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Original Article

MiR-3195 inhibits non-small cell lung cancer malignant behaviors along with cisplatin resistance through targeting PFKFB4

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Abstract

Chemotherapy presents the main therapy of non-small cell lung cancer (NSCLC). Nevertheless, cisplatin-based therapy can be limited by drug resistance. MicroRNA (miRNA) possesses a vital regulatory function in modulating the progression as well as cisplatin resistance of NSCLC, but how miR-3195 influences NSCLC is obscure. In this work, it was discovered that miR-3195 presented definite down-regulation in NSCLC cells. Gain-of function assays revealed that overexpressing miR-3195 hindered NSCLC cell proliferation together with migration whereas induced cell apoptosis. Mechanically, 6-phosphofructo-2-kinase/fructose-2,6-bi-phosphatase 4 (PFKFB4) presented the target gene of miR-3195 and was high-expressed in NSCLC cells. The repressive impacts of overexpressing miR-3195 on NSCLC cells malignant behaviors were reversed via PFKFB4 elevation. Additionally, elevated miR-3195 expression reduced cisplatin resistance of NSCLC both in vitro as well as in vivo. PFKFB4 elevation could offset the reduced cisplatin resistance caused by miR-3195 overexpression in NSCLC cells. In conclusion, this work clarified miR-3195 repressed NSCLC cell proliferation, migration, as well as cisplatin resistance by modulating PFKFB4. Our study might provide a promising clue to promote the anti-tumor effects of chemotherapy.

Keywords: NSCLC, miR-3195, PFKFB4.

1. Introduction

As a frequent malignant tumor possessing the highest incidence as well as death rate all over the world, lung cancer is separated into small-cell along with non-small-cell lung cancer (NSCLC) [1]. In spite of great advances in diagnosis as well as treatment strategies in recent decades, the prognosis of NSCLC patients presents unsatisfactory, and the long-time survival rate presents below 15% [2]. Platinum-based chemotherapy presents an extensively utilized first-line strategy for NSCLC therapy [3]. Cisplatin (DDP) is the most frequently utilized platinum-based chemotherapeutic drug to exert anti-tumor activity [4]. DDP combines with genomic DNA or mitochondrial DNA to trigger DNA injury, hinder DNA duplication, as well as induce transduction pathways and ultimately leads to necrosis or apoptosis [5]. However, under the long-time use of DDP, cancer cells often develop severe chemotherapy resistance, which has emerged to be a main barrier to the clinical application of DDP in NSCLC patients [6]. Hence, reducing the acquired chemotherapy resistance of NSCLC to DDP is a crucial clinical goal [7].

MicroRNAs (miRNAs) belong to non-coding RNAs possessing a length of 19-25 nucleotides. MiRNAs influence the expression of about 60% of human genes via combining with the 3'untranslated region (3'UTR) of downstream target mRNAs and take part in diverse physiological processes, containing cell proliferation, differentiation, as well as apoptosis [8]. It has been revealed that miRNAs have relation with tumorigenesis and development of various tumors, NSCLC included [9, 10]. Moreover, miRNAs are required for inducing DDP resistance in various cancers. For instance, miRNA-27a modulates DDP resistance in bladder cancer via targeting SLC7A11 [11]. MiR-199a overcomes DDP resistance in osteosarcoma through inhibition of HIF-1α [12]. MiR-218 induces DDP resistance in oral cancer via the PPP2R5A/Wnt signaling pathway [13]. Therefore, miRNA may work as an underlying strategy for reversing chemoresistance.

MiR-3195 has been documented to function as a tumor repressor in osteosarcoma SOX4 [14]. Moreover, it has been documented that miR-3195 is underexpressed in NSCLC tissues [15]. Nevertheless, whether miR-3195 can affect the progression as well as chemoresistance of NSCLC is unknown.

In this study, the property of miR-3195 in the progression along with DDP resistance of NSCLC was explored.

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2. Material and methods

2.1. Cell culture and treatment

Procell (Wuha, China) provided human normal lung epithelial cell line (BEAS-2B) as well as NSCLC cell lines (A549, H1975, H1299 and HCC827), and cells received culture in BEGM, Ham's F-12K and RPMI-1640 medium, separately including 10% fetal bovine serum (FBS) together with 1% penicillin and streptomycin at 37°C with 5% CO_2.

To induce DDP resistance in A549 as well as H1975 cells (A549/DDP as well as H1975/DDP), these cells received exposure to 1 μg/mL DDP (Sigma-Aldrich, USA) for 80 days.

2.2. Cell transfection

MiR-3195 mimics and NC mimics were acquired from Sangon Biotech (Shanghai, China). The overexpression vector of PFKFB4 (pcDNA3.1-PFKFB4) and negative control (pcDNA3.1) were obtained from Vazamy (China). Cell transfection could be implemented with Lipofectamine 3000 reagent (Invitrogen, USA) for 48 h.

2.3. Cell counting kit (CCK)-8 assay

For measuring cell viability, cells accepted planting into 96-well plates for growth, 10 μL of CCK-8 solution (Dojindo, Japan) was supplemented at the indicated times. The optical density accepted measurement at 450 nm could be carried out followed by 2-hour incubation.

For IC_{50} analysis, cells accepted planting into 96-well plates overnight, followed by exposure to different doses of DDP (1, 10, 20, 40, 80, as well as 160 μM) for 48 h. Afterwards, 10 μL of CCK-8 solution received addition into each well, and the optical density measured at 450 nm was carried out followed by 2-hour incubation. The calculation of IC_{50} value was carried out using the dose-response survival curve.

2.4. Colony formation assay

Cells received planting into 6-well plates with a density of 600 cells per well. After 14 days of culture, cells received staining with 0.1% crystal violet (Beyotime, China) for 1 h, followed by counting manually the colonies.

2.5. Flow cytometry

After trypsin-digestion, and PBS-rinsing, cells could be gathered. Cell apoptosis could be assessed with the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA). The cells received staining using Annexin V-FITC as well as propidium iodide, followed by analysis with flow cytometry.

2.6. Transwell

Transfected cells (2×10^4 cells) could be placed into upper chambers (pore size of 8 μm, Corning). 500 μL of culture medium including 10% FBS could be adopted to be a chemoattractant to put into lower chambers. After cultivation for 24 h, the cells on the top surface could be wiped off with cotton swabs, and the cells on the bottom received fixation and staining. Later, images were photographed, followed by counting the migrated cells in 5 predetermined fields of view (×200).

2.7. RT-qPCR

TRIzol reagent was utilized for extracting total RNA from cells. Then, RNA reverse transcription was implemented with PrimeScript® RT reagent Kit (Takara, Japan). Next, SYBR Green-based real-time PCR could be conducted. The primers were as below: miR-3195: forward: 5'-TGCAAGCGCGCCGGGCCCGGGT-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'; U6 forward: 5'-TTATGGGTCCTAGCCTGAC-3', reverse: 5'-CACTATTGCGGGTCTGC-3'; PFKFB4: forward: 5'-GGCAGGGAGTTTGCCAAGA-3', reverse: 5'-TCGTTGAGGACCTTCCACTG-3'; GAPDH forward: 5'-TGAAGGTCGGAGTCAACGG-3', reverse: 5'-TCCTGGAAGATGGTGATGGGA-3'. The U6 or GAPDH was adopted to be controlled, respectively. Relative quantification of gene expression was implemented with the $2^{-\Delta\Delta Ct}$ method.

2.8. Dual-luciferase reporter gene assay

Wild-type (WT) together with mutant (MUT) of PFKFB4 3'UTR dual-luciferase reporter plasmids were constructed with the psiCHECK2 reporter vector. Cells were cultured overnight, followed by transfecting with the PFKFB4 3'UTR-WT or PFKFB4 3'UTR-MUT plasmids, together with miR-3195 mimics or NC mimics. Forty-eight h later, the luciferase activity in the cells could be measured with the Dual-Luciferase Reporter Assay System (Promega, USA).

2.9. RNA immunoprecipitation (RIP) assay

The EZ-Magna RIP Kit (Millipore, USA) could be utilized for this assay. Cells received splitting decomposition in RIP lysis buffer which included proteinase as well as RNase inhibitors. Then, cells received co-incubation with RIP lysates together with the human anti-Ago2 antibody or mouse IgG antibody. After digesting the protein by Proteinase K buffer, the immunoprecipitated RNAs received extraction and the enrichment of RNAs received detection with RT-qPCR.

2.10. Western blot analysis

Proteins received isolation with RIPA lysis buffer (Sigma, USA), followed by measuring the protein concentrations. Protein was isolated by SDS-PAGE and then shifted to PVDF membranes. Followed by sealing with 5% skimmed milk powder for 2 h, the membranes were cultivated with the primary antibodies including anti-PFKFB4 (1/500, ab137785, Abcam, UK) as well as GAPDH (1/2500, ab9485, Abcam, UK) at 4°C overnight. Next, the membrane received washing and then hatched with the secondary antibodies (1/2000, ab6728, Abcam, UK) for 2 h. The protein expression was determined with the ECL detection system (Thermo Fisher Scientific, USA).

2.11. Tumor xenograft

Chengdu Dashuo Experimental Animal Co., LTD (Chengdu, China) provided 30 male BALB/c nude mice (4-week-old). Mice were placed in a specific pathogen-free room with eating as well as drinking freely. After 1 week of adaptive feeding, A549 cells that stably expressed miR-3195 mimics, as well as NC mimics, accepted subcutaneous injection into nude mice via the armpit of the right forelimb. The DDP group received injection with DDP (5 mg/kg) two times a week and the other groups received injection with the same dose of normal saline for 4 weeks when apparent tumors occurred. The volume formula was measured every 7 days. After 4 weeks, the nude mice re-

ceived euthanasia, followed by obtaining and weighing the tumor tissues. All animal procedures were in line with the guidelines for the Care and Use of Laboratory Animals.

2.12. Statistical analysis

All assays were performed three times. SPSS 21.0 software was implemented for data analysis. All data could be exhibited to be mean ± standard deviation (SD). The student t-test or one-way analysis of variance (ANOVA) could be adopted for evaluating the difference between groups. P<0.05 meant statistical significance.

3. Results

3.1. Overexpressed miR-3195 represses NSCLC cell proliferation, and migration while promoting apoptosis

According to starBase website (https://rnasysu.com/encori/index.php), miR-3195 expression was apparently declined in NSCLC tissues when compared with normal tissues (P=0.014, Figure 1A). Moreover, miR-3195 expression presented lower in lung cancer cells when compared with normal lung epithelial cell lines (Figure 1B). A549 as well as H1975 possessing the lowest miR-3195 expression were chosen for subsequent assays. MiR-3195 mimics were transfected to overexpress miR-3195 in A549 and H1975 cells, where miR-3195 expression was elevated followed by transfection (Figure 1C). CCK-8 as well as colony formation assays displayed that overexpressed miR-3195 apparently declined NSCLC cell proliferation (Figures 1D-E). Flow cytometry findings uncovered that elevation of miR-3195 accelerated NSCLC cells apoptosis rate (Figure 1F). Moreover, the migratory ability of NSCLC cells presented reduced after miR-3195 increase (Figure 1G).

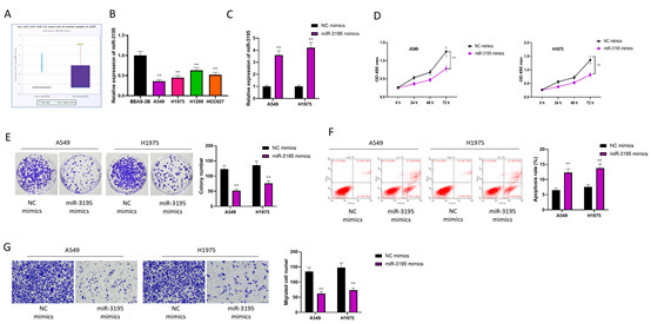

Fig. 1. Overexpressed miR-3195 represses NSCLC cell proliferation, and migration while promoting apoptosis. A. StarBase website showed miR-3195 expression in NSCLC tissues and normal tissues. B. RT-qPCR examined miR-3195 expression in NSCLC cells. C. Overexpression transfection efficiency of miR-3195 in NSCLC cells. D-E. CCK-8 and colony formation assays examined NSCLC cell proliferation after miR-3195 elevation. F. Flow cytometry analysis examined NSCLC cells apoptosis after miR-3195 elevation. G. Transwell assay examined NSCLC cell migration after miR-3195 elevation. **P<0.01.

3.2. PFKFB4 is targeted by miR-3195

The underlying target gene of miR-3195 was predicted through TargetScan database (https://www.targetscan.org/vert_80/). It was found that miR-3195 contained a seed region paired with PFKFB4 3'UTR (Figure 2A). PFKFB4 has been validated to be a proto-oncogene [16]. Herein, we discovered that PFKFB4 presented high expression in NSCLC cells (Figure 2B). Moreover, overexpressed miR-3195 obviously declined PFKFB4 mRNA together with protein levels in NSCLC cells (Figure 2C-D), implying that PFKFB4 could be negatively modulated by miR-3195 in NSCLC, and PFKFB4 might be the target gene of miR-3195. Expectedly, this speculation was certified through dual luciferase reporter gene analyses. The results unveiled that miR-3195 overexpression significantly lessened the luciferase intensity of PFKFB4 3'UTR-WT, whereas not influence PFKFB4 3'UTR-MUT luciferase intensity (Figure 2E). The outcomes of RIP also indicated that miR-3195 could combine with PFKFB4 in NSCLC cells (Figure 2F).

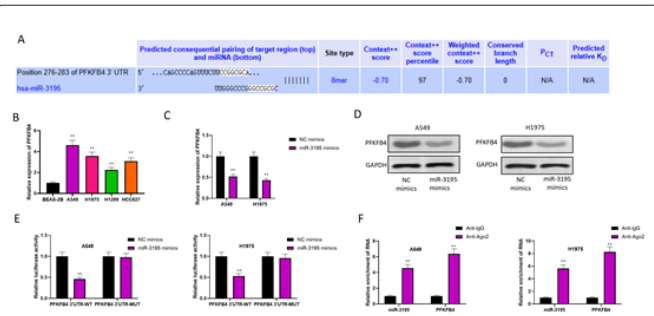

Fig. 2. PFKFB4 is the target gene of miR-3195. A. TargetScan database predicted the binding sites between miR-3195 and PFKFB4 3'UTR. B. RT-qPCR examined PFKFB4 expression in NSCLC cells. C-D. RT-qPCR and western blot examined PFKFB4 mRNA and protein levels in NSCLC cells after miR-3195 elevation. E. Dual-luciferase reporter gene assay examined the luciferase activity of PFKFB4 3'UTR-WT/MUT in NSCLC cells after miR-3195 elevation. F. RIP assay examined the enrichment of miR-3195 and PFKFB4 in Ago2 antibody. **P<0.01.

3.3. MiR-3195 overexpression inhibits DDP resistance of NSCLC cells via targeting PFKFB4

Next, whether miR-3195 affected DDP resistance of NSCLC was examined. Based on CCK-8 experiment, the IC_{50} values of DDP-resistant NSCLC cells were visibly increased versus parental NSCLC cells (Figures 3A-B). Subsequently, it was uncovered that miR-3195 expression declined while PFKFB4 expression presented elevation in DDP-resistant NSCLC cells when compared with the parental cells (Figure 3C-3D). Subsequently, miR-3195 expression presented elevation in DDP-resistant NSCLC cells when transfecting miR-3195 mimics (Figure 3E). The results clarified that IC_{50} values of DDP-resistant NSCLC cells presented decline when miR-3195 presented elevation (Figure 3F). CCK-8 as well as colony formation experiments manifested that miR-3195 elevation reduced DDP-resistant NSCLC cell proliferation (Figure 3G-H). Moreover, miR-3195 promotion promoted DDP-resistant NSCLC cell apoptosis (Figure 3I) and impaired cell migration (Figure 3J).

3.4. MiR-3195 hinders DDP resistance of NSCLC cells

Furthermore, whether miR-3195 took part in reducing DDP resistance of NSCLC cells through regulating PFKFB4 was verified through rescue experiments. PFKFB4 expression was first elevated in DDP-resistant NSCLC cells after transfecting pcDNA3.1-PFKFB4 (Figure 4A). Then, co-transfection of miR-3195 mimics together with pcDNA3.1-PFKFB4 was implemented into DDP-resistant NSCLC cells. The outcomes clarified that

pcDNA3.1-PFKFB4 transfection could offset the IC_{50} values and proliferation of miR-3195 mimics-transfected DDP-resistant NSCLC cells (Figure 4B-D). Likewise, the elevated apoptosis and repressed migration of DDP-resistant NSCLC cells modulated by miR-3195 elevation were counteracted followed by pcDNA3.1-PFKFB4 co-transfection (Figure 4E-F).

3.5. MiR-3195 inhibits tumor growth as well as DDP resistance in vivo

To support in vitro findings, xenotransplantation was implemented in nude mice. It was displayed in Figure 5A-C that miR-3195 overexpression or DDP treatment lessened tumor volume and weight, but simultaneous overexpression of miR-3195 in DDP treatment further weakened tumor volume as well as weight.

4. Discussion

Patients diagnosed with advanced NSCLC lose the chance for surgery and routinely accept chemotherapy. DDP has emerged to be a kind of the most commonly used drugs in chemotherapy for NSCLC. Nevertheless, in most tumors, the development of resistance because of repeated use can undermine their therapeutic effectiveness. Therefore, overcoming drug resistance to DDP-based anti-tumor drugs as well as promoting the sensitivity of tumor tissues and cells to DDP is of great significance for guiding the clinical therapy of NSCLC.

Increasing miRNAs have been identified to have a crucial function in the development of NSCLC, and is proposed to be potent therapeutic targets. MiR-196b-5p promotes tumor progression in NSCLC via down-regulation of TSPAN12 and GATA6 [17]. MiR-621 inhibits the malignant progression of NSCLC via targeting SIX4 [18]. MiR-200a-3p hinders NSCLC cell proliferation, migration, as well as invasion via targeting IRS2 [19]. As for miR-3195, it has been documented to serve as be tumor suppressor in laryngeal cancer [20]. Besides, miR-3195 can be used to potentially indicate the prognosis of ovarian clear cell carcinoma patients [21]. More importantly, miR-3195 is low-expressed in NSCLC patients and is an independent prognostic indicator for NSCLC patients' overall survival [22]. Consistently, our study also indicated that miR-3195 presented down-regulation in NSCLC cells, and further functional assays proved that miR-3195 elevation could impair the proliferation as well as migration whereas promotes the apoptosis in NSCLC cells.

MiRNAs have been revealed to modulate drug resistance to chemotherapy drugs. In NSCLC, it has been documented that miR-328 overexpression confers DDP resistance in NSCLC via targeting of PTEN [23]. MiR-25-3p promotes DDP resistance in NSCLC through adjusting PTEN/PI3K/AKT route [24]. MiR-126-5p targets ADAM9 to promote DDP sensitivity of NSCLC [25]. Nevertheless, until now, no study has looked at the relationship between miR-3195 and DDP resistance in NSCLC. In this research, it was demonstrated that miR-3195 presented down-regulation in DDP-resistant NSCLC cells relative to parental cells. Moreover, miR-3195 overexpression repressed DDP resistance of NSCLC both in vitro as well as in vivo.

MiRNAs are well-known to influence the expression of various target genes. Here, PFKFB4 was manifested to be a target mRNA of miR-3195 through TargetScan database. PFKFB4 is frequently elevated in tumors and has

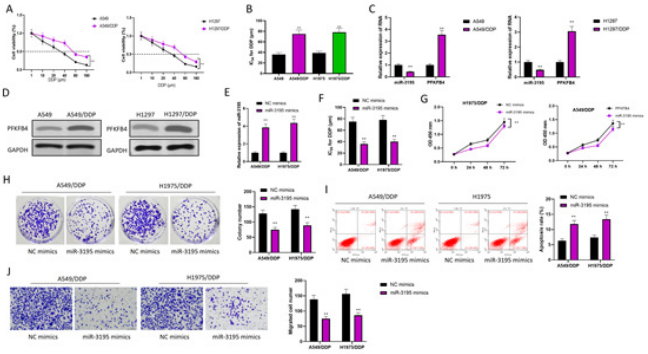

Fig. 3. MiR-3195 overexpression inhibits DDP resistance in NSCLC cells. A. CCK-8 assay-based detection of IC_{50} values in A549 and A549/DDP groups as well as H1975 and H1975/DDP groups. B. Quantitative and statistical analysis of IC_{50} for each group. C-D. RT-qPCR or western blot detected miR-3195 and PFKFB4 expression in A549 and H1975 and A549/DDP and H1975/DDP cells. E. Overexpression transfection efficiency of miR-3195 in A549/DDP and H1975/DDP cells. F. CCK-8 detected IC_{50} value in A549/DDP and H1975/DDP cells after miR-3195 overexpression. G-H. CCK-8 and colony formation assays examined A549/DDP and H1975/DDP cells proliferation after miR-3195 elevation. I. Flow cytometry analysis examined A549/DDP and H1975/DDP cells apoptosis after miR-3195 elevation. J. Transwell assay examined A549/DDP and H1975/DDP cells migration after miR-3195 elevation. $^{**}P<0.01$.

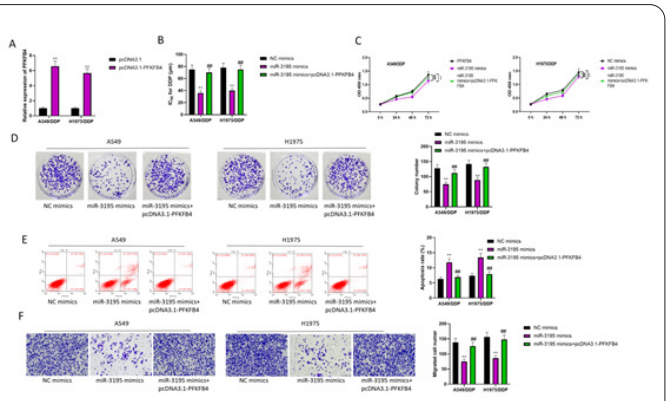

Fig. 4. MiR-3195 inhibits DDP resistance in NSCLC cells via targeting PFKFB4. A. Overexpression transfection efficiency of PFKFB4 in A549/DDP and H1975/DDP cells. MiR-3195 mimics and pcDNA3.1-PFKFB4 were co-transfected into A549/DDP and H1975/DDP cells, followed by B. CCK-8 detected IC_{50} value. C-D. CCK-8 and colony formation assays examined NSCLC cell proliferation. E. Flow cytometry analysis examined NSCLC cells apoptosis. F. Transwell assay examined NSCLC cell migration. $^{**}P<0.01$, compared with NC mimics group, $^{\#\#}P<0.01$, compared with miR-3195 mimics group.

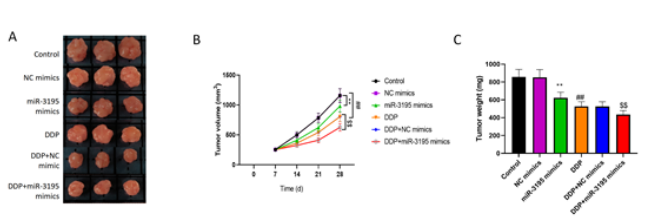

Fig. 5. MiR-3195 inhibits tumor growth and DDP resistance in vivo. A. Representative image of tumor. B. Tumor volume. C. Tumor weight. $^{**}P<0.01$, compared with NC mimics group, $^{\#\#}P<0.01$, compared with control group, $^{\$\$}P<0.01$, compared with DDP group.

been involved in drug resistance to cancers. PFKFB4 is a metabolic driver of hepatocellular carcinoma progression and chemoresistance through ROS mitigation [26]. PFKFB4 is high-expressed in clear-cell renal cell carcinoma, mediating sunitinib resistance through promoting pentose phosphate pathway [27]. PFKFB4 accelerates palbociclib resistance in breast cancer by increasing stemness [28]. In this research, PFKFB4 was validated to be a direct target gene of miR-3195. PFKFB4 presented high expression in NSCLC cells. Previous study has indicated that PFKFB4 promotes lung adenocarcinoma progression [29]. Consistently, our study further proved that elevation of PFKFB4 could offset the repressive impacts of overexpressing miR-3195 on NSCLC cells malignant behaviors. Moreover, our study unveiled that PFKFB4 expression presented elevation in DDP-resistant NSCLC cells versus the parental cells, which was in accordance with previous report [30]. Additionally, our study indicated that PFKFB4 elevation could offset the reduced DDP resistance caused by miR-3195 overexpression in NSCLC cells.

5. Conclusion

In summary, our study clarifies that miR-3195 presents low expression in NSCLC cells, and miR-3195 represses the malignant behavior as well as DDP resistance in NSCLC through targeting PFKFB4. Our study offers a promising target for promoting the anti-tumor effects of chemotherapy.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

We have received approval from the Ethics Committee of The Affiliated Taizhou People's Hospital of Nanjing Medical University, Taizhou School of Clinical Medicine, Nanjing Medical University.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

CR conducted the experiments and wrote the paper; GX analyzed and organized the data; SY conceived, designed the study and revised the manuscript.

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