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The loss of endothelin-2 exhibits an anticancer effect in A549 human lung adenocarcinoma cell line

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Abstract

Lung cancer is the leading cause of cancer-related deaths worldwide, and adenocarcinoma is the most common subtype of lung cancer. Endothelin-2 (ET-2) is expressed in the epithelium of alveoli, and its expression is increased in cancer. However, the role of ET-2 in lung adenocarcinoma remains unclear. This study aimed to investigate the pathophysiological functions of ET-2 in A549 human lung adenocarcinoma cells. We analyzed the expression of ET-2 mRNA in lung adenocarcinoma tissues compared with that in nontumor lung tissues using public online databases. The function of ET-2 in A549 cells was investigated using siRNA. ET-2 mRNA level was upregulated in lung adenocarcinoma tissues, and high ET-2 level was associated with poor overall survival in patients with lung adenocarcinoma. ET-2 silencing reduced the proliferation, migration, and invasion, and enhanced apoptosis in A549 cells. Mechanistically, ET-2 silencing reduced the expression levels of X-linked inhibitor of apoptosis and survivin, which are members of the inhibitor apoptosis protein family. In addition, silencing ET-2 inhibited epithelial–mesenchymal transition, which halted migration. Therefore, the specific targeting of ET-2 may be a potential treatment strategy for lung adenocarcinoma.

Key words: endothelin-2, lung adenocarcinoma, survivin, XIAP

Résumé

Le cancer du poumon est la première cause de mortalité liée au cancer à l'échelle mondiale, et l'adénocarcinome est le sous-type de cancer du poumon le plus fréquent. L'endothéline 2 (ET-2) s'exprime dans l'épithélium des alvéoles et son expression est accrue dans le cancer. Cependant, le rôle de l'ET-2 dans l'adénocarcinome du poumon demeure à clarifier. Cette étude portait sur les rôles physiopathologiques de l'ET-2 dans les cellules A549 d'adénocarcinome pulmonaire chez l'humain. Nous avons étudié l'expression de l'ET-2 en ARNm dans les tissus d'adénocarcinome pulmonaire par rapport à des tissus pulmonaires non tumoraux à l'aide de bases de données publiques en ligne. Nous avons étudié le fonctionnement de l'ET-2 dans les cellules A549 à l'aide d'ARN interférent (siRNA). Les taux d'ARNm de l'ET-2 étaient régulés à la hausse dans les tissus d'adénocarcinome pulmonaire, et l'augmentation des taux d'ET-2 était dans l'ensemble associée avec de mauvais taux de survie chez les patients atteints d'adénocarcinome pulmonaire. L'extinction (« silencing ») de l'ET-2 entraînait une atténuation de la prolifération, de la migration, et de l'invasion des cellules A549, avec un accroissement de leur apoptose. Au point de vue des modes d'action, l'extinction de l'ET-2 entraînait une diminution des taux d'expression d'inhibiteurs de l'apoptose liés au chromosome X (XIAP) et de la survivine, membres de la famille des protéines inhibitrices de l'apoptose. De plus, l'extinction de l'ET-2 entraînait une inhibition de la transition de l'épithélium vers le mésenchyme, ce qui interrompait la migration. Par conséquent, le ciblage spécifique de l'ET-2 pourrait correspondre à une stratégie de traitement éventuelle contre l'adénocarcinome pulmonaire. [Traduit par la Rédaction]

Mots-clés : endothéline 2, adénocarcinome pulmonaire, survivine, XIAP

Introduction

Lung cancer is the leading cause of death in both sexes according to the estimated data of GLOBOCAN 2020 (Sung et al. 2021). Based on its histological type, lung cancer is divided into small cell lung cancer (SCLC, 20% of all lung cancers) and nonsmall cell lung cancer (NSCLC, 80% of all lung cancers). The major types of NSCLC include adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Adenocarcinoma is the most common subtype of lung cancer, accounting for 40% of lung cancers and 60% of NSCLCs. NSCLCs

are managed by surgery and (or) adjuvant treatment (Zheng 2016). However, despite the development of numerous novel treatments, lung cancer remains a serious problem. Therefore, more effective treatment strategies are required.

Endothelin (ET) consists of three isoforms (ET-1, ET-2, and ET-3), which act on two G-protein-coupled receptors, ET receptor type A and type B (ET_A and ET_B). Among these three isoforms, ET-1 has been the most extensively studied and is involved in the pathophysiology of various diseases, including cancer. ET-1 activation to ET_A and ET_B activates G-protein- or β-arrestin-dependent mechanisms to modulate multiple signaling pathways that mediate tumor growth, metastasis, invasion, and chemoresistance (Rosanò and Bagnato 2016).

ET-2 is structurally highly similar to ET-1 and has the same affinity as ET-1 for both ET_A and ET_B receptors. Because ET-1 is more abundant than ET-2, it is often hypothesized that ET-2 mimics the function of ET-1. Unlike ET-1, only a few studies have reported the role of ET-2 in cancer. ET-2 is upregulated in human breast cancer (Grimshaw et al. 2002), and its expression of ET-2 mRNA is three times higher in basal cell carcinoma than in normal skin (Tanese et al. 2010). Additionally, ET-2 reduces apoptosis under hypoxia (Grimshaw et al. 2002) and potentiates the invasiveness of cancer cells (Grimshaw et al. 2004). However, considering that global knockout mice of ET-2 revealed a phenotype distinct from that of ET-1 knockout mice (Chang et al. 2013), we hypothesized that ET-2 may have distinct pathophysiological roles in cancer.

In the present study, we first analyzed public online databases of human cancer and identified that ET-2 mRNA was significantly higher in lung adenocarcinoma (LUAD) tissues compared to normal lung tissues. In addition, high ET-2 expression was associated with poor overall survival (OS) in patients with LUAD. Therefore, in this study, we aimed to explore the role of ET-2 in human adenocarcinoma cells A549 by silencing ET-2 and further investigate the underlying mechanisms.

Materials and methods

Bioinformatics analysis

To measure the levels of ET-1 and ET-2 mRNA expression in LUAD compared to those in normal specimens, we used the online databases Gene Expression database of Normal and Tumor tissues 2 (GENT2) (http://gent2.appex.kr/gent2/) and Gene Expression Profiling Interactive Analysis 2 (GEPIA2) (http://gepia2.cancer-pku.cn/#analysis), interactive web-based tools that comprise normal and tumor samples from the National Center for Biotechnology Information Gene Expression Omnibus database (Park et al. 2019) and The Cancer Genome Atlas (Tang et al. 2019), respectively. The significance test methods used were two-tailed Student's *t* test with significant value for the *P* value <0.05. In GEPIA2, $\log_2$FC was set as 1 and *P* value <0.05 as the cutoff criteria.

To examine the correlation between EDN-1 (222802_at) and EDN-2 (206758_at) mRNA expression and OS in LUAD, a cohort of patients with LUAD cancer (all stages) from the data sets was analyzed using the Kaplan–Meier plotter web tool (http://kmplot.com/analysis/index.php?p=service&cancer=lung) (Győrffy 2021). Patients with lung cancer were followed up for 10 years. To determine the prognostic value, the samples were divided into two groups based on the median expression of the genes. mRNA expression above or below the median separated the cases into high and low expression levels. A *P* value <0.05 was considered statistically significant.

Table 1. Primer sequence for quantitative real-time PCR.

Name	Primer
Human GAPDH F	CGACCACTTTGTCAAGCTCA
Human GAPDH R	AGGGGAGATTCAGTGTGGTG
Human XIAP F	AATAGTGCCACGCAGTCTACA
Human XIAP R	CAGATGGCCTGTCTAAGGCAA
Human survivin F	CACTGCCCCACTGAGAACGA
Human survivin R	AAGGAAAGCGCAACCGGAC
Human E-cadherin F	TGCACCAACCCTCATGAGTG
Human E-cadherin R	GTCAGTATCAGCCGCTTTCAG
Human vimentin F	CGTGAATACCAAGACCTGCTC
Human vimentin R	GGAAAAGTTTGGAAGAGGCAG
Human ET-2 fm	CTTCTCCAAAGGCTGAGGGACATT
Human ET-2 R	TCCTGTTGTCGCTTGGCAAAGA

Cell culture

A human LUAD cell line (A549) was obtained from the American Type Culture Collection (Manasaa, VA, USA). Cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and antibiotic–antimycotic (Gibco) in a 5% CO_2 incubator at 37 °C. The medium was changed every 2 days.

RNA interference

Silencing of ET-2 (Dharmacon Ref#: SO-2521143G) or the negative control (Ambion, Silencer Select Negative Control siRNA) was performed using Lipofectamine RNAiMAX (Invitrogen). Briefly, RNAiMAX mixture was prepared according to the manufacturer's instructions. Transfection was performed at 30%–50% cell confluence. The cells were then incubated for 24 h at 37 °C in a CO_2 incubator.

RNA isolation and real-time polymerase chain reaction

Total RNA was extracted from cells using RNAiso plus (Takara), according to the manufacturer's instructions, followed by purification with Nucleospin RNA clean-up (Macherey-Nagel). cDNA was prepared from ~1 μg of total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara). Polymerase chain reactions (PCRs) were prepared using FastStart SYBR Green Master (Roche Applied Science), followed by real-time PCR analysis using a LightCycler96 (Roche Applied Science). The mRNA levels of target genes relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed. The nucleotide sequences of the primers used (Life Science) are listed in Table 1.

Proliferation assay trypan blue exclusion cell assay

Cell proliferation was evaluated using a trypan blue exclusion assay. The cells were detached and resuspended in equal volumes. Cells were stained with trypan blue (0.4% solution) (Wako) and counted for stained/unviable and unstained/viable cells every 24 h for 3 days.

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and protein concentrations were measured using a DC Protein Assay Kit (Bio-Rad). Briefly, the protein was extracted with RIPA buffer and centrifuged at 15 000 *g* for 15 min at 4 °C. Moreover, ∼20–35 μg of total protein was separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to nitrocellulose membranes and blocked with 5% skim milk powder with 0.1% Tween 20. The membranes were incubated overnight in a rotational incubator at 4 °C with the relevant primary antibodies. The membranes were then rinsed with Tris-buffered saline with 0.1% Tween® 20 (TBS-T) three times for 5–10 min each. This was followed by incubation with secondary goat anti-rabbit IgG horseradish peroxidase (HRP) antibodies at room temperature for 1 h. The membranes were rinsed with TBS-T three times for 5–10 min each time. The membranes were visualized using enhanced chemiluminescence (Bio-Rad). The target protein expression levels were normalized to GAPDH expression levels. The primary antibodies used were as follows: GAPDH (Cell Signaling Technology, 2118S), X-linked inhibitor of apoptosis (XIAP) (Cell Signaling Technology, 2042), survivin (Cell Signaling Technology, 2808S), caspase-7 (Cell Signaling Technology, 9492), cleaved caspase-3 (Cell Signaling Technology, 9661S), caspase-3 (Cell Signaling Technology, 9662S), poly(adenosine diphosphate-ribose) diphosphate (PARP) (Cell Signaling Technology, 9541), and cleaved PARP (Cell Signaling Technology, 9541). The secondary antibody used was anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, 7074S).

Scratch-wound assay

A549 cells treated with the designated siRNAs were cultured in six-well plates. Cells at 90% confluence were scratched with 1 mL pipette tips, and the remaining cells were washed twice with phosphate-buffered saline twice. The cells were incubated with 10% FBS in the RPMI 1640 medium. Wounded areas were measured at time point zero, and after 21 h of incubation, photographs were taken of the same areas recorded at time point zero.

Cell migration and invasion assays

A549 cells transfected with the indicated siRNAs were resuspended and placed in the top layer of a transwell insert membrane with 8 μm pores (Corning). The lower layer of the insert contained 2% FBS in RPMI 1640 medium as a chemoattractant. The cells were maintained at 37 °C in 5% CO_2 overnight. Nonmigrating cells on top of the transwell insert membrane were removed using cotton swabs. Cells that migrated to the bottom were fixed with methanol 100%, stained with Mayer's hematoxylin, washed with distilled water, photographed, and counted. For the invasion assay, Matrigel (Corning) was diluted in RPMI medium to a final concentration of 300 μg/mL and was coated to the inner surface of transwell insert membrane, according to the manufacturer's protocol. After overnight incubation, the cells that invaded the lower layer were fixed, stained, and counted (magnification 100×).

Statistical analyses

All data are presented as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism 8. Differences between groups were calculated using a two-tailed Student's *t* test. Statistical significance was set at $P < 0.05$.

Results

High ET-2 expression in LUAD

We compared the differences in either ET-2 or ET-1 expression between LUAD tissues and normal lung tissues from two independent bioinformatics databases. Analysis from the GEPIA2 (LUAD sample, 483; normal, 59) and GENT2 (LUAD sample, 254; normal, 509) databases identified significantly higher ET-2 expression in LUAD tissues than in normal lung tissues. In contrast, ET-1 expression was significantly lower in LUAD tissues than in normal lung tissues (Figs. 1A–1D).

High ET-2 mRNA levels were associated with a poor prognosis of LUAD

The Kaplan–Meier analysis and log-rank test evaluating the median expression values of ET-2 and ET-1 mRNA demonstrated that the survival rate of patients with LUAD with high ET-2 expression, in terms of OS, was significantly worse than that of patients with low expression (Fig. 1E). The survival rate of patients with LUAD with low ET-1 expression in terms of OS was significantly worse than that in the high-expression group (Fig. 1F). Accordingly, these findings suggest that the role of ET-2 in LUAD progression needs to be further elucidated.

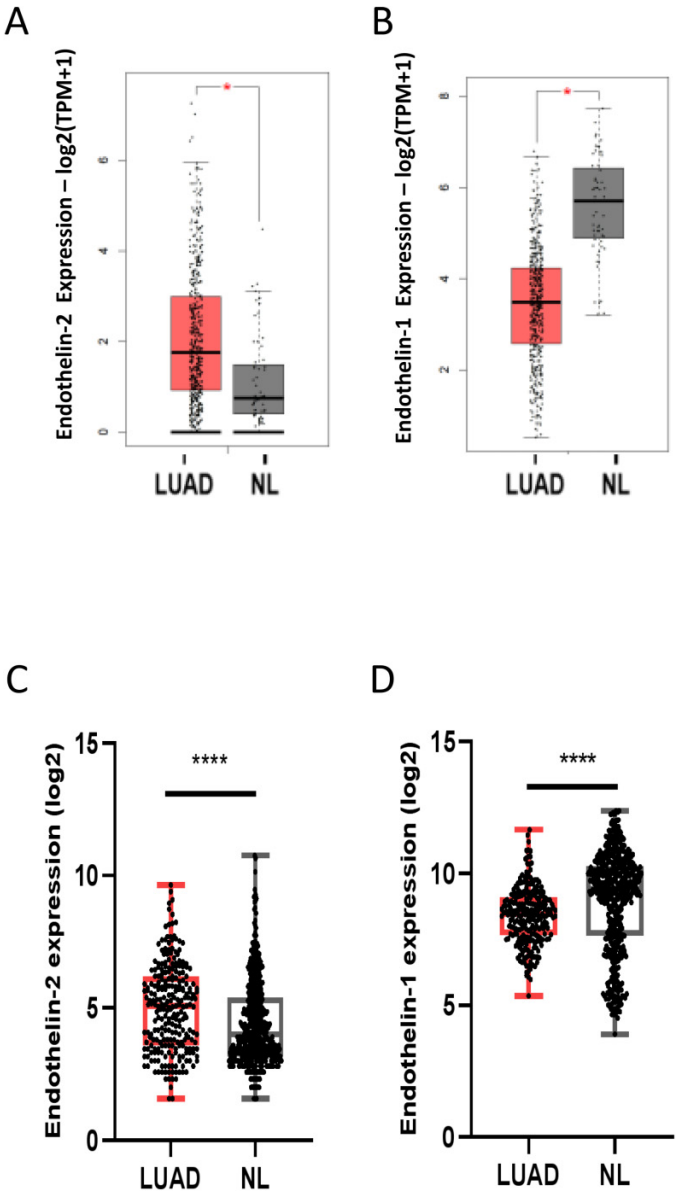
ET-2 downregulation decreased A549 cell proliferation

To study the function of ET-2, we silenced ET-2 in A549 cells (Fig. 2A). ET-2-silenced A549 cells showed a decreased cell growth rate compared to the negative control (Fig. 2B).

ET-2 downregulation retarded A549 cell migration and invasion

To determine the migration ability of A549 cells, we performed wound healing and transwell migration assays. However, for the invasion assay, transwell insert membranes coated with Matrigel were used. A549 cells transfected with ET-2 siRNA exhibited significantly decreased migration and invasion abilities (Figs. 3A–3F).

Figure 1. Endothelin (ET-1) and ET-2 mRNA levels in lung adenocarcinoma (LUAD) from patient's samples. (A, B) Bloxpots showed results with $\log_2$ of transcript count per million [$\log_2$(TPM + 1)] presenting the expression levels of ET-2 and ET-1 from GEPIA2 database. (C, D) ET-2 and ET-1 mRNA expression ($\log_2$) from GENT2 U133plus2 data set. T, tumor; N, normal lung tissue. Data were analyzed using a two-tailed Student's *t* test $^{*}P < 0.05$; $^{****}P < 0.0001$. (E, F) Kaplan–Meier analysis of OS curves in patients with LUAD with low or high ET-2/ET-1 mRNA levels.



Et-2 silencing facilitated A549 cell apoptosis

During apoptosis, cleavage of PARP-1 is a useful hallmark of this type of cell death. This cleavage is well studied and is generated by caspase-3 and caspase-7, proteases activated during apoptosis. The levels of cleaved PARP, caspase-3, and caspase-7 increased in the ET-2-silenced A549 cells (Figs. 4A–4D). Taken together, these data showed that ET-2 plays a role in proliferation, migration, invasion, and apoptosis.

ET-2 silencing reduced XIAP–survivin and epithelial–mesenchymal transition marker in A549 cells

We then evaluated the effect of silencing ET-2 as the main inhibitor of apoptotic proteins and epithelial–mesenchymal transition (EMT) markers. Survivin and XIAP form a complex that binds directly to caspase-9 and plays a role in cancer cell migration. ET-2 ablation reduced the mRNA and protein

Figure 1.

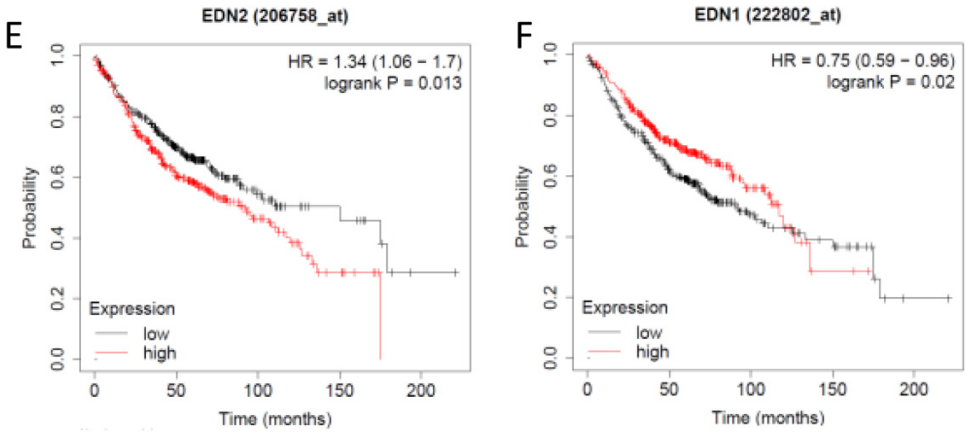
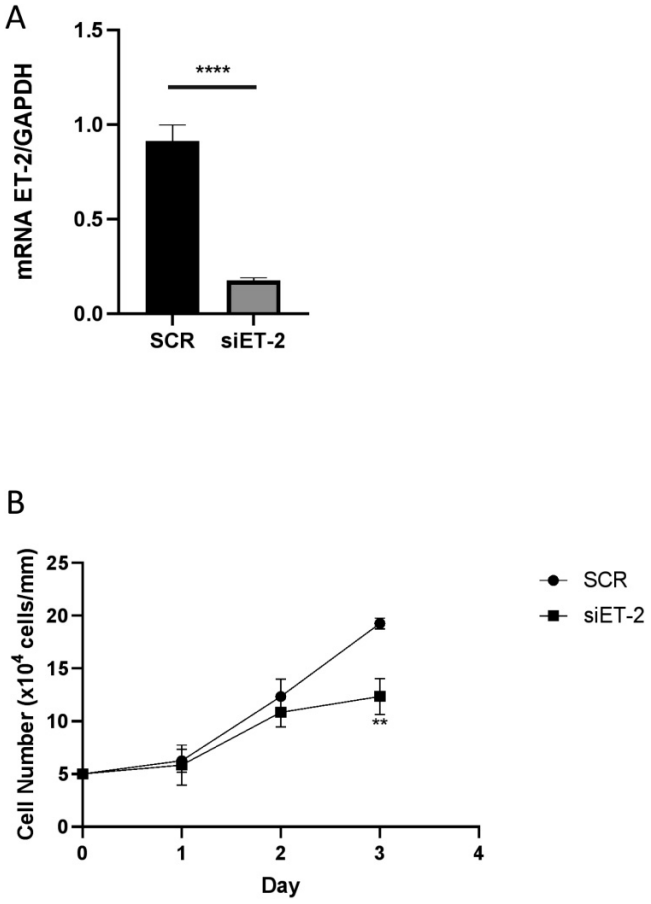


Figure 2. Effect of endothelin (ET)-2 silencing on A549 cell proliferation. (A) Effect of ET-2 silencing on mRNA level in A549 cells. (B) Effect of ET-2 silencing on proliferation in A549 cells. The A549 cells were cultured and transfected with siRNA ET-2/siRNA negative control. The proliferation rate was determined by the trypan blue viability test. Data were analyzed using a two-tailed Student's *t* test. $^{**}P < 0.01$, $^{****}P < 0.0001$.



levels of survivin and XIAP (Figs. 5A–5E). E-cadherin, an epithelial marker, was increased by silencing of ET-2 (Fig. 5F). Vimentin, SM22, and Twist mRNA, mesenchymal markers, were reduced by silencing ET-2 (Figs. 5G–5I).

Discussion

In the present study, we found that ET-2 mRNA expression was significantly elevated in LUAD tissues of patients compared to that in normal lung tissues, according to two bioinformatics human cancer databases. In addition, high ET-2 mRNA levels are associated with poor OS in patients with LUAD. Moreover, ET-2 knockdown in human LUAD A549 cells markedly reduced proliferation, migration, and invasion, and enhanced apoptosis. These anticancer effects were mediated by the dual inhibition of XIAP–survivin mRNA and protein levels and suppression of EMT.

Lung cancer is the most common cancer worldwide, and LUAD is the most common subtype of lung cancer. Among the three ET isoforms, ET-1 is the most studied and is known to be involved in various diseases, including cancer. Different from ET-1, the role of ET-2 in cancer has been less explored. ET-2 expression is increased in breast and basal skin cancers (Grimshaw et al. 2002; Tanese et al. 2010). In several cancer cell lines, ET-2 is upregulated by hypoxia (Koong et al. 2000). In mammary breast cancer cells, ET-2 acts as an autocrine survival factor and potentiates tumor invasion (Grimshaw et al. 2002). However, the effect of ET-2 on LUAD remains unclear.

In the present study, the anticancer effect of ET-2 was mediated by the inhibition of XIAP–survivin mRNA and protein levels. XIAP and survivin are inhibitors of the apoptosis protein (IAP) family members, a group of proteins that modulate the balance between proliferation and apoptosis. In addition, IAPs play an important role in cancer cell migration (Oberoi-Khanuja et al. 2013). XIAP and survivin are significantly increased in NSCLC (Krepela et al. 2009) and LUAD, according to bioinformatics analyses (data not shown). In particular, survivin is a well-known regulator of cell proliferation and

Figure 3. Effect of endothelin (ET)-2 silencing on A549 cell migration and invasion in wound healing and transwell migration/invasion assays. Images showing migration and invasion changes of A549 treated with siRNA ET-2 in (A, B) wound healing, (C, D) transwell migration assay, and (E, F) transwell invasion assay. Data were analyzed using a two-tailed Student's *t* test. $^{***}P < 0.001$, $^{****}P < 0.0001$.

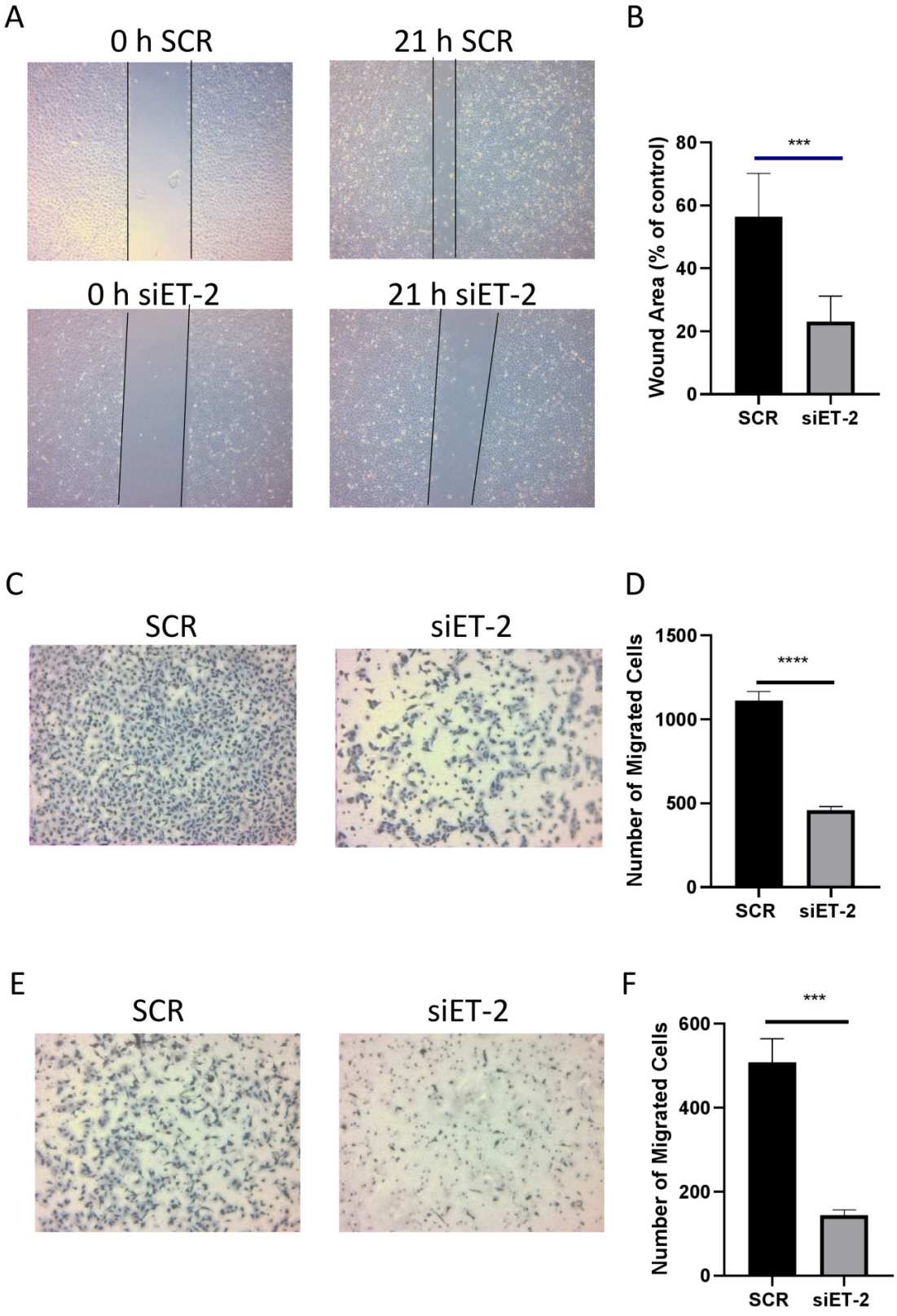
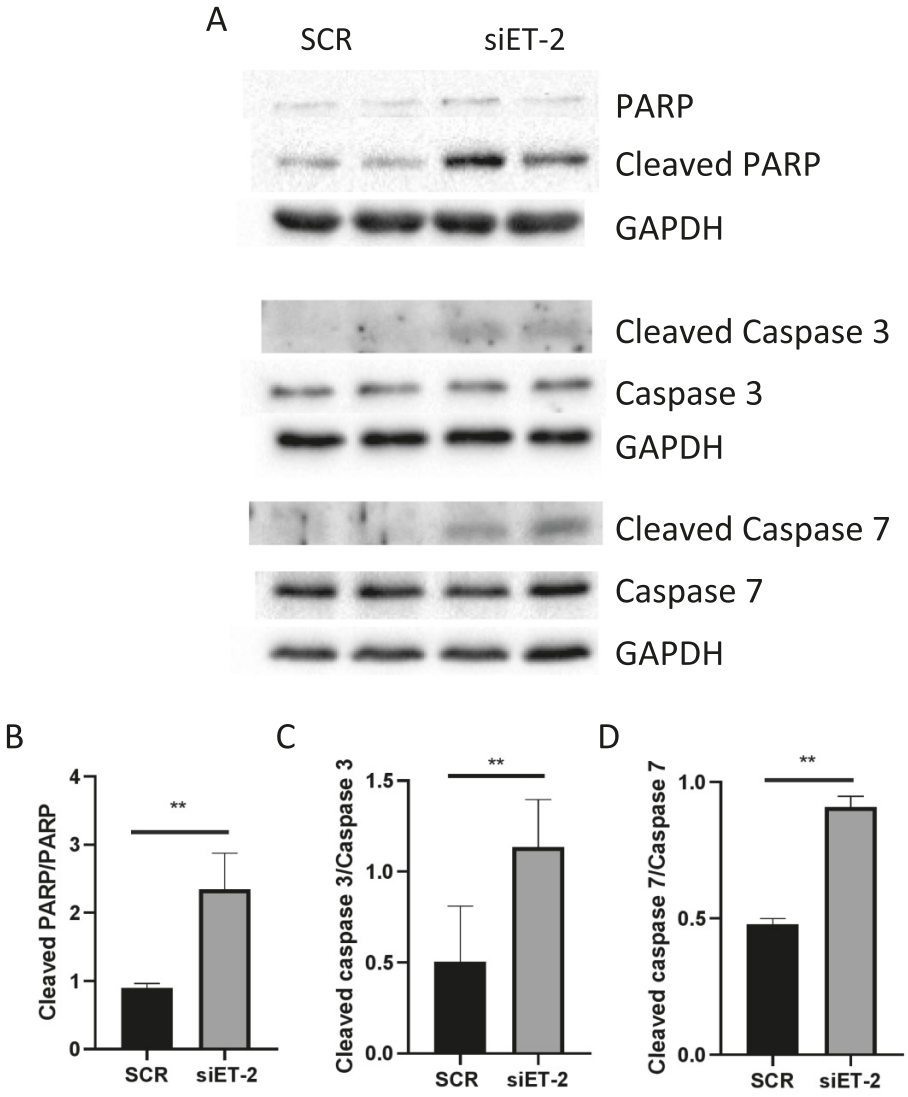


Figure 4. Effect of endothelin (ET)-2 silencing on cleaved poly(adenosine diphosphate-ribose) polymerase (PARP) in A549 cells. (A) Western blot showed ET-2 siRNA increased cleaved PARP, cleaved caspase-3, and cleaved caspase-7. (B–D) Quantification of Western blot. Data were analyzed using a two-tailed Student's *t* test. $^{**}P < 0.01$.

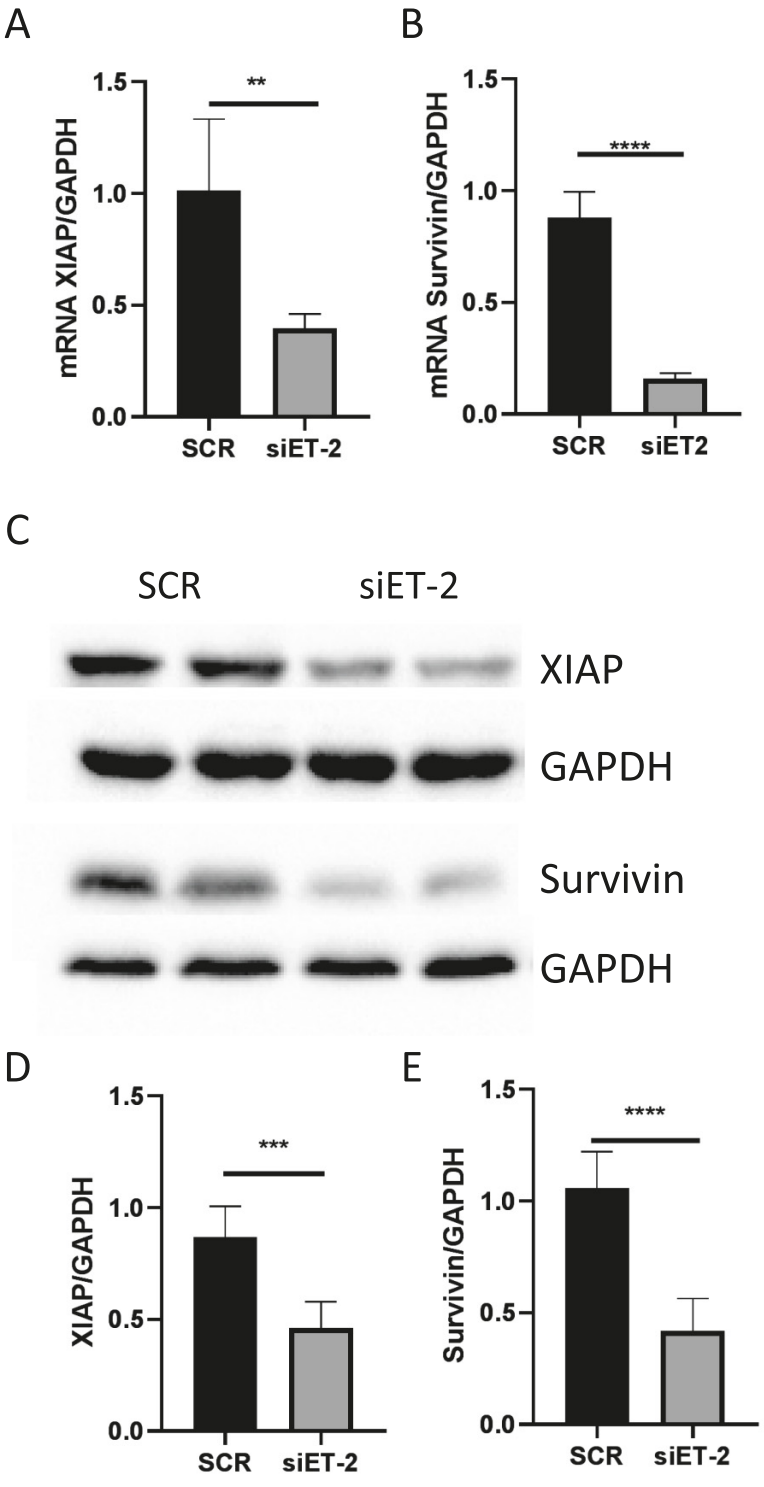


division. Survivin is a component of the heterotetramer chromosomal passenger complex, which is essential for chromosome segregation during mitosis and cytokinesis. XIAP is the most explored and potent member of the IAP family. XIAP interacts with and inhibits the activation of caspase-3, caspase-7, and caspase-9 (Takahashi et al. 1998; Srinivasula et al. 2000; Sun et al. 2000; Chai et al. 2001; Suzuki et al. 2001; Bratton et al. 2002). In addition, XIAP ubiquitinates active caspase-3 and targets it for proteasomal degradation via its E3 ligase activity (Suzuki et al. 2001). Furthermore, survivin and XIAP form a survivin–XIAP complex. This complex promotes XIAP stability against ubiquitination/proteasomal destruction and synergistically inhibits caspase-9 activation (Dohi et al. 2004). Accumulating evidence has shown that dual inhibition of survivin and XIAP effectively inhibits cancer proliferation and migration and induces apoptosis (Cao et al. 2004; Yi et al. 2015; Werner et al. 2017; Li et al. 2018; Chang et al. 2020; Fang et al. 2020). Therefore, the inhibition of ET-2 could be an effective strategy to inhibit LUAD progression through the dual inhibition of XIAP–survivin.

We also observed that ET-2 ablation inhibited the EMT. EMT is an important phenomenon involved in the metastasis, recurrence, and drug resistance of lung cancer. In addition, EMT progression is associated with a significantly poor prognosis in patients with LUAD (Sowa et al. 2015). XIAP has been reported to play a role in the migration of cancer cells, partly via the inhibition of EMT (Jin et al. 2019). Eventually, ET-2 depletion reduced the migration and invasion of A549 LUAD cells, partly through the inhibition of EMT.

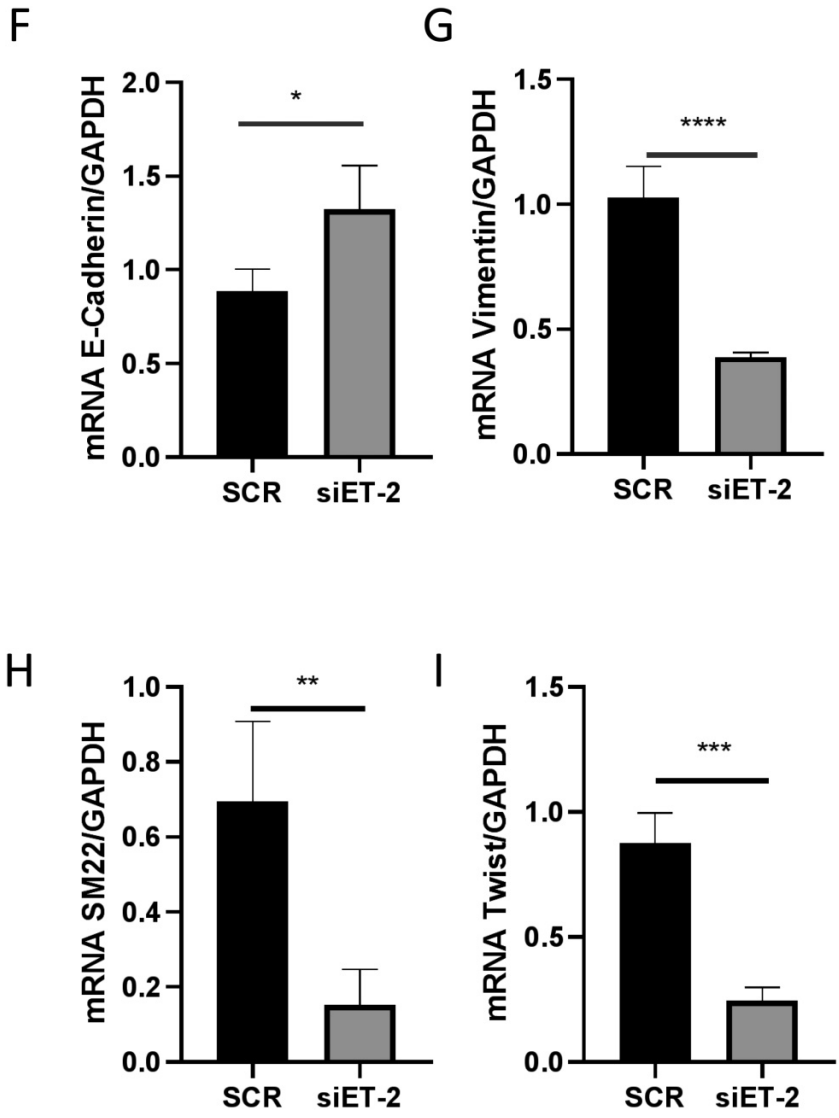
ET signaling elicits multiple pathways that lead to cancer progression and invasiveness. Numerous preclinical studies have revealed the beneficial effects of ET receptor antagonists in cancer treatment. However, clinical trials using ET receptor antagonists have shown poor results in various cancers,

Figure 5. Downregulation of endothelin (ET)-2 suppressed the X-linked inhibitor of apoptosis (XIAP)–survivin and epithelial-mesenchymal transition (EMT) marker. (A, B) XIAP–survivin mRNA and (C–E) protein levels were reduced in ET-2 silencing. EMT markers were measured using quantitative real-time polymerase chain reaction. (F) E-cadherin mRNA was increased by ET-2 silencing. (G–I) Vimentin, SM22, and Twist mRNA were reduced by ET-2 silencing. Data were analyzed using a two-tailed Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.



including NSCLC. This gives rise to other strategies for ET receptor antagonist treatment, such as the development of an anti-ET_B receptor antibody–drug conjugate to the antimitotic agent monomethyl auristatin E (Sandhu et al. 2020), targeting the ET1 receptor/β-arr1/YAP/mutp53 oncogenic network in both tumor and stromal cells (Tocci et al. 2021), and development of a new paradigm for adjuvant therapy, for instance, repurposing ET receptor antagonists as vasodilators, thus

Figure 5.



improving drug distribution to the tumor (Pulido et al. 2020). In this regard, our study showed that loss of ET-2 exhibited dual targeting of XIAP and survivin, which revealed the potency of targeting ET-2 for LUAD treatment.

More in vitro experiments using other LUAD cell lines, in vivo studies, and associated molecular mechanisms including the identification of the receptor subtypes by which the action of ET-2 is mediated in these cells are necessary to further explore the pathophysiological functions of ET-2 in cancer. Nevertheless, our findings provide insights into the participation of ET-2 in LUAD and suggest a plausible therapeutic target for LUAD.

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Data availability

All data are contained within the manuscript.

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Author contributions

RPS and NE, conceptualization; RPS, data curation; RPS, formal analysis; NE, funding acquisition; RPS, YS, and TN, investigation; K-IH and NE, supervision; RPS and NE, writing—original draft.

Competing interests

The authors declare there are no competing interests.

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