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IL-6 stimulates lncRNA ZEB2-AS1 to aggravate the progression of non-small cell lung cancer through activating STAT1

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Abstract. – OBJECTIVE: To illustrate the role of interleukin 6 (IL-6) in the progression of non-small cell lung cancer (NSCLC) *via* activating STAT1.

PATIENTS AND METHODS: The level of IL-6 mRNA in 48 paired NSCLC tissues and matched normal ones was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Kaplan-Meier curves were depicted for assessing the overall survival of NSCLC patients with high or low level of IL-6 mRNA. Subsequently, the ZEB2-AS1 level in A549 cells treated with different doses of IL-6 for different time points was determined. After A549 cells were treated with different doses of IL-6, wound closure assays were performed to assess the migration of cells. Protein levels of pSTAT1 and STAT1 in IL-6-treated A549 cells were detected by Western blot. The regulatory effect of STAT1 on IL-6-induced migration of A549 cells was also evaluated. The interaction between ZEB2-AS1 and STAT1 was explored through Chromatin Immunoprecipitation (ChIP) assay. Finally, the role of ZEB2-AS1/ STAT1 axis in regulating NSCLC cells was investigated through rescue experiments.

RESULTS: Our results indicated that IL-6 was upregulated in NSCLC tissues and cancer cell lines. NSCLC patients with T3-T4 or accompanied with lymphatic metastasis had a higher IL-6 abundance than those with T1-T2 or without metastatic foci. The worse prognosis was identified in NSCLC patients with high expression of IL-6 compared to those with low expression. ZEB2-AS1 showed dose-dependent and time-dependent increase in IL-6-treated A549 cells. IL-6 treatment gradually enhanced the migration ability of A549 cells in a dose-dependent manner. In IL-6-treated A549 cells, protein level of pSTAT1 was remarkably upregulated, and knockdown of STAT1 significantly reversed the promotive effect of IL-6 on migration ability of A549 cells. The results of ChIP assay verified the interaction between ZEB2-AS1 and STAT1. In addition, ZEB2-AS1 could reverse the regulatory effect of STAT1 on the migration ability of A549 cells.

CONCLUSIONS: IL-6 was upregulated in NSCLC and accelerated the progression of NSCLC *via* activating STAT1/ ZEB2-AS1.

Key Words:
NSCLC, IL-6, STAT1, ZEB2-AS1.

Introduction

Lung carcinoma is a kind of respiratory malignant tumor that endangers the health of human beings[1]. Non-small cell lung cancer (NSCLC) is the primary subtype of lung carcinoma and accounts for up to 85% in all cases[2,3]. Despite the tremendous advances in the diagnostic and therapeutic strategies for NSCLC, its prognosis and the 5-year survival are still poor[4]. Therefore, it is urgent to study the molecular mechanisms underlying the development of NSCLC.

The inflammatory response exerts an important role in the occurrence, development, and metastasis of tumors[5]. Multiple members of the interleukin cytokine family are remarkably upregulated in various tumors, particularly interleukin 6 (IL-6) and IL-11. They are considered to be the major regulators in tumorigenesis and tumor metastasis[6]. These cytokines are closely related to the cellular performances and angiogenesis[7].

Long non-coding ribonucleic acids (lncRNAs) are non-coding RNAs with over 200 nt long, which exert transcriptional or post-transcriptional regulation on gene expressions[8]. The role of lncRNAs in tumorigenesis has been identified recently[9,10]; increased lncRNA ZEB2-AS1 stimulates the proliferation and inhibits the apoptosis of lung carcinoma[11]. However, the potential influences of IL-6-induced inflammation and STAT1/ ZEB2-AS1 axis in the progression of NSCLC remain unclear.

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Patients and Methods

Patients and Samples

A total of 48 paired tumor tissues and matched adjacent tissues (3 cm away from the tumor edge) were surgically resected from NSCLC patients treated in Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology from April 2016 to December 2018. The patients were pathologically diagnosed as NSCLC and had not received any preoperative anti-tumor therapy. Clinical data of enrolled NSCLC patients were also collected. All subjects volunteered to participate in the study and signed the written informed consent. This investigation was approved by the Ethics Committee of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology.

Cell Culture

Lung carcinoma cell lines (A549, NCI-H1650, and HCC827) were provided by Cell Bank (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS, Grand Island, NY, USA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a 37°C, 5% CO_2 incubator.

Cell Transfection

Cells were cultured to a confluence of 60% prior to transfection, and cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 6 h of transfection, complete medium was replaced. After 24-48 h of transfection, cells were collected for *in vitro* experiments.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcription. The complementary deoxyribonucleic acids (cDNAs) were applied for PCR using SYBR Green method. QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression of miRNAs was calculated using $2^{-\Delta\Delta CT}$. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. The experiment was repeated for 3 times. Primer sequences were listed in Table I.

Table I. Gene primers table.

Gene	Primers sequence
ZEB2-AS1	F: 5'-CCTGGAAAGGGAAATCCTG-3' R: 5'-AGGATGAATATAGACAGGCCA-3'
IL-6	F: 5′-AGTAGTGAGGAACAAGCCAGA-3′ R: 5′-TACATTTGCCGAAGAGCC-3′
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGT-3' R: 5'-GGGAAGGATCTGTCTCTGACC-3'

Western Blot

Total protein was extracted from cells using radio immunoprecipitation assay (RIPA, Beyotime, Shanghai, China) and quantified by bicinchoninic acid (BCA, Beyotime, Shanghai, China) method. Protein sample was loaded for electrophoresis and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). After blocking in 5% skim milk for 2 h, membranes were incubated with primary and secondary antibodies. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (Media Cybernetics, Silver Springs, MD, USA).

Wound Healing Assay

Cells were seeded in a 6-well plate with 5.0×10^5 cells/well. An artificial wound was created in the confluent cell monolayer using a 1 mL pipette tip. Wound closure images were taken at 0 and 24 h using an inverted microscope, respectively. The percentage of wound closure was calculated.

Chromatin Immunoprecipitation (ChIP)

Cells were subjected to 10 min cross-link with 1% formaldehyde at room temperature. Subsequently, cells were lysed using lysis buffer and sonicated for 30 min. Finally, the sonicated lysate was immuno-precipitated with anti-STAT1 or anti-IgG.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 (Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The *t*-test was performed to analyze the difference between the two groups. Survival analysis was carried out using Kaplan-Meier method, followed by log-rank test for comparing the difference. $p < 0.05$ was considered as statistically significant.

Results

IL-6 Was Negatively Correlated to the Prognosis of NSCLC

We observed a higher abundance of IL-6 in NSCLC tissues compared to matched normal ones (Figure 1A). In particular, NSCLC patients in stage T3-T4 showed an enhanced expression of IL-6 than those in T1-T2 (Figure 1B). NSCLC patients with lymphatic metastasis also presented higher IL-6 level than those without lymphatic metastatic foci (Figure 1C). Kaplan-Meier curves were depicted for assessing the prognostic potential of IL-6 in NSCLC. As the data revealed, the worse prognosis was identified in NSCLC patients with high expression of IL-6 relative to those with low expression (Figure 1D).

IL-6 Induced the Upregulation of ZEB-AS1 Level and the Migration of Lung Carcinoma Cells

To clarify the role of IL-6 in the progression of *NSCLC*, we further treated A549, NCI-H1650, and HCC827 cells with IL-6. Compared with those in blank control, IL-6 induction remarkably upregulated the level of ZEB2-AS1 in lung carcinoma cells (Figure 2A). Besides, ZEB2-AS1 was significantly upregulated in dose-dependent and time-dependent manner in A549 cells after IL-6 treatment (Figure 2B, 2C). Wound healing assays also revealed a dose-dependent elevation of the migration ability of A549 cells after IL-6 treatment (Figure 2D).

IL-6 Aggravated the Malignant Level of NSCLC Through Activating STAT1

To further investigate whether IL-6 could affect the malignant level of NSCLC by activating STAT1, we treated A549 cells with recombinant human IL-6 protein. The results indicated that pSTAT1 expression was remarkably enhanced in IL-6-treated A549 cells (Figure 3A). Subsequently, the transfection efficacy of si-STAT1 was verified in A549 cells (Figure 3B). The pSTAT1 protein was upregulated by IL-6 treatment in A549 cells, while transfection of si-STAT1 then reversed pSTAT1 level (Figure 3C). Moreover, the enhanced migration ability of A549 cells treated with IL-6 was also partially reversed after knockdown of STAT1 *via* si-STAT1 (Figure 3D). The above data demonstrated that IL-6 could influence the malignancy of NSCLC by activating STAT1.

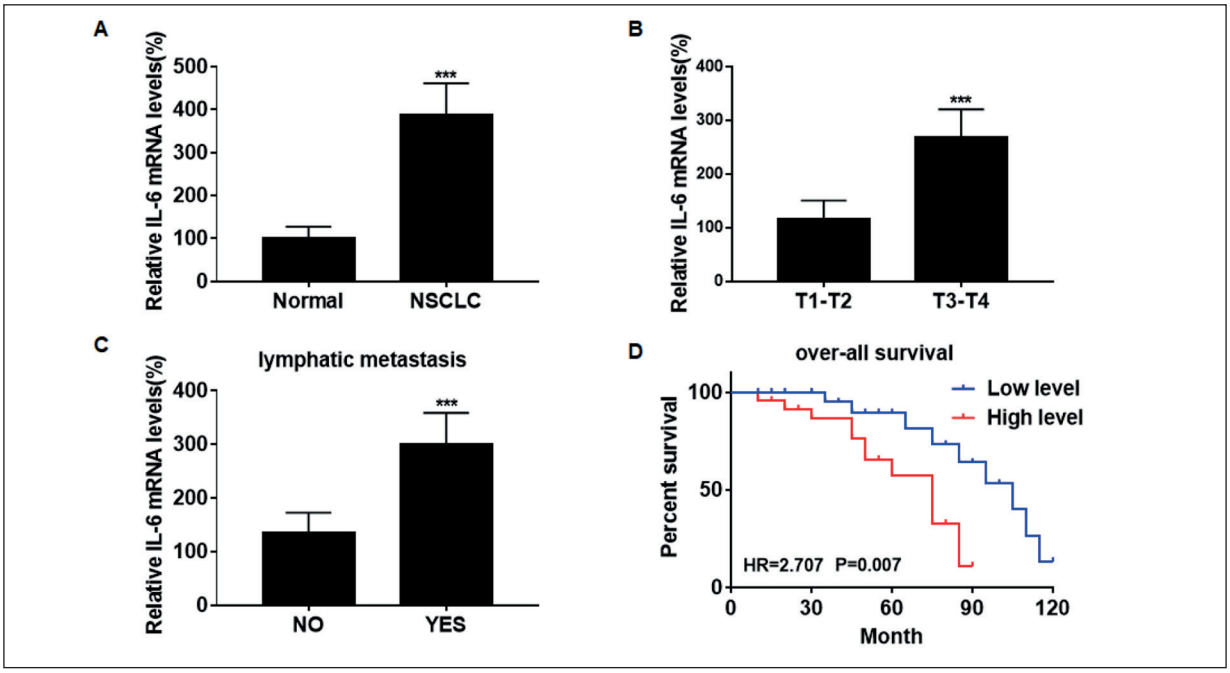

Figure 1. Upregulated IL-6 was negatively correlated to the prognosis of NSCLC. **A,** Relative level of IL-6 in NSCLC tissues and matched adjacent normal tissues. **B,** Relative level of IL-6 in NSCLC patients with T1-T2 and T3-T4. **C,** Relative level of IL-6 in NSCLC patients either with lymphatic metastasis or not. **D,** Kaplan-Meier curves introduced for assessing the overall survival in NSCLC patients with high level or low level of IL-6.

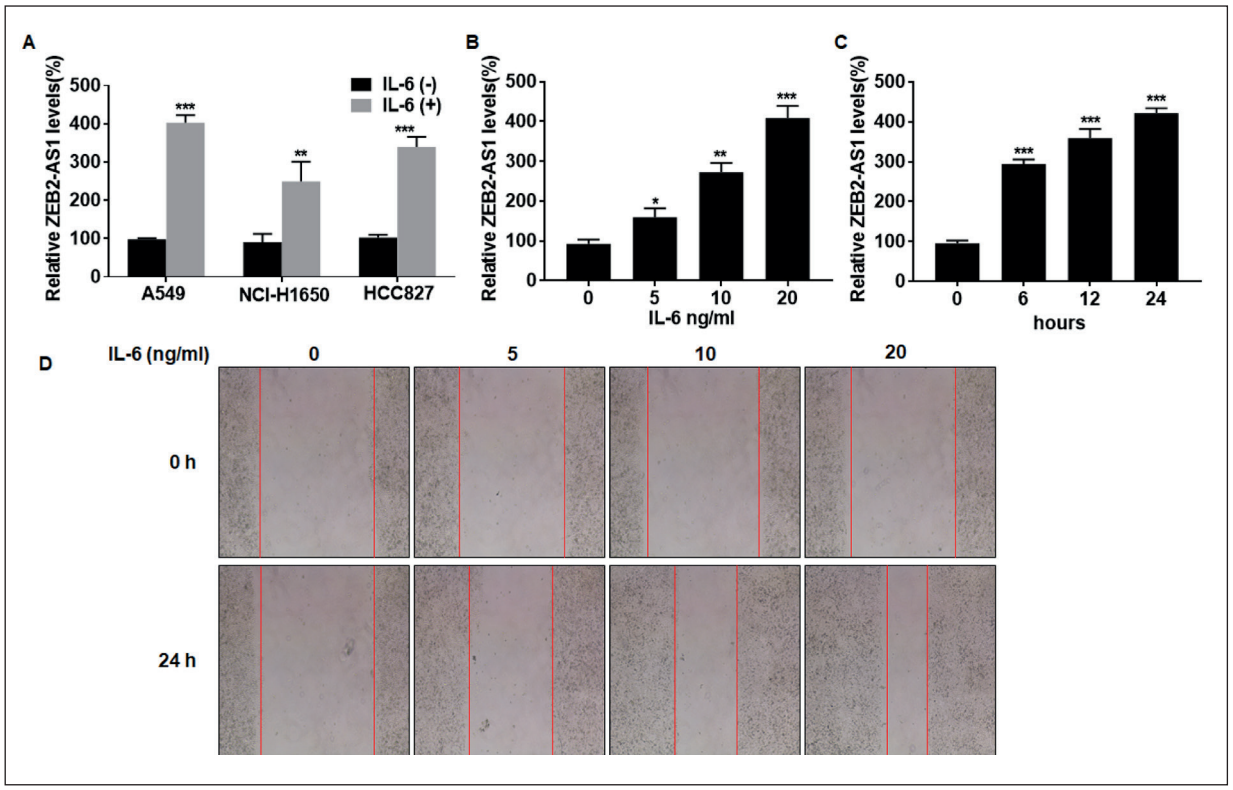

Figure 2. IL-6 induced the upregulation of ZEB-AS1 level and migration ability of lung carcinoma cells. **A,** Relative level of ZEB2-AS1 in A549, NCI-H1650, and HCC827 cells induced with IL-6 or not. **B,** Relative level of ZEB2-AS1 in A549 cells treated with 0, 5, 10, and 20 ng/ml IL-6. **C,** Relative level of ZEB2-AS1 in A549 cells treated with 20 ng/ml IL-6 for 0, 6, 12, and 24 h. **D,** Wound healing assay showed the wound closure in A549 cells treated with 0, 5, 10, and 20 ng/ml IL-6 at 0 and 24 h (magnification × 20).

ZEB2-AS1 Influenced the Migration Ability of NSCLC Through Interacting with STAT1

In IL-6-treated A549 cells, transfection of si-STAT1 markedly downregulated ZEB2-AS1 level (Figure 4A). The results of ChIP assay illustrated that pSTAT1 could bind to promoter region of ZEB2-AS1 (Figure 4B). Transfection of pcDNA-ZEB2-AS1 could greatly upregulate ZEB2-AS1 level in A549 cells (Figure 4C). Subsequently, the rescue experiments were conducted by co-transfection of si-STAT1 and pcDNA-ZEB2-AS1 into A549 cells (Figure 4D). As wound healing assay revealed, IL-6 treatment enhanced the migration ability of A549 cells, while transfection of si-STAT1 remarkably reduced the wound closure. Notably, overexpression of ZEB2-AS1 reversed the regulatory effect of STAT1 on the migratory ability of NSCLC (Figure 4E).

Discussion

Lung carcinoma is a prevalent kind of tumor leading to an extremely high tumor-related mortality throughout the world[12]. Epigenetic regulation is of great importance in the occurrence and progression of tumors[13,14]. Increasing evidence[15-17] has proved the vital function of dysregulated lncRNAs in multiple types of tumors. In this study, ZEB2-AS1 level was dose-dependently and time-dependently upregulated after IL-6 treatment in lung cancer cells. Our results indicated a potentially important role of IL-6 and ZEB2-AS1 in the progression of NSCLC.

Inflammatory response exerts a decisive role in different stages of tumor progression[18]. IL-6 and IL-11 are members of the IL-6 family that are predominantly expressed in solid tumors. Gp130 is a common receptor and signal transducer of cytokines in IL-6 family[19]. Once gp130 binds to

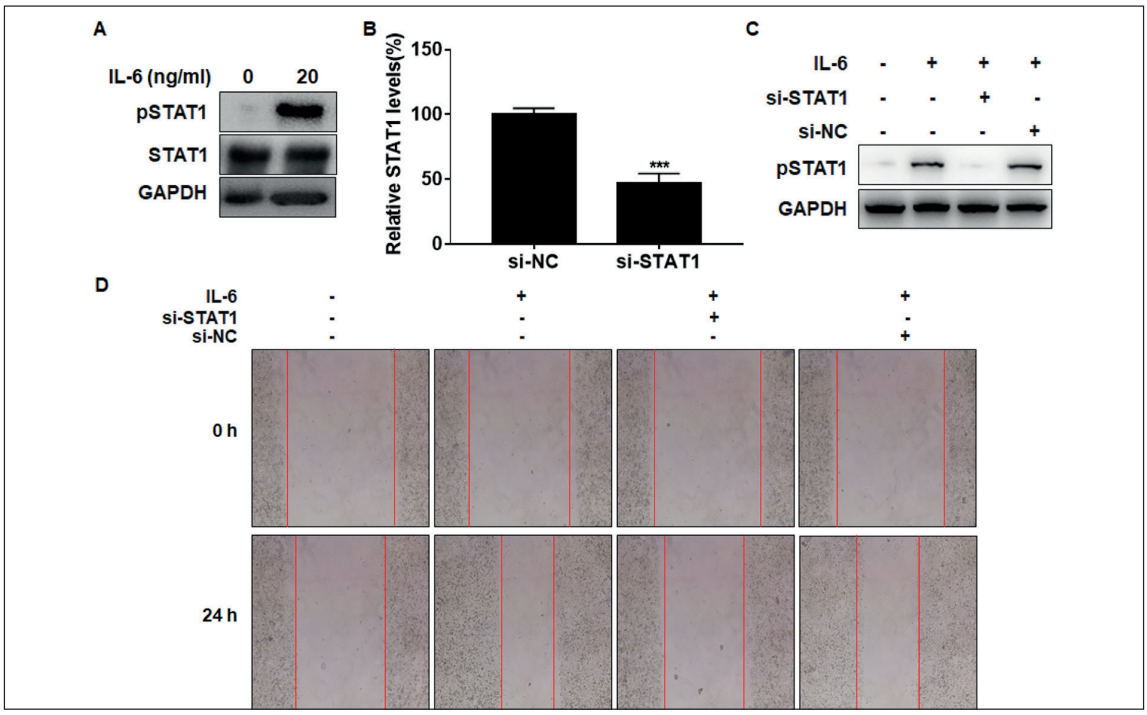

Figure 3. IL-6 aggravated the malignant level of NSCLC through activating STAT1. **A,** Western blot analysis of pSTAT1 and STAT1 in A549 cells induced with 20 ng/ml IL-6 or not. **B,** Transfection efficacy of si-STAT1 in A549 cells. **C,** Western blot analysis of pSTAT1 in A549 cells. **D,** Wound healing assay showed the wound closure in A549 cells (magnification × 20).

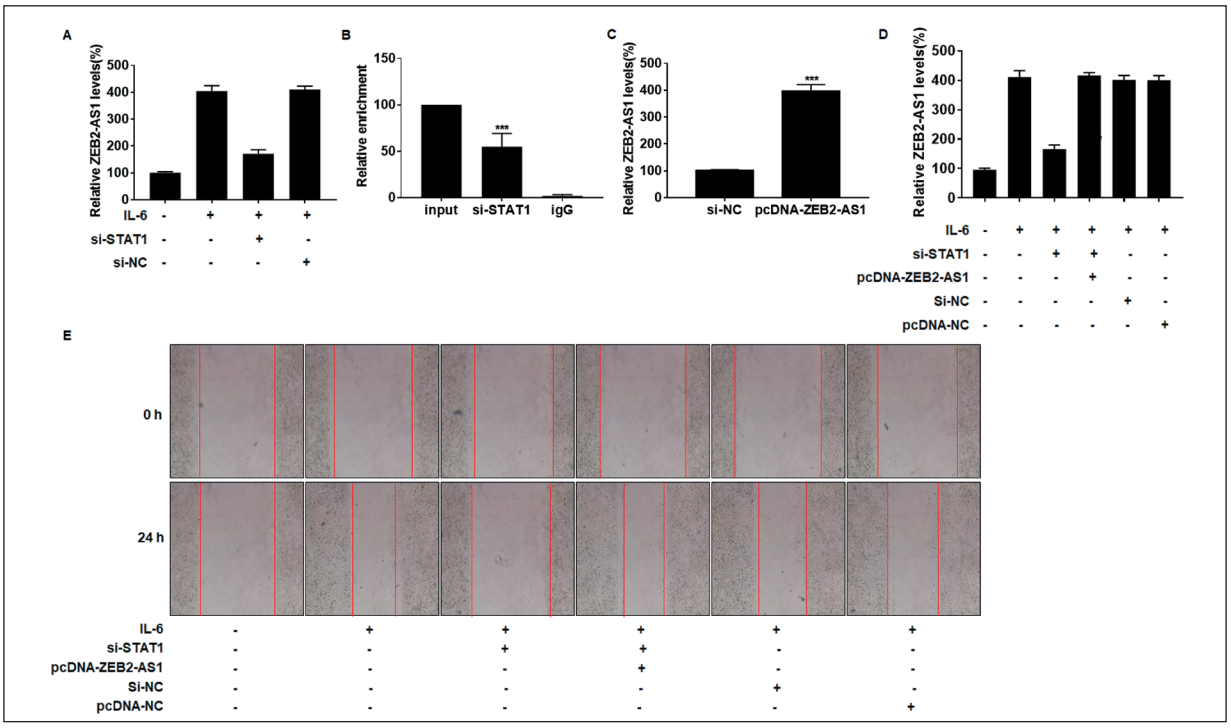

Figure 4. ZEB2-AS1 influenced the migratory ability of NSCLC through interacting with STAT1. **A,** Relative level of ZEB2-AS1 in A549 cells. **B,** ChIP assay showed the enrichment of ZEB2-AS1 in input, STAT1, and IgG group. **C,** Transfection efficacy of pcDNA-ZEB2-AS1 in A549 cells. **D,** Relative level of ZEB2-AS1 in A549 cells. **E,** Wound healing assay showed the wound closure in A549 cells (magnification × 20).

a cytokine-specific receptor, it directly phosphorylates STAT3 and STAT1 by JAK kinase. The phosphorylated STAT forming homologous or heterodimers translocate into the nucleus and act as transcription factors[7, 20].

Signal transducer and activator of transcription-1 (STAT1) is an important member of the STAT family[21]. Recent studies have shown the involvement of STAT1 in tumor invasion and metastasis. Knockdown of STAT1 in melanoma cells results in impaired migration and invasion of cancer cells[22,23]. In breast cancer, STAT1 accelerated the progression by stimulating the transcription of estrogen receptors[24]. Our research suggested that STAT1 could bind to the promoter region of ZEB2-AS1 and promote its transcription, thus influencing the proliferation and migration of A549 cells.

Conclusions

Taken together, these results suggested that the inflammatory factor IL-6 promoted the progression of NSCLC by activating STAT1/ZEB2-AS1. IL-6 might be used as a prognostic marker and therapeutic target for patients with NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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