ACTA ABP BIOCHIMICA POLONICA

Vol. 70, No 3/2023
713–719
https://doi.org/10.18388/abp.2020_6754

Regular paper

Silencing lncRNA EZR-AS1 induces apoptosis and attenuates the malignant properties of lung adenocarcinoma cells

Xianjing Yu[1], Lixue Wu[1], Zhongcui Lu[1], Junli Zhang[2] and Yunfeng Zhou[3]✉

[1]Department of Pathology, West China School of Public Health and West China Forth Hospital, Sichuan University, Chengdu, China; [2]Department of Pathology, The First Affiliated Hospital of Chengdu Medical College, Chengdu, China; [3]Department of Cardiothoracic Surgery, West China School of Public Health and West China Forth Hospital, Sichuan University, Chengdu, China

Adenocarcinoma is one of the major subtypes of lung cancer. This study aimed to investigate the effect of silencing long non-coding RNA (lncRNA) EZR-AS1 on the biological behaviors of lung adenocarcinoma (ADC) cells. EZR-AS1 expression levels in lung ADC tissues and cells, as well as in adjacent non-cancerous tissues, were determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR). EZR-AS1 was knocked down in two lung ADC cell lines using small interfering RNA specific for EZR-AS1 (siEZR-AS1). Proliferation, migration, and apoptosis of EZR-AS1-knockdown cells were assessed using the CCK-8 viability assay, flow cytometry, or wound healing experiments. The levels of proteins related to migration pathways were evaluated using western blotting analysis. EZR-AS1 contents were significantly higher in lung ADC tissues and cells than in the levels in the non-cancerous tissues and cells ($p<0.01$). Transfection of ADC cell lines H1437 and H1975 significantly downregulated EZR-AS1 levels in both cell lines. Cytotoxicity assays revealed that the viability of EZR-AS1-knockdown cells significantly decreased over culture time, and a significant level of apoptosis was induced ($p<0.01$). Wounding healing experiments revealed that EZR-AS1-knockdown significantly reduced the migration rate of both cell lines ($p<0.01$). Furthermore, proteins related to migration pathways such as vimentin, MMP2, and MMP9 were significantly downregulated, whereas the E-cadherin level was significantly increased after EZR-AS1 knockdown. Our work demonstrated that EZR-AS1 is associated with ADC progression, and silencing this gene inhibits proliferation and reduces migration of ADC cells *in vitro*. The altered expression of metastasis-related genes was likely responsible for the reduced migration ability after EZR-AS1 knockdown.

Keywords: lung cancer; long non-coding RNA; cell viability; migration; apoptosis

Received: 26 March, 2023; revised: 02 August, 2023; accepted: 22 August, 2023; available on-line: 18 September, 2023

✉e-mail: c11231354@163.com

Abbreviations: lncRNA, lncRNA long non-coding RNA; ADC, adenocarcinoma; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SCLC, small cell lung cancer; HOTAIR, HOX transcript antisense RNA; cSCC, cutaneous squamous cell carcinoma; ESCC, esophageal squamous cell carcinoma; FBS, fetal bovine serum; TBS, tris buffered saline; HRP, horseradish peroxidase; RIPA, radioimmunoprecipitation assay; SDS-PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; ANOVA, analysis of variance; HCC, hepatocellular carcinoma; MErT, mesenchymal to epithelial reverting transition; IF, intermediate filament; URI, unconventional prefoldin RPB5 interactor; CRC, colorectal cancer; MMP, matrix metalloproteinase; ECM, the extracellular matrix

INTRODUCTION

With the increasing number of cancer patients worldwide in recent years, the total prevalence and mortality rate of lung cancer have always been among the highest, particularly in China (Bade & Dela Cruz, 2020; Bai *et al.*, 2018; Cao & Chen, 2019). Annually, there are about 1.8 million new cases of lung cancer and nearly 1.6 million people die because of lung cancer. Studies show that the five-year survival rate of patients is 4–17% (Klimaszewska-Wisniewska *et al.*, 2018; Musika *et al.*, 2021). Histologically, lung cancer is divided into four types including small-cell lung cancer (SCLC), squamous cell carcinoma, adenocarcinoma (ADC), and large-cell carcinoma (Denisenko *et al.*, 2018). ADC accounts for the highest proportion of lung cancers and is responsible for up to 40% of all lung cancers in China (Wu *et al.*, 2021). However, the specific mechanisms of the occurrence, development, and metastasis of ADC are still largely unclear, and elucidation of these mechanisms will be of great significance in the management of the disease.

Long non-coding RNAs (lncRNAs) are endogenous RNAs that have more than 200 nucleotides in length but do not encode proteins (Saigoh *et al.*, 1998). They play various roles in numerous biological activities and processes, including epigenetic regulation, DNA methylation, histone modification, cell cycle regulation, post-transcriptional and post-translational regulation of DNA expression, and histone modification (Hosseini *et al.*, 2017). LncRNAs have been demonstrated to participate in the occurrence, proliferation, invasion, metastasis and prognosis of many tumors, including lung cancer. For example, the level of lncRNA DANCR is elevated in tissues isolated from high-grade lung cancer and DANCR knockdown reduces the progression of lung cancer in a xenograft mouse model (Figg *et al.*, 1978); HOTAIR level is upregulated in lung cancer and the level is correlated with the degree of metastasis and outcome with poor prognosis (Loewen *et al.*, 2014); and lncRNA ezrin antisense RNA 1 (EZR-AS1) is highly expressed in various cancers such as cutaneous squamous cell carcinoma (cSCC), colon cancer, breast cancer, and esophageal squamous cell carcinoma (ESCC) compared with non-cancer tissues (Bai *et al.*, 2018; Ghaffari *et al.*, 2019; Lu *et al.*, 2021; Xie *et al.*, 2011).

EZR-AS1, 362 bp in length, is an endogenous lncRNA transcribed from the antisense strand of the EZR gene that is located on chromosome 6q25.3 (Liu *et al.*, 2019). As a cytoskeletal-membrane linker protein, *ezrin* is highly expressed in lymph node metastases but not in matched primary tumors, and downregulating *ezrin* with a small-molecule compound could impede the migration

ability of cancer cells (Ghaffari *et al*., 2019). Knockdown of EZR-AS1 results in reduced proliferation ability of breast cancer cells *in vitro* and arrests the cell cycle by inactivating the Wnt/β-catenin pathway (Bai *et al*., 2018). In ESCC, high EZR expression was found to be the cause of poor overall survival and is considered a new molecular marker to predict the prognosis of ESCC patients (Xie *et al*., 2011). However, the role of EZR-AS1 in ADC remains to be elucidated.

In the present work, the effect of silencing lncRNA EZR-AS1 on the growth, migration, and apoptosis of lung ADC cells was investigated in an attempt to explore the therapeutic option of ADC by lncRNA.

MATERIALS AND METHODS

Reagents and instruments

Fetal bovine serum (FBS; Cat. no. 26140079) was purchased from Sigma-Aldrich-Aldrich USA). The RPMI-1640 medium (cat. no. 11875119), Tris-buffered saline (TBS; cat. no. BP2471100), BCA protein assay kit (cat. no. 712853), and Annexin V-FITC kit (cat. no. 331200) was obtained from Thermo Fisher Scientific (USA). The BeyoRT III cDNA Synthesis Kit (with gDNA EZeraser) (cat. no. D7180M), Trizol total RNA extraction reagent (cat. no. R0016), and BeyoFast SYBR Green qPCR Mix (cat. no. D7260), and Lipofectamine 6000 transfection reagent (cat. no. C0526), cell counting kit-8 (cat. no. C0037), and RNase A (Cat. no. ST579) and E-cadherin (cat. no. AF0138; dilution 1:1500) and vimentin (cat. no. AF0318; dilution, 1:1000), MMP9 (cat. no. AF7935, dilution 1:1500), MMP2 (cat. no. AF1420, dilution 1:1500), and GAPDH (cat. no. AF2819, dilution 1:1200), goat horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (cat. no. A0208, dilution 1:2000) and a chemiluminescent kit (cat. no. GS009) was purchased from Beyotime (Shanghai, China). A microplate reader (GloMax® Discover) was purchased from Promega (USA). Flow cytometry (FACS LSR) data were obtained from BD Biosciences (USA). An inverted light microscope (IX73P2F) was obtained from Olympus (Tokyo, Japan). A Real-time PCR Detection System (CFX96) was obtained from Bio-Rad, Hercules, CA, USA.

Cell culture

The human lung adenocarcinoma cell line H1437 (cat. no. CRL-5872), H1975 cells (cat. no. CRL-5908), lung epithelial cell line HBEC3-KT (cat. no. CRL-4051) and NL20 (cat. no. CRL-2503) were obtained from ATCC, Manassas, VA, USA. The cells were grown in RPMI-1640 medium added with 10% FBS, penicillin (100 U/ml), and mycomycin (100 μg/ml) in a humidified incubator with 5% CO_2 at 37°C. The medium was changed every 2–3 days when the cell culture reached the logarithmic phase.

Clinical specimens

Clinical specimens (lung ADC samples and matched paracancerous tissues) were isolated from 50 patients who underwent surgical treatment at The First Affiliated Hospital of Chengdu Medical College in Chengdu, China, between July 2017 and January 2019. All ADC were primary, and the patients had not received any radiotherapy or chemotherapy prior to surgery. This study was approved by the Ethics Committee of Sichuan University (approval no. SU3312D). Informed consent was secured from each patient.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was prepared from tissues and cells using TRIzol reagent based on the manufacturer's protocols. RNA (1 μg) was reversely transcribed into cDNA using the BeyoRT III cDNA synthesis kit and the resultant cDNA was used as a template for RT-qPCR assays with primers designed using Primer Premier 5 (https://www.bioprocessonline.com/doc/primer-premier-5-design-program-0001). RT-qPCR was carried out on CFX96 Real-time PCR System using BeyoFast SYBR Green qPCR mix based on the manufacturer's instructions, with the cycling parameters: pre-denaturing at 95°C for 3 min, followed by 40 cycles of denaturing at 95°C for 10 s, annealing for 30 s with a final extension at 72°C for 30 s. The GAPDH gene was employed as the endogenous expression reference. The expression level relative to GAPDH was determined using the $2^{-\Delta\Delta Ct}$ method as outlined described (Livak & Schmittgen, 2001). The primers used for EZR-AS1 were forward: 5'-CCCTCTCCAATGAAGCCTCTC-3' and reverse: 5'-ACCGAAAATGCCGAAACCAG-3'; and for GAPDH forward: 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse, 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Transfection

Cells were cultured to 70–80% confluency and divided into aliquots for transfection using Lipofectamine 6000 transfection reagent, based on the manufacturer's instructions. The cells were either non-transfected (non-transfected, control), transfected with 0.5 μg siNC (siNC, scrambled control), siEZR-AS1 or pEZR-AS1 (positive control). siRNA (5'-AAA UAA UAC UAC AAU UAA A-3') was synthesized by Wigen Biomedicine (Shanghai, China). After transfection, cells were grown in 96 well plates containing RPMI-1640 medium at 37°C for 48 h and then used for subsequent experiments.

Cell viability assay

The viability of treated cells was determined using a CCK-8 cell counting kit based on the manufacturer's protocols. Briefly, cells were inoculated into the wells of 96-well plates at a density of 5×10^4 cells/well and grown in RPMI-1640 medium at 37°C in 5% CO_2. At predetermined time points, 20 μl CCK-8 solution was dropadded to the wells and the cells were incubated at 37°C with 5% CO_2 for 4 h. After the incubation, optical density (OD) of the plates was determined at 460 nm using a microplate reader. All assays were performed in triplicates. Cell viability was calculated as follows: $OD_{sample}/OD_{control}\times100\%$.

Apoptosis analysis

The apoptosis of treated cells was assessed using the ApoDETECT Annexin V-FITC kit based on the manufacturer's protocols. Briefly, after transfection, the cells were inoculated into the wells of 6-well plates at 5×10^4 cells/well containing RPMI-1640 medium. The cells were cultured at 37°C in 5% CO_2 to a confluence between 50 and 70%, harvested after centrifugation at 750×*g* at 25°C for 10 min, washed with 1 ml PBS three times, and digested with RNase A (100 μg/ml) at 37°C for 20 min. The digested cells were resuspended in pre-chilled binding buffer in an ice bath, and stained with Annexin

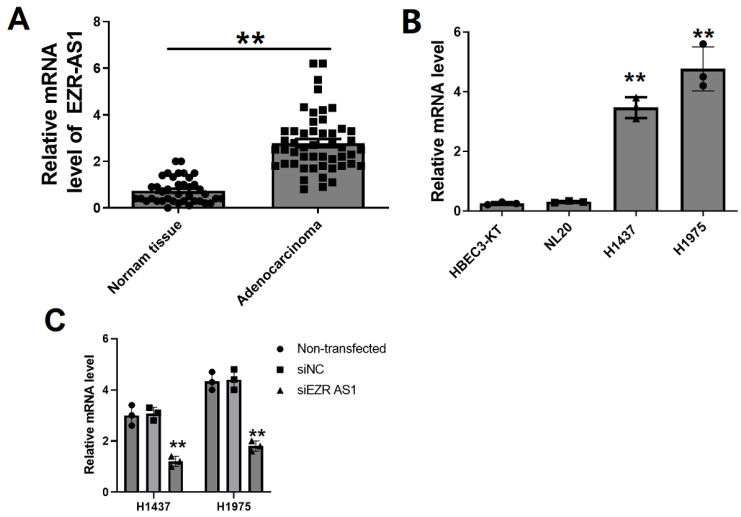

Figure 1. Determination of expression level of long non-coding RNA EZR-AS1 (ezrin antisense RNA 1) in lung ADC tissues and cells by RT-qPCR.
(**A**) EZR-AS1 levels in lung ADC and healthy tissues. (**B**) EZR-AS1 levels in lung ADC cell lines (H1437 and H1975) and non-cancer lines (HBEC3-KT and NL20). (**C**) EZR-AS1 levels in ADC cell lines H1437 and H1975 transfected with siEZR-AS1 (si, small interfering RNA), siNC (NC, negative control). All assays were repeated three times. **denotes $p<0.01$ compared to si-NC or non-transfected cells using one-way ANOVA.

V-FITC (3 µl) and PI-PE (7 µl) in the dark at 25°C for 10 min. After the staining, the cells were loaded onto a FACS LSR flow cytometer for analysis with built-in software. All assays were carried out in triplicates.

Wound healing assay

To assess the migration ability, cells were cultured on slides in the wells of 6-well plate containing RPMI-1640 medium with 10% FBS at 37°C. At 100% confluence, a scratch was created on the monolayer with the tip of a 10 µl pipette as previously described (Justus *et al.*, 2014). Serum-free RPMI-1640 medium was added and cell migration was recorded at 0 and 48 h with an inverted light microscope (at 100× magnification) and cell migration distances were calculated using Image-Pro Plus software obtained from Media Cybernetics, Inc. USA.

Western blot analysis

To assess the levels of specific proteins, treated cells were collected after centrifugation at 500 g at 25°C for 10 min, rinsed twice with pre-chilled PBS, and lysed in the radioimmunoprecipitation assay (RIPA) buffer. The total levels of protein in the lysates were determined using a BCA protein assay kit following the supplier's instructions. After boiling at 100°C for 5 min, 60 µg denatured proteins were loaded to the gels and separated by 12% polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins on the gels were blot-transferred to polyvinylidene fluoride (PVDF) membranes, then blocked by gentle shaking in 5% non-fat milk solution in 1× TBS containing 0.1% Tween 20 (TBST) for 4 h at 25°C. After blocking, the membrane filters were incubated with antibodies to E-cadherin, vimentin, MMP9, MMP2, and GAPDH (served as internal control) at 4°C overnight, rinsed three times at room temperature with TBS buffer, reacted with goat HRP-conjugated anti-rabbit IgG at 25°C for 2 h. Immunoreactive bands on the blots were developed using a chemiluminescent kit in the dark following the manufacturer's protocols. The gray values of the reactive bands were determined using Quantity One software.

Statistical analysis

Data are shown as the means ± standard error derived from three independent experiments. One-way ANOVA with post-hoc Tukey's honest significant difference test was applied to compare the means among the groups. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

EZR-AS1 level is highly elevated in lung ADC tissues and cells

We first determined the level of EZR-AS1 mRNA in lung ADC tissues and cells as well as in non-cancerous tissues and cells. RT-qPCR analysis showed that EZR-AS1 were significantly upregulated in ADC tissues in comparison with the adjacent healthy tissues obtained from patients undergoing surgical resection ($p<0.01$; Fig. 1A). Furthermore, EZR-AS1 mRNA levels in ADC cell lines H1437 and H1975 were significantly elevated compared to those in the non-cancer cell lines HBEC3-KT and NL20 ($p<0.01$; Fig. 1B).

EZR-AS1 knockdown reduces the viability of ADC cells

We then tested whether silencing EZR-AS1 inhibits the growth of ADC cells. H1437 and H1975 cells were transfected with siEZR-AS1 using Lipofectamine-mediated transfection. RT-qPCR analysis showed that siEZR-AS1 transfection resulted in significantly reduced EZR-AS1 levels in both cell lines compared with the controls (non-transfected cells or cells transfected with siNC) ($p<0.01$; Fig. 1C). The cell counting assay showed that the viability of H1437 and H1975 cells decreased over time compared to the controls ($p<0.01$; Fig. 2A). After 72 h of culture, the viability of H1437 and H1975 cells was less than 10%, while that of controls (non-transfected cells or cells transfected with siNC) was over 90% ($p<0.01$; Fig. 2A). Flow cytometry study revealed a significant increase in apoptosis after the cells were

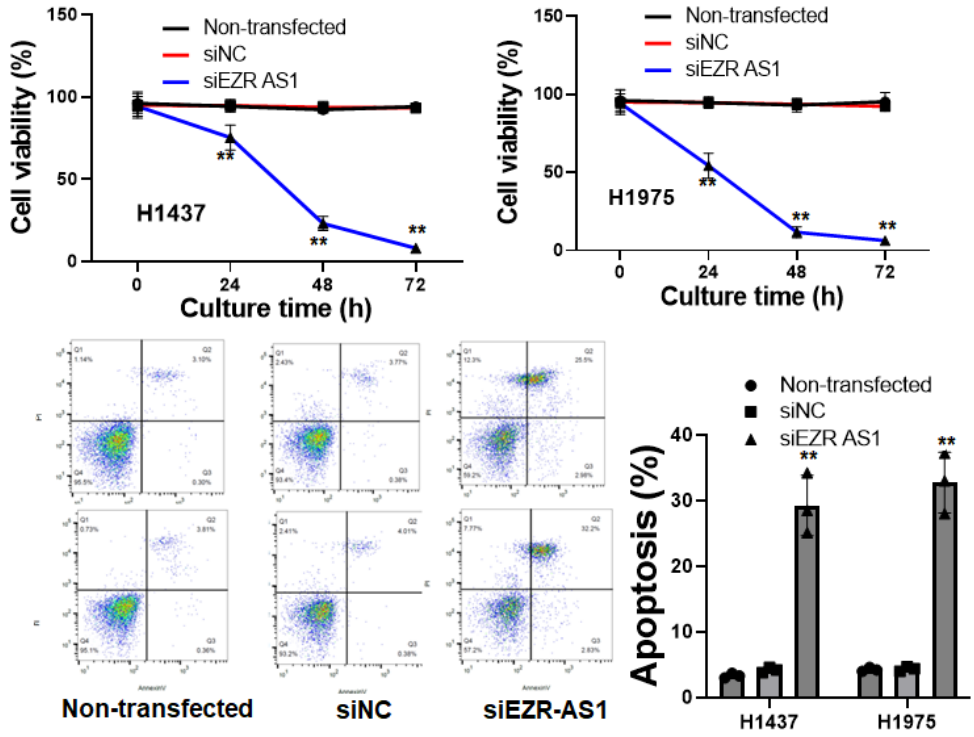

Figure 2. Cell viability and apoptosis of lung ADC cells after transfection with siEZR-AS1.
(A) Cell viability was assessed using the CCK-8 assay at different culture times in lung ADC cell lines H1437 and H1975; (**B**) apoptosis by flow cytometry 48 h after transfection with siEZR-AS1 in lung ADC cell lines H1437 and H1975. Right panel: flow cytometry, left panel: statistical analysis of flow cytometry data. (si, small interfering RNA), siNC (NC, negative control). All assays were repeated three times. **denotes $p<0.01$ compared to si-NC or non-transfected cells using one-way ANOVA.

transfected with siEZR-AS1 in both cell lines at 48 h after transfection compared with the controls ($p<0.01$; Fig. 2B).

EZR-AS1 knockdown suppresses the migration of ADC cells

To evaluate the functions of EZR-AS1 in lung ADC progression, cell migration assays based on wound-healing experiments were performed. The results showed that cell migration rates were significantly reduced after H1437 and H1975 cells were transfected with siEZR-AS1 compared to the controls ($p<0.01$; Fig. 3).

EZR-AS1 knockdown alters the levels of metastasis-related proteins

Since the migration rate of lung ADC cells was reduced after EZR-AS1 knockdown, the levels of a few selected genes that are related to invasion and metastasis pathways were analyzed. Western blotting was used to measure the protein levels of E-cadherin, vimentin, MMP2, and MMP9 in cells after EZR-AS1 knockdown.

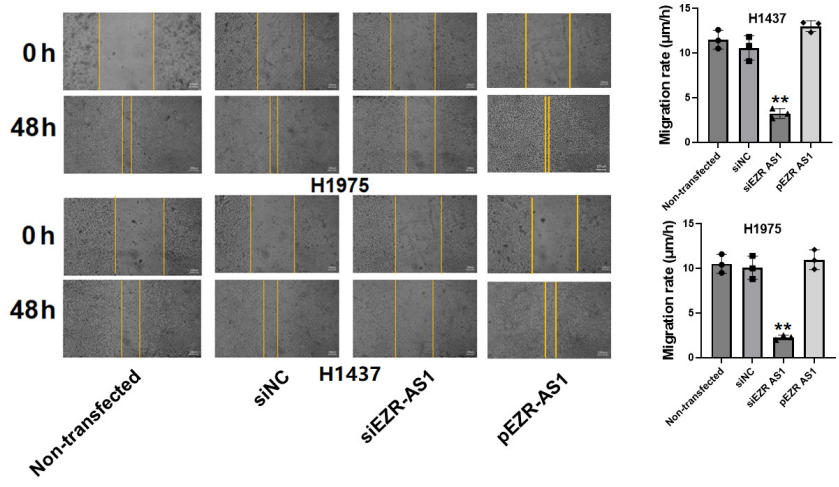

Figure 3. Migration of lung ADC cell lines H1437 and H1975 after transfected with siEZR-AS1.
(**A**) cell migrations in wound healing experiment and (**B**) statistical analysis of migration rate. Scale bar, 100 μm. (si, small interfering RNA), siNC (NC, negative control) and pEZR-AS1 (positive control). All assays were repeated three times. **denotes $p<0.01$ compared to si-NC or non-transfected cells using one-way ANOVA.

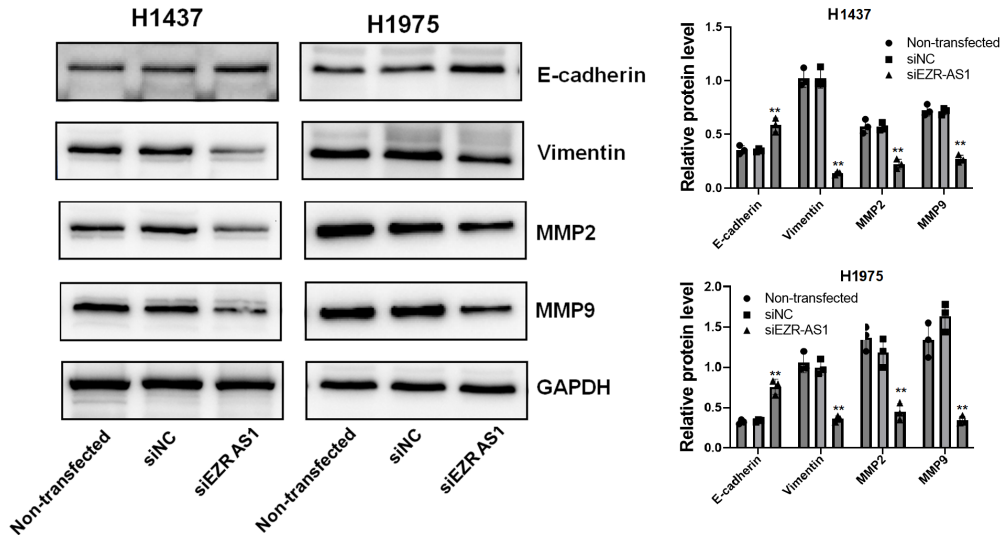

Figure 4. Level of metastasis-related proteins in lung ADC cell lines H1437 and H1975 after transfected with siEZR-AS1.
Right panel: typical Western blots, **left panel**: statistical analysis of relative protein content. All assays were repeated three times. **denotes $p<0.01$ compared to si-NC or non-transfected cells using one-way ANOVA.

The band imaging showed that the levels of these proteins were all significantly downregulated, except for E-cadherin, which was significantly upregulated compared to the controls ($p<0.01$; Fig. 4).

DISCUSSION

EZR-AS1 is implicated in disease progression in various cancers, such as cSCC, ESCC, breast cancer, and colorectal cancer (Bai *et al.*, 2018; Liu *et al.*, 2019; Lu *et al.*, 2021; Peng *et al.*, 2017), its biological role of EZR-AS1 in lung ADC is largely unknown. In the present study, it was found that EZR-AS1 levels were significantly elevated in lung ADC tissues and cells compared to those in non-cancerous tissues and cells. After transfection with siEZR-AS1 specifically targeting EZR-AS1, EZR-AS1 levels in the lung ADC cell lines H1437 and H1975 were significantly downregulated compared to the controls. After EZR-AS1 knockdown, the viability and migration rates of H1437 and H1975 cells were remarkably reduced, while apoptosis was significantly increased. EZR-AS1 knockdown also leads to the altered expression of migration-related genes, thus reducing the migration ability of lung ADC cells and affecting ADC progression.

Previously, it was found that silencing EZR-AS1 inhibits TGF-β-mediated signaling, leading to reduced growth and EMT of colorectal cancer cells (Liu *et al.*, 2019), and EZR-AS1 knockdown significantly reduced phosphorylated (p)-PI3K/PI3K and p-AKT/AKT levels in cSCC, leading to reduced cell viability and migration ability (Lu *et al.*, 2021). Furthermore, silencing EZR-AS1 significantly suppressed the migration, tumorigenicity, and metastatic lymph nodes of ESCC cells in mice (Zhang *et al.*, 2018). In the present experiments, we first profiled the level of EZR-AS1 mRNA in lung ADC and revealed that EZR-AS1 was higher in both ADC tissues from patients and cells established from ADC patients than in non-cancer tissues and cells. This is consistent with previous findings in cSCC, ESCC, breast cancer, and colorectal cancer (Bai *et al.*, 2018; Liu *et al.*, 2019; Lu *et al.*, 2021; Peng *et al.*, 2017), further confirming that EZR-AS1 is deregulated in cancer cells and involved in cancer progression.

To evaluate the effect of EZR-AS1 knockdown on the malignant behavior of ADC cancer cells, we transfected two ADC cell lines with siRNA specific for EZR-AS1 using Lipofectamine-mediated transfection. RT-qPCR analysis revealed that in siEZR-AS1-transfected cells, EZR-AS1 mRNA levels were significantly lower than those in the controls (both non-transfected cells and siNC-transfected cells), demonstrating that siEZR-AS1 is effective in specifically knocking down EZR-AS1. Subsequent analysis revealed that ADC cell viability was significantly reduced following EZR-AS1 silencing, suggesting that EZR-AS1 is likely essential for the occurrence and development of ADC. lncRNAs have been shown to play various roles in cancer occurrence, development, growth, invasion, migration, and prognosis (Bhan *et al.*, 2017; Peng *et al.*, 2017). They can either promote or inhibit the proliferation and metastasis of cancer cells, and knockdown of lncRNAs often results in suppression of cancer cell proliferation(Kong *et al.*, 2019; Zhen *et al.*, 2018). Apoptosis is an important process that regulates tumorigenesis and cancer development (Wong, 2011). Increased apoptosis is often associated with reduced proliferation and viability of cancer cells (Kou *et al.*, 2020). For example, lncRNA NEAT1 knockdown inhibits cell proliferation and increases caspase 3 activity and apoptosis in hepatocellular carcinoma (HCC) cells (Kou *et al.*, 2020), and downregulation of lncRNA MAGI2-AS3 inhibits proliferation and triggers apoptosis in prostate cancer by acting as a microRNA-424-5p sponge (Wei *et al.*, 2022). Previously, downregulation of EZR-AS1 was found to increase apoptosis in breast cancer cells by modulating the Wnt/β-catenin pathway (Bai *et al.*, 2018) and in colorectal cancer cells by inhibiting transforming growth factor-β signaling (Liu *et al.*, 2019). These findings were consistent with our data. Apoptosis is also associated with the expression of specific proteins such as Bax, Bcl2, and cleaved-caspase3. In EZR-AS1 knockdown cSCC cells, Bax was significantly upregulated and Bcl-2 was significantly reduced, suggesting that EZR-AS1 likely functions as an oncogene that contributes to the malignancy of lung cancer (Lu *et al.*, 2021).

Several mechanisms have been reported in EZR-AS1-knockdown-induced loss of cell viability. Lu *et al.* found that the inhibition of the PI3K/AKT signaling path-

way was associated with EZR-AS1-knockdown-induced reduction in cell viability and increase in apoptosis in SCC13 and SCL-1 colon cancer cells (Lu *et al*., 2021). Using LncTar, a software for predicting lncRNA-RNA interactions by means of free energy minimization, they found that focal adhesion kinase (FAK) is a target of EZR-AS1, and FAK is a crucial regulator in PI3K/AKT signaling pathway and its expression was suppressed after EZR-AS1-knockdown. This might be the reason that the PI3K/AKT signaling pathway is deactivated after EZR-AS1-knockdown. This signaling pathway plays a critical role in cell survival and in protecting cancer cells from apoptosis (Xu & Zhang, 2021). In breast cancer, EZR-AS1 protected β-catenin from degradation and EZR-AS1 knockdown inactivated the Wnt/β-catenin pathway (Bai *et al*., 2018). Since cyclin D1 and c-Myc are target genes of β-catenin, EZR-AS1 knockdown would downregulate the expression of cyclin D1 and c-Myc leading to reduced cell viability and migration (Saroha *et al*., 2022). Needless to say, more experiments such as ribonucleoprotein Immunoprecipitation (RIP) and chromatin immunoprecipitation (ChIP) may be carried out to further confirm and elucidate the mechanisms associated with the biological functions of EZR-AS1-knockdown.

Metastasis and treatment resistance are the main reasons for clinical recurrence and poor prognosis in patients with ADC. Several genes, such as RHOB and MMPs, and signaling pathways, such as the TGF-β/Smad signaling pathway, have been identified as metastatic effectors in ADC. Wound-healing experiments showed that after EZR-AS1 downregulation, the migration rate of ADC cells was significantly reduced, suggesting that silencing EZR-AS1 may regulate the expression of metastasis-related genes such as MMP2 and MMP9, as reported previously (Lu *et al*., 2021). To confirm this is true for lung ADC cells, we also examined the levels of several metastasis-related proteins such as E-cadherin, vimentin, MMP2, and MMP9, in the cells after EZR-AS1 knockdown. E-cadherin is important for maintaining cell integrity, and reduced E-cadherin expression can lead to EMT events (Guilford, 1999; Wells *et al*., 2008). Our analysis demonstrated that E-cadherin level was upregulated in EZR-AS1 knockdown-cells with reduced migration ability. It is possible that the increased E-cadherin may result in mesenchymal to epithelial reverting transitions (MErT) (Chao *et al*., 2010). When E-cadherin is upregulated in bladder cancer cells, growth and metastasis are inhibited as a result of deactivation of the β-catenin/TCF target genes (Li *et al*., 2018). As such, the upregulation of E-cadherin has been explored as a therapeutic option in cancer treatment (Howard *et al*., 2008).

In addition, vimentin expression was downregulated following EZR-AS1 knockdown. Vimentin codes for one of the 71 human intermediate filament (IF) proteins that are required for the migration of cancer cells that have undergone EMT. Vimentin can integrate the mechanical input and signals from the surrounding environment to promote cell migration and various other signaling pathways, such as the AKT pathway, also participate in regulating the migration of cancer cells through vimentin (Sim *et al*., 2021). The gene is highly expressed in various epithelial cancers, including prostate, gastrointestinal, and cervical cancers (Lin *et al*., 2017; Satelli & Li, 2011). Overexpression and silencing of vimentin by manipulation of URI (unconventional prefoldin RPB5 interactor) expression increases or decreases the migration and invasion ability of cervical cancer cells, respectively and vimentin knockdown by vimentin small hairpin RNA decreases colorectal cancer (CRC) cell migration (Wang *et al*., 2021).

Matrix metalloproteinases (MMPs) are prominent proteinases associated with tumorigenesis. They regulate signaling pathways that are responsible for cell growth, inflammation, and angiogenesis in a non-proteolytic manner and are involved in EMT, a crucial step in the invasion and migration of tumor cells, because they decompose and modify the extracellular matrix (ECM) and cell-to-ECM and cell-to-cell interface contacts, facilitating the detachment and separation of epithelial cells from the surrounding tissue (Radisky & Radisky, 2010). MMP2 and MMP9 are particularly potent in digesting the components that make up the basement membrane, such as laminin and collagen IV, or interstitial collagen fragments, resulting in cancer metastasis (Barillari, 2020; Cui *et al*., 2017; Vandooren *et al*., 2013). Our study showed that EZR-AS1 knockdown reduced MMP2 and MMP9 expression, which could be partially responsible for the reduced cell migration ability observed in siEZR-AS1-transfected ADC cell lines.

CONCLUSION

Taken together, our results demonstrated that EZR-AS1 is highly expressed in both lung ADC tissues and cells. Knockdown of EZR-AS1 with siRNA led to reduced cell viability, increased apoptosis, and reduced migration ability compared with the control. Alterations in the levels of metastasis-related proteins such as E-cadherin, vimentin, MMP2, and MMP9 likely contribute to the reduced migration ability after EZR-AS1 knockdown. Since silencing of EZR-AS1 results in reduced ADC viability, this strategy should be explored in animal models to confirm the therapeutic effect for potential application in human ADC therapy.

Declarations

Ethics approval: The Ethics Committee of Sichuan University approved this study (Approval no. SU3312D).

Funding: No specific funding.

Availability of data and material: The data and materials are available upon request from the corresponding author.

Competing interests: none.

Authors' contributions: XY, LW, and YZ: project conceptualization, investigation, and data analysis. XY, LW, ZL, and JZ: Data collection, analysis, and methodology development. XY, LW, ZL, and JZ: Investigation and methodology development. All the authors have written, read and approved the final version of the manuscript.

Acknowledgement: Not applicable.

REFERENCES

Bade BC, Dela Cruz CS (2020) Lung Cancer 2020: Epidemiology, etiology, and prevention. *Clin Chest Med* **41**: 1–24. https://doi.org/10.1016/j.ccm.2019.10.001

Bai Y, Zhou X, Huang L, Wan Y, Li X, Wang Y (2018) Long noncoding RNA EZR-AS1 promotes tumor growth and metastasis by modulating Wnt/beta-catenin pathway in breast cancer. *Exp Ther Med* **16**: 2235–2242. https://doi.org/10.3892/etm.2018.6461

Barillari G (2020) The impact of matrix metalloproteinase-9 on the sequential steps of the metastatic process. *Int J Mol Sci*. **21**. https://doi.org/10.3390/ijms21124526

Bhan A, Soleimani M, Mandal SS (2017) Long noncoding RNA and cancer: a new paradigm. *Cancer Res*. **77**: 3965–3981. https://doi.org/10.1158/0008-5472.CAN-16-2634

Cao M, Chen W (2019) Epidemiology of lung cancer in China. *Thorac Cancer* **10**: 3–7. https://doi.org/10.1111/1759-7714.12916

Chao YL, Shepard CR, Wells A (2010) Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. *Mol Cancer* **9**: 179. https://doi.org/10.1186/1476-4598-9-179

Cui N, Hu M, Khalil RA (2017) Biochemical and biological attributes of matrix metalloproteinases. *Prog Mol Biol Transl Sci* **147**: 1-73. https://doi.org/10.1016/bs.pmbts.2017.02.005

Denisenko TV, Budkevich IN, Zhivotovsky B (2018) Cell death-based treatment of lung adenocarcinoma. *Cell Death Dis* **9**: 117. https://doi.org/10.1038/s41419-017-0063-y

Figg DM, Bratt HJ, VanVliet PD, Dean RE (1978) Thyroid cancer: diagnosis and management based on a review of 142 cases. *Am J Surg* **135**: 671–674. https://doi.org/10.1016/0002-9610(78)90133-2

Ghaffari A, Hoskin V, Turashvili G, Varma S, Mewburn J, Mullins G, Greer PA, Kiefer F, Day AG, Madarnas Y, SenGupta S, Elliott BE (2019) Intravital imaging reveals systemic ezrin inhibition impedes cancer cell migration and lymph node metastasis in breast cancer. *Breast Cancer Res* **21**: 12. https://doi.org/10.1186/s13058-018-1079-7

Guilford P (1999) E-cadherin downregulation in cancer: fuel on the fire? *Mol Med Today* **5**: 172–177. https://doi.org/10.1016/s1357-4310(99)01461-6

Hosseini ES, Meryet-Figuiere M, Sabzalipoor H, Kashani HH, Nikzad H, Asemi Z (2017) Dysregulated expression of long noncoding RNAs in gynecologic cancers. *Mol Cancer* **16**: 107. https://doi.org/10.1186/s12943-017-0671-2

Howard EW, Camm KD, Wong YC, Wang XH (2008) E-cadherin up-regulation as a therapeutic goal in cancer treatment. *Mini Rev Med Chem* **8**: 496–518. https://doi.org/10.2174/138955708784223521

Justus CR, Leffler N, Ruiz-Echevarria M, Yang LV (2014) In vitro cell migration and invasion assays. *J Vis Exp* **88**: 51046. https://doi.org/10.3791/51046

Klimaszewska-Wisniewska A, Halas-Wisniewska M, Grzanka A, Grzanka D (2018) Evaluation of anti-metastatic potential of the combination of fisetin with paclitaxel on A549 non-small cell lung cancer cells. *Int J Mol Sci* **19**. https://doi.org/10.3390/ijms19030661

Kong X, Duan Y, Sang Y, Li Y, Zhang H, Liang Y, Liu Y, Zhang N, Yang Q (2019) LncRNA-CDC6 promotes breast cancer progression and function as ceRNA to target CDC6 by sponging microRNA-215. *J Cell Physiol* **234**: 9105–9117. https://doi.org/10.1002/jcp.27587

Kou JT, Ma J, Zhu JQ, Xu WL, Liu Z, Zhang XX, Xu JM, Li H, Li XL, He Q (2020) LncRNA NEAT1 regulates proliferation, apoptosis and invasion of liver cancer. *Eur Rev Med Pharmacol Sci* **24**: 4152–4160. https://doi.org/10.26355/eurrev_202004_20995

Li C, Liu J, Zhang Q, Cui K, Ge Q, Wang C, Chen Z (2018) Up-regulation of Ecadherin expression mediated by a novel dsRNA suppresses the growth and metastasis of bladder cancer cells by inhibiting beta-catenin/TCF target genes. *Int J Oncol* **52**: 1815–1826. https://doi.org/10.3892/ijo.2018.4346

Lin J, Lu J, Wang C, Xue X (2017) The prognostic values of the expression of Vimentin, TP53, and Podoplanin in patients with cervical cancer. *Cancer Cell Int* **17**: 80. https://doi.org/10.1186/s12935-017-0450-6

Liu Z, Wang N, Wang F, Zhang S, Ding J (2019) Silencing of lncRNA EZR-AS1 inhibits proliferation, invasion, and migration of colorectal cancer cells through blocking transforming growth factor beta signaling. *Biosci Rep* **39**. https://doi.org/10.1042/BSR20191199

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402–408. https://doi.org/10.1006/meth.2001.1262

Loewen G, Jayawickramarajah J, Zhuo Y, Shan B (2014) Functions of lncRNA HOTAIR in lung cancer. *J Hematol Oncol* **7**: 90. https://doi.org/10.1186/s13045-014-0090-4

Lu D, Sun L, Li Z, Mu Z (2021) lncRNA EZR-AS1 knockdown represses proliferation, migration and invasion of cSCC *via* the PI3K/AKT signaling pathway. *Mol Med Rep* **23**. https://doi.org/10.3892/mmr.2020.11714

Musika W, Kamsa-Ard S, Jirapornkul C, Santong C, Phunmanee A (2021) Lung cancer survival with current therapies and new targeted treatments: a comprehensive update from the srinagarind hospital-based cancer registry from (2013 to 2017). *Asian Pac J Cancer Prev* **22**: 2501–2507. https://doi.org/10.31557/APJCP.2021.22.8.2501

Peng WX, Koirala P, Mo YY (2017) LncRNA-mediated regulation of cell signaling in cancer. *Oncogene*. **36**: 5661-5667. https://doi.org/10.1038/onc.2017.184

Radisky ES, Radisky DC (2010) Matrix metalloproteinase-induced epithelial-mesenchymal transition in breast cancer. *J Mammary Gland Biol Neoplasia*. **15**: 201-212. https://doi.org/10.1007/s10911-010-9177-x

Saigoh K, Matsui K, Takahashi K, Nishikawa T, Wada K (1998) The stereo-specific effect of D-serine ethylester and the D-cycloserine in ataxic mutant mice. *Brain Res* **808**: 42–47. https://doi.org/10.1016/s0006-8993(98)00810-5

Saroha HS, Kumar Guddeti R, Jacob JP, Kumar Pulukuri K, Karyala P, Pakala SB (2022) MORC2/beta-catenin signaling axis promotes proliferation and migration of breast cancer cells. *Med Oncol* **39**: 135. https://doi.org/10.1007/s12032-022-01728-6

Satelli A, Li S (2011) Vimentin in cancer and its potential as a molecular target for cancer therapy. *Cell Mol Life Sci* **68**: 3033–3046. https://doi.org/10.1007/s00018-011-0735-1

Sim HJ, Song MS, Lee SY (2021) Kv3 channels contribute to cancer cell migration *via* vimentin regulation. *Biochem Biophys Res Commun* **551**: 140–147. https://doi.org/10.1016/j.bbrc.2021.03.019

Vandooren J, Van den Steen PE, Opdenakker G (2013) Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9): the next decade. *Crit Rev Biochem Mol Biol* **48**: 222–272. https://doi.org/10.3109/10409238.2013.770819

Wang Q, Zhu G, Lin C, Lin P, Chen H, He R, Huang Y, Yang S, Ye J (2021) Vimentin affects colorectal cancer proliferation, invasion, and migration *via* regulated by activator protein 1. *J Cell Physiol.* **236**: 7591–7604. https://doi.org/10.1002/jcp.30402

Wei X, Hou Y, Zhang Y, Zhang H, Sun Z, Meng X, Wang Z (2022) Long non-coding RNA MAGI2-AS3 inactivates STAT3 pathway to inhibit prostate cancer cell proliferation *via* acting as a microRNA-424-5p sponge. *J Cancer* **13**: 343–353. https://doi.org/10.7150/jca.60749

Wells A, Yates C, Shepard CR (2008) E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. *Clin Exp Metastasis* **25**: 621–628. https://doi.org/10.1007/s10585-008-9167-1

Wong RS (2011) Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res* **30**: 87. https://doi.org/10.1186/1756-9966-30-87

Wu F, Wang L, Zhou C (2021) Lung cancer in China: current and prospect. *Curr Opin Oncol* **33**: 40–46. https://doi.org/10.1097/CCO.0000000000000703

Xie JJ, Xu LY, Wu ZY, Zhao Q, Xu XE, Wu JY, Huang Q, Li EM (2011) Prognostic implication of ezrin expression in esophageal squamous cell carcinoma. *J Surg Oncol* **104**: 538–543. https://doi.org/10.1002/jso.21909

Xu J, Zhang W (2021) EZR promotes pancreatic cancer proliferation and metastasis by activating FAK/AKT signaling pathway. *Cancer Cell Int* **21**: 521. https://doi.org/10.1186/s12935-021-02222-1

Zhang XD, Huang GW, Xie YH, He JZ, Guo JC, Xu XE, Liao LD, Xie YM, Song YM, Li EM, Xu LY (2018) The interaction of lncRNA EZR-AS1 with SMYD3 maintains overexpression of EZR in ESCC cells. *Nucleic Acids Res* **46**: 1793–1809. https://doi.org/10.1093/nar/gkx1259

Zhen Q, Gao LN, Wang RF, Chu WW, Zhang YX, Zhao XJ, Lv BL, Liu JB (2018) LncRNA DANCR Promotes Lung Cancer by Sequestering miR-216a. *Cancer Control* **25**: 1073274818769849. https://doi.org/10.1177/1073274818769849