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Original Article

Regulation of miR-142-3p on the sensitivity of neuroblastoma cells to CDDP by targeting HMGB1

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Abstract: Objectives: This study aims to investigate the influenceand underlying mechanism of miR-142-3p on the sensitivity of neuroblastoma cells to cis-diamine dichloroplatinum (CDDP). Methods: The CDDP-resistance neuroblastoma cell line SH-SY5Y/CDDP was constructed, and the expression level of miR-142-3p was detected by RT-PCR. The apoptosis level and the IC_{50} value of SH-SY5Y/CDDP were quantified by CCK8 and Elisa. The 3'UTR of HMGB1 was cloned into the luciferase reporter vector to verify that miR-142-3p could target HMGB1. Results: The IC_{50} value of SH-SY5Y/CDDP increased from 4.57±0.65 μg/mL to 23.53±3.87 μg/mL, and it was 5.15 times higher than that of the parental cell line SH-SY5Y. The expression level of miR-142-3p in the SH-SY5Y/CDDP cells was remarkably lower than that in the SH-SY5Y cells. The differences were statistically significant (P<0.01). miR-142-3p mimics significantly increased cell apoptosis, reduced HMGB1 expression and IC_{50} (7.02±1.38 vs 24.27±4.13 μg/mL) to CDDPinSH-SY5Y/CDDP cells. In addition, miR-142-3p effectively inhibited the enzymatic activity of the luciferase driven by HMGB1 3'-UTR (P<0.01). In SH-SY5Y/CDDP cells after the knockdown of HMGB1 by siRNA, the apoptosis level was increased and the IC_{50} value of CDDP was significantly decreased. Conclusions: miR-142-3p can increase the sensitivity of neuroblastoma cells to CDDP by targeting HMGB1.

Keywords: Neuroblastoma, pharmacotherapy, combination, gene expression regulation

Introduction

Neuroblastoma (NB) is the most common malignancy among children with a high malignancy degree and early metastasis, seriously threatening patients' life [1]. The survival rate is only 40% with excision, radiotherapy, chemotherapy, etc. Many children are clinically insensitive to chemotherapy, leading to unsatisfying results despite its significant role in the treatment of neuroblastoma [2]. Studies show that the expression of miR-142-3p is down-regulated in multiple tumors [3-5], and its expression level closely correlates with tumor chemosensitivity [6]. As a potential target gene of miR-142-3p, the high mobility group box-1 protein (HMGB1) displays a high expression level in neuroblastoma, and closely correlates to proliferation and metastasis of neuroblastoma [7]. In this research, the role of miR-142-3p in the regulation of neuroblastomachemosensitivity was primarily studied, and the molecular mechanism of its regulation on neuroblastomachemosensitivity via targeting HMGB1 was also investigated.

Materials and methods

Materials

Neuroblastoma cell line SH-SY5Y (cell bank of Chinese Academy of Sciences, Shanghai, China); RPMI1640 medium, fetal bovine serum (FBS), SYBR, Opti-MEM (Gibco, USA); CPPD (Hansoh Pharmaceutical, Jiangsu, China); ECL system (Syngene, UK); siRNA of HMGB1 (QIAGEN, Germany); antibody of HMGB1 (Abcam, UK); miR-142-3p mimics (GenePharma, Shanghai, China); Trizol, RT-PCR kit, PCR primers, LipofectamineRNAi MAX (Invitrogen, USA); X-tremeGENE (Roche, USA); Dual Luciferase Reporter Gene Assay Kit (Promega, USA); CCK8 (DOJINDO, Japan); Apoptosis Assay Elisa Kit (Roche, USA)

Methods

Culture of neuroblastoma cells and construction of SH-SY5Y/CDDP: The neuroblastoma cells (SH-SY5Y) were cultured with RPMI1640 medium containing 10% FBS, 100 U/mL peni-

cillin and 100 U/mL streptomycin, and incubation was performed at 37°C in 5% CO_2. IC_{50} of SH-SY5Y was detected. The cells were cultured originally with 0.5 μg/mL CDDP for 48 h. Then, the concentration of CDDP was raised to 1, 2, 4, 6, 8, 10 μg/mL, and the incubation was maintained with 10 μg/mL CDDP. Then the CDDP-resistance neuroblastoma cell line SH-SY5Y/CDDP was initially obtained.

Detection of miR-142-3p expression with RT-PCR: The total RNA of SY5Y/CDDP was extracted with Trizol and the cDNA of miR-142-3p was synthesized with the RT-PCR primer: 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAA-3'. Real time-PCR was performed with the template cDNA. The upstream primer: 5'-GGGTGTAGTGTTTCCTAC-3'; the downstream primer: 5'-CAGTGCGTGTCGTGGAG-3'. Conditions: 95°C for 3 min, 95°C for 10 s, 60°C for 30 s, 72°C for 30 s, 40 circles, with U6 as the internal reference. The expression of miR-142-3p was detected in the SH-SY5Y/CDDP cell line.

Transfection: The SH-SY5Y/CDDP cells were cultured with RPMI1640 medium containing 10 μg/mL CDDP, 100 U/mL penicillin, 100 U/mL streptomycin and 10% FBS, and incubation was performed at 37°C in 5% CO_2. The cells at the logarithmic phase were digested and inoculated on a six-well plate (0.7×10^6/well). Using LipofectamineRNAi MAX, the SH-SY5Y/CDDP cells were transfected with miRNA con (control vector) and miR-142-3p mimics (miR-142-3p overexpression vector), respectively. The sequence of miR-142-3p mimics: 5'-UGUAGUGUUUCCUACUUUAUGGA-3'. Three repetitions were set for each group.

Construction of the luciferase reporter vector: The 3'UTR of HMGB1 that may interact with miR-142-3p was figured out by Targetscan. Primer5.0 was employed to design the PCR primers for the 3'UTR of wild-type HMGB1 and mutant HMGB1: 5'-UUUUGUAUAGUUAACACACUACC-3' HMGB1 wt 3'UTR; 3'-AGGUAUUUCAUCCUUUGUGAUGU-5' has-miR-142-3p; 5'-UUUUGUAUCAUUAACAGUGUUGU-3' HMGB1 mut 3'UTR. The cDNA of the SH-SY5Y/CDDP cells was used as the template for PCR amplification. The PCR products were purified and recovered, and then inserted into the luciferase reporter vector pMIR-REPORT. The sequencing result verified the successful construction of the wild-type and the mutant luciferase reporter vector: wt-HMGB1-pMIR-REPORT and mut-HMGB1-pMIR-REPORT.

Detection of luciferase activity: Using X-treme GENE, the SH-SY5Y cells were co-transfected with wt-HMGB1-pMIR-REPORT and miR-142-3p mimics or mut-HMGB1-pMIR-REPORT and miR-142-3p mimics, with miRNA con mimic as the control. The cells were lysed 48 h after transfection, and the luciferase activity was detected with Dual Luciferase Reporter Gene Assay Kit. Three repetitions were set for each group.

Detection of IC_{50} with CCK8: The SH-SY5Y/CDDP cells were divided into three groups: the blank group, the miRNA con group and the miR-142-3p mimics group. The cells at the logarithmic phase were digested and inoculated on a 96-well plate (5×10^4/well). Using Lipofectamine 2000, the SH-SY5Y/CDDP cells were transfected with miRNA con and miR-142-3p mimics, respectively. After 24 h, the absorption values at 450 nm (A450) were measured and the values of IC_{50} were calculated. Three repetitions were set for each group.

Detection of apoptosis with Elisa: The cells were treated in the same way and then treated with 15 μg/mL CDDP 24 h after transfection. Apoptosis Assay Elisa Kit was used according to the instructions and the absorption values at 405 nm (A405) were measured. The content of nucleosome fragments in cytoplasm was measured to analyze apoptosis. Three repetitions were set for each group.

Western blot: The cells were collected and lysed. Then the total protein was extracted and separated with SDS-PAGE, and then transferred to the PVDF membrane at 300 mA. After blocking with 5% skim milk for 2 h, the first antibody was added and incubated at 4°C overnight. After the membrane was washed with TBST buffer, the second antibody was added and incubated at room temperature for 2 h. The membrane was washed with TBST buffer again. Then electrochemiluminescence (ECL) reagent was added for development, and the gel imaging system was applied for imaging.

Statistics

The data were statistically processed with SPSS 12.0 and the results were expressed as $\bar{x}\pm s$. The intergroup comparison was conduct-

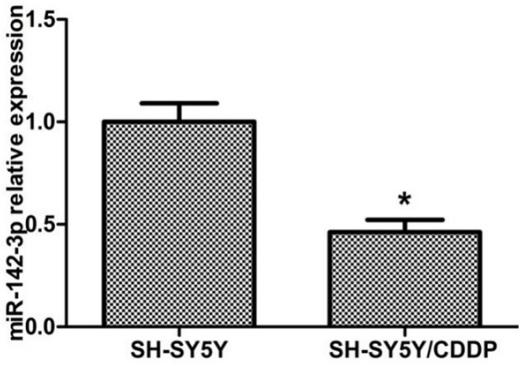

Figure 1. The expression level of miR-142-3p in SH-SY5Y and SH-SY5Y/CDDP cells were determined by RT-PCR (*P<0.01).

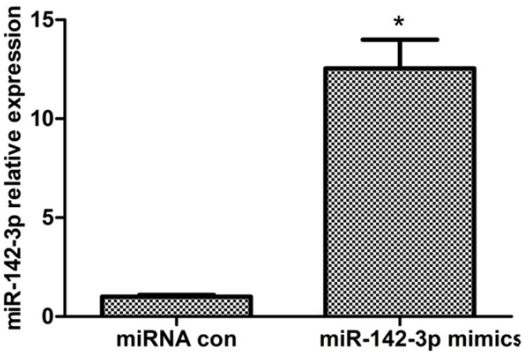

Figure 2. The expression level of miR-142-3p in SH-SY5Y/CDDP cell before and after transfection of miR-142-3p were determined by RT-PCR (*P<0.01).

ed with t-test. P<0.05 indicated statistical significance.

Results

Construction of the CDDP-resistance neuroblastoma cell line SH-SY5Y/CDDP

After the CDDP-resistance neuroblastoma cell line SH-SY5Y/CDDP was stably constructed, the expression level of miR-142-3p was firstly detected. The RT-PCR results are shown in **Figure 1**. Compared with normal SH-SY5Y cells, the expression level of miR-142-3p in the SH-SY5Y/CDDP cells was significantly lower (P<0.01). Then, by means of CCK8, the IC_{50} value of SH-SY5Y/CDDP was found to increase from (4.57±0.65) to (23.53±3.87) μg/mL, and it was 5.15 times higher than that of the parental cell line SH-SY5Y, with significant difference (P<0.05).

Impact of miR-142-3p overexpression on the sensitivity of SH-SY5Y/CDDP cells to CDDP

The successfully prepared SH-SY5Y/CDDP cells were transfected with miR-142-3p and the transfection result is shown in **Figure 2**. Then the IC_{50} values of CDDP were measured with CCK8: the intergroup difference of IC_{50} between the miRNA con mimic group and the blank control group (24.27±4.13 vs 23.53±3.87) μg/mL was not statistically significant, while the miR-142-3p mimics group showed a significantly lower IC_{50} than the miRNA con mimic group (7.02±1.38 vs 24.27±4.13) μg/mL (P<0.05). The drug resistance index of the SH-SY5Y/CDDP cells transfected with miR-142-3p mimics remarkably decreased, indicating a possible relationship between the expression of miR-142-3p and the resistance of neuroblastoma cells to CDDP (**Table 1**).

Impact of miR-142-3p overexpression on apoptosis

The Elisa result is shown in **Figure 3**. Among the SH-SY5Y/CDDP cells without the treatment of CDDP, the cells transfected with miR-142-3p mimics showed a higher apoptosis level compared with those transfected with miRNA con; among the SH-SY5Y/CDDP cells treated with 15 μg/mL CDDP, the cells transfected with miR-142-3p mimics showed a much higher apoptosis level compared with those transfected with miRNA con, with significant difference (P<0.05), which indicated that miR-142-3p overexpression not only promoted apoptosis of the SH-SY5Y/CDDP cells but also increased cellular sensitivity to CDDP.

Targeting regulation of miR-142-3pon HMGB1

Investigation of the targeting effect of miR-142-3p on HMGB1 via detection of luciferase activity: The targeting sequence of miR-142-3p on HMGB1 was figured out by Targetscan. The SH-SY5Y cells were co-transfected with wt-HMGB1-pMIR-REPORT and miR-142-3p mimics or mut-HMGB1-pMIR-REPORT and miR-142-3p mimics, and then luciferase activity was detected. The result is shown in **Figure 4**. The luciferase activity of the reporter vector of wild-type HMGB1 3'UTR was down-regulated by miR-142-3p, indicating the targeting effect of miR-142-3p on HMGB1, while the luciferase activity of

Table 1. IC_{50} value of CDDP of SH-SY5Y cell (x±s)

Groups	IC_{50} (ug/ml)
SH-SY5Y	4.57±0.65
SH-SY5Y/CDDP	23.53±3.87*
SH-SY5Y/CDDP miRNA con mimic	24.27±4.13
SH-SY5Y/CDDP miR-142-3p mimic	7.02±1.38#

*P<0.05, compared with sh-sy5y group; #P<0.05, compared with sh-sy5y/CDDP group.

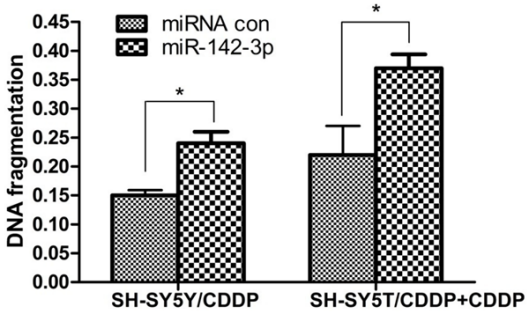

Figure 3. The effects of miR-142-3p on the apoptosis level of SH-SY5Y/CDDP (*P<0.05).

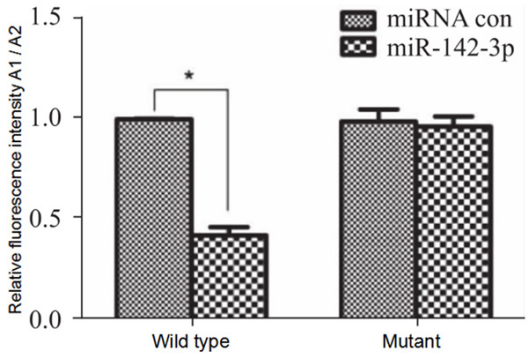

Figure 4. MiR-142-3p can inhibit the enzymatic activity of luciferase reporter vector of HMGB1.

the reporter vector of mutant HMGB1 3'UTR was not obviously decreased. This result suggested the complementary combination between miR-142-3p and HMGB1 3'UTR.

Negative regulation of miR-142-3p on HMGB1 expression in neuroblastoma cells: To further verify this targeting effect, the SH-SY5Y cells were transfected with miR-142-3p mimics (see **Figure 5**), and the expression of HMGB1 was found to be remarkably down-regulated, revealed by Western blot (P<0.05) (see **Figure 6**).

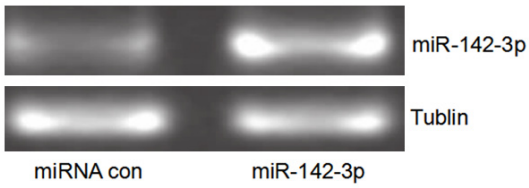

Figure 5. The expression level of miR-142-3p in SH-SY5Y cell after transfection of miR-142-3p.

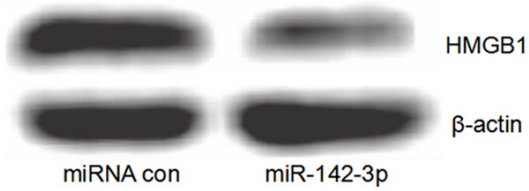

Figure 6. The expression level of HMGB1 protein in SH-SY5Y after transfection of miR-142-3p.

Figure 7. siRNA knockdown results.

Impact of HMGB1 knockdown on the apoptosis of SH-SY5Y/CDDP cells

To investigate the effect of HMGB1 on the sensitivity of SH-SY5Y/CDDP to CDDP, the impact of HMGB1 knockdown on apoptosis after treatment with CDDP was firstly studied. The knockdown result is shown in **Figure 7**. The Elisa result showed that after treatment with CDDP, nucleosome fragments in the cells with HMGB1 knockdown significantly increased compared with the controls, consistent with the impact of miR-142-3p overexpression on apoptosis. The result indicated that the knockdown of HMGB1 could increase the sensitivity of SH-SY5Y/CDDP cells to CDDP (**Figure 8**).

Impact of HMGB1 on the CDDP-resistance of SH-SY5Y/CDDP cells

To further corroborate that the knockdown of HMGB1 could increase the sensitivity of SH-SY5Y/CDDP cells to CDDP, the change of IC_{50} of CDDP was investigated in the cells after knockdown. The result showed that the IC_{50} value of CDDP decreased from (24.61±4.07) to

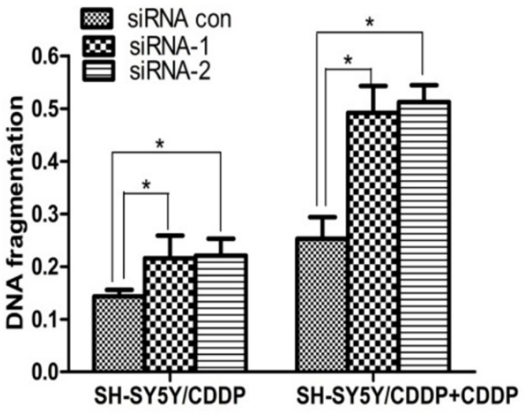

Figure 8. The effects of knockdown of HMGB1 on apoptosis level of SH-SY5Y/CDDP (*P<0.05).

Table 2. The IC_{50} value of SH-SY5Y cell after knockdown of HMGB1 (x±s)

Groups	IC_{50} (ug/m)
SH-SY5Y/CDDP siRNA con	24.61±4.07
SH-SY5Y/CDDP siRNA-1	9.65±1.39*
SH-SY5Y/CDDP siRNA-2	8.43±1.76*

*P<0.05, compared with sh-sy5y/CDDP group.

(9.65±1.39) and (8.43±1.76) μg/mL after knockdown with significant differences (P<0.05), which indicated that the sensitivity of SH-SY5Y/CDDP cells to CDDP remarkably decreased after the knockdown of HMGB1 (**Table 2**).

Discussion

miRNA is a group of non-coding double-stranded RNA synthesized in vivo, which regulates gene expression on a post-transcriptional level via binding the target mRNA 3'UTR complementarily. Now researchers have found that miRNA can affect cellular proliferation, differentiation, infiltration, migration, etc., playing an extremely important role in tumor occurrence and development. Multiple miRNAs engage in the proliferation, invasion and metastasis of neuroblastoma, such as miR-15a, miR-338-3p, etc. [8-10]. As tumor molecular biological research continues, more miRNAs have been found to participate in tumor occurrence, development, invasion and metastasis as well as regulation of the sensitivity to radiotherapy and chemotherapy. Studies show that miR-18-1b overexpression can enhance the sensitivity of glioma cells to VM-26, and miR-21 can enhance the sensitivity of glioblastoma to VM-26 via targeting LRRFIP1 [11, 12]. As one of the mature forms of miR-142, miR-142-3p engages in the regulation of tumor development and its expression is down-regulated in multiple tumors. For example, miR-142-3p overexpression can inhibit clone formation as well as invasion and metastasis of hepatoma cells [13]; miR-142-3p overexpression can inhibit cellular proliferation and induce apoptosis in pancreatic ductal adenocarcinoma [14]. Researches have indicated that HMGB1 closely relates to tumor occurrence, development, metastasis and invasion, which is regarded as a new anti-cancer target [15, 16]. As a potential target gene of miR-142-3p, HMGB1 closely relates to tumor occurrence, development, metastasis and invasion. A high expression level of HMGB1 in neuroblastoma has been indicated; however, the relationship between miR-142-3p and neuroblastoma has not been reported by far. In this study, the effect of miR-142-3p on the sensitivity of neuroblastoma cells to CDDP and the molecular mechanism were investigated.

To investigate the regulation of miR-142-3p on the sensitivity of neuroblastoma cells to CDDP, the CDDP-resistance neuroblastoma cell line SH-SY5Y/CDDP was constructed and then the effect of miR-142-3p on drug resistance and apoptosis was detected. The result showed that compared with the normal SH-SY5Y cells, the expression of miR-142-3p was remarkably down-regulated in the SH-SY5Y/CDDP cells; in the SH-SY5Y/CDDP cells transfected with miR-142-3p mimics, IC_{50} significantly decreased and apoptosis was promoted, which indicated that miR-142-3p could increase the sensitivity of CDDP-resistance neuroblastoma cells to CDDP.

The mechanism of miR-142-3p regulating the sensitivity of CDDP-resistance neuroblastoma cells to CDDP was further investigated. HMGB1 was found to be a potential target gene of miR-142-3p by Targetscan. The direct interaction between miR-142-3p and HMGB1 was corroborated with the luciferase reporter vector firstly. Then, the effect of miR-142-3p overexpression on HMGB1 expression was detected, and the results verified the direct effect of miR-142-3p on HMGB1 and the negative regulation of miR-142-3p on HMGB1 expression. The above results altogether corroborated the targeting

effect of miR-142-3p on HMGB1. Finally, the regulation of HMGB1 on the SH-SY5Y/CDDP cells to CDDP was further verified by siRNA knockdown, and the result showed that in the SH-SY5Y/CDDP cells with the knockdown of HMGB1, the IC_{50} value significantly decreased and apoptosis was significantly promoted, which indicated that the down-regulation of HMGB1 could increase the sensitivity of the SH-SY5Y/CDDP cells to CDDP.

In conclusion, it is verified in this study that miR-142-3p can increase the sensitivity of neuroblastoma SH-SY5Y cells to CDDP via its negative regulation on HMGB1 expression. Whether miR-142-3p can display the same effect on the overall level remains to be further studied. This study contributes new perspectives and reveals a new target for the treatment of neuroblastoma on the miRNA level.

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Disclosure of conflict of interest

None.

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