MIR-486-5P ENHANCES THE SENSITIVITY OF HUMAN NEUROBLASTOMA SH-SY5Y CELLS TO CISPLATIN BY DOWNREGULATING PIM1

HANG SUN[1, 3, #], XIAO WANG[1, #], XIAONING ZHANG[1, 2], ZHEN YUE[1], YANAN YANG[1], YOUJIE LI[1, *], YUNXIAO SUN[3, *]
[1]Key Laboratory of Tumor Molecular Biology in Binzhou Medical University, Department of Biochemistry and Molecular Biology, Binzhou Medical University, Yantai, Shandong 264003, P.R. China - [2]Laboratory of Binzhou maternal and Child Health Hospital, Binzhou, Shandong 256600, P.R. China - [3]Department of Pediatrics, Yantai Affiliated Hospital of Binzhou Medical University, Yantai, Shandong 264100, P.R. China
[#]*Contributed equally to this work*

ABSTRACT

Objective: *As an antitumor drug, cisplatin plays a huge role in the treatment of various types of cancer. In this study, we observed the mechanism of cisplatin in the treatment of human neuroblastoma SH-SY5Y cells.*

Methods: *This experiment uses reverse transcription-polymerase chain reaction (RT-PCR) technology, western blot analysis, and flow cytometry to detect the expression of miR-486-5p and PIM1 after cisplatin acts on SH-SY5Y cells, and predict the binding site of miR-486-5p and PIM1 using the prediction website.*

Results: *Reverse transcription-polymerase chain reaction (RT-PCR) results showed that cisplatin can promote the expression of miR-486-5p and found that miR-486-5p can inhibit cell proliferation. In addition, we transfect miR-486-5p into cells, and the results showed that PIM1 expression in the cells transfected with miR-486-5p was significantly lower than that in the control group. We combined cisplatin and miR-486-5p on SH-SY5Y cells and found that cell proliferation decreased, apoptosis increased, and PIM1 expression decreased compared with cisplatin or miR-486-5p alone.*

Conclusion: *According to the present evidence, miR-486-5p can enhance the sensitivity of human neuroblastoma SH-SY5Y cells to cisplatin by downregulating PIM1. The findings of this study can provide a basis for drug design or new targets for cancer treatment.*

Keywords: *MiR-486-5p, human neuroblastoma cell, cisplatin, PIM1.*

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Introduction

Neuroblastoma is the most common extracranial tumor among children and the most common tumor among infants and young children. It is characterized by an undifferentiated cell phenotype, a high recurrence rate, and poor prognosis[(1)]. Cisplatin is a well-known chemotherapy drug. It has been used to treat a variety of human cancers, including bladder, head and neck, lung, ovarian, and testicular cancers[(2)]. Cisplatin also plays a huge role in the treatment of neuroblastoma. MicroRNA (miRNA) belongs to a class of small non-coding RNA with a length of 20–22 nucleotides. They regulate the expression of target genes by binding to the three prime untranslated region (3'UTR) of mRNA and play an important role in the post-transcriptional regulation of expression[(3, 4)]. Mirna affects nearly all genetic pathways ranging from cell cycle and proliferation to cell apoptosis; it has a wide range of target genes[(5, 6)]. The dysregulation of miRNA expression is associated with various types of cancer by acting as tumor suppressors and oncogenes[(7)]. For example, miR-149 inhibits cell proliferation

and enhances chemosensitivity by targeting CDC42 and BCL2 in neuroblastoma[8]. It can be used as a potential molecular target for cancer[9]. Meanwhile, miR-21 regulates the TETs/PTENp1/PTEN pathway to promote hepatocellular carcinoma growth[10].

It exhibits potential carcinogenic functions in nearly all types of cancer[11]. Our research found that cisplatin can play a role in SH-SY5Y cells by upregulating miR-486-5p. Another study found that PIM1 kinase is a true oncogene implicated in early transformation and tumor progression in hematopoietic malignancies and prostate carcinomas[12]. In our study, miR-486-5p can downregulate PIM1 by targeting the 3'UTR end of PIM1. In recent clinical studies, the combination of drugs and miRNA has become a new type of treatment for tumors.

For example, miR-34a is combined with doxorubicin to treat triple-negative breast cancer[13], miR-34a and paclitaxel synergistically inhibit cancer cells[14], and gemcitabine combined with miR-205 is used to treat advanced pancreatic cancer[15]. In the current study, we will explore the apoptosis and proliferation effects of cisplatin combined with miR-486-5p on human neuroblastoma SH-SY5Y cells. We will observe whether such combination is better than cisplatin or miR-486 alone as mentioned earlier.

Materials and methods

Cell lines and culture

Human neuroblastoma cell lines SH-SY5Y were obtained from the Institute of Medical Molecular Genetics of Binzhou Medical University (Yantai, China). The cells were cultured in Dulbecco's modified Eagle's medium(DMEM)/F12 (1:1) (HyCLone, Logan, UT, USA) with 10% fetal calf serum (HyClone, Logan, UT, USA) and maintained in a 5% CO_2 humidified incubator at 37 °C, supplemented with penicillin (20 U/mL) and streptomycin (20 μg/mL).

miRNA transfection

The cells were plated in a 6-well plate 1 day in advance to make the cell density 40% at the time of transfection. The cells were treated with 1 μg of miRNA and 2.5 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and then incubated for 6-8 h in a 37 °C 5% CO2 incubator.

Thereafter, the transfection solution was aspirated into the 6-well plate, replaced with 2 mL of fresh medium, and incubated in a 37 °C 5% CO_2 incubator for 48 h to prepare for subsequent RNA or protein extraction and to detect apoptosis.

Cisplatin treatment

The cells were counted and plated in a 6-well plate. After 16-18 h, different concentrations (0, 1, 2, 3 μg/mL) of cisplatin were dissolved in the medium in the 6-well plate during cell exchange. The cells were incubated in a 37 °C 5% CO_2 incubator for 48 h and then collected and prepared for the follow-up experiments.

MTT detection

SH-SY5Y cells were plated in a 96-well plate at a density of 5000 cells/well. Culturing was continued at 37 °C in a 5% CO_2 incubator. On the second day, each group was added with cisplatin at concentrations corresponding to the experimental design.

After culturing for 48 h, 10 μL of 5 mg/mL MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and the cells were incubated for 4 h at 37 °C in a 5% CO_2 incubator. The medium was discarded, and then 100 μL DMSO (Sigma Aldrich; Merck KGaA) was added to each well. Absorbance was measured at 491 nm in a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA) after shaking.

Real-time polymerase chain reaction (RT-PCR)

Mirna was extracted from SH-SY5Y cells using RNAiso for small RNA (Takara Biotechnology Co., Ltd., Dalian, China), and RNA concentration was detected using a spectrophotometer (Nano Drop 2000; Nano Drop Technologies, Inc., Wilmington, DE, USA) at 260/280 absorbance values. Poly(A) polymerase with poly(A) tail was used at the 3'UTR end of miRNA. RNA (1–2 μg) was used for the real-time (RT) primer 5'-aacatgtacagtcatgggd (T) 30N (A, G, C, or T)-3' guided synthesis of first-strand complementary DNA (cDNA).

The forward primer used to amplify miR-486-5p was 5'-UCCUGUACUGAGCUGCCCCGAG-3'. The reverse primer was 5'-CGGGGCAGCUCA-GUACAGGAUU-3'.

The forward primer of 5S ribosomal RNA (rRNA) was 5'-GCCATACCACCCTGAACG-3', and the reverse primer was 5'-AACATGTACAGTCCAT-GGATG-3'.

Prime Script™ RT reagent kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan) was used to perform RT reaction. Then, SYBR Premix Ex

Taq™ (Takara Bio, Inc., Otsu, Japan), the forward primer, the reverse primer, template cDNA, and nuclease-free distilled water are mixed, and PCR amplification was performed on a PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The PCR products were isolated on 1% agarose gel, and the results were observed on a Tanon gel imaging system. Human 5S rRNA served as the control. The results were calculated using the $2^{-\Delta\Delta Cq}$ value.

Western blot analysis

The cells were cultured in a 6-well plate. After transfection, cell pellets were collected in a 1.5 mL EP tube and washed twice with cold phosphate-buffered saline (PBS). Then, 100 μL of radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) with 1 mM of PMSF was added to the EP tube to extract protein. Protein samples were loaded onto each lane of 10% or 12% polyacrylamide gel for SDS-PAGE (Beyotime Institute of Biotechnology, Shanghai, China). The voltage was first adjusted to 80 V and then to 120 V after the marker presented clear bands. Then, the protein was transferred to a PVDF membrane through a membrane transfer device under a constant current of 250 mA.

Time depended on the molecular weight of the protein, which was generally between 60 min and 120 min. The PVDF membrane was sealed with 2.5% skim milk powder at room temperature for 2 h. Then, the membrane was placed in diluted anti-PIM1 polyclonal antibody (1:500; rabbit no. bs-3540R; BIOSS, Beijing, China) and incubated overnight at 4 °C. The following day, the membranes were washed three times with TBST for 10 min each time and then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:5,000; cat no. 13278; Bioworld Technology, Inc.) for 2 h. Lastly, chemiluminescent liquid was added to the image in a FluorChem FC2 gel imaging system (Protein Simple, San Jose, CA, USA). The protein expression was quantified using Image cal software.

Flow cytometry analysis

Cisplatin and miR-486-5p were transfected simultaneously into SH-SY5Y cells. After 48 h, the supernatant was discarded and the cells were washed twice with cold PBS. The cells were digested and collected in a 1.5 mL EP tube. Then, 400 μL of binding buffer (Nanjing KeyGen Biotech. Co. Ltd., Nanjing, China) was added to the collected cells to resuspend the cells. Subsequently, 4 μL of annexin V-FITC (Nanjing KeyGen Biotech. Co. Ltd., Nanjing, China) and 4 μL of PI (Nanjing KeyGen Biotech. Co. Ltd., Nanjing, China) staining solution were added. After 10 min, wavelength was detected via flow cytometry (BD FACSCantoTMII; BD Bioscience, Franklin Lakes, NJ, USA). The stimulation wavelength was 488 nm, and the emission wavelength was 530 nm.

Statistics

Statistical analyses were performed using SPSS 22.0 software (IBM Corporation, Armonk, NY, USA). Independent two-sample t-tests were conducted to analyze differences between two groups. One way ANOVA was used to analyze differences among three or more groups. A post hoc test of ANOVA was conducted by performing Tukey's test. Data were expressed as mean ± standard deviation. Statistical significance was accepted at $P<0.05$.

Results

Cisplatin inhibits the proliferation of SH-SY5Y cells by upregulating miR-486-5p

To determine the role of cisplatin in SH-SY5Y cells, the MTT technique was used to detect the effect of different concentrations (0, 1, 2, 3 μg/mL) of cisplatin on proliferation in SH-SY5Y cells. The results showed that as concentration increased, cell proliferation rate gradually decreased (Figure 1A). To find the downstream target genes of cisplatin that affect cell proliferation, RT-PCR was adopted to assess miR-486-5p expression in different concentrations (0, 1, 2, 3 μg/mL) of cisplatin. The results showed that miR-486-5p expression increased in SH-SY5Y cells with increasing cisplatin concentration, and the change would be insignificant at 3 μg/mL (Figure 1B). These findings indicated that cisplatin can play a role through miR-486-5p.

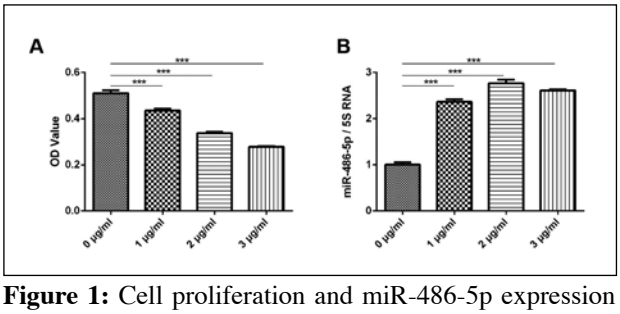

Figure 1: Cell proliferation and miR-486-5p expression after cisplatin treatment. *(A) MTT indicated that cell proliferation decreases with increasing cisplatin concentration. (B) RT-PCR indicated that the expression of miR-486-5p increased with an increase in cisplatin concentration. ***P<0.001.*

MiR-486-5p can inhibit the proliferation of SH-SY5Y cells

Different concentrations (25 nM and 50 nM) of miR-486-5p were transfected into SH-SY5Y cells. The results of RT-PCR showed that miR-486-5p was overexpressed in the cells (Figure 2A). To understand the role of miR-486-5p in SH-SY5Y cells, we examined the effect of different concentrations (25 nM and 50 nM) of miR-486-5p cell proliferation by using the MTT technique. The result showed that the cell proliferation rate gradually decreased as miR-486-5p concentration increased (Figure 2B).

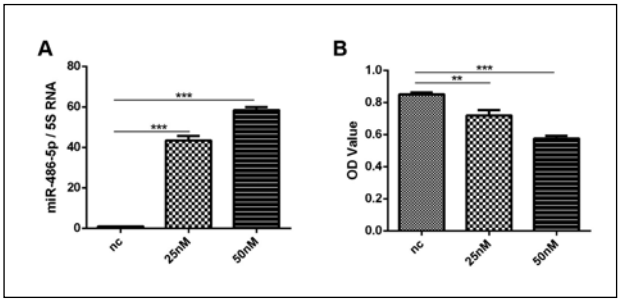

Figure 2: Determination of transfection efficiency and cell proliferation.
*(A) RT-PCR results showed that miR-486-5p treated cells expressed high levels of miR-486-5p. (B) MTT results showed that miR-486-5p can affect cell proliferation; the higher the concentration, the more evident the inhibition of proliferation. **P<0.01; ***P<0.001.*

MiR-486-5p regulates PIM1 mRNA expression by directly binding to 3'-UTR

Through the website (http://www.targetscan.org), we predicted that miR-486-5p can be directly combined with PIM1's 3'UTR (Figure 3A). To further explore the relationship between miR-486-5p and PIM1, the transfection of miR-486-5p into SH-SY5Y cells indicated that PIM1 expression was reduced (Figures 3B and 3C). Further research showed that miR-486-5p negatively regulated PIM1.

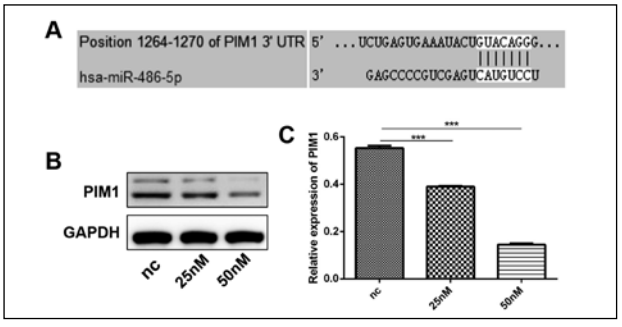

Figure 3: PIM1 expression is regulated by miR-486-5p.
*(A) The sequence of PIM1-3'UTR is targeted by miR-486-5p. (B) Western blot results showed that PIM1 expression was decreased in the miR-486-5p-treated cells compared with that in the control cells. (C) Relative values for PIM1 vs. GAPDH in B. ***P<0.001 vs. NC cells.*

Cisplatin combined with miR-486-5p inhibits the proliferation of SH-SY5Y cells

To evaluate the role of cisplatin combined with miR-486-5p in SH-SY5Y cells, we observed the proliferation of cells under an optical microscope (Figure 4A). We found that cisplatin combined with miR-486-5p inhibited cell proliferation better than cisplatin or miR-486-5p acting on the cells alone. Similarly, the same phenomenon was observed in the MTT experiment (Figure 4B). These results indicated that miR-486-5p can enhance the proliferation inhibition of SH-SY5Y cells via cisplatin.

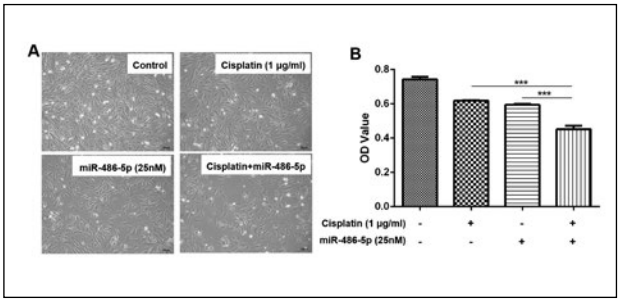

Figure 4: Cell proliferation slows down under the actions of cisplatin and miR-486-5p.
*(A) The inverted microscope showed that the combined effects of cisplatin and miR-486-5p inhibited cell proliferation most significantly compared with NC and cisplatin or miR-486-5p alone. Scale bar: 100 μm. (B) MTT analysis presented the same trend. ***P<0.001.*

Cisplatin combined with miR-486-5p promotes the apoptosis of SH-SY5Y cells

To examine the roles of cisplatin and miR-486-5p in apoptosis, we used flow cytometry to analyze the effects of cisplatin and miR-486-5p on SH-SY5Y cells. The results showed that the combined use of cisplatin and miR-486-5p can promote cell apoptosis more than using miR-486-5p or cisplatin alone (Figure 5).

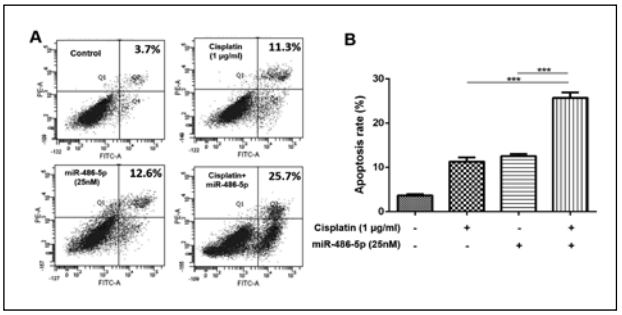

Figure 5: Increased apoptosis under the actions of cisplatin and miR-486-5p.
*(A) Flow cytometry results indicated that cisplatin or miR-486-5p alone had a higher percentage of apoptosis than NC, and the combination of cisplatin and miR-486-5p had the highest percentage of apoptosis. (B) Apoptosis rate increased significantly after performing cisplatin combined with miR-486-5p treatment. ***P<0.001..*

Cisplatin combined with miR-486-5p works by negatively regulating PIM1

Cisplatin combined with miR-486-5p can play a role in cell proliferation and apoptosis. We detected the levels of miR-486-5p transfected with different types of substances via qRT-PCR, and the results showed that miR-486-5p presented the highest expression in the combined group (Figure 6A). Further study of the downstream target gene PIM1 found that cisplatin combined with miR-486-5p can reduce PIM1 most significantly (Figure 6B). This finding is also consistent with previous research results.

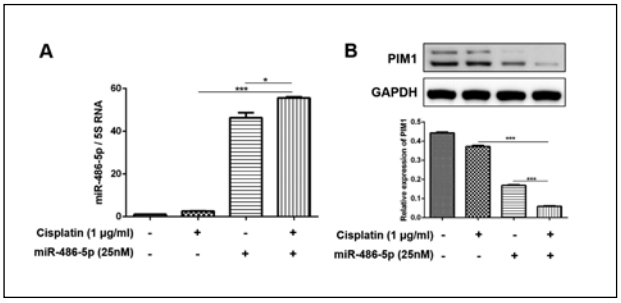

Figure 6: Expression of miR-486-5p and PIM1.
*(A) RT-PCR results showed that cisplatin combined with miR-486-5p achieved a higher expression level of miR-486-5p compared with that of the control group. *P<0.05. (B) Western blot results indicated that the expression level of PIM1 in combined cisplatin and miR-486-5p was lower compared with that in the control group. *P<0.05 ,***P<0.001.*

Discussion

As an anti-cancer drug, cisplatin has been widely used in the clinical treatment of tumors. It can play a role in many types of cancer, such as lung cancer[16], colorectal cancer[17], and breast cancer[18]. Similarly, we found that cisplatin can inhibit the proliferation of human neuroblastoma in our research. In recent studies, cisplatin typically inhibits cell proliferation, migration, or apoptosis by downregulating or upregulating a certain gene or signaling pathway molecule. In accordance with previous reports, cisplatin inhibits the proliferation of SH-SY5Y cells by downregulating the expression of BDNF[19]. As an anti-liver cancer drug, cisplatin is related to the downregulation of miR-21 expression and the upregulation of miR-122[20]. Our research found that cisplatin can increase the expression of miR-486-5p and inhibit the proliferation and apoptosis of SH-SY5Y cells by affecting the expression of miR-486-5p.

As a popular non-coding RNA that has been studied in recent years, miRNA plays an important role in tumorigenesis and development and as a proto-oncogene or a tumor suppressor gene. It can also play a role in RNA silencing and the posttranscriptional regulation of gene expression. Given these characteristics, miRNA can regulate various signal transduction pathways involved in cancer development, such as cell proliferation, apoptosis, and migration[21]. Reports indicate that miR-146a-5p can play a role in a variety of tumors, and studies have found that miR-146a-5p can be used as a non-invasive biomarker and targeted therapy for various cancer types[22]. Evidence is increasing that miRNA-214 may act as a biomarker in certain types of cancer[23]. In accordance with the results obtained in the last 10 years, some types of miRNA have gradually become biomarkers for breast cancer diagnosis (i.e., miR-9, miR-10b, and miR-17-5p) and prognosis (i.e., miR-148a and miR-335) and the prediction of treatment outcome (i.e., miR-30c, miR-187, and miR-339-5p)[24]. Research has confirmed that miR-486-5p is a tumor suppressor, and it can exert a tumor suppressor effect on lung squamous cell carcinoma, non-small cell lung cancer, and breast cancer[25-27]. We found through the current research that miR-486-5p can inhibit the proliferation of SH-SY5Y cells, further proving its tumor suppressor function.

The classic approach for miRNA to work is to combine it with the 3'UTR end to enable it to regulate. In the current research, we determined that miR-486-5p can bind to the 3'UTR end of PIM1 to regulate SH-SY5Y cells. As a tumor-promoting factor, PIM1 is highly expressed in various tumors and plays a role in promoting cancer[28-30]. In the present study, the expression of PIM1 in SH-SY5Y cells decreased under the action of miR-486-5p or cisplatin alone, but the decrease was more pronounced under the combined actions of cisplatin and miR-486-5p.

Considering the drug resistance and considerable side effects of cisplatin in the treatment of tumors, the combination therapy of cisplatin and miRNA has been used as a new treatment strategy for many types of human cancer. In this study, we used cisplatin combined with miR-486-5p to act on SH-SY5Y cells, and the results indicated that the combined effect was better than cisplatin or miR-486-5p alone. The cell proliferation of the combined group was slower and the apoptosis rate increased compared with the single action group. Similarly, Shi et al[14] found that miR-34a and paclitaxel can synergistically inhibit cancer cells. In another study, the authors found that miR-let7b and hedgehog inhibitor GDC-0449 can be used to treat pancreatic

cancer. In animals treated with a combination of miR-let7b and GDC-0449, tumor cell proliferation decreased and apoptosis increased[31]. This result further confirmed that the combination of drugs and miRNA can have an excellent effect in the treatment of tumors. The aforementioned findings provide new methods and strategies for the future clinical treatment of cancer and offer a good prospect for the cure of cancer.

In summary, cisplatin in human neuroblastoma SH-SY5Y cells works by upregulating miR-486-5p and downregulating PIM1, and the combined effects of cisplatin and miR-486-5p improve the treatment effect. The results indicate that miR-486-5p can enhance the effect of cisplatin on human neuroblastoma SH-SY5Y cells by downregulating PIM1. The findings of this study provide new targets and evidence for neuroblastoma treatment.

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Corresponding Authors:
Yunxiao Sun
Department of Pediatrics, Yantai Affiliated Hospital of Binzhou Medical University, Yantai, Shandong 264003, P.R. China
Email: sunyunxiao1979@163.com
(China)
Youjie Li
Key Laboratory of Tumor Molecular Biology in Binzhou Medical University, Department of Biochemistry and Molecular Biology, Binzhou Medical University, Yantai, Shandong 264003, P.R. China
Email: youjie1979@163.com
(China)