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MiR-195 Regulates cell apoptosis of human hepatocellular carcinoma cells by targeting LATS2

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Recently, we have reported tissue- and stage-specific expression of miR-195 in human hepatocellular carcinoma cells and so far, not many reports discuss the function of this microRNA (miRNA). 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 miR-195 in BEL-7402/5-FU cells. Western blots were performed to determine protein levels of LATS2, P53 and CDK2. MTT analysed the cell proliferation activity. Flow cytometry were performed to determine apoptosis rate. Up-regulation of miR-195 increased expression of LATS2 and increased apoptosis of HCC cells, while Anti-miR-195 treatment inhibited expression of LATS2. miR-195 over-expression inhibited the luciferase activity of a LATS2 3' untranslated region-based reporter construct in BEL-7402/5-FU cells. These results indicate that miR-195 could increase cell apoptosis by targeting LATS2 in hepatocellular carcinoma cells.

1. Introduction

MicroRNAs (miRNAs) are endogenous, noncoding, ~22 nt small RNAs which are involved in sequence-specific negative regulation of the stability and translation of target mRNAs (Lee et al. 1993) miRNAs exhibit cell- and tissue-specific expression(Lee et al. 2001) and play important regulatory roles in cell cycle, apoptosis, and development (Bartel 2004; Carleton et al. 2007; He et al. 2004). It has been shown that miRNAs are aberrantly expressed or mutated in cancer, suggesting that they may play a role as a novel class of oncogenes or tumor suppressor genes (Bentwich. et al. 2005; Zamore and Haley et al. 2005). 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 (Hobert 2008). The first evidence that miRNAs are involved in cancer came from the finding that miR-15 and miR-16 (chromosome 13q14) were deleted or missing in B cell chronic lymphocytic leukemia (CLL) cells (Cimmino et al. 2005). These miRNAs promote apoptosis by targeting mRNA of the anti-apoptotic BCL-2 gene and, thus, function as tumor suppressors. Let-7 is another miRNA with tumor-suppressing properties and its expression is reduced in human lung cancer associated with poor postoperative prognosis (Takamizawa et al. 2004). In contrast, the miR-17-92 cluster at chromosome 13q31.3 is overexpressed in lung cancers, and its introduction dramatically increases lung cancer cell growth (Hayashita et al. 2005). The oncogenic properties of miR-17–92 can be partly explained by its predicted targets including the tumor suppressor genes PTEN and RB2 (Lewis et al. 2003). Multiple G1/S transition-related molecules, including cyclin D1, CDK6 and E2F3, were characterized as direct functional targets of miR-195 (Xu et al. 2009). miR-195 was further suggested to exert its proapoptotic function mainly through targeting Bcl-2 expression (Liu et al. 2010).

Hepatocellular carcinoma (HCC) is one of the top 10 most prevalent cancers worldwide (Seeff et al. 2006) and accounts for 80–90% of liver cancers (Di Bisceglie et al. 2004). Like other cancers, aberrant gene regulation features are significantly present in HCC. 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 (Thorgeirsson et al. 2006). In particular, apoptosis regulation (Fabregat et al. 2007) has been extensively described as a crucial event in the carcinogenetic process that leads to HCC development.

LATS2 (also known as KPM) is also considered a tumor suppressor gene since frequent LOH of this gene has been reported in various human cancers including breast, ovary, and liver (Lee et al. 1988; Sato et al. 1991; Wang et al.1988) and *in vitro* over expression of LATS2 was seen to cause G1/S arrest through the inhibition of CDK2 activity (Li et al. 2003). Additionally, as mentioned earlier, LATS2 is a tumor suppressor genes, which is implicated in the regulation of the cell cycle. The p53 tumor suppressor gene is subject to frequent mutational alterations in human cancer. The p53 protein is a sequence-specific transcription factor, present in very low amounts in normal cells. In response to various types of stress, p53 is hyperactivated to modulate the expression of numerous target genes. p53 activation can lead to a variety of changes in cell fate, most notably induction of cell cycle arrest and apoptosis. Furthermore, the Lats2 gene is itself a target for positive transcriptional regulation by p53 (Aylon et al. 2006).

In the present study, we focused on the expression and roles of miR-195 in HCC development and found that miR-195 was down-regulated in in HCC cell lines. And miR-195 was further identified to function as a tumor suppressor in HCC, as restoration of miR-195 expression in HCC cell lines could reduce cell viability and promote cell apoptosis *in vitro*. Thus, our data sug-

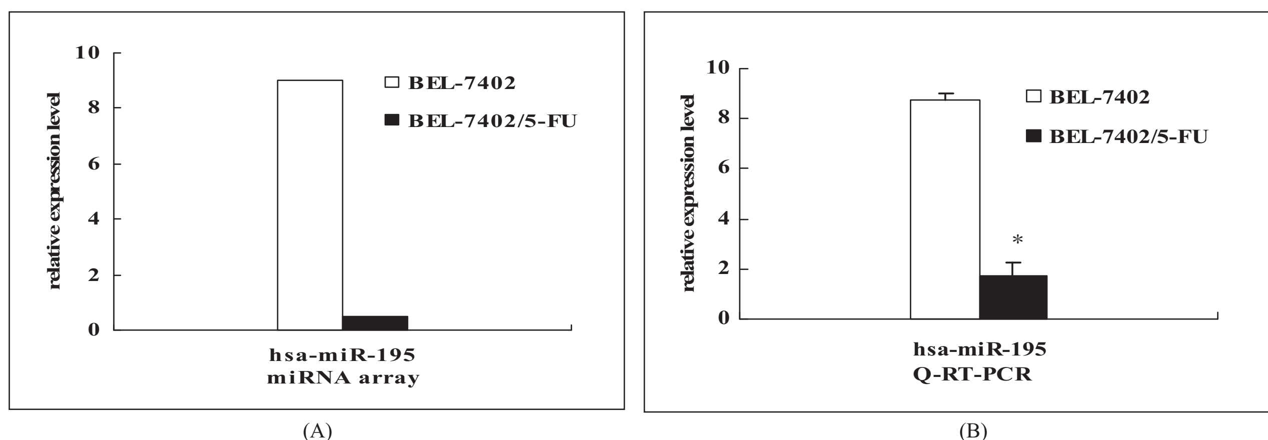

Fig. 1: BEL-7405/5-FU cells expressing lower miR-195 than BEL-7402 cells. (A) RNA isolated from BEL-7402/5-FU and BEL-7402 cell were tested miRNA microarray. (B) Real-time RT-PCR analysis was carried out to validate the microarray results. Triplicate assays were performed for RNA sample and the relative 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)

gest the important roles of miR-195 in HCC pathogenesis and implicate its potential application in cancer therapy.

2. Investigations and results

2.1. BEL-7402/5-FU cell displays altered expression of miRNAs from BEL-7402

To identify miRNAs expressions in BEL-7402 and BEL-7402/5-FU cells, we performed miRNAs expression profiling using MiRCURYTM LNA Arrays. As shown in Fig. 1A, 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. 1B).

2.2. MiR-195 sensitizes BEL-7402/5-FU cells to 5-FU

We wanted to determine whether miR-195 regulates apoptosis in HCC-derived cell lines. Sense and anti-sense miR-195 sequences were inserted into a miRNA vector, generating pcDNA6.2 miR-195, and Mature antisense oligodeoxynucleotides specifically against miR-195 (Anti-miR-195) and Control anti-miRNA were chemically synthesized. A vector that expresses a hairpin shRNA with limited homology to any known sequences of human genome was used as a control miRNA. As expected. miR-195 dramatically increased miR-195 expression in both BEL-7402 and BEL-7402/5-FU cells (Fig. 2).

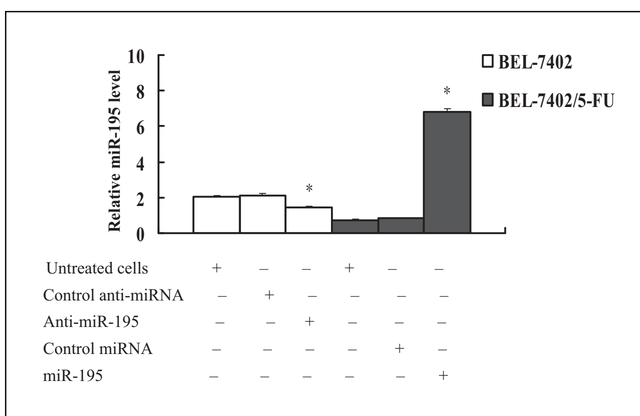

Fig. 2: Tranfection of different miR-195 vectors altering the expression of miR-195. BEL-7402 and BEL-7402/5-FU were transfected with miR-195, Anti-miR-195 or control for 48 h, and RNA were isolated and analyzed by real-time PCR. *P<0.05 compared to other groups. (n=3)

2.3. MiR-195 increases LATS2, p53 expression and decreases CDK2 expression

miR-195 has been reported to be tumor suppressor of testicular germ cell tumors. Next, we wanted to determine whether miR-195 is involved in regulating the expression of LATS2, p53 and CDK2. We transfected BEL-7402 with either Anti-miR 195 or Control anti-miRNA and BEL-7402/5-FU cells with either miR-195, or Control miRNA, respectively, were quantitated by real time PCR. The miR-195 significantly increases LATS2, p53 mRNA. However, the CDK2 mRNA level was decreased significantly (Fig. 3A, B).
Western blots were performed to determine protein levels. We transfected BEL-7402 with either Anti-miR-195 or Control anti-miRNA and BEL-7402/5-FU cells with either miR-195, or Control miRNA. The miR-195 significantly increases the protein level of LATS2, p53. However, the CDK2 protein level was decreased significantly (Fig. 3C,D).

2.4. MiR-195 inhibited the cell proliferation activity of hepatocellular carcinoma cells

It has been demonstrated that LATS2 is an essential mitotic regulator required for the coordination of cell division (Li et al. 2003). Our results have shown that the LATS2 protein level was modulated by miR-195 in hepatocellular carcinoma cells. To determine whether miR-195 regulates cell proliferation in HCC-derived cell lines. We assayed its effect on cell proliferation activity. The proliferation activity of BEL-7402, cells transfected with Anti-miR-195 or Control anti-miRNA and BEL-7402/5-FU cells transfected with miR-195 or Control miRNA was determined by MTT assay, respectively. As shown in Fig. 4A, B, BEL-7402 cells treated with anti-miR-195 oligonucleotides showed a significant increase in cell viability compared with the control anti-miRNA oligonucleotides transfected cells. In contrast, BEL-7402/5-FU cells treated with miR-195 reduced significant proliferation activity compared with Control miRNA. These results indicated that hepatocellular carcinoma cell proliferation can be modulated through miR-195 mediated LATS2 expression.

2.5. MiR-195 increased the cell apoptosis rate of hepatocellular carcinoma cells

Flow cytometry was performed to determine apoptosis rate. Anti-miR-195 transfection reduced apoptosis rate of BEL-7402, while the transfection of MiR-195 increased apoptosis rate of BEL-7402/5-FU cells (Fig. 5A,B). These results indicated that

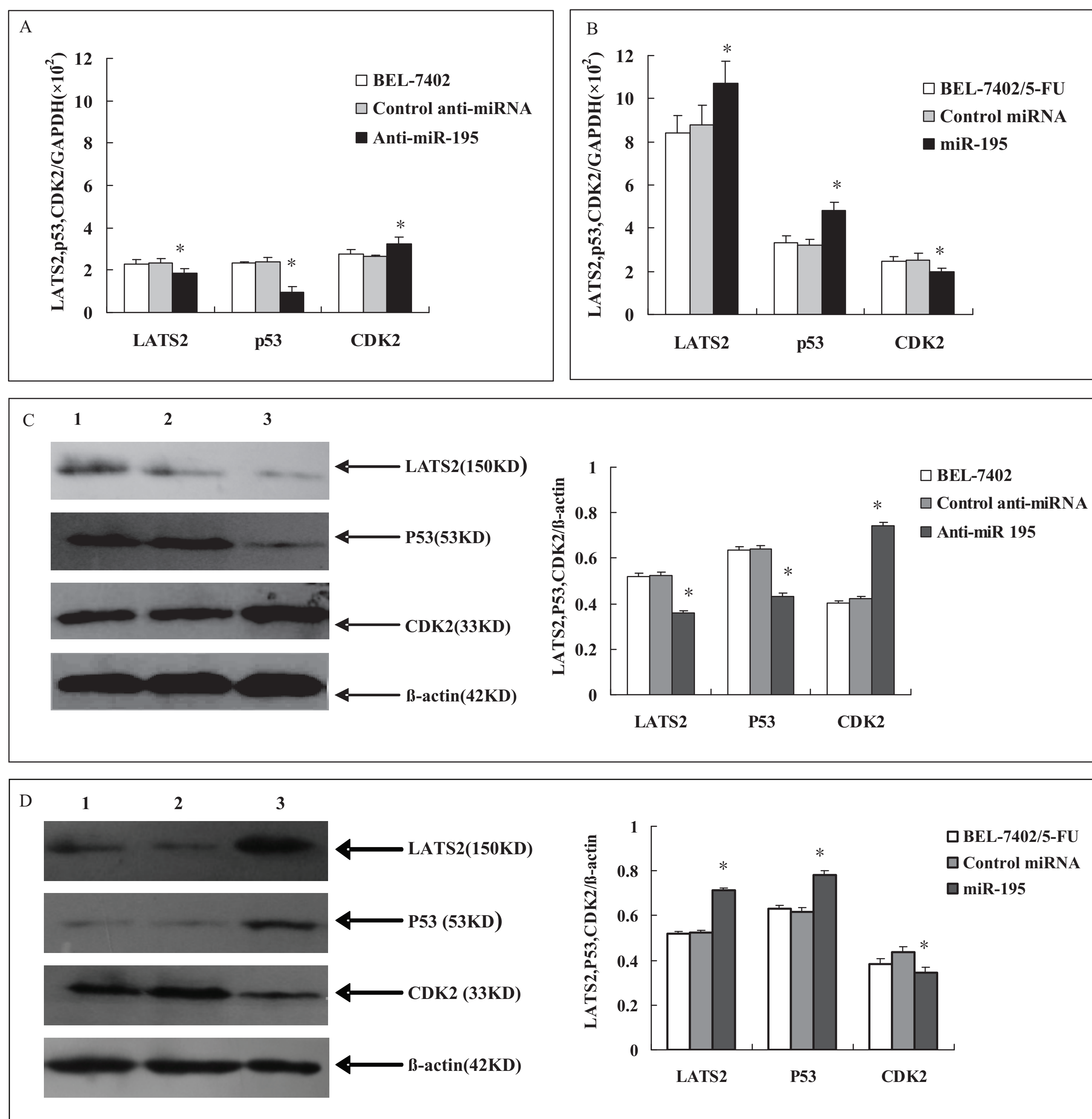

Fig. 3: miR-195 regulates the expression LATS2, p53 and CDK2 genes. BEL-7402 was transfected with Anti-miR-195 or Control anti-miRNA or non-treatment and BEL-7402/5-FU was transfected with miR-195, Control miRNA or non-treatment. (A) and (B) RNA were isolated and real-time PCR were performed to detect relative mRNA level of detected LATS2, p53 and CDK2 genes. (C) and (D) Western blot were applied to determine the protein level of LATS2, p53 and CDK2 genes. Relative levels of mRNA and protein were normalized GAPDH and actin, respectively. *$P<0.05$ compared to other groups. (n=3)

up-regulated miR-195 can increase hepatocellular carcinoma cell apoptosis.

2.6. MiR-195 acts directly at the LATS2 3'-UTR

We next investigated whether the 3'-UTR of LATS2 was a functional target of miR-195 in HCC cell lines. MiR-195 was predicted to bind two sites in the 3'-UTR of LATS2 (Fig. 6A). We first cloned a 1832-bp fragment of the 3' UTR of LATS2 containing the putative miR-195 binding site into the psiCHECK-2 reporter vector (Fig. 6B marked as WT 3'UTR). To identify which miR-195 binding sites of LATS2-3' UTR was critical for miR-195 binding. The two conserved targeting regions for miR-195 binding were specifically mutated (Fig. 6B marked as Mut 1st 3'UTR and Mut 2nd 3'UTR), respectively. BEL-7402/5-FU cells transiently transfected with the WT 3'UTR-reporter construct and miR-195 led to a significant decrease of reporter activity when compared to the control (Fig. 6C 48% decreased). In addition, we also performed Mut.1st-3'UTR, Mut.2nd-3'UTR and co-transfection with Mut.1st-3'UTR and Mut.2nd-3'UTR luciferase reporter assay (Fig. 6C). The luciferase activity of the reporter that carried Mut.1st-3'UTR reporter construct was decreased about 27% of reporter activity when compared to the control and the luciferase activity of Mut.2nd-3'UTR was decreased about 3%. The activity of the reporter was with synergistic effect on the abolishment of miR-195 mediating decrease of reporter activity in co-transfection with Mut.1st-3'UTR and Mut.2nd-3'UTR in the cells which was not decreased by miR-195 mediating LATS2–3'UTR luciferase activity. This result indicated that miR-195 target site 1 of LATS2–3'UTR plays a more important role than miR-195 target site 2 in miR-195 mediating LATS2 protein expression.

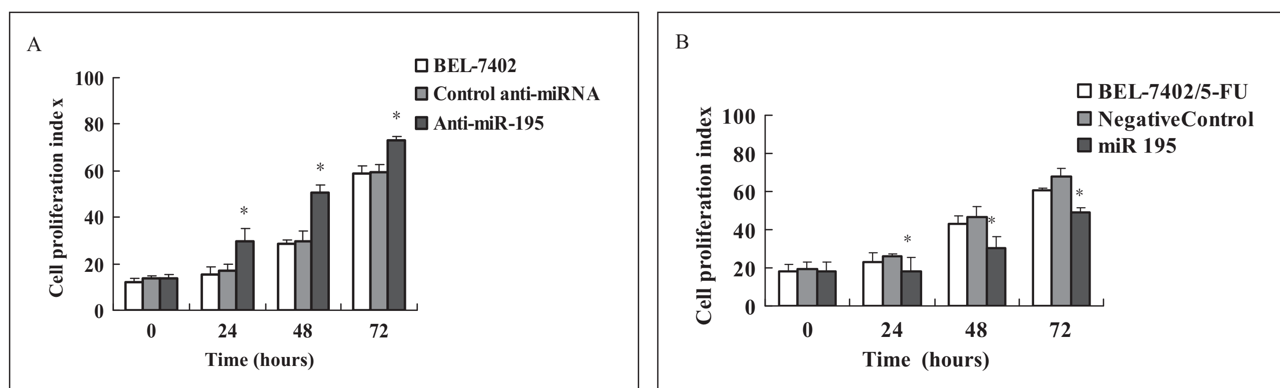

Fig. 4: Effects of miR-195 on cell proliferation of hepatocellular carcinoma cells. (A) The growth-promoting effect of Anti-miR-195, BEL-7402 cells was transfected with control ant-miRNA or anti-miR-195. Cell viability assay was measured by MTT assay at 0, 24, 48 and 72 h. (B) The growth-inhibitory function of miR-195 was measured by MTT assay. Cell viability assay was performed at 0, 24, 48 and 72 h after transfection of BEL-7402/5-FU cells with Negative Control ormiR-195. *P<0.05 compared to other groups. (n=3)

3. Discussion

It is well demonstrated that a defect in cell cycle control is an essential step during carcinogenesis. Therefore, it is reasonable to expect that deregulation of cell cycle-related miRNAs may facilitate tumorigenesis. In this study we showed that miR-195 was frequently down-regulated in HCC cell lines. Ectopic expression of miR-195 suppressed HCC cells to form colonies *in vitro*. We propose that reduced expression of miR-195 may disrupt cell cycle control, in turn promote cell proliferation, and consequently facilitate the development of cancers like HCC. The underlying mechanism responsible for decreased expression of miR-195 in HCC is still unknown. Notably, miR-195 is located at chromosome 17p13.1, a region that is frequently deleted in human cancers, especially HCC.
miRNAs are a class of small noncoding RNAs that play an important role in regulating gene expression in various living systems (Mertens-Talcott et al. 2007). Recent evidence indicates that miRNAs can function as tumor suppressors and oncogenes and play a crucial role in the initiation and progression of human cancer (Saito et al. 2009). In addition, expression profiles of miRNAs have been found to be correlated with cancer pathogenesis and useful in diagnosis and prognosis of cancers (Volinia et al. 2006; He and Hannon 2004; He et al. 2005; Wu et al. 2007). Identification of cancer-specific miRNAs and their targets is critical for understanding their role in tumorigenesis (O'Donnell et al. 2005; Volinia et al. 2006).
In this study, we found that miR-195 also supports the growth of hepatocellular carcinoma cell line. In order to understand the mechanism by which miR-195 supports the growth of HCC cells, it is crucial to identify its target genes. In addition, forced expression of miR-195 had a similar effect to suppress hepatocellular carcinoma cell proliferation, blocked cell cycle G1 progression, and induced apoptosis. Such results suggest that miR-195 have similar effects to play a tumor-suppressor role in hepatocellular carcinoma by the same cluster of gene regulation. Recently, LATS2 was found to be a target of miR-372 and miR-373 (Wha Ja Cho et al. 2009).
It is not yet clear how tumor suppressor LATS2 exerts its effects on cells. However, previous reports suggest that LATS2 overexpression results in cell cycle arrest at the G2/M phase via inhibition of Cdc2-Cyclin B kinase activity (Kamikubo et al. 2003),

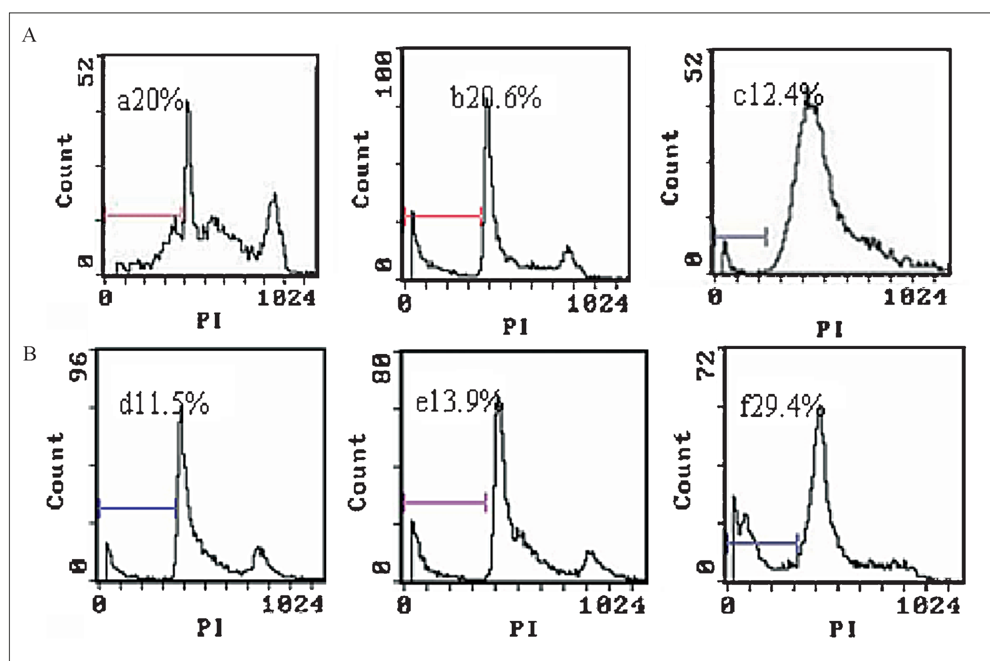

Fig. 5: miR-195 increases the cell apoptosis rate of BEL-7402 and BEL-7402/5-FU cells. (A) BEL-7402 was (a) non-treatment and transfected with (b) Control anti-miRNA or (c) Anti-miR-195 (B) BEL-7402/5-FU was (d) non-treatment and transfected with (e) Control miRNA or (f) miR-195. Cells apoptosis rates were evaluated by flow cytometry, and the percentage of apoptotic cells was calculated. *P<0.05, compared to other groups (n=3)

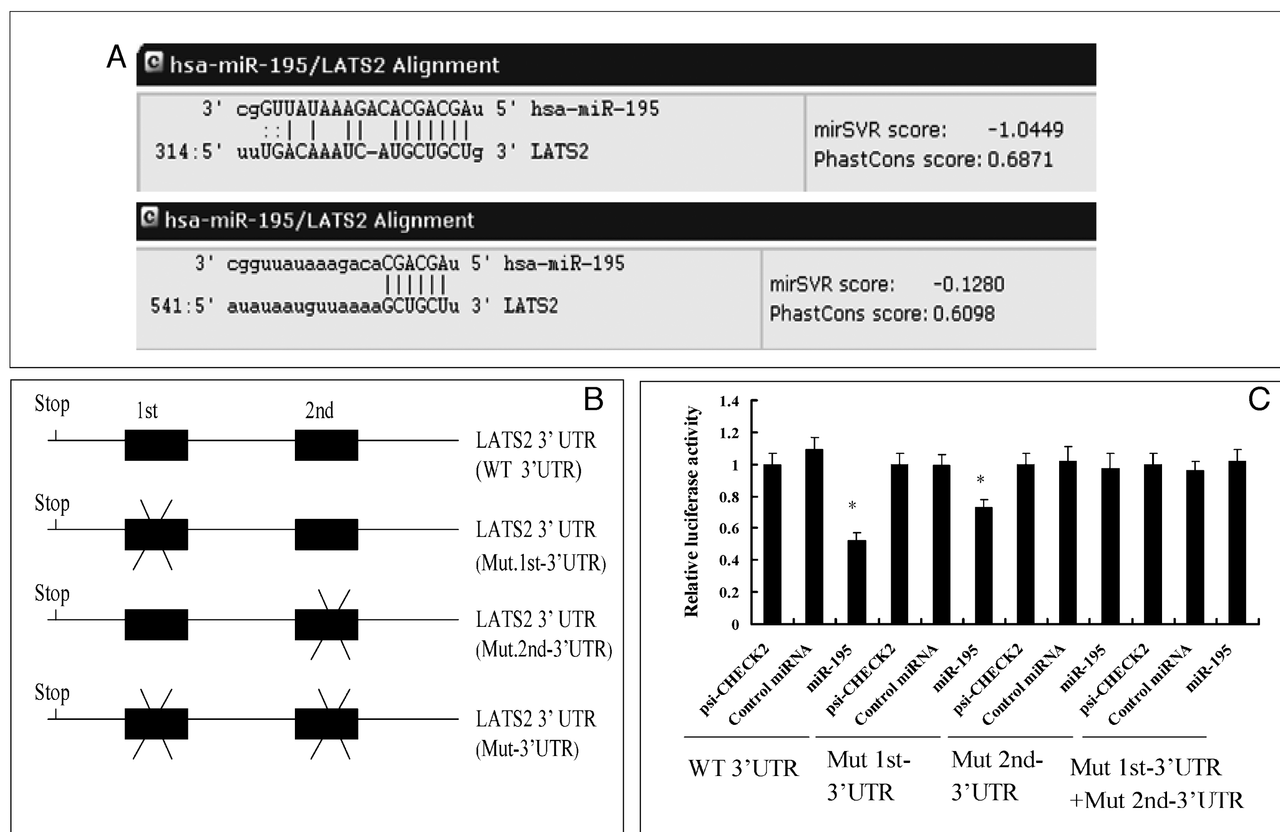

Fig. 6: Effect of the putative miR-195 binding site derived from the LATS2 3'-UTR on luciferase expression. (A) Putative binding sites of miR-195 in the LATS2 3'-UTR regions are determined by TargetScan 5.1 and microrna.org. (B) The two conserved targeting regions for miR-195 binding were specifically mutated, respectively. (C) Dual luciferase assay was performed in BEL-7402/5-FU cells transfected with luciferase construct alone, cotransfected with miR-195, or Control miRNA. Firefly luciferase construct containing mutant target site of the LATS2 3'UTR was generated and transfected as indicated. Firefly luciferase activity was normalized to Renilla luciferase activity for each sample (n=3). *$p<0.05$ compared to Control miRNA

cell cycle arrest at G1/S phase via inhibition of E3 ubiquitin ligase activity of Mdm2 (Li et al. 2003), and apoptosis via down-regulation of Bcl-2 and Bcl-Xl (Ke et al. 2004). Down-regulation of tumor suppressor LATS2 expression has been reported to provide an environment favorable for proliferation of cancer cells (Lee et al. 2009). Our results suggest that miR-195 plays an important role in the up-regulation of LATS2 expression in hepatocellular carcinoma cells. In the overexpression of LATS2, HCC cells were inhibited of cell growth and triggered to apoptosis when LATS2 expressions were independently regulated by miR-195. In many primary cancers, a loss of heterozygosity has been frequently observed in a chromosomal region where the LATS2 gene resides (Yabuta et al. 2000). In conclusion, we have shown that miR-195 is down-regulated in human BEL-7402/5-FU cells and its down-regulation supports growth of these cells through down-regulation of tumor suppressor LATS2. These findings suggested that miR-195 down-regulation may play a crucial role in the tumorigenesis of hepatocellular carcinoma through suppression of LATS2 expression.

4. Experimental

4.1. Material

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).

4.2. 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 MDR phenotype, 5-FU (with final concentration of 2 μg/ml) was added to the culture media for BEL-7402/5-FU cells.

4.3. 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) following the manufactuer's protocol, where opropanol was replaced by ethanol for RNA precipitation. RNA quality was measured using denaturing agarose gel electrophoresis.
MiRNA labeling: miRCURYTM Array labeling ke, 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.
Microarray preparation and hybridization: miRCURYTM 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 was 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.

4.4. Construction of miR-195 expression plasmids

pcDNATM6.2-GW/miR linear vector was purchased from GenePharma (Shanghai, China), and hsa-miR-195$^{(TM)}$ insert sequence with the follow-

ing sense: 5'- CACTGACTGACGCCAATATCTGTGCTGCTA-3'. Mature antisense oligodeoxynucleotides specifically against miR-195 (Anti-miR-195) and Control anti-miRNA 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.

4.5. Cell culture and transfection

BEL-7402 and BEL-7402/5-Fu cells were cultured in RPMI- 1640 medium (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum (Hyclone, Logan), and for BEL-7402/5-Fu 2 μg/ml (QiLu, Shandong, 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% confluent. Transfection was carried out according to the manufacturer's protocol. hsa-miR-195 (4 μg) and Control miRNA (4 μg) carrying green fluorescent protein (GFP) and Anti -miR-195 (4 μg) and Control anti-miRNA (4 μg) 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.

4.6. Real-time quantitative RT-PCR

The expression level of miR-195 was measured in cells transfected with miR-195, Anti-miR-195, or Control miRNA by using the NCodeTM miRNA First-Strand cDNA Synthesis Kit and NCodeTMSYBR 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 $2-\Delta CT$, where $\Delta CT = (CTmiRNA - CT5\,s)$. Relative gene expression was multiplied by U6 to simplify the presentation of the data.
The expression levels of LATS2, p53 and CDK2 were measured in cells 48 h post-transfection using High Capacity cDNA Reverse Transcription kit and Power SYBR Green PCR master Mix (Applied Biosystems).

4.7. Western blotting

Cells were lysed in PRO-PREP lysis buffer (iNtRON BIOTECHNOLOGY). Proteins were resolved by SDS-PAGE, transferred onto PVDF transfer membranes (PIERCE), and probed with appropriate dilutions of anti-human LATS2 polyclonal antibody (sc-23065, Santa Cruz Biotechnology), anti-p53 monoclonal antibody (#2524, Cell Signaling), anti-CDK2 monoclonal antibody (#2546S, Cell Signaling), or anti-β-actin monoclonal antibody (BM0627, Boster Bio-Technology).

4.8. MiRNA and anti-miR transfection and cell proliferation assay

The transfection of hepatocellular carcinoma cells was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Lipofectamine 2000 transfections were carried out in 60 mm plates containing 1.2 μl of 30 nM or 1.8 μl 60 nM miRNA, 990 μl Opti-MEM (GIBCO^{TM}, Invitrogen, Carlsbad, CA, USA) and 10 μl of lipofectamine 2000 reagent. The cells were incubated with the transfection-complexes for 6 h. Then 3 ml fresh cell growth medium was added and incubated in the incubator for 16–18 h. BEL-7402 cell was transfected with Anti-miR-195 or Control anti-miRNA and BEL-7402/5-FU cell was transfected with miR-195 or Control miRNA. Cells were then seeded into 6-well culture dishes containing 2 ml of culture media. After 24 h of recovery, the cells were subjected to cell proliferation experiments. Cell proliferation was assessed using the MTT assay. After transfection, cells (1×10^5/well) were plated in a 96-well plate and incubated at 37 °C, and cell proliferation was assessed at indication time.

4.9. Cell apoptosis analysis

Cells were harvested and washed twice in PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na2HPO4 7H20; 1.4 mM KH2PO4; pH 7.2) at room temperature and resuspended at 2×10^6 cells/ml in PBS. For propidium iodide staining, washed cells were fixed in pure ethanol at −20 °C overnight and the rest of the steps were performed based on the manufacture's procedure (Molecular Probes Inc.). Cells were washed twice in PBS, resuspended in FACS buffer (PBS, 0.2% BSA and 1% sodium azide) and analyzed using a FACS flow cytometer (Becton Dickinson, Inc.).

4.10. MiR-195 target prediction

Given the limitations of any single prediction program, we used two separate prediction programs (TargetScan Human 5.1 and microrna.org) to identify common predicted targets for miR-195.

4.11. Construction of the LATS2 3'-UTR-luciferase plasmids and reporter assays

The LATS2 3'-UTR target site was amplified by PCR using the primers Fwd-5'-GCAAGCTTATGGGGGCCAGGCAC-3' and Rev-5'-GGCACTAGTCATTATTGCACAGAGATTT-3' and cloned into downstream of the luciferase gene in the psi-CHECK2 luciferase vector. This vector was sequenced and named WT-3' UTR. Site-directed mutagenesis of the miR-195 target-site in the LATS2–3'-UTR was carried out using Quick change-mutagenesis kit (Stratagene, Heidelberg, Germany) and named Mut.1st-3' UTR and Mut.2nd-3' UTR, respectively, in which used WT-3' UTR as a template. For reporter assays, the cells were transiently transfected with wildtype or mutant reporter plasmid and miR-195 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 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.

4.12. Statistical analysis

Statistical analysis was performed using SPSS software (Version 13.0, 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 $P < 0.05$(*).

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