Over-regulation of microRNA-133b inhibits cell proliferation of cisplatin-induced non-small cell lung cancer cells through PI3K/Akt and JAK2/STAT3 signaling pathway by targeting EGFR

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Abstract. The present study determined the anticancer activity and its mechanism of microRNA-133b on cell proliferation of cisplatin-induced non-small cell lung cancer cells. The expression of microRNA-133b cisplatin-induced non-small cell lung cancer (NSCLC) tissue was lower than that of para-carcinoma tissue in patients. Overall survival of higher expression in cisplatin-induced NSCLC patients was higher than that of lower expression in cisplatin-induced NSCLC patients. Over-regulation of microRNA-133b inhibited cell proliferation and LDH activity, induced apoptosis and caspase-3 activity, suppressed the protein expression of EGFR, PI3K, p-Akt, p-JAK2 and p-STAT3, decreased cyclin D1 and increased Bax protein expression in cisplatin-induced A549 cells. EGFR inhibitor (lapatinib) suppressed EGFR protein expression, inhibited cell proliferation and LDH activity, and induced apoptosis and caspase-3 activity in cisplatin-induced A549 cells by over-regulation of microRNA-133b. When EGFR protein expression was suppressed, PI3K, p-Akt, p-JAK2 and p-STAT3, decreased cyclin D1 and increased Bax protein expression in cisplatin-induced A549 cells by over-regulation of microRNA-133b. Altogether, our results indicated that over-regulation of microRNA-133b inhibits cell proliferation of cisplatin-induced NSCLC by PI3K/Akt and JAK2/STAT3 signaling pathway by targeting EGFR.

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Introduction

Lung cancer is ranked the first in terms of morbidity among all human tumors, which also has short survival (1). Although the treatment is continually updated, the 5-year survival rate has not improved significantly over the past 25 years, which is ~15%. Lung cancer cells are resistant to chemotherapy, which is one of the major causes short survival for patients (2). Certain factors are known to be associated with tumor tolerance to chemotherapeutic agents. The regulation of chemotherapeutic uptake and elimination by lung carcinoma cells, which is mediated by membrane translocation mechanism-related proteins (such as the P-glycoprotein and multidrug resistance-associated proteins). In addition, other important physiological activities of cells, such as apoptosis, proliferation, intracellular environment abnormalities, are also important ways to induce the occurrence of drug resistance (2,3). The former is referred to as the classical resistance pathway while the latter is as the atypical pathway of drug resistance (4).

Cisplatin (CDDP) is a non-cell cycle-specific cytotoxic drug. Its main mechanism is to form hydrates in the body after crosslinking with DNA and replication and transcription inhibition, thereby promoting tumor cell apoptosis to achieve the purpose of killing tumor cells (5). It is the first-line drug for clinical application. At present, treatment for lung cancer is dominated by platinum-based chemotherapy (such as cisplatin and carboplatin), which is supplemented by other chemotherapeutic drugs (6). That lung cancer cells are resistant to cisplatin is an important cause of chemotherapy failure, the mechanism of which has not been fully elucidated yet.

Biological information data showed that miRNA molecules control more than one third of human genes, which are considered to be the most predominant factors of gene expression in the eukaryotic genome (7). Abundant evidence has shown that gene expression profile in different tissues and at various differentiation stages is closely related to tumor occurrence and development (8). It has been predicted that using miRNA as target molecule for tumor biotherapy may be more effective than coding gene as a target molecule (8). Therefore, treating cancer by regulating miRNA expression

may open up new roads for the targeted therapy of lung cancer (7).

Abnormal miRNA expression may lead to the loss or enhancement of miRNA function, thus affecting the expression levels of the target protein (9). Receptors affecting gene expression on drug uptake, metabolism and distribution pathways, as well as targeting clinical function may significantly affect the therapeutic effects of antitumor drugs (10). It is of great significance to elucidate the mechanism of action of miRNA in the metabolism of antitumor drugs, so as to improve the efficacy and safety, to reverse the drug resistance of the tumor and to guide the personalized medication (11).

Studies have shown that the signal channels of Janus kinase signal transducers 2 and activator of transcription 3 (JAK2/STAT3) can regulate the expression of pro-angiogenesis factors, such as vascular endothelial growth factor (VEGF). In this way, it can promote the formation of capillaries in lung cancer. VEGF expression is closely related to the hypoxia inducible factor-1α (HIF-1α) (12).

The PI3K/AKT pathway is a widely existing pathway for signal transduction. It plays an important role in the cell proliferation, cell apoptosis, as well as cell metabolism. In recent years, it has been found that the PI3K/AKT pathway plays an important role in the development and progression of tumors (13). It mainly affects the energy metabolism, growth as well as the proliferation of tumor cells, inhibits apoptosis and affects invasion ability of tumor cells (14). A variety of antitumor drugs have been developed in clinical application with the key members such as PI3K, AKT, mTOR, p70s6k as drug targets (13). The PI3K/AKT pathway also plays an important role in the resistance of tumor cells to cisplatin. For instance, the overexpression of AKT1 leads the resistance of lung cancer cells to cisplatin. The inhibition of the AKT1 expression can reverse the resistance of lung adenocarcinoma MDR cells A549/CDDP to cisplatin (15). Further studies have shown that AKT1-induced resistance of lung cancer to cisplatin is mediated by the signal pathway mTOR-P70S6K1 (15). The present study demonstrated anticancer activity and its mechanism of microRNA-133b on cell proliferation of cisplatin-induced non-small cell lung cancer cells.

Patients and methods

Patients. In the present study, NSCLC and para-carcinoma tissues (>5 cm from cancer tissue) from patients (n=24) were collected at operation. At every three months, we re-evaluated each patient. All experimental procedures were performed in accordance with the guidelines of Fourth Hospital of Hebei Medical University (Hebei, China).

Cell culture and miRNA transfection. A549/cisplatin cells were obtained from the Cell Biology Tesearch Laboratory and Modern Analysis Testing Center of Central South University (Changsha, China) and cultured in RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) with 2 mg/l cisplatin (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO_2. MicroRNA-133b and negative control mimics were obtained

synthesized by GenePharma (Shanghai, China). A549/cisplatin was transfected using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA).

Cell viability assay. Cell proliferation was documented every 24 h after transfection, and MTT solution was added into each well and incubated at 37°C for 4 h. The purple colored precipitates of formazan were dissolved in 200 µl dimethyl sulfoxide (DMSO) at 37°C for 15 min. The absorbance was measured using an automatic multi-well spectrophotometer (Bio-Rad, Richmond, CA, USA) at 490 nm.

Apoptosis assay. Cell proliferation was documented every 48 h after transfection, and washed with phosphate-buffered saline (PBS). Cell was stained with 10 µl Annexin V-FITC and 5 µl propidium iodide (PI) apoptosis detection kit (BD Biosciences, San Jose, CA, USA) for 15 min in the dark at 4°C. Analysis rate was carried out on FACSCanto II (BD Biosciences).

Western blot analysis. The cells were harvested with ice-cold PBS. The total protein was prepared using radioimmunoprecipitation assay lysis buffer and determined using the Bradford protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Equal quantities of protein (50 µg) were loaded onto an 8-10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked in 5% shimmed milk diluted with Tris-buffered saline with Tween-20 (TBST) at 37°C for 1 h, and incubated overnight at 4°C with anti-EGFR, anti-PI3K, anti-p-Akt, anti-p-JAK2, anti-p-STAT3, anti-cyclin D1, anti-Bax and anti-GAPDH (all from Cell Signaling Technology, Inc., Danvers, MA, USA). The membranes were then incubated with a goat anti-rabbit IgG-conjugated to horseradish peroxidase secondary antibody (1:5,000; Cell Signaling Technology, Inc.) for 1 h at 37°C and visualized using ECLplus reagents (Amersham Biosciences Corp., USA).

Statistical analysis. Data from each group are expressed as mean ± standard error of the mean (SEM). Two-way ANOVA with Bonferroni multiple comparison adjustments were used to assess differences across the investigational groups. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Cisplatin-induced NSCLC microRNA-133b expression. First the endogenous levels of microRNA-133b expression in cisplatin-induced NSCLC were examined. Fig. 1 shows that the expression of microRNA-133b cisplatin-induced non-small cell lung cancer (NSCLC) tissue was lower than that of para-carcinoma tissue in patients. Moreover, overall survival of higher expression in cisplatin-induced NSCLC patients was higher than that of lower expression in cisplatin-induced NSCLC patients.

Over-regulation of microRNA-133b inhibits cell proliferation and LDH activity in cisplatin-induced NSCLC. To investigate the roles of miRNA-133b in cisplatin-induced
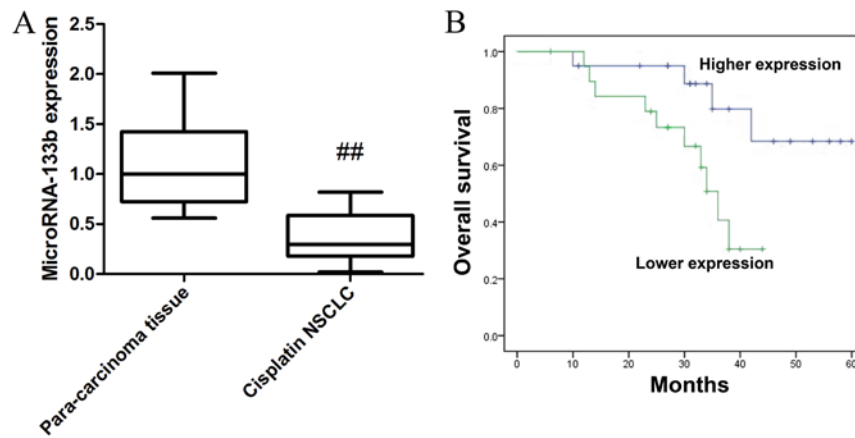
Figure 1. microRNA-133b expression in cisplatin-induced NSCLC.
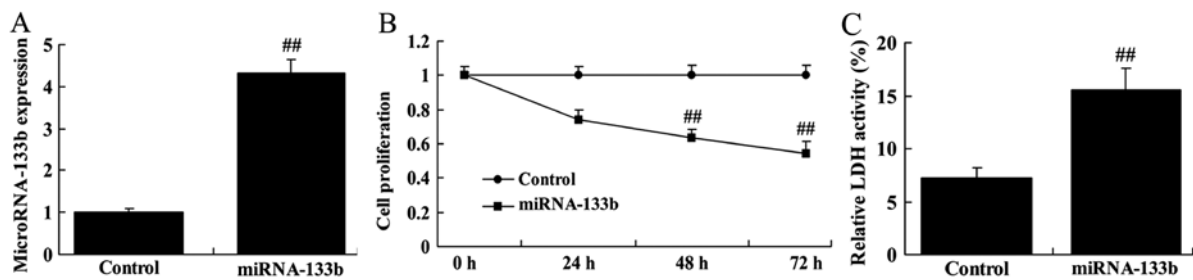

Figure 2. Over-regulation of microRNA-133b inhibits cell proliferation and LDH activity in cisplatin-induced NSCLC. (A) MicroRNA-133b expression, (B) cell proliferation and (C) LDH activity in cisplatin-induced NSCLC. Control, control negative mimics group; microRNA-133b, microRNA-133b mimics group; ##p<0.01 compared with negative mimics group.
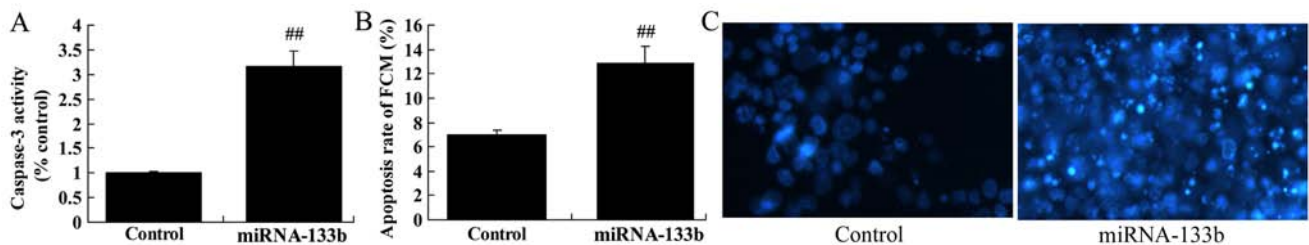

Figure 3. Over-regulation of microRNA-133b induces apoptosis and caspase-3 activity in cisplatin-induced NSCLC. (A) Caspase-3 activity, (B) apoptosis rate and (C) cell nucleus staining. Control, control negative mimics group; microRNA-133b, microRNA-133b mimics group; ##p<0.01 compared with negative mimics group.

NSCLC apoptosis, A549/cisplatin cells were transfected with microRNA-133b mimics. As shown in Fig. 2, over-regulation of microRNA-133b inhibited cell proliferation and increased LDH activity in cisplatin-induced NSCLC.

Over-regulation of microRNA-133b induces apoptosis and caspase-3 activity in cisplatin-induced NSCLC. We measured apoptosis and caspase-3 activity in cisplatin-induced NSCLC by microRNA-133b. Notably, the results from the present study demonstrated over-regulation of microRNA-133b-induced apoptosis and caspase-3 activity in cisplatin-induced NSCLC (Fig. 3).

Over-regulation of microRNA-133b suppresses EGFR, PI3K and p-Akt protein expression in cisplatin-induced NSCLC. To investigate the mechanism of microRNA-133b on

cisplatin-induced NSCLC apoptosis, EGFR, PI3K and p-Akt protein expression was measured using western blot analysis. The results of western blot analysis showed that over-regulation of microRNA-133b suppressed EGFR, PI3K and p-Akt protein expression in cisplatin-induced NSCLC (Fig. 4).

Over-regulation of microRNA-133b suppresses p-JAK2 and p-STAT3 protein expression in cisplatin-induced NSCLC. To verify the mechanism of microRNA-133b on cisplatin-induced NSCLC apoptosis, JAK2/STAT3 signaling pathway was selected and analyzed. As expected, p-JAK2 and p-STAT3 protein expression in cisplatin induced NSCLC by microRNA-133b over-regulation (Fig. 5).

Over-regulation of microRNA-133b suppressed cyclin D1 protein expression and induced Bax protein expression in
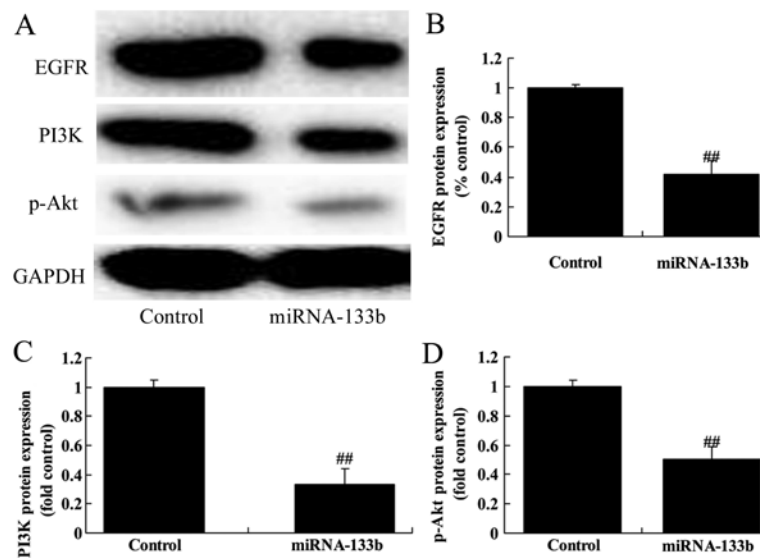
Figure 4. Over-regulation of microRNA-133b suppresses EGFR, PI3K and p-Akt protein expression in cisplatin-induced NSCLC. Over-regulation of microRNA-133b suppressed EGFR, PI3K and p-Akt protein expression using (A) western blot and (B) statistical analyses of EGFR, (C) PI3K and (D) p-Akt protein expression in cisplatin-induced NSCLC. Control, control negative mimics group; microRNA-133b, microRNA-133b mimics group; ##$p < 0.01$ compared with negative mimics group.
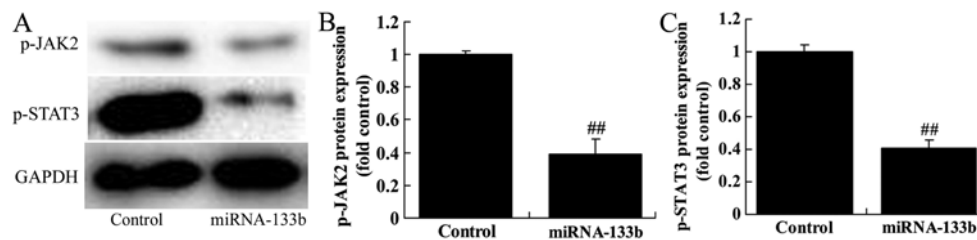

Figure 5. Over-regulation of microRNA-133b suppresses p-JAK2 and p-STAT3 protein expression in cisplatin-induced NSCLC. Over-regulation of microRNA-133b suppressed p-JAK2 and p-STAT3 protein expression using (A) western blot and (B) statistical analyses of p-JAK2, and (C) p-STAT3 protein expression in cisplatin-induced NSCLC. Control, control negative mimics group; microRNA-133b, microRNA-133b mimics group; ##$p < 0.01$ compared with negative mimics group.
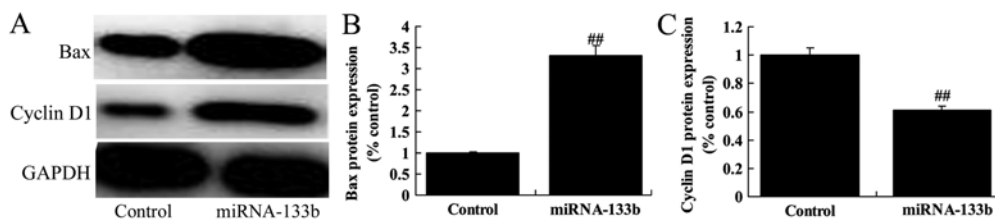

Figure 6. Over-regulation of microRNA-133b suppresses cyclin D1 protein expression and induced Bax protein expression in cisplatin-induced NSCLC. Over-regulation of microRNA-133b suppressed cyclin D1 protein expression and induced Bax protein expression using (A) western blot and (B) statistical analyses of cyclin D1, and (C) Bax protein expression in cisplatin-induced NSCLC. Control, control negative mimics group; microRNA-133b, microRNA-133b mimics group; ##$p < 0.01$ compared with negative mimics group.

cisplatin-induced NSCLC. Next, we measured the function of microRNA-133b on cisplatin-induced NSCLC apoptosis and cell cycle. As shown in Fig. 6, over-regulation of microRNA-133b suppressed cyclin D1 protein expression and induced Bax protein expression in cisplatin-induced NSCLC, suggesting that the upregulation of microRNA-133b on apoptosis and cell cycle of cisplatin-induced NSCLC is subsequent to PI3K/Akt and JAK2/STAT3 signaling pathway by targeting EGFR.

The suppression of EGFR on cell proliferation and LDH activity in cisplatin-induced NSCLC following microRNA-133b. We investigated the role of EGFR in the function of microRNA-133b on cell growth of cisplatin-induced NSCLC. As shown in Fig. 7, the suppression of EGFR (lapatinib, 5 nM) significantly inhibited cell proliferation and increased LDH activity in cisplatin-induced NSCLC following microRNA-133b, compared to that of microRNA-133b group.
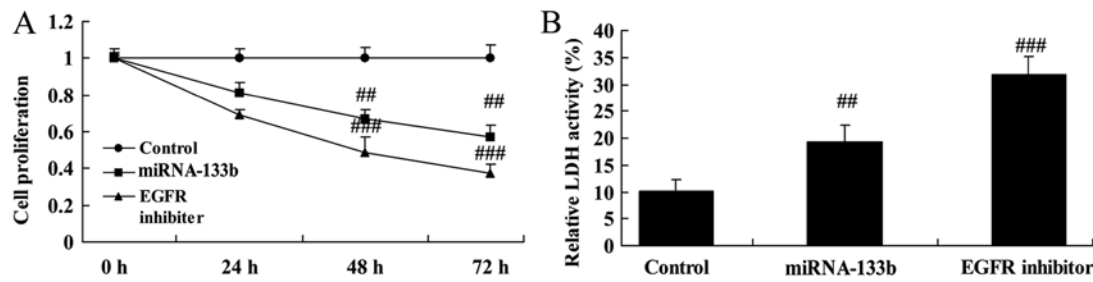
Figure 7. The suppression of EGFR on cell proliferation and LDH activity in cisplatin-induced NSCLC following microRNA-133b. The suppression of EGFR on (A) cell proliferation and (B) LDH activity in cisplatin-induced NSCLC following microRNA-133b. Control, control negative mimics group; microRNA-133b, microRNA-133b mimics group; EGFR inhibitor, lapatinib, 5 nM + microRNA-133b mimics group; ##p<0.01 compared with negative mimics group, ###p<0.01 compared with microRNA-133b mimics group.
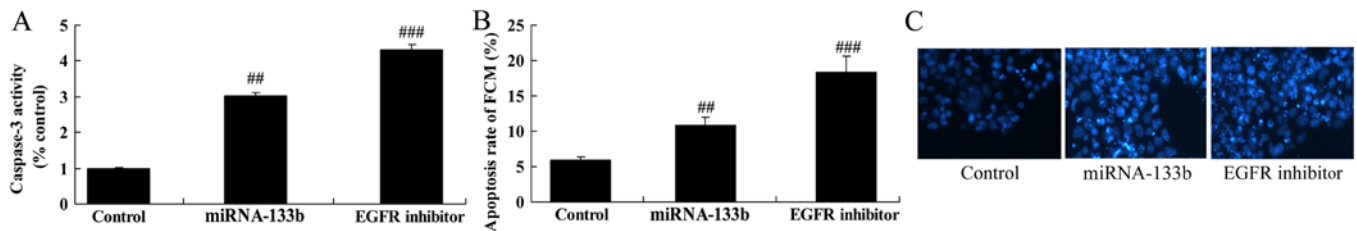

Figure 8. The suppression of EGFR on apoptosis and caspase-3 activity in cisplatin-induced NSCLC following microRNA-133b. (A) Caspase-3 activity, (B) apoptosis rate and (C) cell nucleus staining. Control, control negative mimics group; microRNA-133b, microRNA-133b mimics group; EGFR inhibitor, lapatinib, 5 nM + microRNA-133b mimics group; ##p<0.01 compared with negative mimics group, ###p<0.01 compared with microRNA-133b mimics group.
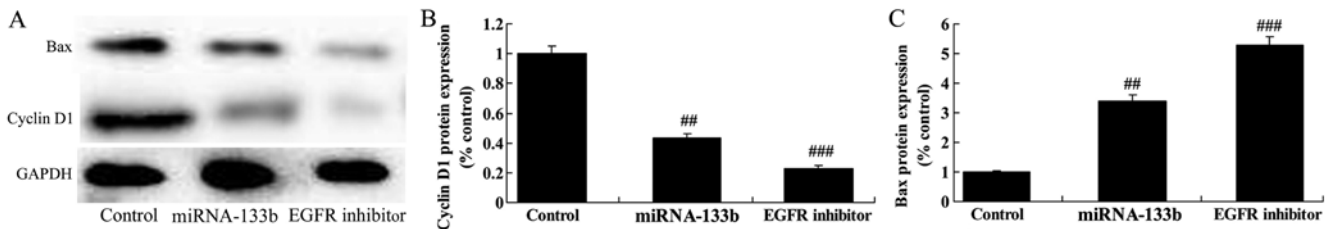

Figure 9. The suppression of EGFR on cyclin D1 and Bax protein expression in cisplatin-induced NSCLC following microRNA-133b. The suppression of EGFR on cyclin D1 protein expression and induced Bax protein expression using (A) western blot and (B) statistical analyses of cyclin D1 and (C) Bax protein expression in cisplatin-induced NSCLC following microRNA-133b. Control, control negative mimics group; microRNA-133b, microRNA-133b mimics group; EGFR inhibitor, lapatinib 5 nM + microRNA-133b mimics group; ##p<0.01 compared with negative mimics group, ###p<0.01 compared with microRNA-133b mimics group.

The suppression of EGFR on apoptosis and caspase-3 activity in cisplatin-induced NSCLC following microRNA-133b. Then, the suppression of EGFR significantly also induced apoptosis rate and caspase-3 activity in cisplatin-induced NSCLC following microRNA-133b, compared to that of microRNA-133b group (Fig. 8).

The suppression of EGFR on EGFR, PI3K and p-Akt protein expression in cisplatin-induced NSCLC following microRNA-133b. However, we measured EGFR, PI3K and p-Akt protein expression using western blot analysis. As shown in Fig. 9, the suppression of EGFR significantly also suppressed EGFR, PI3K and p-Akt protein expression in cisplatin-induced NSCLC following microRNA-133b, compared to that of microRNA-133b group (Fig. 9).

The suppression of EGFR on p-JAK2 and p-STAT3 protein expression in cisplatin-induced NSCLC following

microRNA-133b. Our findings suggested that p-JAK2 and p-STAT3 protein expression in cisplatin-induced NSCLC following microRNA-133b by EGFR suppression, compared to that of microRNA-133b group (Fig. 10).

The suppression of EGFR on cyclin D1 and Bax protein expression in cisplatin-induced NSCLC following microRNA-133b. Lastly, cyclin D1 protein expression was significantly suppressed, and Bax protein expression significantly induced in cisplatin-induced NSCLC following microRNA-133b by EGFR suppression, compared to that of microRNA-133b group (Fig. 11).

Discussion

The resistance of lung cancer cells to chemotherapy is one of the main reasons for the treatment failure in lung cancer patients (3). Among the current therapeutic schemes of lung
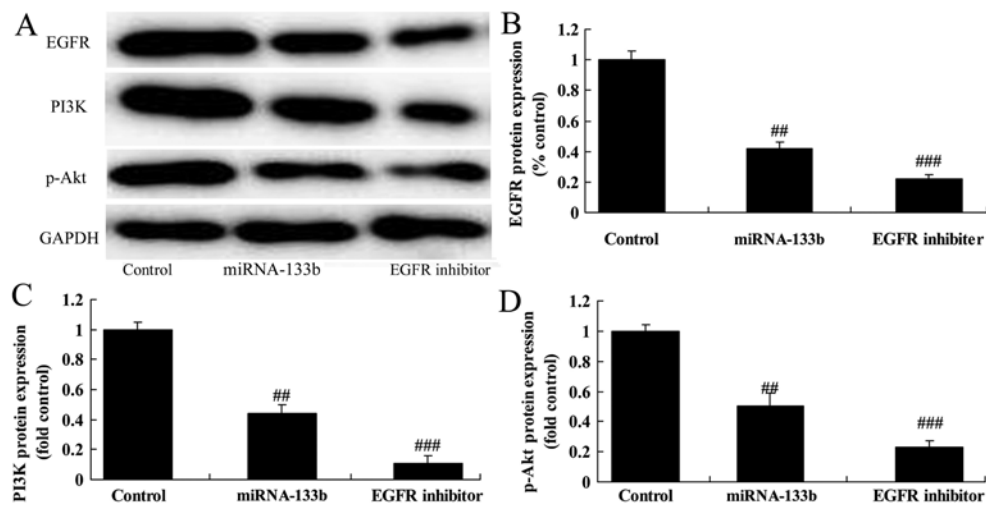
Figure 10. The suppression of EGFR on EGFR, PI3K and p-Akt protein expression in cisplatin-induced NSCLC following microRNA-133b. The suppression of EGFR on EGFR, PI3K and p-Akt protein expression using (A) western blot and (B) statistical analyses of EGFR, (C) PI3K and (D) p-Akt protein expression in cisplatin-induced NSCLC following microRNA-133b. Control, control negative mimics group; microRNA-133b, microRNA-133b mimics group; EGFR inhibitor, lapatinib, 5 nM + microRNA-133b mimics group; ##p<0.01 compared with negative mimics group, ###p<0.01 compared with microRNA-133b mimics group.
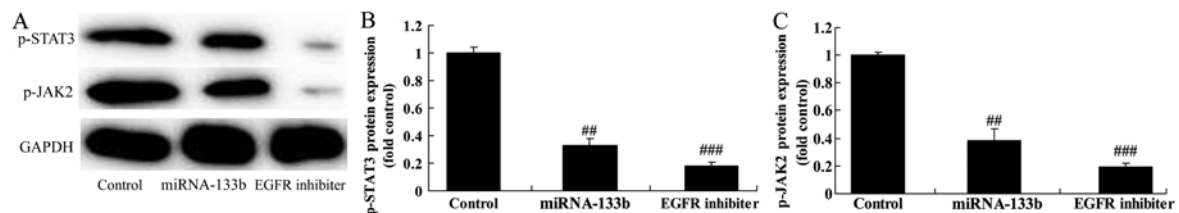

Figure 11. The suppression of EGFR on p-JAK2 and p-STAT3 protein expression in cisplatin-induced NSCLC following microRNA-133b. The suppression of EGFR on p-JAK2 and p-STAT3 protein expression using (A) western blot and (B) statistical analyses of p-JAK2 and (C) p-STAT3 protein expression in cisplatin-induced NSCLC following microRNA-133b. Control, control negative mimics group; microRNA-133b, microRNA-133b mimics group; EGFR inhibitor, lapatinib, 5 nM + microRNA-133b mimics group; ##p<0.01 compared with negative mimics group, ###p<0.01 compared with microRNA-133b mimics group.

cancer, cisplatin is the first-line chemotherapeutic commonly used. Its main mechanism is to form adducts with DNA, to inhibit cell transcription and translation, and to promote apoptosis of tumor cells. In recent years, studies have shown that lung cancer is susceptible to resistance to cisplatin, leading to treatment failure (16). This is one of the hot spots of the research on lung cancer, the main mechanism of which has not been fully elucidated, yet (17). In the present study, we explored the expression of microRNA-133b cisplatin-induced non-small cell lung cancer (NSCLC) tissue and para-carcinoma tissue in patients. Notably, overall survival of higher expression in cisplatin-induced NSCLC patients was higher than that of lower expression in cisplatin-induced NSCLC patients.

In 1993, the first miRNA (miRNA-line4) was found in the *Caenorhabditis elegans*, which was confirmed to play a role in regulating the timing of cell development (18). Later, the researchers continued to find the corresponding miRNA from fruit flies, human beings, plants and other eukaryotes (9). Growing evidence has suggested that they are mediators of the genetic expression, modification, transcription as well as translation (9). It was found that the miRNA polymorphism, abnormal miRNA expression, as well as receptors affecting gene expression on drug uptake, metabo-

lism and distribution pathways, as well as targeting clinical function may significantly affect the therapeutic effects of antitumor drugs. This may lead to the sustaining drug resistance of the tumor (9). Our results confirmed that over-regulation of microRNA-133b inhibited cell proliferation and LDH activity, induced apoptosis and caspase-3 activity, decreased cyclin D1 and increased Bax protein expression in cisplatin-induced A549 cells. Our results suggested that microRNA-133b played critical roles in regulation of proliferation of NSCLC and may be potential diagnostic and predictive biomarkers. Chen *et al* showed that microRNA-133b may be used as valuable prognostic biomarker for colorectal cancer (19). In the present study, we only used cisplatin-induced A549 cells, which is a limitation in the present study. We may use more cisplatin-induced NSCLC cell lines in further study.

STAT3 is involved in the proliferation, angiogenesis, migration, invasion and transformation of tumor cells (12). STAT3 is an effective target site to inhibit VEGF expression and tumor angiogenesis (20). It was found that there is a site for binding to STAT3 protein on the VEGF promoter (21). The activation of STAT3 protein allows VEGF to promote migration and angiogenesis of vascular endothelial cells. It shows that the STAT3 protein is at the core position in tumor angio-

genesis (22). In the present study, we found that over-regulation of microRNA-133b suppressed EGFR, p-JAK2 and p-STAT3 protein expression in cisplatin-induced A549 cells. Zhou *et al* demonstrated that microRNA-133b induces apoptosis of human renal carcinoma cells via the JAK2/STAT3 signaling pathway (23). Our findings revealed that microRNA-133b may be associated with EGFR/JAK2/STAT3 signaling pathway downstream in cisplatin-induced A549 cells.

The main members of the PI3K/AKT pathway are PI3K, AKT, mTOR, p70S6K1 and PTEN. PI3K is activated by a variety of factors, including the insulin-like growth factor (IGF-I). Later, PIP2 is transformed into PIP3, which induces the translocation of AKT into the cytoplasm. P-AKT is an important phosphorylase *in vivo* that is known as phosphokinase B (PKB) (24). There are many downstream active substrates (24). Phosphorylation can directly or indirectly affect (activate or inhibit) downstream mTOR as well as Bcl-2, thus playing extensive and complex physiological effects. It is believed that the PI3K/AKT/mTOR pathway is highly expressed in many tumor tissues (25). Moreover, many gene mutations can be seen, which leads to the enhancement or abnormality. Thus, this may affect the regulation of the proliferation, apoptosis as well as invasion of tumor cells and other important physiological activities (26). To be specific, the upregulation of PI3K/AKT/mTOR pathway can promote the proliferation of tumor cells, induce the invasiveness and inhibit apoptosis of tumor cells, and promote tumor growth (27). Therefore, a variety of PI3K/AKT/mTOR pathway inhibitors have been developed. Some of them affects inhibition of tumor growth improving the 5-year survival rate in animal and clinical experiments. This belongs to the new field of tumor treatment (28). Our results indicated that over-regulation of microRNA-133b suppressed PI3K and p-AKT protein expression in cisplatin-induced A549 cells, which may also clarify the molecular mechanisms of microRNA-133b on suppression of NSCLC proliferation. Liu *et al* showed that microRNA-133b inhibits proliferation of ovarian cancer cells via Akt by EGFR (21). However, the limitation of the study is not to sufficiently demonstrate that microRNA-133b regulates EGFR, and we may use luciferase assay to analyze the binding site of miR-133b in EGFR-3'UTR in further study.

In conclusion, to the best of our knowledge, this is the first study to demonstrate that expression of microRNA-133b cisplatin-induced NSCLC tissue was lower than that of para-carcinoma tissue in patients, and made a positive impact on overall survival of cisplatin-induced NSCLC patients. Consequently, over-regulation of microRNA-133b inhibits cell proliferation of cisplatin-induced NSCLC by PI3K/Akt and JAK2/STAT3 signaling pathway by targeting EGFR. These findings showed that microRNA-133b may be exploited further for treatment of cisplatin-induced NSCLC.

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