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RESEARCH

miR-135a/b Modulate Cisplatin Resistance of Human Lung Cancer Cell Line by Targeting MCL1

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Abstract microRNAs (miRNAs) are short non-coding RNA molecules, which post-transcriptionally regulate genes expression and play crucial roles in diverse biological processes, such as development, differentiation, apoptosis, and proliferation. Here, we investigated the possible role of miRNAs in the development of drug resistance in human lung cancer cell line. We found that miR-135a/b were downregulated while MCL1 was upregulated in A549/CDDP (cisplatin) cells, compared with the parental A549 cells. In vitro drug sensitivity assay demonstrated that overexpression of miR-135a/b sensitized A549/CDDP cells to cisplatin. The luciferase activity of MCL1 3′-untranslated region-based reporter constructed in A549/CDDP cells suggested that MCL1 was the direct target gene of miR-135a/b. Enforced miR-135a/b expression reduced MCL1 protein level and sensitized A549/CDDP cells to CDDP-induced apoptosis. Taken together, our findings first suggested that hsa-miR-135a/b could play a role in the development of CDDP resistance in lung cancer cell line at least in part by modulation of apoptosis via targeting MCL1.

Keywords miR-135a/b · Cisplatin resistance · Apoptosis · MCL1 · Lung cancer

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Introduction

Worldwide, lung cancer is the most common cancer in terms of both incidence and mortality. It is estimated that there are 1.61 million new cases and 1.38 million deaths annually due to lung cancer [1]. Despite recent advances in diagnostic methods, surgical technique, radiotherapy, chemotherapy and targeting therapy, prognosis is still poor, mainly due to the occurrence of acquired drug resistance. Accumulating studies indicate that there are several major mechanisms of drug resistance in cancer cells, such as increased detoxification of anticancer drugs by glutathione system, defective apoptosis pathway, increased levels of DNA repair or DNA tolerance, decreased uptake of water-soluble drugs and enhanced drug efflux from cancer cells mediated by ATP-binding cassette (ABC) transporters [2–5]. In addition, recent studies have shown that cancer stem cells and epithelial–mesenchymal transition-type cells could play critical roles in drug resistance [6–9]. Finally, researchers have demonstrated that microRNAs (miRNAs) were involved in the regulation of drug resistance [10].

MicroRNAs (miRNAs) are a group of non-coding, single-stranded RNAs that are approximately 22 nucleotides in length. These small RNAs modulate protein expression post-transcriptionally by interacting with complementary sites within the 3′UTR of a target mRNA [11]. miRNAs are implicated in a variety of cellular processes, including

cell differentiation, proliferation, migration, metabolism, and apoptosis [12–14]. miRNAs are recognized as key players in carcinogenesis by acting as oncogene or tumor suppressor gene. Emerging evidences have shown that knock-down or re-expression of specific miRNAs by synthetic anti-sense oligonucleotides or miRNAs precursors or mimics could modulate drug resistance. For instance, miR-200c expression was observed to be down-regulated in human breast cancer cells resistant to doxorubicin MCF-7/ADR. Up-regulation of miR-200c with transfection of miR-200c mimics in breast cancer cells could enhance the chemosensitivity to epirubicin [15]. Elevated levels of miR-1915 in the mimics-transfected HCT116/L-OHP cells sensitized these cells to anticancer drugs [16]. The miR-181a was downregulated in the K562/A02 cells, and the over-expression of miR-181a could sensitize K562/A02 cells to Daunorubicin by targeting BCL2 [17]. On the other hand, miR-21 was upregulated in glioblastoma cells, and miRNA-21 inhibitor could sensitize human glioblastoma U251 Stem Cells to chemotherapeutic drug Temozolomide [18]. Knockdown of miR-203 upregulated SOCS3 expression and enhanced cisplatin (CDDP) chemosensitivity in breast cancer cells [19]. miR-93 was significantly upregulated in CDDP-resistant ovarian cancer cells, and downregulating miR-93 sensitized cells to undergo apoptosis [20]. Collectively, these reports suggested a role of miRNAs in drug resistance.

However, it remained unknown whether miR-135a/b were involved in the drug resistance of lung cancer. In this study, we reported that miR-135a/b were downregulated in human lung cancer cell line A549/CDDP compared with the parental A549 cell line. We first demonstrated that miR-135a/b might play a role in the development of drug resistance in human lung cancer cell line by targeting the antiapoptotic MCL1 partly.

Materials and Methods

Cell Culture

Human lung cancer cell line A549 (http://a549.com/) and its drug-resistant variant A549/CDDP (both obtained from Biosis Biotechnology Company, Shanghai, China) were all cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum (Gibco BRL, Grand Island, NY) in a humidified atmosphere containing 5 % CO_2 at 37 °C. To maintain the drug-resistant phenotype, CDDP (with final concentration of 4 μg/ml) was added to the culture media for A549/CDDP cells.

Quantitative Real-Time PCR Analysis for miRNA

Total RNA from A549 and A549/CDDP cell lines was isolated with Trizol reagent (Invitrogen, Carlsbad, CA), and miRNA fraction was further purified using a mirVana™ miRNA isolation kit (Ambion, Austin, TX). The concentration and purity of the RNA samples were determined spectroscopically. Expression of mature miRNA was assayed using stem-loop RT followed by real-time PCR analysis [21]. The SYBR and U6 gene were used for detecting the gene amplification and normalizing the each sample, respectively. EzOmics™ miRNA qPCR Detection

Primer Set (Catalog No.BK1010) and EzOmics™ One-Step qPCR Kit (Cat No. BK2100), which were purchased from Biomics Biotechnologies Co., Ltd (Nantong, China), were used for quantitative real-time PCR analysis for miR-135a/b and U6 snRNA, respectively. The fold change for miRNA from A549/CDDP cells relative to control A549 cells was calculated using the $2^{-\Delta\Delta Ct}$ Method [22], where $\Delta\Delta Ct = \Delta Ct$ A549/CDDP-ΔCt A549 and $\Delta Ct = Ct$ miRNA−Ct U6 snRNA. PCR was performed in triplicate.

In Vitro Drug Sensitivity Assay

A549/CDDP and A549 cells were plated in 6-well plates (6×10^5 cells/well), 100 nM of the miR-135a/b mimics or 100 nM miRNA mimic control was transfected in A549/CDDP cells, while 100 nM of the miR-135a/b inhibitors or 100 nM miRNA inhibitor control was transfected in A549 cells, using lipofectamine 2000 (Invitrogen, Long Island, NY, USA) according to the manufacturer's protocol, respectively. The miR-135a/b mimics, miRNA mimic control, 2′-O-methyl (2′-O-Me) modified miR-135a/b inhibitors, and miRNA inhibitor control were chemically synthesized by Shanghai GenePharma Company (Shanghai, China). The sequence of each was shown in Online Resource 1.

Twenty-four hours after, transfection cells were seeded into 96-well plates (5×10^3 cells/well) for next step experiment. After cellular adhesion, freshly prepared anticancer drug CDDP was added with the final concentration being 0.01, 0.1, 1, and 10 times of the human peak plasma concentration as previously described [21]. The peak serum concentration is 2.0 μg/ml for CDDP [23, 24]. Forty-eight hours after the addition of the drug, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The absorbance at 490 nm (A490) of each well was read on a spectrophotometer. The concentration at which the drug produced 50 % inhibition of growth (IC50) was estimated by the relative survival curve. Three independent experiments were performed in quadruplicate.

Dual Luciferase Activity Assay

The 3′UTR of human MCL1 cDNA containing the putative target site for the miR-135a/b (sequence shown in Online Resource 2) was chemically synthesized and inserted at the XbaI site, immediately downstream of the luciferase gene in the pGL3-control vector (Promega, Madison, WI) by

Biomics Biotechnologies Co., Ltd (Nantong, China). Twenty-four hours before transfection, cells were plated at 1.5×10^5 cells/well in 24-well plates. Two hundred nanograms of pGL3-MCL1-3′-UTR plus 80 ng pRL-TK (Promega) was transfected in combination with 60 pmol of the miR-135a/b mimics or miRNA mimic control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol as described [21]. Luciferase activity was measured 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. Three independent experiments were performed in triplicate.

Western Blot Analysis

A549/CDDP cells were plated in 6-well plates (6×10^5 cells/well). Seventy-two hours after the transfection of miR-135a/b mimics or miRNA mimic control, cells were harvested and homogenized with lysis buffer. Total protein was separated by denaturing 10 % SDS–polyacrylamide gel electrophoresis. Western blot analysis was performed as described [25]. The primary antibodies for MCL1 and β-tubulin were purchased from Cell signaling Technology and Bioworld Technology, respectively. Protein levels were normalized to β-tubulin. Fold changes were determined.

Apoptosis Assay

Cells were plated in 6-well plates (6×10^5 cells/well). Twenty-four hours after the transfection of miRNA mimics as described above, A549/CDDP cells were treated by CDDP, with final concentration of 5 and 20 μg/ml, respectively. Forty-eight hours after the treatment of CDDP, flow cytometry was used to detect apoptosis of the transfected A549/CDDP cells by determining the relative amount of AnnexinV-FITC-positive-PI-negative cells as previously described [25].

Statistical Analysis

Each experiment was repeated at least 3 times. Numerical data were presented as mean ± SD. The difference between means was analyzed with Student's *t* test. All statistical analyses were performed using SPSS11.0 software (Chicago, IL). Differences were considered significant when $P<0.01$.

Results

miR-135a/b were Downregulated in A549/CDDP Cells Compared with A549 Cells

Quantitative real-time PCR for miR-135a/b verified that miR-135a/b were significantly downregulated in A549/CDDP cells compared with parental cells and the decreased fold changes were 1.66±0.06 and 5.56±0.09, respectively (Fig. 1a, Fig. 1b).

miR-135a/b Regulated CDDP Resistance of Human Lung Cancer Cell Line

In A549/CDDP cells, MTT assay revealed that those transfected with miR-135a/b mimics exhibited greatly decreased resistance to CDDP compared with the miRNA mimic control transfected cells (Fig. 2a), while in A549 cells, those transfected with miR-135a/b inhibitors exhibited greatly enhanced resistance to CDDP compared with the miRNA inhibitor control–transfected cells (Fig. 2b).

These results suggested that miR-135a/b might regulate CDDP resistance of human lung cancer cell line.

The Anti-Apoptotic MCL1 was the Target Gene of miR-135a/b

TargetScanHuman 5.1 (http://www.targetscan.org) predicted that MCL1 was the target gene of the miR-135a/b conservely between different species (Online Resource 3). To explore whether MCL1 was the target gene of the miR-135a/b, we constructed a luciferase reporter vector with the putative MCL1 3′UTR target site for the miR-135a/b downstream of the luciferase gene (pGL3-MCL1-3′-UTR). Luciferase reporter vector together with the miR-135a/b mimics or the miRNA mimic control was transfected into A549/CDDP cells. In this cell line, a significant decrease in relative luciferase activity was noted when pGL3-MCL1-3′-UTR was cotransfected with the miR-135a/b mimics but not with the miRNA mimic control, suggesting that MCL1 was the target gene of the miR-135a/b (Fig. 3).

miR-135a/b Modulated CDDP Resistance by Repressing MCL1 Protein Expression

One attraction was that the decreased expression of miR-135a/b in A549/CDDP cells was concurrent with the overexpression of MCL1 protein, compared with the parental A549 cells in our study (Fig. 4a). Since the antiapoptotic MCL1 protein was the target of the miR-135a/b, we hypothesized that the miR-135a/b might modulate drug resistance of cancer cells by repressing the MCL1 protein expression. To ascertain our hypothesis, we transfected the miR-135a/b mimics and the control miRNA mimic into A549/CDDP cells to detect the MCL1 expression level changes. In A549/CDDP cells, 72 h after the transfection, Western Blot demonstrated significantly decreased MCL1 protein level in miR-135a/b mimics transfected cells compared with the miRNA mimic control transfected cells (Fig. 4b).

These results showed that miR-135a/b might modulate CDDP resistance of cancer cells at least in part by repressing the MCL1 protein expression.

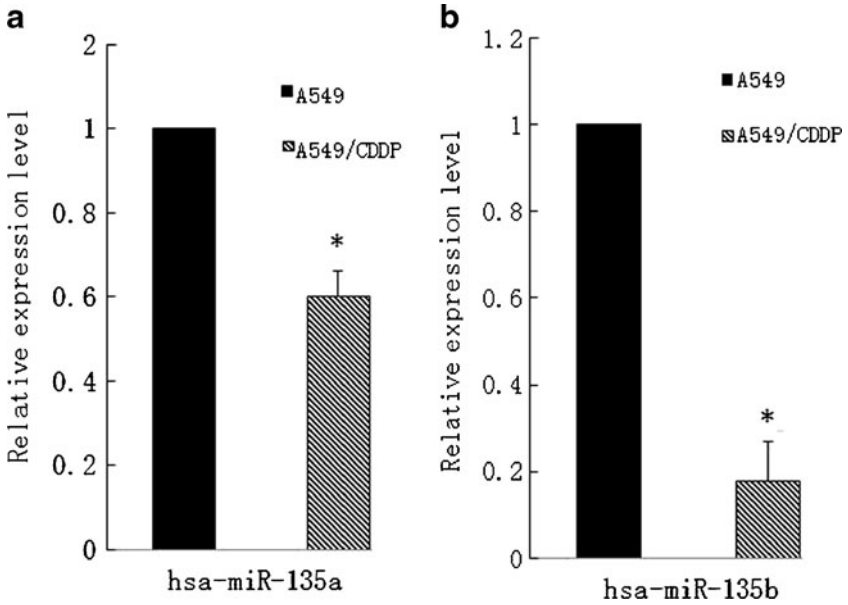

Fig. 1 Real-time quantification of miR-135a/b by stem-loop RT-PCR showed that miR-135a/b were downregulated in A549/CDDP cell line compared with A549 cell line. Triplicate assays were performed for each RNA sample, and the relative amount of miR-135a/b was normalized to U6 snRNA. Data was shown as fold changes of miR-135a/b levels in A549/CDDP cell line relative to A549 cell line, which was set as 1 (mean ± SD). * $P<0.01$

miR-135a/b Sensitized A549/CDDP Cells to CDDP-Induced Apoptosis

The development of drug resistance in various cancer cells has been linked to a reduced susceptibility to drug-induced apoptosis, which was shown to be a consequence of overexpression of antiapoptotic proteins, such as BCL2, IAPs, and BCL-XL. Since the miR-135a/b might regulate drug resistance of cancer cells at least in part by repressing the MCL1 protein expression, considering the well-characterized role of MCL1 in apoptosis and drug resistance, we suggested a hypothesis that miR-135a/b might play a role in the development of drug resistance at least in part by modulation of apoptosis by targeting MCL1. To confirm this hypothesis, we evaluated CDDP-induced apoptosis after transfection with A549/CDDP cells with the miR-135a/b mimics and the miRNA mimic control. In A549/CDDP cells, a marked increase in apoptosis, as assessed by flow cytometry, was observed in the miR-135a/b mimics transfected cells after CDDP treatment, compared with the miRNA mimic control transfected cells (Fig. 5).

Discussion

CDDP has been used clinically for nearly 30 years as part of the treatment of many different tumor types, including lung cancer. However, treatment with this agent was characterized by resistance, both acquired and intrinsic. Recently, according to the increased number of findings

miRNAs could modulate drug sensibility of cancer cells, at least in part [15–20]. Besides, anti-apoptosis was a common challenge in human malignancies contributing to both progress of cancer and resistance to conventional therapeutics. The MCL1 protein, a member of the B-cell lymphoma 2 (BCL2) family, played a pivotal role in protecting cells from apoptosis and was overexpressed in a variety of human

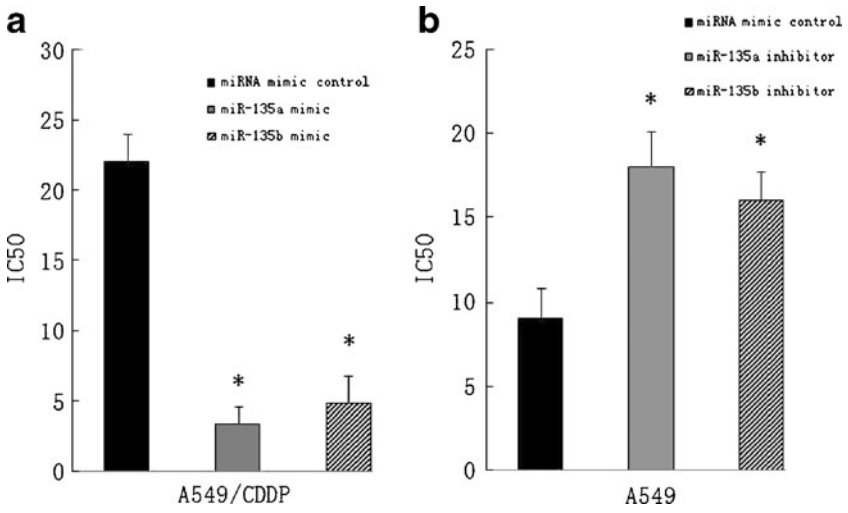

Fig. 2 miR-135a/b sensitize A549/CDDP cells to CDDP. In A549/CDDP cells, those transfected with miR-135a/b mimics exhibited greatly decreased resistance to CDDP (**a**). In A549 cells, those transfected with miR-135a/b inhibitors exhibited greatly enhanced resistance to CDDP (**b**). * $P<0.01$

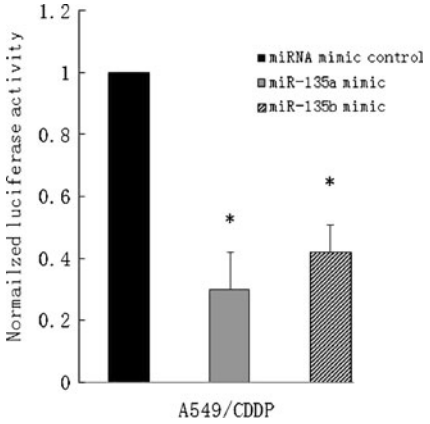

Fig. 3 Dual luciferase assay performed in A549/CDDP cells suggested that MCL1 is the target gene of the miR-135a/b. In A549/CDDP cells, a significant decrease in relative luciferase activity was noted when pGL3-MCL1-3′-UTR was cotransfected with the miR-135a/b mimics but not with the miRNA mimic control. * $P<0.01$

cancers [26]. In our study, we found that the antiapoptotic protein MCL1 was upregulated while the miR-135a/b were downregulated in A549/CDDP cells compared with A549 cells. The mechanistic connection of miR-135a/b dysregulation with the establishment of drug resistance in A549/CDDP cells was evidenced by the correlation between exogenous overexpression of miR-135a/b and corresponding changes in the protein levels of the target MCL1.

miR-135a is encoded in 2 genes: MIRN135A1 and MIRN135A2, located at 3p21.1 and 12q23.1, respectively. miR-135b is located on chromosomal band 1q32.1. They are based on the sequence AGCAGC starting at the second nucleotide from the 5′ end of the mature (~22 nt, single stranded) miRNA, a motif which referred to as AGC×2. Recently, there is increasing interest in understanding the role of miR-135a/b in cancers. Wu S et al. found that glia-enriched miR-135a was dramatically downregulated in malignant glioma and correlated with the pathological grading. It was capable of inducing mitochondria-dependent apoptosis of malignant glioma by regulating various genes including STAT6, SMAD5 and BMPR2 [27]. In addition, Navarro A et al. identified that overexpression of miR-135a could significantly downregulate the expression of JAK2 protein and decrease cell growth [28]. In concordance with these studies, we also found that miR-135a/b could enhance apoptosis of drug-resistant lung cancer cells via targeting MCL1, which was involved in mitochondria-dependent apoptosis by inhibiting the release of cytochrome c. However, because a miRNA could target more than one gene, the function of miRNAs might still exist cell-type specificity. For instance, the study by Chen Y et al. suggested that miR-135a could promote breast cancer cells migration and invasion by targeting HOXA10 [29]. Nevertheless, Matsuyama H et al. found that miR-135b mediated nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)-

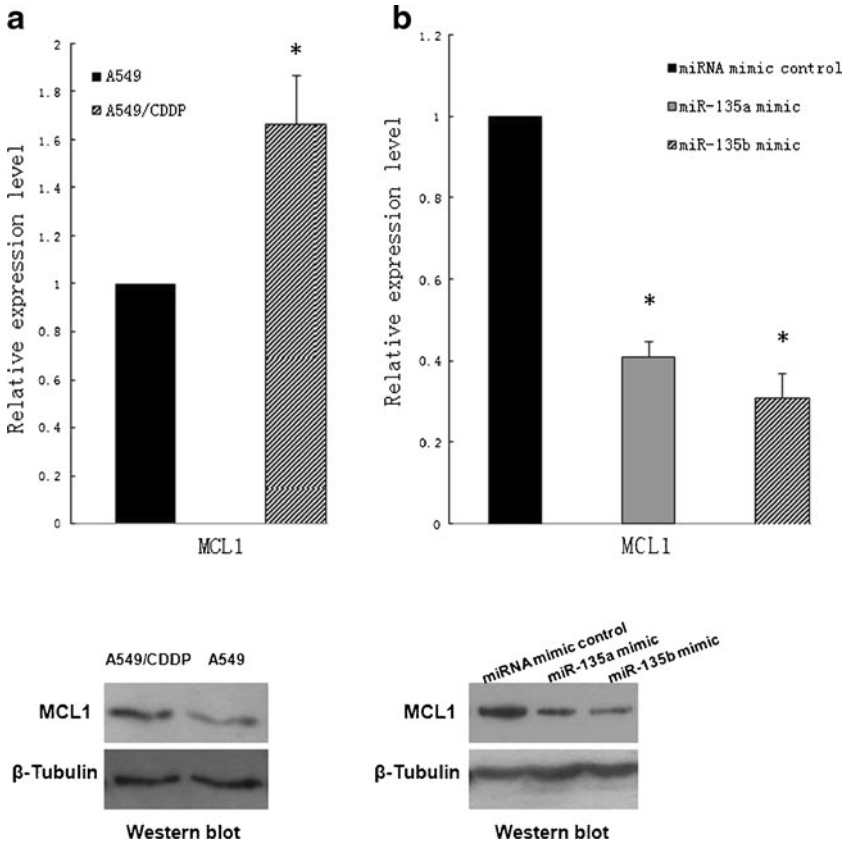

Fig. 4 **a** The antiapoptotic MCL1 protein was overexpressed in A549/CDDP cells compared with the parental A549 cells. Representative Western blot was attached beside the graph. **b** In A549/CDDP cells, 72 h after the transfection, Western Blot demonstrated significantly decreased MCL1 protein level in miR-135a/b mimics transfected cells compared with the miRNA mimic control transfected cells. Representative Western blot was attached beside the graph. The results represent the mean ± SD from 3 independent experiments. * P<0.01

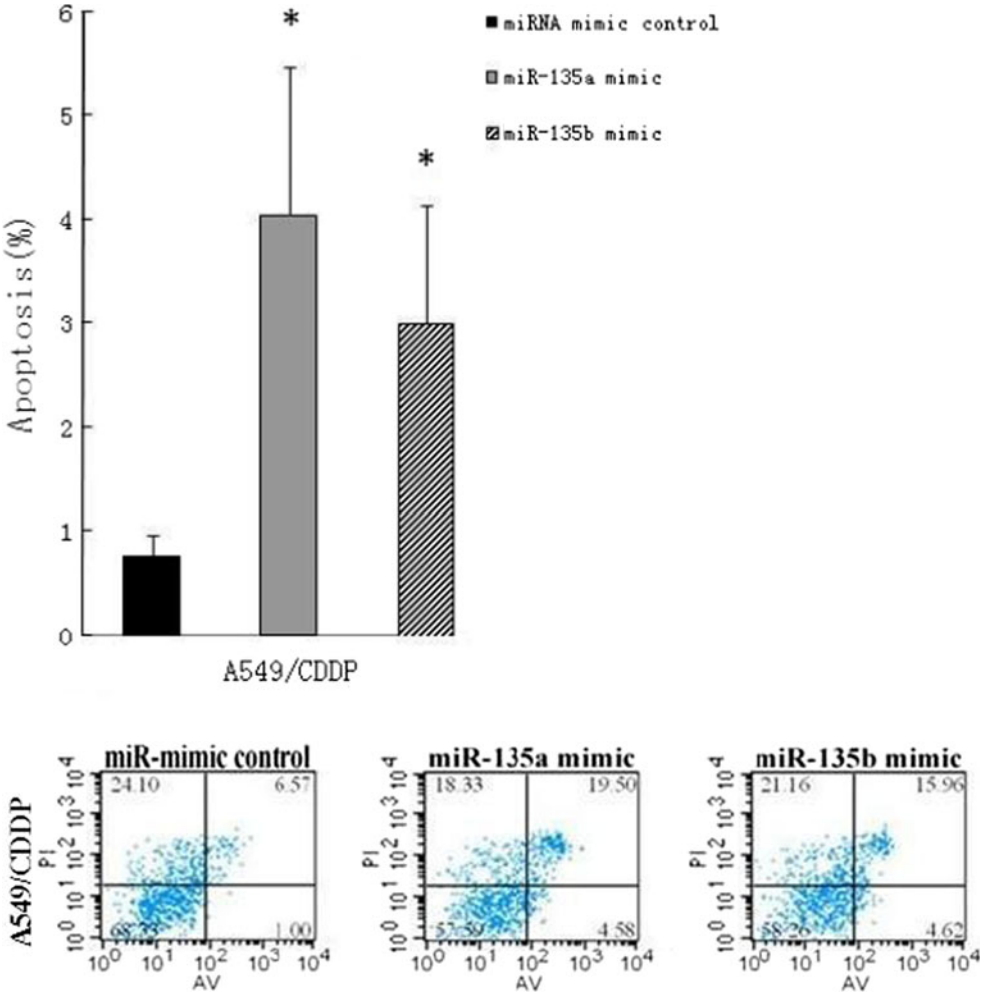

Fig. 5 miR-135a/b mimics sensitized A549/CDDP cells to CDDP-induced apoptosis. In A549/CDDP cells, apoptosis evaluated by flow cytometry showed a marked increase in apoptosis in miR-135a/b mimics transfected cells after CDDP treatment, compared with the miRNA mimic control transfected cells. Representative flow cytometry report was attached beside the graph. The results shown represent the mean ± SD from 3 independent experiments. * $P<0.01$

driven oncogenicity in anaplastic large cell lymphoma (ALCL), and miR-135b introduction could decrease chemosensitivity in Jurkat cells [30]. One possible explanation for this was that the contrary function of miR-135a/b in different cell types might rely on the balance of the expression level between its target inhibiting and promoting apoptosis or proliferation genes [31].

Currently, there are few studies about the modulation of miR-135a/b on cell apoptosis. Our study found that miR-135a/b were downregulated in human drug-resistant lung cancer cell line A549/CDDP compared with the parental cell line. According to the data from UCSC (http://genome.ucsc.edu), DNA methylation of CpG islands in the promoter region was detected in MIRN135A1, MIRN135A2, and MIRN135B of multiple cancer cell lines, respectively. This might lead to the downregulation of miR-135a/b in drug resistant cells. However, more researches were needed to elucidate the underlying mechanism. In recent years, researchers have developed several kinds of drug resistance reversal agents [32]. Nevertheless, these agents failed to be applied in clinic because of their severe side effects or poor pharmacokinetics in vivo [33]. Therefore, increasing the drug sensitivity by the modulation of epigenetic changes, such as abnormal expression of miRNAs, might be a key step toward improving therapy.

In conclusion, the findings we reported here presented the first evidence that hsa-miR-135a/b might be involved in the development of CDDP resistance in human lung cancer cell line. hsa-miR-135a/b could sensitize human drug-resistant lung cancer cell line to CDDP, at least in part, by targeting MCL1 expression. Our study might have implications for cancer chemotherapy which was often failed due to the appearance of drug resistance. Besides, the application of combinatorial approaches that involve inhibiting MCL1 and manipulation of additional signaling pathways to enhance therapeutic outcomes could also be considered. However, it should be noted that our data were derived from cell lines which have been removed from their in vivo context and could not be considered accurate surrogates for clinical tumors. Thus, further studies to assess the roles of hsa-miR-135a/b in vivo and in clinical context were warranted.

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