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Correlations of the ZEB1 expression with the incidence and prognosis of non-small cell lung cancer

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Abstract. – OBJECTIVE: In order to investigate the role of the zinc finger E-box-binding homeobox 1 (ZEB1) expression in the incidence of non-small cell lung cancer (NSCLC), the effects of the ZEB1 expression on the pathogenesis and prognosis of NSCLC were investigated.

PATIENTS AND METHODS: Correlations of the expression of ZEB1 in 88 clinical patients with NSCLC with clinicopathological features were determined. The patients were followed up for 60 months to study the correlation between the ZEB1 expression and the prognosis of NSCLC patients. To further explore the role of the ZEB1 expression in the incidence of NSCLC, the expressions of ZEB1 and E-cadherin in three NSCLC cell lines (A549, NCI-H1299 and NCI-H1975) and human normal lung epithelial BESA-2B cell line were measured, and the cell invasion ability was detected. The role of ZEB1 in NSCLC cell invasion was verified through the knockdown of ZEB1 by the short hairpin ribonucleic acids (shRNAs). In addition, its effects on the proliferation and apoptosis of NSCLC cells were confirmed by the overexpression of ZEB1.

RESULTS: The expression of ZEB1 in NSCLC tissues was remarkably higher than that in normal tissues, and the expression level of ZEB1 was significantly related to the cancer stage and tumor size. The lower the expression of ZEB1 was, the higher the overall survival rate and the longer the survival time would be. The expression of ZEB1 was negatively correlated with that of E-cadherin in cell lines, and ZEB1-shRNAs markedly reduced the invasion ability of NSCLC cells. The overexpression of ZEB1 resulted in an increase in the proliferation activity and a significant decrease in the apoptosis of A549 cells.

CONCLUSIONS: The expression of ZEB1 is closely related to the incidence and prognosis of NSCLC. Increased expression of ZEB1 is helpful for promoting the invasion of NSCLC and enhancing the proliferation activity of NSCLC.

Key Words:
ZEB1, NSCLC, Incidence, Prognosis.

Introduction

Non-small cell lung cancer (NSCLC) is the main form of lung cancer and one of the major cancers causing death worldwide[1]. NSCLC mainly causes the death of patients by the constant invasion and metastasis of tumors[2,3]. The zinc finger E-box-binding homeobox 1 (ZEB1) is a transcription factor containing two Kruppel1 zinc finger clusters, a mediator factor of target DNA binding EB-like sequences[4-6]. ZEB1 can be used as a transcription inhibitor or activator. ZEB1 participates in the formation of various tissues during development and plays an important role in tumor progression[7,8]. During the development of the germ layer and the nerve tissue in the body, the ZEB1 level changes significantly during differentiation[9]. It participates in not only lymphocyte and cartilage formation, but also nerve cell development[10-12]. Besides, EMB1 exerts a regulatory effect in the epithelial-mesenchymal transition (EMT). ZEB1 is a biomarker of invasive cancer with a high risk of recurrence. However, the regulatory role of ZEB1 in malignant tumors deserves further study. In this work, the regulatory role of ZEB1 in NSCLC was investigated and discussed, the correlations of the ZEB1 expression with the pathogenesis and prognosis of NSCLC were analyzed, and the effects of the ZEB1 expression on the invasion and apoptosis of NSCLC cells were explored.

Patients and Methods

Data and Cell Line Sources

A total of 88 patients with clinical NSCLC from Peking Union Medical College Hospital signed the informed consent, and they were fol-

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lowed up for 60 months to observe their prognosis. Three NSCLC cell lines (A549, NCI-H1299 and NCI-H1975) and human normal lung epithelial BESA-2B cell line were purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd., (Shanghai, China).

Main Test Reagents and Instruments

Rabbit anti-ZEB1 antibody was purchased from Abcam (Cambridge, MA USA), VECTASTAIN Elite ABC Kit (Universal, Burlingame, CA, USA) Kit from Shanghai Sangge Biotechnology Co., Ltd., (Shanghai, China), Oligotex Messenger RNA (MRNA) Maxi Kit from Shanghai Haoran Biological Technology Co., Ltd., (Shanghai, China), Diff-Quik dyeing reagent from Beijing Biolab Technology Co., Ltd., (Beijing, China) BD BioCoat Matrigel Invasion Chambers 24-well plates from BD Biosciences (Franklin Lakes, NJ, USA), 3-(4,5)-dimethylthiahiazo(-z-yl)-3,5-diphenytetrazoliumromide (MTT) Cell Proliferation and Cytotoxicity Assay Kit from Beyotime Biotechnology (Shanghai, China).

Methods

Immunohistochemistry

The prepared paraffin sections were cut at 4 μm and thermally fixed at 60°C for 60 min. After dewaxing with 10 mM citrate buffer, the sections were treated with 3% hydrogen peroxide and then reacted with rabbit anti-ZEB1 antibody (1:4000) for 1 h. VECTASTAIN Elite ABC Kit (Universal) (Burlingame, CA, USA) was used for detection. After that, the sections were incubated with 3, 3'-diaminobenzidine for 4 min, counterstained with diluted hematoxylin and dehydrated. Finally, the lid was covered for open-field microscopy.

Determination of the Expressions of ZEB1 and E-cadherin Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNAs were isolated using Oligotex mRNA Maxi Kit (Qiagen, Valencia, CA, USA) Forward and reverse primers and probes were designed. The primers and probe sequences used are as follows: ZEB1: 5'-TCCATGCTTAGGCTACT-3' and 5'-ACCGTAGTTGAGTATGCCA-3', the probe: FAM-CCAATAAGCAAACGATTCTGATTCCCCAG-BHQ, and E-cadherin: 5'-AGTGTCCCCCGGTATCTTCC-3', 5'-CAGCCGCTTTCAGATTTTCAT-3', the probe: FAM-TGCCAATCGAAATTGGAAATTT-BHQ. Quantitative PCR (qPCR) was performed according to the conditions and procedures suggested by the manufacturer who synthesized primer and standardized with 18S ribosomal RNAs.

Determination of the Expressions of ZEB1 and E-Cadherin via Western Blotting

The protein concentration was determined, and it was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto the polyvinylidene difluoride (PVDF) membrane. The rabbit anti-ZEB1 antibody (1:2000), E-cadherin (1:1500) and β-actin (1:2000) were used. Then, the rabbit anti-rat horseradish peroxidase (HRP) (1:5000) was applied for detection, followed by imaging, photographing and recording.

Determination of Cell Invasion

The self-explosion attack test was carried out using BD BioCoat Matrigel Invasion Chambers 24-Well Plate (BD Biosciences, Franklin Lakes, NJ, USA) with the pore size of 8 μm. The specific operation was as follows: firstly, the target cells were washed with serum-free minimum essential media (MEM), and then serum starvation was carried out for 12 h. Secondly, 1×10^5 cells/mL were added to the upper chamber. In the lower chamber, Dulbecco's modified Eagle' medium (DMEM) containing 10% fetal bovine serum (FBS) and L-glutamine was added for incubation at 37°C for 48 h. Invasive cells were stained with Diff-Quik and counted manually using ImagePro Plus. Datum graphs were plotted, and GraphPad Prism (La Jolla, CA, USA) was applied for statistical analysis. Percentage of cell invasion = average number of cells invading Matrigel insertion wells/average number of cells crossed through control membranes not coated with Matrigel.

Determination of Cell Activity

MTT assay was adopted to determine the cell proliferation activity. A549 cells transfected with plasmid complementary DNA (pcDNA)-ZEB1 grew for 3 days. The cells were incubated at 37°C in 0.1 mg/mL MTT, and the cell activity was detected at the specified time points. The cells were lysed in 150 mL dimethyl sulfoxide (DMSO) at room temperature for 15 min. The absorbance of the well was read with a microplate reader. Each experiment was con-

ducted in triplicate.

Detection of Cell Apoptosis

1×10^5 cells were inoculated into a 6-well plate, transfected with pcDNA-ZEB1 and incubated for 48 h. The cells were then washed twice with PBS and resuspended in Annexin V binding buffer. The cell suspension was incubated with 5 mL Annexin V-fluorescein isothiocyanate (FITC) and 5 mL propidium iodide (PI) at room temperature for 15 min in the dark. Then the samples on the FACSCalibur™ flow cytometer, and the results were calculated. The apoptosis ratio of cells was compared between the pcDNA-ZEB1 transfection group and the control group. Each group of samples was measured in triplicate.

Statistical Analysis

Data were counted and analyzed by Statistical Product and Service Solutions 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (La Jolla, CA, USA) and expressed as mean ± standard deviation (n=3). The intergroup comparison was conducted using the *t*-test. The difference was significant when $p<0.05$ and was extremely significant when $p<0.01$. The correlations of the high and low expressions of ZEB1 with survival were statistically analyzed by Kaplan-Meier method.

Results

Correlations of the ZEB1 Expression with Clinicopathological Features

Immunohistochemical assay was adopted to detect the expression of ZEB1 in 88 clinical NSCLC patients and to analyze the difference in the ZEB1 expression between NSCLC cancer tissues and normal tissues. The results revealed that the ZEB1 expression in NSCLC tissues was markedly up-regulated compared with that in normal tissues ($p<0.01$) (Figure 1). Besides, the correlations of the ZEB1 expression with clinicopathological features (age, gender, tumor-node-metastasis stage, tumor size and lymph node metastasis) were analyzed by summarizing the clinical data of patients. The results manifested that the expression level of ZEB1 in NSCLC was significantly correlated with the cancer stage ($p=0.001$) and the tumor size ($p=0.001$). The expression of ZEB1 had no significant correlations with other clinicopathological features in NSCLC, such as gender, age and lymph node metastasis (Table I).

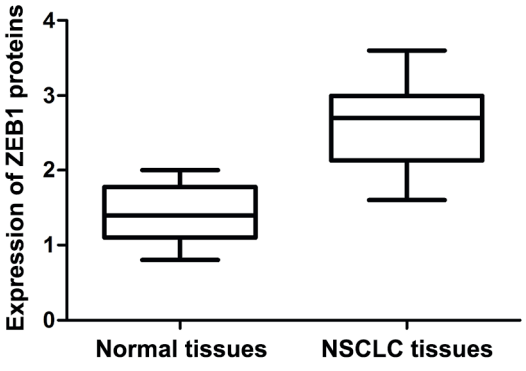

Figure 1. Expression of ZEB1 proteins in normal tissues and NSCLC tissues. Compared with that in normal tissues, the expression of ZEB1 proteins in NSCLC tissues is significantly elevated ($p<0.01$).

Relationship Between the ZEB1 Expression and the Patient's Survival

Kaplan-Meier survival analysis was performed to study the correlation between the ZEB1 expression and the prognosis of NSCLC patients. According to the relative expression of ZEB1 in tumor tissues, 88 NSCLC patients were divided into the high ZEB1 group (n=36) and the low ZEB1 group (n=52). The overall 5-year survival rate was 40.1% in the low ZEB1 group and 21.7% in the high ZEB1 group. The median survival time was 39 months in the low ZEB1 group and 25 months in the high ZEB1 group (Figure 2).

Correlation Between the ZEB1 Expression and the E-cadherin in Expression in NSCLC Cell Lines Cultured in vitro

ZEB1 and E-cadherin were analyzed on three NSCLC cell lines (A549, NCI-H1299 and NCI-H1975) and human normal lung epithelial BESA-2B cell line. The results showed that the ZEB1 expression was the highest in A549 cells, followed by NCI-H1299 and NCI-H1975 cells, all of which were significantly higher than that in BESA-2B cells (Figures 3A and 3C). However, the expression of E-cadherin was the opposite (Figure 3B and 3C). The results demonstrated that the expression of ZEB1 was negatively correlated with that of E-cadherin in cell lines.

Cell Invasion Detection

Cell invasion ability was measured on A549, NCI-H1299, NCI-H1975 and human normal

Table I. Correlations of the ZEB1 expression with clinicopathological features.

Feature	Case (n)	ZEB1 expression		p
		No. of high expression cases (n=36) [n (%)]	No. of low expression cases (n=52) [n (%)]	
Age (years old)				0.713
≤ 60	46	19 (52.7)	27 (51.9)	
> 60	42	17 (47.3)	25 (48.1)	
Gender				0.367
Male	53	21 (58.3)	32 (61.5)	
Female	35	15 (41.7)	20 (38.5)	
Cancer stage				0.001*
I or II	44	11 (30.6)	33 (63.5)	
III or IV	44	25 (69.4)	19 (36.5)	
Tumor size				0.001*
≤ 5 cm	48	13 (36.1)	35 (67.3)	
> 5 cm	40	23 (63.9)	17 (32.7)	
Lymph node metastasis				0.128
Negative	48	18 (50.0)	30 (57.7)	
Positive	40	18 (50.0)	22 (42.3)	

Note: * indicates a significant correlation (p<0.01).

lung epithelial BESA-2B cell lines. According to the results, A549 cells (with the high ZEB1 expression) displayed the highest percentage of cell invasion (88.2%), followed by NCI-H1299 cells (67.1%) and NCI-H1975 cells (53.2%) compared with that of BESA-2B. The invasion ability of three NSCLC cell lines was significantly stronger than that of BESA-2B cell line (3.4%) (Figure 4).

Effects of the Knockdown of ZEB1 by Short Hairpin Ribonucleic Acids (shRNAs) on the Invasion Ability of NSCLC Cells

In order to study the effect of ZEB1 gene silencing on NSCLC cell migration, A549 cells were transfected with ZEB1-shRNAs and non-silencing controls. Real-time RT-PCR was adopted to detect the effect of ZEB1-shRNAs on the expression of ZEB1 in A549 cells, the expression level of E-cadherin was measured, and the cell invasion ability was evaluated. The results showed that ZEB1-shRNAs evidently decreased the expressions of ZEB1 genes and proteins in A549 cells, and promoted the expression of E-cadherin (Figure 5A-5B). Furthermore, in terms of cell invasion, the invasion ability of A549 cells was markedly reduced in the ZEB1-shRNA group compared with those in the blank group and the non-silent control group (Figure 5C).

Effects of ZEB1 on the Proliferation and Apoptosis of NSCLC Cells

To investigate the effects of ZEB1 on the ability and apoptosis of NSCLC cells, the effects of ZEB1 overexpression on the proliferation and apoptosis of A549 cells were explored. MTT assay results manifested that the activity of A549 cells overexpressed with ZEB1 was enhanced compared with that of the control cells (Figure

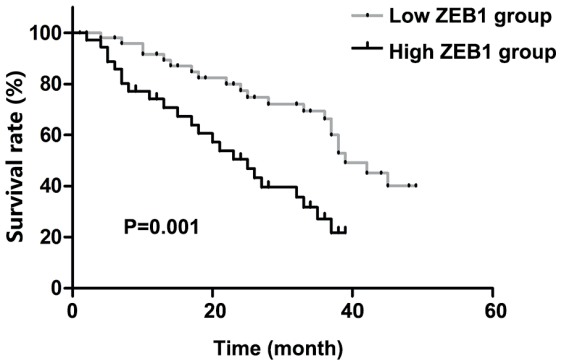

Figure 2. Relationship between the ZEB1 expression and the patient's survival rate. The overall survival rate is 40.1% in the low ZEB1 group and 21.7% in the high ZEB1 group. The median survival time is 39 months in the low ZEB1 group and 25 months in the high ZEB1 group.

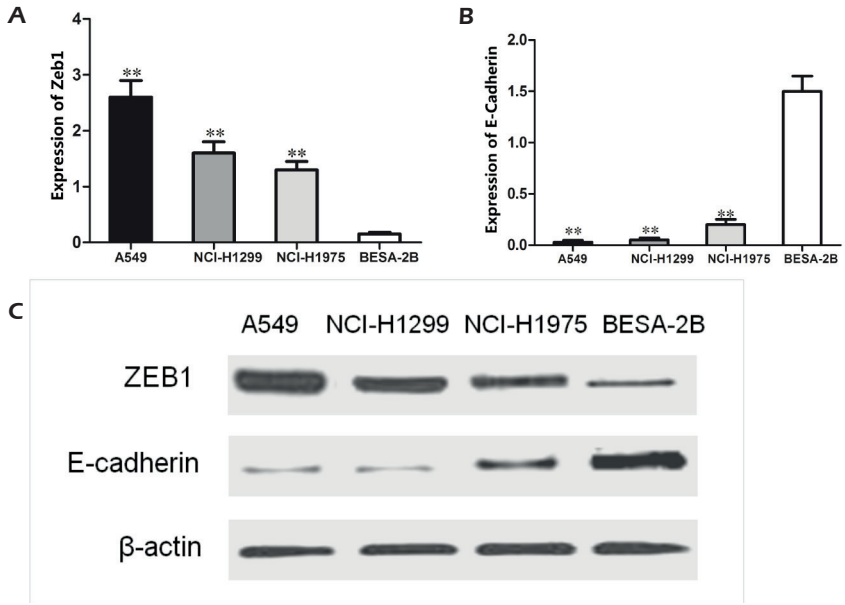

Figure 3. Correlation between the expression of ZEB1 and that of E-cadherin in NSCLC cell lines. ***A,*** Detection of the relative expression of ZEB1 in different cell lines via RT-PCR. ***B,*** Detection of the relative expression of E-cadherin in different cell lines via RT-PCR. ***C,*** Detection of the expressions of ZEB1 and E-cadherin in different cell lines via Western blotting. ** Represents an extremely significant difference ($p<0.01$).

6A). Flow cytometry analysis revealed that the ZEB1 overexpression significantly reduced the cell apoptosis (2.3%) compared with control cells (12.8%) (Figure 6B).

Discussion

Before this study, some researchers[13-18] studied the mechanism of ZEB1 proteins in other cancers, such as colon cancer, gastric cancer, small cell lung cancer, thyroid cancer, prostate cancer and endometrial cancer. In this study, the effects of Zeb1 on the incidence and prognosis of NSCLC were researched for the first time. It was observed that the expression level of ZEB1 in NSCLC was notably correlated with the cancer stage and tumor size ($p=0.001$). The results of 60-month follow-up and analysis of survival time and survival rate showed that the expression of ZEB1 was negatively related to the prognosis, proving that ZEB1 can be used as a potential tumor marker and prognostic indicator in NSCLC patients. In addition, in order to further explore the correlations of ZEB1 with the incidence and prognosis of NSCLC, NSCLC cell lines *in vitro* were also investigated. *In vitro* studies confirmed that the ZEB1 expression was negatively correlated with the E-cadherin expression. The EMT is an important process that allows cells and cell populations to migrate in developing tissues during the embryo development. The deletion of E-cadherin is a marker of the EMT. In adults, the EMT is considered as a potential cancer invasion and metastasis pro-

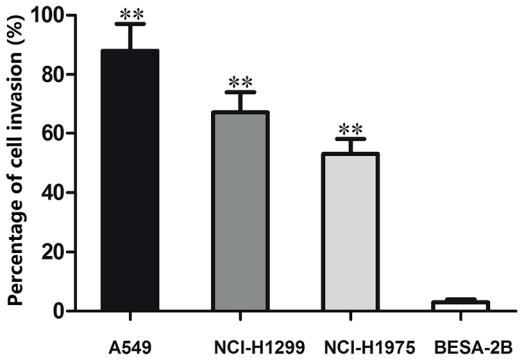

Figure 4. Detection of the invasion ability of A549, NCI-H1299, NCI-H1975 and human normal lung epithelial BESA-2B cell lines. ** Represents an extremely significant difference ($p<0.01$).

cess[19-21]. During the EMT, epithelial cells actively down-regulate the cell adhesion system and make it lose polarity. Thuault et al[22] reported that the transcription factor ZEB1 can induce the EMT in malignant tumors. The expression of ZEB1 in cancer cells may be related to the loss of differentiation markers, increased proliferation or EMT[23,24]. EMT remains controversial as a potential mechanism for cancer progression. E-cadherin is usually expressed in epithelial cells and participates in maintaining the polarity and cell-cell contact[25,26]. Loss of the E-cadherin expression is observed during tumor progression in most cancers. The down-regulation of E-cadherin in epithelial cells can be achieved by binding to the transcription factors, ZEB1, Snail, Slug, E12/E47, etc., of the EB element in the promoter[25,26]. Specifically, ZEB1 has been shown to bind to EB in the E-cadherin promoter in lung cancer and breast cancer cells[23,24]. In this study, the effects of the knockdown of ZEB1 by shRNAs on the E-cadherin expression and cell invasion were observed. The results denoted that the down-regulation of ZEB1 significantly promoted the expression of E-cadherin proteins and decreased the invasion ability of A549 cells. Finally, the effects of the ZEB1 overexpression on the proliferation and apoptosis of NSCLC A549 cells were determined. The results detected the role of ZEB1 in promoting the proliferation and reducing the apoptosis of NSCLC cancer cells. It has also been proved that ZEB1 is confined to the interstitial region of the normal endometrium and low-grade endometrial carcinoma. Moreover, a study of Singh et al[18] indicated that ZEB1 is abnormally expressed in epithelial-derived tumor cells of highly invasive uterine serous carcinoma and type II endometrial carcinoma. In addition, Li et al[27] demonstrated that the E-cadherin expression is inhibited when ZEB1 is inappropriately expressed in epithelial-derived tumor cells, and this reverse relationship was associated with increased migration and invasion potential. The forced expression of ZEB1 in non-migrating, low-grade and relatively dif-

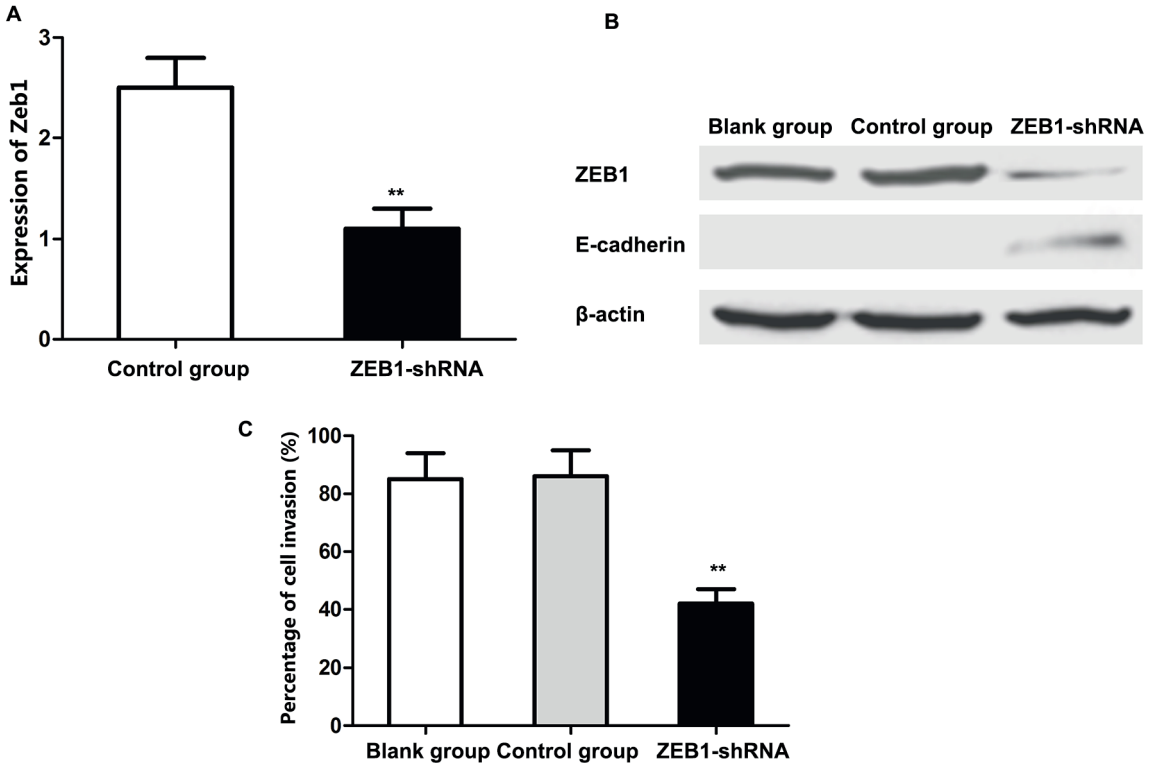

Figure 5. Effects of the knockdown of ZEB1 by shRNAs on the invasion ability of NSCLC cells. ***A,*** Detection of the effect of ZEB1-shRNAs on the expression of ZEB1 in A549 cells using Real-time RT-PCR. ***B,*** Detection of the effects of ZEB1-shRNAs on the expressions of ZEB1 proteins and E-cadherin by Western blotting. ***C,*** Effect of ZEB1-shRNA on the invasion ability of A549 cells. ** Represents an extremely significant difference ($p<0.01$).

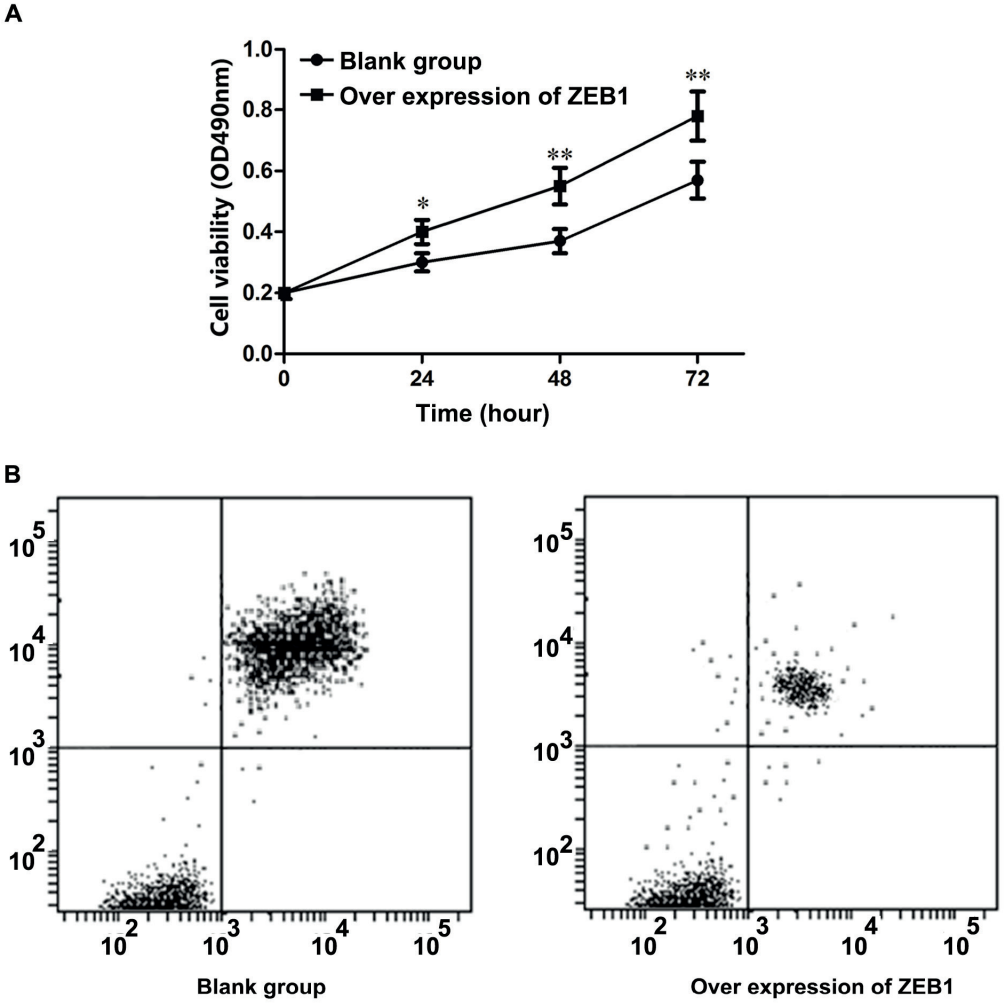

Figure 6. Effects of the ZEB1 overexpression on the proliferation and apoptosis of NSCLC cells. ***A***, Determination of the effects of A549 cells transfected with pcDNA-ZEB1 and blank plasmids on the cell activity via MTT assay. * Indicates a significant difference ($p<0.05$), and ** indicates an extremely significant difference ($p<0.01$). ***B***, Determination of the cell apoptosis by flow cytometry. Compared with the control group, the overexpression of ZEB1 significantly reduces the cell apoptosis.

ferentiated Ishikawa cell lines causes them to migrate. In contrast, in HEC50co, a highly migratory and invasive type II cell line, the decrease in ZEB1 results in a decrease in migration ability[18].

Conclusions

ZEB1 plays an important role in the incidence and prognosis of NSCLC and may have the potential to be a molecular marker of recurrence risk of NSCLC. Besides, it exerts a direct regulatory effect on cell metastasis and NSCLC progression and can be used as a new marker for prognosis or progression of NSCLC. The abnormal overexpression of ZEB1 in NSCLC can be used as a marker for the high invasion of NSCLC and a basis for chemotherapy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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