Upregulation of MiR-340-5p Reverses Cisplatin Sensitivity by Inhibiting the Expression of CDK6 in HepG2 Cells

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	Cisplatin (CDDP) has been successfully used in chemotherapy for liver cancer. However, the development of CDDP resistance in HepG2 cells usually leads to relapse and a worsening prognosis. MiR-340-5p has attracted much attention because of its ability to affect cell resistance. This project is intended to explore the role of miR-340-5p and CDK6 in CDDP-R HepG2 cells and provide new ideas for the treatment of liver cancer. A dual-luciferase reporter assay was used to confirm the targeting relationship between miR-340-5p and CDK6. We constructed a CDDP-resistant model of HepG2 cells to examine the effect of miR-340-5p on the drug sensitivity of HepG2 cells. CDDP-R HepG2 cells were transfected with miR-340-5p overexpression plasmid and CDK6 silencing plasmid. QRT-PCR was used to detect the expression of miR-340-5p and CDK6. A western blot was performed to determine the expression of CDK6, CyclinD1, and CyclinD2, CCK-8, flow cytometry, TUNEL and Clonogenic assays were also carried out to detect CDDP-R HepG2 cells. There is a targeting relationship between miR-340-5p and CDK6. The drug resistance of CDDP-R HepG2 cells was significantly higher than that of CDDP-S HepG2 cells. CDDP-R HepG2 cells transfected with both miR-340-5p overexpressing plasmid and CDK6 silencing plasmid showed a lower proliferation ability, cell cycle arrest in the G0/G1 phase, and lower drug resistance compared with single CDDP-R HepG2 cells. Overexpression of miR-340-5p aggravated CDDP-R HepG2 cells' apoptosis and inhibited cell viability. Overexpression of miR-340-5p could reverse the sensitivity of HepG2 cells to CDDP by inhibiting the expression of CDK6 in HepG2 cells.
	Key words: miR-340-5p, CDK6, CDDP, HepG2, CyclinD1. Sha TIAN. Ruoxia WU, Xiaodi HUANG, Zicheng LIANG, Zhen ZHANG, Xuefei TIAN [™] , College of Integrated
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Liver cancer is one of the most common malignant tumors in the world and the second leading cause of cancer death (ZHAI *et al.* 2018). Especially in the developing countries of Asia, the incidence of liver cancer has increased significantly in recent years (ARGYROU *et al.* 2017). Globally, about 630,000 new cases of liver cancer occur every year, and more than half of these new cases occur in China (CHAI *et al.* 2019). Currently, chemotherapy is the main method for treating advanced liver cancer and treating patients with poor liver function. Cisplatin (CDDP) is one of the most commonly used chemotherapy drugs for advanced liver cancer and postoperative patients, combined with arterial chemotherapy (GENG *et al.* 2019). CDDP chemotherapy drugs will circulate throughout most of the organs and tissues of the body along with blood circulation, and is a means of systemic treatment. It is very effective in the treatment of cancer, but the subsequent side effects of chemotherapy and the adverse reactions of chemotherapy have brought intense pain to patients (CHU *et al.* 2016). Due to the existence of self or acquired chemotherapy

© Institute of Systematics and Evolution of Animals, PAS, Kraków, 2021 Open Access article distributed under the terms of the Creative Commons Attribution License (CC-BY) <u>http://creativecommons.org/licences/by/4.0</u> resistance, its efficacy may be limited (CUI *et al.* 2018; SHAH 2015). Therefore, we urgently need to explore a combination therapy that could reduce the drug resistance of liver cancer cells and restore the sensitivity of HepG2 cells to CDDP.

The side effects and drug resistance of CDDP limit its application and effectiveness. Side effects are mainly reflected in nephrotoxicity, ototoxicity, and neurotoxicity (SONG et al. 2016). Previous studies have shown that drug resistance is a complex process involving many genes, including microRNA (miRNA) (CHENG et al. 2015). Recent studies have shown that miRNAs are key regulators of tumorigenesis and development (POWERS et al. 2016). They usually regulate tumor cell proliferation, migration, invasion, and drug resistance by targeting oncogenes, tumor suppressor genes, transcription factors, and other regulatory factors involved in cell death and survival (DVINGE et al. 2013; SHI et al. 2016; SHINDO et al. 2018). miR-340-5p inhibits cell proliferation and drug resistance in breast cancer cells by downregulating the LGR5 expression of the Wnt/ β -catenin pathway (SHI et al. 2019). miR-340-5p reduces cell chemoresistance by targeting ZEB1 in osteosarcoma (YAN et al. 2018). miR-340-5p exerts its tumor suppressor function by directly targeting ANXA3 in liver cancer, and may be a new prognostic biomarker and therapeutic target (YANG et al. 2018). Our previous work confirmed that miR-340-5p is abnormally expressed in CDDP-R HepG2 cells, and found that most HepG2 cells have a low G1/S ratio. However, we are not certain about the role and mechanism of miR-340-5p involved in CDDP-R HepG2 cells. We speculate that miR-340-5p may interfere with cell division by regulating cell cycle factors.

Cyclin-dependent kinase 6 (CDK6) is a protein kinase that binds to cyclin and has kinase activity (BELLUTTI et al. 2018). CDK6 is indispensable in the normal progress of the G1/S process (ROMERO-POZUELO et al. 2020). There is evidence that cell cycle arrest is related to CDK6 and D-type cyclin. D-type cyclin competes with CDK inhibitor family 2 to bind to monomeric CDK4/6 to form active cyclin D-CDK4/6 complex an (ALVAREZ-FERNÁNDEZ & MALUMBRES 2020). A cyclin D-CDK4/6 complex could directly phosphorylate the transcription factor forkhead box protein M1 (FOXM1), and then induce the transcription of other genes involved in the G2/M phase of the cell cycle (ANDERS et al. 2011). There are three D-type cyclins in mammals (CyclinD1, CyclinD2, and CyclinD3) (LIU et al. 2016). The cyclinD-CDK4 complex is a regulator of the stability of CDC25A in the G1 phase, which generates a negative feedback loop to control the G1/S transition (DOZIER et al. 2017). CDK6 may mediate the cell cycle progression of HepG2 cells through the cyclin pathway, thereby changing cell proliferation, drug resistance, and apoptosis.

Although the results of previous studies suggest that miR-340-5p may affect the drug resistance of HepG2 cells, there is no relevant research on whether miR-340-5p could reverse the sensitivity of HepG2 cells to CDDP through CDK6. Through the research of this subject, we clarified the regulation mechanism of miR-340-5p on the HepG2 cell cycle and it's drug resistance through CDK6 so as to provide new ideas and a new basis for a CDDP combined treatment of liver cancer.

Material and Methods

Cell culture and treatment

HepG2, SMMC-7721, Huh-7, 293A, and Hep3B cells were purchased from HonorGene (Changsha Aibiwei Biotechnology Co., Ltd.). HepG2 cells were cultured in DMEM medium (D5796, Sigma, China) containing 10% FBS (10099141, Gibco, China) and 100 U/ml penicillin/streptomycin (SV30010, Biyuntian, China) at 37°C with 5% CO₂ and humidity. HepG2 were induced to CDDP-R cells resistant to CDDP. IC50 was calculated through a CCK-8 experiment to determine whether the induction was successful. The cells were grouped as follows: CDDP-S group (CDDP-sensitive HepG2 cells), CDDP-R group (successfully induced CDDP-resistant HepG2 cells), control group (CDDP-R cells), mimic-NC group (CDDP-R cells transfected with mimic-NC plasmid), miR-340-5p mimic group (CDDP-R cells transfected with miR-340-5p overexpression plasmid), si-NC group (CDDP-R cells transfected with si-NC plasmid) and si-CDK6 group (CDDP-R cells transfected with si-CDK6 plasmid).

Cell Counting Kit-8 (CCK-8) assay

Cells in different groups were inoculated on 96-well plates (NU679, Dojindo, Japan) with 1×10^4 cells/well density, and 100 µl per well. After adherent culture, 10 µl/well of CCK-8 was added to each well for the corresponding time as described above. The CCK-8 solution was prepared in a complete medium. The Optical Density (OD) value of the absorbance of all samples at 450 nm was measured with a microplate reader (MB-530, China) after incubation at 37°C and 5% CO₂ for 4 h. Cell viability = (OD_{Drug}-OD_{Blank})/(OD _{Control}-OD_{Blank}) ×100%. The IC50 of cells was calculated by the SPSS18.0 software profit regression model.

Clonogenic assay

The cells in the exponential growth stage were inoculated in a 6-well plate with 200 cells per well and cultured in an incubator at 37° C with 5% CO₂ and 95% humidity. The culture medium was replaced once every 5-7 days. Cell culture was terminated when macroscopic clones were observed. Then, the culture medium was discarded and the cells were carefully soaked in PBS twice. 1 ml of 4% paraformaldehyde was added to each well to fix the cells for 15 min. The fixation solution was removed and 1 ml of dye solution was added for dyeing at room temperature for 30 min. After decolorization, the OD value at 550 nm was determined by a microplate reader.

Quantitative real-time PCR (qRT-PCR)

The total RNA of the HepG2 cells (500 µl) was extracted after 3 min of cell lysis with Trizol. In the next step, the total extracted RNA was reversely transcribed into cDNA in accordance with the instruction of a Reverse transcription kit (CW2569, CWBIO, China). Subsequently, real-time PCR was performed on a Fluorescence quantitative RCP instrument (QuantStudio1, Thermo, USA) with reference to the instructions of the UltraSYBR Mixture kit (CW2601, CWBIO, China). Reaction conditions were: predenaturation at 95°C for 10 min, denaturation at 94°C for 15 s, annealing at 60°C for 30 s and 40 cycles. The internal references of the primer were U6 and β -actin, and the primer sequences are listed in Table 1. The relative transcription level of the target gene was calculated using 2 µg total cDNA as a template by the relative quantitative method $(2^{-\Delta\Delta C})$ method): $\triangle \triangle Ct = \triangle$ experimental group $-\triangle$ Control group, \triangle Ct = Ct(target gene) – Ct(β -actin). The experiment was repeated three times.

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Primer sequences

Gene	Sequences (5'-3')
miR-340-5p	F: GCGGTTATAAAGCAATGAGA
	R: TGTGCGTGTCGTGGAGTCG
CyclinD1	F: ACCTCTTCACCTTATTCATGGCT
	R: GCCTTTCCCGACCCTGCTAC
CyclinD2	F: GTGCTGGGGAAGTTGAAGTG
	R: GATCATCGACGGTGGGTACA
CDK6	F: CCGACTGACACTCGCAGC
	R: TCCTCGAAGCGAAGTCCTCA
CDK6 siRNA1	CCAACGTGGTCAGGTTGTTTGATGT
CDK6 siRNA2	CACAGTGTCACGAACAGACAGAGAA
CDK6 siRNA3	GAGGTCTGGACTTTCTTCATTCACA
β-actin	F: ACAGCCTCAAGATCATCAGC
	R: GGTCATGAGTCCTTCCACGAT
U6	F: GCTTCGGCAGCACATATACTAAAAT
	R: CGCTTCACGAATTTGCGT

Western blot (WB)

The total protein of the HepG2 cells $(1 \times 10^6 \text{ cells})$ each group) was extracted with a RIPA kit (P0013B, Beyotime, China). The protein extracts concentration was determined according to the instructions of the BCA protein quantitative kit. The protein extracts concentration was calculated according to the standard curve. The protein extracts were separated by 10% SDS-PAGE electrophoresis. Proteins extracts were electrostatically transferred to the NC membrane. The membrane was blocked with 5% skim milk for 2 h at room temperature and incubated with the primary antibody overnight at 4°C. For primary antibodies, we used rabbit anti-CDK6 (1:2000, 14052-1-AP, Proteintech, USA), rabbit anti-CyclinD1 (1:200, Orb453523, Biorbyt, UK), rabbit anti-CyclinD2 (1:1000, Orb413965, Biorbyt, UK), and anti- β -actin (1:5000, 66009-1-Ig, Proteintech, USA). The membrane was then incubated with HRP-conjugated Affinipure Goat Anti-Mouse IgG (H+L) (1:5000, SA00001-1, Proteintech, USA) and HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H+L) (1:6000, SA00001-2, Proteintech, USA) for 1 h, respectively. The film was immersed in SuperECL Plus (BW-61, Jiaxin, China) for luminescence development. The β -actin was used as the internal control. Image J was used to analyze the grayscale.

Dual-luciferase reporter assay

293A cells were inoculated in 24-well plates and grew for 10-24 hrs (80% confluence). PHG-miRTarget CDK6-3U plasmid (1 μ g/ μ l) was co-transfected with miRNA NC (NC+CDK6 group) and hsa-miR-340-5p mimics (hsa-miR-340-5p+CDK6 group), respectively, into the 293A cells. 20 μ l of cell lysate was added into each plate, followed by 100 μ l of Luciferase Assay Reagent II (LAR II) working fluid. After mixing, the mixture was put into the luminescent detector to detect the Firefly luminescence value. Then, we added 100 μ l of Stop&Glo detection solution to stop the first light emission and detected the Renilla luciferase value.

Cell cycle assay

Cell suspension was collected and centrifuged at 800 rpm for 5 min to obtain cell precipitation. PBS was washed 2-3 times to prepare a single cell suspension with a cell concentration adjusted to 1×10^6 cells/ml, which was fixed with 70% ethanol. After the residual ethanol was removed, 150 µl of propidium iodide (PI) solution was added and dyed in the dark at 4°C for 30 min. Finally, the cell cycle was measured by flow cytometry (A00-1-1102, Beckman, USA) at 488 nm. The adherent cells and debris were excluded by the gating technique, and the percentage of each cell cycle of the PI fluorescence histogram was analyzed.

Apoptosis assay

The cells were digested and collected by trypsin without EDTA. About 5×10^5 cells were collected after washing with PBS twice and centrifuged at 2000 rpm for 5 min. 500 µl of binding buffer was added to resuspend the cells. 5 µl of annexin V-FITC (KGA108, KeyGen, China) and 5 µl of propidium iodide were added to the cell suspension and mixed. Then, the reaction was kept at room temperature and kept away from light for 15 min. Within 1 hr, flow cytometry (A00-1-1102, Beckman, USA) was utilized to detect cell apoptosis.

TUNEL assay

Cells were inoculated in 24-well plates to make cell slides. The slide was fixed with 4% paraformaldehyde for 30 min and washed with PBS for 5 min x 3 times. TUNEL staining was performed using the Annexin V-EGFP Apoptosis Kit (KGA702, KeyGen, China) according to the manufacturer's instructions. DAPI (Wellbio, China) solution was used to restain the nuclei at 37°C for 10 min, and PBS was used to wash the nuclei for 5 min x 3 times. Cell images were obtained under fluorescence microscope (BA410T, Motic, China) to count TUNEL positive cells.

Statistical analysis

All data were analyzed using Graphpad Prism 8.0 software (GraphPad Software, San Diego, California, USA). The unpaired T test was used between the two groups conforming to the normal distribution. Comparisons among multiple groups were conducted by a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. A value of p<0.05 was considered to be statistically significant.

Results

IC50 value of HepG2 in CDDP

To investigate the drug resistance of liver cancer cell lines with cisplatin, we compared the CDDP resistance of four different HCC cell lines. We found that the drug resistance of HepG2 was significantly lower than other liver cancer cell lines, including SMMC-7721, Huh-7, and Hep3B (Fig. 1A). Then, we constructed CDDP-R HepG2 cells to investigate whether there is a substance to reverse CDDP-resistance. CDDP-R HepG2 cells were selected by gradually increasing the concentration of CDDP in the medium. After 6 months, we calculated the IC50 value of sensitive and resistant CDDP HepG2. Compared with the IC50 value of 5.8 μ M of CDDP-S HepG2 cells as shown in Figure 1B, the IC50 value of CDDP-R cells was significantly increased to 115 µM (Fig. 1C). The results indicated that the CDDP-R HepG2 cells were successfully constructed.

Expression of miR-340-5p and CDK6 in CDDP-S and CDDP-R HepG2 cells

In order to study whether miR-340-5p and CDK6 are related to drug resistance of hepatocellular carcinoma cells, we did qRT-PCR and WB experiments. We first detected the expression of miR-340-5p and CDK6 in CDDP-R HepG2 cells by qRT-PCR. The data showed that compared with CDDP-S HepG2 cells, the expression of miR-340-5p in CDDP-R HepG2 cells was much lower, but the mRNA and protein expression levels of CDK6 increased. Figure 2 shows that miR-340-5p and CDK6 are abnormally expressed in CDDP-R HepG2 cells. This may suggest that the drug resistance of HepG2 cells is related to miR-340-5p and CDK6.



Fig. 1 A-C. Inhibition concentration of HepG2 in CDDP. A – Cell viability of four different HCC cell lines HepG2, Huh-7, Hep3B, and SMMC-7721. Among them, HepG2 cells showed the lowest viability. B – The relative cell viability of CDDP-S HepG2 cells and the IC50 value of CDDP. The IC50 value of CDDP-S HepG2 cells is $5.8 \ \mu$ M. C – Cell viability and the IC50 value of CDDP in CDDP-R HepG2 cells. The IC50 value of CDDP-R HepG2 cells is $115 \ \mu$ M. * – compared with the HepG2 group, p<0.05. # – compared with the 0 μ M group, p<0.05.



Fig. 2. Expression of miR-340-5p and CDK6 in HepG2 cells. A. CDDP-R HepG2 had low expression of miR-340-5p and high expression of CDK6 at the mRNA level. B. CDK6 was highly expressed in CDDP-R HepG2 at the protein level. * – compared with the CDDP-S group, p<0.05.

Overexpression of miR-340-5p could inhibit CDK6 expression in HepG2 cells

Biological information showed that miR-340-5p could specifically target the 3'UTR of CDK6. We verify through experiments whether miR-340-5p could regulate CDK6 expression. We used a dualluciferase report assay and the results showed that miR-340-5p could target CDK6. Figure 3A and 3B suggested that miR-340-5p may affect HepG2 cells by regulating the expression of CDK6. We constructed the CDDP-R HepG2 cell miR-340-5p over-The data expression group. indicated that miR-340-5p is highly expressed in the miR-340-5p mimic group. Figure 3C shows that the miR-340-5p overexpression group was successfully constructed. We further detected the expression of the cyclerelated genes CDK6, CyclinD1, and CyclinD2. The results demonstrated that compared with the Control and mimic-NC groups, the mRNA and protein expression of CDK6, CyclinD1, and CyclinD2 in the miR-340-5p mimic group were suppressed (Fig. 3D and 3E). We finally used flow cytometry to detect the cell cycle. It can be seen from Figure 3F, compared with the Control and mimic-NC group, the miR-340-5p mimic group showed a higher cell cycle staying in the G0/G1 phase, and the S phase cell ratio was significantly reduced. The above results indicated that miR-340-5p could inhibit CDK6 expression, thus affecting the cycle of CDDP-R HepG2 cells.

Upregulation of miR-340-5p could reverse the sensitivity of HepG2 cells to CDDP

In the above results, it was found that miR-340-5p was abnormally expressed in CDDP-R HepG2 cells. We then investigated whether the upregulation of miR-340 had an effect on the CDDP sensitivity of HepG2 cells. First, we observed the proliferation capacity of HepG2 treated in different ways. Figure 4A shows that compared with the Control and mimic-NC groups, the cell proliferation ability of the miR-340-5p mimic group was lower. It suggested that the overexpression of miR-340-5p significantly inhibited the activity of CDDP-R HepG2 cells. We then examined the apoptosis and cloning of the cells. Compared with the Control and mimic-NC groups, the apoptosis rate of the miR-340-5p mimic group increased and cloning was inhibited. Figure 4B and 4C more fully display that CDDP-R HepG2 was less active and more prone to apoptosis after transfection with miR-340-5p overexpression. In brief, a high expression of miR-340-5p could reverse the CDDP sensitivity of HepG2 cells.

Inhibition of CDK6 could reverse the sensitivity of HepG2 cells to CDDP

The above experimental results indicate that CDK6 is abnormal in CDDP-R HepG2 cells. We further investigate whether CDK6 silencing has an effect on the CDDP sensitivity of HepG2 cells. We transfected CDDP-R HepG2 cells with CDK6 silencing plasmid. CDK6 expression was substantially inhibited in the si-CDK6 group. The data in Figure 5A reflected that the transfection of si-CDK6 plasmid was successful. Next, we used CCK-8 to detect cell proliferation in Figure 5B. Compared with the si-NC group, proliferation of CDDP-R HepG2 cells in the si-CDK6 group decreased. The clone number of the si-CDK6 group decreased. The experiment of Figure 5C and 5D confirm that inhibition of CDK6 reduces the activity of CDDP-R HepG2 cells. Figure 5E is the result of the



Fig. 3 A-F. miR-340-5p targets CDK6 expression. A – The TargetScan website showed miR-340-5p had interaction with CDK6. B – The dual-luciferase reporter assay proved that miR-340-5p could target CDK6. Luciferase activity in 293A cells was significantly inhibited by the CDK8-WT + miR-340-5p mimic group. C – miR-340-5p was overexpressed in the miR-340-5p mimic group. D – The mRNA expression of CDK6, CyclinD1, and CyclinD2 in HepG2 was inhibited by miR-340-5p. E – The protein expression of CDK6, CyclinD1, and CyclinD2 was inhibited by miR-340-5p. F – The over-expressed miR-340-5p of HepG2 cells had a higher cell cycle staying in the G0/G1 phase, while the proportion of cells in the S phase was significantly reduced. * – compared with the Control group, p<0.05. # – compared with the mimic-NC group, p<0.05.



Fig. 4 A-C. Overexpression of miR-340-5p reverses CDDP sensitivity in HepG2 cells. A – A CCK8 assay was used to detect cell proliferation. Cell activity was decreased by miR-340-5p interference. B – Annexin V-FITC and PI were used to evaluate the apoptosis rate. The apoptotic rate of CDDP-R HepG2 cells transfected with overexpressing miR-340-5p was increased. C – The Clonogenic assay was used to detect the number of cloned cells. The cloning ability of HepG2 cells was inhibited by miR-340-5p. * – compared with the Control group, p<0.05. # – compared with the mimic-NC group, p<0.05.

flow cytometry cycle test. Compared with si-NC, the ratio of G0/G1 cells in the si-CDK6 group increased, while the ratio of S-phase cells decreased. After inhibiting CDK6, the cells stayed in the G0/G1 phase. Finally, we conducted a TUNEL assay, and the data showed that compared with si-NC, the apoptosis of CDDP-R HepG2 cells in the si-CDK6 group was considerably increased. In short, when CDK6 was inhibited, it could affect the CDDP sensitivity of HepG2 cells by changing the cell cycle.

Discussion

Incidences of liver cancer are increasing year by year. Surgical resection supplemented with chemotherapy is the main treatment for early patients (CHENG *et al.* 2018). Cisplatin chemotherapy works by interfering with cell metabolism and DNA replication (LU *et al.* 2016; OH *et al.* 2014). Since the efficacy of chemotherapy is mainly affected by the natural or acquired drug resistance of tumor cells, the research on the mechanism of cisplatin resistance and



Fig. 5 A-E. Silencing CDK6 could reverse the sensitivity of HepG2 cells to CDDP. A – CDK6 siRNA transfection efficiency. B – A CCK8 assay was used to detect cell proliferation. C – A Clonogenic assay was used to detect the number of cloned cells. HepG2 cell cloning ability was inhibited after CDK6 silencing. D – A TUNEL assay was used to detect the apoptosis rate (×100, Scale bar=100 μ m.). HepG2 cell apoptosis increased after CDK6 silencing. E – Cell cycle was detected by flow cytometry. The ratio of G0/G1 cells in the si-CDK6 group increased, while the ratio of S-phase cells decreased. * – compared with the si-NC group, p<0.05.

the strategy of reversing drug resistance have always been hotspots in the field of cancer research (TIAN *et al.* 2017). miRNAs could directly affect the target gene by recognizing and complementing the 3'UTR end of the target gene, down-regulating the expression of the target gene, thereby exerting its biological function (BA *et al.* 2019; ZHANG *et al.* 2019). This topic mainly explores the expression level of miR-340-5p and the CDDP-R HepG2 cells.

Recent studies have reported that CDK6, a key protein that regulates the cell cycle, could be specifically bound by SUMO1 in the SUMO family, thereby regulating the tumor cell cycle. CDK6 protein is degraded by ubiquitin, which significantly reduced the protein expression level, and then induced tumor cells from rapid proliferation cycle to slow proliferation cycle state (REN et al. 2019). In the bioinformatics prediction of miR-340-5p targeting CDK6 signal to reverse the molecular mechanism of cisplatin resistance in HepG2 cells, we found that miR-340-5p has species conservation. In the 5' UTR of miR-340-5p, 8 bases were completely complementary to the CDK6 mRNA3'UTR, and the free energy between the two was relatively low, so we speculated that CDK6 might be the target gene of miR-340-5p. In this study, by transiently translating si-CDK6 and miR-340-5p mimics, it was found that both CDK6 and miR-340-5p could affect the sensitivity of HepG2 cells to CDDP, and through computer biological information prediction, it was found that CDK6 may be the downstream target of miR-340-5p. In order to verify the results of bioinformatics detection, a luciferase report test found that the expression levels of miR-340-5p and CDK6 were synchronized, that is, the expression level of overexpressed miR-340-5p could inhibit the increase of CDK6 expression level. It further demonstrated that miR-340-5p could directly act on CDK6, that is, by directly targeting the 3'UTR region of CDK6, CDK6 could be inhibited, which results in the reduction of CDDP resistance and the increased chemotherapeutic sensitivity of HepG2 cells.

The cell cycle is divided into different time stages, G1, G0, S, and G2 and M. Cyclin D binds to cyclindependent kinases (CDKs) to the phosphorylate pRb protein, which plays an important role in regulating cell cycle progression (LI et al. 2018). In this experiment, the concentration of CDDP-R HepG2-resistant miR-340-5p was changed by the transfection of the miR-340-5p mimic, and then fluorescent quantitative PCR and western blotting were used to detect the changes in the transcription and translation levels of the CDK6 gene and its downstream genes, CyclinD1 and CyclinD2. After the overexpression of miR-340-5p in CDDP-R HepG2 cells, the mRNA and protein expressions of CDK6 and its downstream genes, CyclinD1 and CyclinD2, were effectively inhibited. The above studies fully show that miR-340-5p has a certain targeting relationship with CDK6, and miR-340-5p

could target the CDK6 signal transduction pathway to partially reverse the occurrence of cisplatin resistance in HepG2 cells. Since this research is still in the basic research stage, there are still a lot of problems waiting to be overcome before clinical application. Under the effect of CDDP, the apoptosis of hepatoma cells increased and proliferation ability decreased. In future work, we will clarify the expression characteristics and laws of CDK6 and CyclinD1 in HepG2, and explore the mechanism of CDK6 protein action and its role in the malignant transformation and radiotherapy resistance of liver cancer, so as to provide new methods and ideas for the future treatment of liver cancer.

In conclusion, miR-340-5p was significantly down-regulated in CDDP-R HepG2 cells. Overexpression of miR-340-5p leads to enhancing CDDP sensitivity. CDK6 is the direct target of miR-340-5p, and high expression of CDK6 improves CDDP sensitivity. Overall, our results indicated that miR-340-5p played a critical role in regulating CDDP chemosensitivity by targeting CDK6 in HepG2 cells. In this study, we discussed the expression pattern and biological effects of miR-340-5p in the CDDP chemosensitivity of HepG2, which may provide a new perspective for the treatment of liver cancer.

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Author Contributions

Research concept and design: S.T., X.T.; Collection and/or assembly of data: S.T., X.T.; Data analysis and interpretation: Zh.L., Q.Z., R.W., X.H.; Writing the article: Zh.L., Q.Z., R.W., X.H., Zi.L.; Critical revision of the article: Zi.L., Z.Z; Final approval of article: Zi.L., Z.Z.

Conflict of Interest

The authors declare no conflict of interest.

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