lncRNA ZEB1-AS1 is downregulated in diabetic lung and regulates lung cell apoptosis

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Received April 12, 2019; Accepted January 9, 2020

DOI: 10.3892/etm.2020.9355

Abstract. The present study aimed to investigate the role of ZEB1-antisense RNA 1 (AS1) in diabetic lung (pneumonia with excluded causes other than diabetes). In the present study, the expression of ZEB1-AS1 in plasma was detected by performing reverse transcription-quantitative PCR. A receiver operating characteristic curve was used for diagnostic analysis. Lung cell apoptosis under the treatment of high glucose was analyzed by a cell apoptosis assay. p53 expression in lung cells was detected by performing western blotting. The present data demonstrated that ZEB1-AS1 was downregulated in the plasma of patients with diabetic lung (DL) compared with diabetic patients without complications (~1.6-fold) and healthy controls (~2.4-fold), and downregulation of ZEB1-AS1 distinguished patients with DL from healthy controls. ZEB1-AS1 in lung cells was downregulated by high glucose treatment, and overexpression of ZEB1-AS1 resulted in inhibited lung cancer cell apoptosis and downregulated p53. p53 overexpression attenuated the effects of ZEB1-AS1 overexpression on lung cell apoptosis. In conclusion, the present study demonstrated that ZEB1-AS1 was downregulated in patients with DL and regulates lung cancer cell apoptosis by downregulating p53.

Introduction

The incidence of diabetes mellitus (DM) ranks first among all metabolic disorders and is still rising (1). In 2014, ~387 million patients were suffering from diabetes and it is predicted that >200 million new cases will be diagnosed by 2035 (2). A hyperglycemic state affects cell functions, which induce the development of macro- and microvascular diseases, such as diabetic nephropathy, retinopathy, atherosclerosis, neuropathy and dysregulation of other major organs (3-5). The lungs are also a target of diabetes, and altered lung volume, ventilation control, capacity of pulmonary diffusing and neuroadrenergic bronchial innervation were frequently observed in diabetic patients with a long course of the disease (6). However, to the best of the authors' knowledge, studies on diabetic lung (DL) are rare and the pathogenesis is still relatively unknown.

Genetic factors contribute to the development of diabetic complications, and aberrant gene expression patterns are closely correlated with the pathological changes in diabetic patients (3-6). Genes are dysregulated under a hyperglycemic state and the altered gene expression participates in the development of diabetic complications (7). Long non-coding RNAs (lncRNAs; >200 nt) have critical roles in controlling development of pathological changes under a hyperglycemic state by regulating gene expression at multiple levels (8,9). It was identified that the activation of p53 in diabetic mice induced a pro-apoptotic gene expression pattern, suggesting the enhancing effect of the gene on the progression of diabetic complications (10). p53 may interact with lncRNAs to achieve its functions (11). In a previous study, Li *et al* (12) demonstrated that lncRNA ZEB1-antisense RNA 1 (AS1) promoted the development of non-small cell lung cancer by regulating Wnt/β-catenin signaling, which is known to be involved in diabetic complications (3-5). There is a hypothesis that this lncRNA may also participate in other types of lung disease. The present study investigated the role of ZEB1-AS1 in DL.

Materials and methods

Research subjects. The patients of the present study included 78 cases of DL, pneumonia with excluded causes other than diabetes (41 males and 37 females; 38 to 62 years old, with a mean age of 51.8±7.7 years), 78 patients with diabetes (DB) with obvious complications (DB group; 41 males and 37 females; 37 to 65 years old, with a mean age of 52.1±7.9 years) and 78 healthy volunteers (control group; 41 males and 37 females; 36 to 63 years old, with a mean age of 52.2±7.0 years). All the participants were selected from The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University between October 2016 and October 2018. Inclusion criteria for DL and DB group consisted of the following: i) Newly diagnosed pneumonia cases; ii) normal conditions of major organs except lung in the DL group; and iii) no therapies initiated before admission. Exclusion criteria for the DL and

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Key words: diabetes, diabetic lung, long non-coding RNA ZEB1-antisense RNA 1, p53, apoptosis

DB groups consisted of the following: i) Other obvious clinical disorders were observed; and ii) lung diseases other than diabetic lung. The three groups of participants showed similar sex and age distributions, while white blood cell count and C-reactive protein were significantly higher in the DL group compared with the DB and control groups (P<0.05; Table I). The general clinical data of the three groups of participants are presented in Table I. The Ethics Committee of The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University approved the present study (Nanjing, China). All patients were informed of the experimental principle, and all patients signed informed consent.

Plasma and cells. Fasting blood (~5 ml) was extracted from the elbow vein of each participant on the day of admission and before the initiation of any therapies. Blood was transferred to EDTA-treated tubes, followed by centrifugation at 1,200 x g for 10 min at room temperature to prepare the plasma samples.

The BEAS-2B normal human lung cell line (American Type Culture Collection) was used to perform all *in vitro* cell experiments. Eagle's MEM (Sigma-Aldrich; Merck KGaA) was mixed with 10% FBS (Sigma-Aldrich; Merck KGaA) and the mixture was used to cultivate BEAS-2B cells. Cells were cultivated under conditions of 37˚C and 5% CO_2.

Reverse transcription-quantitative PCR (RT-qPCR). Plasma and BEAS-2B cells were mixed with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to extract total RNA. Following DNase I digestion, AMV Reverse Transcriptase XL (Takara Bio, Inc.) was used to synthesize cDNA by reverse transcription through the following temperature protocol: 25˚C for 10 min, 55˚C for 10 min and 85˚C for 10 min. SYBR®-Green Realtime PCR Master Mix (Toyobo Life Science) was used to prepare all PCR mixtures to detect the expression of ZEB1-AS1 and p53 mRNA with 18S rRNA, and GAPDH was used as an endogenous control. PCR reactions were performed using the following thermocycling conditions: Initial denaturation at 95˚C for 1 min, followed by 40 cycles of 95˚C for 10 sec and 55˚C for 40 sec. Primer sequences were as follows: ZEB1-AS1: 5'-CCG TGGGCACTGCTGAA-3' (forward) and 5'-CTGCTGGCA AGCGGAAC-3' (reverse); p53: 5'-CTGTCATCTTCTGTC CCTT-3' (forward) and 5'-TGGAATCAACCCACAGCTG-3' (reverse); 18S rRNA: 5'-CTACCACATCCAAGGAAGC-3' (forward) and 5'-TTTTTCGTCACTACCTCCCC-3' (reverse); and GAPDH: 5'-GGTCTCCTCTGACTTCAAC-3' (forward) and 5'-TGAGGGTCTCTCTCTTCCT-3' (reverse). All reactions were repeated three times and Cq values were processed using the $2^{-\Delta\Delta Cq}$ method (13).

ELISA. The Human p53 ELISA kit (cat. no. ab46067; Abcam) was used to measure the levels of p53 in plasma of all three groups of participants. Plasma p53 levels were normalized to pg/ml.

Transient cell transfection. ZEB1-AS1 and p53 expression vectors were constructed by Sangon Biotech Co., Ltd. using pcDNA3.1. BEAS-2B cells were harvested at a confluence of 70-90%, and 10 nM ZEB1-AS1 (NR_024284.1) and p53 (AB082923.1) expression vector or 10 nM empty pcDNA3.1 (negative control; NC) were transfected into 10^5 BEAS-2B cells using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.). The control group included cells without transfections. All subsequent experiments were performed using cells collected 24 h after transfections.

Table I. General clinical data of the three groups of participants.

Characteristic	DL n=78	DB n=78	Control n=78
Age, years	51.8±7.7	52.1±7.9	52.2±7.0
Sex			
Male	41	41	41
Female	37	37	37
Disease duration (years)	9.9±2.3	3.1±1.1	N/A
White blood cell count, 10^9/l	12.2±1.9[a,b]	8.9±2.1	7.1±2.5
C-reactive protein, mg/l	55.4±9.2[a,b]	3.4±1.7	9.5±3.1

[a]P<0.05 vs. Control and [b]P<0.05 vs. DB. DL, diabetic lung; DB, diabetes with complications.

Cell apoptosis assay. BEAS-2B cells ($3x10^4$) were harvested 24 h after transfections and mixed with 1 ml Eagle's MEM, which was supplemented with 10% FBS to prepare single cell suspensions. Cell suspensions were transferred to 6-well plates with 2 ml per well, followed by the addition of 0, 10 and 30 mM D-glucose. A total of three replicates were set for each concentration. Cells were cultivated under the conditions of 37˚C and 5% CO_2 for 48 h. After that, 25% trypsin digestion was performed and Annexin V-FITC (Dojindo Molecular Technologies, Inc.) and propidium iodide (PI; Dojindo Molecular Technologies, Inc.) were used to stain the cells. All operations were performed in strict accordance with the manufacturer's instructions. Finally, apoptotic cells were detected by performing flow cytometry using BD Biosciences 2 Laser FACSCalibur Flow Cytometer w/HTS Autosampler (BD Biosciences). Data were processed using BD FACSDiva™ software (version 8.0; BD Biosciences).

Western blotting. BEAS-2B cells (10^5) were collected 24 h after transfections and mixed with 1 ml RIPA solution (Thermo Fisher Scientific, Inc.) to extract total protein. Bicinchoninic Acid assay (Thermo Fisher Scientific, Inc.) was performed to determine the protein concentration. Following protein denaturing and 10% SDS-PAGE, proteins (30 μg per lane) were transferred to PVDF membranes. After blocking in 5% non-fat milk (2 h at 25˚C), p53 (1:1,200; cat. no. ab131442; Abcam) and GAPDH (1:1,000; cat. no. ab9485; Abcam) rabbit polyclonal primary antibodies were used to incubate with membranes (4˚C overnight), followed by incubation with IgG-HRP goat anti-rabbit secondary antibody (1:1,000; cat. no. MBS435036; MyBioSource, Inc.) for 2 h at 25˚C. Signals were developed using ECL (Thermo Fisher Scientific, Inc.) and densitometry was performed using ImageJ v1.46 software (National Institutes of Health).

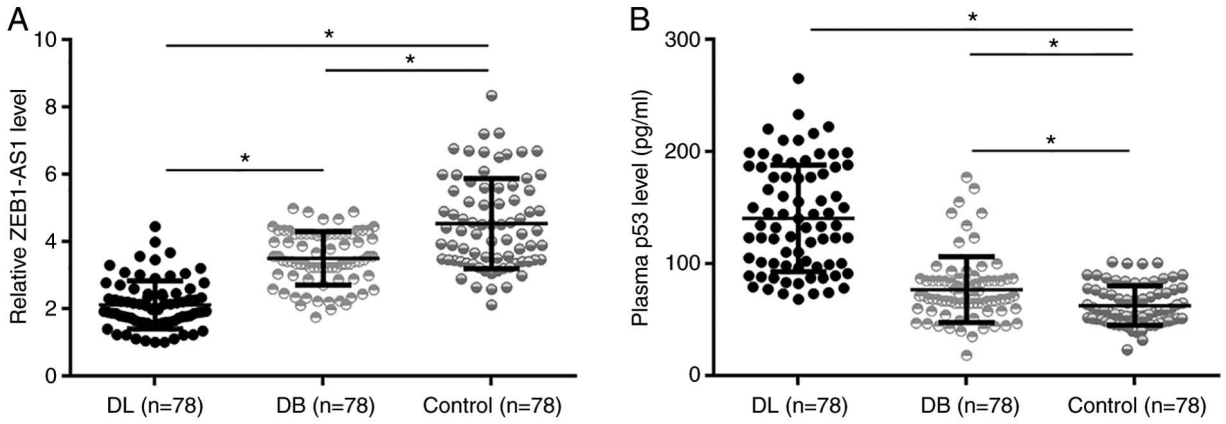

Figure 1. ZEB1-AS1 and p53 are dysregulated in the DL group. Reverse transcription-quantitative PCR and ELISA were performed to measure plasma levels of ZEB1-AS1 and p53 in the DL (n=76), DB (n=76) and control (n=76) groups. (A) Data analyzed by one-way ANOVA and Tukey's test demonstrated that ZEB1-AS1 levels were significantly lower in the DL group than in the other two groups, and the DB group also demonstrated lower ZEB1-AS1 levels than the control group. (B) In contrast, p53 levels were significantly higher in the DL group than in the other two groups, and the DB group also demonstrated higher p53 levels than the control group. Mean values of three replicates are presented. *P<0.05. AS1, antisense RNA 1; DL, diabetic lung; DB, diabetes with complications.

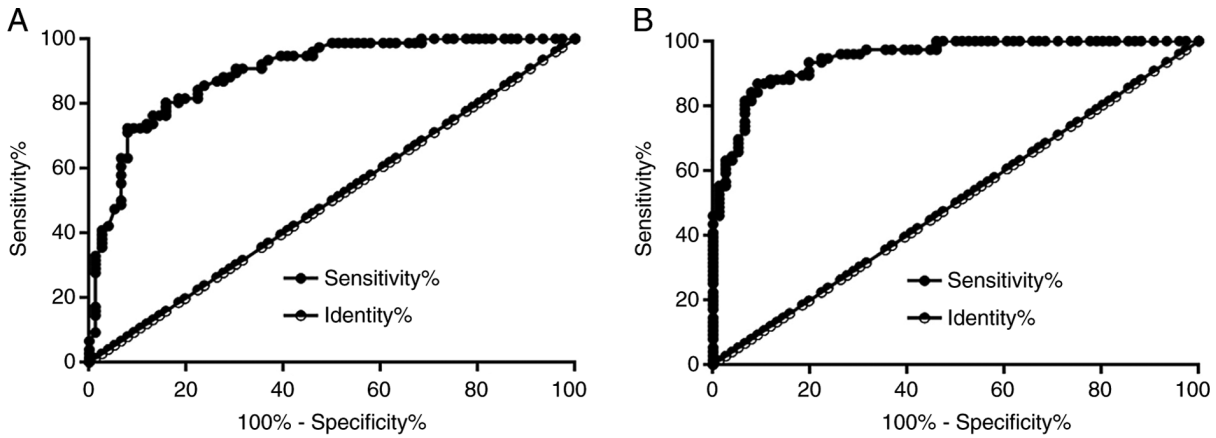

Figure 2. Downregulated plasma ZEB1-AS1 distinguishes patients with DL from the DB and control groups. ROC curve analysis showed that downregulated plasma ZEB1-AS1 distinguished patients with DL from (A) DB and (B) Control groups. In the ROC curve, participants in the DB or control groups were used as true negative cases and patients with DL were true positive cases. AS1, antisense RNA 1; DL, diabetic lung; DB, diabetes with complications; ROC, receiver operating characteristic.

Statistical analysis. Sample sizes in the present study provide sufficient statistical power in all cases (0.87-0.93). Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc.). All mean ± SD values presented in the present study represent the data from three biological replicates. Differences among different patient and cell groups were analyzed by performing one-way ANOVA and Tukey's test. Correlations were analyzed by performing Pearson's correlation coefficient. Diagnostic analyses were performed using receiver operating characteristic (ROC) curve analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

ZEB1-AS1 and p53 are dysregulated in the DL group. ZEB1-AS1 and p53 in plasma were detected using RT-qPCR and ELISA, respectively. The data were compared among the DL (n=78), DB (n=78) and control (n=78) groups by performing one-way ANOVA and Tukey's test. It was identified that ZEB1-AS1 levels were significantly lower in the DL group than in the other two groups (1.6- and 2.4-fold, respectively), and the DB group also showed lower ZEB1-AS1 levels than the control group (1.5-fold, Fig. 1A; P<0.05). In contrast, p53 levels were significantly higher in the DL group than in the other two groups (1.9 and 2.9-fold, respectively), and the DB group also showed higher p53 levels than the control group (1.5-fold; Fig. 1B; P<0.05).

Downregulated plasma ZEB1-AS1 distinguishes patients with DL from the DB and control groups. The diagnostic value of ZEB1-AS1 for DL was evaluated by performing ROC curve analysis. In the present study, participants in the DB or the control group were used as true negative cases and the patients with DL were true positive cases. With DB as true negative cases, the area under the curve (AUC) was 0.89, with a standard error of 0.025 and 95% confidence interval of 0.84-0.94 (Fig. 2A). With the control and the true negative cases, the AUC was 0.95, with a standard error of 0.016 and 95% confidence interval of 0.92-0.98 (Fig. 2B).

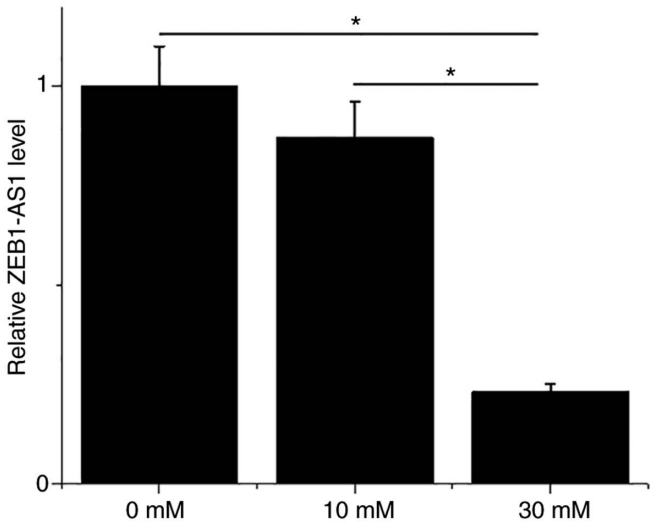

Figure 3. D-glucose treatment downregulates ZEB1-AS1 in BEAS-2B cells. Reverse transcription-quantitative PCR was performed to detect the expression of ZEB1-AS1 in BEAS-2B cells treated with 0, 10 and 30 mM glucose for 48 h. It was observed that, comparing with 0 and 10 nM glucose, treatment with 30 mM glucose significantly downregulated the expression levels of ZEB1-AS1. However, treatment with 10 nM D-glucose only led to slightly downregulated ZEB1-AS1 in BEAS-2B cells compared with the untreated cells. Mean values of three replicates are presented. *P<0.05. AS1, antisense RNA 1.

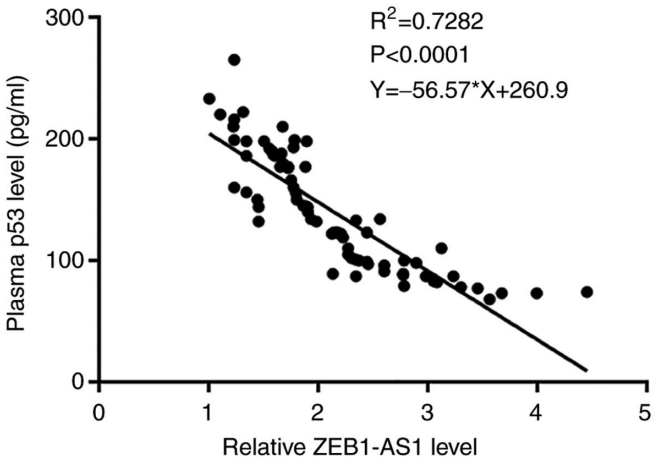

Figure 4. Plasma ZEB1-AS1 and p53 are negatively correlated in the DL group. Pearson's correlation coefficient indicated that ZEB1-AS1 and p53 in the DL group are negatively and significantly correlated. AS1, antisense RNA 1; DL, diabetic lung.

D-glucose treatment downregulates ZEB1-AS1 in BEAS-2B cells. BEAS-2B cells were treated with 0, 10 and 30 mM glucose for 48 h, followed by the detection of ZEB1-AS1 by performing RT-qPCR. It was observed that compared with 0 and 10 nM glucose, treatment with 30 mM glucose significantly downregulated the expression levels of ZEB1-AS1 (Fig. 3; P<0.05). However, treatment with 10 nM D-glucose only led to slightly downregulated ZEB1-AS1 in BEAS-2B cells compared with the untreated cells.

Plasma levels of ZEB1-AS1 and p53 are negatively correlated in the DL group. The correlation between the plasma levels of ZEB1-AS1 and p53 in the DL group was analyzed by performing Pearson's correlation coefficient. R^2>0.65 indicates a promising linear correlation. It was observed that the plasma levels of ZEB1-AS1 were negatively and significantly correlated with the plasma levels of p53 (Fig. 4; R^2=0.7282; P<0.0001).

ZEB1-AS1 downregulates p53 to inhibit BEAS-2B cell apoptosis under glucose treatment. The ZEB1-AS1 and p53 expression vectors were transfected into BEAS-2B cells. At 24 h after transfections, expression levels of ZEB1-AS1 and p53 were significantly increased compared with the control (C) and negative control (NC) groups (Fig. 5A; P<0.05). Moreover, ZEB1-AS1 overexpression led to the downregulation of p53 at both the mRNA and protein levels, while p53 overexpression failed to affect ZEB1-AS1 (Fig. 5B; P<0.05). A cell apoptosis assay was performed to analyze the effects of transfections on cell apoptosis. Cells apoptosis data were compared among the groups by performing one-way ANOVA and Tukey's test. Compared with the two controls, overexpression of ZEB1-AS1 inhibited apoptosis, while p53 overexpression promoted lung cancer cell apoptosis. Moreover, p53 overexpression attenuated the effects of ZEB1-AS1 overexpression on lung cell apoptosis (Fig. 5C; P<0.05).

Discussion

The expression pattern and functions of ZEB1-AS1 in DL were investigated in the present study. ZEB1-AS1 was downregulated in DL and it was identified that ZEB1-AS1 overexpression may inhibit the apoptosis of lung cells in a high glucose environment by downregulating p53.

p53 is a well-characterized tumor suppressor that inhibits the progression of different types of cancer by inducing the apoptosis of cancer cells (14). However, the pro-apoptotic roles of p53 may not be beneficial for use in humans. In the development of other types of diseases, such as diabetic complications, the activation of p53 in patients with diabetes promotes the apoptosis of cells in many major organs, including the lungs, which thereby promotes the development of diabetic complications (15). In effect, inhibition of p53 could provide a novel approach for the prevention and treatment of diabetic complications (16). In the present study, upregulated p53 in the DL group and the increased apoptotic rate of lung cells in a high glucose environment after p53 overexpression was observed. Therefore, p53 may promote the development of DL by inducing lung cell apoptosis.

ZEB1-AS1 is upregulated in patients with lung cancer and it may promote cancer development by interacting with the Wnt/β-catenin pathway (12). It is widely accepted that Wnt/β-catenin signaling participates in the development of diabetic complications (3-5). Therefore, ZEB1-AS1 may also participate in the development of diabetic lung.

The present study demonstrated that ZEB1-AS1 was downregulated in patients with DL, and downregulated ZEB1-AS1 may serve as a marker for the diagnosis of DL. ZEB1-AS1 was identified as an inhibitor of p53 in lung cells, and the inhibition of p53 by ZEB1-AS1 was involved in the regulation of lung cell apoptosis in a high glucose environment. Therefore, the overexpression of ZEB1-AS1 may be a potential target to

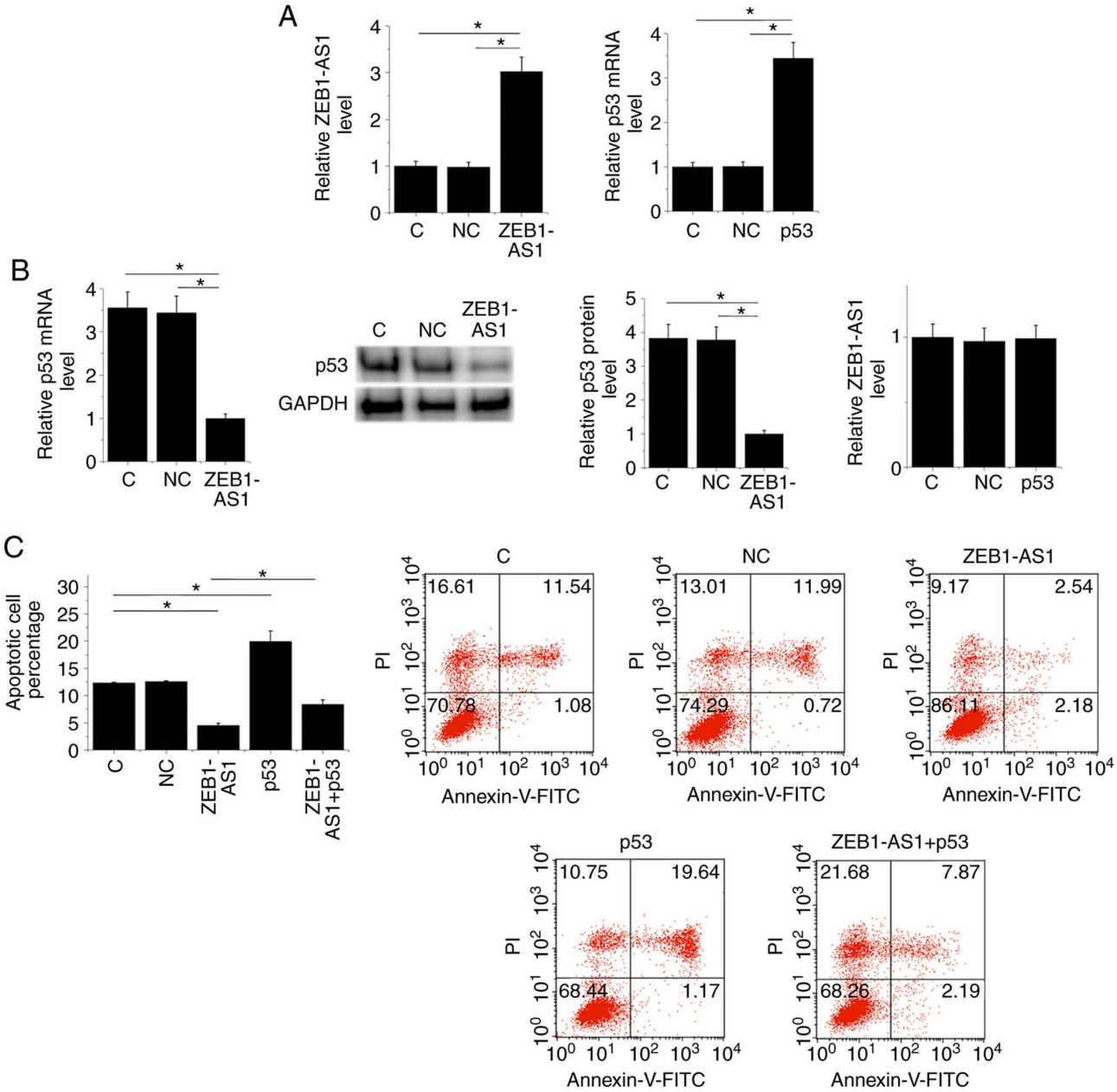

Figure 5. ZEB1-AS1 downregulates p53 to inhibit BEAS-2B cell apoptosis under glucose treatment. Western blotting and reverse transcription-quantitative PCR were performed to analyze the interaction between ZEB1-AS1 and p53 using an overexpression approach. (A) Expression levels of ZEB1-AS1 and p53 were significantly increased compared with the C and NC groups at 24 h after transfections. (B) ZEB1-AS1 overexpression led to the downregulation of p53 at both the protein and mRNA levels, as determined by western blotting and reverse transcription-quantitative PCR, respectively, while p53 overexpression failed to affect ZEB1-AS1. A cell apoptosis assay was performed to analyze the effects of transfections on cell apoptosis. (C) Overexpression of ZEB1-AS1 inhibited cell apoptosis, while p53 overexpression promoted lung cancer cell apoptosis. Moreover, p53 overexpression attenuated the effects of ZEB1-AS1 overexpression on lung cell apoptosis. Mean values of three replicates are presented. *P<0.05. AS1, antisense RNA 1; C, control; NC, negative control; PI, propidium iodide.

inhibit the apoptosis of lung cells in patients with diabetes. It is known that Wnt/β-catenin has cross-talk with p53 in many types of diseases (17,18). Therefore, Wnt/β-catenin may be a mediator for the interactions between Wnt/β-catenin and p53; however, this requires further investigation.

The present study investigated the involvement of an lncRNA in DL. To the best of the authors' knowledge, studies on lncRNAs in DL are rare. However, the present study is limited by the small sample size and the lack of animal model studies. In conclusion, ZEB1-AS1 was downregulated in DL, and ZEB1-AS1 overexpression may improve DL by downregulating p53.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LG designed all experiments. LG and HS performed experiments. ZY collected and analyzed data. LG drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

Ethics approval and consent to participate

The Ethics Committee of The Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University (approval no. 2016-181; Nanjing, China) approved the present study. All patients were informed of the experimental principle, and all patients signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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