwell Assay

After transfection, 1×10^4 cells in 200 µL of serum-free RPMI were added to top chamber of a 8 µm pore size insert (Corning, Lowell, MA, USA) with or without 50 µg Matrigel (BD Biosciences, San Jose, CA, USA). RPMI-1640 and FBS were added to the lower chamber. 48 h later, the top surface of the chambers was treated by methanol for 30 min after being wiped by a cotton swab. They were then stained in crystal violet for 20 min. Three fields were used to count the data for invasion membrane.

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was used for separating RNA. By the Transcription Kit, the RNA was reverse-transcribed to complementary deoxyribose nucleic acids (cDNAs). The $2^{-\Delta\Delta Ct}$ method was utilized for calculating the relative expression. Following are the primers used for RT-PCR. ROR1-AS1 primers forward 5′-CAGACGA CACTGGAACTC-3′ and reverse 5′-TGATTTG-GTAGCTTGGATC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward 5′-CCAAAAT GATGGC CTGG-3′ and reverse ATGGCAT TGTGGT-CATTCA.

Luciferase Assays

ROR1-AS1 3′-UTR was cloned into the pGL3 vector (Promega, Madison, WI, USA) as wild-type (WT) 3′-UTR. Then, site-directed mutagenesis of the miR-375 binding site in ROR1-AS1 3′-UTR was performed by quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as mutant (MUT) 3′-UTR. Following was transfection of WT-3′-UTR or MUT-3′-UTR and miR-ctrl or miR-375 for 48 h. The Luciferase assay was conducted on the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

RNA Immunoprecipitation (RIP) Assay

To confirm the endogenous relationship between ROR1-AS1 and miR-375, Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was conducted. Briefly, treated CRC cells were collected and lysed in RIP lysis buffer containing protease inhibitor and RNase inhibitor, and were incubated for 2 h at 4°C with the RIP buffer containing magnetic beads coated with Ago2 antibodies (Millipore, Billerica, MA, USA). IgG acted as a negative control (input group). Then, coprecipitated RNAs were gathered and monitored by RT-qPCR analysis.

Statistical Analysis

All analyses were performed with Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA). Student's *t*-test was performed to the data. The statistical significance was defined as $p<0.05$.

Results

ROR1-AS1 Expression Level in CRC Tissues and Cells

First, ROR1-AS1 expression was detected *via* RT-qPCR in 52 patients' tissues and 4 CRC cell lines. As a result, ROR1-AS1 was significantly upregulated in CRC tissue samples (Figure 1A). The ROR1-AS1 expression level of CRC cells was higher than that of NCM460 (Figure 1B).

Loss of ROR1-AS1 Inhibited Cell Migration and Invasion of CRC Cells

In this study, we chose the HCT116 CRC cell line for the knockdown of ROR1-AS1. Then, the transfection efficiency of ROR1-AS1 was detected by RT-qPCR (Figure 2A). Moreover, wound healing assay results showed that knockdown of ROR1-AS1 repressed the ability of migration in CRC cells (Figure 2B). The outcome of the transwell assay also revealed that migrated and invaded cells were markedly decreased after ROR1-AS1 was knocked down in CRC cells (Figure 2C and 2D).

Gain of ROR1-AS1 Enhanced Cell Migration and Invasion of CRC Cells

In this study, we chose the SW620 CRC cell line for the overexpression of ROR1-AS1. ROR1-AS1 expression was detected by RT-qPCR (Figure 3A). Wound healing assay results showed that overexpression of ROR1-AS1 enhanced the ability of migration in CRC cells (Figure 3B). The outcome of the transwell assay also revealed that migrated and invaded cells were remarkably reduced after ROR1-AS1 was overexpressed in CRC cells (Figure 3C and 3D).

MiR-375 Was a Direct Target of ROR1-AS1 in CRC

The RT-qPCR results showed that the expression level of miR-375 in CRC cells was significantly lower in ROR1-AS1 (lncRNA ROR1-AS1) group when compared with the miR-375 level in the negative control group (Figure 4A). The expression level of miR-375 in CRC cells was markedly lower in the ROR1-AS1 lentivirus group when compared with the miR-375 level in the scramble control group (Figure 4A). The results of the Western blot assay revealed that after ROR1-AS1 was knocked down, miR-375 could be downregulated at the protein level (Figure 4B). Furthermore, the Luciferase assay revealed that co-transfection of ROR1-AS1-WT and miR-375 markedly decreased the Luciferase activity, while co-transfection of ROR1-AS1-MUT and miR-375 had no effect on the Luciferase activity either (Figure 4C). Meanwhile, RIP assay identified that ROR1-AS1 and miR-375 were remarkably enriched in Ago2-containing beads compared to control group (Figure 4D).

Discussion

Compelling evidence has suggested that lncRNAs play a crucial role in the carcinogenesis of CRC through the regulation of

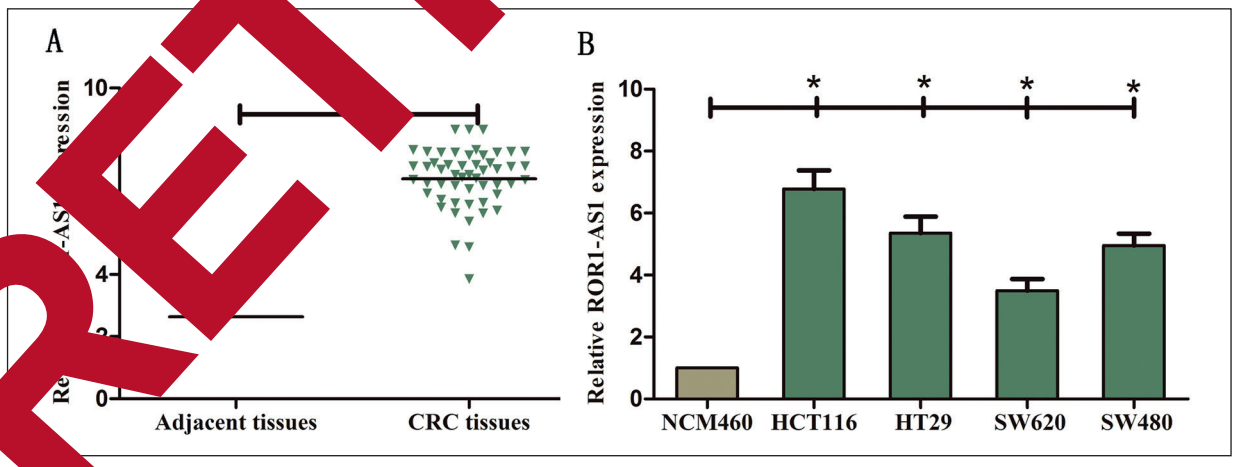

Figure 1. ROR1-AS1 expression in CRC tissues and cells. ***A,*** ROR1-AS1 expression was significantly increased in the CRC tissues compared with the adjacent tissues. ***B,*** The expression levels of ROR1-AS1 were determined in the human CRC cell lines and normal human colonic epithelial cells (NCM460) by RT-qPCR. GAPDH was used as an internal control. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

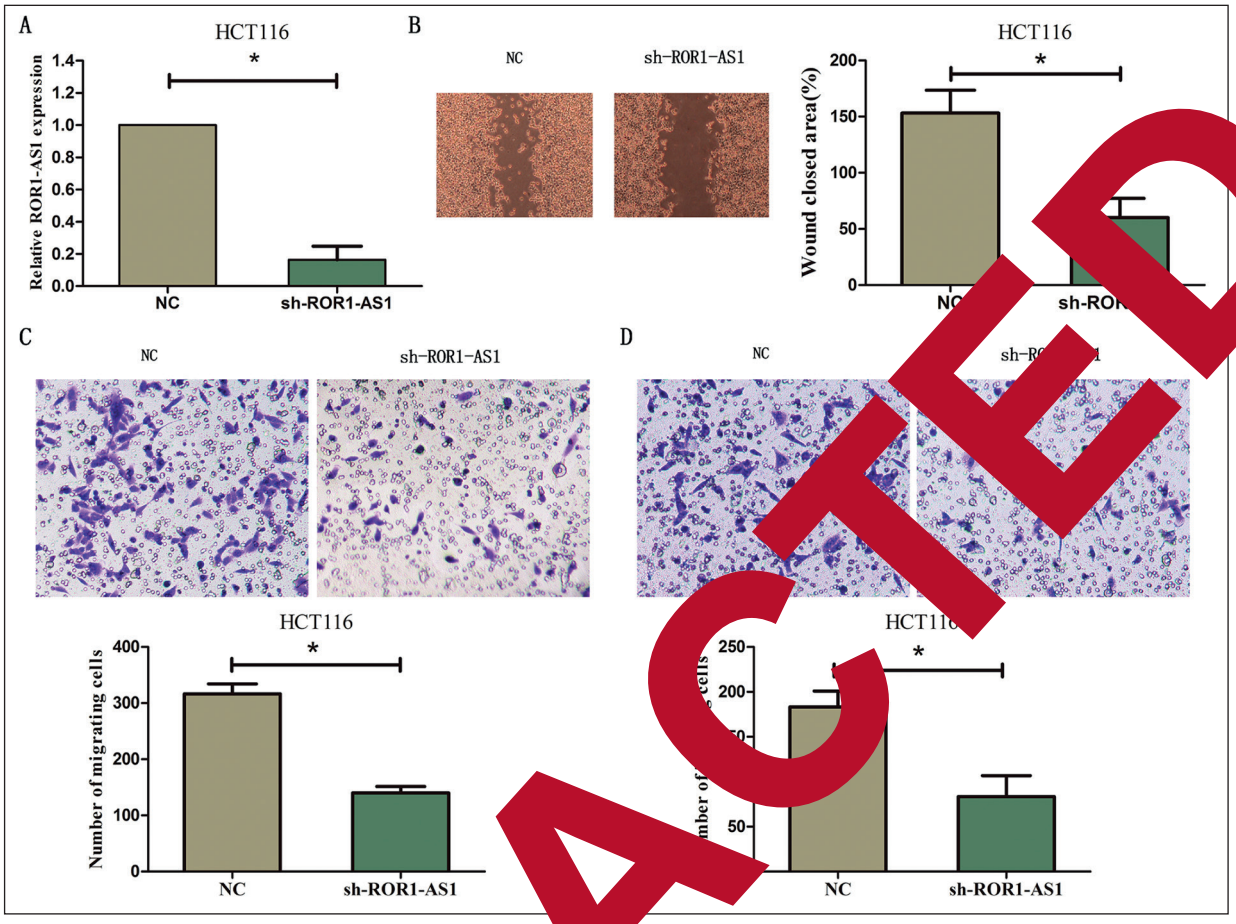

Figure 2. Loss of ROR1-AS1 repres cell migra nd invasion. ***A,*** ROR1-AS1 expression in HCT116 CRC cells transduced with ROR1-AS1 shRNA 1) and ne control (NC) was detected by RT-qPCR. GAPDH was used as an internal control. ***B,*** Wound ng assa owed that kdown of ROR1-AS1 significantly reduced cell migration in CRC cells (magnification: 40× he trans ay showed that the number of migrated cells was markedly decreased *via* knockdown of ROR1-AS1 in CR (m . ***D,*** The transwell assay showed that the number of invaded cells was remarkably decrease kno ROR1-AS1 in CRC cells (magnification: 40×). The results represent the average of three independent ex ents (mc ndard error of the mean). *p<0.05.

various c iological be rs. For instance, lncRN P73AS1 promote ll apoptosis of CRC spongi miR103 and further modulat exp ion of PTEN[9]. LncRNA H19 promo resis e in CRC *via* spong- to 194-5 LncRNA RUNX1-IT1 ts ce ion and cell proliferation in C[11].

AS1 located in 1p31.3, is a newly cRNA. It is first discovered in antle cell lymphoma[12]. However, the role of 1-AS1 in malignant cancers including CRC re s unknown. In this work, we first found that ROR1-AS1 was upregulated both in CRC samples and CRC cells. Besides, loss of ROR1-AS1 repressed cell migration and invasion in CRC cells, while gain of ROR1-AS1 promoted cell migration and invasion in CRC cells. The above results indicated that ROR1-AS1 promoted tumorigenesis of CRC and might act as an oncogene.

To further identify the underlying mechanism of how ROR1-AS1 affects CRC cell metastasis, we predicted and picked miR-375 as the potential binding microRNAs of ROR1-AS1 by using bioinformatic analysis and experimental verification. As is known to all, miR-375 is a tumor suppressor in many carcinomas which regulates diverse biological processes. For instance, miR-375 is associated with prognosis of esophagus cancer patients in Chinese people[13]. Moreover, miR-375 could be a prognosis marker of liver

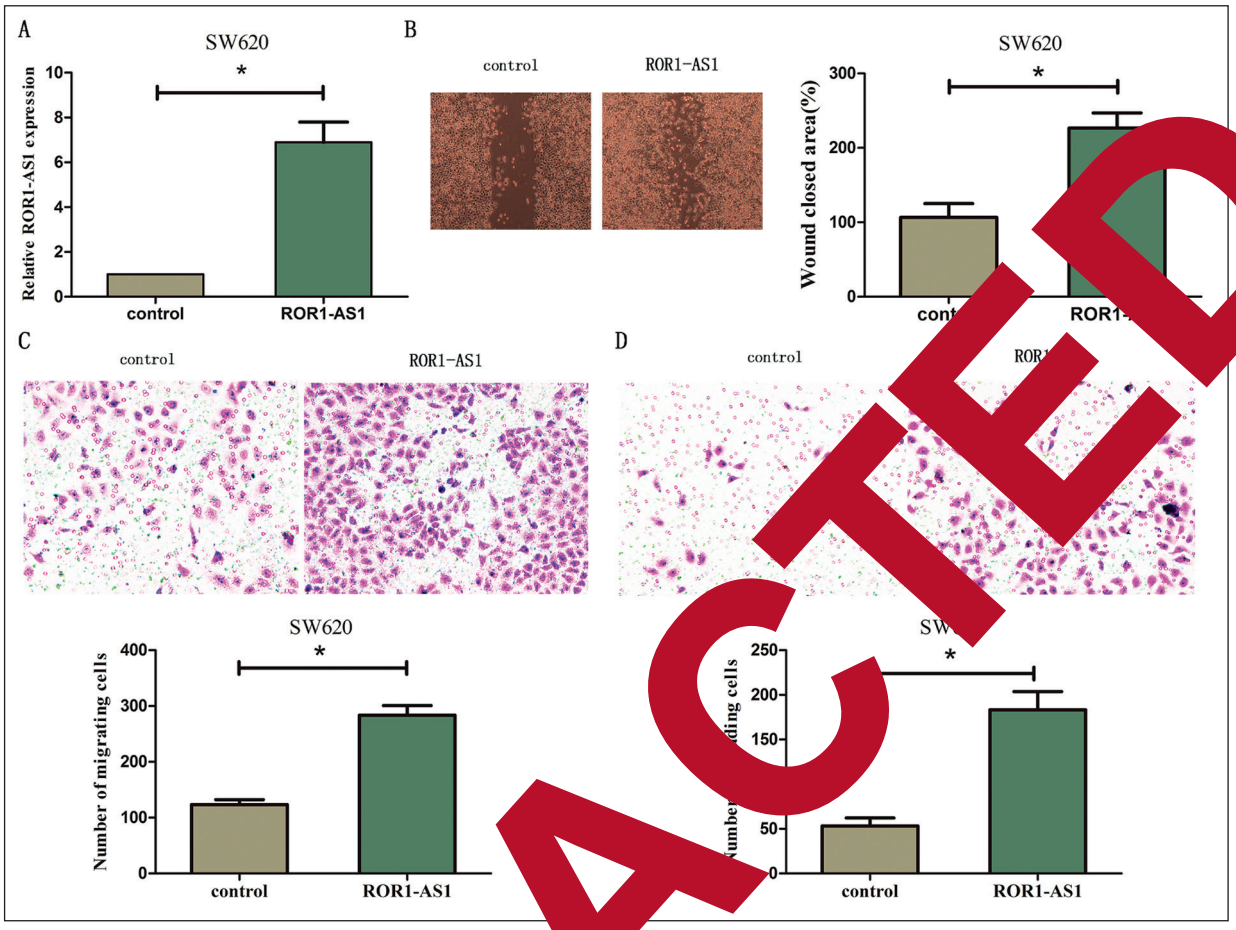

Figure 3. Gain of ROR1-AS1 enha[illegible]ell migra[illegible]nd invasion. ***A,*** ROR1-AS1 expression in SW620 CRC cells transduced with ROR1-AS1 lentivi[illegible]ROR[illegible]) and the [illegible]ble vector (control) was detected by RT-qPCR. GAPDH was used as an internal control. ***B,*** [illegible]d healin[illegible]say showe[illegible]at overexpression of ROR1-AS1 significantly enhanced cell migration in CRC cells (magn[illegible]n: 40×). [illegible]nswell assay showed that the number of migrated cells was markedly increased *via* overexpressio[illegible]of [illegible]AS[illegible]agnification: 40×). ***D,*** The transwell assay showed that the number of invaded cells was rem[illegible]ly in[illegible]*ia* overexpression of ROR1-AS1 in CRC cells (magnification: 40×). The results represent the average [illegible]e indepen[illegible]periments (mean ± standard error of the mean). *$p<0.05$.

cancer p[illegible]ts[14]. MiR-3[illegible]hances resistance to doc[illegible]l by regulating [illegible]23A in prostate canc[illegible] MiR-[illegible] enhances cell invasion *via* tar[illegible] SI[illegible]11 in oral squamous cell carcinoi[illegible]ently, [illegible]375 inhibits the development [illegible]lorec[illegible]carcinoma *via* targeting [illegible][17].

[illegible]he pres[illegible]study, we first discovered the int[illegible] between miR-375 and ROR1-AS1. [illegible]owed that the expression level of [illegible]R-375 could be upregulated by knockdown of [illegible]1-AS1, and the expression level of miR-375 c[illegible]be downregulated by overexpression of ROR1-AS1. Furthermore, miR-375 could directly bind to ROR1-AS1 through a Luciferase assay. MiR-375 was significantly enriched by ROR1-AS1 RIP assay. All the results above suggested that ROR1-AS1 functioned as a competing endogenous RNA for miR-375 in CRC.

Conclusions

We identified that ROR1-AS1 could enhance prostate cancer cell migration and invasion by sponging miR-375. These findings suggest that ROR1-AS1 may contribute to therapy for CRC as a candidate target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

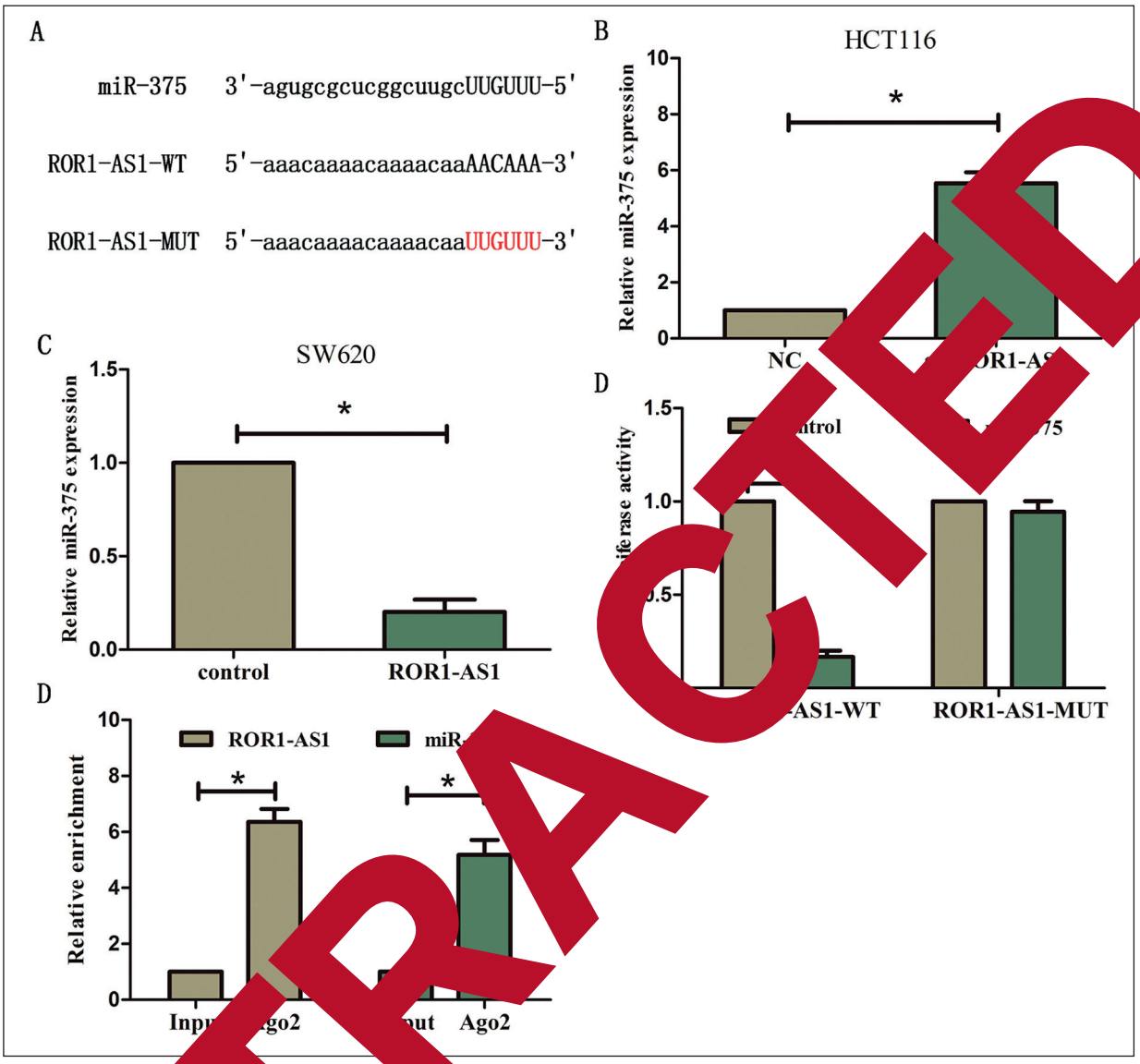

Figure 4. Inter[illegible] between ROR1-AS1 and miR-375. ***A,*** The binding sites of miR-375 on ROR1-AS1. ***B,*** RT-qPCR results showed that miR-375 expression was increased in ROR1-AS1 shRNA (sh-ROR1-AS1) compared with negative control (NC). ***B,*** RT-qPCR results showed that miR-375 expression was decreased in ROR1-AS1 lentivirus (ROR1-AS1) compared with scramble vector (control). ***C,*** Co-transfection of miR-375 and ROR1-AS1-WT strongly decreased the Luciferase activity, while co-transfection of miR-375 control and ROR1-AS1-WT did not change the Luciferase activity. ***D,*** RIP assay identified that ROR1-AS1 and miR-375 were significantly enriched in Ago2-containing beads compared to input group. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

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