miRNA-195 sensitizes human hepatocellular carcinoma cells to 5-FU by targeting BCL-w

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Abstract. The role of microRNA-195 in developing acquired drug resistance in hepatocellular carcinoma cells was investigated. Expression profiling of miRNAs revealed a limited set of miRNAs with altered expression in drug resistant hepatocellular carcinoma cell line BEL-7402/5-FU compared to its parental BEL-7402 cell line. Real-time PCR confirmed down-regulation of miRNA-195 in BEL-7402/5-FU cells. Overexpression of miRNA-195 sensitized BEL-7402/5-FU cells to anticancer drugs. Consistent with these findings, miR-195 antisense oligonucleotide induced drug resistance in BEL-7402/5-FU cells. Also, the basal levels of the anti-apoptotic protein Bcl-w were high in BEL-7402/5-FU cells and miR-195 overexpression repressed Bcl-w protein level and inhibited the luciferase activity of a Bcl-w 3' untranslated region-based reporter construct in both BEL-7402/5-FU and BEL-7402 cells. These results indicate that miR-195 could improve the drug sensitivity at least in part by targeting Bcl-w to increase cell apoptosis in hepatocellular carcinoma cells.

Introduction

Hepatocellular carcinoma (HCC) is one of the top 10 most prevalent cancers worldwide (1) and accounts for 80-90% of liver cancers (2). Like other cancers, aberrant gene regulation features are significantly present in HCC. However, effective chemotherapeutic agents for this disease have not been developed. Recent studies have focused on the role of drug resistance in HCC treatment. Most chemotherapeutic drugs act as an apoptosis inducer. Consequently, the development of resistance to cytotoxic drugs by cancer cells may be due to cellular resistance to apoptosis. The commitment of apoptosis is largely a mitochondrial event controlled by proteins in the Bcl-2 family (3). Bcl-XL is an anti-apoptotic member of the Bcl-2 family that is located mainly on the outer membrane of mitochondria (4). The overexpression of Bcl-w, Bcl-XL and Bcl-2 is considered to be the mechanism by which tumor cells acquire resistance to apoptosis.

Numerous pathways (e.g., proliferation, cell cycle regulation, apoptosis, and angiogenesis) were identified to be dysregulated during hepatocarcinogenesis in HCC patients by using microarray analysis (5). In particular, apoptosis regulation (6) has been extensively described as a crucial event in the carcinogenetic process that leads to HCC development. Recently, a new class of small non-coding RNAs (miRNA) has been discovered (7) and is shown to be implicated in carcinogenesis (8). By binding to the complementary sequences of their target mRNAs (mostly in the 3'-UTR), miRNAs were able to induce mRNA degradation or translational repression (9). Multiple G1/S transition-related molecules, including cyclin D1, CDK6 and E2F3, were characterized as direct functional targets of miR-195 (10). miR-195 was further suggested to exert its proapoptotic function mainly through targeting Bcl-2 expression (11). Bcl-w, an anti-apoptotic Bcl-2 family member (12) was identified as harboring a putative miR-195 binding site in its 3'-UTR by using online prediction algorithmas. Bcl-w expression was modulated by Met/HGF receptor (c-met) in human colorectal cancers (13). Moreover, Bcl-w was up-regulated in autoimmune hepatitis (AIH)-associated cirrhosis (14) and may play a role in hepatocarcinogenesis.

In the present study, we demonstrated that a limited set of miRNAs were differentially expressed in a drug-resistant human hepatocellular carcinoma cell line BEL-7402/5-FU, compared to its parental cell, BEL-7402. MiR-195, one of the down-regulated miRNAs in BEL-7402/5-FU cells, was demonstrated to play a role in the development of drug resistance in hepatocellular carcinoma cells by targeting the antiapoptotic gene, Bcl-w.

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Abbrevations: BEL-7402/5-FU, 5-fluorouracil-resistant human liver adenocarcinoma cell line; Bcl-2, B cell lymphoma/leukemia-2; Bcl-XL, B-cell lymphoma/leukemia-extra long; miRNA, microRNA; MTT, 3-[4,5-Dimethylthiazolzyl]-2,5-diphenyl tetrazolium bromide; Q-RT-PCR, quantitative real-time reverse transcription polymerase chain reaction

Key words: miR-195, Bcl-w, 5-Fluorouracil, hepatocellular carcinoma

Materials and methods

Materials. PcDNATM6.2-GW/miR linear vector was purchased from GenePharma (Shanghai, China). Human hepatocellular carcinoma cell line BEL-7402 and its drug resistant line BEL-7402/5-FU were purchased from Nanjing KeyGen Biotech. Co., Ltd. (Nanjing, China).

Cell lines and cultures. Both human hepatocellular carcinoma cell line, BEL-7402 and its drug resistant line BEL-7402/5-FU were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY) in a humidified atmosphere containing 5% CO_2 at 37˚C. To maintain the drug resistance phenotype, 5-FU (with final concentration of 20000 ng/ml) was added to the culture media for BEL-7402/5-FU cells.

MicroRNA microarray and hybridization analysis. Microarray was performed at KangChen Bio-tech (Shanghai, China). RNA extraction: total RNA from BEL-7042 cells and BEL-7042/5-Fu cells were prepared using TRIzol (Invitrogen CA, USA) following the manufacturer's protocol, where propanol was replaced by ethanol for RNA precipitation. RNA quality was measured using denaturing agarose gel electrophoresis.

MiRNA labeling: miRCURY™ Array labeling ke, Exiqon: total RNA (5 μg) was ligated to an RNA-linker (p-rUrUrUdA-Cy3-dye Dharmacon) labeled at 3'-end with 3™ fluorescent T4 RNA ligase overnight at 37˚C.

Microarray preparation and hybridization: miRCURY™ Array microarray kit (cat# 208002V9.2) was purchased from Exiqon (Denmark); Hybridization Chamber (Cat#40080) was obtained from Corning (Japan), and Bioarray LifterSlip coverslip was purchased 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 kinetics of DNA and LNA RNA-modified capture probes, the hybridization conditions were individually set optimally for LNA-RNA and DNA-based arrays. Microarrays with immobilized RNA-LNA-modified capture probes were hybridized at 55˚C using microarray hybridization solution containing 30% formamide. Microarrays with immobilized DNA-oligonucleotides were hybridized at 42˚C in hybridization solution containing 10% formamide.

Image acquisition and quantification: the slides were scanned using the Genepix 4000B (Axon Instruments). The 635 nm laser was used. The 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 2-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.

Construction of miR-195 expression plasmids. PcDNATM6.2-GW/miR linear vector was purchased from GenePharma (Shanghai), and hsa-miR-195™ insert sequence with the following sense: 5'-CACTGAC TGACGCCAATATCTGTGCTGCTA-3' and antisense sequences were: 5'-ACTGACTGACTAGCAGCAGA AATATTGGC-3' were chemically synthesized. A negative control vector that expresses a hairpin shRNA with limited homology to any known sequences of human genome was commercially available (GenePharma). 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.

Cell culture and transfection. BEL-7402 and BEL-7402/5-FU cells were cultured in RPMI-1640 medium (Gibco-BRL) with 10% fetal bovine serum (Hyclone, Logan), and for BEL-7402/5-FU 2 μg/ml (QiLu, Shangdong, China) was added. Eighteen hours before transfection, cells were seeded into wells of a 6-well plate that contained antibiotic-free medium; at the time of transfection, the cell confluence was routinely 90-95%. Transfection was carried out according to the manufacturer's protocol. hsa-miR-195™ (4 μg), anti-hsa-miR-195 (4 μg) and negative plasmid (4 μg) carrying green fluorescent protein (GFP) were diluted with 100 μl OPTI-MEM (Invitrogen) or 10 μl Lipofectamine 2000 (Invitrogen) with 100 μl OPTI-MEM. After 5 min, the dilutions were mixed together and incubated at 37˚C for 20 min, then dispensed into each well. Forty-eight hours after transfection, fluorescence microscope was used to detect the transfection efficiency.

Real-time quantitative RT-PCR. The expression level of miR-195 was measured in cells transfected with pcM-195s, pcM-195a, or miR-NC by using the NCode™ miRNA First-Strand cDNA Synthesis Kit and NCode SYBR Green miRNA qRT-PCR Kit (Invitrogen, USA). The level of U6 RNA was measured and used to normalize the relative abundance of miR-195. 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-ΔCT, where ΔCT = (CTmiRNA - CT5s) (15). Relative gene expression was multiplied by U6 to simplify the presentation of the data.

The expression levels of Bcl-w, Bcl-2, Bcl-XL and Bax were measured in cells 48 h post-transfection using High Capacity cDNA Reverse Transcription kit and Power SYBR Green PCR master Mix (Applied Biosystems).

MiR-195 target prediction. Given the limitations of any single prediction program, we used two separate prediction programs (Targetscan 5.1 and PicTar) to identify common predicted targets for miR-195. Target Scan 5.1 utilizes matching in the 3' UTR for only 7mer and 8mer interactions sites.

Construction of 3'-UTR reporter plasmids and luciferase assays. The part of the 3'UTR of Bcl-w mRNA containing the intact miR-195 recognition sequence (at nucleotide 777-783 of 3'UTR) was amplified by a pair of primers (F: 5'-CCGCTCGAGCTTTCCAGAAAGTGATTGGCAAG-3'; R: 5'-ATAAGAATGCGGCCGCCCTTGGAAAGTGCAG CAGTGAG-3') and subcloned into *Xho*I and *Not*I sites of psiCheck-2 vector (Promega, USA) immediately downstream of the luciferase gene to form a psi-Bcl-w-3'UTR construct. The psi-Bcl-w /mut-3'UTR reporter construct

with point mutations in the seed sequence as underlined (5'-TGCTGCT-3') was synthesized using the site-directed mutagenesis kit (Stratagene, La Jolla, CA). For the luciferase assay, BEL-7402 or BEL-7402/5-Fu cells were cultured in 24-well plates and each transfected with 0.1 μg of either miR-195s, miR-195a or negative control, which contained the Renilla luciferase gene, and 30 pmol miR-195s, miR-195a, or negative control. Transfection was done using Lipofectamine 2000 (Invitrogen) and Opti-MEM I reduced serum medium (Life Technologies) in a final volume of 0.6 ml. At 24 h after transfection, firefly and Renilla luciferase activity were measured using the Dual Luciferase Reporter Assay (Promega). Each transfection was repeated twice in triplicate. Relative protein levels were expressed as Renilla/firefly luciferase ratios.

Western blotting. Cell lysates were centrifuged at 10,000 x 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 10% SDS-PAGE and transferred to PVDF membranes (Amersham-Pharmacia Biotech). After blocking, we then incubated PDVF membranes with rabbit, anti-Bcl-w, anti-Bcl-XL, and anti-Bcl-2 primary monoclonal antibody at dilution of 1:1000 and rabbit anti-Bax, polyclonal antibody at dilution of 1:1000 (Cell Signaling Technology, USA) overnight at 4˚C and further incubated for 1 h with horseradish peroxidease conjugated 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).

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

Cytotoxicity analysis. Cells from the above groups were seeded into a 96-well plate at $1x10^4$ cells per well. After 24 h, various concentrations of 5-FU were added and the cells were incubated for another 24 h. Then the cells were treated with MTT (5 g/l, Sigma, USA) for 4 h at 37˚C, and 200 μl dimethysl sulphoxide (DMSO) was added in each well for 10 min. The reaction was optically monitored at 570 nm (A_{570}) using a 96-well microtitre plate reader (Pharmacia, Piscataway, NJ). All experiments were carried out in triplicate. The inhibitory rate (IR) of Bel-7402/5-FU cells was calculated according to the equation as following: IR (%) = [A_{570} (control) - A_{570} (drug)]/A_{570} (control) x 100%, where A_{570} (control) was the absorbance in miR-195s or miR-195a or negative vector transfected cells or untreated cells, and A_{570} (drug) was the absorbance in the drug-treated group.

Statistical analysis. Statistical analysis was performed using SPSS software (Version 14.0, SPSS Incorporation, USA. Data were expressed as mean ± SD and analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) test. Statistical significance is displayed as $^*P<0.05$.

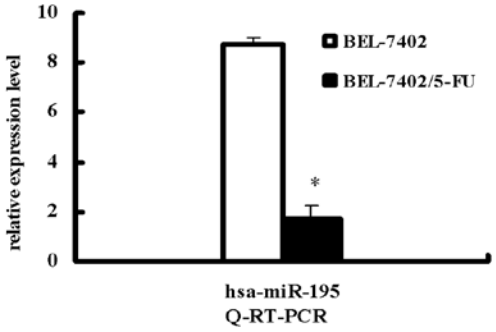

Figure 1. BEL-7405/5-FU cells express lower miR-195 than BEL-7402 cells. Real-time RT-PCR analysis was carried out to validate the microarray results. Triplicate assays were performed for RNA sample and the relative amount of miRNA was normalized to U6 snRNA. $^*P<0.01$, relative fold changes of miRNA levels in BEL-7402/5-FU cells relative to BEL-7402 cells (n=3).

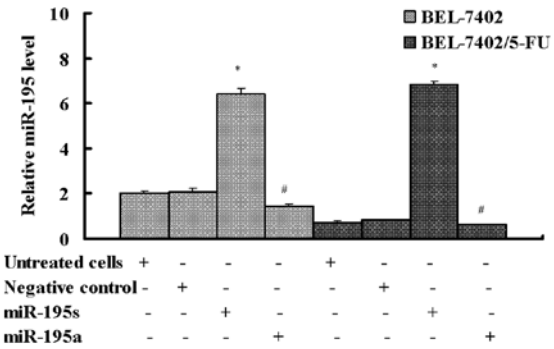

Figure 2. Transfection of different miR-195 vectors altering the expression of miR-195. BEL-7402 and BEL-7402/5-FU were transfected with miR-195s, miR-195a or control for 48 h, and RNA were isolated and analyzed by real-time PCR. $^*P<0.05$ and $^\#P<0.05$ compared to other groups (n=3).

Results

BEL-7402/5-FU cells display altered expression of miRNAs from BEL-7402. To identify miRNA expression in BEL-7402 and BEL-7402/5-FU cells, we performed miRNAs expression profiling using MiRCURY LNA Arrays. As shown in Table I, hsa-miR-195 was down-regulated in BEL-7402/5-FU cells as compared to BEL-7402 cells. Real-time PCR confirmed the down-regulation of miRNA-195 in (Fig. 1).

MiR-195 sensitizes BEL-7402/5-FU cells to 5-FU. We determined whether miR-195 regulates apoptosis in HCC-derived cell lines. Sense and anti-sense miR-195 sequences were inserted into a miRNA vector, generating pcM-195s and pcM-195a that expressing mature and anti-sense sequences of miR-195, respectively. A vector that expresses a hairpin shRNA with limited homology to any known sequences of human genome was used as a negative control. As expected. miR-195s dramatically increased miR-195 expression in both

Table I. Data of the microarray between BEL-7402 and BEL-7402/5-FU tumor cells.

A, Up-regulated miRs		
MiR name	Fold change	P-value
hsa-miR-192	2.4632	0.0797
hsa-miR-194	2.0494	0.1094
B, Down-regulated miRs		
MiR name	Fold change	P-value
hsa-miR-195	0.0818	0.0292
hsa-miR-199a-5p	0.0669	0.0186
hsa-miR-18a	0.4218	0.0299
hsa-miR-127-3p	0.0841	0.0067
hsa-miR-122	0.2007	0.0070
hsa-miR-486-3p	0.2929	0.0032
hsa-miR-371-5p	0.3115	0.0056
hsa-miR-20b*	0.3224	0.0047
hsa-miR-32*	0.4390	0.0068
hsa-miR-429	0.0397	0.0007
hsa-miR-335	0.1180	0.0012
hsa-miR-518c*	0.1183	0.0024
hsa-miR-32	0.1561	0.0054
hsa-miR-374b	0.2841	0.0033
hsa-miR-361-3p	0.0841	0.0006
hsa-miR-98	0.1114	0.0021
hsa-miR-31	0.2779	0.0044
hsa-miR-151-5p	0.2789	0.0052
hsa-miR-10a*	0.0015	0.00002
hsa-miR-378	0.0693	0.0009
hsa-miR-130b	0.0711	0.0007
hsa-miR-503	0.1175	0.0011
hsa-miR-30e	0.4438	0.0078
hsa-miR-96	0.4622	0.0067
hsa-miR-7	0.4652	0.0071
hsa-miR-801	0.4676	0.0058
hsa-miR-10a	0.0007	0.00001
hsa-miR-500	0.0481	0.0005
hsa-miR-590-5p	0.0778	0.0009
hsa-miR-491-5p	0.3687	0.0026
hsa-miR-301a	0.0558	0.0002
hsa-miR-99b*	0.0895	0.0006
hsa-miR-146-5p	0.3380	0.0019
hsa-miR-519d	0.3617	0.0027
hsa-miR-18b	0.4339	0.0079
hsa-miR-148b	0.0592	0.0004
hsa-miR-18a*	0.8180	0.0089
hsa-miR-340	0.1920	0.0017
hsa-miR-584	0.4592	0.0028
hsa-miR-17*	0.2485	0.0098
hsa-miR-210	0.1327	0.0077
hsa-miR-492	0.1811	0.0013
hsa-miR-193a-3p	0.2543	0.0041
hsa-miR-422a_MM2	0.1369	0.0016

Down-regulated and up-regulated miRNAs with fold changes and p-values in BEL-7402/5-FU relative to BEL-7402 cells. Out of 536 detected miRNA, 46 miRNAs were differentially expressed between BEL-7402 and BEL-7402/5-FU cells. Among these miRNA, 2 were up-regulated and 44 down-regulated. Of note, miR-195 was the one of expressed miRNA in BEL-7402/5-FU compared to BEL-7402 cell line.

BEL-7402 and BEL-7402/5-FU cells, and miR-195a repressed miR-195 in BEL-7402 (Fig. 2).

MTT and flow cytometry were performed to determine metabolic activity and apoptosis rate, respectively. MiR-195s transfection reduced metabolic activity and increased apoptosis rate of BEL-7402 and BEL-7402/5-FU cells induced by 5-FU. The transfection of miR-195a had no significant effect on the cell lines (Fig. 3). These results suggest miR-195 improves drug sensitivity of 5-FU on BEL-7402 and BEL-7402/5-FU cells.

BEL-7402/5-FU cells express different protein level of Bcl-w, Bcl-2, Bcl-XL, and Bax from BEL-7402. The development of drug resistance in various cancer cells has been linked to a reduced susceptibility to drug-induced apoptosis. This has been attributed in some part to the overexpression of anti-apoptotic proteins, such as Bcl-2 and Bcl-XL (18). Therefore, we sought to investigate whether the resistance of BEL-7402/5-FU cells to 5-FU is a consequence of overexpression of anti-apoptotic members of the Bcl-2 family. We performed Western blot analysis to examine the expression of Bcl-w, Bcl-2 and Bcl-XL in BEL-7402 and BEL-7402/5-FU cells. BEL-7402/5-FU cells had a higher expression of Bcl-w, Bcl-2 and Bcl-XL than the BEL-7402 cells. We also found that the BEL-7402/5-FU cells had a lower expression of Bax (a pro-apoptotic member of the Bcl-2 family) as compared to BEL-7402 cells (Fig. 4). Previous studies have shown that the pro-apoptotic members of the Bcl-2 family mediate cytochrome c release and cell apoptosis. These observations imply that decreased miR-195 levels might be involved in the regulation of Bcl-w, Bcl-2, Bcl-XL and Bax expression. Thus, we hypothesized that the removal of miRNA-195 inhibition on anti-apoptotic gene expression would facilitate the development of drug resistance in BEL-7402/5-FU.

MiR-195 decreases Bcl-w, Bcl-2, Bcl-XL expression and increases Bax expression. Next, we determined whether miR-195 is involved in regulating the expression of Bcl-w, Bcl-2, Bcl-XL and Bax, and the sensitivity to 5-FU. We transfected

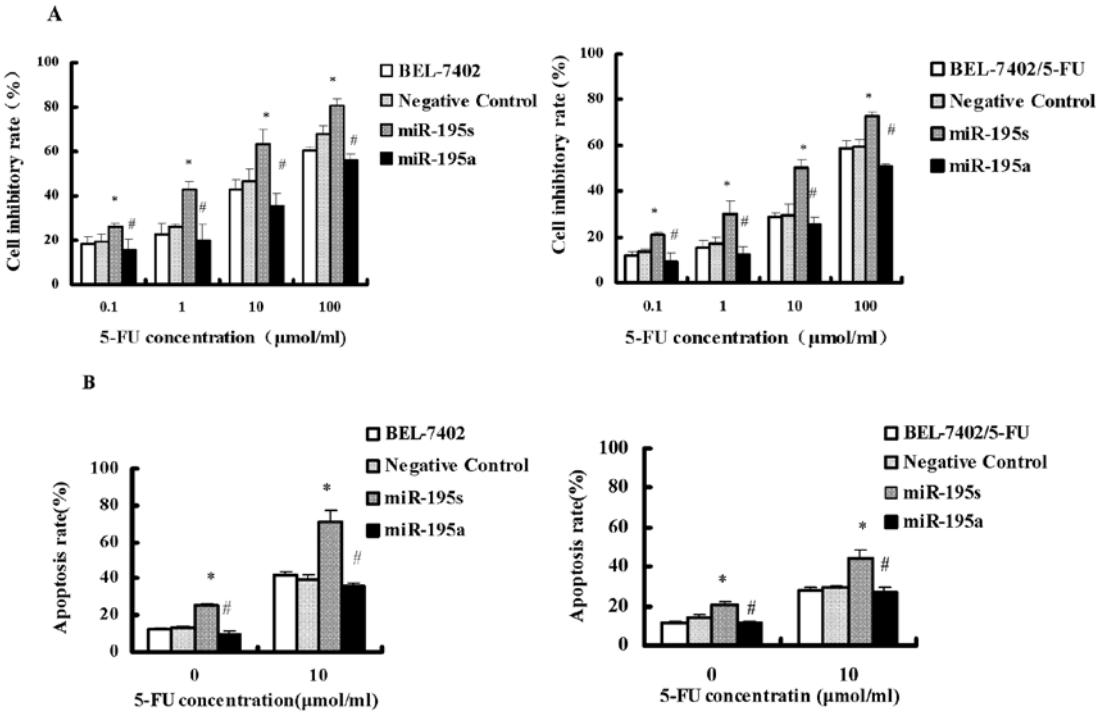

Figure 3. miR-195 increases the sensitivity of 5-FU on BEL-7402 and BEL-7402/5-FU cells. (A) Stable transfected BEL-7402 and BEL-7402/5-FU cells with miR-195s or miR-195a, negative control or non-treatment were seeded on 96-well plates, respectively. Next day, cells were exposed to different concentration of 5-FU for 24 h. Cells were harvested and the percentage of cell inhibition was determined by MTT assays. (B) BEL-7402 and BEL-7402/5-FU cells were transfected with miR-195s, miR-195a, negative control, or non-treatment, and 24 h later cells were treated with 10 μmol/ml of 5-FU for 48 h. Cell apoptosis rates were evaluated by flow cytometry, and the percentage of apoptotic cells was calculated. *P<0.05 and #P<0.05, compared to other groups (n=3).

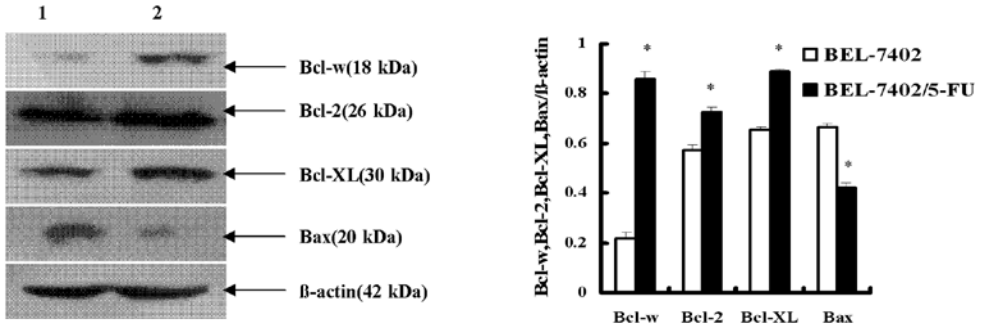

Figure 4. (A) Expression difference of Bcl-w, Bcl-2, Bcl-XL and Bax in BEL-7402 and BEL-7402/5-FU cells. Western blot analysis was performed to detect protein expression difference from two cells, relative protein level was normalized to actin. *P<0.05, BEL-7402/5-FU cells compared to BEL-7402 cells (n=3).

BEL-7402 and BEL-7402/5-FU cells with either miR-195s, miR-195a or negative plasmid. The miR-195s significantly decreased Bcl-w, Bcl-2 and Bcl-XL mRNA. However, the Bax mRNA level was significantly elevated in both cell lines. As expected, transfection of miR-195a increased the expression of Bcl-w, Bcl-2 and Bcl-XL mRNA in BEL-7402 cells (Fig. 5A and B).

Western blots were performed to determine protein levels. Bcl-w, Bcl-2 and Bcl-XL protein levels were repressed in miR-195s transfected BEL-7402 and BEL-7402/5-FU cells, whereas Bax protein levels were increased in both cell lines. In addition, miR-195a increased protein expression of Bcl-w, Bcl-2 and Bcl-XL in both cell lines (Fig. 5C and D). These results suggest that miR-195 down-regulates anti-apoptotic Bcl-2 family members and up-regulates Bax genes in BEL-7402 and BEL-7402/5-FU cells.

MiR-195 acts directly on the Bcl-w 3'-UTR. In order to identify putative miR-195 targets that may contribute to tumor genesis, an *in silico* strategy was applied. By querying PicTar (16) and TargetScan (17), Bcl-w was identified as a putative miR-195 target gene by both programs. The prediction results and score are listed in Fig. 6A and B; as a validated miR-195 target, CCNG1 is shown as a reference. To validate the observation from Pictar and TargetScan querying, a 750-bp fragment of the 3'-UTR of Bcl-w containing the putative miR-195 binding site was cloned into the psiCHECK-2 reporter vector. As shown in Fig. 6C, the miR-195 expression vector significantly repressed the expression of reporter

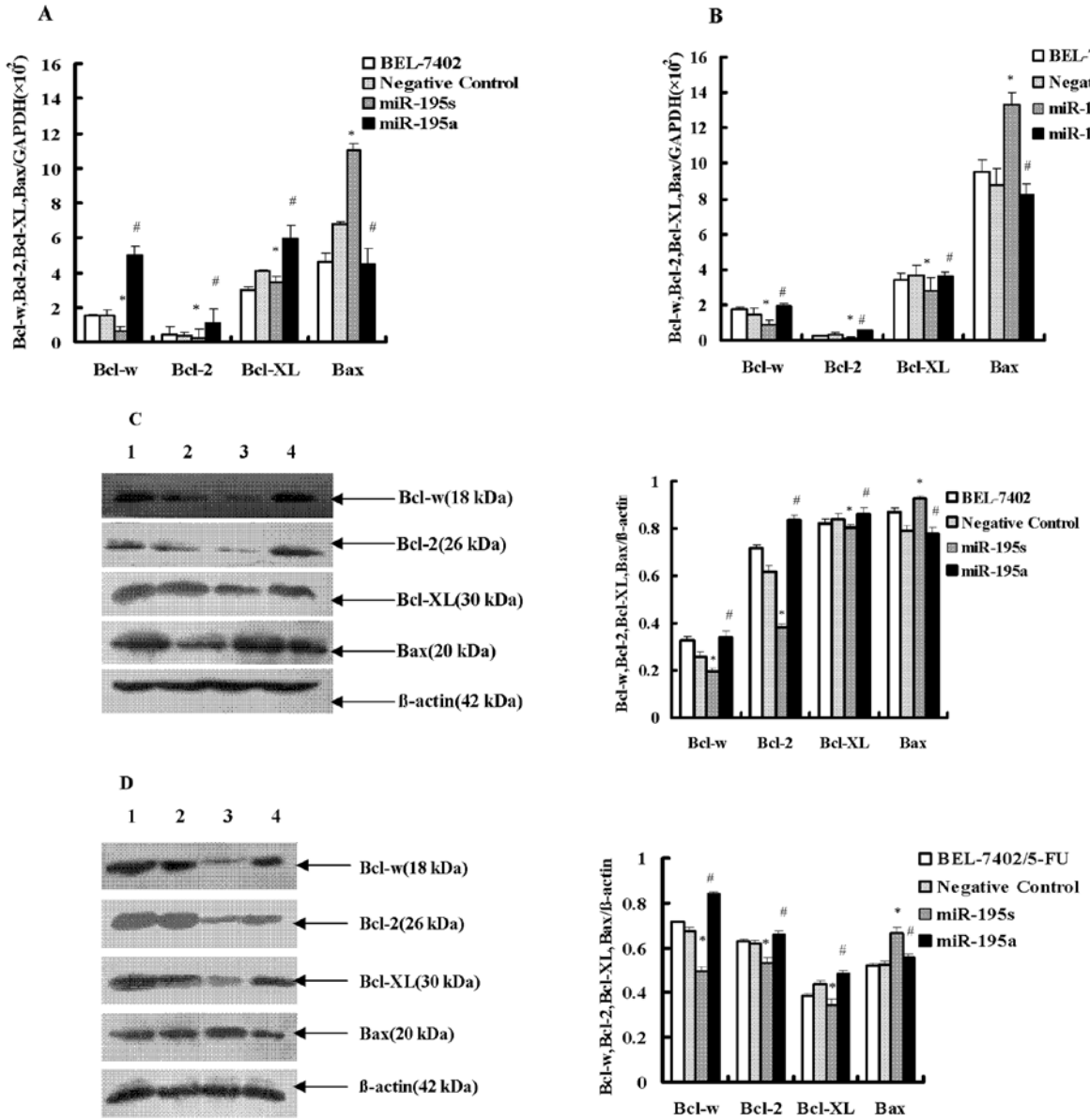

Figure 5. miR-195 regulates the expression Bcl-2 family members and Bax genes. BEL-7402 and BEL-7402/5-FU were transfected with miR-195s, miR-195a, negative control or non-treatment. (A) RNA were isolated and real-time PCR were performed to detect relative mRNA level of detected Bcl-w, Bcl-2, Bcl-XL and Bax genes. (B and C) Western blot analysis was applied to determine the protein level of Bcl-w, Bcl-2, Bcl-XL and Bax genes. Relative levels of mRNA and protein were normalized GAPDH and actin, respectively. $^{*}P<0.05$ and $^{\#}P<0.05$, compared to other groups (n=3).

construct, and anti-sense miR-195 increased the expression of the reporter construct in both BEL-7402 and BEL-7402/5-FU cells. These results demonstrate that miR-195 could directly inhibit Bcl-w expression at post-transcriptional level through its 3'-UTR.

Discussion

Research effort has been intensely focused on studying 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 (19). In addition, approaches interfering with miRNA function are considered in cancer research (20). It is known that certain microRNAs impart drug resistance (21) or sensitivity (22) to cancer cells. However, drug resistance, an obstacle to curative treatment of 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; one is the anti-apoptotic proteins such as Bcl-2 and Bcl-XL, and the other is the pro-apoptotic proteins such as Bax, Bad and Bid (23). Among the Bcl-2 protein family members, overexpression of Bcl-2 prevent chemotherapeutic agent-induced apoptosis associated with altered mitochondria membrane (24).

The balance between Bcl-2 family members defines whether a cell will live or die. As the ratio between each 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

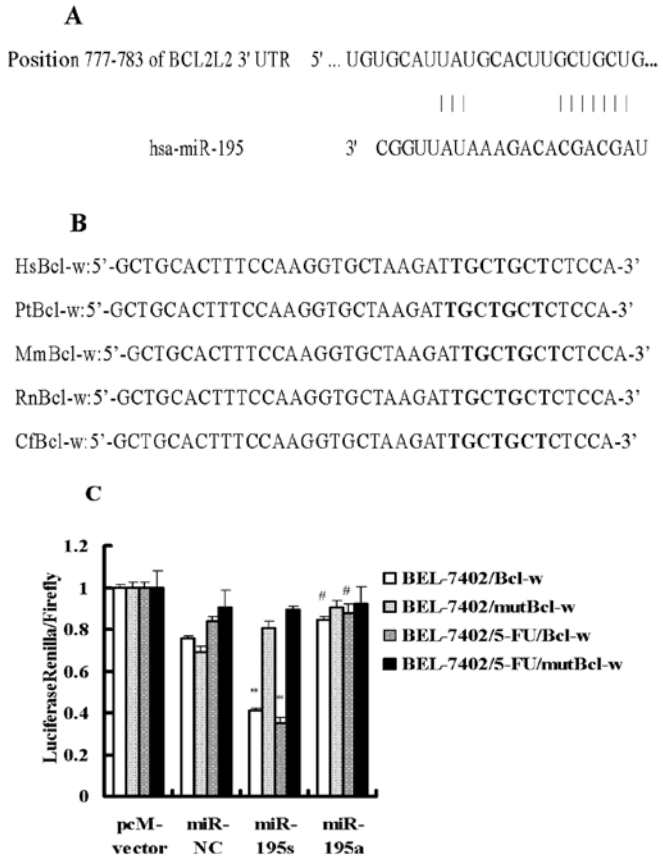

Figure 6. Effect of the putative miR-195 binding site derived from the Bcl-w 3'-UTR on luciferase expression. (A) Putative binding sites of miR-195 in the Bcl-w 3'-UTR regions are determined by TargetScan and Pictar. (B) Bcl-w 3'-UTR sequences that contain the putative miR-195 targeting site are highly conserved in human, chimpanzee (Ensembl Transcript), mouse, rat, dog. (C) Dual luciferase assay was performed in BEL-7402 and BEL-7402/5-FU cells transfected with luciferase construct alone, cotransfected with miR-195s, miR-195a or negative control. Firefly luciferase construct containing mutant (mutpsi-Bcl-w) target site of the Bcl-w 3'-UTR was generated and transfected as indicated. Firefly luciferase activity was normalized to Renilla luciferase activity for each sample (n=3). *P<0.05 and #P<0.05, compared to BEL-7402/Bcl-w or BEL-7402/5-FU/Bcl-w.

family proteins in liver cancer cells may be involved in the resistance (25).

Several miRNAs have been reported to be involved in controlling apoptosis and cancer formation (8,26), and the validated target genes of miR-195, which is down-regulated in HCC, are CCND1,CDK6, E2F3 (10). Here, miR-195 is suggested to function as a tumor suppressor in CRC development, which is consistent with its roles in hepatocellular carcinoma and adrenocortical carcinoma (27). However, in chronic lymphocytic leukemia and breast cancer, miR-195 expression is reported to be elevated in these malignancies (28-30). Hence, deregulation of miR-195 may be different in different types of cancer, and the roles of miR-195 in carcinogenesis and progression can not be simply concluded as a tumor suppressor or oncogene. In this study, we showed that miR-195 was frequently down-regulated in HCC cell lines. Ectopic expression of miR-195 suppressed HCC and colorectal carcinoma cells to form colonies *in vitro* and to develop tumors *in vivo* (reference). The underlying mechanism that is responsible for the decreased expression of miR-195 in HCC is still unknown. Here, we reported that miR-195 represses Bcl-w expression by directly targeting the 3'-UTR region of Bcl-w mRNA. MiR-195 was shown to inhibit Bcl-w at the post-transcriptional level in HCC-derived BEL-7402 and BEL-7402/5-FU cell lines.

We observed the effect of miR-195 on cellular sensitivity to anti-cancer drugs. 5-fluorouracil (5-FU) was used to treat advanced primary hepatocellular carcinoma in clinic, but drug resistance is usually problematic. In this study, BEL-7402/5-FU cells showed a high expression of Bcl-w, Bcl-2 and Bcl-XL and a lower expression of Bax than in BEL-7402 cells. Transfecting miR-195 vector into BEL-7402/5-FU cells resulted in the down-regulation of Bcl-w, Bcl-2 and Bcl-XL. MiR-195 transfected cells have a higher rate of 5-FU induced cell death than in normal, negative vector and miR-195 inhibitor transfected cells. It could be hypothesized that miR-195 might inhibit cell proliferation mainly through suppressing the expression Bcl-w, Bcl-2 and Bcl-XL.

In summary, our results strongly suggest that miR-195 might sensitize cell to 5-FU-induced apoptosis by suppressing the expression of Bcl-w via a direct binding of miR-195 to the 3'UTR of Bcl-w mRNA. The elevated miR-195 expression shows promise as a new strategy for the treatment of hepatocellular carcinoma.

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