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miR-495 Enhances the Sensitivity of Non-Small Cell Lung Cancer Cells to Platinum by Modulation of Copper-Transporting P-type Adenosine Triphosphatase A (ATP7A)

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ABSTRACT

Copper-transporting P-type adenosine triphosphatase A (ATP7A) is associated with platinum drug resistance in non-small cell lung cancer (NSCLC). microRNAs (miRNAs) are a class of small non-coding RNA molecules that regulate gene expression at post-transcriptional level. In this study, the aim is to explore which miRNAs might participate in the platinum resistance by targeting ATP7A in NSCLC. Using real-time PCR-based miRNA expression profiling and bioinformatics, we selected miR-495 as a candidate miRNA. EGFP reporter assay, real-time PCR, and Western blot validated that ATP7A was a direct target for miR-495. The drug sensitivity assay indicated that miR-495 enhanced the cell response to cisplatin (CDDP) in NSCLC cells, while inhibition of miR-495 led to the opposite effects. Importantly, either overexpression or knockdown of ATP7A could override the effect of miR-495 on chemosensitivity. We also demonstrated that miR-495 increased the intracellular CDDP accumulation and overexpression of ATP7A can reduce the increased drug concentration induced by miR-495. Finally, we discovered that there was a converse relationship between miR-495 and ATP7A levels in NSCLC tissues sensitive or resistant to CDDP. In conclusion, our data demonstrate that miR-495 regulates the multi-drug resistance by modulation of ATP7A expression in NSCLC and suggest that miR-495 may serve as a potential biomarker for the treatment of multi-drug resistant NSCLC patients with high ATP7A levels. J. Cell. Biochem. 115: 1234–1242, 2014.

KEY WORDS: microRNAs; miR-495; ATP7A; PLATINUM; CISPLATIN (CDDP)

Non-small cell lung cancer (NSCLC) is the most common malignant cancer worldwide and accounts for 80% of all lung cancers [Li et al., 2012b]. Platinum-related chemotherapy, including cisplatin (CDDP), is the main treatment method for NSCLC patients. CDDP has been used as the key medicine for NSCLC, but the overall response rate of the NSCLC patients to CDDP is less than 20% [Tiseo et al., 2006; Inoue et al., 2010].

Copper-transporting P-type adenosine triphosphatase A (ATP7A) is involved in the Cu transport from the cytoplasm to the trans-Golgi network. Recent study shows that copper transporter may participate in the transport of platinum drugs [Li et al., 2012b]. ATP7A mediates the ovarian cancer cell resistance to cisplatin [Samimi et al., 2004]. Another study indicates that ATP7A may be responsible for the intracellular cisplatin sequestration during the process of cell resistance to cisplatin in ovarian cancer cells [Kalayda et al., 2008]. In addition, ATP7A is reported to be related to the cisplatin resistance in NSCLC cell lines [Inoue et al., 2010; Li et al., 2012b], but the molecular mechanism responsible for the regulation of ATP7A in NSCLC is yet to be determined.

microRNAs (miRNAs) are a class of small non-coding RNA molecules and post-transcriptionally regulate gene expression in various cancers through complete or incomplete complementarity with the binding sites in the 3′ untranslated region (3′UTR) of the target gene mRNA [Bartel, 2004]. Depending on the biological roles of target genes, miRNAs function as either oncogenes or tumor suppressor genes in cancers [Ying et al., 2011]. miRNAs are involved in various biological processes in cancers, including cell proliferation, cell death, apoptosis, and chemoresistance [Brennecke et al., 2003;

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Ambros, 2004; Xia et al., 2008]. For instance, miR-451 increases the sensitivity of the doxorubicin-resistant MCF-7 cells to doxorubicin in breast cancer [Kovalchuk et al., 2008]. The inhibition of miR-519c enhances ABCG2 expression and contributes to drug resistance [To et al., 2009]. miR-215 contributes to cell chemoresistance to both methotrexate (MTX) and TS inhibitor Tomudex (TDX) by targeting denticleless protein homolog (DTL) in osteosarcoma and colon cancer cells [Song et al., 2010]. In addition, miR-135a is associated with the paclitaxel resistance partly through downregulating adenomatous polyposis coli gene (APC) in non-small cell lung cancer [Holleman et al., 2011]. However, whether miRNAs are involved in ATP7A-mediated CDDP resistance is yet to be elucidated.

In this study, we found that ATP7A is a direct target gene of miR-495 and can be negatively regulated by miR-495. The inhibition of miR-495 reduced the cell sensitivity to CDDP in A549 and H1299 cells; however, the overexpression of miR-495 increased the cell sensitivity to CDDP in A549/CDDP cells. Importantly, ATP7A can restore the cell response to multi-drug resistance induced by miR-495. Our results indicate that miR-495 regulates the cell response to platinum drug resistance by modulation of ATP7A expression and miR-495 may serve as an important biomarker for the NSCLC patients with CDDP resistance problem.

MATERIALS AND METHODS

CELL LINES AND TISSUE SPECIMENS

The lab of the Department of Respiratory Medicine in Fourth Military Medical University supplied human non-small cell lung cancer (NSCLC) cell lines, H1299, A549 and its multi-drug resistant variant A549/CDDP. All the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin. All the cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. To maintain the multi-drug resistance phenotype, CDDP (with final concentration of 4 lg/ml) was added to the culture medium for A549/CDDP cells. Each time 10^6 cells were seeded in six-well plates and the next day, the cell transfection was performed with Lipofectamine™ 2000 reagent (Invitrogen). The miRNA mimics, ASO of miRNAs and the pcMV6/ATP7A plasmid were purchased from the GenePharma Company (Shanghai, China).

Studied materials included 10 tissue specimens from patients with NSCLC. Five patients had the CDDP resistance characteristics, and the other five were sensitive to CDDP treatment. All patients signed an informed consent sheet for this study. Clinical and pathological data are described in Table I.

RNA ISOLATION AND REAL-TIME PCR ANALYSIS

The total RNAs were isolated from the cells and tissues using Trizol (Invitrogen) according to the manufacturer's protocol. The RNA concentration and purity was determined using the Nano-Drop ND-1000 spectrophotometer (Thermo Scientific). For miRNA reverse transcription (RT) reaction, special miRNA RT primers were used, while for the RT reaction of mRNAs, Oligo (dT) was used as the common primer. The real-time PCR assay was performed by the SYBR Premix Ex Taq system (TaKaRa, Madison, WI) and the PCR reaction entailed 95°C for 3 min, 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. Each sample was run in triplicates to ensure quantitative accuracy. The U6 small nuclear B non-coding RNA (RNU6B) was used as the endogenous control to normalize the expression of miRNAs, while β-actin was used as the endogenous control to normalize the expression of ATP7A.

REAL-TIME PCR-BASED miRNA EXPRESSION PROFILING

Total miRNA was reverse-transcribed using Megaplex RT Primers (Applied Biosystems). The produced cDNAs were preamplified using Megaplex PreAmp Primers (Applied Biosystems) and the pre-amplified products were applied to a TaqMan Human microRNA Array Panel. Real-time PCR was performed using a 7900HT Fast Real-Time System (Applied Biosystems). The real-time PCR data obtained using the TaqMan microRNA Panel was analyzed with Applied Biosystems software (SDS ver. 2.3 and RQ manager ver. 1.2). For quantification, the relative C_t method ($-\Delta\Delta C_t$ method) was applied. U6 small nuclear B non-coding RNA (RNU6B) was used as the endogenous control. Each sample was run in triplicate to ensure quantitative accuracy.

IN VITRO DRUG SENSITIVITY ASSAY

The cells were seeded into 48-well plates at a density of 3×10^4 cells/well. At 24 h after transfection, the transfected cells were seeded into 96-well plates (5×10^3 cells/well) for the next step experiment. After cellular adhesion, CDDP was added with the final concentration being 0.01, 0.1, 1, and 10 times of the human peak plasma concentration for the drug. The peak serum concentration of CDDP was 2.0 lg/ml [Zhu et al., 2012a]. At 48 h after drug treatment, cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The absorbance at $A_{490\,nm}$ was read on a spectrophotometer. The concentration at which the drug produced

TABLE I. Clinical Characteristics of 10 Patients Whose Sample Were Used for miR-495 and ATP7A Analysis

Patient ID	Age	Sex	Histology	Stage	T	N	M	CIR(%)	S or R
NSCLC-1	70	F	SCC	Ib	2	0	0	58.1	S
NSCLC-4	58	M	Adenocarcinoma	IIb	2	1	0	63.6	S
NSCLC-6	62	M	Adenocarcinoma	Ib	2	0	0	23.4	R
NSCLC-7	60	M	Adenocarcinoma	IIIa	3	2	0	17.7	R
NSCLC-10	67	F	SCC	IIIb	4	1	0	53.3	S
NSCLC-14	53	M	Adenocarcinoma	IIb	2	1	0	21.9	R
NSCLC-16	72	M	SCC	Ia	1	0	0	68.2	S
NSCLC-17	55	F	SCC	IIb	2	1	0	13.5	R
NSCLC-20	59	M	SCC	IIIa	3	1	0	9.2	R
NSCLC-23	50	M	SCC	Ib	2	0	0	71.2	S

SCC, squamous cell carcinoma; CIR(%), CDDP inhibition rate (%); S, sensitive; R, resistance.

50% inhibition of growth (IC_{50}) was estimated by the concentration–effect curve.

INTRACELLULAR CDDP CONCENTRATION MEASUREMENT

The cells were seeded into six-well plates at a density of 3×10^5 cells/well. The cells were transfected when the cell confluence reached about 80%. At 24 h after transfection, the cells were treated with CDDP with 0.2 or 0.4 μg/ml. At 24 or 48 h after CDDP treatment, the intracellular CDDP concentration using high performance liquid chromatography (HPLC) was determined according to the modified method [Hanada et al., 1995].

PLASMID CONSTRUCT AND EGFP REPORTER ASSAY

The 3′UTR of ATP7A containing the miR-495 binding sites was synthesized and cloned into the pcDNA3/EGFP vector (at the downstream of EGFP gene). The mutant 3′UTR of ATP7A containing four point mutations in miR-495 binding site was synthesized and cloned into the downstream of pcDNA3/EGFP vector. The cells were cotransfected with miRNA mimics and pcDNA3/EGFP-ATP7A 3′UTR or mutant 3′UTR construct, or anti-sense oligonucleotides (ASO) of miRNAs and pcDNA3/EGFP-ATP7A 3′UTR or mutant 3′UTR construct. The vector pDsRed2-N1 (Clontech) expressing RFP was cotransfected together with the above vectors and was used as the spiked-in control. At 48 h after transfection, the cells were lysed using RIPA buffer (150 mM NaCl, 1% NP-40, 1% Triton X-100, 1 mM $MgCl_2$, 0.1% SDS and 10 mM Tris–HCl at pH 7.4) and then the fluorescent intensity was measured using an F-4500 Fluorescence Spectrophotometer (HITACHI).

WESTERN BLOT ANALYSIS

The cells were transfected with miRNA mimics or miRNA ASOs when the cells reached about 80% confluence. At 48 h after transfection, the cells were washed with 1× PBS and then lysed by RIPA buffer for 30 min at 4°C. The protein concentration was measured using Nano-Drop ND-1000 spectrophotometer (Thermo Scientific) and 20 μg protein was loaded into the SDS–PAGE gel for Western blot assay. The first antibody was mouse monoclonal anti-ATP7A antibody (Abcam, 1:500 dilutions) or rabbit monoclonal anti-GAPDH antibody (Abcam, 1:1,000 dilution). The secondary antibody was the goat anti-mouse or anti-rabbit IgG conjugated with HRP (horseradish Peroxidase) at a dilution of 1:1,000. The bound antibodies were detected with the use of ECL Plus Western Blotting Detection system (GE Healthcare) and the chemiluminiscent signals were detected with the use of high-performance chemiluminescence film (GE Healthcare). GAPDH was used as the endogenous control to normalize the expression of ATP7A.

IMMUNOHISTOCHEMISTRY

For the immunohistochemical staining of ATP7A between CDDP resistant and CDDP sensitive tissues, 4 μm sections were cut on slides coated with poly-L-lysine (Sigma–Aldrich, St. Louis, MO) and incubated overnight at 37°C. The slides were deparaffinized, hydrated, and treated with 0.5% pepsin (2000 FIP-U/g, Merck, Darmstadt, Germany) at 37°C. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in absolute methanol and non-specific binding was blocked with 10% goat serum. Mouse monoclonal antibody for ATP7A (Abcam) was used as the first antibody. The slides were incubated with the first antibody at room temperature overnight after which the Histostain bulk kit was used (Zymed Laboratory, Inc., San Fransisco, CA). The sections were counterstained with haematoxylin and mounted with Immuno-mount (Shanon, Inc., Pittsburgh, PA). For negative controls, the first antibody was replaced by mouse non-immune IgG or PBS.

STATISTICAL ANALYSIS

All the data were presented as the mean ± SD. The significance of differences was carried out by two-paired Student′s *t*-test, and for the comparison between multiple groups, we used one-way ANOVA analysis. $P < 0.05$ was considered statistically significant.

RESULTS

miR-495 IS DOWNREGULATED IN A549/CDDP CELLS AND MODULATES THE CELL DRUG SENSITIVITY

To determine the miRNAs that are involved in the regulation of CDDP drug resistance induced by ATP7A in NSCLC cells, we used the bioinformatics, TargetScan, miRBase, and miRDB, for miRNAs prediction. We also performed real-time PCR-based miRNA profiling to explore the differentially expressed miRNAs between A549 and A549/CDDP cells. Combining the bioinformatics and miRNA profiling analysis, we selected miR-497, miR-495, miR-200b, and miR-367 as the candidate miRNAs. As shown in Figure 1A, real-time PCR results validated that miR-497, miR-495, miR-200b, and miR-367 were significantly downregulated in A549/CDDP cells, especially for miR-495 which was reduced by about 75%, compared to A549 cells. To determine whether these miRNAs affect cell sensitivity to CDDP, A549, and A549/CDDP cells were exposed to different CDDP concentration after transfection with the miRNA ASOs and the miRNAs mimics, respectively. As shown in Figure 1B, the inhibition of miR-497, miR-495, and miR-200b increased the A549 cell survival rate upon different CDDP treatment, respectively, compared to the controls. In contrast, overexpression of miR-497, miR-495, and miR-200b reduced the A549/CDDP cell survival rate upon different concentration of CDDP treatment, respectively. However, neither the inhibition nor overexpression of miR-367 had an effect on cell sensitivity to CDDP treatment (Fig. 1C). Taken together, these results suggest miR-497, miR-495, miR-200b may potentially play roles in CDDP resistance in NSCLC cell lines. Previous studies report that miR-200b is involved in the docetaxel- and cisplatin resistance in lung adenocarcinoma [Wu et al., 2011; Feng et al., 2012] and miR-497 modulates the cisplatin resistance in NSCLC [Zhu et al., 2012b], but few studies focus on miR-495 regulation in CDDP resistance in NSCLC. Hence, we choose miR-495 that has different expression level between A549 and A549/CDDP for further study.

miR-495 DIRECTLY TARGETS ATP7A AND NEGATIVELY REGULATES ATP7A EXPRESSION ON mRNA AND PROTEIN LEVELS

To verify whether ATP7A is a direct target for miR-495 in NSCLC, EGFP reporter assay was performed. Figure 2A showed that there were three putative binding sites for miR-495 in the 3′UTR of ATP7A mRNA. Then we cloned the 3′UTR of ATP7A and inserted it into the

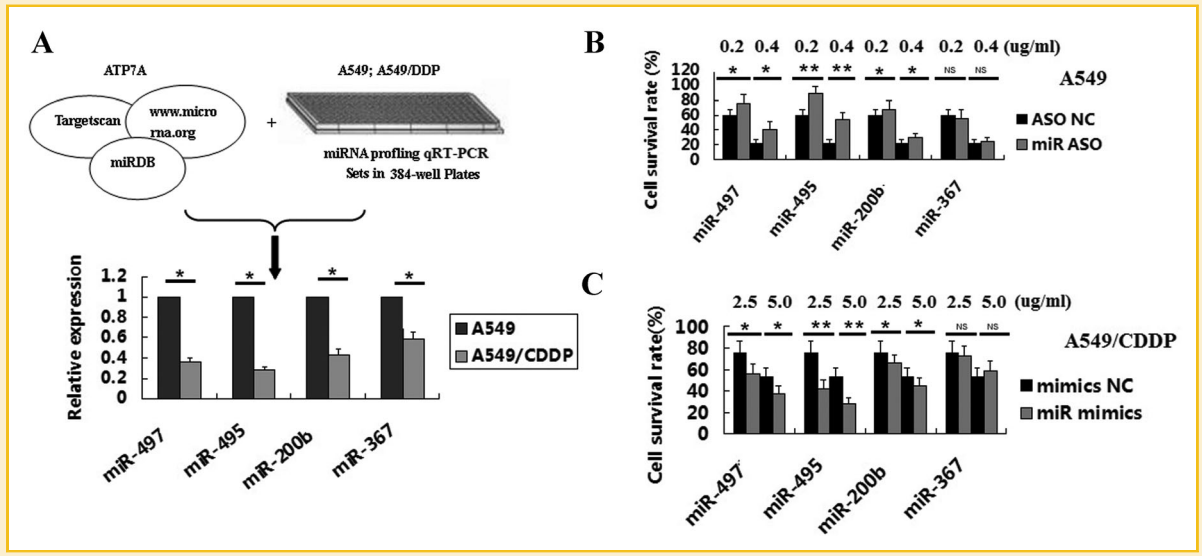

Fig. 1. The validation of miRNAs downregulated in A549/CDDP cells. A: The expression of the downregulated miRNAs were analyzed by real-time PCR in A549 and A549/CDDP cells. B: The cell viability was analyzed using MTT assay in A549 cells after transfection with anti-sense oligonucleotides (ASO) of miRNAs or the control. The transfected cells were treated with an increase dose of CDDP. C: The cell viability was analyzed using MTT assay in A549/CDDP cells transfected with miRNAs mimics or the control. The transfected cells were treated with an increase dose of CDDP. $^{*}P < 0.05$, $^{**}P < 0.01$. All the experiments were repeated three times and all data are representative of three independent experiments. "NS" represented no significance.

EGFP expression vector. The EGFP reporter assay indicated that miR-495 reduced the GFP intensity and the inhibition of miR-495 increased the GFP intensity controlled by the ATP7A 3′UTR in both A549 and H1299 cells, compared to the control groups (Fig. 2B). In addition, real-time PCR and Western blot showed that the inhibition of miR-495 increased the ATP7A protein and mRNAs levels in both A549 and H1299 cells compared to the control groups. In contrast, overexpression of miR-495 had the opposite effects (Fig. 2C). To further define miR-495 binding sites in ATP7A 3′UTR, we constructed four vectors (Fig. 2D): vector1 (only site 1 was mutated), vector 2 (only site 2 was mutated), vector 3 (only site 3 was mutated), and vector 4 (site 1, 2, and 3 were mutated together), respectively. Then, we co-transfected these reporter vectors and miRNA mimics or miRNA ASOs into A549 cells. As shown in Figure 2E,F, the inhibition of miR-495 had no effect on the GFP intensity in the vector bearing the mutation of the first and second miR-495 binding region, but blocking miR-495 affected the intensity of EGFP in the mutant vector containing a mutation in the third binding site. It may be explained that the third binding site play a minor role in the regulation of ATP7A by miR-495. Taken together, these data suggest that ATP7A is a direct target gene of miR-495 and miR-495 suppresses ATP7A expression directly.

miR-495 REGULATES THE CELL RESPONSE TO CDDP IN A549 AND A549/CDDP CELLS VIA TARGETING ATP7A

To determine whether miR-495 regulates the cell response to CDDP via modulation of ATP7A rather than other genes, we performed the rescue experiment in NSCLC cell lines. In the A549 and H1299 cells, we cotransfected the cell lines with miR-495 ASO and siRNA of ATP7A. Figure 3A showed that miR-495 expression was reduced significantly by antisense blocking and silencing ATP7A had no effect on miR-495 levels. As shown in Figure 3B, silencing ATP7A can restore the ATP7A protein levels increased by the inhibition of miR-495. Then, the drug sensitivity analysis of A549 and H1299 cells exposed to CDDP showed that the inhibition of miR-495 increased the IC_{50} value of CDDP, and silencing ATP7A can abrogate the decrease in chemosensitivity to CDDP by miR-495 inhibition (Fig. 3C).

A similar rescue experiment was performed in multi-drug resistant A549/CDDP cells. We cotransfected the cells with miR-495 mimics and the ATP7A overexpression plasmid, pcMV6/ATP7A. As shown in Figure 3D,E, we found that transfection of miR-495 mimics could result in a high level of miR-495 and that overexpression of ATP7A had no effect on miR-495, while ATP7A overexpression can restore the ATP7A expression reduced by miR-495 overexpression. Furthermore, we observed that miR-495 reduced the IC_{50} of CDDP in A549/CDDP cells and overexpression of ATP7A can restore the A549/CDDP cell resistance to CDDP reduced by miR-495 (Fig. 3F). In short, the data suggest that miR-495 modulates the cell resistance to CDDP via regulating ATP7A.

miR-495 REGULATES THE CELL RESPONSE TO OTHER PLATINUM DRUGS IN A549 AND H1299 CELLS VIA TARGETING ATP7A

Besides CDDP, CBDCA, L-OHP, and Taxol are other platinum drugs for NSCLC chemotherapy, and previous report shows ATP7A is associated with multi-drug resistance in NSCLC, so we assume ATP7A and miR-495 might be involved in the regulation of the drug resistance induced by CBDCA, L-OHP, and Taxol in A549 and H1299 cells. We transfected the cells with miR-495 ASO and siRNA of ATP7A. As shown in Figure 4A,B, we discovered that the inhibition of miR-495 increased the IC_{50} of both CBDCA and L-OHP in A549 and

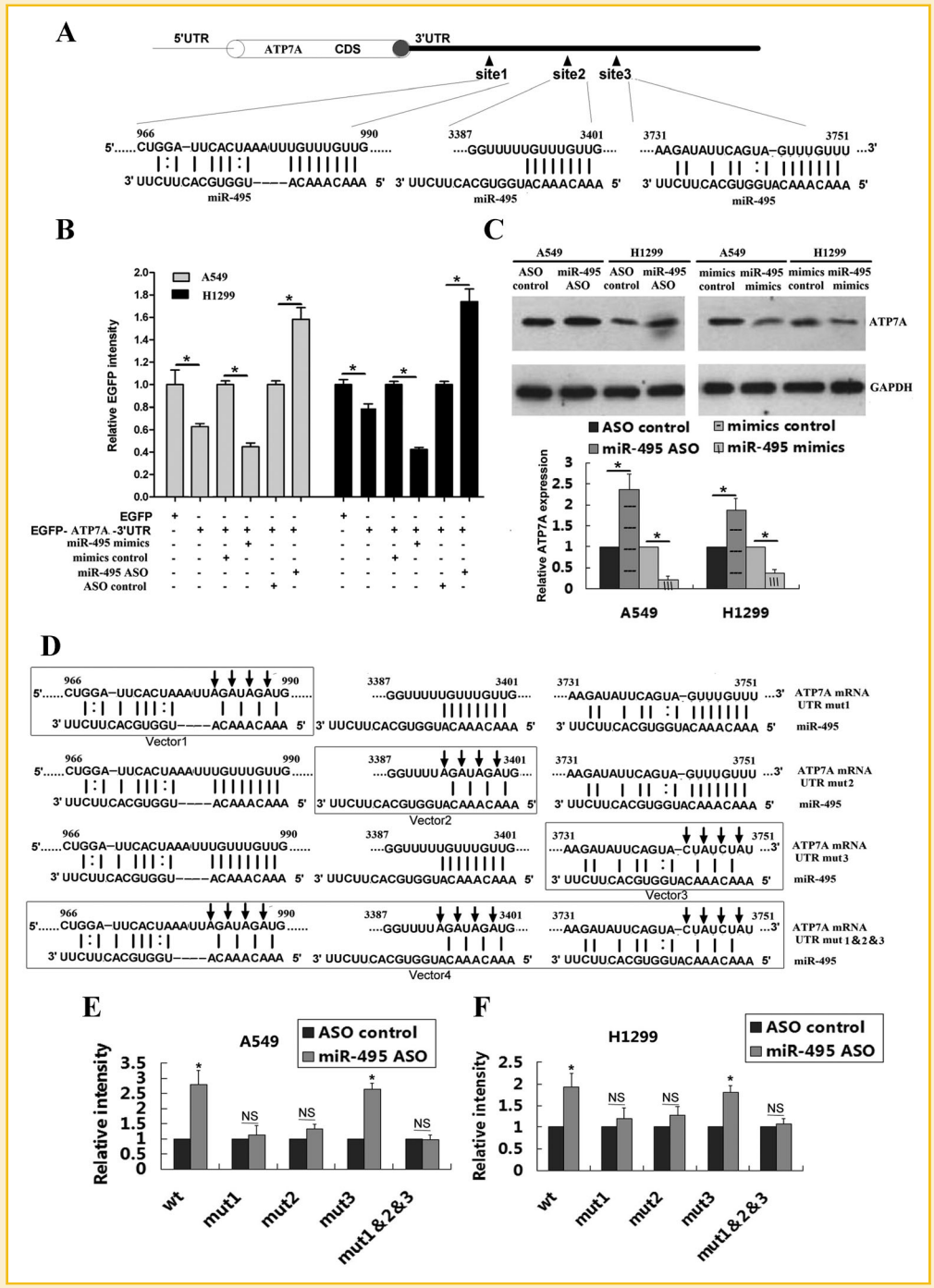

Fig. 2. The validation of ATP7A as a direct target for miR-495. A: The alignment of miR-495 binding sites in the ATP7A 3′UTR was shown. B: The effect of miR-495 on the GFP intensity controlled by the ATP7A 3′UTR was analyzed in A549 and H1299 cells. The GFP intensity of EGFP control, EGFP-ATP7A-3′UTR plus control mimic and EGFP-ATP7A-3′UTR plus ASO control was normalized as 1. C: The effect of miR-495 on ATP7A protein and mRNA levels was analyzed by Western blot and real-time PCR in A549 and H1299 cells. D: The mutated nucleotides in different binding sites were shown. E, F: The impact of miR-495 on the GFP intensity controlled by the mutant ATP7A 3′UTR was analyzed in A549 and H1299 cells. $^{*}P < 0.05$. All the experiments were repeated three times and all data are representative of three independent experiments. "NS" represented no significance.

H1299 cells compared to the control, while knockdown of ATP7A decreased the IC_{50} value of CBDCA and L-OHP. All these results were similar to the effect of miR-495 and ATP7A on the IC_{50} of CDDP. However, neither miR-495 nor ATP7A had an effect on the cell response to Taxol (Fig. 4C).

miR-495 REGULATES THE INTRACELLULAR CDDP ACCUMULATION IN A549 AND H1299 CELLS

Based on the above results, we concluded that miR-495 was related with the cell sensitivity to CDDP. Hence, we tried to explore whether miR-495 modulates the intracellular CDDP accumulation. As

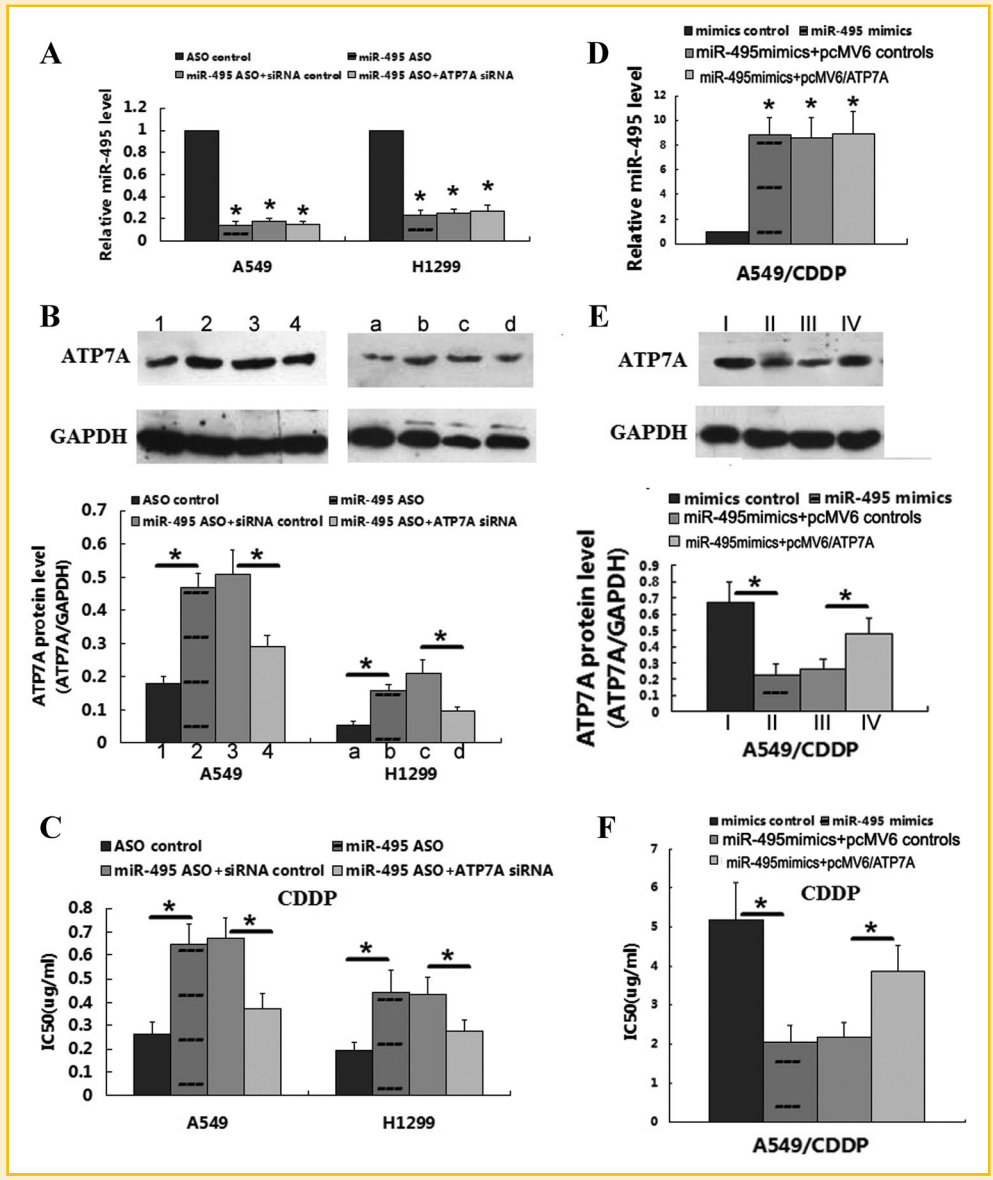

Fig. 3. The effect of miR-495 on the cell response to CDDP in A549, H1299, and A549/CDDP cells. A, B: The expression of miR-495 and ATP7A was analyzed by real-time PCR and Western blot, respectively, in A549 and H1299 cells after transfection with ASO of miR-495 and the ATP7A siRNA, or the controls. C: The cell sensitivity to CDDP was measured in A549 and H1299 cells after transfection with ASO of miR-495 and the ATP7A siRNA, or the controls. D, E: The expression of miR-495 and ATP7A was analyzed by real-time PCR and Western blot, respectively, in A549/CDDP cells after transfection with miR-495 mimics and ATP7A, or the controls. F: The cell sensitivity to CDDP was measured in A549/CDDP cells transfection with miR-495 mimics and ATP7A, or the controls. $^{*}P < 0.05$. All the experiments were repeated three times and all data are representative of three independent experiments.

shown in Figure 5A, we found that the inhibition of miR-495 reduced the intracellular CDDP concentration in both A549 and H1299 cells; importantly, silencing ATP7A can restore the CDDP concentration reduced by miR-495 inhibition. In contrast, over-expression of miR-495 led to an opposite effect on the intracellular CDDP concentration (Fig. 5B). Taken together, these results indicate that miR-495 regulates the intracellular CDDP concentration through ATP7A that might contribute to the change of chemo-sensitivity to DDP.

miR-495 AND ATP7A EXPRESSION AND THEIR RELATIONSHIP WITH CDDP DRUG SENSITIVITY IN NSCLC TISSUES

According to the real-time PCR analysis for miR-495 and ATP7A expression in CDDP-sensitive and CDDP-resistant NSCLC tissues, we noticed a high expression of miR-495 and a significant down-regulation of ATP7A in CDDP-sensitive tissues, compared to the CDDP-resistant tissues, as shown in Figure 6A,B. In addition, we also detected the expression of ATP7A using Western blot and immunohistochemistry in the CDDP-sensitive and CDDP-resistant

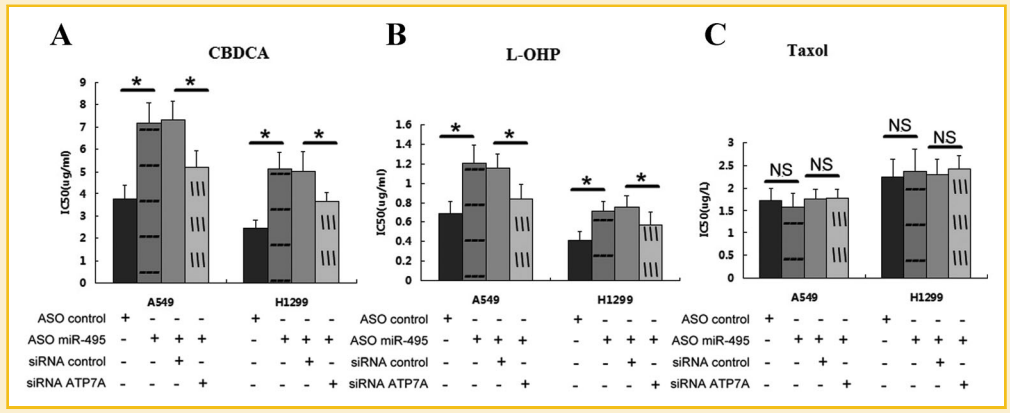

Fig. 4. The effect of miR-495 on the cell response to other drugs in A549 and H1299 cells. A, B: The cell sensitivity to CBDCA, L-OHP, and Taxol was measured in A549 and H1299 cells after transfection with ASO of miR-495 and the ATP7A siRNA, or the controls. $^{*}P < 0.05$. All the experiments were repeated three times and all data are representative of three independent experiments. "NS" represented no significance.

tissues. In accord with the real-time PCR results, we found that ATP7A protein levels were also higher in CDDP-resistant tissues than in the CDDP-sensitive tissues (Fig. 6B,C). Taken together, these data further validated that the upregulation of ATP7A regulated by lower expression of miR-495 may contribute to the platinum resistance in NSCLC.

DISCUSSION

miRNAs function as regulators of gene expression in various biological processes in cancers. Dysregulation of miRNAs may lead to the development of cancers and the multi-drug resistance to the chemotherapeutic reagents. Previous study indicates that miR-495 is dysregulated during the multi-drug resistance, including CDDP, but the molecular mechanism of the regulation of miR-495 during CDDP resistance is not fully clarified [Guo et al., 2010]. In this study, we showed that miR-495 was downregulated in A549/CDDP cells and CDDP-resistant tissues, compared to A549 cells and CDDP-sensitive tissues, respectively. We also indicated that miR-495 increased the cell sensitivity to CDDP in NSCLC and increased the intracellular CDDP concentration.

Although several studies focus on the mechanism of the regulation of multi-drug resistance by miRNA in various cancers, the mechanism of most miRNAs is yet to be further studied. For example, the inhibition of miR-21 increases the cell sensitivity to gemcitabine in human cholangiocarcinoma cell lines [Meng et al., 2006]. miR-345 and miR-7 modulate cisplatin resistance of MCF-7 human breast adenocarcinoma cells via downregulation of the human multi-drug resistance-associated protein 1 [Pogribny et al., 2010]. miR-214 suppresses the expression of PTEN, leading to the activation of Akt pathway and the cell resistance to cisplatin in human ovarian cancer cells [Yang et al., 2008]. In accord with the previous studies, our data showed that miR-495 regulated the NSCLC cell sensitivity to CDDP via suppression of ATP7A.

miR-495 is known to function as either an oncogene or a tumor suppressor. miR-495 can be promoted by the transcription factor E12/E47 and contributes to the cell invasion and cell proliferation via the

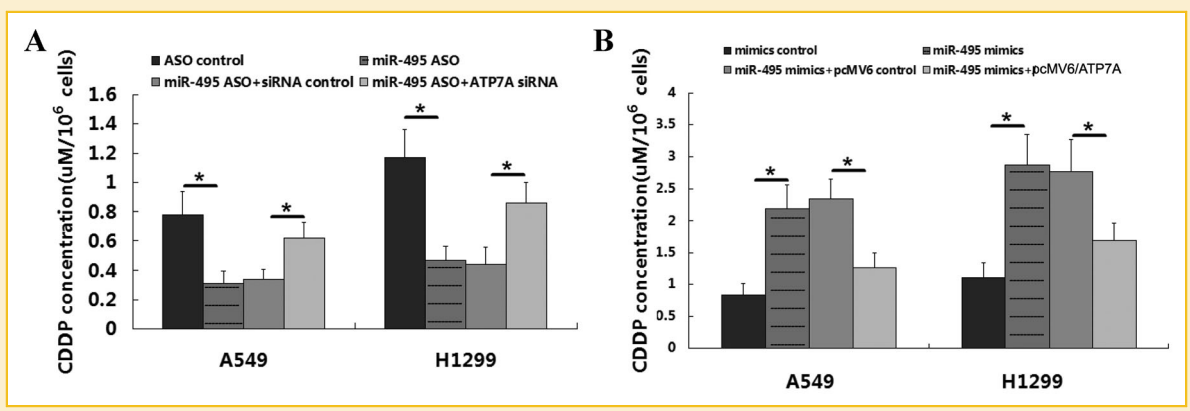

Fig. 5. The effect of miR-495 on the intracellular CDDP accumulation in A549 and H1299 cells. A, B: The intracellular CDDP accumulation was measured in A549 and H1299 cells after transfection with ASO of miR-495 and the ATP7A siRNA, or miR-495 mimics and ATP7A, respectively. $^{*}P < 0.05$. All the experiments were repeated three times and all data are representative of three independent experiments.

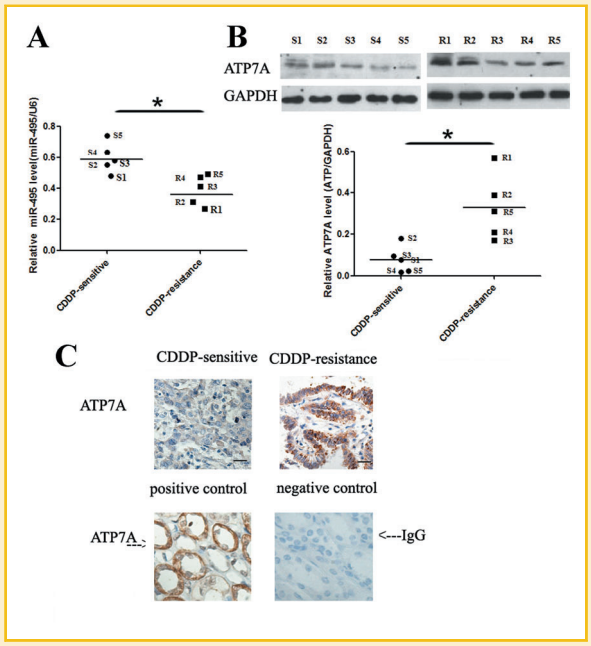

Fig. 6. The expression of miR-495 and ATP7A in CDDP-sensitive and CDDP-resistant NSCLC tissues. A: The expression of miR-495 was analyzed by real-time PCR in CDDP-sensitive and CDDP-resistant NSCLC tissues. B, C: The expression of ATP7A was analyzed by real-time PCR, Western blot and immunohistochemistry, respectively, in CDDP-sensitive and CDDP-resistant NSCLC tissues. The human kidney tissues were used as the positive control (ATP7A) and negative control (IgG). $^{*}P < 0.05$. Bar, 50 μm. All the experiments were repeated three times and all data are representative of three independent experiments.

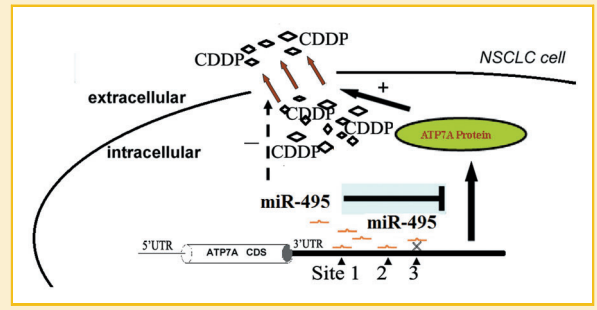

Fig. 7. A model of the regulation of ATP7A by miR-495 in the NSCLC cell resistance to CDDP. miR-495 suppresses the ATP7A expression and ATP7A contributes to the CDDP transport, leading to the reduction of intracellular CDDP accumulation.

suppression of E-cadherin and REDD1 in breast cancer stem cells, functioning as an oncogenic miRNA [Hwang-Verslues et al., 2011]. However, in MLL-rearranged leukemia, miR-495 inhibits the cell viability and promotes the apoptosis by targeting PBX3 and MEIS1, suggesting that miR-495 functions as a tumor suppressor gene [Jiang et al., 2012]. In addition, miR-495 suppresses the migration and invasion abilities of gastric cancer cells by downregulation of phosphatase of regenerating liver-3 (PRL-3) [Li et al., 2012a]. In our study, we showed that miR-495 reduced the cell viability under the CDDP treatment and it probably functioned as a tumor suppressor gene.

In our study, we validated that ATP7A was a direct target gene for miR-495. There are three binding sites for miR-495 in the 3′UTR of ATP7A. Using EGFP reporter assay for detection of the effect of miR-495 on the GFP intensity controlled by the mutated 3′UTR of ATP7A, we found that miR-495 had no effect on the GFP intensity controlled by the first and second mutated ATP7A 3′UTR. However, for the third mutated binding sites, miR-495 reduced the GFP intensity. What is more, there is only one different nucleotide among the three binding sites. Therefore, we assume that the only one different nucleotide plays a crucial role in the interaction between miR-495 and ATP7A, but the mechanism needs to be further explored. Previous study shows that there is one different nucleotide between miR-107 and miR-103 in their 3′ end. The study further demonstrates that miR-107 can inhibit let-7 activity but miR-103 only has marginal effect on let-7, so the difference of only one nucleotide between miR-107 and miR-103might contribute to their distinct modes of target recognition [Chen et al., 2011]. Similar to the previous study, our study needs to try to detect the affinity between miR-495 and ATP7A.

The mechanism of multi-drug resistance is complex, including multiple processes such as drug transport, intracellular drug accumulation, DNA repair, cell survival and apoptosis [Huang et al., 2004; Kuo et al., 2007; Guo et al., 2010]. In this study, we found that miR-495 increased the intracellular CDDP concentration through the suppression of ATP7A, leading to the sensitivity of NSCLC cells to CDDP. The results are consistent with the previous study that ATP7A is responsible for the CDDP resistance in human NSCLC, ovarian cancer, oral squamous cancer, and epidermoid cancer cells [Kuo et al., 2007; Matsumoto et al., 2007; Yoshizawa et al., 2007; Kalayda et al., 2008; Inoue et al., 2010]. Although one miRNA can regulate more than one target genes, our study, at least partially, illustrated that miR-495 regulated the cell response to CDDP, CBDCA, and L-OHP via the modulation of ATP7A. While for the cell resistance to taxol, other miRNAs or genes might participate in the drug resistance, except miR-495 and ATP7A.

In conclusion, our study showed that miR-495 regulated the intracellular CDDP concentration and modulate the NSCLC cells resistance to CDDP at least partly through the direct target of ATP7A (Fig. 7). The findings suggest that miR-495 may serve as an important molecular biomarker for the NSCLC patients with CDDP resistance problem. Although the miRNA signatures need to be further validated in the clinical NSCLC patients, targeting these miRNAs can provide more beneficial strategy for practical treatment for the patients.

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