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Overexpression of miR-4443 promotes the resistance of non-small cell lung cancer cells to epirubicin by targeting INPP4A and regulating the activation of JAK2/STAT3 pathway

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We aimed to elucidate the roles and regulatory mechanism of miR-4443 in regulating the resistance of non-small cell lung cancer (NSCLC) cells to epirubicin (EPI). Fifty-four advanced NSCLC patients were classified as "insensitive" or "sensitive" according to patient's responses following EPI-based chemotherapy and then the expression of miR-4443 was determined. The EPI-resistant H1299 cells were collected and transfected with miR-4443 mimics, whereas parental H1299 cells were transfected with miR-4443 inhibitors. The inhibition of growth (IC_{50}), cell cycle or apoptosis of different transfected groups were investigated. Additionally, the potential target of miR-3188 was identified and verified by luciferase reporter assay. Besides, the regulatory relationship between miR-3188 and JAK2/STAT3 pathway was explored. miR-4443 was highly expressed in insensitive NSCLC patients to EPT-based chemotherapy and EPI-resistant H1299 cells. Inhibition of miR-4443 increased the sensitivity of EPI-resistant H1299 cells to EPI by decreasing IC_{50} of EPI, inducing cell apoptosis and G0/G1 cell cycle arrest, while overexpression of miR-4443 promoted the resistance of parental H1299 cells to EPI. Furthermore, inositol polyphosphate 4-phosphatase type I gene (INPP4A) was a target of miR-4443 and its expression could be negatively regulated by miR-4443. Overexpression of miR-4443 promoted the resistance of parental H1299 cells to EPI by targeting INPP4A. Besides, overexpression of miR-4443 activated JAK2/STAT3 pathway in parental H1299 cells to EPI. Overexpression of miR-4443 may promote the resistance of NSCLC cells to EPI by targeting INPP4A and regulating the activation of JAK2/STAT3 pathway. miR-4443 may serve as a drug target for NSCLC.

1. Introduction

Lung cancer is a common malignant tumor with high morbidity and mortality worldwide (Zeyadi 2014), of which non-small-cell lung cancer (NSCLC) is the predominant subtype accounting for approximately 85% (Sève et al. 2010). Moreover, NSCLC is always diagnosed at an advanced stage with the 5-year overall survival rate of only 11 % (Sun et al. 2014; Thomson and Forman 2009). Epirubicin (EPI) is the 4' epimer of the anthracycline antibiotic doxorubicin, which has been used to treat a variety of malignancies, including advance NSCLC alone or in combination with other cytotoxic agents (Plosker and Faulds 1993). Phase I-II study of high-dose epirubicin (EPI) has confirmed that EPI is considered a tolerable and active single agent for advanced NSCLC (Feld et al. 1992). Therefore, elucidation of key molecular mechanism mediating the sensitivity of NSCLC cells to EPI will help to greatly improve the clinical outcomes.

MicroRNAs (miRNAs) are small regulatory RNA about 22 nucleotides in length, interacting with target mRNAs to regulate gene expression either through inhibiting translation or degrading target mRNAs (Garzon et al. 2009; Morris and Mattick 2014). miRNAs are emerging as key players implicated in a wide range of biological functions, such as cell proliferation, growth, migration and apoptosis (Vidigal and Ventura 2015; Zhang et al. 2015b). miRNAs also have pathological roles in the regulation of a variety of diseases, including various kinds of cancers (Acunzo et al. 2015; Chiam et al. 2015; Yu et al. 2015). Moreover, the roles of miRNAs in mediating drug resistance in several cancer have also been reported (Zhang et al. 2016; Kutanzi et al. 2011). MiR-4443 was first detected in enterovirus 71-infected cells in 2015 by Xun et al. (Xun et al. 2015) and is increasingly discovered to participate in cancerous malignancies, including breast cancer (Xiu et al. 2016), and colon cancer (Meerson and Yehuda 2016). However, the role of miR-4443 in the development and drug resistance of NSCLC has not been reported.

In the present study, we advanced NSCLC patients were classified as ''insensitive'' or ''sensitive'' according to patient's responses following EPI-based chemotherapy. Then, the expression of miR-4443 was determined in these patients. In addition, we investigated the effects of miR-4443 dysregulation on the inhibition of growth (IC_{50}), cell cycle or apoptosis of parental H1299 cells and EPI-resistant H1299 cells, respectively. Further, the potential target of miR-3188 was identified and the regulatory relationship between miR-3188 and the JAK2/STAT3 pathway was explored. All efforts of this study were to elucidate the roles and regulatory mechanism of miR-4443 in regulating the resistance of NSCLC cells to EPI, thus providing a new insight for the treatment of NSCLC.

2. Investigations and results

2.1. miR-4443 is highly expressed in insensitive patients to EPI-based chemotherapy

To explore the effects of miR-4443 on EPI-based chemotherapy for NSCLC patients, we enrolled 54 advanced NSCLC patients who

were divided into ''insensitive'' and ''sensitive'' groups according to patient's responses following EPI-based chemotherapy. The results showed that miR-4443 was highly expressed in insensitive patients to EPI-based chemotherapy than that in sensitive patients (P<0.05, Fig. 1), indicating that miR-4443 may play a crucial role in the development of EPI-resistant NSCLC.

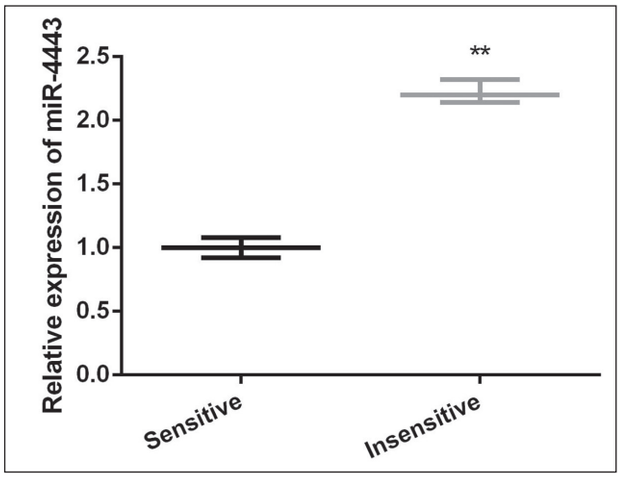


Fig. 1: miR-4443 is highly expressed in insensitive patients to EPI-based chemotherapy compared with sensitive patients. * P<0.05; **P <0.01.

2.2. miR-4443 is highly expressed in EPI-resistant H1299 cells

We then preformed *in vitro* cell experiments to investigate the roles of miR-4443 in the development of EPI-resistant NSCLC. The parental H1299 cells were treated with increasing concentrations of EPI for selection of EPI-resistant H1299 cells. As shown in Fig. 2A, the IC_{50} of EPI of EPI-resistant H1299 cells were significantly higher than parental H1299 cells (P<0.05), indicating that the sensitivity of EPI-resistant H1299 cells to EPI was lower than parental H1299 cells. Moreover, the results of flow cytometry showed that compared with parental H1299 cells, the percentage of EPI-resistant H1299 cells at G0/G1 phase was significantly decreased, while the percentage of EPI-resistant H1299 cells at S phase was markedly increased (both P<0.05, Fig. 2B). Furthermore, with the increased concentration of EPI treatment, the colonies of parental H1299 cells were significantly lower than the colonies of parental H1299 cells (P<0.05, Fig. 2C). These data indicate that the EPI-resistant H1299 cell model was successfully constructed. We therefore detected the expression of miR-4443 in EPI-resistant H1299 cells. The results showed that miR-4443 is highly expressed in EPI-resistant H1299 cells in comparison with that in parental H1299 cells (P<0.05, Fig. 2D).

2.3. Inhibition of miR-4443 increased the sensitivity of EPI-resistant H1299 cells to EPI

To detect the role of miR-4443 in regulating the sensitivity of EPI-resistant H1299 cells to EPI, EPI-resistant H1299 cells were transfected with miR-4443 inhibitor or inhibitor NC. Forty-eight hours after transfection, the expression of miR-4443 was significantly decreased in EPI-resistant H1299 cells after transfection with miR-4443 inhibitor (P<0.01, Fig. 3A). The results of the MTT assay showed that the IC_{50} values of EPI of miR-4443 inhibitor-transfected EPI-resistant H1299 cells were significantly lower than theta of inhibitor NC-transfected cells (P<0.05, Fig. 3B), indicating that the sensitivity of EPI-resistant H1299 cells to EPI was increased after inhibition of miR-4443. In addition, after treatment with different concentrations of EPI, the apoptosis rates of miR-4443 inhibitor-transfected EPI-resistant H1299 cells were all higher than that of inhibitor NC-transfected cells (P<0.05, Fig. 3C), indicating that inhibition of miR-4443 in EPI-resistant H1299 cells induced cell apoptosis. Also, compared with the inhibitor NC group, the expression levels of BCL-2 and pro-caspase-3 were decreased in miR-4443 inhibitor group, while Bax and cleaved caspase-3 were increased (Fig. 3D). Besides, in comparison with the inhibitor NC group, the percentage of miR-4443 inhibitor-transfected EPI-resistant H1299 cells at the G0/G1 phase was significantly increased, while percentage of miR-4443 inhibitor-transfected EPI-resistant H1299 cells at S phase was markedly decreased (P<0.05, Fig. 3E), indicating that inhibition of miR-4443 in EPI-resistant H1299 cells arrested cell cycles at the G0/G1 phase.

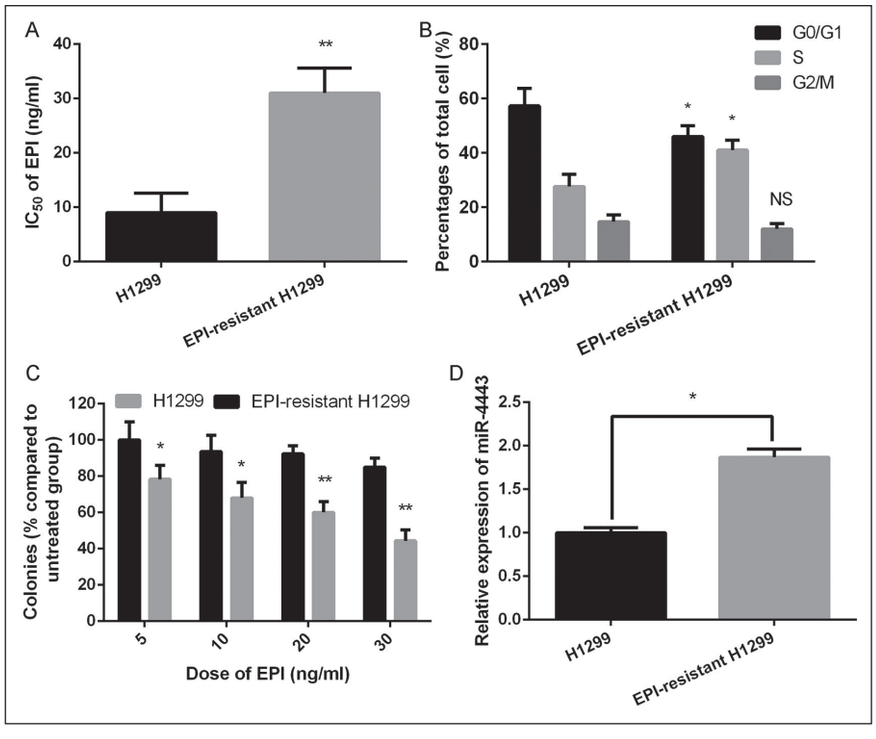


Fig. 2: miR-4443 is highly expressed in EPI-resistant H1299 cells. A: IC_{50} of EPI of EPI-resistant H1299 cells and parental H1299 cells. B: The percentage of total cells at different phase of cell cycle. C: The formatted colonies after treatment with different concentration of EPI. D: The expression of miR-4443 in EPI-resistant H1299 cells and parental H1299 cells. * P<0.05; **P <0.01.

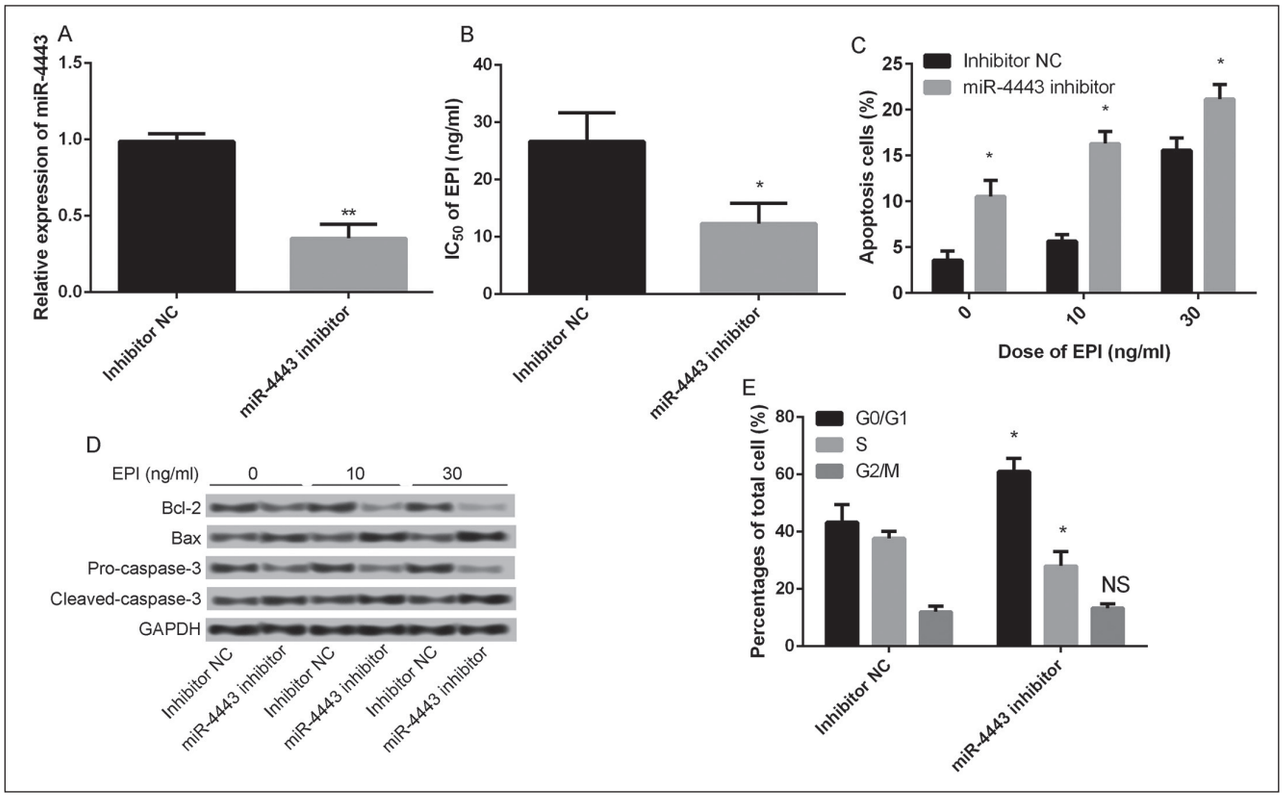


Fig. 3: Inhibition of miR-4443 increased the sensitivity of EPI-resistant H1299 cells to EPI. A: The expression of miR-4443 in EPI-resistant H1299 cells which were transfected with miR-4443 inhibitor or inhibitor NC. B: IC_{50} of EPI of different transfected groups. C: The percentage of apoptosis of different transfected groups under different concentration of EPI. D: The expression of apoptosis-related proteins in different transfected groups under different concentration of EPI. E: The percentage of total cells at different phase of cell cycle in different transfected groups. * P<0.05; **P <0.01.

2.4. Overexpression of miR-4443 promoted the resistance of parental H1299 cells to EPI

We also detected the effects of mR-4443 on the resistance of parental H1299 cells to EPI. As shown in Fig. 4A, the expression of miR-4443 was significantly increased in parental H1299 cells after transfection with miR-4443 mimic after 48 h of transfection (P<0.01, Fig. 4A), indicating that miR-4443 was successfully overexpressed in parental H1299 cells. The IC_{50} values of EPI in the miR-4443 mimic group was markedly higher than in the mimic NC group (P<0.05, Fig. 4B), indicating that the resistance of parental H1299 cells to EPI was significantly enhanced after overexpression of miR-4443. Apoptosis of miR-4443 mimic-transfected H1299 cells were markedly inhibited after treatment with different concentrations of EPI (P<0.05, Fig. 4C), accompanied with increased expression levels of BCL-2 and pro-caspase-3, and decreased expression levels of Bax and cleaved caspase-3 (Fig. 3D). Furthermore, the percentage of H1299 cells at the G0/G1 phase was significantly decreased after overexpression of miR-4443, while the percentage of H1299 cells at S phase was markedly increased (P<0.05, Fig. 4E).

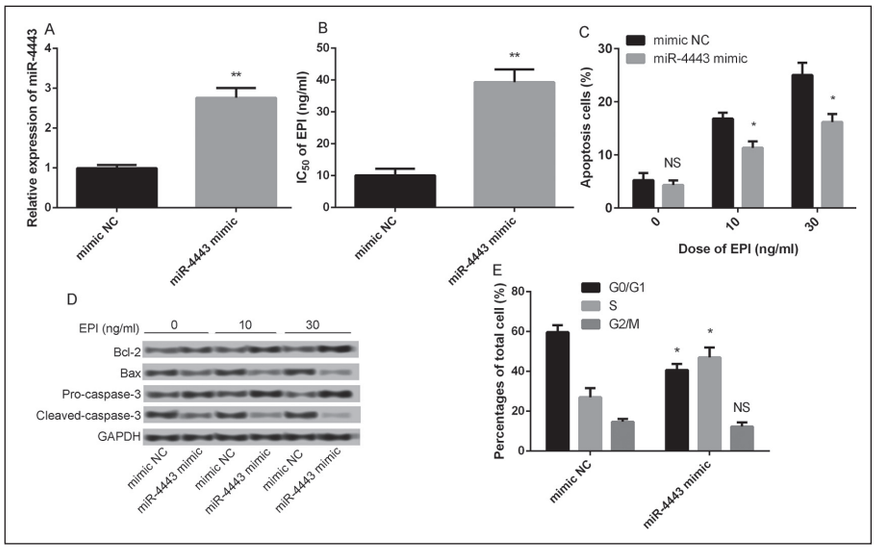


Fig. 4: Overexpression of miR-4443 promoted the resistance of parental H1299 cells to EPI. A: The expression of miR-4443 in parental H1299 cells which were transfected with miR-4443 mimic or mimic NC. B: IC_{50} of EPI of different transfected groups. C: The percentage of apoptosis of different transfected groups under different concentration of EPI. D: The expression of apoptosis-related proteins in different transfected groups under different concentration of EPI. E: The percentage of total cells at different phase of cell cycle in different transfected groups. * P<0.05; **P <0.01.

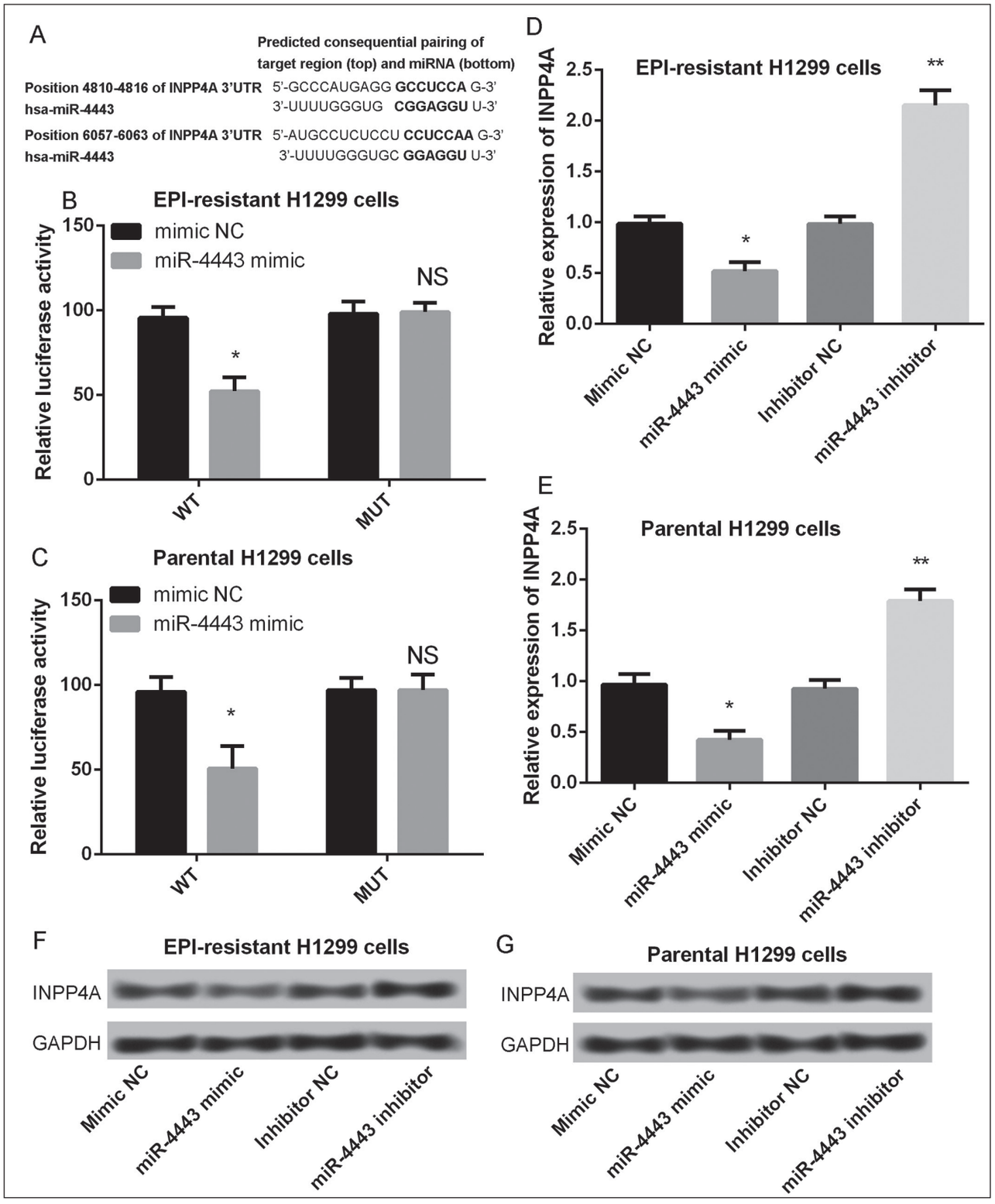
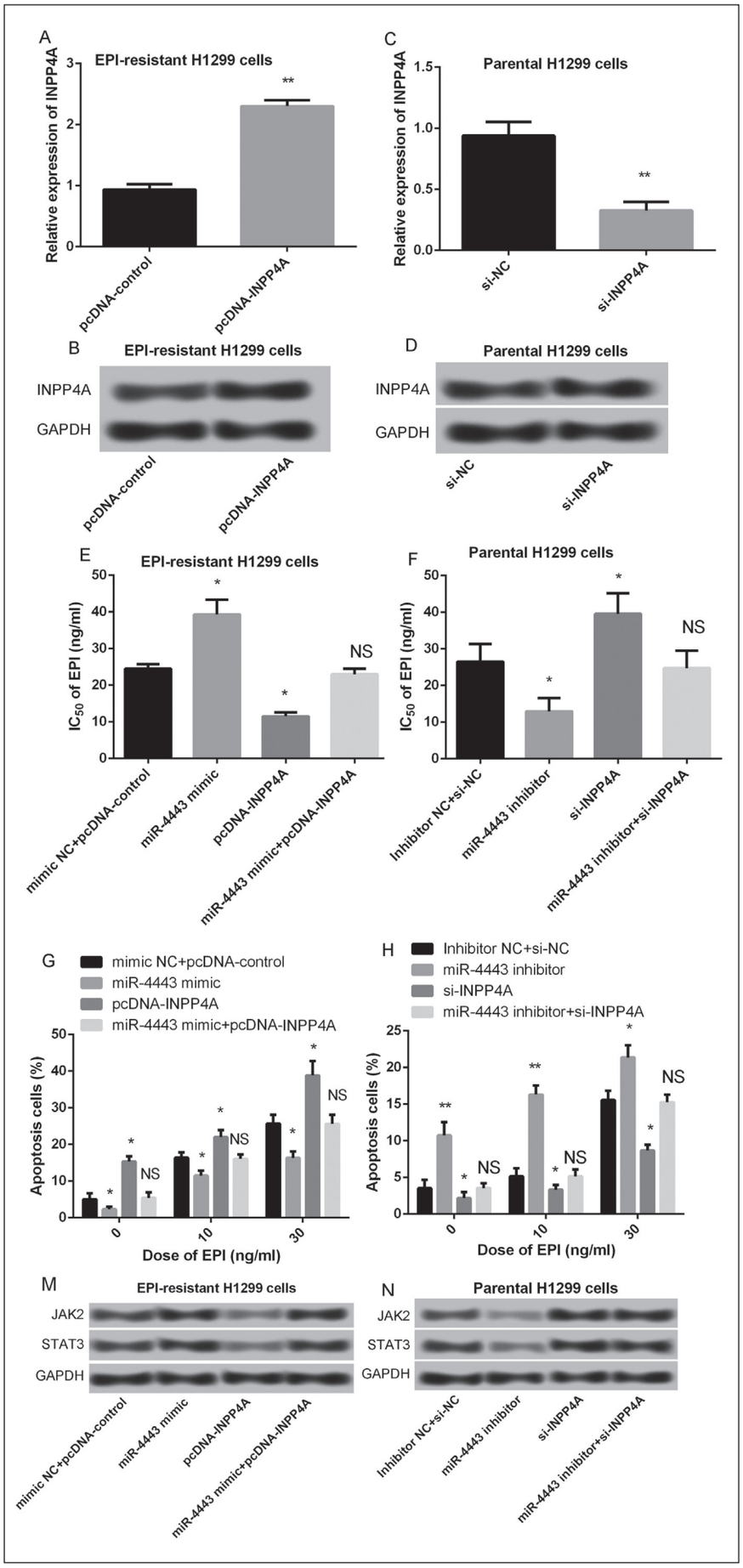


Fig. 5: INPP4A was a target of miR-4443 and its expression could be negatively regulated by miR-4443. A: The predicted binding sequence of INPP4A and miR-4443. B and C: Dual luciferase activity assay confirmed that miR-4443 could directly interact with INPP4A 3'UTR in both EPI-resistant H1299 cells and parental H1299 cells. D and E: The mRNA expression levels of INPP4A in different transfected EPI-resistant H1299 cells and parental H1299 cells. F and G: The protein expression levels of INPP4A in different transfected EPI-resistant H1299 cells and parental H1299 cells.

2.5. INPP4A was a target of miR-4443

The target of miR-4443 was identified in this study to explore the regulatory mechanism of miR-4443. As shown in Fig. 5A, INPP4A was predicted as a potential target of miR-4443. The results of dual luciferase activity assay confirmed that miR-4443 could directly interact with INPP4A 3'UTR in both EPI-resistant H1299 cells and parental H1299 cells (Fig. 5B-C). In addition, the mRNA expression levels of INPP4A were significantly decreased in miR-4443 mimic transfected group and markedly increased in miR-4443 inhibitor transfected group ($P<0.05$, Fig. 5D-E). The



protein expression levels of INPP4A in different transfected groups displayed similar results (Fig. 5F-G). These data indicated that INPP4A was a target of miR-4443 and its expression could be negatively regulated by miR-4443.

2.6. Overexpression of miR-4443 promoted the resistance of parental H1299 cells to EPI by targeting INPP4A

To determine whether the effects of miR-4443 on the sensitivity of H1299 cells to EPI by targeting INPP4A, the EPI-resistant H1299 cells were transfected with pcDNA-INPP4A or pcDNA-control, while the parental H1299 cells were transfected with si-INPP4A or si-NC. We found that INPP4A was significantly upregulated in EPI-resistant H1299 cells after transfection of pcDNA-INPP4A ($P<0.05$, Fig. 6A-B) and markedly downregulated in parental H1299 cells after transfection with si-INPP4A ($P<0.05$, Fig. 6C-D). Overexpression of INPP4A significantly decreased the IC_{50} of EPI in EPI-resistant H1299 cells, which reversed the increased IC_{50} of EPI caused by miR-4443 overexpression. Oppositely, knockdown of INPP4A markedly increased the IC_{50} of EPI in parental H1299 cells, which counteracted the effects of miR-4443 inhibitor on the decreased IC_{50} of EPI ($P<0.05$, Fig. 6F). Besides, overexpression of INPP4A significantly induced EPI caused cell apoptosis in EPI-resistant H1299 cells, which were opposite and neutralized the effects of miR-4443 overexpression ($P<0.05$, Fig. 6G). On the other hand, knockdown of INPP4A significantly inhibited EPI caused cell apoptosis in parental H1299 cells and neutralized the miR-4443inhibition-induced cell apoptosis ($P<0.05$, Fig. 6H).

Fig. 6: Overexpression of miR-4443 promoted the resistance of parental H1299 cells to EPI by targeting INPP4A and regulating JAK2/STAT3. A and B: The mRNA and protein expression levels of INPP4A in EPI-resistant H1299 cells which were transfected with pcDNA-INPP4A or pcDNA-control. C and D: The mRNA and protein expression levels of INPP4A in parental H1299 cells after transfection with si-INPP4A or si-NC. E and F: IC_{50} of EPI of different transfected EPI-resistant H1299 cells or parental H1299 cells. G and H: The percentage of apoptosis of different transfected EPI-resistant H1299 cells or parental H1299 cells under different concentration of EPI. M and N: The protein expression of JAK2 and STAT3 in different transfected EPI-resistant H1299 cells or parental H1299 cells.* $P<0.05$; **$P<0.01$.

2.7. Overexpression of miR-4443 promoted the resistance of parental H1299 cells to EPI by regulating JAK2/STAT3 pathway

To investigate the regulatory mechanism of miR-4443 on the resistance of parental H1299 cells to EPI, we further elucidated the relationship between miR-4443 and the JAK2/STAT3 pathway. In EPI-resistant H1299 cells, we found that the expression levels of JAK2 and STAT3 were significantly increased after overexpression of miR-4443 compared with control, while decreased after overexpression of INPP4A (Fig. 6M). Also, the effects of overexpression of miR-4443 on the increased expression levels of JAK2 and STAT3 were neutralized by overexpression of INPP4A (Fig. 6M). In parental H1299 cells, JAK2 and STAT3 were significantly downregulated after inhibition of miR-4443 compared with control, while upregulated after knockdown of INPP4A (Fig. 6N). The effects of miR-4443 inhibition on the decreased expression levels of JAK2 and STAT3 were also counteracted by knockdown of INPP4A (Fig. 6N). These data indicate that overexpression of miR-4443 may activate the JAK2/STAT3 pathway promote the resistance of parental H1299 cells to EPI.

3. Discussion

In this study, we found that miR-4443 is highly expressed in insensitive NSCLC patients to EPT-based chemotherapy and EPI-resistant H1299 cells. Inhibition of miR-4443 increased the sensitivity of EPI-resistant H1299 cells to EPI, while overexpression of miR-4443 promoted the resistance of parental H1299 cells to EPI. In addition, INPP4A was a target of miR-4443 and overexpression of miR-4443 promoted the resistance of parental H1299 cells to EPI by targeting INPP4A. Besides, overexpression of miR-4443 activated the JAK2/STAT3 pathway in parental H1299 cells to EPI. These data imply the role of miR-4443 in regulation of EPI resistance in NSCLC cells and merit further discussion.

Intriguingly, INPP4A was identified as a direct target of miR-4443 and its expression could be negatively regulated by miR-4443. INPP4A, a PtdIns(3,4)P2 phosphatase, is found to suppress glutamate excitotoxicity in the central nervous system and thus protect neurons from excitotoxic cell death (Sasaki et al. 2010). Aich et al. (2012) suggested that resveratrol could restore the expression of INPP4A, thus attenuating experimental allergic asthma in mice by downregulating PI3K/Akt pathway. In addition, a study reported that miR-940 could regulate the malignant behavior of bladder cancer cells *via* targeting INPP4A (Wang et al. 2017). Upregulation of miR-935 could contribute to the development of pancreatic carcinoma through repressing INPP4A (Wang et al. 2018). In our study, we found that inhibition of miR-4443 increased the sensitivity of parental H1299 cells to EPI, which was markedly counteracted after knockdown of INPP4A. Also, the effects of miR-4443 overexpression on enhanced resistance of EPI-resistant H1299 cells were neutralized by overexpression of INPP4A. Therefore, we speculate that highly expression of miR-4443 may promote resistance of NSCLC cells to EPI via targeting INPP4A.

Furthermore, another important aspect of the present analysis is that the expression levels of JAK2 and STAT3 were significantly increased in miR-4443-overexpressed EPI-resistant H1299 cells, while markedly decreased in miR-4443-suppressed parental H1299 cells. It has been reported that JAK2/STAT3 pathway plays a key role in mediating sorafenib resistance of hepatic stellate cell coculture in Huh7 cells (Chen et al. 2014). Silencing of the immunoregulatory protein B7-H3 can increase paclitaxel sensitivity through abrogating the phosphorylation of JAK2/STAT3 signaling (Liu et al. 2011). In addition, JAK2/STAT3 signaling is found to regulate growth and/or progression of lung tumors and the addition of a JAK2 inhibitor is considered as a therapy for improving clinical outcomes of NSCLC patients (Looyenga 2012). TG101348, a JAK2 inhibitor, is found to overcome erlotinib-resistance and inhibit tumor growth of erlotinib-resistant NSCLC cells (Zhang et al. 2015a). Besides, inhibition of the JAK/STAT pathway with ruxolitinib can overcome cisplatin resistance NSCLC (Hu et al. 2014). These findings suggest a key role of the JAK/STAT pathway in drug resistance. Thus, based on our results, we hypothesize that high expression of miR-4443 may promote resistance of NSCLC cells to EPI possible *via* activating JAK2/STAT3 pathway.

In conclusion, our findings indicate that a high expression of miR-4443 may promote the resistance of NSCLC cells to EPI by targeting INPP4A and regulating the activation of JAK2/STAT3 pathway. miR-4443 may serve as a drug target for the treatment of NSCLC.

4. Experimental

4.1. Patients and tissue samples

Between April 2014 and November 2016, a total of 54 patients who were diagnosed with advanced NSCLC and received epirubicin (EPI)-based chemotherapy at our hospital were enrolled in this study. The inclusion criteria were as follows: primary NSCLC; a clinical stage of IIIB to IV; and a histological diagnosis of NSCLC with at least one measurable lesion. Patients with unresectable NSCLC received 120 mg/sm of epirubicin (EPI; Pharmacia, Italy) as iv bolus plus 60 mg/sm of cisplatinum (CP) every 28 days up to the maximum cumulative dose of 840 mg/sm of EPI. According to patient's responses following EPI-based chemotherapy which were assessed by medical image analysis and detection of serum tumor markers, patients were divided into ''insensitive'' (stable or progressive disease) and ''sensitive'' (complete or partial response) groups. NSCLC tissues were collected from clinically ongoing surgical specimens and then snap frozen in liquid nitrogen. This study was approved by the Chinese Medical Association Society of Medicine's Ethics Committee in accordance with the Helsinki Declaration and all patients or their guardians provided written informed consent.

4.2. Cell culture and treatment

The human lung cancer cell line H1299 (American Type Culture Collection, Rockville, MD, USA) was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal calf serum and 1% antibiotic-antimycotic solution at 37 °C in a humidified incubator with 5 % CO_2. When monolayer confluence reached 75 %, cells were harvested and used for subsequent analysis. The parental H1299 cells were treated with increasing concentrations of EPI until the cells were able to propagate in 30 ng/mL of drug, consequently, the EPI-resistant H1299 cells were obtained by stepwise selection (Jonsson et al. 1999). The resistant cells were then grown in medium lacking EPI until use.

4.3. Cell transfection

The miR-4443 mimics, mimic control, 2'-O-methyl (2'-O-Me) modified miR-4443 inhibitors, and inhibitor control were synthesized by Shanghai GenePharma Company (Shanghai, China). The EPI-resistant H1299 tumor cells and parental H1299 cells were plated in 6-well plates at a density of 6×10^5cells/well. The EPI-resistant H1299 cells were then transfected with 100 nM of miR-4443 mimics or mimic control using lipofectamine 2000 (Invitrogen, Long Island, NY, USA) following the manufacturer's protocol, whereas parental H1299 cells were transfected with 100 nM of miR-4443 inhibitors or inhibitor control using the same method.

4.4. In vitro drug sensitivity assay

Twenty-four hours after transfection, cells in different treatment groups were seeded into 96-well plates at a density of 5×10^3cells/well. After cellular adhesion, cells were treated with different concentrations of freshly prepared anticancer drug EPI (0, 5, 10, 20, or 30 ng/ml, respectively). After incubation for 48 h, cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, each well was added with 20 mL of MTT solution to incubate cells for 4 h and then the media was replaced with 150 mL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to dissolve the dark blue crystals. The absorbance of each well at 490 nm (A490) was measured with a spectrophotometer (Olympus, Tokyo, Japan). By the relative survival curve, the concentration of EPI which produced 50% inhibition of growth (IC_{50}) was determined. Each experiment was independently performed three times with quadruplicate.

4.5. Flow cytometric analysis of cell cycle or apoptosis

For cell apoptosis analysis, cells were double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Shanghai, China), followed by the classification of cells as viable cells, early apoptotic cells, apoptotic cells and dead cells was completed and analyzed by means of a FACScan flow cytometer (BD Biosciences, Shanghai, China) equipped with Cell Quest software (BD Biosciences). Three independent experiments were performed.

For cell-cycle analysis, cells were stained with PI using the BD Cycletest Plus DNA Reagent Kit (BD Biosciences) and the percentage of cells in G0/G1, S or G2/M phase was counted and compared by means of a FACScan flow cytometer (BD Biosciences). Each experiment was performed at least in triplicate.

4.6. Colony formation assay

After treatment with IC_{50} of EPI, the EPI-resistant H1299 cells or parental H1299 cells were placed in a 6-well plate and maintained in RMPI 1640 containing 10 %

FBS for 2 weeks. Afterwards, colonies were washed with PBS, fixed with methanol and stained with 0.1 % crystal violet in 20 % methanol for 15 min. This assay was repeated three times.

4.7. Dual luciferase activity assay

The 3' UTR of inositol polyphosphate 4-phosphatase type I gene (INPP4A) cDNA containing the wild type or mutated putative target site for the miR-4443 was chemically synthesized and inserted into the downstream of the luciferase gene in the pGL3-control vector (Promega, Madison, WI). For luciferase reporter assay, cells with a density of 1.5×10^5 cells/well were plated in a 24-well plate and then transfected with pGL3-INPP4A -3'-UTR plus pRL-TK (Promega) in combination with 60 pmol of the miR-4443 mimics or mimic control using Lipofectamine 2000 (Invitrogen). After 24 h of transfection, luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to renilla luciferase activity. All experiments were conducted in triplicate.

4.8. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen, USA) and then reverse transcribed to cDNA with a Reverse Transcription Kit (Takara, Shiga, Japan). qRT-PCR analyses were carried out using the Power SYBR Green kit (Takara, Shiga, Japan) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA). The expression levels of mRNAs and miRNAs were respectively normalized to β-actin and U6 and calculated with the $2^{-\Delta\Delta Ct}$ method.

4.9. Western blot assay

Total protein was extracted from cells with the mammalian protein extraction reagent RIPA (Beyotime, Beijing, China) containing a protease inhibitor cocktail (Roche, CA, USA) and phenylmethylsulfonyl fluoride (PMSF) (Roche, CA, USA). Equal amount of protein extraction (50 μg) was then separated by 10% SDS-PAGE, transferred to nitrocellulose membrane (0.22 mm; Sigma, USA), blocked with 5% non-fat milk and incubated with specific antibodies. Primary antibodies to GAPDH, Bcl-2, Bax, pro-caspase-3, cleaved-caspase-3, pro-caspase-9, cleaved-caspase-9, INPP4A, JAK2, STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the prepared in a dilution of 1:1000 for incubating the members overnight at 4 °C, followed by the addition of horseradish-peroxidase labeled secondary antibodies (1:1000). GAPDH was used as a control. The protein blots were finally visualized using enhanced chemiluminescence (Pierce, Bradenton, FL, USA) and their densitometry were quantified using Quantity One software (Bio-Rad, CA, USA).

4.10. Statistical analysis

All data are presented as means±SD and were analyzed with SPSS 16.0 software (IBM, IL, USA). Student's t-test or one-way ANOVA were carried out to analyze the differences between groups using SPSS 16.0 software (IBM, IL, USA). A p-value of less than 0.05 was considered significant.

Conflicts of interest: None declared.

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