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ORIGINAL ARTICLE

Long noncoding RNA ROR1-AS1 enhances lung adenocarcinoma metastasis and induces epithelial-mesenchymal transition by sponging miR-375

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Summary

Purpose: *The vital role of long noncoding RNAs (lncRNAs) in tumor progression have been identified in numerous studies. In this research, lncRNA ROR1-AS1 was explored to verify its function during the development of lung adenocarcinoma (LAC).*

Methods: *Real-time quantitative polymerase chain reaction (RT-qPCR) was utilized to measure ROR1-AS1 expression of LAC tissues. Function assays including wound healing assay and transwell assay were conducted to detect the effect of knockdown of ROR1-AS1 on the metastasis of LAC, and luciferase assays and RNA immunoprecipitation assay (RIP) were also performed to explore the underlying mechanism.*

Results: *ROR1-AS1 expression level was significantly higher in LAC samples compared with that in adjacent tissues, which was associated with patients' prognosis. Knockdown of ROR1-AS1 inhibited cell migration and cell invasion of LAC cells via suppressing epithelial-mesenchymal transition (EMT) process. Furthermore, it was discovered that ROR1-AS1 acted as a competing endogenous RNA via sponging miR-375 in LAC.*

Conclusions: *These results suggested that ROR1-AS1 could act as a sponge for miR-375 and promo//e cell migration and invasion through suppressing the process of EMT in LAC, which may offer a potential therapeutic target in LAC.*

Key words: *long noncoding RNA, ROR1-AS1, lung adenocarcinoma, miR-375*

Introduction

Lung cancer is the leading cause of cancer-related deaths in the world. Approximately 224,390 patients were estimated to be newly diagnosed with lung cancer in America in 2016 [1]. Non-small-cell lung cancer (NSCLC) is the predominant subtype of lung cancer which accounts for approximately 85% of all cases, while lung adenocarcinoma (LAC) accounts for more than half of NSCLC cases and leads to more than 500,000 deaths each year [2,3]. The lack of effective systemic treatments contributes to the poor prognosis of LAC patients with 5-year survival rate of only 18%. These facts highlight the need for better understanding the molecular mechanism of metastasis underlying lung cancer and developing novel therapeutic managements.

Noncoding RNA (ncRNA) accounts for more than 98% of all the sequences. As one subtype of ncRNA, long noncoding RNAs (lncRNAs) regulate a variety of cellular processes and pathways in the development of cancers. For instance, lncRNA PVT1 is significantly upregulated in gastric cancer and contributes to poor prognosis in patients with gastric cancer [4]. LncRNA 91H exerts oncogenic properties by upregulating expression of H19/IGF2, which increases the aggressive phenotype of breast cancer cells [5]. LncRNA NR_036575.1 acts as an

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oncogene in papillary thyroid cancer by contributing to enhancing cell proliferation and cell migration, which suggests lncRNA NR_036575.1 could be applied as a potential biomarker and therapeutic target [6]. LncRNA linc00261 functions as a tumor suppressor in gastric cancer through depressing the stability of Slug proteins and inhibiting epithelial-mesenchymal transition (EMT) [7]. However, the role of lncRNA ROR1-AS1 in metastasis of LAC and its underlying molecular mechanism have not been studied so far.

In this study, we found out that the expression of ROR1-AS1 was remarkably higher in LAC tissues and was associated with LAC patients' survival. Moreover, the migration and invasion of LAC cells were changed *via* knockdown of ROR1-AS1 *in vitro*. The EMT-related proteins were also significantly changed by knocking down ROR1-AS1. Then we further explored the underlying mechanism on how ROR1-AS1 functioned in LAC development.

Methods

Cell lines and clinical samples

Fifty LAC patients operated at our hospital were enrolled. All fresh tissues were stored at –80°C. No radiotherapy or chemotherapy was performed before surgery. The Ethics Committee of our hospital approved this study protocol, and all participants in this study provided written informed consents.

Cell culture

Human LAC cell lines (SPCA1, H1299, A549, and H358) and normal human bronchial epithelial cell line (16HBE), provided by Chinese Academy of Science (Shanghai, China), were cultured with 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA), Roswell Park Memorial Institute 1640 medium (RPIM-1640) (HyClone, South Logan, UT, USA) and penicillin 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, which was then used for transfection of A549 LAC cells.

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cultured lung adenocarcinoma cells or patients' tumor tissues by using TRIzol reagent (TaKaRa Bio, Inc., Shiga, Japan) and then reverse-transcribed to complementary DNA (cDNA) through reverse Transcription Kit (TaKaRa, Shiga, Japan). $2^{-\Delta\Delta Ct}$ method was utilized for calculating relative expressions. Following are the primers used for RT-qPCR: ROR1-AS1 primers forward 5'-CTGACGAAACACTGGAACTC-3', reverse 5'-GTCTGATTTGGTAGCTTGGATG-3'; Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) primers forward 5'-CCAAAATCAGATGGGGCAATGCTGG-3'and reverse 5'-TGATGGCATGGACTGTGGTCATTCA-3'.

Wound healing assay

After transfection, cells were cultured in RPIM-1640 medium overnight. When growing to about 80% confluence, cells were scratched by a sterile 10 μL pipette tip and incubated in a humidified incubator with 5% CO_2 atmosphere at 37°C. Then, the open wound area was measured at 24 h. The experiments were repeated three times.

Transwell assay

After transfection, 5 ×10^4 cells in 200 μL serum-free RPIM-1640 were added to top chamber of an 8μm pore size insert (Corning, Corning, NY, USA) with or without 50 μg Matrigel (BD, Bedford, MA, USA) and RPIM-1640 and FBS were added to the lower chamber. Forty-eight h later, the top surface of chambers was treated by methanol for 30 min after wiped by cotton swab. Then, they were stained with crystal violet for 20 min. Three fields were used to count the data for membrane invasion.

Western blot analysis

Reagent radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) was utilized to extract protein from cells. Bicinchoninic acid (BCA) protein assay kit (TaKaRa, Shiga, Japan) was chosen for quantifying protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, they were incubated with antibodies after being replaced to the polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). Cell Signaling Technology (CST, Danvers, MA, USA) provided us rabbit anti-GAPDH and rabbit anti-N-cadherin, anti-Vimentin and anti-E-cadherin, as well as goat anti-rabbit secondary antibody. Image J software (NIH, Bethesda, MD, USA) was applied for assessment of protein expression.

Luciferase assays

ROR1-AS13′-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. This was followed by transfection of WT-3′-UTR or MUT-3′-UTR and miR-ctrl or miR-375 for 48 h. Then, the luciferase assay was performed 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 LAC 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.

Statistics

All analyses were carried out using the SPSS 19.0 (SPSS, Chicago, IL, USA). Kaplan-Meier analysis followed by Log-rank test were conducted for patient survival. The experiments were performed in triplicate and the results are shown as standard error of the mean (SEM). Student t-test was performed to analyze the data between 2 groups. The statistically significance was set at $p<0.05$.

Results

ROR1-AS1 expression level in LAC tissues and cells

Firstly, ROR1-AS1 expression was detected *via* RT-qPCR in 50 patients' tissues and 4 LAC cell lines. ROR1-AS1 was significantly upregulated in LAC tissue samples (Figure 1A). ROR1-AS1 expression level of LAC cells was higher than that of 16HBE (Figure 1B). Patients' survival after surgery was analyzed through Kaplan-Meier method. We divided 50 cervical cancer patients into two groups, high-ROR1-AS1 and low-ROR1-AS1, according to the median expression. Kaplan-Meier analysis followed by Log-rank test showed that LAC patients had a poorer overall survival with higher ROR1-AS1 level (Figure 1C).

Knockdown of ROR1-AS1 inhibited cell migration and invasion of LAC cells

In this study, we chose A549 LAC cell line for the knockdown of ROR1-AS1. Then, transfection efficiency of ROR1-AS1 was detected by RT-qPCR (Figure 2A). Wound healing assay showed that knockdown of ROR1-AS1 repressed the ability of migration in LAC cells (Figure 2B). The outcome of transwell assay also revealed that migrated and invaded cells were obviously decreased after ROR1-AS1 was knocked down in LAC cells (Figure 2C and 2D).

Knockdown of ROR1-AS1 inhibited the EMT process of LAC cells

RT-qPCR results showed that through knockdown of ROR1-AS1, the mRNA expression of N-cadherin and Vimentin was downregulated, and E-cadherin was upregulated in LAC cells (Figure 3A). Moreover, Western blot assay also showed the same effect on EMT-related proteins through knockdown of ROR1-AS1 (Figure 3B).

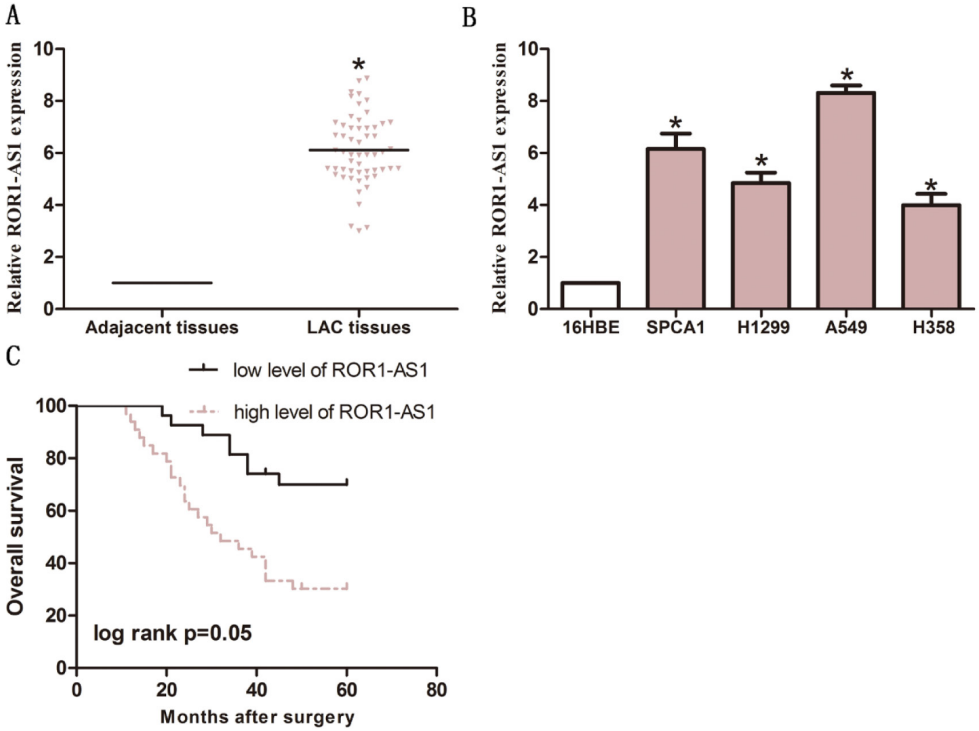

Figure 1. ROR1-AS1 expression in LAC tissues and cells. **(A):** ROR1-AS1 expression was significantly increased in the LAC tissues compared with adjacent tissues (*$p<0.05$). **(B):** The expression levels of ROR1-AS1 were determined in the human LAC cell lines and normal human bronchial epithelial cell (16HBE) by RT-qPCR. GAPDH was used as an internal control. Data are presented as the mean ± standard error of the mean (*$p<0.05$); **(C):** Kaplan-Meier analysis showed that LAC patients had a poorer overall survival with higher ROR1-AS1 level.

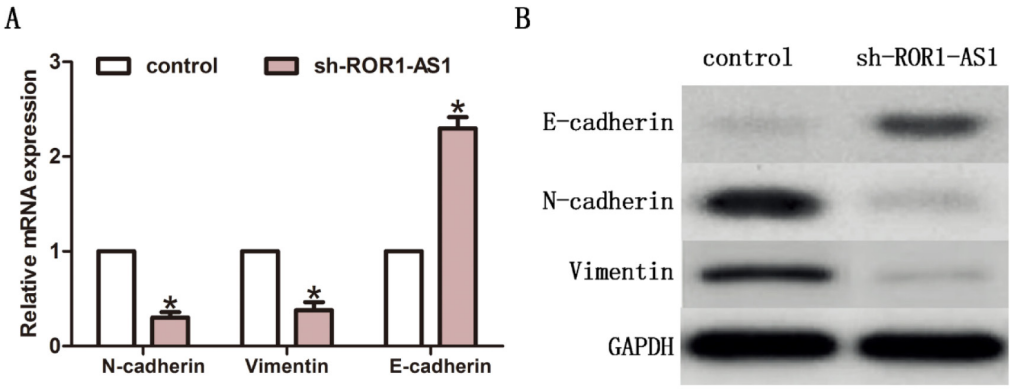

Figure 3. Knockdown of lncRNA ROR1-AS1 inhibited the process of EMT in LAC cells. **(A):** RT-qPCR results showed that N-cadherin and Vimentin were downregulated, and E-cadherin was upregulated after ROR1-AS1 was knocked down in LAC cells. **(B):** Western blot results showed that N-cadherin and Vimentin were downregulated, and E-cadherin was upregulated after ROR1-AS1 was knocked down in LAC cells. The results represent the average of three independent experiments. Data are presented as the mean ± standard error of the mean. *p<0.05.

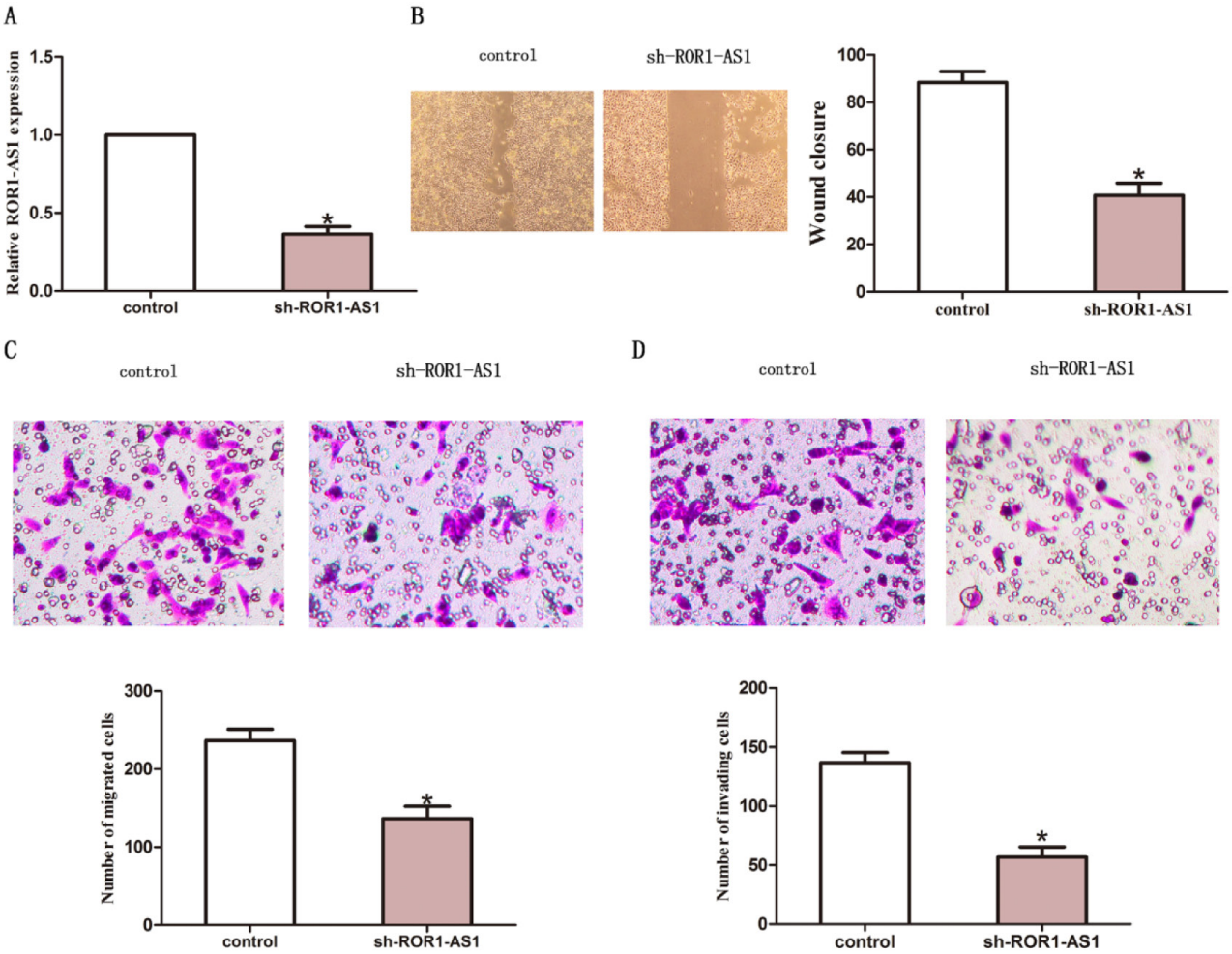

Figure 2. Loss of ROR1-AS1 repressed LAC cell migration and invasion. **(A):** ROR1-AS1 expression in A549 LAC cells transduced with ROR1-AS1 shRNA (sh-ROR1-AS1) and negative control was detected by RT-qPCR. GAPDH was used as an internal control. **(B):** Wound healing assay showed that knockdown of ROR1-AS1 significantly reduced cell migration in LAC cells (magnification: 40×). **(C):** Transwell assay showed that the number of migrated cells was significantly decreased *via* knockdown of ROR1-AS1 in LAC cells (magnification: 40×). **(D):** Transwell assay showed that the number of invaded cells was significantly decreased *via* knockdown of ROR1-AS1 in LAC cells (magnification: 40×). The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05.

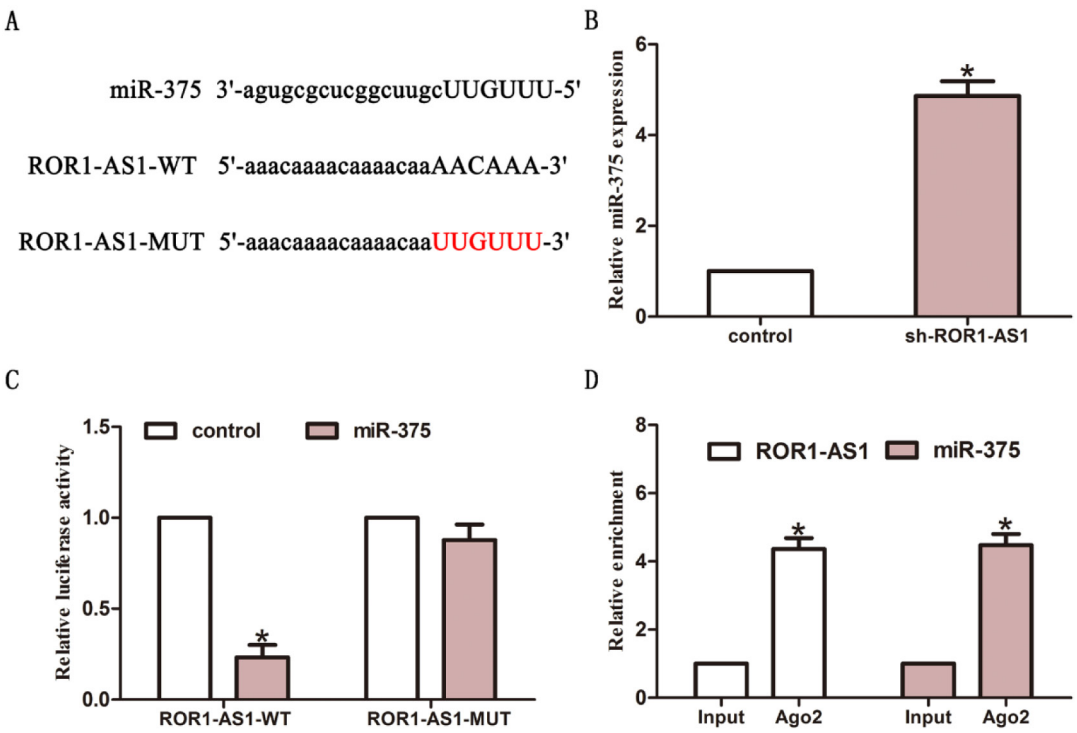

Figure 4. Interaction between ROR1-AS1 and miR-375. **(A):** The binding sites of miR-375 on ROR1-AS1. **(B):** RT-qPCR results showed that miR-375 expression was increased in ROR1-AS1 shRNA (sh-ROR1-AS1) compared with the negative control. **(C):** Co-transfection of miR-375 and ROR1-AS1-WT strongly decreased the luciferase activity, while co-transfection of miR-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 the 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.

MiR-375 was a direct target of ROR1-AS1 in LAC

Bioinformatics analysis showed that miR-375 might be a potential target of ROR1-AS1, which contained the binding area of miR-375 (Figure 4A). RT-qPCR results showed that the expression level of miR-375 in LAC cells was significantly higher in ROR1-AS1 shRNA (sh-ROR1-AS1) group when compared with the miR-375 level in the negative control group (Figure 4B). Furthermore, the luciferase assay revealed that co-transfection of ROR1-AS1-WT and miR-375 largely 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 significantly enriched in Ago2-containing beads compared to the input group (Figure 4D).

Discussion

Metastasis is the major cause of mortality in LAC. Evidence has demonstrated that lncRNAs participate in the metastasis of LAC. For instance, through targeting the expression of p21, lncRNA CRNDE/PRC2 functions as an oncogene in LAC and contributes to the radiotherapy resistance of LAC [8]. LncRNA CASC2 suppresses cell metastasis and EMT of LAC through inhibiting SOX4 [9]. Downregulation of lncRNA SFTA1P inhibits cell migration and cell invasion in LAC [10]. LncRNA SNHG3 functions as an oncogene in LAC and enhances the tumor proliferation, which may provide a potential new therapeutic and prognostic target for LAC [11].

Located in 1p31.3, ROR1-AS1 is a newly discovered lncRNA which is firstly discovered in mantle cell lymphoma [12]. However, the role of ROR1-AS1 in cancers including LAC remains unknown. In this study, we firstly found that ROR1-AS1 was upregulated in LAC samples and was associated with patients' prognosis. Besides, knockdown of ROR1-AS1 repressed cell migration and invasion in LAC cells.

EMT, a vital process in tumor metastasis, has been reported to be regulated by several lncRNAs in various cancers including LAC. It could be regulated by a variety of different cytokines and growth factors during tumor development and contributes to the loss of epithelial markers (such as E-cadherin) and the upregulation of mesenchymal markers (such as N-cadherin and vimentin), which can enhance the ability of cancer cells to migrate and invade [13,14]. Our study showed that E-cadherin could be upregulated and N-cadherin and Vimentin

could be downregulated after knockdown of ROR1-AS1, which suggest that the EMT process could be induced by ROR1-AS1. The above results indicated that ROR1-AS1 promoted tumorigenesis of LAC through inducing EMT and might act as an oncogene.

To further identify the underlying mechanism of how ROR1-AS1 affected LAC cell metastasis and induced EMT process, we predicted the potential binding microRNAs of ROR1-AS1 by using bioinformatics analysis and experimental verification. As it is known, miR-375 functions as a tumor suppressor in many cancers and regulates diverse biological processes including EMT process. For instance, miR-375 accelerates docetaxel-resistance through regulating SEC23A in prostate cancer [15]. Moreover, miR-375 inhibits the development of colorectal carcinoma *via* targeting KLF4 [16]. MiR-375 suppresses EMT process by repressing the RNA-binding protein Quaking [17]. LncRNA MLK7-AS1 promotes the EMT process of ovarian cancer by regulating miR-375 [18].

Previous studies have discovered that miR-375 could suppress the process of EMT and further affect the progression of tumors. In the present study, we firstly discovered the interaction among EMT process, miR-375 and ROR1-AS1. The results showed that the expression level of miR-375 could be upregulated by knocking down ROR1-AS1. Furthermore, miR-375 could directly bind to ROR1-AS1 through a luciferase assay, and miR-375 was significantly enriched by ROR1-AS1 RIP assay. Furthermore, EMT process could be suppressed *via* knocking down ROR1-AS1. All the results above suggest that ROR1-AS1 functioned as a competing endogenous RNA for miR-375 and further induced EMT process of LAC.

Conclusions

The data above identified that ROR1-AS1 acted as a sponge of miR-375 and could enhance lung adenocarcinoma cancer cell migration and invasion through inducing EMT process. These findings suggest that ROR1-AS1 may contribute to therapy for LAC as a candidate target.

Conflict of interests

The authors declare no conflict of interests.

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