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Let-7c sensitizes acquired cisplatin-resistant A549 cells by targeting ABCC2 and Bcl-XL

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Received January 24, 2013, accepted February 8, 2013

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Pharmazie 68: 955–961 (2013) *doi: 10.1691/ph.2013.3538*

Cancer cells that develop resistance to cisplatin (DDP) are a major clinical obstacle to the successful treatment of cancer, including non-small cell lung cancer (NSCLC). Recent studies have implicated dysregulation of microRNAs (miRNAs) function in chemoresistance. Here, we explored the role of let-7c in the acquisition of DDP-resistant phenotype in A549 cells. Let-7c was downregulated in A549/DDP cell compared with A549 cells. Modulation of let-7c altered the sensitivity of A549/DDP cells to DDP through regulating DDP-induced apopotis. Furthermore, ABCC2 and Bcl-XL were identified as targets of let-7c. ABCC2 and Bcl-XL knockdown increased DDP sensitivity and DDP-induced apoptosis in A549/DDP cells. In conclusion, our findings suggested for the first time that let-7c modulate DDP response in A549/DDP cells, and one of the mechanisms was through targeting ABCC2 and Bcl-XL. Thus, let-7c could be considered for potential therapeutic application for modulating DDP-based therapy.

1. Introduction

NSCLC accounts for approximately 85% of all cases of lung cancer and is the leading cause of human cancer-related deaths worldwide (Jemal et al. 2007). Currently, about 51% of NSCLC patients were diagnosed in the advanced stages (Travis et al. 1995). Cisplatin-based doublet regimens are the generally accepted standard treatment for most of the advanced NSCLC patients with good performance status (Cobo et al. 2007). Usually, the preliminary success related with partial response or disease stabilization precedes the selection of chemotherapy-resistant tumor cells, resulting in chemotherapeutic failure (Galluzzi et al. 2010). Although several critical biomarkers of drug resistance have been identified in the last decade, additional knowledge on the molecular mechanisms of chemoresistance is needed to improve the effectiveness of chemotherapeutic agents (Sun et al. 2007).

MicroRNAs are a class of endogenous single-strand and highly conserved non-coding small RNAs. They regulate negatively the expression of target genes by translational repression or mRNA degradation through binding to the 3′UTR of target genes. Several lines of evidence suggest that miRNAs are frequently deregulated in human malignancies and function as tumor suppressors or ontogenesis (Calin and Croce 2006). The lethal-7 (let-7) is the second discovered miRNA after lin-4 in *Caenorhabditis elegans* and the first known human miRNA. Let-7 family members are found decreased in various human cancers with few exceptions and function as tumor suppressors (Barh et al. 2010). Furthermore, recent evidence suggests that some members of the let-7 family regulate the sensitivity of cancer cells to chemotherapeutic agents and may be associated with resistance to chemotherapy (Ma et al. 2010).

Let-7c, one member of the let-7 family, is down-regulated in various including lung, liver and prostate cancers (Ozen et al. 2008; Navarro et al. 2009; Pineau et al. 2010). Let-7c inhibited cancer growth in many cancer types (Johnson et al. 2007; Motoyama et al. 2008; Roush and Slack 2008; Shimizu et al. 2010) and also functioned as a tumor metastasis suppressor in colorectal cancer by targeting MMP11 and PBX3 (Han et al. 2012). Previous studies have shown that let-7c negatively regulates expression of c-myc, HMGA2 and BcL-XL (Shell et al. 2007; Shimizu et al. 2010; Nadiminty et al. 2012). These genes are frequently upgraduated and have effects on initiation, progression, prognosis or response to chemotherapy in NSCLC (Ikuta et al. 2005; Karczmarek-Borowska et al. 2006; Sarhadi et al. 2006). In addition, Kumar et al. (2011) have shown that let-7c expression is significantly lower in DDP-resistant ovarian cancer cell line A2780/CP70 cells than in A2780 cells by microRNA array. However, the effect of let-7c on acquired DDP resistance in NSCLC remains elusive.

In the present study, we examined the effect of let-7c on the DDP resistance of A549 cells. The results show let-7c alters the sensitivity of A549/DDP cells to DDP at least in part via directly targeting ABCC2 and Bcl-XL.

2. Investigations and results

2.1. Let-7c is underexpressed in A549/DDP cells

The IC_{50} of DDP in A549 and A549/DDP cells was $18.03 \pm 1.418\,\mu M$ and $69.03 \pm 5.086\,\mu M$, respectively (Fig. 1A). QRT-PCR demonstrated that the expression of let-7c was reduced by about 86% in A549/DDP cells compared with A549 cells (Fig. 1B). These results show that let-7c is involved in A549 resistance to DDP.

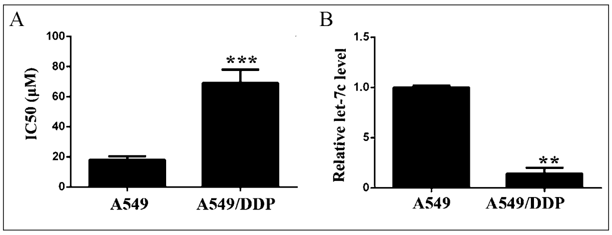

Fig. 1: Let-7c was downregulated in A549/DDP cells compared with A549 cells. (A) The IC50 of DDP detected in A549 and A549/DDP cells by MTS assay. (B) let-7c level in A549/DDP and A549 was assessed by qRT-PCR. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with A59 cells.

2.2. *Effect of let-7c on the sensitivity of A549/DDP cells to DDP*

A549/DDP cells were transfected with let-7c mimics, let-7c inhibitor or NC for 24 h, and then MTS assay was performed to determine IC_{50}. As shown in Fig. 2C, the IC_{50} value of DDP in A549/DDP cells transfected with let-7c mimics, NC and let-7c inhibitor was 29.83 ± 1.810 μM, 66.84 ± 2.870 μM and 99.03 ± 5.446 μM, respectively, which suggested that forced expression of let-7c sensitized A549/DDP cells to DDP. In contrast, downregulation of let-7c confered DDP resistance to A549/DDP cells. These results support a functional role of let-7c in regulating cell sensitivity to DDP in A549/DDP cells.

2.3. *Involvement of let-7c in DDP-induced apoptosis*

Cellular apoptosis was tested by Annexin V-FITC Flow cytometry and Hoechst 33342 staining. Flow cytometry showed that DDP-induced apoptotic cells in let-7c transfected A549/DDP cells was markedly increased compared to NC-treated cells, and downregulation of endogenous let-7c prevented DDP-induced apoptosis in A549/DDP cells (Fig. 3A, 3B). Similar results also were obtained by Hoechst 33342 staining (Fig. 3C).These results show that let-7c affects DDP sensitivity in A549/DDP cells by regulating the DDP-induced apoptosis.

2.4. *ABCC2 and Bcl-XL are targets of let-7c in A549/DDP cells*

DDP is a substrate of ABCC2 (Cui et al. 1999). Furthermore, ABCC2 was predicted to be a putative target of let-7c by Targetscan and miRanda. Bcl-XL has been validated as a target of let-7c in Huh7 cells (Shimizu et al. 2010; Qin et al. 2012). Bcl-XL expression was increased in the DDP-resistant lines compared with the parental lines and knockdown of Bcl-XL increased sensitivity of DDP-resistant cells to DDP treatment, including A549/DDP cells (Bauer et al. 2005; Lei et al. 2007). So we selected ABCC2 and Bcl-XL as the targets of further studies. As shown in Fig. 4A, A549/DDP cells exhibited significantly higher levels of ABCC2 and Bcl-XL protein expression than A549 cells, consistent with our expectation.
Further, the mRNA levels of ABCC2 and Bcl-XL were unaffected neither by let-7c mimics nor let-7c inhibitor (Fig. 4B, 4C). ABCC2 and Bcl-XL protein were reduced in cells treated by let-7c mimics compared with NC, whereas let-7c inhibitor increased ABCC2 and Bcl-XL protein levels when compared with NC (Fig. 4D). These data suggest that it is quite likely that the expression of ABCC2 and Bcl-XL protein are modulated by let-7c at the posttranscriptional level, thus we proposed that ABCC2 and Bcl-XL were direct targets of let-7c in A549/DDP cells.

2.5. *ABCC2 and Bcl-XL are direct targets of let-7c*

To test whether ABCC2 and Bcl-XL are regulated by let-7c through direct binding to their 3′UTR, we constructed reporter genes containing 248 bp of ABCC2 and 377 bp of Bcl-XL, respectively (Fig. 5A, 5B). The luciferase reporter plasmids were transfected into A549/DDP cells along with let-7c mimics, NC or let-7c inhibitor, and luciferase activity was determined. We found that enhanced expression of let-7c significantly reduced reporter activity of both constructs compared with NC and let-7c inhibitor significantly increased reporter activity of both constructs compared with NC (Fig. 5C, 5E). Furthermore, we mutated the reporter constructs by altering bases in the predicted let-7c target sequence (Fig. 5A, 5B). Mutation abolished the effect of let-7c and let-7c inhibitor on the luciferase activity of ABCC2 and Bcl-XL constructs (Fig. 5D, 5F). Taken as a whole, these results suggest that ABCC2 and Bcl-XL are the direct targets of let-7c in A549/DDP cells.

2.6. *Knockdown of ABCC2 and Bcl-XL mimic the effect of let-7c on A549/DDP cells*

To further assess whether the effect of let-7c on A549/DDP cells sensitivity to DDP could be mediated by ABCC2 and Bcl-XL, the IC_{50} was detected by MTS assay in A549/DDP cells treated with si-ABCC2, si-Bcl-XL or si-control. As shown in Fig. 6E, the IC_{50} value of DDP in A549/DDP/si-ABCC2 and A549/DDP/si-Bcl-XL cells were significantly reduced compared with that of DDP in A549/DDP/si-control (38.23 ± 2.598 μM vs 43.57 ± 3.466 μM vs 80.18 ± 10.76 μM, respectively). Furthermore, inhibition of ABCC2 and Bcl-XL expression increased the apoptosis induced by DDP in A549/DDP cell by Flow cytometry analysis (Fig. 6F, 6G) and Hoechst 33342 staining (Fig. 6H). All these results demonstrate that knockdown of ABCC2 and Bcl-XL reverses the resistance of A549/DDP cells to DDP and increase DDP-induced apoptosis, similar to the ectopic expression of let-7c in A549/DDP cells.

3. Discussion

The present study showed a significantly decreased expression of let-7c in A549/DDP cells compared with parental A549 cells. It is similar with the previous findings that let-7c, let-7e and let-7i were lower in DDP-resistant cancer cells versus in their parental cells (Sorrentino et al. 2008; Yang et al. 2008; Kumar et al. 2011; Sugimura et al. 2012). Forced overexpression of let-7c sensitized A549/DDP cells to DDP. Similarly, Ectopic let-7i expression resulted in attenuated chemoresistance to DDP in ovarian and breast cells.
miRNA microarray has been used to screen the distinguished microRNA in A549/DDP and A549. Zhang et al. (2010) reported that miR-186* and miR-136 were downregulated more than 2-fold and four microRNAs were unregulated more than 2-fold in A549/DDP cells compared with A549 cells. Wang et al. (2011) reported that miR-224, miR-886, miR-138, miR-27b and miR-194 were downregulated more than 2-fold and 10 microRNAs were upregulated more than 2-fold. In these two reports, let-7c was not found to be downregulated. We suspect the difference between our result and the two previous reports is contributed by the method to detect the microRNA level because qRT-PCR is a more sensitive technique than miRNA microarray (Chen et al. 2009). And we could not exclude other possible factors responsible for this difference such as resistance index, cell condition.
Further we analyzed possible molecular mechanisms how let-7c modulates DDP sensitivity. For the first time we validated that

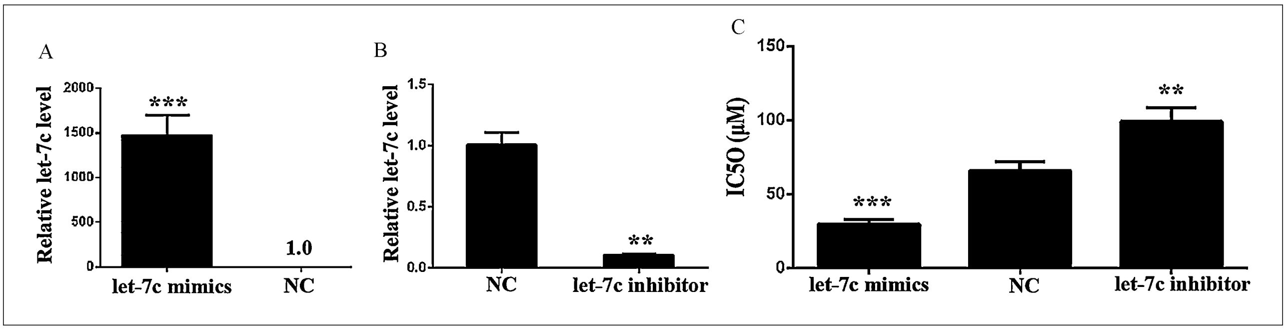

Fig. 2: Effect of let-7c expression on the IC50 of A549/DDP cells to DDP. (A) Expression of let-7c in A549/DDP cells treated with let-7c mimics or NC 24 h after transfection by qRT-PCR. (B) Let-7c level in A549/DDP cells transfected with NC or let-7c inhibitor 24 h after transfection by qRT-PCR. (C) The IC50 of A549/DDP cells to DDP by MTS assay. A549/DDP cells were transected with let-7c mimics, NC or let-7c inhibitor respectively and different concentration DDP was added to A549/DDP cells at 24 h post-transfection. After 24 h incubation, MTS assay was performed. **P < 0.01. ***P < 0.001, compared with NC-treated group.

ABCC2 was a direct target of let-7c by bioinformatics assay, western blot and luciferase report assay. ABCC2, also designated as MRP2 or cMOAT, has been found to be overexpressed in various DDP-resistant human cancer cell lines (Chen and Tiwari 2011). Overexpression of ABCC2 in HEK-293 cells and MDCK cells enhanced the resistance to a number of anticancer agents, including DDP (Cui et al. 1999). ABCC2 Knockdown by RNA interfering or ABCC2 inhibitor led to an increased sensitivity to DDP (Wang et al. 2000; Horibe et al. 2011). To date, few studies have explored the role of ABCC2 in A549/DDP cells. Horibe et al. (2011) reported that ABCC2 protein expression was comparable between A549 and A549/DDP cells. In contrast, here we found that ABCC2 expression was higher in A549/DDP cells than in A549 cells. Our A549/DDP cell line was established from A549 cells by stepwise selection with increasing concentration of DDP (Wang et al. 2000), whereas the A549/DDP cell line used in Horibe's study was generated from A549 cells exposed to 4 μm/L DDP for 3 months and further cloned by limited dilution methods. We suspected that two distinct methods to get the two A549/DDP sublines were responsible for the differential profile of ABCC2 protein. Furthermore, knockdown of ABCC2 by si-ABCC2 led to increased sensitivity of A549/DDP cells to DDP, providing support for the role of ABCC2 in the acquired DDP resistance of A549 cells.

Another mechanism probably responsible for DDP resistance is the overexpression of Bcl-XL, an antiapoptotic protein in the Bcl-2 family (Ikuta et al. 2005). The Previous studies have demonstrated that overexpression of Bcl-XL provided tumor cells with resistance to a variety of anti-cancer drugs including DDP (Minn et al. 1995). Let-7c modulated the expression of Bcl-XL by directly interacting with the 3′UTR of Bcl-XL (Shimizu et al. 2010). Furthermore, si-Bcl-XL increase A549/DDP cells sensitivity to DDP (Lei et al. 2007). Similarly to previous findings, in the present study we demonstrated that let-7c targeted Bcl-XL and then increased the sensitivity of A549/DDP cells to DDP.

Our study identified ABCC2 and Bcl-XL as targets of let-7c in A549/DDP cells. As we know, a single miRNA could target multiple messenger RNAs (Bartel 2004). Let-7c modulated c-myc (Nadiminty et al. 2012) or has been predicted to target other genes by bioinformatics analysis (Targetscan or miRanda), such as STAT3, SLC31A1 or cyclin D1. Overexpression of these genes has been shown to be involved in DDP resistance (Stewart 2007). Therefore, our study does not exclude the possibility that

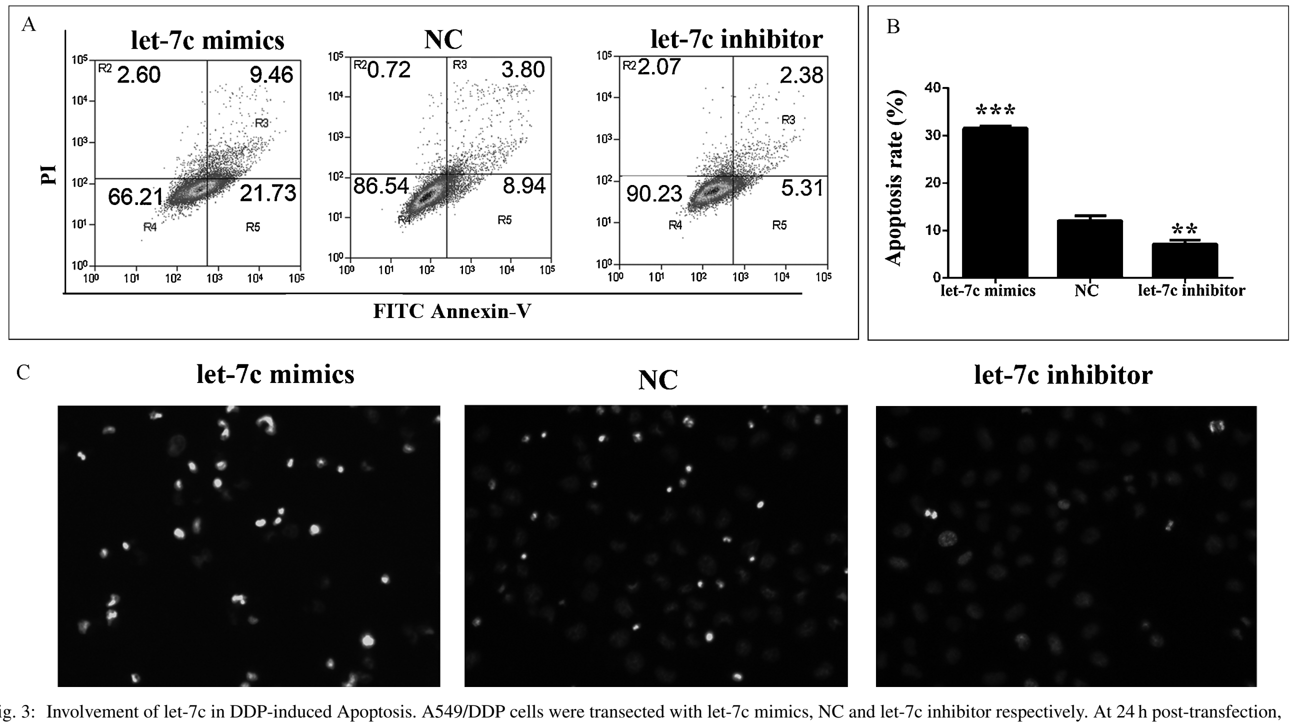

Fig. 3: Involvement of let-7c in DDP-induced Apoptosis. A549/DDP cells were transected with let-7c mimics, NC and let-7c inhibitor respectively. At 24 h post-transfection, DDP was added to A549/DDP cells with a final concentration of 25 μM. After 24 h incubation, cells were treated for flow cytometry and Hoechst 33342 staining analysis. (A) The representative Flow cytometry results of three independent experiments. (B) The apoptosis rate by Flow cytometry. (C) The representative image by hoechst 33342 staining. **P < 0.01. ***P < 0.001, compared with NC-treated group.

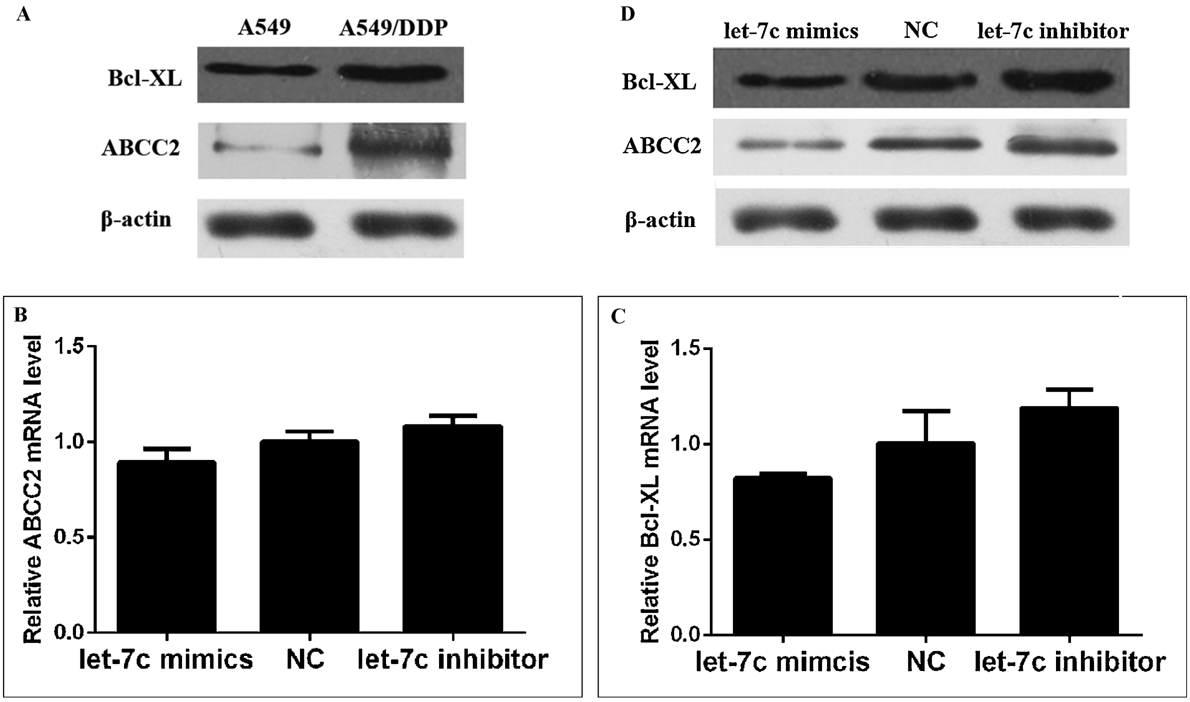

Fig. 4: ABCC2 and Bcl-XL were regulated by let-7c. (A) ABCC2 and Bcl-XL protein levels detected by Western blot in A549 and A549/DDP cells. (B, C) ABCC2 (B) and Bcl-XL (C) mRNA levels detected by qRT-PCR in A549/DDP cells transfected with let-7c, NC or let-7c inhibitor 48 h after transfection. (D) Western blot analysis of ABCC2 and Bcl-XL protein levels in A549/DDP cells transfected with let-7c, NC or let-7c inhibitor for 48 h.

additional target genes play roles in the function of let-7c in A549/DDP cells.

Taken together, we demonstrate the role of let-7c in contribution to sensitivity of A549/DDP cells to DDP. This effect may be mediated by, at least in part, targeting ABCC2 and Bcl-XL. With the potential of miRNA therapy, restoring let-7c expression may constitute a novel therapeutic strategy to sensitize DDP resistance.

4. Experimental

4.1. Cell lines and cell culture

Human lung adenocarcinoma cell line A549 was purchased from American Type Culture Collection (ATCC) and its cisplatin-resistant subline (A549/DDP) was obtained from Cancer Institute and Hospital, Chinese Academy of Medical Sciences. Cells were maintained in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C with 5% CO2. For A549/DDP cells, 6 μM DDP was added to maintain the drug-resistant phenotype. Three replicate tests were performed for all the experiments.

4.2. Transfection

Cells were transfected with let-7c mimics (100 nM), let-7c inhibitor (100 nM), negative control (NC, 100 nM), small interfering RNA targeting ABCC2 (si-ABCC2, 100 nM), small interfering RNA targeting Bcl-XL (si-Bcl-XL, 100 nM) and si-control (si-control, 100 nM) respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. All these products were purchased from GenePharma (Shanghai, China). The target sequences of si-ABCC2 and si-Bcl-XL are GCTGGCCTTTAGTCAACTACA and GGAGATGCAGGTATTGGTG, respectively, according to the former reports (Lei et al. 2007; Xie et al. 2010).

4.3. MiRNA target prediction

Identification of potential miRNA targets of let-7c was conducted using Targetscan (http://www.targetscan.org) or miRanda (http://www.microrna.org/microrna/home.do).

4.4. Total RNA extraction and quantitative RT-PCR (qRT-PCR)

TRIzol® reagent (Invitrogen) was used to extract total RNA, including miRNAs. Expression of ABCC2 and Bcl-XL were measured by quantitative RT-PCR (qRT-PCR) using SYBR green I (invitrogen) with ABI7500 system (Applied Biosystems, CA, USA) as described previous. The primer sequences for qRT-PCR were as following: ABCC2: forward, 5′-CTCACTTCAGCGAGACCG-3′, reverse, 5′-CCAGCCAGTT CAGGGTTT-3′; Bcl-XL: forward, 5′-GGAGCTGGTGGTTGACTTTC-3′; reverse, 5′-ACAATGCGACCCCAGTTTAC-3′; β-actin: forward: 5′-CACCCAGCACAATGAAGAT-3′; reverse: 5′-CAAATAAAGCCATGCC AAT-3′ (Brantley et al. 2008; Xie et al. 2010).

Let-7c expression was determined by a miRNA-specific Taqman MiRNA Assay kit (Applied Biosystems, Foster City, CA, USA). U6 small nuclear RNA was used as an internal control. The fold-change for miRNA and mRNA expression was calculated using $2^{-\Delta\Delta Ct}$ Method (Livak and Schmittgen 2001).

4.5. MTS assay

Cells were seeded into 96-well plates (3×10^3 viable cells/well) and allowed to attach overnight. After cellular adhesion, freshly prepared anticancer drugs including DDP were added (DDP: 0, 6.25, 12.5,25, 50, 100 μM). After 24, cell viability was measured using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS) kit (Promega, Madison, USA) following the manufacturer's instruction and 50% inhibitory concentration (IC_{50}) to DDP were calculated as previously described (Wang et al. 2010). The absorbance at 490 nm (A490) of each well was read in a Multiskan Ascent 354 microplate reader (Thermo, Labsystems).

4.6. Flow cytometry

Cells were harvested and stained with Annexin V-FITC (BD Pharmingen, Heidelberg, Germany) according to the manufacture's protocol. A fluorescence-activated cell-sorting (FACS, Dickinson, USA) assay was performed immediately after staining.

4.7. Hoechst 33342 staining

Cells were fixed with 4% formaldehyde in PBS for 10 min at 4 °C and then incubated with 5 μg/mL Hoechst 33342 (Sigma, St. Louis, USA) to stain the nuclei for 5 min. After washed with PBS, the apoptotic cells were observed under a fluorescence microscope (Leica TCS SMD FCS, Leica, Germany). Cells exhibiting condensed chromatin nuclei were judged as apoptotic cells.

4.8. Western Blot

Cells were lysed in RIPA buffer with 1 mM PMSF (Sigma, St. Louis, USA). The total protein concentration was determined by BCA Protein Assay Kit

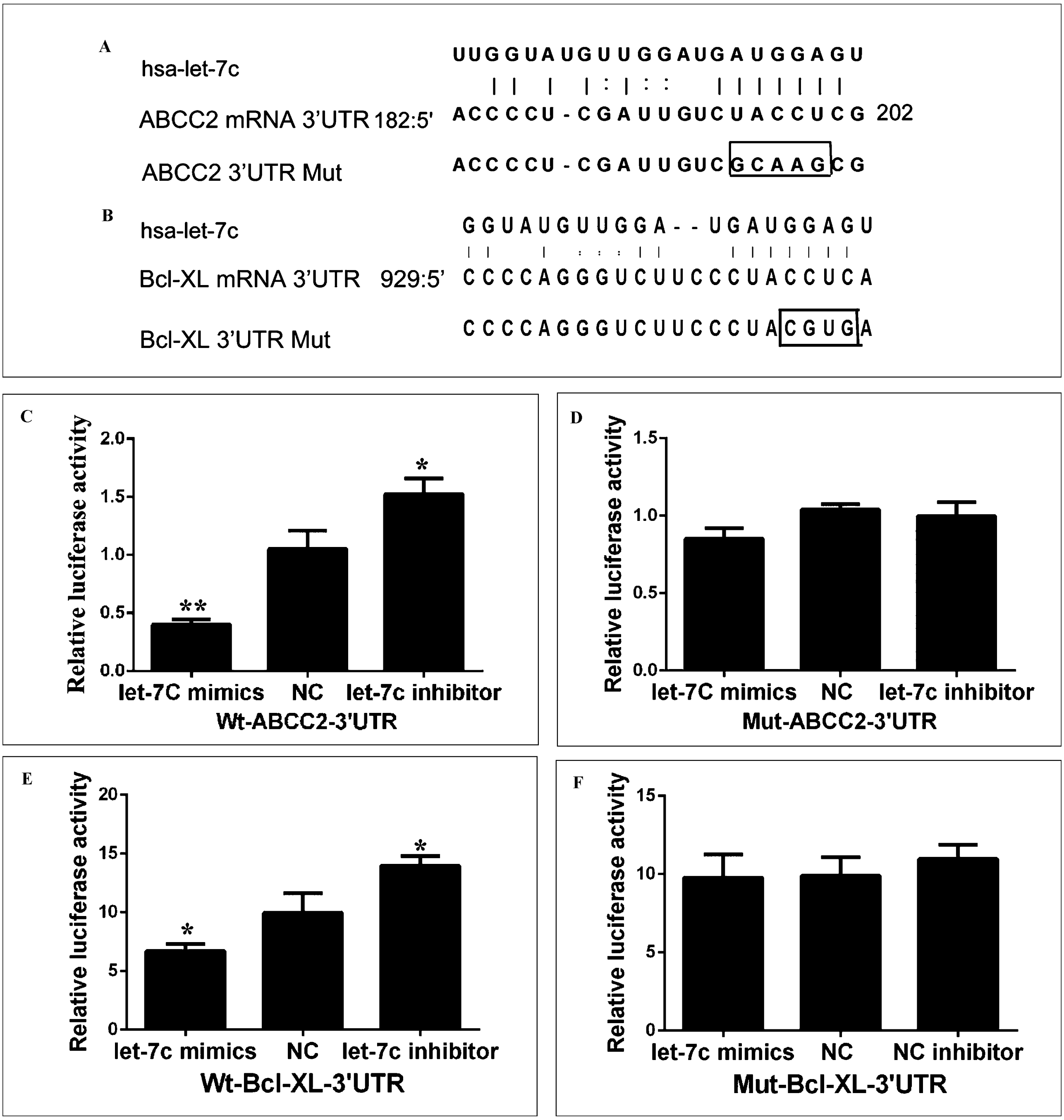

Fig. 5: ABCC2 and Bcl-XL are direct targets of let-7c. (A, B) Schematic of predicted let-7c site in the 3′UTR of human ABCC2 (A) and Bcl-XL (B). (C-F) 200 ng of Wt-ABCC2 (C), Mut-ABCC2 (D), Wt-Bcl-XL(E) or Mut-Bcl-XL (F) plus 20ng pRL-TK were transfected into A549/DDP cells with let-7c mimics, NC or let-7c inhibitor as indicated. After 24 h, luciferase activity was measured. *P < 0.05, **P < 0.01, compared with NC-treated group.

(Pierce, Rockford, Italy). Western blot was performed as described previously (Meng et al. 2007). The following primary antibodies were used: rabbit polyclonal antibody against ABCC2 and Bcl-XL, mouse monoclonal antibody against β-actin (all from Cell Signaling Technology, Beverly, USA).

4.9. *Construction of 3′UTR reporter plasmids*

For reporter gene assays, the 3′UTR sequences of ABCC2 and Bcl-XL containing the putative let-7c-binding sites were amplified by PCR from gDNA and inserted into the pGL3.0 control vector (Promega, Madison, WI, USA) using the XBA I site. Amplification of 3′UTR of ABCC2 and Bcl-XL used the following primer sequences: ABCC2: Forward, CCATGGGTTA-GAAAAGGACT; Reverse, TGAAATTCAGGACAGTGGTTG; Bcl-XL: Forward: ACCATACTGAGGGACCAACT; Reverse: GAGCAGAAGA-GAGAGGGAGG. The mutated 3′ UTR fragments of ABCC2 and Bcl-XL were synthesized after point mutations were made in several bases within the binding sites. All constructs were confirmed by sequencing.

4.10. *Dual luciferase reporter assay*

A549/DDP cells were seeded in 24-well plates at 5×10^4 cells/well and allowed to grow overnight. 200 ng luciferase reporter plasmid plus 20 ng pRL-TK (Promega, Madison, WI, USA) were transfected combination with let-7c or NC. Luciferase activity was measured at 24 h after transfection using the dual luciferase reporter assay system (Promega, Madison, WI, USA) on a SIRIUS luminometer (Berthold Detection System, Pforzheim, Germany).

4.11. *Statistical analysis*

Data are expressed as means ± SD, unless otherwise stated. The differences between two groups were analyzed by using a Student's two-tailed t test. P-

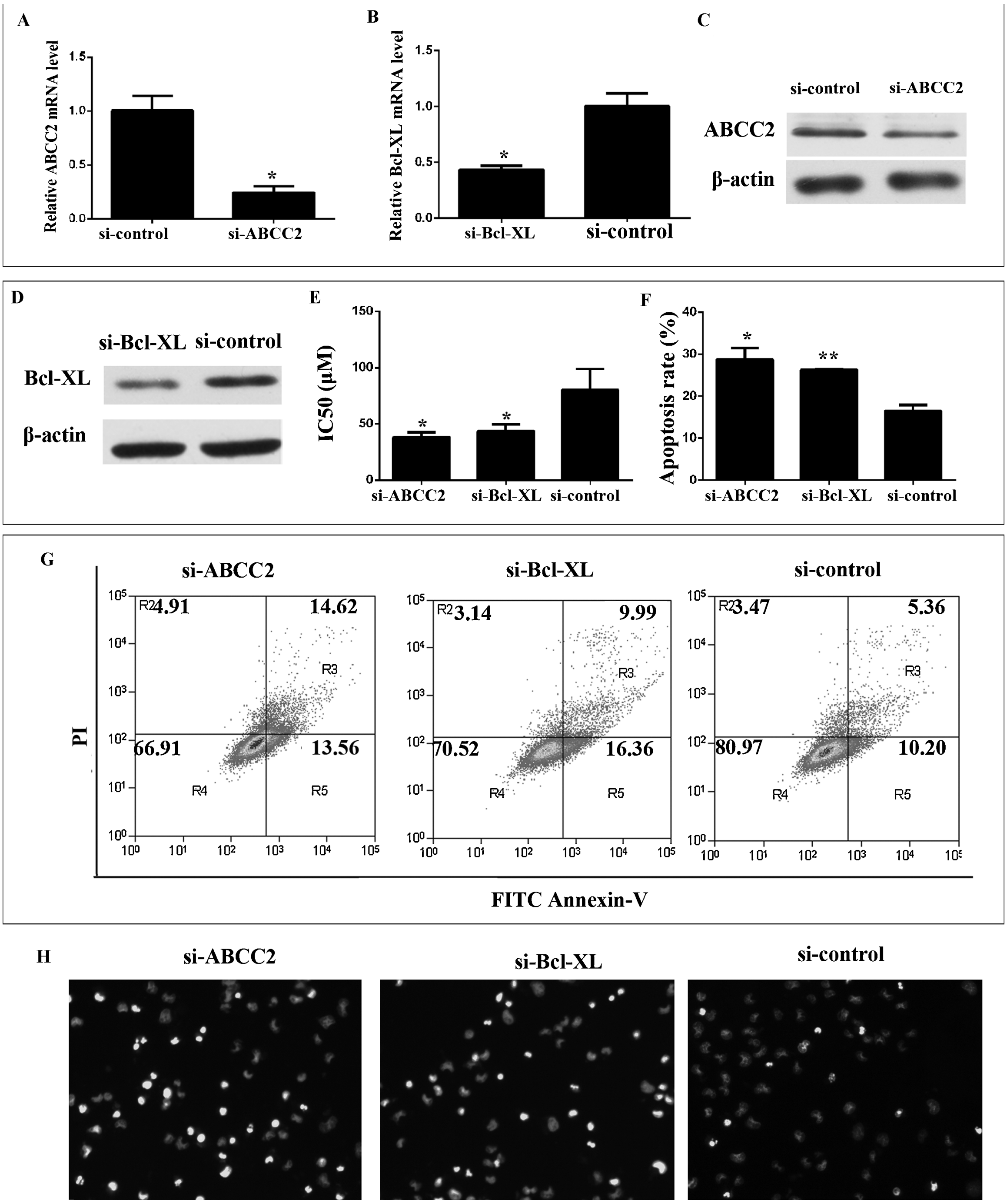

Fig. 6: ABCC2 and Bcl-XL knockdown mimics the effect of let-7c on sensitivity of A549/DDP cells to DDP. A549/DDP cells were transfected with si-ABCC2, si-Bcl-XL or si-control. At 24 h after transfection, cells were treated with different concentration DDP (MTS assay) or 25 μM DDP (Flow cytometry) or 25 μM DDP (Hoechst 33342 staining) for additional 24 h. (A, B) ABCC2 (A) and Bcl-XL (B) mRNA levels were detected by qRT-PCR 48 h after transfection with siRNA as indicated. (C, D) ABCC2 (C) and Bcl-XL (D) protein levels were detected by Western blot 48 h after transfection with siRNA as indicated. (E) MTS assay was used to measure the IC50. (F-H) Apoptosis was assayed by Annexin V-FITC Flow cytometry (F, G) and Hoechst 33342 staining (H). *$P < 0.05$, **$P < 0.01$, compared with si-control-treated group.

value <0.05 was considered to be statistically significant. Statistical analysis was performed using SPSS18.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (No. 30801421, 30901834, 81001476, 81072706), Scientific Foundation of Hunan (No.11K073, 10JJ4020), 863 Project (No. 2009AA022710, 2009AA022703, 2009AA022704) and NCET-10-0843.

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