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# MiR-122 increases sensitivity of drug-resistant BEL-7402/5-FU cells to 5-fluorouracil via down-regulation of Bcl-2 family proteins

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To investigate the changes in drug sensitivity of miR-122 transfected BEL-7402/5-FU cells. MiR-122 and negative miRNA expression vectors were constructed and stably transfected into BEL-7402/5-FU cells. Real-time RT-PCR was used to detect the level of miR-122, Bcl-XL, Bcl-2 and P53 mRNA. Western Blotting was used to detect Bcl-2, Bcl-XL and P53 protein expression. Drug sensitivity of the cells to 5-fluorouracil (5-FU) was analyzed with MTT and flow cytometry. Compared with negative miRNA transfectants or untreated cells, mRNA and protein expression level of Bcl-2, Bcl-XL in stable miR-122 transfectants were decreased. Accordingly, P53 protein expression showed a significant up-regulation; MTT results showed that after incubation with 5-FU, miR-122 transfectants had higher cell inhibitory rates than negative miRNA or untreated cells; flow cytometry results demonstrated that apoptosis rate increased in miR-122 transfected cells, compared with negative miRNA or untreated cells. After addition of 5-FU (10 and 100 μmol/l), miR-122 transfected cells showed higher apoptosis rate than negative miRNA or untreated cells. MiR-122 can specifically down-regulate the expression of Bcl-2 and Bcl-XL, and increase P53 activity in BEL-7402/5-FU cells, which increased cells spontaneous apoptosis and sensitize cells to 5-FU. Therefore, MiR-122 can be used as a potential therapy agent against human hepatoblastoma.

## 1. Introduction

Primary hepatocellular carcinoma (HCC) is one of the ten most common human carcinomas Levin et al. 1995). The treatment options of HCC are limited and prognosis is poor (Lou et al. 2000). Moreover, effective chemotherapeutic agents for this disease have not been developed. Recent studies have focused on drug resistance. Most chemotherapeutic drugs act primarily by inducing apoptosis. Consequently, the 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). Recent studies have focused on the role programmed cell death (apoptosis) plays in both normal and neoplastic growth; certain genes can either suppress (e.g., Bcl-2, Bcl-XL) or promote (e.g., Bik, Bax and Bak) apoptosis. Bcl-XL is an anti-apoptotic member of the Bcl-2 family, which is located mainly on the outer membrane of mitochondria (Takehara et al. 2001). Over expression of Bcl-XL and Bcl-2 is considered to be the mechanism by which tumor cells acquire resistance to apoptosis (Yu et al. 2004; Minn et al. 1995).

Recently, the successful use of miRNAs in down-regulating gene expression in several model systems had led to many attempts to explore this methodology in potentially therapeutic settings (Bartel et al. 2004). By binding to the complementary sequences of their target mRNA (mostly in the 3'UTR), miRNA are able to induce mRNA degradation or translation repression (Hobert et al. 2004). MiR-122 is a liver-specific miRNA that is expressed in the developing liver and at high levels in the adult liver, where it makes up 70% of all miRNAs (Lagos-Quintana et al. 2001; Chang et al. 2004). The best known function of miR-122 is to regulate lipid and cholesterol metabolism (Esau et al. 2006). MiR-122 down-regulation has been reported in rodent and human HCCs (Kutay et al. 2006; Gramantieri et al. 2007), suggesting that its function is associated with hepatocarcinogenesis. However, its role in cancer cell drug resistance still remains unexplored. In this thesis, we investigate the role of miRNA in the resistance of human BEL-7402 hepatocellular carcinoma cells to 5-FU. BEL-7402/5-FU exhibits a considerable deregulation of the miRNA profile and alters expression of miRNA. The mechanistic link of miRNA deregulation and the drug-resistance phenotype of BEL-7402/5-FU were evidenced by a remarkable correlation between specific miRNA expression and corresponding changes in protein levels of their targets, especially for those playing a documented role in cancer drug resistance. Experiments now are designed to better understand the role of Bcl-2 family proteins' over-expression in malignant tumors acquiring resistance to apoptosis. So Bcl-2 family proteins have involved in drug resistance. Therefore, we used the particularly miRNA to interrupt Bcl-2 family mRNAs or proteins and investigated the effect of miRNA on drug sensitization in BEL-7402/5-FU cells.

**Table 1: Validation of miR-122 among untreated cells, negative vector transfected cells and miR-122 transfected cells**

| Group | U6Ct | (hsa-miR-122)Ct | (hsa-miR-122)Ct-U6Ct | [(hsa-miR-122)Ct-U6Ct]other sample-[(hsa-miR-122)Ct-U6Ct]1 | $2^{-\Delta\Delta CT}$ |
|---|---|---|---|---|---|
| Normal | 12.84 | 24.75 | 11.91 | 0 | 1.00 |
| Negative | 12.63 | 24.36 | 11.73 | −0.18 | 1.13 |
| MiR-122 | 12.74 | 24.17 | 11.43 | −0.48 | 1.39 |

**Table 2: Inhibition rate (IR) of miR-122 transfected cells, negative vector transfected cells and untreated cells after 5-FU treatment**

| Group | 5-FU (μmol/ml) | | | |
|---|---|---|---|---|
| | 0.1 | 1 | 10 | 100 |
| IR for normal (%) | 8.85 ± 1.50 | 10.43 ± 1.82 | 12.01 ± 1.10 | 42.65 ± 0.49 |
| IR for negative (%) | 8.41 ± 1.56 | 9.29 ± 2.94 | 12.61 ± 3.43 | 41.13 ± 1.91 |
| IR for miR-122(%) | 23.40 ± 1.83* | 24.00 ± 1.67* | 40.50 ± 2.91* | 65.54 ± 1.00* |

ANOVA analysis showed the mean difference of miR-122 transfected cells were significant at the concentration of 0.1, 1, 10 and 100 μmol/ml of 5-fluroracil (5-FU). ANOVA analysis showed the mean difference is significant at the 0.05 levels. *$p < 0.05$ compared with negative vector transfected cells or untreated cells combined 5-FU (n = 3).

## 2. Investigations and results

### *2.1. Overexpression of Bcl-2 and Bcl-XL in 5-FU resistant cells*

To evaluate the underlying mechanisms of cellular resistance to 5-FU, we performed Western blot analysis to evaluate the expression of Bcl-2 and Bcl-XL. As shown in Fig. 1, BEL-7402/5-FU cells had a higher expression of Bcl-2 and Bcl-XL compared with BEL-7402 cells, while P53 showed a lower expression level in BEL-7402/5-FU cells.

### *2.2. Microarray expression*

MiRCURYTM LNA Arrays was used to detect the different expression profiles of miRNAs in BEL-7402 and BEL-7402/5-FU cells. Every sample was repeated four times to improve miChip accuracy. It showed an image of a microarray hybridized with BEL-7402 and BEL-7402/5-FU RNAs. The profiles of miRNAs expressions were obtained based on the data of the microarray. Compared with BEL-7402 and BEL-7402/5-FU, there were 2 differentially up-regulated (>2) miRNAs and 331 down-regulated miRNAs (<0.5) (Fig. 2). Up-regulated miRNAs included hsa-miR-192 and hsa-miR-194, while down-regulated miRNAs included hsa-miR-122, hsa-miR-195, hsa-miR-199a, hsa-miR-127–3p and so on. The temporal regulation of these miRNAs indicated that they might play important roles in celluar resistance to 5-FU.

### *2.3. Computational identification of microRNA target*

We used TARGETSCAN (http://www. targetscan.org) and PICTAR (http://pictar.bio.nyu.edu) to determine the predicted accessible miRNA binding sites. The functional importance of a predicted miRNA/mRNA interaction can be validated, which led to the conclusion that miR-122 may be bound to 3'UTR of Bcl-2 family mRNA (Fig. 3).

### *2.4. Validation of miR-122 expression results using Real-time RT-PCR*

Real-time RT-PCR was used to confirm that the miRNA-122 expression vector is expressed in comparison to a control vector. This method was proved to be quantitative and sensitive and specific enough to discriminate the single nucleotide difference between miRNAs. To determine the utility of the miRNA transfection and quantitative measure expression ratios, miR-122 untreated cells, negative vector transfected cells and miR-122 transfected cells were identified. The expression levels in untreated cells, negative vector transfected cells and miR-122 transfected cells were compared and validated using Real-time RT-PCR (SYBR MicroRNA Reverse Transcri- ption Assay from Ambion). The Real-time RT-PCR results showed that miR-122 expression in miR-122 transfected cells was higher than negative vector transfected cells and untreated cells (Table 1), which indicated that miR-122 vector were constructed and transfected successfully.

### *2.5. Down-regulation of Bcl-2 family increased the susceptibility of cells to 5-fluorouracil to induce cell death*

Recent studies showed that the over expression of Bcl-2 family proteins could inhibit apoptosis and increase tumor cell's chemotherapeutical resistance. To determine whether miR-122 induced Bcl-2 family protein down-regulation could influence the sensitivity of cells to chemotherapeutical drugs. Cells were subjected to MTT assay for the assessment of cell viability. MiR-122 transfected cells showed a significantly higher rate of 5-FU induced cell death than normal or transfected negative vector cells. Their $A_{570}$ showed a dose-dependent effect on drugs. After treatment with 0.1, 1, 10, 100 μmol/ml of 5-FU, the inhibitory rates of transfected miR-122 cells, negative vector and untreated control changed considerably (Table 2). By $IC_{50}$ value calculation, we found that miR-122 transfected cells combined with 5-FU showed significantly lower value than that of negative vector transfected cells or untreated cells (Table 3). Moreover, flow cytometry results demonstrated that apoptosis rate was increased in transfected miR-122 cells compared with negative vector transfected cells or untreated

**Table 3: MiR-122 decreased the $IC_{50}$ value of 5-FU**

| Group | $IC_{50}$ |
|---|---|
| Normal | 206774.7 ± 1926.8 |
| Negative | 208988.6 ± 1865.6 |
| MiR-122 | 2789.52 ± 186.1* |

$IC_{50}$ value of 5-FU was decreased after combining with miR-122.ANOVA analysis showed the mean difference is significant at the 0.05 levels. *$p < 0.05$ compared with negative vector transfected cells or untreated cells combined 5-FU (n = 3).

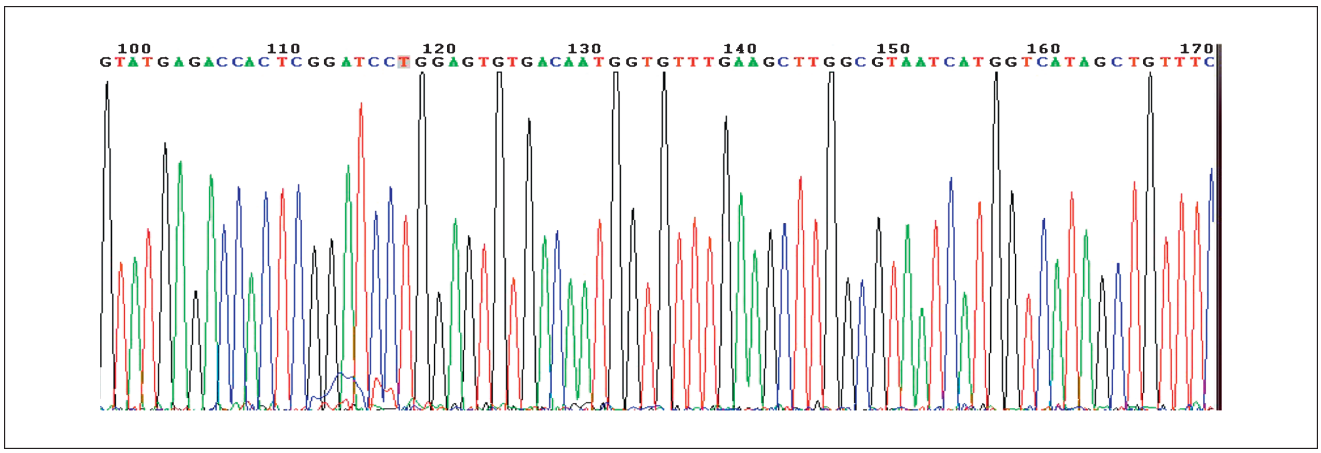


Fig. 1: Expression of Bcl-2, Bcl-XL and P53 in human liver adenocarcinoma cell line BEL-7402 and 5-fluorouracil (5-FU)-resistant cell line BEL-7402/5-FU. (A) Western blotting detected Bcl-2, Bcl-XL and P53 protein expression. 1. BEL-7402; 2. BEL-7402/5-FU. (B) Bcl-2 and Bcl-XL protein expression were increased and P53 protein expression was decreased in BEL-7402/5-FU cells. * $p < 0.05$, vs. BEL-7402 cells

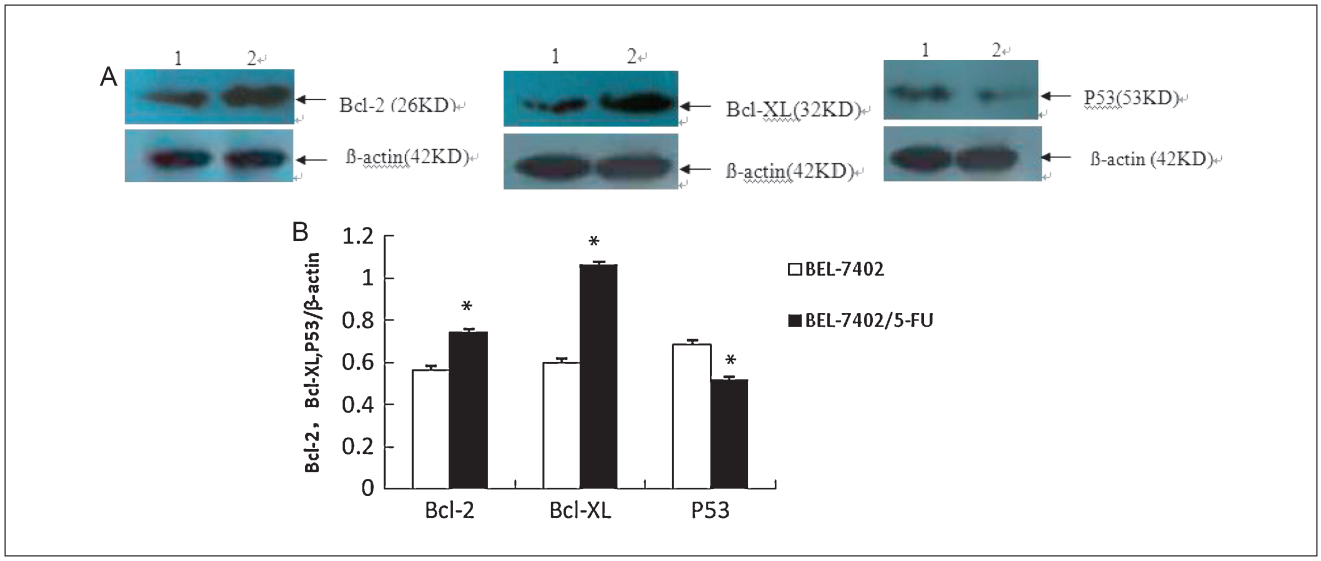


Fig. 2: Genome-wide miRNA profiling using miChip. miRNA expression profiles were monitored across BEL7402 and BEL-7402/5-FU cells. Data were organized according to the expression levels of individual miRNA. The key color bar indicates miRNA expression levels (dark red indicates high expression, whereas, dark green indicates no detectable expression). 7402: BEL-7402; 5-FU:BEL-7402/5-FU

cells ($5.64 \pm 0.35$ vs. $2.23 \pm 0.48$ or $1.03 \pm 0.52\%$, $^*P < 0.05$ (Fig. 4 A). When treated with 5-FU (1 and 10 μmol/ml), miR-122 cells showed higher apoptosis rate than that of negative vector transfected cells or untreated cells ($25.0 \pm 1.22$ vs. $9.19 \pm 0.78$ or $7.55 \pm 0.86\%$; $42.0 \pm 2.0$ vs.$11.9 \pm 1.0$ or $9.22 \pm 0.99\%$, $^*P < 0.05$ (Fig. 4 B, C). Therefore, a significant difference was between miR-122 transfected cells and negative vector transfected cells or untreated cells (Fig. 4 D).

### *2.6. MiR-122 efficiently inhibited Bcl-XL, Bcl-2 and increased P53 mRNA expression*

We transfected BEL-7402/5-FU cells with either negative vector or miR-122 vector. After transfection, the mRNA levels of Bcl-XL, Bcl-2 and P53 were assayed by Real-time RT-PCR. The Bcl-XL mRNA level in miR-122 transfected cells was significantly lower than that of the negative or untreated group, while the P53 mRNA level in miR-122 transfected cells was significantly higher than that of the negative or untreated groups. (Table 4).

### *2.7. MiR-122 inhibited Bcl-2, Bcl-XL and increased P53 activity*

Accumulated results indicated that Bcl-2 family played key roles in drug induced cytochrome c release. Thus, we checked the Bcl-2, Bcl-XL and P53 expression. Bcl-XL and Bcl-2 protein expression levels were higher in normal or negative vector transfected cells than in miR-122 transfected cells (Fig. 5 A, B). Accordingly, P53 was activated in miR-122 transfected cells. These results suggested that miR-122 might induce apoptosis through down-regulation of Bcl-2 family proteins and that the activation of P53 might mediate mitochondrial apoptosis pathway.

## 3. Discussion

Effective therapy for hepatic cancer has not been developed. However, drug resistance, an obstacle to curative treatment of

**Table 4: Validation of Bcl-XL, Bcl-2 and P53 mRNA expression among untreated cells, negative vector transfected cells and miR-122 transfected cells. n = 3. Mean ± SD, $^b p < 0.05$ vs normal**

| Group | Bcl-XL/GAPDH | Bcl-2/GAPDH | P53/GAPDH |
|---|---|---|---|
| Normal | $2.02 \times 10^{-2}$ | $9.67 \times 10^{-4}$ | $1.45 \times 10^{-2}$ |
| Negative | $2.05 \times 10^{-2}$ | $1.43 \times 10^{-3}$ | $2.06 \times 10^{-2}$ |
| MiR-122 | $1.83 \times 10^{-2\,b}$ | $1.31 \times 10^{-3}$ | $2.12 \times 10^{-2\,b}$ |

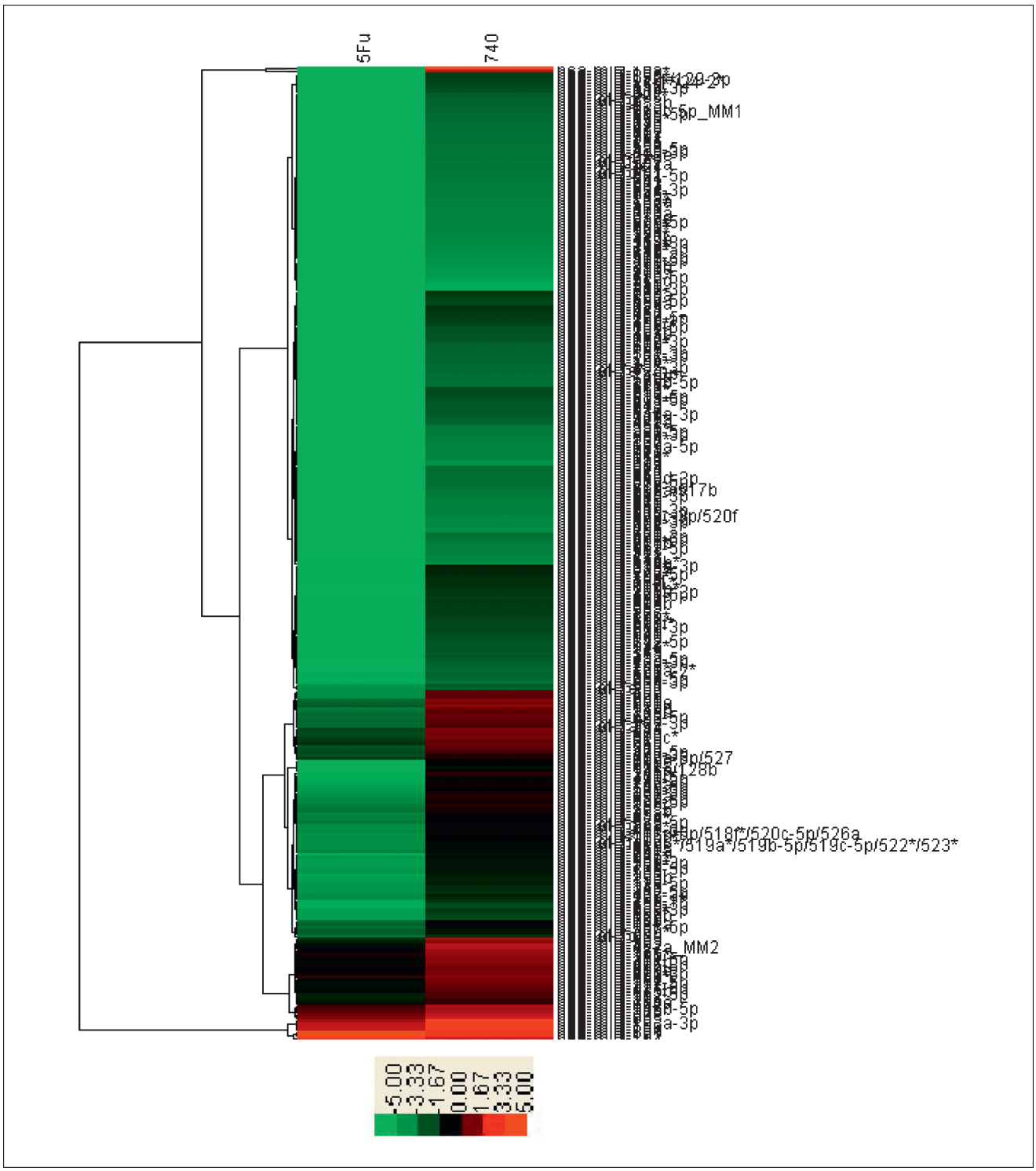


Fig. 3: Predicted targeting of the Bcl-2 family gene by miR-122 (TARGETSCAN)

solid tumors, occurs frequently via suppression of apoptosis, a process controlled by pro and anti-apoptotic members of the Bcl-2 protein family.

Members of the Bcl-2 family proteins can be divided into two subfamilies: anti-apoptotic protein such as Bcl-2 and Bcl-XL, and pro-apoptotic protein such as Bax, Bad and Bid (Decaudin et al. 1997). Among Bcl-2 protein family members, it has been repeatedly shown that over expression of Bcl-2 prevent chemotherapeutic agent-induced apoptosis associated with altered mitochondria membrane potential (Xuhu et al. 2004). The balance between Bcl-2 family members defines whether a cell will live or die. As the ratio between repressors and death promoters in the Bcl-2 family will determine the sensitivity of cells to apoptotic stimuli, the findings suggest that the changed expression patterns of Bcl-2 family proteins in liver cancer cells may be involved in chemo resistance (Luo et al. 1999).

MiRNAs have emerged as a class of genes involved in human tumorigenesis and will likely be identified as mediators of drug resistance. Although clear evidence that miRNAs play a role in drug resistance has yet to be provided, the plethora of mechanisms via Bcl-2 family levels can be modulated by miRNAs, together with the fact that Bcl-2 family are expressed in many tumors, and the evidence that Bcl-2 family expression have been shown to correlate with drug resistance means that evidence

| | predicted consequential pairing of target region (top) and miRNA (bottom) | seed match | site-type contribution | 3' pairing contribution | local AU contribution | position contribution | context score | context score percentile |
|---|---|---|---|---|---|---|---|---|
| Position 1331-1337 of BCL2L2 3' UTR | 5' ...GAGGAAGAAAUUAUU-CACUCCAG... | 7mer-1A | -0.099 | -0.007 | -0.026 | 0.064 | -0.07 | 48 |
| hsa-miR-122a | 3' UGUUUGUGGUAACAGUGUGAGGU | | | | | | | |

Fig. 4: Flow cytometry analysis of BEL-7402/5-FU cells after treatment with 1and 10 μmol/ml 5-FU for 24 h. (A) Flow-cytometirc analysis of propidium iodide-stained cells was performed 14 days after transfection; (B) Flow-cytometirc analysis of propidium iodide-stained cells was performed 14days after transfection and treatment with 1 μmol/ml 5-FU for 24 h; (C) Flow-cytometirc analysis of propidium iodide-stained cells was performed 14 days after transfection and treatment with 10 μmol/ml 5-FU for 24 h. The percentage of hypodiplaid cells are shown. The experiments were performed in triplicate and representative data are shown. (a) untreated cells; (b) negative vector transfected cells; (c) miR-122 transfected cells; (d) untreated cells add 5-FU(1 μmol/ml); (e) negative vector transfected cells add 5-FU (1 μmol/ml); (f) miR-122 transfected cells add 5-FU (1 μmol/ml); (h) untreated cells add 5-FU (10 μmol/ml); (I) negative vector transfected cells add 5-FU (10 μmol/ml); (J) miR-122 transfected cells add 5-FU (10 μmol/ml); (D) The mean ± SD of hypodiploid cells from three determinations were shown. ANOVA analysis the mean difference was significant. $*p < 0.05$, compared with negative vector transfected cells or untreated cells (n = 3).

implicating the role of miRNAs in drug resistance will soon emerge.

Evidence to date indicates that miRNAs negatively regulate the transcription of targeting mRNAs by binding in the 3'UTR. We used TARGETSCAN (http://www. Targetscan.org) and PICTAR (http://pictar.bio.nyu.edu) to determine the predicted accessible miRNA binding Bcl-2 family genes (such as miR-182, miR-143, miR-195, miR-15a, miR-16, miR-122 and so on). MiR-122 is the most abundant miRNA in hepatocytes, and its expression in BEL-7402/5-FU cell lines is much lower than in BEL-7402 (ratio = 0.2 < 0.5). The down-regulation of miR-122 might contribute to the 5-FU resistance of BEL-7402/5-FU cell through inhibiting the expression of Bcl-2 family genes.

To further test this speculation, we observe whether miR-122 can increase cells sensitivity of anti-cancer drugs. 5-Fluorouracil (5-FU) is used to treat advanced primary hepatocellular carcinoma, but drug resistance is usually a big problem. So we demonstrated a dose-dependent effect of the drugs on cell viability. This study revealed that BEL-7402/5-FU cells showed a higher expression of Bcl-2 and Bcl-XL and a lower expression of P53 than BEL-7402 cells. Transfecting miR-122 vector into BEL-7402/5-FU cells resulted in the down regulation of Bcl-2 and Bcl-XL. MiR-122 transfected cells have a significantly greater death rate of 5-FU induced cells than normal or negative vector transfected cells. It could be predicted that miR-122 might inhibit cell proliferation mainly through suppressing the expression of Bcl-2 and Bcl-XL. We also found that miR-122 transfectants are more sensitive to 5-FU than normal or negative vector transfected cells, which might be due to the down regulation of Bcl-2 family proteins (Bcl-2 and Bcl-XL), the increased Bax/Bcl-2 ratio and the activation of P53.

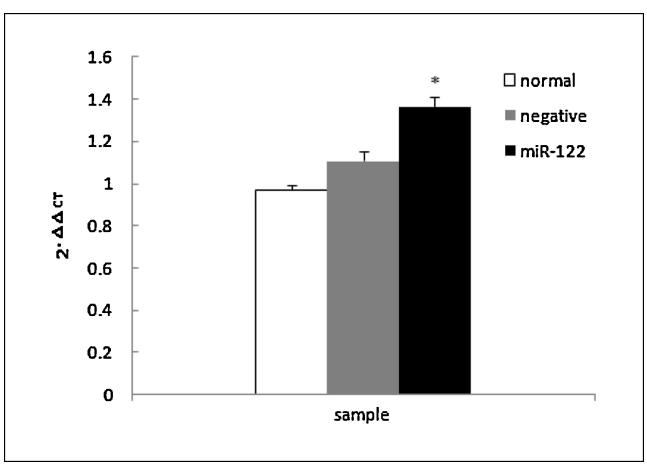


Fig. 5: Effects of miR-122 on Bcl-2, Bcl-XL and P53 protein expression. (A) Western blot detected Bcl-2, Bcl-XL and P53 proteins expression. (1) Untreated cells; (2) Negative vector transected cells; (3) miR-122 transfected cells. (B) Bcl-2, Bcl-XL or P53/ß-actin ratio. Bcl-2 and Bcl-XL proteins expression significantly was reduced at miR-122 transfectants, P53 protein was up-regulated. $*p < 0.05$, compared with negative vector transfected cells or untreated cells (n = 3)

It was demonstrated that expression of Bcl-2 family genes could be regulated by miR-122. More importantly, expression of miR-122 in BEL-7402/5-FU resulted in increased sensitivity of cells to 5-FU, indicating that alteration of miRNA expression may be of implications for cancer therapeutic strategies.

Our results on resistantance of human hepatoma cells to 5-FU suggest that miR-122 is a potential target for cancer therapy/chemotherapy. Nevertheless, *in vivo* delivery and tumor specificity are challenging issues for the use of miRNA as an anticancer therapeutic agent. Development of genetic vectors or formulations for *in vivo* delivery of miRNA will be necessary before miRNA being used as a therapeutic agent. For example, Ectpic miR-34a expression resulted in cell cycle arrest and growth inhibition and attenuated chemoresistance to anticancer drug camptothecin by inducing apoptosis, suggesting a potential role of miR-34a for the treatment of p53-defective prostate cancer. MiR-140 is involved in the chemoresistance by reduced cell proliferation through G(1) and G(2) phase arrest mediated in part through the suppression of HDAC4 (Fujita et al. 2008). MiR-140 may be a candidate target to develop novel therapeutic strategy to overcome drug resistance, and so on. Development of genetic vectors or formulations for *in vivo* delivery of miRNA will be necessary before miRNA being used as a therapeutic agent.

In conclusion, miR-122 can down-regulate Bcl-2 family expression, and enhance the hepatocelluar sensitivity to 5-FU treatment. MiR-122 may be used as an effective chemotherapy-eutic and chemopreventive strategy to fight against human cancer.

## 4. Experimental

### *4.1. MiRNA microarray and hybridization*

MiRNA microarray was performed at KangChen Bio-tech (421 HongCao Rd Building 63, 2nd floor Shanghai, P.R China 200233). Total RNA from BEL-7402 cells and BEL-7402/5-FU was prepared using Trizol (Invitrogen) following the manufacturer's protocol, where opropanol was replaced by ethanol for RNA precipitation. RNA quality was measured using denaturing agarose gel electrophoresis. MiRCURY™ Array labeling kit, Exiqon: total RNA (5 μg) 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. MiRCURY™ Array microarray kit (Cat#208002V9.2) was purchased from Exiqon; Hybridization Chamber (Cat#40080) was got from Corning, and Bioarray LifterSlip coverslip was obtained from Ambion. Spotted microarray slides were processed using an automated hybridization station, which facilitated sample processing and hybridization standardization. Taking into account the different hybridization kinetic of DNA and LNA RNA-modified capture probes, the hybridization conditions were individually set optimally for LNA-RNA and DNA-based arrays. Microarrays

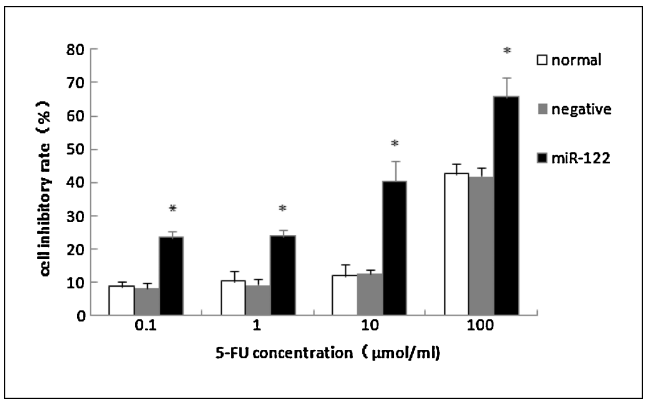


Fig. 6: Identifying miR-122 expression vetor. Sequencing confirmed the insert sequence (from 118 to 139 bp)

with immobilized RNA-LNA-modified capture probes were hybridized at 55 °C using microarray hybridization solution containing 30% formamide. Micro-arrays with immobilized DNA-oligonucleotides were hybridized at 42 °C in hybiridization solution containing 10% formamide. The slides were scanned using the Genepix 4000B (Axon Instruments). The 635 nm laser is used. Data were analyzed in Genepix Pro 6.0 (Axon Instruments), and saved as Excel files. 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.

### 4.2. MiR-122 vector construction

pSilencer 3.1-H1 linear Vector was purchased from Ambion (Austin, USA), and miR-122 insert sequence was equivalent to microrna.sanger.ac.uk accession MIMAT0000421, with the following sense and antisense sequences: sense 5'- TGG AGT GTG ACA ATG GTG TTTG-3' and antisense 5'- CAA ACA CCA TTG TCA CAC TCCA-3'. Negative control vector was pSilencer 3.1-H1 linear vector; it was provided by vector kit. Plasmid DNA was purified with Cs-Cl ethidium bromide gradient centrifugation. The purified DNA was diluted to 1 mg/ml and stored at −20 °C until used. Extracted plasmids were primarily confirmed by agarose gel electrophoresis. Sequencing was used to verify the miR-122 inserted templates (Fig. 6).

### 4.3. Cell lines and transfection

The human hepatoblastoma cell line BEL-7402 and the 5-fluorouracil-resistant cell line BEL-7402/5-FU were purchased from Kaiji Cell Center (Nanjing, 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% $CO_2$ and for BEL-7402/5-FU, 2 μg/ml 5-FU was added. Twelve hours before transfection, cells were seeded into wells of a 24-well plate that contained antibiotic-free medium; at the time of transfection, the cell confluence was routinely 90∼95% confluent. Transfection was carried out according to the manufacturer's protocol. MiR-122 and negative plasmid (1 μg) were diluted with 50 μl OPTI-ME-M (Invitrogen) or 2.5 μl lipofectamine 2000 (Invitrogen) with 50 μl OPTI-MEM. After 5 min, the dilutions were mixed together and incubated at 37 °C for 25 min, then dispensed into each well. Forty-eight hours later, transfection with the pSilencer 3.1-H1-miR-122 plasmid was performed using a standard lipofection method. Geneticin (800 μg/ml, G418; Invotrigen, USA) was added to the medium to select transfected miR-122 and negative vector cells. Three to five cell clones formed in each microplate during the 14 d incubation and the clones were picked and amplified in medium containing 400 g/ml G418. MiR-122 and negative vector were assessed in BEL-7402/5-FU cells transfected with miR-122 and negative vector.

### 4.4. Real-time RT-PCR

Total RNA was extracted from the cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Real-time RT-PCR was performed using standard protocols on an Applied Biosystem's 7500 HT Sequence Detection System. 5 μL of a 1/100 dilution of cDNA in water was briefly added into 12.5 μL of the 2 × SYBR green PCR master mix (Ambion), with 800 nmol/L of each primer in a total volume of 25 μL. All reactions were run in triplicate and included no template or 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.

### 4.5. Western blot analysis

We homogenized cells in a lysis buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 g/ml aprotinin, 100 g/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 50 mM sodium fluoride and PBS (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, IL). Equal amounts of lysate protein were run on 12% SDS-PAGE and transferred to PVDF membranes (Amersham-Pharmacia Biotech). After blocking, we incubated PDVF membranes with mouse anti-Bcl-XL, anti-Bax, and anti-P53 primary monoclonal antibody at dilution of 1:200 and rabbit anti-Bcl-2 polyclonal antibody at dilution of 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C and further incubated for 1 h with horseradish peroxidase conjugated anti-mouse and anti-rabbit secondary antibody at dilution of 1:1000. Bound antibodies were detected by Enhanced Chemiluminescence (ECL) kit with a Lumino Image Analyzer (Taitec, Tokyo, Japan).

### 4.6. Flow cytometry to analyze apoptosis

All cells were treated with 5-fluorouracil (5-FU), washed twice with PBS and fixed with 70% ethanol overnight at 4 °C. Then they were washed once again with PBS and stained with 800 μl propidium iodide (50 mg/l, Sigma, St. Louis, USA) at room temperature for 30 min to determine apoptosis by using flow cytometry (EPICS-XL, Beckman Coulter, Fullerton, USA). The data were analyzed with Cell Quest software version 3.3 (Becton Dickinson, San Jose, USA).

### 4.7. MTT analyzes cells drug sensitivity

Cells from the above groups were seeded into a 96-well plate at $1 \times 10^4$ cells per well. After 24 h, various concentrations of 5-FU were added. And cells were incubated for another 24 h. Then cells were treated with MTT (5 g/l, Sigma) for 4 h at 37 °C, and 200 μl dimethysl sulphoxide (DMSO) was added into each well for 10 min. The reaction was optically monitored at 570 nm ($A_{570}$) using a 96-well microtitre plate reader (Pharmacia, Piscataway, USA). All experiments were carried out in triplicate. The inhibitory rate (IR) of Bel-7402/5-FU cells was calculated according to the following equation:

$IR\ (\%) = [A_{570}\ (control) - A_{570}\ (drug)]/A_{570}\ (control) \times 100\%$

where $A_{570}$ (control) was the absorbance in miR-122 or negative vector transfected cells or untreated cells, and $A_{570}$ (drug) was the absorbance in the drug-treated group.

### 4.8. Statistical analysis of the data

Statistical analysis was performed using SPSS software (Version 11.0, 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, $P < 0.05$ was considered significant.

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