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Original Article

lncRNA ASBEL and lncRNA Erbb4-IR reduce chemoresistance against gemcitabine and cisplatin in stage IV lung squamous cell carcinoma via the microRNA-21/LZTFL1 axis

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Abstract: Drug resistance is a major cause of treatment failure and post-treatment disease progression in patients with cancer. This study aimed to investigate the mechanisms of chemoresistance to gemcitabine (GEM) plus cisplatin (cis-diamminedichloroplatinum, DDP) combination therapy in stage IV lung squamous cell carcinoma (LSCC). It also examined the functional role of lncRNA ASBEL and lncRNA Erbb4-IR in the malignant progression of LSCC. The expression of lncRNA ASBEL, lncRNA Erbb4-IR, miR-21, and LZTFL1 mRNA was examined in human stage IV LSCC tissues and adjacent normal tissues, human LSCC cells and normal human bronchial epithelial cells using qRT-PCR. Furthermore, LZTFL1 protein levels were also examined using western blots. Cell proliferation, cell migration and invasion, and cell cycle progression and apoptosis were evaluated *in vitro* using the CCK-8, transwell, and flow cytometry assays, respectively. Based on the treatment response, LSCC tissues were classified as GEM-, DDP-, and GEM+DDP-sensitive/resistant. The MTT assay was performed to assess the chemoresistance of human LSCC cells to GEM, DDP, and GEM+DDP following transfection experiments. The results showed that lncRNA ASBEL, lncRNA Erbb4-IR, and LZTFL1 were down-regulated in human LSCC tissues and cells, whereas miR-21 was up-regulated. In stage IV human LSCC tissues, miR-21 levels were negatively correlated with those of lncRNA ASBEL, lncRNA Erbb4-IR, and LZTFL1 mRNA. The overexpression of lncRNA ASBEL and lncRNA Erbb4-IR inhibited cell proliferation, migration, and invasion. It also blocked cell cycle entry and accelerated apoptosis. These effects were mediated by the miR-21/LZTFL1 axis and reduced chemoresistance to GEM+DDP combination therapy in stage IV human LSCC. These findings indicate that lncRNA ASBEL and lncRNA Erbb4-IR function as tumor suppressors in stage IV LSCC and attenuate chemoresistance to GEM+DDP combination therapy via the miR-21/LZTFL1 axis. Hence, lncRNA ASBEL, lncRNA Erbb4-IR, and LZTFL1 may be targeted to enhance the efficacy of GEM+DDP combination chemotherapy against LSCC.

Keywords: Advanced lung squamous cell carcinoma, tumor suppressor, gemcitabine and cisplatin combination therapy, chemoresistance, lncRNA ASBEL/lncRNA Erbb4-IR/miR-21/LZTFL1 axis

Introduction

Lung cancer is a common malignancy with a high global incidence and mortality, making it an important public health concern worldwide. According to estimates, there were 2.1 million cases of newly diagnosed lung cancer and 1.8 million deaths due to lung cancer in 2018 [1]. Non-small-cell lung carcinoma (NSCLC) accounts for approximately 85% of all lung cancer cases, and it is associated with a high risk of metastasis and poor outcomes [2]. The second most common type of NSCLC is lung squamous cell carcinoma (LSCC), which alone accounts for about 40% of all lung cancers. The prognosis of LSCC is poor, and few targeted agents are available for its treatment [3, 4]. Moreover, the early symptoms of LSCC are non-specific, and the disease is often only diagnosed at an advanced stage. As a result, most patients cannot benefit from surgical treatment. Radiotherapy and chemotherapy are the main therapeutic options for advanced LSCC. Recently, several studies have shown that first-line treatment with a com-

bination of gemcitabine (GEM) and cisplatin (cis-diamminedichloroplatinum, DDP) provides good treatment efficacy against advanced LSCC [5]. However, owing to chemoresistance and high metastasis rates, the 5-year survival rate in patients with advanced LSCC remains dismal (less than 15%) [6, 7]. Hence, new strategies are urgently required to prevent chemoresistance against GEM and DDP in cases of advanced LSCC.

Accumulating evidence indicates that the dysregulation of non-coding RNAs, including long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), plays a role in the mechanisms of chemoresistance. Non-coding RNAs are known to regulate genes involved in drug resistance and can accelerate malignant behaviors such as cell proliferation and suppressed apoptosis in various cancers [8]. Several studies have confirmed that microRNA-21 (miR-21) is closely involved in the development of lung cancers (including LSCC) and can serve as a diagnostic marker and therapeutic target for this malignancy [9, 10]. miR-21 overexpression has been observed in the serum, plasma, and pleural lavage fluid of patients with lung cancer. It has also been detected in lung tumor-associated fibroblasts and lung cancer cells and tissues. miR-21 overexpression can increase cell proliferation, migration, and invasion and decrease apoptosis in lung cancer cells [2, 9-15]. Additionally, miR-21 overexpression can promote resistance to DDP in lung cancer cells [16]. Interestingly, miR-21 up-regulation facilitates GEM resistance in cancer cells and its down-regulation is known to promote GEM chemosensitivity [17, 18]. Nevertheless, the effect of miR-21 on the sensitivity of LSCC to GEM alone or GEM+DDP has not been investigated, and the corresponding mechanisms remain unclear. The lncRNAs ASBEL and Erbb4-IR have been reported to be upstream regulators of miR-21 and play a critical role in the malignant progression of osteosarcoma and prostate carcinoma via the regulation of miR-21 expression [19-22]. However, the expression and function of lncRNA ASBEL and lncRNA Erbb4-IR have not been examined in lung cancer. Moreover, their effects on the sensitivity of cancer cells to GEM and/or DDP have not been explored. Leucine zipper transcription factor-like 1 (*LZTFL1*), a verified target gene of miR-21 [23], is known to exert an inhibitory effect on pulmonary tumor formation [24]. However, no previous study has investigated the correlation between LZTFL1 expression and GEM or DDP sensitivity, and whether LZTFL1 is regulated by lncRNA ASBEL or lncRNA Erbb4-IR is also unclear. Furthermore, the expression and functions of LZTFL1 in LSCC have not been reported.

Based on previous studies, we hypothesized that the lncRNA ASBEL/lncRNA Erbb4-IR/miR-21/LZTFL1 axis can modulate the malignant behavior of LSCC cells and thereby regulate chemoresistance to GEM and DDP combination therapy in stage IV LSCC. We tested this hypothesis in the present study to uncover the mechanisms underlying GEM/DDP chemoresistance in LSCC.

Materials and methods

Patients and clinical samples

In this study, 120 randomly selected pairs of LSCC tissue samples and adjacent normal tissue samples were examined. In all cases, patients had stage IV LSCC (graded according to the TNM staging system). Of the 120 patients, 82 were men and 38 were women (mean age, 58.4 ± 11.2 years; range, 44-75 years). All patients had been diagnosed with LSCC between July 2019 and July 2020 in our hospital. All diagnoses were based on the clinical histopathology of fiberoptic bronchoscopy specimens.

The patients received treatment with DDP alone, GEM alone, or DDP+GEM and did not undergo any surgery, radiotherapy, or other anti-cancer treatment. Patients were excluded if they: 1) Were frail and old (> 75 years); 2) Had severe heart, liver, or kidney dysfunction; 3) Had poor bone marrow function, low leukocyte levels, or thrombocytopenia; 4) Had complications, infection, or fever or were prone to bleeding; 5) Had metastatic lung cancer or other malignant tumors; 6) Had incomplete medical records; or 7) Had mental illness.

The patients were randomly divided into three groups of 40 patients each and received drug treatment based on previously published literature [25]: DDP alone (75 mg/m^2, administered once a day), GEM alone (1000 mg/m^2, administered once a week), or DDP plus GEM (combination of both DDP and GEM). Three courses of

treatment, each lasting 21 d, were conducted. Patients were further subdivided into sensitive (complete or partial response) and resistant groups (stable or progressive disease) according to their drug response.

Tissue samples were collected before and after treatment under fiberoptic bronchoscopy, immediately placed in liquid nitrogen, and then stored at -80°C for further experiments. Ethics approval was obtained from the ethics committee of the Affiliated Hospital of Chengde Medical University (Ethical approval number: LL2021028), and informed consent was obtained from the patients or their families before study initiation.

Cell lines and cell culture

Human LSCC cell lines (H226 and H2170) and a normal human bronchial epithelial cell line (16HBE) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium (Gibco: Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone: GE Healthcare Life Sciences, Logan, UT, USA), penicillin (100 U/ml, Gibco, BRL), and streptomycin (0.1 mg/ml, Gibco, BRL) at 37°C in humidified air containing 5% CO_2.

Cell transfection

A pcDNA3.1 vector containing lncRNA Erbb4-IR, pcDNA3.1 vector containing lncRNA ASBEL, and empty vectors (as negative controls, NCs) were constructed by Sangon Biotech Co., Ltd. A miR-21 mimic and NC miRNA were purchased from Sigma-Aldrich (Merck KGaA). A pcDNA3.1-LZTFL1 overexpression vector and the corresponding NC (empty vector) were purchased from Genechem (Shanghai, China). After 12 h of culture, cells were transfected with one of the following vector combinations or their corresponding NCs: pcDNA3.1-lncRNA ASBEL, pcDNA3.1-lncRNA Erbb4-IR, miR-21 mimic, pcDNA3.1-LZTFL1, pcDNA3.1-lncRNA ASBEL+miR-21 mimic, pcDNA3.1-lncRNA Erbb4-IR+miR-21 mimic, pcDNA3.1-lncRNA ASBEL+miR-21 mimic+pcDNA3.1-LZTFL1, or pcDNA3.1-lncRNA Erbb4-IR+miR-21 mimic+pcDNA3.1-LZTFL1. Transfection was performed using the Lipofectamine 2000 reagent (Invitrogen, USA) based on the manufacturer's instructions. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed in order to verify the transfection efficiency. Follow-up experiments were performed 24 h after transfection.

qRT-PCR

The expression levels of lncRNA ASBEL, lncRNA Erbb4-IR, miR-21, and LZTFL1 mRNA were measured using qRT-PCR in human LSCC tumor tissues and paired adjacent tissues before and after treatment. They were also examined in normal cells and human LSCC cells transfected with different constructs. Total RNA was extracted from tissues and cells using the TRIzol reagent (Invitrogen, USA) and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, CA, USA) based on the manufacturer's instructions. The First Chain cDNA synthesis kit (Thermofizrre) was used to reverse transcribe the cDNA. DNA contamination was removed using RNAse-free DNase I. qRT-PCR was performed based on previously published protocols [22]. The human U6 gene was used as the endogenous control for miR-21, and GAPDH was used as the endogenous control for lncRNA ASBEL, lncRNA Erbb4-IR, and LZTFL1. All experiments were repeated thrice and relative expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

Western blotting (WB)

The protein expression of LZTFL1 was examined in human LSCC tumor tissues and matched adjacent normal tissues before and after treatment using WB. It was also examined in H226, H2170, and 16HBE cells before and after the transfection of various constructs (pcDNA3.1-lncRNA ASBEL, pcDNA3.1-lncRNA Erbb4-IR, pcDNA3.1-LZTFL1, and empty vector). WB experiments were performed as described previously [23, 26]. Briefly, the total protein was extracted from cells and tissues using the RIPA lysis buffer (Beyotime, Shanghai), and protein concentrations were determined with a BCA assay kit (Beyotime, Shanghai). A protease inhibitor cocktail (Beyotime, China) was added to the extracts, and the proteins were separated using SDS-PAGE. Separated proteins were transferred onto PVDF membranes (Millipore, USA), which were blocked and incubated with a primary antibody against LZTFL1 (1:1,000, Abcam, USA) at 4°C overnight. Next, the membranes were incubat-

ed with a goat anti-rabbit IgG-HRP secondary antibody (1:5,000, Abcam, USA) at 37°C for 1 h. The protein expression was normalized using GAPDH as the internal control (Santa Cruz, Dallas, TX, USA; sc-32233) and quantified using Image J software (National Institutes of Health, Bethesda, Maryland).

Pearson correlation coefficient analysis

Briefly, the relationship between the expression of miR-21 and that of lncRNA ASBEL, lncRNA Erbb4-IR, and LZTFL1 in human LSCC tumor tissues was analyzed using Pearson correlation coefficient analysis.

Cell proliferation assay

Eight hours after transfection, H226 and H2170 cells were seeded into a 96-well culture plate (BD Biosciences, USA) (2500 cells/well) for cell proliferation assays, as described previously [27]. After incubation for 0, 24, 48, and 72 h, 10 μL of Cell Counting Kit-8 (CCK-8, Dojindo, Japan) solution was added to each well to examine cell proliferation according to the manufacturer's directions. Cell proliferation was quantified based on optical density (OD) values at 450 nm, which were measured using a Varioskan Flash Microplate reader (Thermo Fisher Scientific, USA).

Cell migration and invasion assay

Cell migration and invasion capacities were examined using transwell assays without and with Matrigel, respectively. Cell treatments were performed as described for the proliferation assay. To specifically evaluate the cell migration and invasion capacities of treated cells, transwell assays were performed using previously published protocols [23, 28].

Cell apoptosis and cell cycle assay

Before flow cytometry cells were treated as described for the proliferation assay. Flow cytometry was performed to detect apoptotic cells and examine cell cycle progression as described previously [29, 30]. For apoptosis analysis, cells were double stained with FITC-labeled Annexin V and propidium iodide (PI) (Sigma-Aldrich). Apoptotic cell counts were obtained using FACsorter (BD Biosciences, San Jose, CA, USA). For cell cycle analysis, cells were stained with PI alone, and cell cycle distribution was observed using FACsorter.

MTT cell viability assay

In order to determine the effects of the lncRNA ASBEL/lncRNA Erbb4-IR/miR-21/LZTFL1 axis on the sensitivity of H226 and H2170 cells to GEM, DDP, and GEM+DDP, the viability of H226 and H2170 cells was examined after transfection with the following constructs: empty vector, pcDNA3.1-lncRNA ASBEL, pcDNA3.1-lncRNA Erbb4-IR, pcDNA3.1-lncRNA ASBEL+miR-21 mimic, pcDNA3.1-lncRNA Erbb4-IR+miR-21 mimic, pcDNA3.1-lncRNA ASBEL+miR-21 mimic+ pcDNA3.1-LZTFL1, or pcDNA3.1-lncRNA Erbb4-IR+miR-21 mimic+pcDNA3.1-LZTFL1. Cells were treated with DDP, GEM, or DDP+GEM, and their viability was assessed using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 25 μl, 5 mg/ml, M2128, Sigma) assay based on previous protocols [29].

Briefly, cells were seeded in a 96-well culture plate (BD Biosciences, USA) (2500 cells/well) and treated with different concentrations of DDP (0, 0.5, 1, 2, 4, 8, 16, 32, and 64 mg/ml) (Shandong Qilu Co. China) for 48 h [31], different concentrations of GEM (0, 12.5, 25, 50, 100, and 200 μM) (Jiang-su, HaoSeng Pharmaceutical, China) for 24 h [32], or with GEM plus DDP for 24 h. Following 4 h of incubation with MTT in the dark at 37°C, the absorbance of each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The viability of control cells was considered to be 100%. The sensitivity of the cells to DDP/GEM was assessed based on the half-maximal inhibitory concentration (IC50).

Statistical analysis

All data management and analyses were performed using SPSS software (version 20), and data were presented as the mean ± standard deviation (SD) or number (n) and percentage/rate (%). Differences in quantitative data were analyzed using independent-samples t-tests and Student's t-tests (for comparing two groups) or one-way ANOVA (for comparing more than two groups). The differences in percentages/rates were analyzed using the χ^2 test, non-parametric Mann-Whitney U test, or Fisher's exact test. All continuous data were subjected to normality tests. Correlations analyses for quantitative data were performed using Pearson correlation analysis, and linear

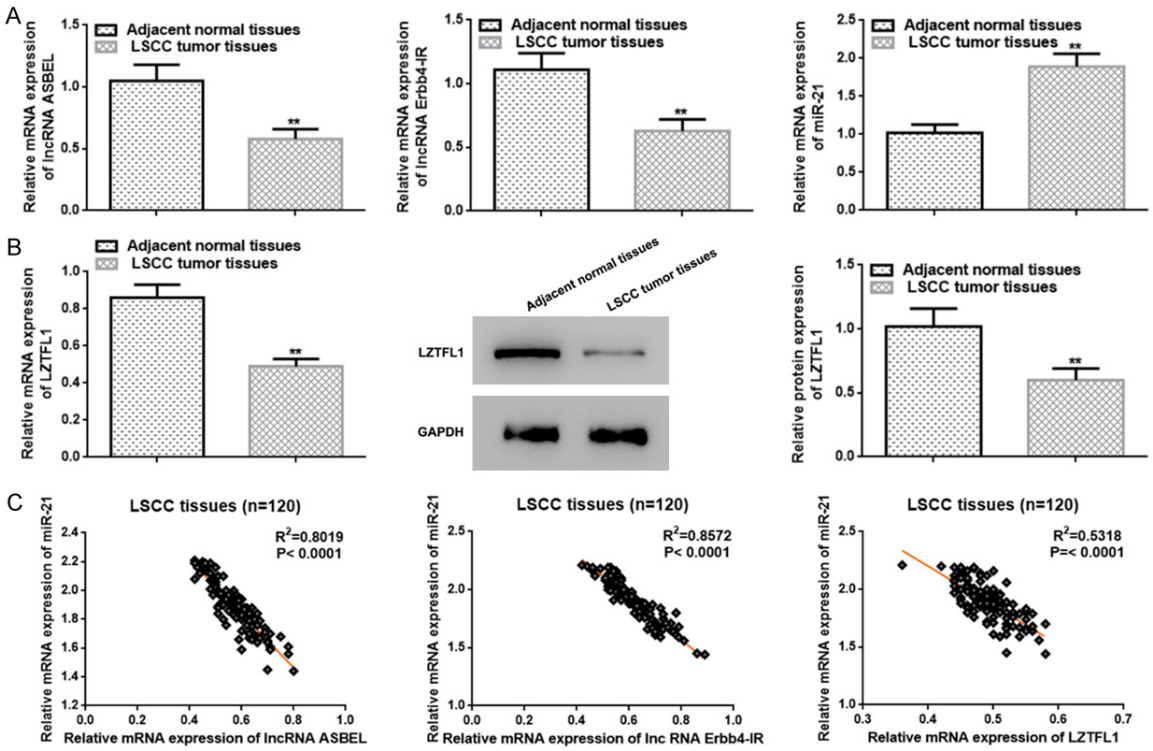

Figure 1. Expression of lncRNA ASBEL, lncRNA Erbb4-IR, miR-21, and LZTFL1 in stage IV human LSCC tumor tissues. A. Expression of lncRNA ASBEL, lncRNA Erbb4-IR, and miR-21 in clinical stage IV LSCC tissue specimens examined using qRT-PCR. B. LZTFL1 mRNA and protein levels in stage IV LSCC tumor tissues examined using qRT-PCR and western blot, respectively. C. Correlation between the expression levels of miR-21 and the expression levels of lncRNA ASBEL, lncRNA Erbb4-IR, and LZTFL1 mRNA in LSCC tissues (Pearson correlation analysis). ** vs adjacent normal tissues, $P < 0.01$.

regression analysis was also carried out. The significance level was set at a *P*-value < 0.05.

Results

Expression of lncRNA ASBEL, lncRNA Erbb4-IR, LZTFL1, and miR-21 in stage IV LSCC tissues

The expression levels of lncRNA ASBEL, lncRNA Erbb4-IR, miR-21, and LZTFL1 mRNA were detected in untreated stage IV human LSCC tumor tissues and matched adjacent normal tissues using qRT-PCR. Moreover, the protein level of LZTFL1 was also estimated using WB. The results showed that lncRNA ASBEL and lncRNA Erbb4-IR were down-regulated in clinical stage IV LSCC tumor tissues when compared with matched adjacent normal tissues, while miR-21 was up-regulated ($P < 0.01$) (**Figure 1A**). Meanwhile, LZTFL1 mRNA and protein levels were markedly lower in LSCC tumor tissues than in normal tissues ($P < 0.01$) (**Figure 1B**). Interestingly, a negative correlation was observed between the expression of miR-21 and the expression of lncRNA ASBEL, lncRNA Erbb4-IR, and LZTFL1 mRNA in LSCC tumor tissues ($P < 0.0001$) (**Figure 1C**).

Expression of lncRNA ASBEL, lncRNA Erbb4-IR, LZTFL1, and miR-21 in human LSCC cells

We measured lncRNA ASBEL, lncRNA Erbb4-IR, miR-21, and LZTFL1 mRNA levels in human LSCC cells using qRT-PCR. Furthermore, LZTFL1 protein levels were measured using WB. The findings were similar to those observed in clinical tumor tissues. The levels of lncRNA ASBEL, lncRNA Erbb4-IR, and LZTFL1 mRNA were lower in H226 and H2170 cells (human LSCC cells) than in 16HBE cells (normal human bronchial epithelial cells), while the levels of miR-21 were higher ($P < 0.01$) (**Figure 2A**). Furthermore, LZTFL1 protein levels were lower in H226 and H2170 cells than in 16HBE cells ($P < 0.01$) (**Figure 2B**).

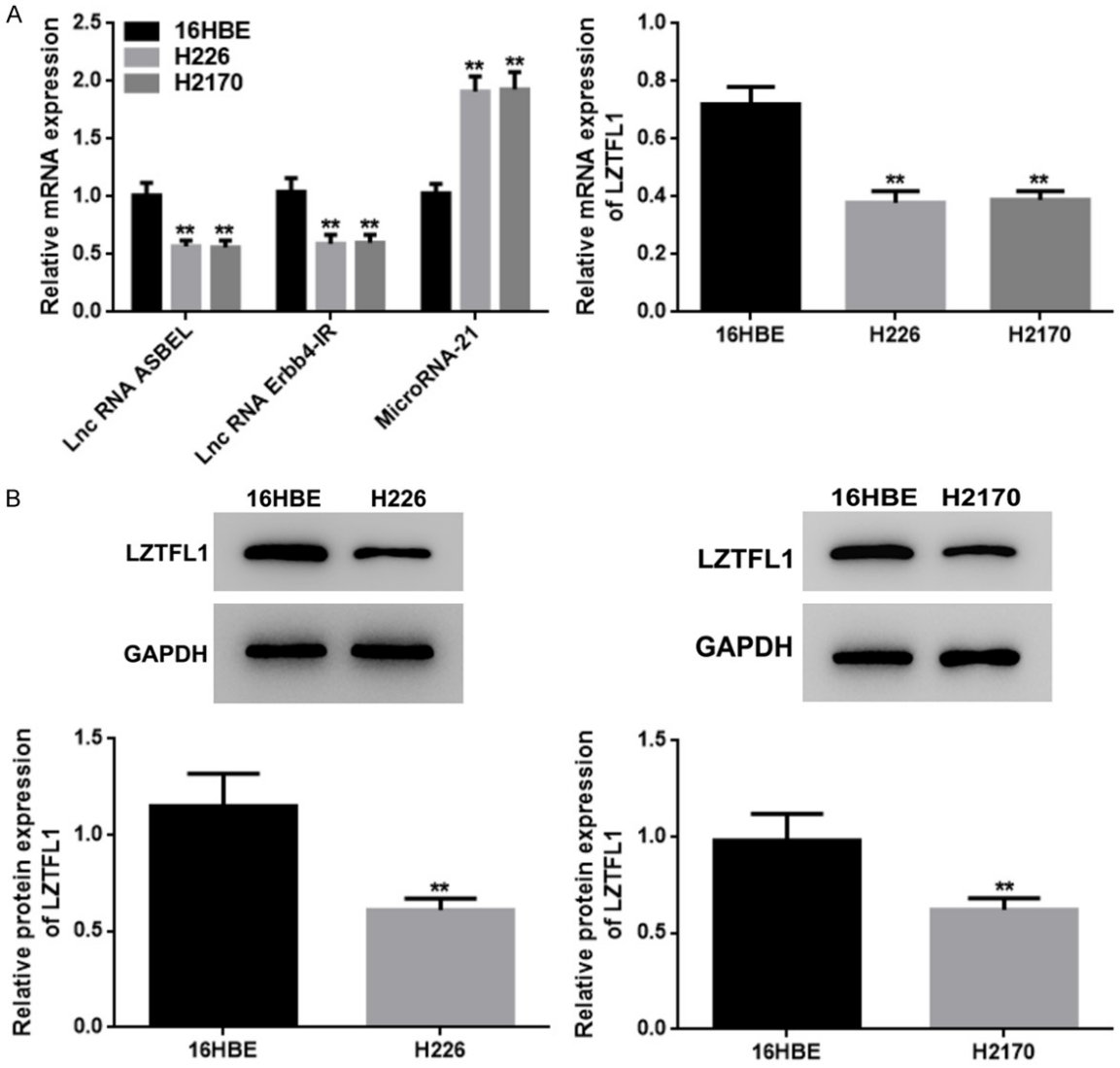

Figure 2. Expression of lncRNA ASBEL, lncRNA Erbb4-IR, miR-21, and LZTFL1 in human LSCC cells. A. Expression of lncRNA ASBEL, lncRNA Erbb4-IR, miR-21, and LZTFL1 mRNA in human LSCC cells examined using qRT-PCR. B. Protein levels of LZTFL1 in human LSCC cells examined using western blot. ** vs 16HBE cells, $P < 0.01$.

Effect of lncRNA ASBEL and lncRNA Erbb4-IR overexpression on miR-21 expression, LZTFL1 expression, and malignant behaviors in LSCC cells

As shown in **Figure 3A**, the expression of lncRNA ASBEL and lncRNA Erbb4-IR was significantly higher in H226 and H2170 cells transfected with pcDNA3.1-lncRNA ASBEL and pcDNA3.1-lncRNA Erbb4-IR, respectively, than in the cells transfected with the empty vector ($P < 0.01$). These results indicated that lncRNA ASBEL and lncRNA Erbb4-IR were successfully overexpressed in H226 and H2170 cells. As shown in **Figure 3B-D**, the overexpression of lncRNA ASBEL and lncRNA Erbb4-IR led to a decrease in miR-21 levels and an increase in LZTFL1 mRNA and protein levels in H226 and H2170 cells ($P < 0.05$). The lncRNA ASBEL and lncRNA Erbb4-IR in H226 and H2170 cells significantly reduced cell proliferation at 48 h ($P < 0.05$) and 72 h ($P < 0.01$) (**Figure 3E**) post-transfection, as indicated by lower OD at 450 nm in the CCK-8 assay. As shown in **Figures 4** and **5A**, the number of migratory and invasive cells was remarkably lower after the overexpression of lncRNA ASBEL and lncRNA Erbb4-IR than after vector transfection, while the

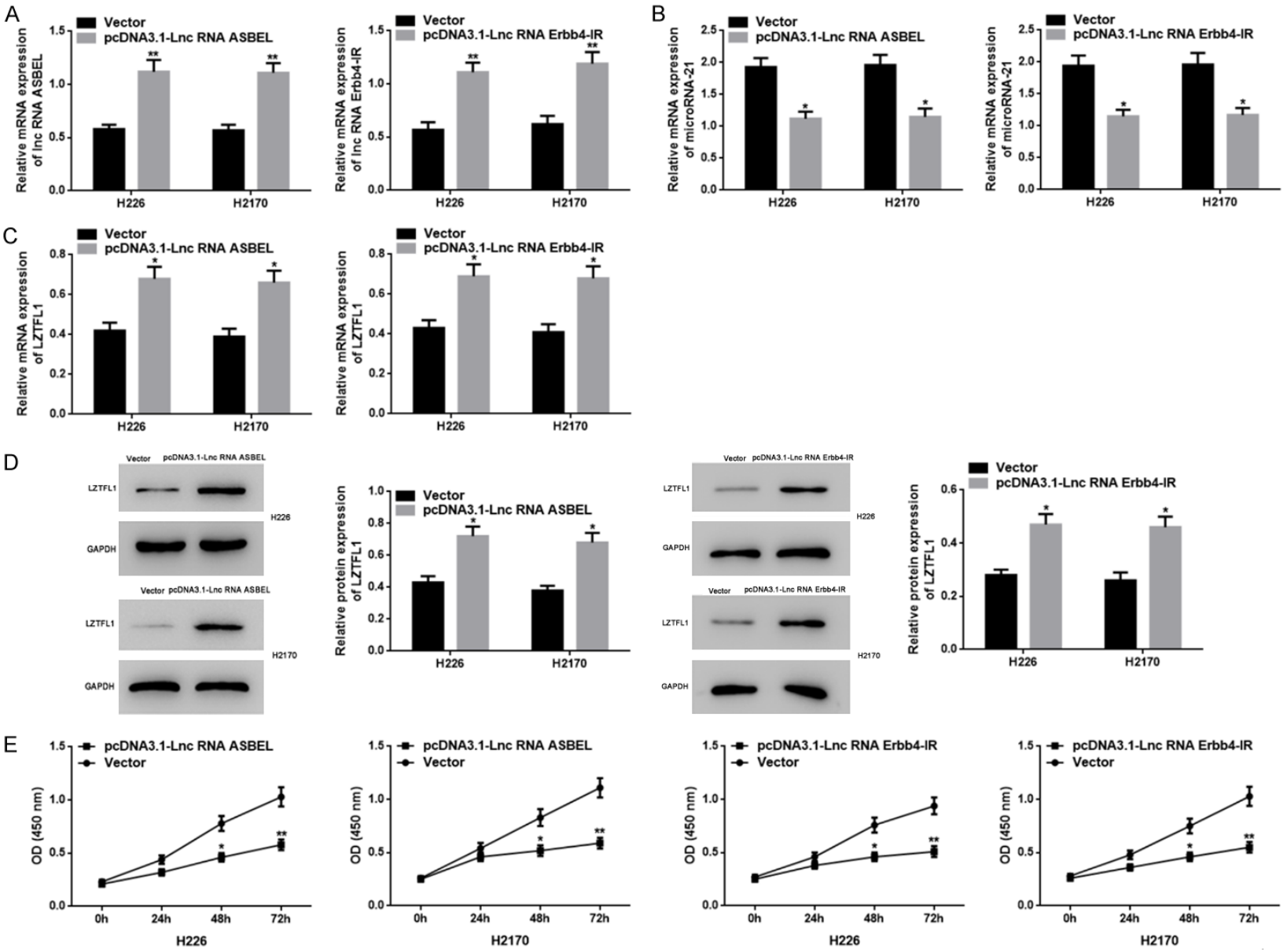

Figure 3. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR decreased miR-21 expression, increased LZTFL1 expression, and suppressed cell proliferation in human LSCC cells. A. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR in human LSCC H226 and H2170 cells via the transfection of pcDNA3.1-lncRNA ASBEL

and pcDNA3.1-lncRNA Erbb4-IR, respectively. B. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR decreased miR-21 expression in H226 and H2170 cells. C. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR increased LZTFL1 mRNA levels in H226 and H2170 cells. D. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR increased in LZTFL1 protein levels in H226 and H2170 cells. E. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR suppressed the proliferation of H226 and H2170 cells, as demonstrated by the CCK-8 assay. * vs Vector group, $P < 0.05$; ** vs Vector group, $P < 0.01$.

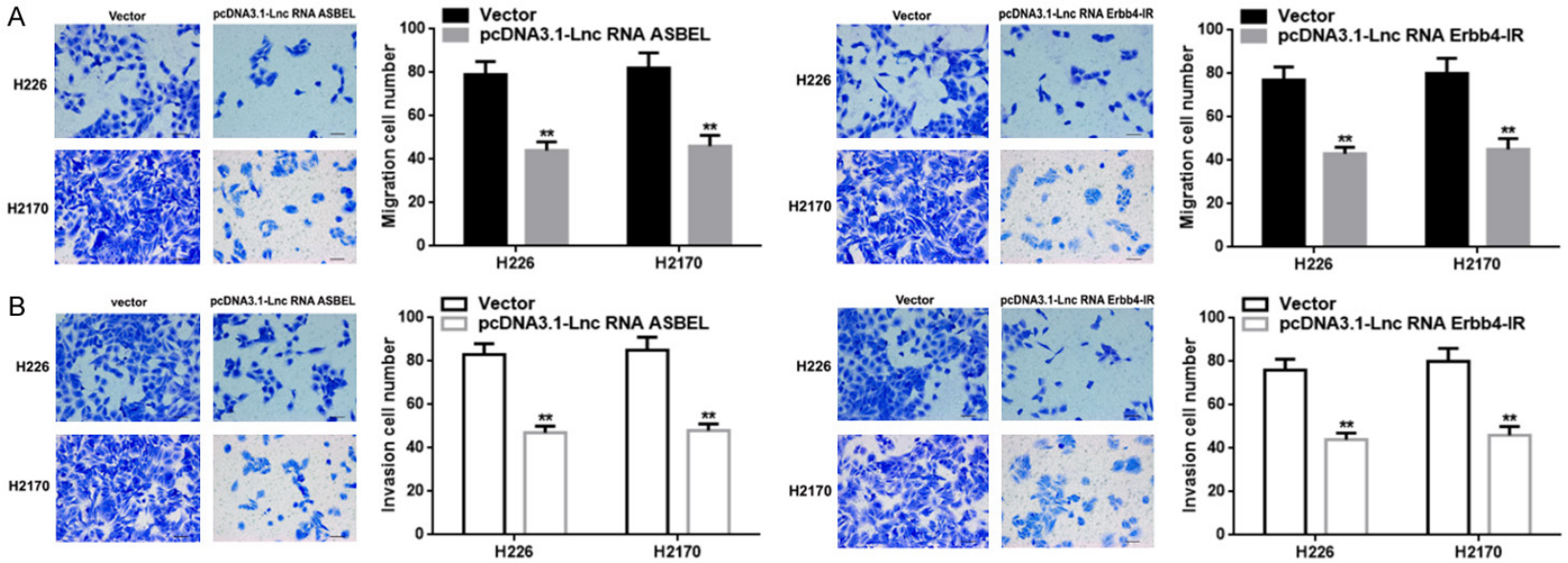

Figure 4. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR significantly inhibited cell migration and invasion in human LSCC cells. (A, B) Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR reduced the number of migratory (A) and invasive cells (B), as demonstrated by the transwell assay. Scale bar = 8 μm. ** vs Vector group, $P < 0.01$.

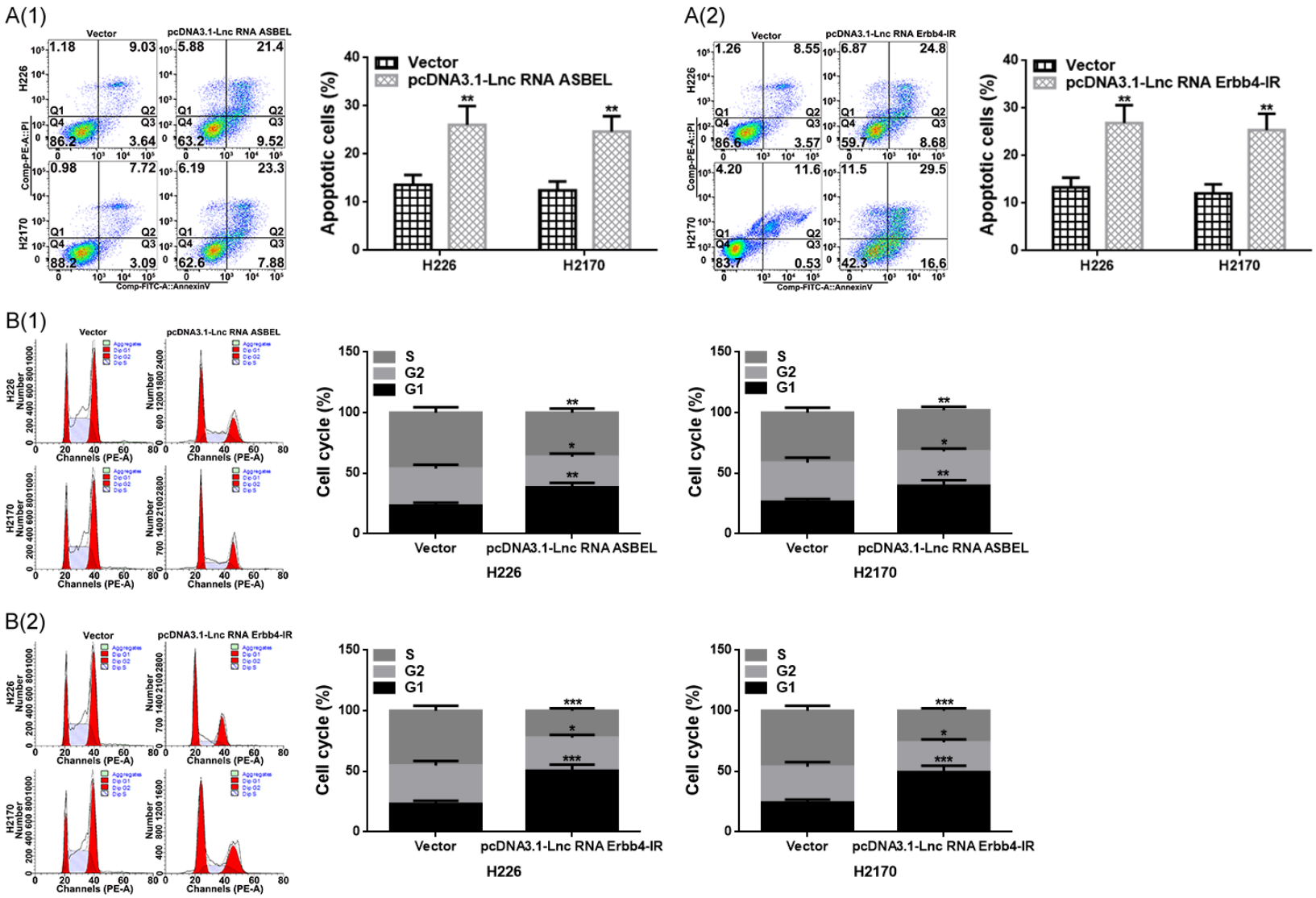

Figure 5. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR facilitated apoptosis and inhibited cell cycle entry. (A, B) Flow cytometry showed that the overexpression of lncRNA ASBEL and lncRNA Erbb4-IR increased the rate of cell apoptosis (A) and inhibited cell cycle progression (B), leading to an increased proportion of cells in G1 arrest and a decreased proportion of cells in the S and G2 phase. * vs Vector group, $P < 0.05$; ** vs Vector group, $P < 0.01$.

number of apoptotic cells was significantly higher ($P < 0.01$). Both the lncRNA ASBEL and lncRNA Erbb4-IR groups showed a clear trend of decreased cell cycle entry, with fewer cells in the S and G2 stages and more cells showing cell cycle arrest in the G1 phase ($P < 0.05$ or $P < 0.01$; **Figure 5B**).

Role of the miR-21/LZTFL1 axis in mediating the effects of lncRNA ASBEL and lncRNA Erbb4-IR overexpression on malignant behaviors in LSCC cells

As shown in **Figure 6A** and **6B**, H226 and H2170 cells transfected with the miR-21 mimic showed markedly higher miR-21 levels and lower LZTFL1 mRNA levels than the cells transfected with miR-NC ($P < 0.01$). However, the miR-21 mimic did not affect the expression levels of lncRNA ASBEL and lncRNA Erbb4-IR ($P > 0.05$, **Figure 6C**, **6D**). H226 and H2170 cells transfected with pcDNA3.1-LZTFL1 showed remarkedly higher LZTFL1 mRNA and protein levels than the cells transfected with an empty vector (**Figure 6E**, **6F**) ($P < 0.01$). However, they did not show altered levels of lncRNA ASBEL and lncRNA Erbb4-IR ($P > 0.05$, **Figure 6G**, **6H**). Compared with the vector group, the pcDNA3.1-lncRNA ASBEL and pcDNA3.1-lncRNA Erbb4-IR groups showed lower rates of cell proliferation at 48 and 72 h post-transfection. These groups also showed a lower number of migratory and invasive cells and a higher number of apoptotic cells. Cell cycle entry was inhibited in these cells, and there were fewer cells in the S and G2 phases and more cells in the G1 phase ($P < 0.05$ or $P < 0.01$). Co-transfection with the miR-21 mimic could partially reverse the effects of pcDNA3.1-lncRNA ASBEL and pcDNA3.1-lncRNA Erbb4-IR transfection in H226 and H2170 cells ($P < 0.05$). The addition of a pcDNA3.1-LZTFL1 vector to pcDNA3.1-lncRNA ASBEL/pcDNA3.1-lncRNA Erbb4-IR+miR-21 mimic transfection attenuated the effects of miR-21 mimic transfection in H226 and H2170 cells ($P < 0.05$) (**Figures 6I-K**, **7** and **8**).

Modulation of chemoresistance to GEM plus DDP combination therapy in LSCC and the role of the miR-21/LZTFL1 axis

After chemotherapy, clinical LSCC tumor tissues were classified into drug-sensitive or drug-resistant groups based on their response to GEM alone, DDP alone, or GEM+DDP. In the GEM alone group, there were 18 GEM-resistant tumors and 22 GEM-sensitive tumors. In the DDP alone group, there were 16 DDP-resistant tumors and 24 DDP-sensitive tumors. In the GEM+DDP group, there were 15 (GEM+DDP)-resistant tumors and 25 (GEM+DDP)-sensitive tumors. Twenty adjacent normal tissue samples obtained before any treatment were randomly selected as controls for subsequent experiments. As shown in **Figure 9**, the levels of lncRNA ASBEL and lncRNA Erbb4-IR were lower in GEM-, DDP-, and (GEM+DDP)-sensitive tumor tissues than in normal tissues, while the levels of miR-21 were higher ($P < 0.05$ or $P < 0.01$). GEM/DDP-resistant tumor tissues showed lower levels of lncRNA ASBEL and lncRNA Erbb4-IR and higher levels of miR-21 than GEM/DDP-sensitive tumor tissues ($P < 0.05$). Similarly, (GEM+DDP)-sensitive tumor tissues exhibited lower expression levels of lncRNA ASBEL and lncRNA Erbb4-IR as well as higher expression levels of miR-21 than (GEM+DDP)-resistant tumor tissues ($P < 0.05$). Further analysis showed that the levels of lncRNA ASBEL and lncRNA Erbb4-IR were higher in (GEM+DDP)-sensitive tumor tissues than in GEM/DDP-resistant tumor tissues, while the levels of miR-21 were lower ($P < 0.05$). Similarly, the levels of lncRNA ASBEL and lncRNA Erbb4-IR were lower in (GEM+DDP)-resistant tumor tissues than in GEM/DDP-resistant tumor tissues, while the levels of miR-21 were higher ($P < 0.05$). These findings indicated that the curative effect of combination therapy with GEM plus DDP was superior to that of single-agent therapy with GEM or DDP alone. The most interesting finding (**Figure 9A(1)** and **9A(2)**) was that the trends of lncRNA ASBEL and lncRNA Erbb4-IR expression were consistent with the differences in LZTFL1 mRNA and protein levels between the groups ($P < 0.05$ or $P < 0.01$) (**Figure 9B**). The MTT assay was performed to obtain the IC50 values of GEM and DPP in H226 and H2170 cells following transfection experiments (**Figure 9C-E**). The overexpression of lncRNA ASBEL and lncRNA Erbb4-IR could reduce the IC50 value of DDP alone, GEM alone, and DDP+GEM in H226 and H2170 cells ($P < 0.05$ or $P < 0.01$). This trend was partly attenuated when the cells were transfected with a miR-21 mimic ($P < 0.05$). However, transfection with pcDNA3.1-LZTFL1 could partially reverse this attenuation effect ($P < 0.05$).

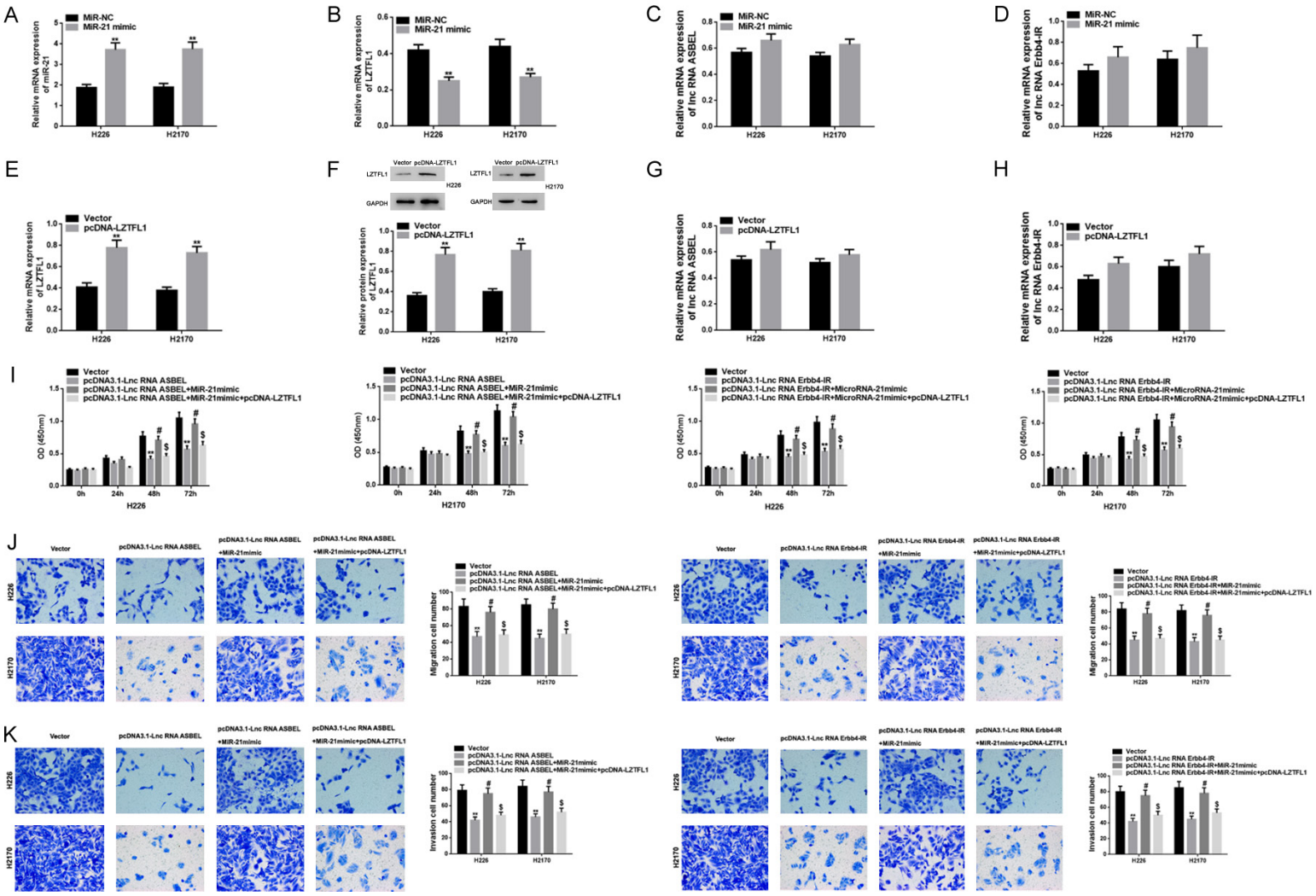

Figure 6. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR inhibited cell proliferation, migration, and invasion via the miR-21/LZTFL1 axis. A. Overexpression of miR-21 in human LSCC cells (H226 and H2170) through the transfection of a miR-21 mimic. ** vs miR-NC group, $P < 0.01$. B. Overexpression of miR-21 decreased LZTFL1 expression in H226 and H2170 cells, as demonstrated using qRT-PCR. ** vs miR-NC group, $P < 0.01$. C, D. Overexpression of miR-21 did not influence the expression of lncRNA ASBEL and lncRNA Erbb4-IR. E, F. LZTFL1 overexpression was successfully achieved following the transfection of pcDNA3.1-LZTFL1 into H226 and H2170 cells. ** vs Vector group, $P < 0.01$. G, H. Overexpression of LZTFL1 did not influence the expression of lncRNA ASBEL and lncRNA Erbb4-IR. I-K. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR inhibited cell proliferation, migration, and invasion in human LSCC cells by regulating the miR-21/LZTFL1 axis, as demonstrated using the CCK-8 assay, transwell assay without Matrigel, and transwell assay with Matrigel, respectively. Scale bar = 8 μm. ** vs Vector group, $P < 0.01$; # vs pcDNA3.1-lncRNA ASBEL, $P < 0.05$; $ vs pcDNA3.1-lncRNA ASBEL+miR-21 mimic, $P < 0.05$.

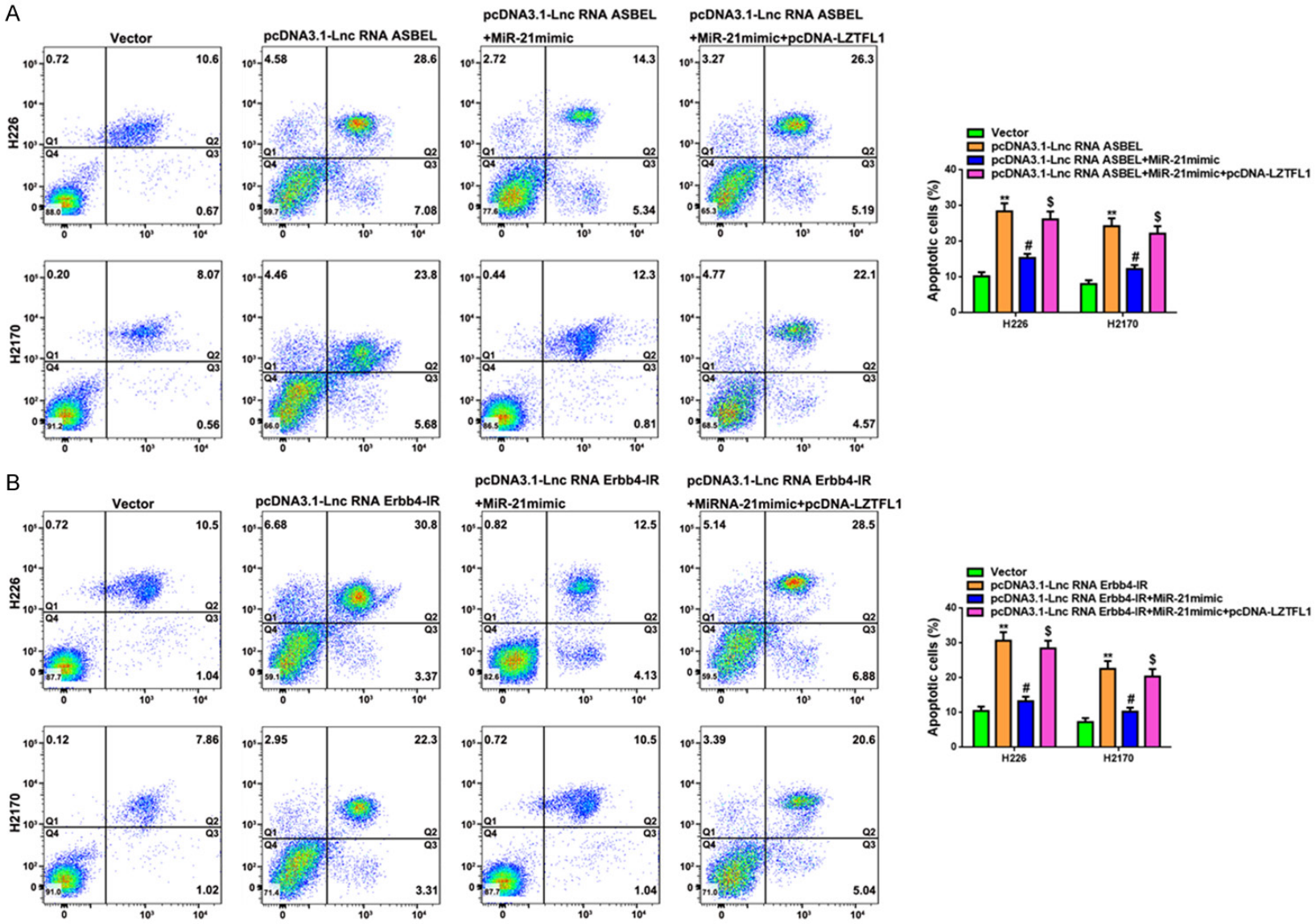

Figure 7. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR promoted apoptosis via the miR-21/LZTFL1 axis. A, B. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR increased the percentage of apoptotic H226 and H2170 cells by regulating the miR-21/LZTFL1 axis, as observed using flow cytometry. ** vs Vector group, $P < 0.01$; # vs pcDNA3.1-lncRNA ASBEL, $P < 0.05$; $ vs pcDNA3.1-lncRNA ASBEL+miR-21 mimic, $P < 0.05$.

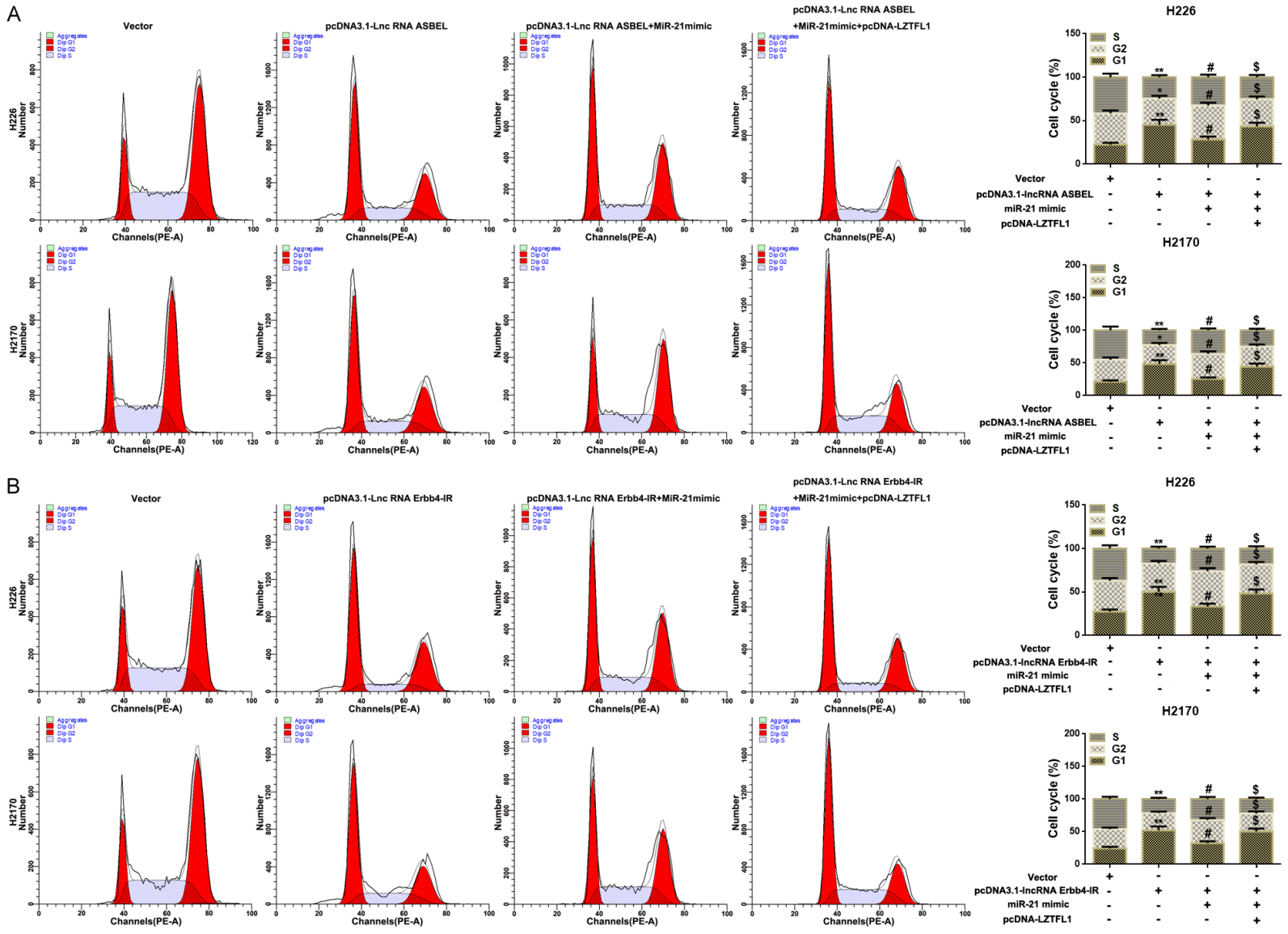

Figure 8. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR inhibited cell cycle entry via the miR-21/LZTFL1 axis. A, B. Flow cytometry showed that the overexpression of lncRNA ASBEL and lncRNA Erbb4-IR inhibited cell cycle entry in H226 and H2170 cells, leading to an increased proportion of cells in G1 arrest and a decreased proportion of cells in the S and G2 phase. This effect was achieved by the modulation of the miR-21/LZTFL1 axis. * vs Vector group, $P < 0.05$; ** vs Vector group, $P < 0.01$; # vs pcDNA3.1-lncRNA ASBEL, $P < 0.05$; $ vs pcDNA3.1-lncRNA ASBEL+miR-21 mimic, $P < 0.05$.

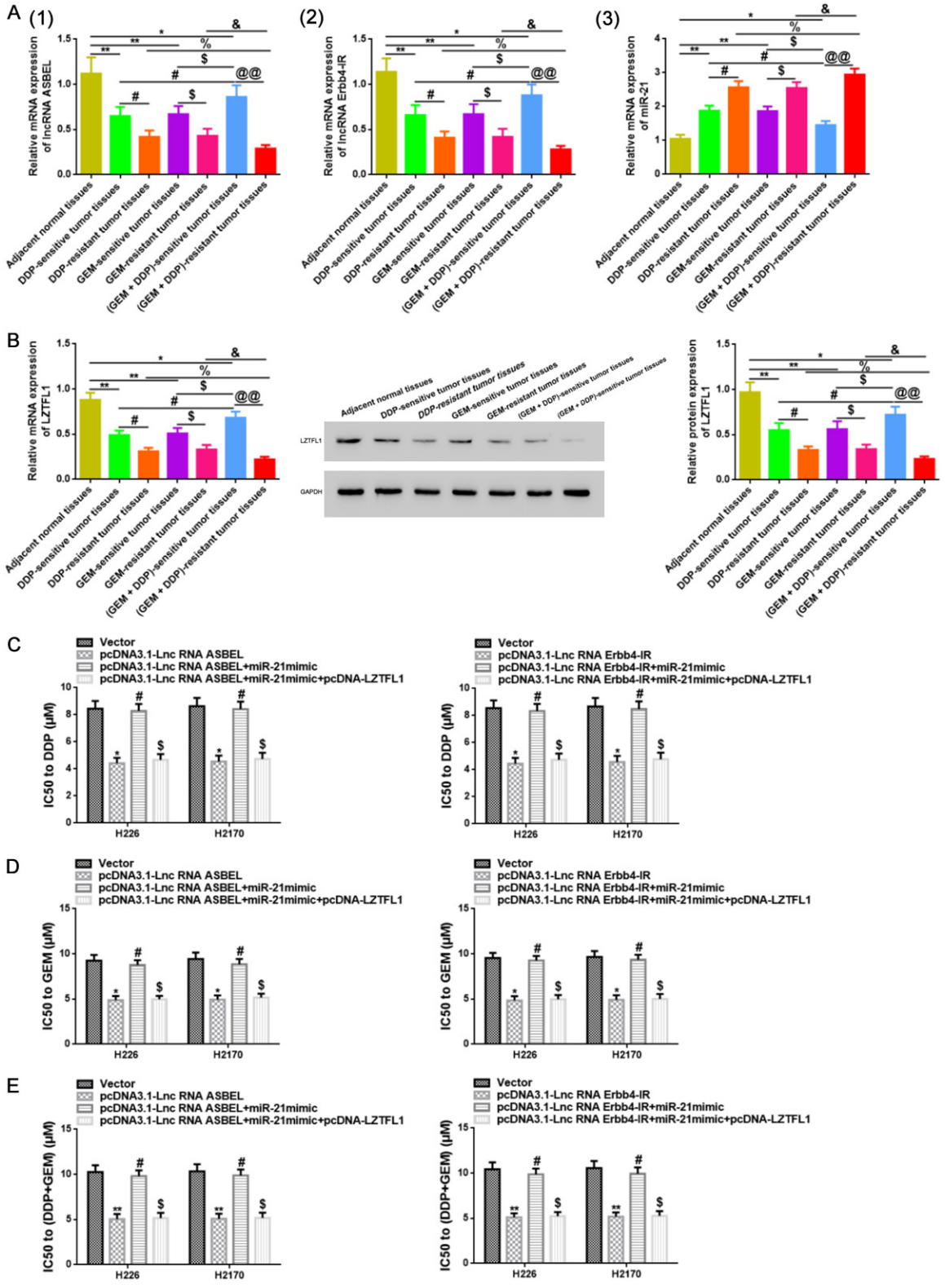

Figure 9. lncRNA ASBEL and lncRNA Erbb4-IR modulated chemoresistance to combination therapy with GEM and DDP in LSCC via the miR-21/LZTFL1 axis. A. Expression of lncRNA ASBEL, lncRNA Erbb4-IR, and miR-21 in clinical stage IV DDP/GEM/(DDP+GEM)-sensitive/resistant LSCC tumor tissues, examined using qRT-PCR. B. mRNA and protein levels of LZTFL1 in clinical DDP/GEM/(DDP+GEM)-sensitive/resistant LSCC tumor tissues examined using qRT-PCR and western blot, respectively. * vs Adjacent normal tissues, $P < 0.05$; ** vs Adjacent normal tissues, P <

0.01; # vs DDP-sensitive tumor tissues, P < 0.05; $ vs GEM-sensitive tumor tissues, P < 0.05; @@ vs (DDP+GEM)-sensitive tumor tissues, P < 0.01; % vs DDP-resistant tumor tissues, P < 0.05; & vs GEM-resistant tumor tissues, P < 0.05. C-E. Overexpression of lncRNA ASBEL and lncRNA Erbb4-IR enhanced the sensitivity of human LSCC cells (H226 and H2170) to DDP, GEM, and DDP+GEM via miR-21/LZTFL1 axis regulation, decreasing the IC50 values of these chemotherapeutic agents. These changes were observed using the MTT assay. * vs Vector group, P < 0.05; ** vs Vector group, P < 0.01; # vs pcDNA3.1-lncRNA ASBEL, P < 0.05; $ vs pcDNA3.1-lncRNA ASBEL+miR-21 mimic, P < 0.05.

Discussion

Previous studies on malignant tumors have suggested that the dysregulation of lncRNAs and miRNAs is involved in chemotherapeutic resistance. In cancer cells, the altered expression of genes involved in drug resistance can affect malignant behaviors such as proliferation, cell cycle progression, and apoptosis [8, 33]. First-line treatment with GEM plus DDP is the most commonly utilized strategy for LSCC treatment [34]. However, the objective response rate of this regimen is less than 30%, and the median progression-free survival (PFS) is only 3 to 5 months [5, 35]. A report published in 2018 indicated that the median PFS and overall survival (OS) in LSCC patients treated with a GP regimen (GEM plus DDP) are 6.0 and 13.6 months, respectively. Patients with tumors sensitive to the GP regimen show the longest OS (20.0 months), while those with resistant tumors exhibit the shortest OS (11.2 months) [36]. Therefore, the GP regimen does not show good clinical efficacy in all patients [37], and most patients with GP-resistant tumors eventually die [38]. In order to improve the efficacy of the GP regimen against LSCC, the mechanism of drug resistance must be delineated.

LSCC is usually diagnosed at an advanced stage (stage III or IV). Therefore, the present study aimed to assess the role of the lncRNA ASBEL/lncRNA Erbb4-IR/miR-21/LZTFL1 axis in chemoresistance to GEM and DDP combination therapy in stage IV LSCC. lncRNA ASBEL and lncRNA Erbb4-IR were down-regulated in human stage IV LSCC tissues and cells. However, in human LSCC cells, their overexpression could suppress cell proliferation, migration, invasion, and cell cycle entry and promote apoptosis via the miR-21/LZTFL1 axis. Our results showed that lncRNA ASBEL and lncRNA Erbb4-IR can reduce chemoresistance to GP (GEM plus DDP) in stage IV human LSCC by modulating the miR-21/LZTFL1 pathway.

Several reports have shown that miR-21 is overexpressed in tumors and plasma in patients with LSCC. The overexpression of miR-21 is associated with poor patient prognoses and enhanced malignant behaviors, including increased cell proliferation, invasion, and migration and reduced apoptosis. In LSCC, these effects are mediated by the miR-21-dependent regulation of PTEN, RECK, and Bcl-2; therefore, miR-21 is considered as a potential biomarker for the diagnosis of LSCC [10, 14, 15]. In the present study, miR-21 was found to be up-regulated in stage IV LSCC tumor tissues and human LSCC cell lines (H226 and H2170). Moreover, miR-21 overexpression enhanced malignant behaviors such as cell proliferation, migration, invasion, and cell cycle entry and reduced apoptosis in H226 and H2170 cells. A previous study showed that the overexpression of miR-21 can promote DDP chemoresistance in NSCLC cell lines, including SPC-A1, A549, and H2170 cells [16]. In the present study, the overexpression of miR-21 promoted DDP chemoresistance in LSCC cells. This was consistent with the finding that of miR-21 levels are higher in DDP-resistant than in DDP-sensitive LSCC tissues. Moreover, co-transfection with a miR-21 mimic increased the IC50 of DDP in H226 and H2170 cells overexpressing lncRNA ASBEL or lncRNA Erbb4-IR. A previous study showed that miR-21 up-regulation is strongly linked to increased chemoresistance against GEM in pancreatic cancer [17, 18]. However, the present study is the first to report that the up-regulation of miR-21 promotes chemoresistance against GEM and GEM plus DDP in LSCC.

GEM, a cytosine nucleoside derivative, is an anti-metabolic anticancer drug that alters the cell cycle. This drug mainly affects DNA synthesis (S stage) in tumor cells and can inhibit the G1 to S transition under certain conditions. Thereby, it inhibits cell proliferation, induces cell apoptosis, and suppresses tumor growth. In contrast, DDP is a platinum-based compound that suppresses DNA replication by forming intra- and inter-strand DNA adducts [39-41]. Hence, the miR-21-induced increase in chemoresistance against DDP, GEM, and DDP+GEM observed in the present study may

be associated with the miR-21-induced increase in cell cycle entry (reduced S and G2-phase arrests and increased G1-phase arrest), cell proliferation, migration, and invasion and reduction in apoptosis.

Regarding the molecular mechanism of miR-21-mediated chemoresistance, previous evidence indicates that lncRNA ASBEL and lncRNA Erbb4-IR can negatively regulate miR-21 and thereby modulate the malignant progression of different types of theriomas [19, 22]. A similar negative regulatory relationship was also observed for stage IV LSCC in the present study. The expression of lncRNA ASBEL and lncRNA Erbb4-IR was negatively correlated with that of miR-21 in human LSCC tissues, and their overexpression decreased miR-21 levels in H226 and H2170 cells. Notably, the present study is the first to demonstrate that lncRNA ASBEL and lncRNA Erbb4-IR are down-regulated in human stage IV LSCC tissues and H226 and H2170 cells. This study is also the first to reveal that the overexpression of these lncRNAs can inhibit cell proliferation, migration, invasion, and cell cycle progression (G1 to S stage) as well as promote apoptosis in LSCC cells, while also inhibiting chemoresistance to GEM and DDP via the miR-21/LZTFL1 axis. Moreover, the overexpression of lncRNA ASBEL and lncRNA Erbb4-IR can also increase LZTFL1 mRNA and protein levels *in vitro*. Compared with GEM-, DDP-, and (GEM+DDP)-resistant LSCC tissues, GEM-, DDP-, and (GEM+DDP)-sensitive tissues show higher levels of lncRNA ASBEL and lncRNA Erbb4-IR. Furthermore, the overexpression of these lncRNAs can decrease the IC50 values of DDP and GEM plus DDP against human LSCC cells.

Few studies have reported the expression and function of lncRNA ASBEL and lncRNA Erbb4-IR in cancers. Only one study has shown that lncRNA ASBEL is down-regulated in colorectal cancer cells and that its overexpression can remarkably inhibit cell proliferation and tumor growth in colorectal cancer by down-regulating transcription factor 3 (TCF3) [42]. Further, only one study has reported that lncRNA Erbb4-IR is down-regulated in prostate carcinoma tissues and is associated with prognosis, and that its overexpression can dramatically inhibit cell proliferation and promote apoptosis in prostate carcinoma cells by weakening the expression of miR-21 in vitro [22]. These previous findings support the results of the present study to a certain extent. Interestingly, Wang et al reported that plasma miR-21 levels are elevated in breast cancer patients, and miR-21 suppression can inhibit cell proliferation and metastasis in vivo via LZTFL1 [23]. LZTFL1 is down-regulated in epithelial tumors (including NSCLC), and its down-regulation is associated with recurrence and poor survival in NSCLC. LZTFL1 acts as a tumor suppressor in gastric cancers and NSCLC by inhibiting cell migration, epithelial-to-mesenchymal transition, and tumorigenesis [24]. However, so far, no studies have examined the expression and role of LZTFL1 in LSCC and its effects on sensitivity to GEM, DDP, and GEM plus DDP. The present study is the first to demonstrate that LZTFL1 mRNA and protein levels are down-regulated in human LSCC tissues and cells, and that the overexpression of LZTFL1 exerts an antagonistic effect on cell proliferation, migration, invasion, and cell cycle entry while promoting cell apoptosis in LSCC cells. In addition, our study showed that the up-regulation of LZTFL1 exerts an inhibitory effect on chemoresistance to GEM, DDP, and GEM plus DDP in LSCC cells. Therefore, the present study demonstrates that the lncRNA ASBEL/lncRNA Erbb4-IR/miR-21/LZTFL1 axis has an important role in regulating chemoresistance to stage IV of LSCC against combination therapy with GEM and DDP in stage IV LSCC.

Conclusions

In summary, the present study reveals-for the first time-that lncRNA ASBEL, lncRNA Erbb4-IR, and LZTFL1 are down-regulated in human LSCC tissues and cells and function as tumor suppressors, preventing the malignant progression of LSCC. More interestingly, the results show that these molecules can inhibit chemoresistance to GEM, DDP, and GEM plus DDP in LSCC cells by inhibiting cell proliferation, migration, invasion, and cell cycle entry and promoting apoptosis. lncRNA ASBEL and lncRNA Erbb4-IR can regulate the chemoresistance of stage IV LSCC to combination therapy with GEM and DDP by modulating the miR-21/LZTFL1 axis. Therefore, lncRNA ASBEL, lncRNA Erbb4-IR, miR-21, and LZTFL1 can serve as novel markers for predicting chemoresistance against the GP regimen (GEM plus DDP) in patients with

advanced LSCC and may also be potential chemotherapeutic targets.

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Signed informed consent was obtained from patients or their family members before the initiation of clinical experiments.

Disclosure of conflict of interest

None.

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