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MicroRNA-98 sensitizes cisplatin-resistant human lung adenocarcinoma cells by up-regulation of HMGA2

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This study was done to explore the role of microRNA-98 (miR-98) in cisplatin sensitization in human lung adenocarcinoma cell line. Differential expressions of miRNAs were analysed between cisplatin-resistant human lung adenocarcinoma cell line A549/DDP and its parental cell A549 by miRNAs microarray, of which 14 miRNAs were showed to be significantly (> 2-fold) up-regulated and 8 miRNAs had marked down-regulation (< 0.5-fold) in A549/DDP cells compared with in A549 cells. MiR-98, a member in the let-7 family, acts as a negative regulator in the expression of HMGA2 (high mobility group A2) oncogene, and it has been shown to have a nearly 3-fold decrease in A549/DDP cells. We found that elevated expression of miR-98 led to a higher sensitivity of A549/DDP cells to cisplatin, and the protein level of HMGA2, was clearly up-regulated in both A549/DDP and A549 cells by miR-98. Moreover, both Bcl-XL and Bcl-2, were down-regulated in the Pre-miR-98$^{(TM)}$ transfectants cells. We for the first time demonstrated that the expression of miR-98 increases cells spontaneous apoptosis and sensitizes cells to cisplatin at least in part via HMGA2 up-regulation. Our findings provided insight into some specific miRNAs in lung cancer as potential therapeutic targets.

1. Introduction

Lung cancer kills more people than any other cancer worldwide (Henschke et al. 2008). The high mortality from this disease stems from the lack of effective responses to conventional therapies in advanced cancer patients, substantiating the need to develop more effective targeted therapies and chemoprevention (Tessema et al. 2008). Non-small-cell lung cancer (NSCLC) constitutes approximately 80% of all lung cancers, with 40% being at an advanced stage at the time of diagnosis. Cisplatin (DDP), a commonly used therapeutic agent in NSCLC, is the standard regimen used in the first-line treatment of advanced NSCLC. Systemic chemotherapy is warranted, but is hampered by the chemoresistance of most NSCLC (Nishio et al. 1999). Clinical multidrug resistance to chemotherapeutic agents is a major obstacle to potentially curative treatments for advanced NSCLC. Thus, new methods to improve the clinical response to chemotherapy are required.

MicroRNAs (miRNA) are small, non-coding RNA molecules identified in plants, animals, and viruses and are primarily involved in genesilencing (Du et al. 2005). Recently verification for translation regulation by microRNPs (micro-ribonucleo proteins) oscillates while repression and activation was proposed (Vasudevan et al. 2007). MiRNAs play a key role in regulating diverse cellular processes that include development, differentiation, cell growth, apoptosis, viral infection, and metabolism (Ambros 2004). Expression profiling of miRNA has revealed signatures associated with cancer classification, diagnosis, staging, and progression, as well as prognosis and response to treatment (Calin et al. 2004, 2006; Castoldi et al. 2006). The discovery of roles of miRNA in the initiation and progression of human cancer may provide additional targets for anticancer treatments. Gene therapy for malignant disease is a promising approach. Recently, accumulating evidence has indicated that altered miRNA level resulted from mutation or aberrant expression was correlated with various human cancers (Esquela-Kerscher et al. 2006; Harfe 2005; Hwang et al. 2005; Chen et al. 2004). It is suggested that the abnormally expressed miRNAs in human cancers target transcripts of essential protein-coding genes involved in tumorigenesis, including oncogenes and tumor-suppressor genes (Calin et al. 2004). While the role of miRNA in cancer development is being extensively investigated and becomes increasingly well defined, their involvement in tumor cell response to chemotherapy has only been suggested by a few reports. Si et al. (2007) found that suppression of miR-21 using antisense oligonucleotides sensitized MCF-7 cells to the anti-cancer drug topotecan. In addition, it was shown that gemcitabine and 5-FU treatment could alter the expression of a surprising number of diverse miRNAs in cholangiocarcinoma tumor cells and colon cancer cells, respectively (Meng et al. 2006; Rossi et al. 2007). Moreover, the modulation of some miRNAs increases the sensitivity of cholangiocarcinoma tumor

Abbreviations: A549/DDP, cisplatin-resistant human lung adenocarcinoma cell line; DDP, cisplatin; HMGA2, high mobility group-A2; Bcl-XL, B-cell lymphoma/leukemia-extra long; Bcl-2, B cell lymphoma/leukemia-2; miRNA; microRNA; MTT, 3-[4,5-Dimethylthiazolzyl]-2,5-diphenyl tetrazolium bromide; Q-RT-PCR, Quantitative real-time reverse transcription polymerase chain reaction.

cells to gemcitabine *in vitro*. More recently, the effect of miRNAs on chemotherapy was systematically studied as part of the Molecular Targets Program aimed at elucidating molecular targets and understanding mechanisms of chemosensitivity and chemoresistance (Blowe et al. 2007). However, there have been little available data on the potential role of miRNAs in the chemoresistance of lung cancer.

In this study, experiments were designed to screen a set of differentially expressed miRNA between cisplatin-resistant human lung adenocarcinoma cells A549/DDP and parental A549 cells. MiR-98 as a potential target against cisplatin resistance in lung adenocarcinoma cells was investigated.

2. Investigations and results

2.1. miRNA differential expression between A549 and 549/DDP cell

To investigate differential expression in miRNA between A549 and 549/DDP cells, MiRCURYTM LNA arrays were used for sensitive, specific, and comprehensive profiling of miRNAs. Every sample was repeated four times to improve miChip accuracy. Fourteen upregulated miRNAs (> 2) and 8 downregulated miRNAs (< 0.5) were observed in A549 cells compared to that in 549/DDP. Differentially up-regulated miRNAs included hsa-miR-382 and hsa-miR-801, and differentially down-regulated miRNAs included hsa-miR-183, hsa-miR-98, and hsa-miR-886-3p. The heat map showed that miR-98 is nearly 3-fold decreased in A549/DDP cells (Table 1) The specific targets of these miRNAs indicated that they might play important roles to resistance to cisplatin.

Table 1: miRNA microarray expression data between A549 and A549/DDP cells

A. Down-regulated miRNAs

miRNA name	Fold change	P-value
hsa-miR-601	0.4820	0.0085
hsa-miR-10a*	0.3894	0.0055
hsa-miR-195*	0.5003	0.0131
hsa-miR-876–3p	0.4154	0.0074
hsa-miR-186*	0.4278	0.0039
hsa-miR-132*	0.4357	0.0211
hsa-miR-147	0.3927	0.0087
hsa-miR-198	0.4589	0.0037
hsa-miR-136	0.4190	0.0027
hsa-miR-518c*	0.3898	0.0026
hsa-miR-551b*	0.3653	0.0067
hsa-miR-125a-3p	0.4558	0.0124
hsa-miR-106b*	0.4723	0.0045
hsa-miR-181d	0.2845	0.0003

B. Up-regulated miRNAs

miRNA name	Fold change	P-value
hsa-miR-183	3.5666	0.0023
hsa-miR-98	2.9508	0.0065
hsa-miR-320	2.2583	0.0068
hsa-miR-886-3p	4.6915	0.0089
hsa-miR-374a	3.2254	0.0007
hsa-miR-886-5p	32.5087	0.0076
hsa-miR-541*	4.6520	0.0031
hsa-miR-155*	3.0760	0.0048

Shown were all arrayed in which the miRNAs change significantly, and the direction of change (A. down-expression or B. up-expression) in A549 relative to A549/DDP cells. MiR-98 had a nearly 3-fold decreased in A549/DDP cells comparing with parental A549 cells.

2.2. Computational identification of microRNA target

We used TargetScan (http: //www. targetscan.org) and PicTar (http: //pictar. bio. nyu. edu) software to determine the predicted accessible miRNA binding sites,and the functional importance of a predicted miRNA/mRNA interaction can be validated. We identified that miR-98 may be able to bind to 3'UTR of HMGA2 and BCL-2 family mRNA (Table 2).

2.3. Expression of HMGA2, Bcl-XL and Bcl-2 in cisplatin-resistant cells

To understand the mechanisms of cellular resistance to cisplatin in lung adenocarcinoma cells, Western blot analysis was used to detect HMGA2, Bcl-XL and Bcl-2 protein expressions. As shown in Fig. 1A, B, Bcl-xl and Bcl-2 showed a higher expression and HMGA2 had a lower expression level in A549/DDP cells compared with that in A549 cells.

2.4. Validation of miR-98 expression in Pre-miR-98$^{(TM)}$ vector transfected A549/DDP cells

PcDNATM6.2-GW/miR linear vector was sequenced to verify the miR-98 inserted template. The vector carrying luciferase gene was transfected into A549/DDP cells. An inverted fluorescence microscope (Olympus, Tokyo, Japan) was used to detect the transfection efficiency. Q-RT-PCR was used to assess the miRNA-98 expression level in A549/DDP cells. This method has proved to be quantitative and sensitive and is specific enough to discriminate the single nucleotide difference between miRNAs. The miR-98 expression levels in all experimental groups were compared and validated using Q-RT-PCR (SYBR MicroRNA Reverse Transcription Assay from Ambion). The Q-RT-PCR results showed that miR-98 expression level in miR-98 transfected cells was higher than that in negative miRNA transfected cells and untreated cells, which indicated that miR-98 vector were constructed and transfected successfully (Fig. 2).

2.5. MiR-98 expression may lead to HMGA2 upregualetion and Bcl-XL and Bcl-2 down regulation in mRNA level in A549/DDP cells

A549/DDP cells were tranfected with either Pre-miR-98$^{(TM)}$ vector or negative miRNA and the expression level of HMGA2, Bcl-XL and Bcl-2 mRNA was assessed by Q-RT-PCR. It was found that the HMGA2 mRNA expression in miR-98 transfected cells was significantly increased than that in the negative miRNA or untreated groups, while Bcl-XL, Bcl-2 mRNA expression did not show significant difference in those three groups (Fig. 3A, B).

2.6. MiR-98 regulates HMGA2, Bcl-XL and Bcl-2 expression level in A549 and A549/DDP cells

To determine whether down regulation of HMGA2 expression by miR-98 is mediated through a HMGA2 3'UTR, a luciferase reporter gene linked to the HMGA2 3'UTR in the psiCHECK2 vector, psiCHECK2- HMGA2 3'UTR, was used. A construct containing a mutant form of the miR-98 (Mut-miR-98), psiCHECK2- HMGA2 3'UTR, AS-miR-98 and the empty vector psiCHECK2 were used as control. When A549/DDP cells were transfected with miR-98, the luciferase expression from the HMGA2 3'UTR was reduced to 59% of that of psiCHECK2 control vector. However, Mut-miR-98 treatment did not affect luciferase expression from the the HMGA2 3'UTR (Fig. 4A). To monitor the protein level of HMGA2, Bcl-XL and Bcl-2 protein in miR-98 transfected A549 and A549/DDP cells, Western blot was performed. The negative miRNA had no significant effect on the three protein levels. HMGA2 protein was

Table 2: Predicted targeting of HMGA2 and BCL2 family gene by miR-98 (PicTar)

human Refseq Id	PicTar score	microRNAs with Anchor sites	annotation
NM_003483	23.25	hsa-miR-98	Homo sapiens high mobility group AT-hook 2 (HMGA2), mRNA.
NM_001191	2.12	hsa-miR-98	Homo sapiens BCL2-like 1 (BCL2L1), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA

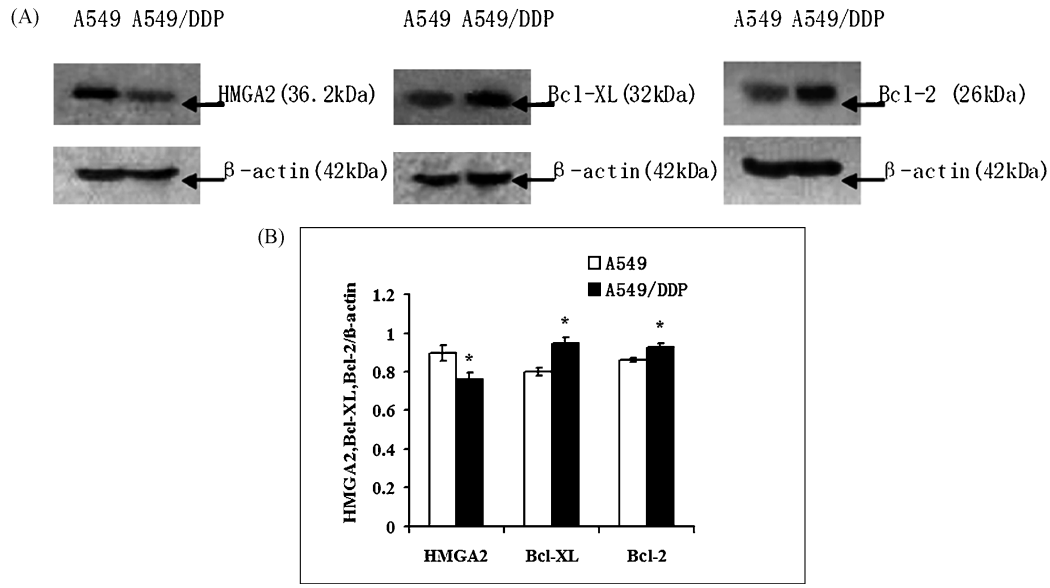

Fig. 1: Protein expression of HMGA2, Bcl-2 in human lung adenocarcinoma cell line A549 and cisplatin-resistant cell line A549/DDP (A) Western blotting detected HMGA2, Bcl-xl and Bcl-2 protein expression. (B) HMGA2 protein expression was decreased and Bcl-xl and Bcl-2 protein expression were increased in A549/DDP cells. Data were Mean ±SD (n=3) * $P < 0.05$ vs. A549 cells.

increased markedly in the cells transfected with miR-98 compared with untreated cells and cells transfected with negative miRNA, whereas Bcl-XL and Bcl-2 expression were found to be decreased in cells transfected with miR-98 (Fig. 4B, C, D, E).

2.7. Up-regulation of HMGA2 increased cellular susceptibility to cisplatin treatment in A549/DDP cells

Recent studies showed that the down expression of HMGA2 protein and over expression of Bcl-2 family could increase tumor cells cisplatin resistance and inhibit apoptosis. To determine whether miR-98 induced HMGA2 protein up-regulation and Bcl-2 family protein down-regulation could influence the sensitivity of cells to cisplatin, cells were subjected to MTT assay for the assessment of cell viability. MiR-98 transfected cells had a significantly higher rate of cisplatin induced cell death than untransfected cells or transfected negative miRNA cells (Fig. 5). After treatment with 0, 0.2, 2, 20, 200 μg/ml of cisplatin, the inhibitory rate of transfected miR-98 cells, untransfected cells and negative miRNA transfected cells were: 0.2 μg/ml DDP ($18.12 \pm 0.78\%$ vs. $3.39 \pm 0.24\%$, 3.44 ± 0.45 % and $4.41 \pm 0.65\%$), 2 μg/ml DDP ($19.35 \pm 0.95\%$ vs. $5.37 \pm 0.43\%$, $3.78 \pm 0.5\%$ and $5.39 \pm 0.72\%$), 20 μg/ml DDP ($53.24 \pm 1\%$ vs. $28.59 \pm 0.55\%$, $25.2 \pm 0.78\%$ and $26.4 \pm 0.95\%$), 200 μg/ml DDP ($59.76 \pm 0.68\%$ vs. $40.1 \pm 0.73\%$, $34.17 \pm 0.88\%$ and $36.2 \pm 0.79\%$). miR-98 transfected cells combined with cisplatin showed significantly lower IC_{50} values than those of negative miRNA transfected cells or untransfected cells (Table 3). Moreover, flow cytometry results demonstrated that apoptosis rate was increased in miR-98 transfected cells in combination with 20 μg/ml cisplatin treatment for 48 h compared with negative miRNA transfected cells or untransfected cells (Fig. 6).

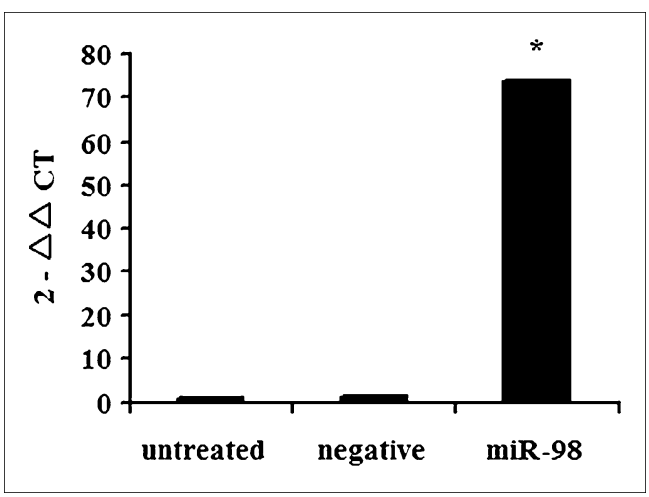

Fig. 2: Validation of miR-98 among untreated cells, negative miRNA transfected cell and Pre-miR-98$^{(TM)}$ transfected cells MiR-98 were increased in A549/DDP cells data were Mean±SD (n=3) * $P < 0.05$ vs. untreated cells or negative miRNA transfected cells.

3. Discussion

Recently research has focused on the role of altered miRNA expression in human malignancies. miRNA expression signatures seem to hold great promise in tumor characterization and could be potential diagnostic and prognostic markers for cancer diagnosis and treatment. In addition, approaches to interfering with miRNA function are considered to offer novel therapeutic opportunities for cancer (Wu et al. 2007). In this study,

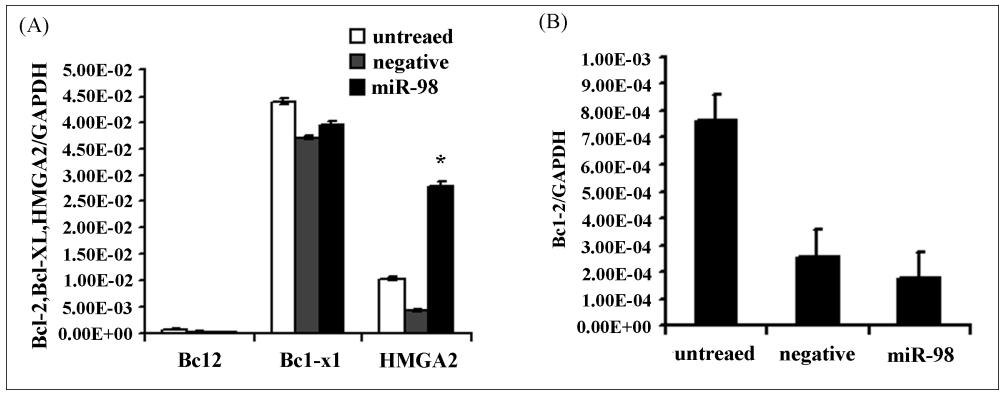

Fig. 3: Validation of HMGA2, Bcl-XL and Bcl-2 mRNA expression among untreated cells, negative miRNA transfected cells and Pre-miR-98$^{(TM)}$ transfected cells (A) HMGA2 mRNA expression was significantly increased at miR-98 transfected cells. Data were Mean±SD (n=3) * $P < 0.05$ vs. Untreated cells or negative miRNA transfected cells. Separated the Bcl-2 mRNA expression into figure (B).

we investigated the role of miRNA in acquiring resistance to chemotherapeutics, which could also serve as potential targets for chemosensitizing strategies.

Dozens of differentially expressed mRNAs and proteins have been identified between A549/DDP cell line and its parental A549 cell line (Lei et al. 2007; Huang et al. 2007). Here we intended to explain the mechanisms of chemoresistance at miRNA level. The model of miRNA-mediated gene silencing has been widely accepted, however, a new model of action of miRNA was also found. Vasudevan et al. (2007) certified that miRNA not only function as negative regulation factor of transcript, but also as positive regulation factor of target gene. One miRNA can target hundreds of downstream target mRNAs or proteins, while one miRNA may also influence the target gene by a different model of action. Accordingly, the identification of some specific miRNAs may provide critical links among those chemoresistance-related mRNAs and proteins and help to delineate a more comprehensive picture of the molecular network underlying chemoresistance of lung adenocarcinoma.

In this study, a set of differentially expressed miRNAs were found between A549/DDP cells and its parental A549 cells. Some differentially expressed miRNAs, such as miR-98 (Hebert et al. 2007), miR-183 (Zhang et al. 2008), miR-382 (Li et al. 2008), and miR-150 (Lin et al. 2008), are well characterized cancer associates. Some "oncomirs" have been shown to play critical roles in cancer biology and chemoresistance. In A549/DDP cells, we observed the downregulation of miR-98. Since miR-98 potentially regulate high mobility group A2 (HMGA2) target genes, and BCL2, BCL2L1 as a culprit of drug resistance in A549/DDP cells, we proposed a role of miR-98 in the development of chemoresistance by regulating HMGA2, BCL2L1 and BCL2.

It is generally accepted that HMGA2 is a small non-histone chromosomal protein, and a member of the HMGA family of architectural transcription factors. HMGA proteins have been implicated in the regulation of transcription, differentiation, neoplastic transformation, integration and expression of viral genomes (Sarhadi et al. 2006). Recently, HMGA2 expression has been found in various malignant tumors. Furthermore, HMGA2 expression has been shown to be associated with enhanced selective chemosensitivity towards the topoisomerase (topo) II inhibitor, doxorubicin, in breast cancer HS578T and salivary Pa-4/ HMGA2 cells (Sgarra et al. 2004). Some researchers verified the relation between cisplatin-resistance and HMGA2 in head and neck squamous cell carcinoma (SCC) cell line, by comparing cell lines grown in normoxic and hypoxic atmospheres (Hebert et al. 2007). Our study demonstrated that transfection of miR-98 into A549 and A549/DDP cells increased protein expression level of HMGA2, which depended on the presence of FXR1 and AGO2. MiRNA switch to translation activation may be related to growth-arrest conditions. Vasudevan's studies also revealed that let-7 induced up-regulation of HMGA2 on cell cycle arrest in HeLa cells (Vasudevan et al. 2007). High sequence homologous is conserved between MiR-98 and let-7. So it may be indicated that miR-98 sensitized A549/DDP cells to cisplatin through the up-regulation of HMGA2.

The proto-oncogene Bcl-2, first discovered in low-grade Burkitt cell lymphomas, is a critical regulator of apoptosis (Boo et al. 2004). Most of chemotherapeutic drugs act primarily by induction of apoptosis. Consequently, the development of resistance of cancer cells to cytotoxic drugs may be a result of resistance to apoptosis. The commitment of apoptosis is largely a mitochondria event controlled by proteins in the Bcl-2 family (Adams et al. 1998; Tahir et al. 2007), which comprise suppress (Bcl-2, Bcl-XL) or promote (Bik, Bax and Bak) apoptosis (Yu et al. 2004). Bcl- XL is a functional and structural homolog of Bcl-2, which also provides protection against a wide variety of chemotherapeutic agents (Adams et al. 1998). Overexpression of Bcl-XL is linked with chemoresistance and occurrence of malignancies including NSCLC (Karczmarek-Borowska et al. 2006). Using both inducible and constitutive expression systems, we identified that miR-98 expression may lead to cisplatin sensitization in A549/DDP cell by decreasing the expression of Bcl-XL and Bcl-2. The findings suggested that the down regulation of Bcl-XL and Bcl-2 proteins by miR-98 is critical not only for tumor development but also for tumor progression and resistance to chemotherapy.

Our studies showed that miR-98 is a good target for cancer therapy, especially for cancers resistant to conventional chemotherapy. Nevertheless, *in vivo* delivery and tumor specificity are challenging issues for the use of miR-98 as an anticancer therapeutic agent. Development of genetic vectors or formulations for *in vivo* delivery of miRNA will be necessary before miRNA can be used as a therapeutic agent.

Table 3: MiR-98 decreased the IC50 value of DDP

Group	IC_{50}
A549	351 ± 7.2 μg/ml
A549/DDP	694.9 ± 9.4 μg/ml
Negative	647.7 ± 10.3 μg/ml
miR-98	42.7 ± 1.3 μg/ml *

IC_{50} value of DDP was decreased after combining with miR-98. ANOVA analysis showed the mean difference is significant at the 0.05 levels. *P < 0.05 compared with A549 cells, A549/DDP cells or negative miRNA transfected cells Mean ± SD, n = 3.

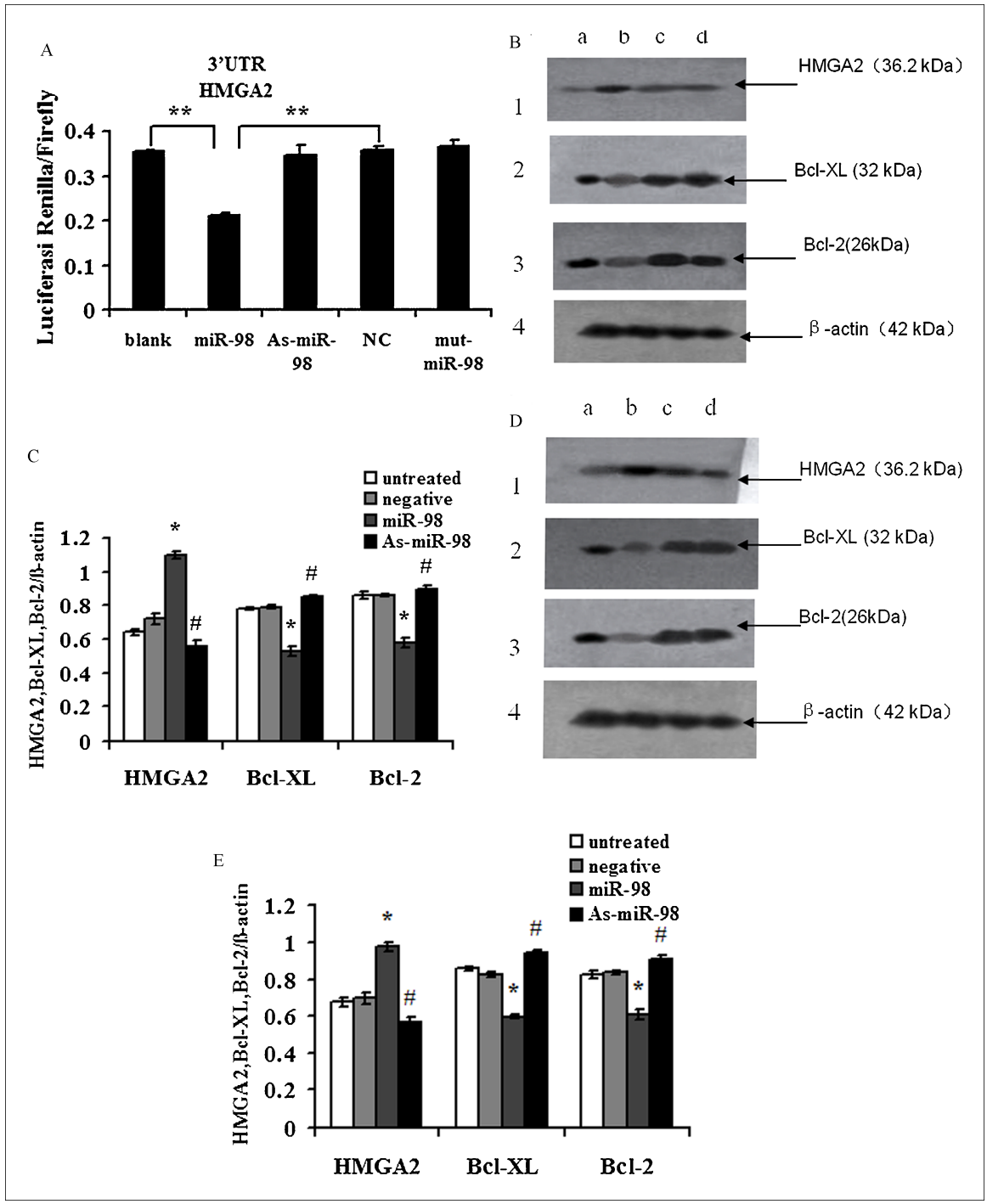

Fig. 4: Effects of miR-98 on HMGA2, Bcl-xl and Bcl-2 protein expression (A) A549/DDP cells were contransfected with renilla luciferase constructs containing the HMGA2 3'UTR and miR-98 or Mut-miR-98 for 48 h. AS-miR-98, NC and the empty vector psiCHECK2 was used as a negative control. Western blotting detected HMGA2, Bcl-xl and Bcl-2 protein expression in A549 cells (B) (C) and A549/DDP cells (D) (E). (a) As-miR-98 transfected cells; (b) miR-98 transfected cells; (c) negative miRNA; (d) untreated. HMGA2 protein expression was increased and Bcl-xl, Bcl-2 protein expression were decreased in cells transfected with miR-98. As-miR-98 treatment attenuated the action of miR-98 in A549 and A549/DDP cells. HMGA2, Bcl-xl and Bcl-2/β-actin ratio. Data were Mean± SD (n=3) * P <0.05 and # P <0.05, compared to other groups (n=3).

4. Experimental

4.1. Cell culture and cultures

The human lung adenocarcinoma cells A549 and the cisplatin-resistant human lung adenocarcinoma cells A549/DDP were purchased from Xiangya Medical College Cell Center (Changsha, China). The cells were routinely maintained in phenol red-free 1640 (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, USA), at 37 °C in a humidified incubator containing 5% CO_2. To maintain the resistance to chemotherapeutics phenotype, cisplatin (QiLu, Shangdong, China. with final concentration of 2 μg/ml) was added to the culture media for A549/DDP cells.

4.2. MiRNA microarray and hybridization

Prior to experimentation, A549/DDP cells were cultured 1 week with cisplatin. Total RNA from A549 and A549/DDP cell lines was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol, where isopropanol was replaced by ethanol for RNA precipitation. RNA quality was measured using denaturing agarose gel electrophoresis. Microarray was performed at KangChen Bio-tech (421 HongCao Rd Building 63, 2nd floor Shanghai, P.R China 200233). Briefly, 5 μg of RNA from each sample was ligated to an RNA-linker (p-rUrUrUdA-Cy3-dye Dharmacon) labeled at 3'-end with 3TM fluorescent T4 RNA ligase overnight at 37 °C, and hybridized respectively on a miRCURYLNATM Array v.10.0 (Exiqon, Ved baek, Denmark) as described. Microarray images

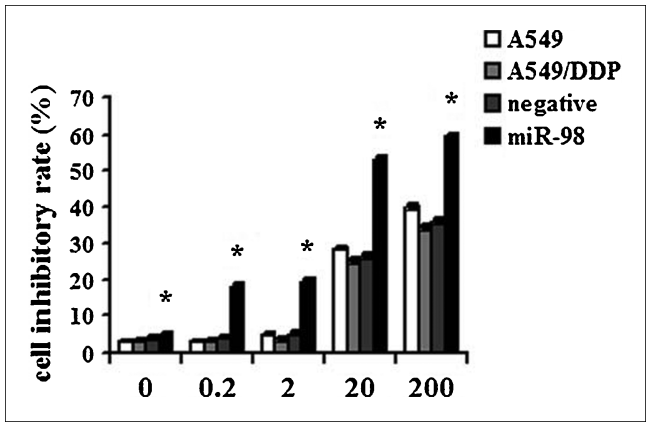

Fig. 5: Inhibition of Pre-miR-98$^{(TM)}$ combined with DDP on cisplatin (DDP)-resistant human lung adenocarcinoma cell line A549/DDP ANOVA analysis shows the mean difference of miR-98 transfected group is significant at the concentration of 0, 0.2, 2, 20, and 200 μg/ml of DDP. Shown are representative of three independent experiments. Data were Mean±SD (n=3) * $P<0.05$ vs. A549 cells, A549/DDP cells or negative miRNA transfected cells combined DDP.

were acquired using a Genepix 4000B scanner (Axon Instruments, Union City, CA) and processed and analyzed with GenePix Pro6.0 software (Axon Instruments) and Excel. GeneSpring 7.2(Silicon Genetic) was used for further data analysis. Changes in expression, either two-fold greater or less, were considered to be differentially expressed. All data used for analysis had a signal-to noise ratio of > 2, an average sum intensity 50% higher than that of the negative control spots, and a regression ratio of < 0.5, as previously reported. All data used for analysis had a signal to noise ratio of > 2, an average sum intensity 50% higher than that of the negative control spots, and a regression ratio of < 0.5, as previously reported.

4.3. *Pre-miR-98$^{(TM)}$ vector construction and transfection*

PcDNATM6.2-GW/EmGFP-miR linear vector carrying luciferase gene was purchased from jima GenePharma (Sanghai, China), Pre-miR-98$^{(TM)}$ insert sequence with the following sense: 5'-TGCTGTGAGGTAGTA-AGTTGTATTGTTGTTTTGGCCACTGACTGACAACAATACCTTACT-ACCTCA-3' and antisense sequences were: 5'-CCTGTGAGGTAGTAA-GGTATTGTTGTCAGTCAGTGGCCAAAACAACAATACAACTTACT-ACCTCAC-3' which can form a hairpin and processed into mature miR-98 5'-TGAGGTAGTAAGTTGTATTGTT-3'. A negative control miRNA-NC with limited homology to any known sequences of human genome was commercially available (GenePharma). Negative control sequence 5'-AAATGTACTGCGCGTGGAGAC-3'. MiR-98 inhibitor containing 5'-AACAAUACAACUUACUACCUCA-3' was chemically synthesized. Plasmid DNA was purified by cesium chloride bromide gradient centrifugation. The purified DNA was diluted to 1 mg/ml and stored at −20 °C until used. A549/DDP and A549 cells at the time of transfection were the cell confluence routinely 80∼85% confluent after being seeded in 6-well plates. Transfection was carried out according to the manufacturer's protocol. Pre-miR-98$^{(TM)}$ (4 μg) or negative plasmid (4 μg) carrying green fluorescent protein (GFP) in 100 μl OPTI-MEM (Invitrogen) were mixed with 10 μl of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) dissolved in 100 μl of OPTI-MEM (Invitrogen) and allowed to stand at room temperature for 20 min. The resulting 200 μl transfection solutions were then added to each well containing 1.8 ml of medium. Forty-eight hours after transfection, fluorescence microscopy detected transfection efficiency.

4.4. *MiRNA target prediction*

The analysis of miRNA predicted targets was determined using the algorithms TargetScan (http://genes.mit.edu/targetscan/) (Lewis et al. 2003) and PicTar (http://pictar.bio. nyu.edu/) (Krek et al. 2005).

4.5. *Luciferase assay*

To generate the luciferase reporter constructs psiCHECK2-HMGA2 3'UTR, complimentary oligonucleotides containing the target site of miR-98. Primer sequence for fishing HMGA2 from human peripheral blood were as following sense: 5'-CCGCTCGAGGGGGCGCCAACGTTC GATTTCTA-3' and antisense sequences: 5'-ATAAGAATGCGGCCGC TCTGTTTTGACCAAACTTTATTACTCATTTAATG-3'. Mut-miR-98 (5'-UAUCGAUCUAAGUUGUAUUGUU-3') was mismatched with HMGA2 3'UTR. Mature antisense oligodeoxynucleotides specifically against miR-98 (AS-miR-98) were used as Inhibitior. FF-luciferase and RL-luciferase were measured using Promega's dual-luciferase reporter assay system (E1910) protocol and detected by a GloMax Luminometer (Promega).

4.6. *Q-RT-PCR*

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Q-RT-PCR was performed using standard protocols on an Applied Biosystem's 7500 HT Sequence Detection System. Briefly, 5 μl of a 1/100 dilution of cDNA in water was added into 12.5 μL of the 2 × SYBR green PCR master mix (Ambion), with 10 nmol/L of each primer in a total volume of 25 μl. For miR-98 gene, the sense and antisense primers were GSP:5'GGGGTGAGG-

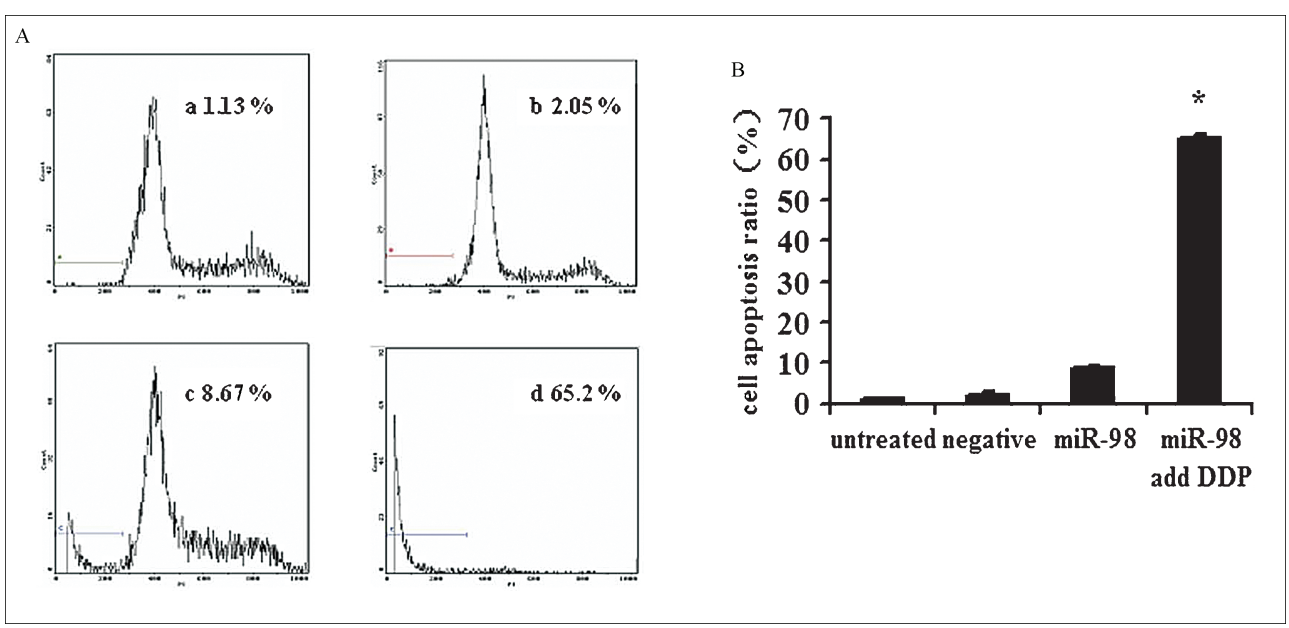

Fig. 6: Fow cytometry analysis of A549/DDP after treatment with miR-98 combined with 20 μg/ml DDP for 48 h (A) Flow cytometry analysis of propidium iodide (PI)-stained cells was carried out. The percentages of hypodiploid cells are shown. The experiments were carried out in triplicate and representative data are shown. (a), untreated cells; (b), negative miRNA transfected cells; (c), miR-98 transfected cells; (d), miR-98 transfected cells added (20 μg/ml); (B) The mean±SD of hypodiploid cells from three determinations are shown. ANOVA analysis is used. Data were MeanμSD (n=3) * $P< 0.05$ vs. miR-98, negative miRNA or untransfected group.

TAGTAAGTTGT3' and R:5'TGCGTGTCGTGGAG TC3'(amplification product 63 bp; GSP stands for specific primer of corresponding miRNA, R represents a primer that matching RT primer). For U6 gene, the sense and antisense primers were F:5'GCTTCGGCAGCACATATACTAAAAT3' and R:5'CGCTTCACGAATTTGCGTGTCA T 3' (amplification product 89 bp). The common cycling conditions were amplified for 15 s at 95 °C, 60 s at 60 °C, for 40 cycles. For HMGA2 gene, the sense and antisense primers were 5'-CTAC TCTGTCCTCTGCCTGTG-3' and 5'-GAGATTGAGATTGAAAGTGCC T-3'(amplification product 104 bp), respectively. The cycling conditions were amplified for 10 s at 95 °C, 15 s at 59 °C, 20 s at 72 °C and 5 s at 85 °C for 40 cycles. For Bcl-XL gene, the sense and antisense primers were 5'-CACCACATCCTCCGTCCA-3' and 5'-AACCCTTCCATACCTGCCA-3' (amplification product 225 bp), respectively. The cycling conditions were amplified for 5 s at 95 °C, 15 s at 59 °C, 20 s at 72 °C, and 5 s at 85 °C for 40 cycles. For Bcl-2 gene, the sense and antisense primers were 5'-CAACTCCCAATACTGGCTC T-3' and 5'-CGGACTTCGGTCTCCTAA A-3'(amplification product 151 bp), respectively. The cycling conditions were amplified for 5 mins at 95 °C, 10 s at 95 °C, 10 s at 59 °C, 20 s at 72 °C and 5 s at 82 °C for 40 cycles. For GAPDH gene, the sense and antisense primers were 5'-GGGAAACTGTGGCGTGAT-3' and 5'-GAGTGGGTGTCGCTGTTG A-3' (amplification product 299 bp), respectively. The cycling conditions were amplified for 5 mins at 95 °C, The cycling conditions were amplified for 10 s at 95 °C, 15 s at 59 °C, 20 s at 72 °C and 5 s at 85.5 °C for 40 cycles. All reactions were run in triplicate and included no template and no reverse transcription controls for each gene. The cycle number at which the reaction crossed an arbitrarily placed threshold (CT) was determined for each gene, and the relative amount of each miRNA to U6 RNA was calculated using the equation $2-\Delta CT$, where $\Delta CT = (CTmiRNA - CT5\,s)$ (Lu et al. 2005). Relative gene expression was multiplied by 106 to simplify the presentation of the data.

4.7. Western blot

Cells were homogenized in a lysis buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS (Sodium dodecyl sulfate), 50 mg/ml aprotinin, 100 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 50 mM sodium fluoride and PBS (Phosphate Buffered Saline, pH 7.4). Cell lysates were centrifuged at 10,000 g for 10 min at 4 °C. Protein content in the supernatants was determined by a BCA protein assay kit (Pierce, Rockford, USA). Equal amounts of lysate protein were run on 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and transferred to PVDF membranes (Amersham-Pharmacia Biotech). After blocking, we incubated PDVF membranes with mouse anti-HMGA2 (ABNOVA 20153 Paseo Del Prado Walnut, CA 91789 USA), anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (MultiSciences Biotech Co,. Ltd) primary monoclonal antibody at dilution of 1:500 and rabbit anti-Bcl-XL (ABZOOM BIOLABS, Inc P.O. Box 726578 Dallas, TX, USA) polyclonal antibody at dilution of 1:200 for overnight at 4 °C and further incubated for 1 h with horseradish peroxidase conjugated anti-mouse (MultiSciences Biotech Co,. Ltd) and anti-rabbit secondary antibody at dilution of 1:1000. Bound antibodies were detected by Enhanced chemiluminescence ECL kit (Santa Cruz Biotechnology) with a Lumino Image Analyzer (Taitec, Tokyo, Japan).

4.8. Flow cytometry

All cells were treated with 20 μg/ml DDP, washed twice with PBS and fixed with 70% ethanol overnight at 4 °C. Then the cells were washed once again with PBS and stained with 800 μl propidium iodide (50 μg/ml, Sigma-Aldrich, St.Louis, USA) at room temperature for 30 min to determine apoptosis by flow cytometry (EPICS-XL, Beckman Coulter, Fullerton, USA) and data were analyzed with Cell Quest software version 3.3 (Becton Dickinson, San Jose, USA).

4.9. MTT analyses

Cells from the above groups were seeded into a 96-well plate at 6 × 103 cells per well. After 24 h, various concentrations of cisplatin were added, and cells were incubated for another 24 h. Then cells were treated with MTT (5 mg/ml, Sigma) for 4 h at 37 °C, and 200 μl dimethyl sulphoxide (DMSO) was added in each well for 10 min. The reaction was optically monitored at 570 nm (A570) using a 96-well microtitre plate reader (Pharmacia, Piscataway, USA). All experiments were carried out in triplicate. The inhibitory rate (IR) of A549/DDP cells was calculatedaccording to the equation as following:$IR(\%) = [A570(control) - A570(drug)]/A570(control) \times 100\%$ Where A570 (control) was the absorbance in Pre-miR-98$^{(TM)}$ or negative vector transfected cells or untreaed cells, and A570 (drug) was the absorbance in the drug-treated group.

4.10. Statistical analysis

Statistical analysis was performed using SPSS software (Version 13.0; SPSS Incorporation, Chicago, USA). Data were expressed as mean ± SD and analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) test, and $P < 0.05$ was considered significant.

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