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Mir-22-3p Enhances the Chemosensitivity of Gastrointestinal Stromal Tumor Cell Lines to Cisplatin through PTEN/PI3K/Akt Pathway

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ABSTRACT

Mir-22-3p is associated with many important biological processes, including neuroprotection, tumorigenesis, and various other tumor progressions. Our study aimed to investigate the roles of Mir-22-3p in chemosensitivity of gastrointestinal stromal tumor (GIST-T1) cells to cisplatin and explore its underlying mechanisms.

Mir-22-3p high-expressing cell line was established by transfecting GIST-T1 cell line cells with Mir-22-3p mimic. After treatment with cisplatin (10 μM), Cell counting kits-8 (CCK-8) method was used to detect the cell viability. Flow cytometry was applied to measure the degree of cell apoptosis. Scratch wound healing test was used to detect the migration ability of cells. The protein and mRNA levels of the phosphatase and tensin homolog deleted on chromosome ten (PTEN)/phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) pathway-related factors were analyzed by Western blot and qRT-PCR.

The mRNA level of Mir-22-3p was increased in transfected GIST-T1 cells compared with that in control cells. The survival rate and Bcl-2/Bax ratio of GIST-T1 cells treated with both Mir-22-3p analogue and cisplatin were significantly decreased, while the apoptosis rate and protein level of caspase-3 were significantly increased ($p<0.05$). In addition, the mRNA and protein levels of PTENwere significantly increased in cells treated with both Mir-22-3p analogue and cisplatin ($p<0.05$), while the expression levels of PI3K and Akt were significantly decreased ($p<0.05$).

Mir-22-3p overexpression can increase the chemosensitivity of cisplatin in human gastrointestinal stromal tumor cells by PTEN/PI3K/Akt pathway.

Keywords: Chemosensitivity; Cisplatin; Gastrointestinal stromal tumor; Mir-22-3p

INTRODUCTION

Cell growth, differentiation and development are regulated by a variety of proteins and signaling pathways.[1] Balance between cell division, growth, differentiation and apoptosis provides the basis for

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development.[2] The occurrence of carcinogenesis process may be caused by the disturbed balance of those processes.[3] Phosphatidylinositol 3 kinase (PI3K)/protein kinase B(Akt) signaling pathway, which are frequently disturbed in many human cancers,[4] have been shown to play pivotal role not only in the development of cancer but also in the response of tumor to various cancer treatments.[5] Phosphatidylinositol-3 kinases (PI3Ks) can produce PI-3, 4, 5-trisphosphate (PIP3) using PIP2,[6] PIP3 can activate Ser/Thr kinase Akt. The activated Akt can phosphorylate the targets proteins that involved in the regulation of cell survival, growth, proliferation, and other cellular processes. It can affect the phosphorylation and inactivation of the inhibitors that involved in cell cycle progression, survival, glycolysis, angiogenesis, and protein synthesis. It unlocks most of the processes involved in oncogenesis in turn.[7-11] Conversely, the phosphatase and tensin homolog deleted on chromosome ten (PTEN) dephosphorylates phosphoinositide in cells and functions as a tumor suppressor by negatively regulation PI3Ks signaling pathway. Based on the opposite functionalities of PI3K/Akt signaling pathways and PTEN in cancer development, it will be possible to inhibit cancer development and improve the efficiency of cancer treatment by upregulating the expression of PTEN and downregulating the expression of PI3K and Akt.

Cisplatin is the commonly used metal platinum complexes in clinic, it has broad anti-tumor spectrum characteristics. Nowadays, new anticancer drugs emerge in an endless stream, but cisplatin is still the most commonly used chemotherapy drugs in clinical practice, especially the killing effect of cancer cells in high-dose application is more significant. Therefore, in the clinical application of cisplatin to overcome the tumor of drug resistance, still need to carry out a large number of systematic experiments.[12] The PI3K/Akt pathway is well known to be a major cell survival pathway in many cancers. As a key molecule of this pathway, Akt regulates maybe related to cisplatin resistance Previous studies have shown that Mir-22-3p was involved in PI3K pathway in colorectal cancer cells,[13] indicating that Mir-22-3p may be a good candidate that can be used to regulate PI3K/Akt pathway. In this study, the roles of Mir-22-3p in chemosensitivity of gastrointestinal stromal tumor (GIST-T1) cells to cisplatin were explored by specifically increasing its expression. We aimed to investigate the roles of Mir-22-3p in chemosensitivity of GIST-T1 cells to cisplatin and to explore its potential underlying mechanisms by investigating its relationship with the PTEN/PI3K/Akt pathway.

MATERIALS AND METHODS

Cell Culture and miRNA Transfection

GIST-T1 cell line was cultured in DMEM medium (Biowit, Shenzhen, China) with 10% fetal bovine serum (FBS) (Beyotime Biotechnology, Shanghai, China) at 37°C in a humidity atmosphere containing 5% CO_2. For subsequent experiments, cells in log growth phase were collected and re-suspended in phosphate buffer saline (PBS, pH 7.4).

The Mir-22-3p mimic was purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). GIST-T1 cell line was divided into three groups. The GIST-T1 cells were seeded at 1×10^5 cells/well in 24 well plates. After 5 h incubation at 37°C, the Mir group cells were transfected with 100 nM mimic. Negative control (NC) group was transfected with empty vector. The transfection reagents Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection in strict accordance with the manufacturers' instructions. Control group without any treatment. The quality of transfection was detected by qRT-PCR.

Cell Proliferation Was Detected by Cell Counting Kits-8 (CCK-8) Assay

The GIST-T1 cells were plated in 96-well plates (1×10^3 cells/mL) and cultured in DMEM medium with 10% FBS for 24 h. GIST-T1 cell line were randomly divided into 4 groups: control, Mir, Cisplatin (Cis)and Mir + Cis group. In control group was used cells trated with Lipofectamine 2000. In Mir and Mir + Cis groups were used cells transfected with Mir-22-3p mimic. After 5 h incubation at 37°C, Cis and Mir + Cis cells were exposed to 10 μM cisplatin for 24h, other cells did not expose to cisplatin.[14] Then the medium was removed and added 100 μL fresh medium containing 10% CCK-8 (Dojindo, Kumamoto, Japan) to each well. The plate was incubated for 1-4 hours, and the absorbance at 450 nm was determined using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). All tests were repeated three times.

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Annexin V/PI Staining Assay

Apoptosis was detected by Fluoroscein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (BD Biosciences, San Diego, USA) using FITC V-FITC/PI double staining. The GIST-T1 cells in log growth phase were collected and plated on 96-well plates （2×10^5 cells/mL）and cultured in DMEM medium for 24 h. GIST-T1 cells were divided into 4 groups and treated as described in CCK8 assay. After 5 h incubation at 37°C, the cells were washed with phosphate buffered saline (PBS), digested with trypsin, and resuspended in calcium-enriched HEPES buffer. Finally, cells were stained with Annexin V-FITC and PI for 15 min and then were analyzed by flow cytometry (CyAn ADP9, Beckman Coulter, Fullerton, USA).

Cells Migration Ability Was Detected by Scratch Wound Healing Assay

Cells were plated onto a 6-well plate (3×10^5/well) and cultured in DMEM medium for 24 h. The groups and treatment methods were as described previously. After 5 h the medium was removed and cells were washed with PBS, medium with different concentrations of drug was added into each well according to the design. Photos were taken at 0 h, 24 h, 48 h, 60 h post- scratching.

qRT-PCR

Total RNA was extracted from cultured cells with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA with the PrimeScript RT-PCR Kit (TaKaRa, Shiga, Japan). Real-time PCR was performed with a SYBR Premix Ex Taq™ II kit (TaKaRa, Shiga, Japan), according to the manufacturer's protocol, on CFX96 Real Time PCR detection system (Bio Rad, Hercules, CA, USA) with SYBR green II. The following primers were used: 5'-CTCGCTTCGGCAGCACA-3' and for U6; 5'-AAGCTGCCAGTTGAAGAACTGTA-3' (sense) and universal primer (anti-sense) for Mir-22-3p; 5'-TCCACAAACAGAACAAGATG-3' (sense) and 5'-CTGGTCCTGGTATGAAGAAT-3' (anti-sense) for PTEN; 5'-ACCACAGTCCATGCCATCAC-3' (sense) and 5'-TCCACCACCCTGTTGCTGTA-3' (anti-sense) for GAPDH. The PCR cycling condition is following: 94°C for 1 min; and 40 cycles at 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s. The relative expression of genes was presented as fold change and calculated using the $2^{-\Delta\Delta CT}$ method.

Western Blot

GIST-T1 cells were plated on 6-well plates and treated with different drugs. The protein concentration was measured using a bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific Inc.). Then proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by the transmembrane step to transfer proteins to polyvinylidence fluoride (PVDF) membrane (Merck, Darmstadt, Germany) After washing, the membrane was blocked by tris buffered saline-tween (TBST)solution containing 5% skim milk. TBST solution containing 3% bovine serum albumin was used to dilute the antibodies of PTEN (1:1000 dilution, Dallas Area Kitefliers Organization, Denmark), PI3K (1:1000), Bax (1:1000), Akt (1:1000), Bcl-2 (1:1000), Caspase-3 (1:1000) and β-actin (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was incubated with primary antibody overnight at 4°C. After washing, the membrane was incubated with the secondary antibody for 1 hour. After washing, the color development step was performed with ECL luminous substrate for 3-5 min. QUANTITY ONE gel electrophoresis image analyzer was used for data analysis.

Statistical Analysis

Statistical analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, IL). All data were described as mean±standard deviation (SD). One-way ANOVA was used to compare data among multiple groups. SNK method was used to compare the data between two groups. $p<0.05$ was considered to be statistically significant.

RESULTS

Mir-22-3p mRNA Expression

The expression of Mir-22-3p mRNA in transfected GIST-T1 cells were detected by qRT-PCR. We found that the Mir-22-3p mRNA level of the transfected GIST-T1 cells was significantly higher than that in control group ($p<0.01$). As shown in Figure 1, Mir-22-3p mRNA level increased by 67.93±8.81%. The Mir-22-3p mRNA expression in NC group had no difference compared with control group. Therefore, in the next experiment, we used NC group as a control.

Cell Proliferation

We used CCK-8 assay to detect the cell proliferation. As shown in Figure 2, the survival rate of GIST-T1 cells with Cis group was significantly lower than that in control group (p<0.05).

The survival rate in Mir group was significantly lower than that in control group (p<0.05) and the survival rate in Mir+Cis group was significantly lower than that of the cells with cispltin treatment alone (p<0.05).

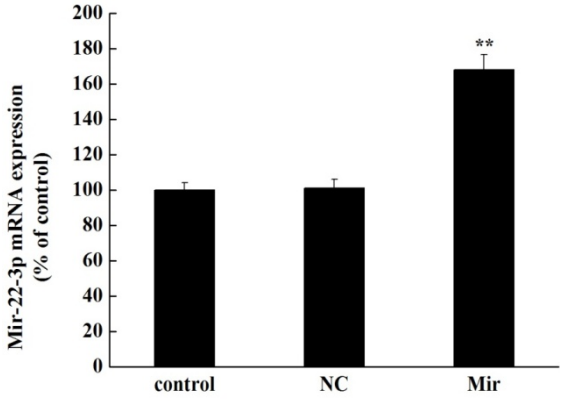

Figure 1. mRNA level of Mir-22-3p in each group after transfection. Control group was gastrointestinal stromal tumor (GIST-T1) cells without any treatment; Negative control (NC) group was GIST-T1 cells transfected with empty vector Lipofectamine 2000; Mir group was GIST-T1 cells transfected with Mir-22-3p mimic. All the data were described as Mean±Standard (SD), $^{**}p$<0.01 vs control group.

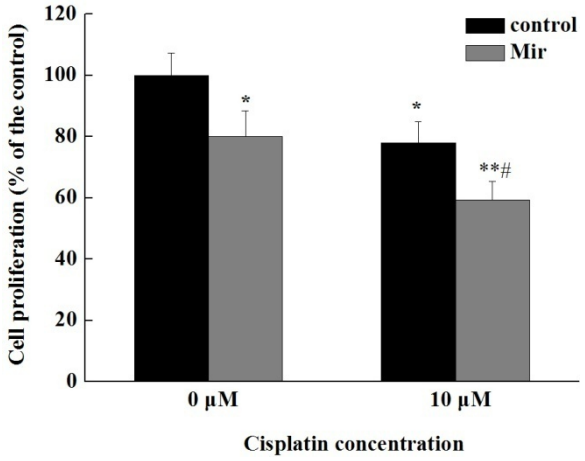

Figure 2. Cell survival rate of each group in CCK8 assay. All the data were described as Mean±SD, $^{*}p$<0.05 vs control group, $^{**}p$<0.01vs control group; $^{\#}p$< 0.05 vs cisplatin (Cis) treatment alone group.

Cell Apoptosis and Protein Expressions

The apoptosis rates of transfected GIST-T1 cells and normal GIST-T1 cells after cisplatin treatment were both significantly higher than that of normal GIST-T1 cells, especially for the transfected GIST-T1 cells, apoptosis ate became much higher after cisplatin treatment（Figure 3A）. Compared with the Bcl-2/Bax ratio in normal GIST-T1 cells, Bcl-2/Bax ratio in transfected GIST-T1 cells and cells with cisplatin treatment alone were significantly decreased (decreased by 76.62±4.12% and 54.61±4.11%, respectively, p<0.05). The lowest Bcl-2/Bax ratio, which was lower than that in GIST-T1 cells with cisplatin treatment alone (p<0.05) and normal GIST-T1 cells (p<0.01) was found in transfected GIST-T1 cells with cisplatin treatment. Protein levels of caspase-3 in transfected GIST-T1 cells and cisplatin treated GIST-T1 cells were both significantly higher than that in normal GIST-T1 cells (increased by 21.5±2.01% and 33.62±9.59%, respectively, p<0.05), while highest caspase-3 protein level, which is higher than that in GIST-T1 cells with cisplatin treatment alone (p<0.05) and normal GIST-T1 cells (increased by 69.37±5.08%, p<0.01) was found in transfected GIST-T1 cells with cisplatin treatment (Figure 3B).

The Migration Ability of GIST-T1 Cells

The distances of the wound in all the groups at 0 h were almost the same. The distance of the wound in Mir group and Cis group is significant bigger than those of the blank control group (p<0.05) 24 h after the scratching, while the distance of the wound of Mir+Cis group was the biggest among all four groups. After 48 h and 60 h, the distance of the wound in Mir group and Cis group is bigger than those of the blank control group, but there was no significant difference. Mir+Cis group was still the biggest compared with the other groups (Figure 4).

Relative mRNA Expressions of PTEN

The mRNA level of PTEN in Mir group and Cis group were significantly higher than that in control group (increased by 21.74±1.03%, 19.53±1.17%, respectively, p<0.05), the mRNA level of PTEN in Mir+Cis group was significantly higher than that Cis group and control group (p<0.01) (Figure 5).

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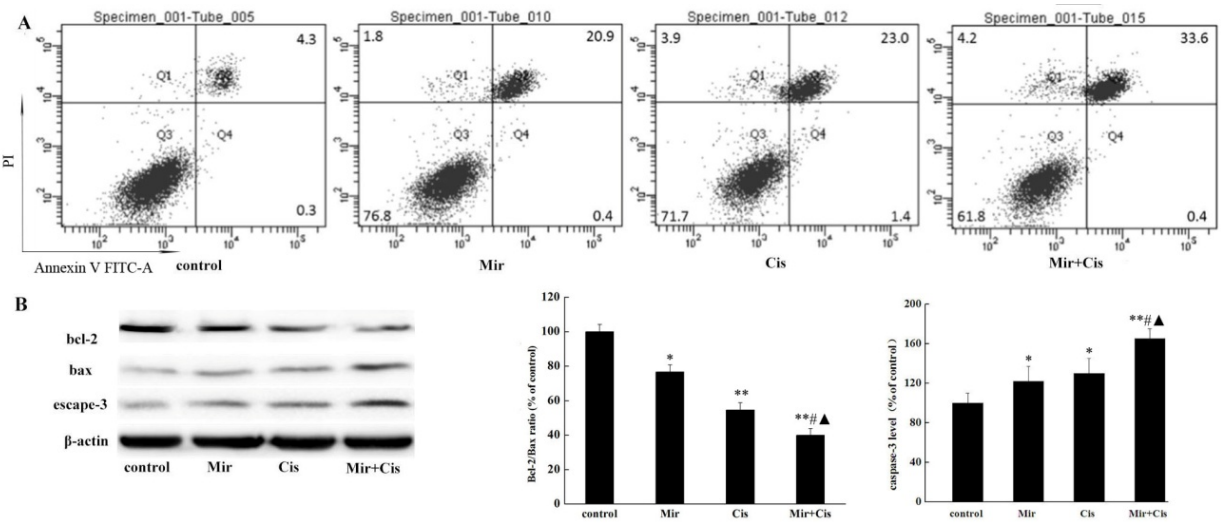

Figure 3. The effects of up-regulation of Mir-22-3p combined with cisplatin treatment on the apoptosis of gastrointestinal stromal tumor (GIST-T1) cells. A. Cell apoptosis measured by flow cytometry; B. Expression of apoptosis related proteins, the bars indicated the relative quantity data obtained by density analysis of triple experiments. All the data were described by Mean±SD, $^{*}p<0.05$ vs control group; $^{**}p<0.01$ vs control group; $^{\#}p<0.05$ vs transfection group (Mir); $^{\blacktriangle}p<0.05$ vs cisplatin (Cis) treatment alone group.

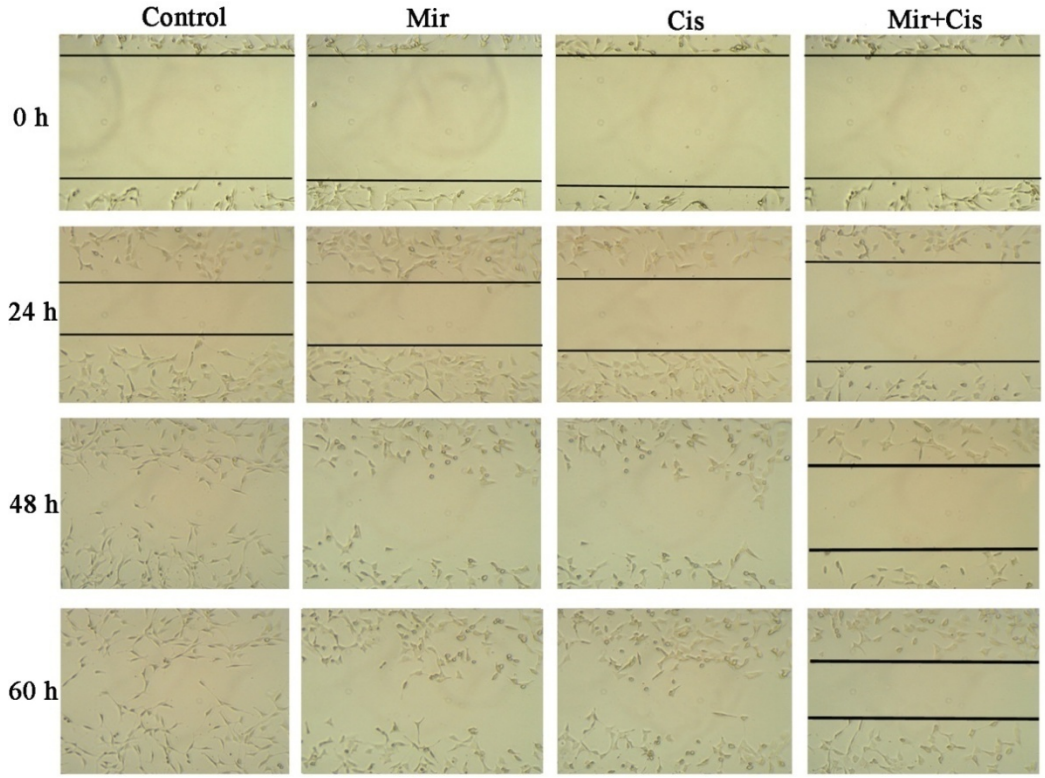

Figure 4. Mir-22-3p up-regulation combined with cisplatin treatment on the migration ability of gastrointestinal stromal tumor (GIST-T1) cells. Wound-healing assay of GIST-T1 cells transfected with Mir-22-3p and treated with Cisplatin.

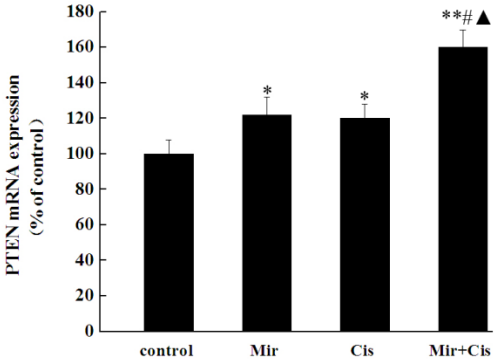

Figure 5. The phosphatase and tensin homolog deleted on chromosome ten (PTEN) mRNA level of each group. All the data were described as mean±SD, $^{*}p<0.05$ vs control group; $^{**}p<0.01$ vs control group; $^{\#}p<0.05$ vs transfection group (Mir); $^{\blacktriangle}p< 0.05$ vs cisplatin (Cis) treatment alone group (Cis).

Relative Protein Expressions of PTEN/PI3K/Akt Pathway

PTEN protein in Mir group and Cis group were significantly higher than that in control group (increased by 21.74±1.19%, 35.34±2.35%, respectively, $p<0.05$) PTEN protein in Mir+Cis group is significantly higher than Cis group (173.68±9.05 vs. 135.34±7.92, $p<0.05$) and control group ($p<0.01$). Compared with the PI3K and Akt proteins in control group, the proteins expression in Mir group and Cis group were significantly decreased ($p<0.05$). The proteins level was the lowest in Mir+Cis group (Figure 6).

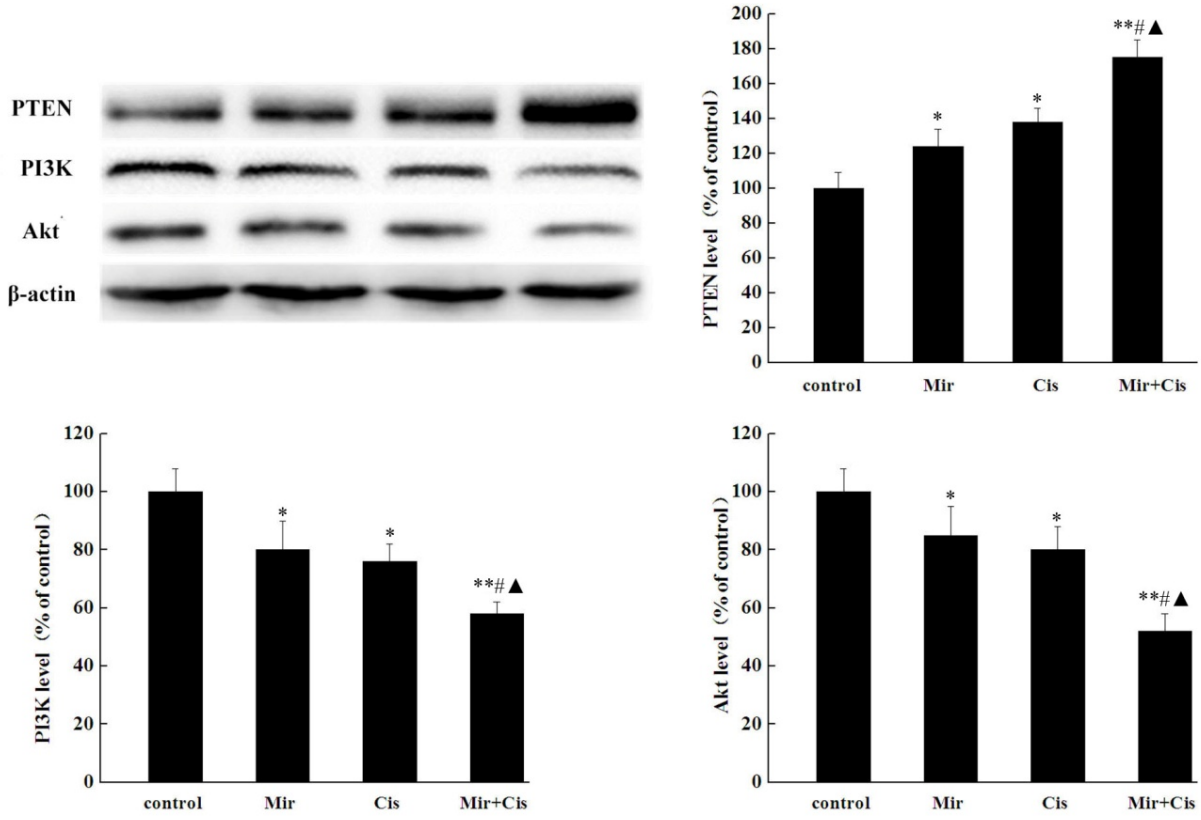

Figure 6. The effect of up-regulation of Mir-22-3p combined with cisplatin treatment on the expression of gastrointestinal stromal tumor (GIST-T1) cell the phosphatase and tensin homolog deleted on chromosome ten (PTEN)/phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) related protein detected by western blot. All the data were described by Mean ± SD, $^{*}p<0.05$ vs control group (control); $^{**}p<0.01$ vs control group (control); $^{\#}p<0.05$ vs transfection group (Mir); $^{\blacktriangle}p<0.05$ vs cisplatin (Cis) treatment alone group.

DISCUSSION

The activation of PI3K/Akt signaling pathway can be induced by many types of toxic insults or cellular stimuli.[15,16] PI3K/Akt signaling pathway, have been shown to be closely related to the development of various diseases such as diabetes mellitus, autoimmunity and cancer.[16-20] PI3K/Akt signaling pathways play pivotal roles in various cellular functions such as proliferation, growth, transcription, translation, and survival. Previous studies have shown that disturbed PI3K/Akt signaling pathway can lead to the disturbance in the balance between cell proliferation and survival, and eventually lead to the development and progression of cancer.[2]PI3K and Serine/threonine kinase Akt are the two key players in this pathway. PI3Ks are lipid kinases with the ability to phosphorylate PI, PI-4-phosphate (PI-4-P), or PI-4, 5-bisphosphate (PI-4,5-P2 or PIP2).[6] The members of PI3Ks can be divided into 3 groups based on their functions. Group 1 PI3Ks can produce PIP3 using PIP2. PIP3 can bind to the pleckstr in homology (PH) domain of Ser/Thr kinase Akt to active it. Then the activated Akt will be able to phosphorylate a variety targets involved in the regulation of cell survival, growth, proliferation, and other cellular processes.[21]The phosphorylation activities of Akt on RxRxxS/T motifs of the targets can usually lead to the phosphorylation and inactivation of inhibitors of cell cycle progression, glycolysis, survival, protein synthesis, and angiogenesis, which in turn unlock most of the processes involved in oncogenesis.[7-11]

In our study, we found that the Bcl-2/Bax, which indicates the activity of apoptosis in Mir+Cis group was significantly higher than the other three groups. The caspase-3 protein level in Mir+Cis group was higher than that in Cis group and control group, indicated that the apoptosis rate of transfected GIST-T1 cells with cisplatin treatment was much higher than that with cisplatin treatment alone and normal GIST-T1 cells. This was confirmed by calculating the survival rate of each group- lowest survival rate was found in transfected GIST-T1 cells with cisplatin treatment among all the four groups. In addition, wound healing assay has shown that the combined treatment of Mir-22-3p overexpression and cisplatin treatment has much stronger negative effect on cell migration ability than that of Mir-22-3p overexpression or cisplatin treatment alone.

PTEN protein in humans is encoded by the PTEN gene, and mutations happened in this gene can usually lead to the development of many cancers. PTEN, as a tumor suppressor, can play an opposite of PI3Ks to producing PIP2 by hydrolyzing PIP3. In addition, PTEN can also dephosphorylate phosphoinositides produced by PI3Ks on position 3′of PI, PI-4-P, or PI-4,5-bisphosphate to inhibit the tumor development.[21-24] In our study, we found that the Mir-22-3p overexpression and cisplatin treatment alone was enough to increase both the mRNA and protein levels of PTEN. However the combined treatment of Mir-22-3p analogue transfection and cisplatin treatment increased both the mRNA and protein levels of PTEN to higher levels than that of the treatment with Mir-22-3p analogue transfection or cisplatin treatment alone, indicating that Mir-22-3p can positive regulate the expression of PTEN, and Mir-22-3p overexpression combined with cisplatin treatment played a better role in this process than that of cisplatin treatment alone. We also found that protein levels of PI3K and Akt were lower in transfected GIST-T1 cells with cisplatin treatment that those in normal GIST-T1 cells and GIST-T1 cells with cisplatin treatment alone, indicating that Mir-22-3p overexpression combined with cisplatin treatment can significantly inhibit the activity of PI3K/Akt signaling pathway and this inhibition effect is much stronger than that of cisplatin treatment alone. The data here suggested that Mir-22-3p overexpression can inhibit the tumor development possibly through a similar way of cisplatin.

In summary, our data suggest that Mir-22-3p overexpression can increase sensitivity of human GIST-T1 cells to chemotherapy, and Mir-22-3p achieved this function possibly though upregulating PTEN expression and suppressing PI3K/Akt signaling pathway.

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