

Original Article

MicroRNA-520b targets interleukin-2 to regulate the growth, metastasis and drug sensitivity of liver cancer cells

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Abstract: Liver cancer is ranked as the 5th major type of cancer and is responsible for significant number of human deaths across the world. Recent investigations have shown microRNAs (miRs) to be involved in diverse cellular processes. Additionally, they have been shown to exhibit therapeutic implications in treatment of different diseases including cancer. This study investigated the role and therapeutic implications of miR-520b in liver cancer. The results of the present study revealed significant downregulation of miR-520b in liver cancer cell lines. Overexpression of miR-520b suppresses the growth of the SNU-182 and HepG2 cells by triggering apoptosis. This was also accompanied by upsurge of Bax and Caspase-3 and depletion of Bcl-2 in SNU-182 and HepG2 cells. Additionally, miR-520b caused a significant decrease in the migratory and invasive potential of the SNU-182 and HepG2 cells and enhanced their chemosensitivity to Gemcitabine. Bioinformatic analysis and the dual luciferase showed that miR-520b targets IL2 in HepG2 cells. Suppression of IL2 inhibits the growth of the HepG2 cells while as IL2 overexpression could avoid the tumor suppressive effects of miR-520b in HepG2 cells. Taken together, miR-520b may prove to be essential therapeutic target in liver cancer treatment and warrants further research endeavours.

Keywords: Liver cancer, microRNA, cell cycle arrest, proliferation

Introduction

The implications of microRNAs (miRs) as therapeutic targets is currently one of the promising aspects in cancer research [1]. The miRs are around 18-25 nucleotides long endogenous molecules [2]. They regulate the expression of the target genes by binding to 3'UTR causing degradation of mRNA or repression of translation [2]. The miRs play vital roles in fundamental cellular processes which include, but are not limited to proliferation, development, differentiation, apoptosis and autophagy [3]. There are strong evidences which have shown that many miRs show dysregulation in cancerous tissues and play important part in the development of cancer [4]. Hence, it is believed that miRs may serve as therapeutic targets and will allow targeted therapy for the treatment of cancer [5]. The miR-520b has been shown to control the growth of different types of cancers. For example, miR-520b targets cyclin D1 and MEKK2 to suppresses the proliferation of the of hepatoma cells [6]. Wang *et al.*, reported that miR-

520b inhibits the growth and metastasis of spinal osteosarcoma cells [7]. Nonetheless, the role and therapeutic potential of miR-520b has not been studied in liver cancer. Against this back drop, present study investigated the function and therapeutic utility of miR-520b in liver cancer. Ranked as the fifth most prevalent type of cancer, liver cancer is responsible for significant proportion of cancer related mortality. In 2012, liver cancer accounted for more 9% of the all the cancer related mortalities [9]. Because of the very aggressive nature and poor five year survival, liver cancer is one of the major health issues around the globe [9]. Majority of the liver cancers have been reported to be of epithelial origin and upto 90% of the liver cancers are hepatocellular carcinomas [10]. Owing to the lethality of the liver cancer, it is imperative to identify novel therapeutic targets for its treatment. Herein, we report that miR-520b is significantly suppressed in liver cancer and controls the growth and metastasis of the liver cancer by targeting IL2. Taken together, we miR-520b acts a tumor

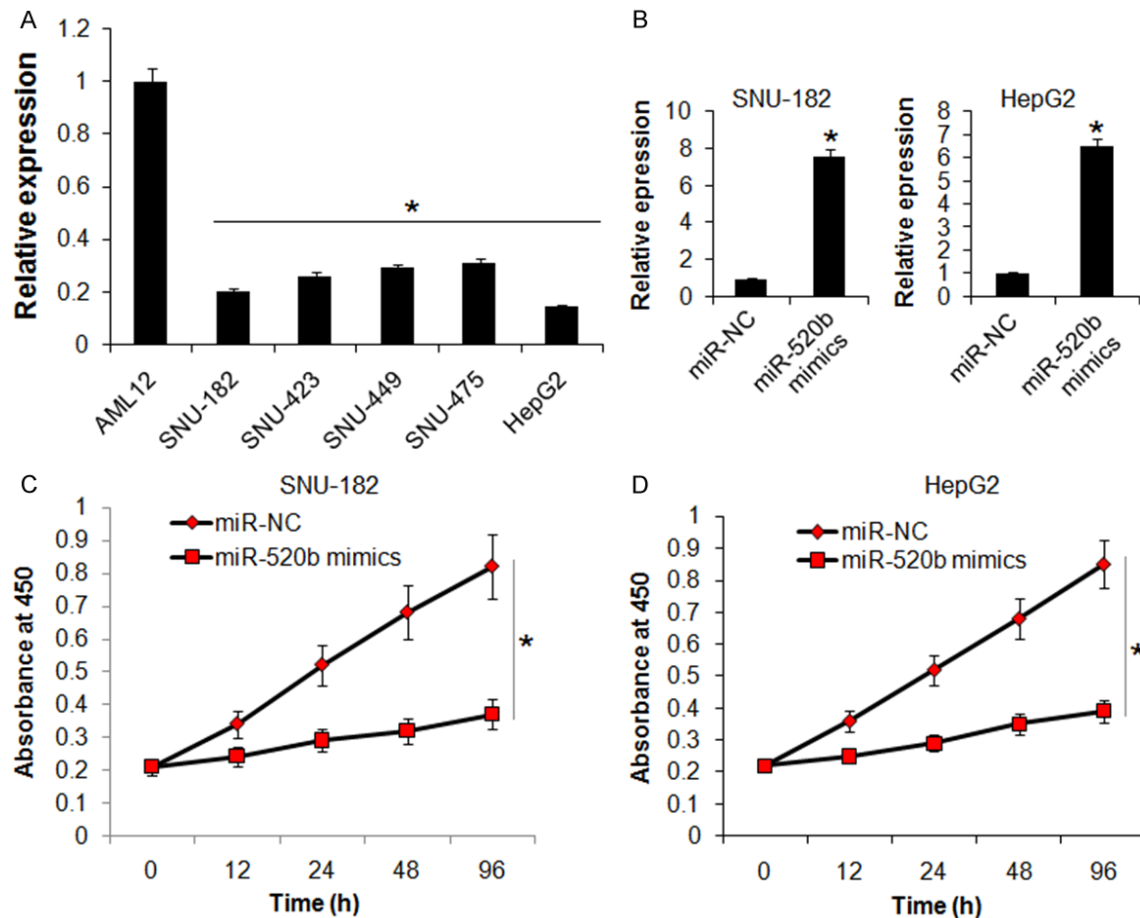


Figure 1. The miR-520b inhibits the proliferation of the human liver cancer cells. A. Expression of miR-520b in normal and liver cancer cell lines. B. Expression of miR-520b in miR-NC and miR-520b mimics transfected SNU-182 and HepG2 liver cancer cells. C. Cell viability of miR-NC and miR-520b mimics transfected SNU-182 liver cancer cells. D. Cell viability of miR-NC and miR-520b mimics transfected HepG2 liver cancer cells. The experiments were performed in triplicate and expressed as mean $\pm$ SD (* $P < 0.05$).

suppressor in liver cancer may point to a novel therapeutic target in liver cancer.

Materials and methods

Cell lines and culture conditions

The liver cancer cell lines (SNU-182, SNU-423, SNU-449, SNU-475, HepG2) and three normal liver cell line (AML-12) were procured from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 μ g/ml streptomycin and 100 U/ml penicillin and a humidified atmosphere containing 5% CO₂.

Expression analysis

For gene expression studies, the total RNA was isolated using RNAiso plus (Total RNA extraction reagent, Takara) which was quantified us-

ing NanoDrop spectrophotometer and subsequently reverse transcribed to cDNA using RevertAid First strand cDNA synthesis kit (Invitrogen). The quantitative real time-PCR (qRT-PCR) was performed on QuantStudio 5.0 Real Time PCR system (Applied Biosystems) using SYBR Green reagent (Invitrogen). The relative expression levels were estimated by $2^{-\Delta\Delta CT}$ method using human U6 gene as internal control for normalization.

MTT assay

The SNU-182 and HepG2 cells were cultured in a 96-well tissue culture plates with approximately 2500 cells/well. The viability of the cells was evaluated at different time intervals (0, 12, 24, 48 and 96 h) by Vybrant MTT Cell Proliferation Assay (Invitrogen, USA) as the manufacturer's guidelines. Finally, the optical density was measured at 570 nm.

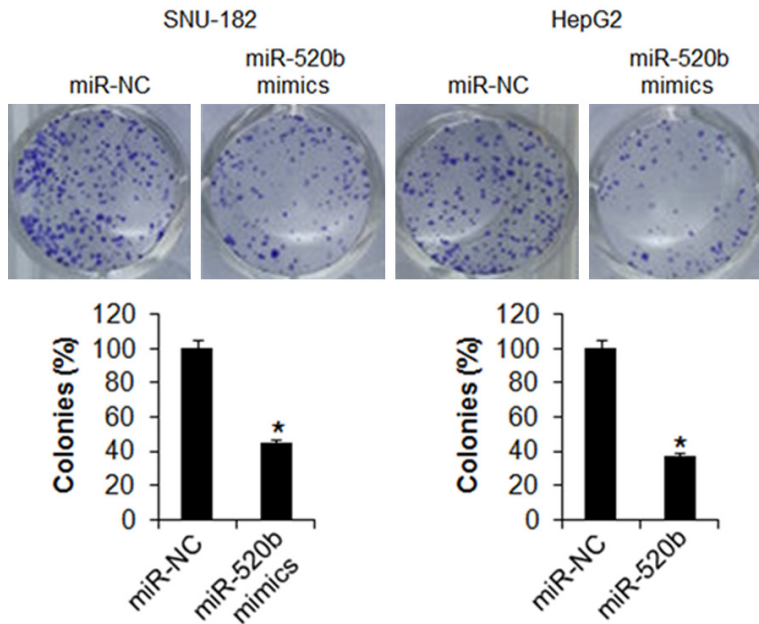


Figure 2. The miR-520b inhibits the colony formation potential of the SNU-182 and HepG2 liver cancer cells. The experiments were performed in triplicate and expressed as mean $\pm$ SD (* $P < 0.05$).

Analysis of cell death

The SNU-182 and HepG2 cells were transfected with suitable constructs and cultured for 24 h at 37°C and then fixed with ethanol (70%) for 20 min. The cells were then subjected to PBS washing and subsequently stained with AO/EB solution. Finally, the cells were examined under microscope to detect the induction of apoptosis. The SNU-182 and HepG2 cells were transfected with appropriate constructs and then incubated for 48 h at 37°C. The cells were then dissociated with the help of trypsin and then PBS washed. The cells were then resuspended in 1X binding buffer which was followed by the addition of 5 μ L of annexin V-FITC and propidium iodide (PI). The cell culture was then placed in dark room for 15 min. The apoptosis percentage was then evaluated by a flow cytometer.

Wound heal assay

After transfection with suitable constructs, the SNU-182 and HepG2 cells were placed in twelve well plates with approximately 1×10^5 cells per well. A scratch was made by a pipette tip at 24 h after transfection. The cells were PBS washed and fresh media was added. Following 24 h incubation at 37°C, the cells were subjected to fixation with methanol. The initial wound width and final wound width was deter-

mined from photomicrographs.

Cell invasion assay

Transwell chambers with Matrigel was employed to monitor the SNU-182 and HepG2 cell invasion. In brief, the cells were transfected with appropriate constructs and 48 h post-transfection, the cells were harvested and suspended in fresh media. While as 200 μ L of the cell suspension containing approximately 5×10^4 cells was placed onto the upper compartment, a fresh 500 μ L media was placed in the lower compartment. After 24 hours cells present at the upper compartment were removed by swabbing while as the cells that invaded to the lower surface were fixed

and then subsequently stained with 0.05% crystal violet. Finally, ten random fields were selected to determine cell invasion under the light microscope.

Western blotting

The liver cancer cell lines were lysed and the protein concentration in each sample was measured by Bradford assay. Equal concentrations of the proteins from each sample were loaded on 10% SDS polyacrylamide gels (SDS-PAGE) and then followed by shifting to polyvinylidene fluoride membranes. Blocking of the membrane was then performed by fat-free milk (5%) in TBST. This was followed by incubation with a primary antibody for 24 h at 4°C. Subsequently, a secondary antibody was added at 25°C for about 2 h. The bands of interest were finally observed by chemiluminescence.

Statistical analysis

The experiments were carried out in triplicate and the values shown as the mean $\pm$ standard deviation (SD). Student's t-test (for comparison between two samples) and one way analysis of variance followed by Tukey's test (for comparison between more than two samples) were used for statistical analysis using GraphPad Prism software (version 7; GraphPad Software,

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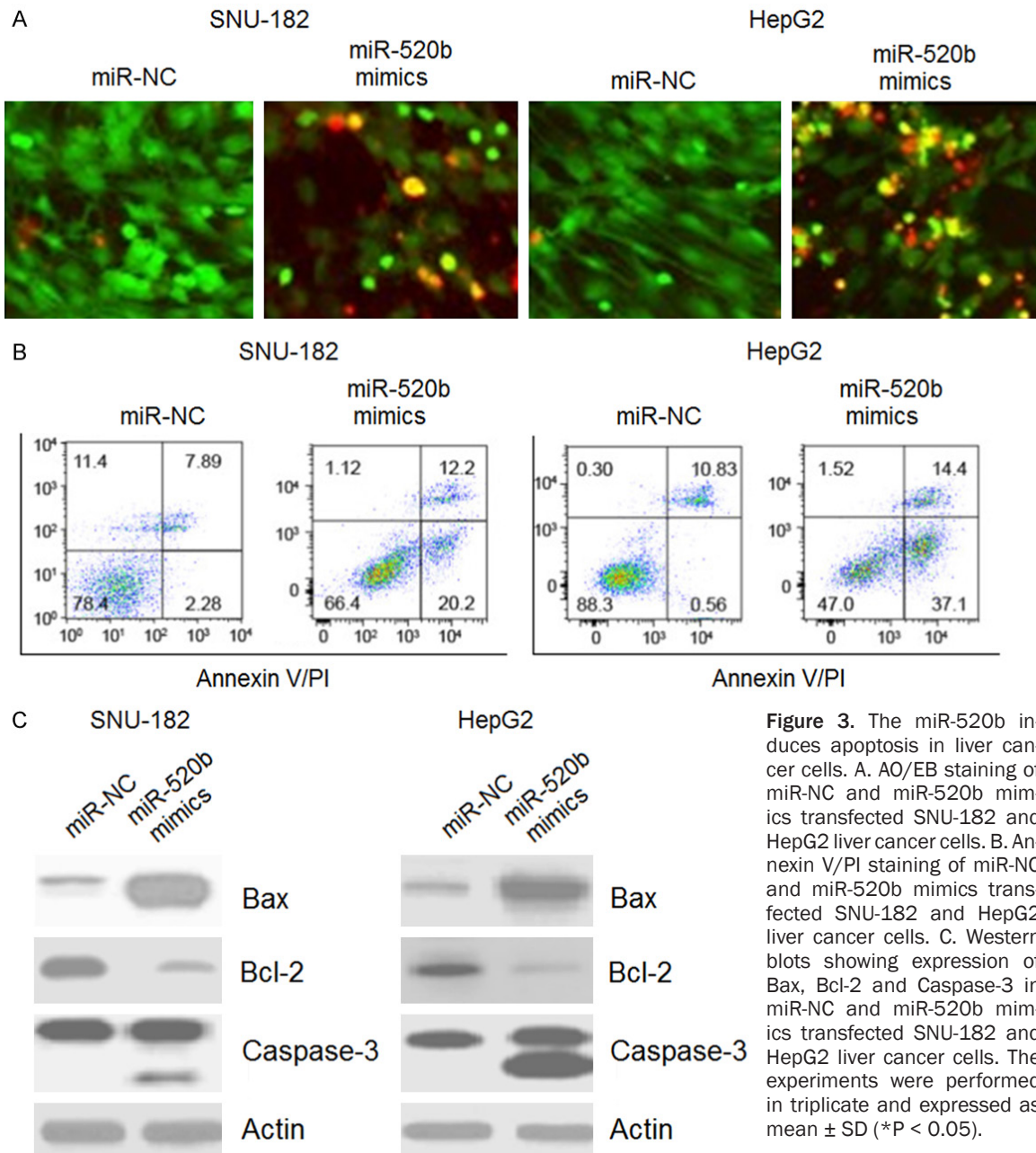


Figure 3. The miR-520b induces apoptosis in liver cancer cells. A. AO/EB staining of miR-NC and miR-520b mimics transfected SNU-182 and HepG2 liver cancer cells. B. Annexin V/PI staining of miR-NC and miR-520b mimics transfected SNU-182 and HepG2 liver cancer cells. C. Western blots showing expression of Bax, Bcl-2 and Caspase-3 in miR-NC and miR-520b mimics transfected SNU-182 and HepG2 liver cancer cells. The experiments were performed in triplicate and expressed as mean $\pm$ SD (* P < 0.05).

Inc., La Jolla, CA, USA. P < 0.05 was considered to indicate a statistically significant difference.

Results

The miR-520b is suppressed in liver cancer

The expression of miR-520b was determined in different liver cancer cell lines and in one normal cell line. Gene expression analysis of miR-520b in liver cancer cells showed miR-520b to be significantly (upto 7.14) downregulated in liver cancer cell lines (**Figure 1A**). The highest

downregulation was observed in SNU-182 and HepG2 cells. These cell lines were therefore used for further studies.

The miR-520b inhibits growth of liver cells via induction of apoptosis

Next, we sought to understand the role of miR-520b in liver cancer and for that we overexpressed miR-520b in SNU-182 and HepG2 liver cancer cells (**Figure 1B**). The MTT assay showed a remarkable decline in the proliferation rate of the liver SNU-182 and HepG2 cancer cells upon

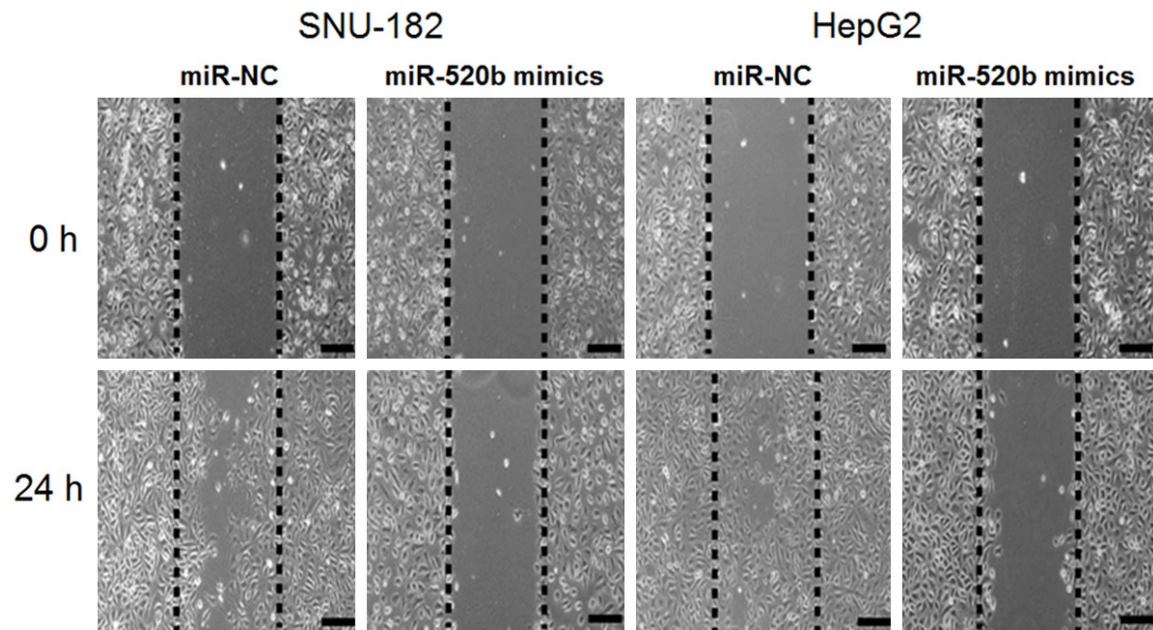


Figure 4. The miR-520b inhibits the migration of the SNU-182 and HepG2 cells. Wound heal assay of miR-NC and miR-520b mimics transfected SNU-182 and HepG2 liver cancer cells was performed and showed inhibition of cell migration. The experiments were performed in triplicate.

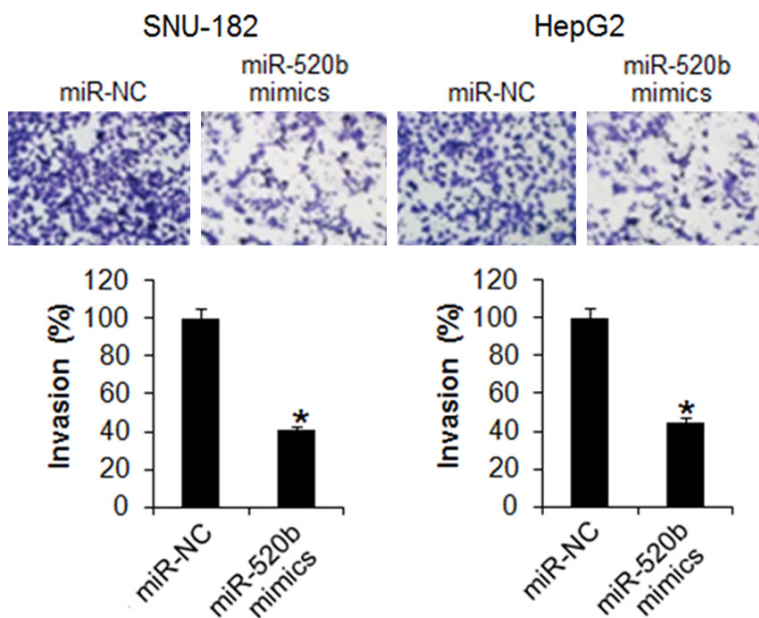


Figure 5. The miR-520b inhibits the invasion of the human liver cancer cells. Transwell assay of miR-NC and miR-520b mimics transfected SNU-182 and HepG2 liver cancer cells was performed and showed inhibition of cell invasion. The experiments were performed in triplicate and expressed as mean $\pm$ SD (*P < 0.05).

miR-520b overexpression (**Figure 1C**). The colony formation assay also confirmed the antiproliferative effects of miR-520b overexpression on the SNU-182 and HepG2 cancer cells. The

colony formation in SNU-182 and HepG2 cells was inhibited by upto 60% in comparison to the control (**Figure 2**). Next we carried out the AO/EB staining of the miR-520b mimics and miR-NC transfected SNU-182 and HepG2 cells and we found that miR-520b overexpression triggers apoptosis in SNU-182 and HepG2 cells (**Figure 3A**). The Annexin V/PI assay also confirmed the induction of apoptosis which was accompanied by enhancement of Bax and caspase-3 and suppression of Bcl-2 (**Figure 3B and 3C**).

MiR-520b suppresses the metastasis of liver cancer cells

The wound heal assays showed that overexpression of miR-520b suppressed the migration of the SNU-182 and HepG2 cells as evident from the wound width (**Figure 4**). The transwell assays used assess the effects of miR-520b on the invasion of SNU-182 and HepG2 cells.

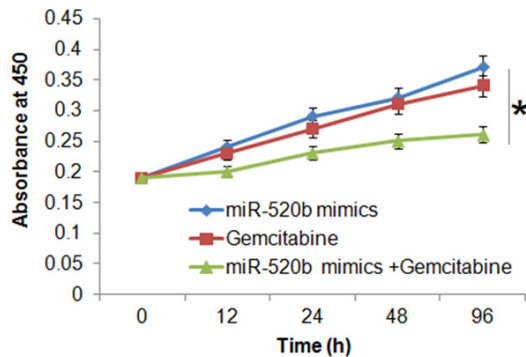


Figure 6. Overexpression of miR-520b in HepG2 cells enhances their drug sensitivity to gemcitabine. The experiments were performed in triplicate and expressed as mean $\pm$ SD (*P < 0.05).

We found that the invasion of the SNU-182 and HepG2 cells was suppressed upon miR-520b overexpression. The invasion of the SNU-182 and HepG2 cells was suppressed by 60 and 55% relative to the control respectively (**Figure 5**).

The miR-520b enhances the drug sensitivity of the liver cancer cells

The impact of miR-520b were also evaluated on the Gemcitabine sensitivity of the HepG2 cells. The results showed that miR-520b caused remarkable enhancement in the Gemcitabine sensitivity of the HepG2 cells (**Figure 6**).

The miR-520b targets IL2 in liver cancer cells

The bioinformatic approaches such as TargetScan (<http://www.targetscan.org>) were employed to identification of miR-520b targets. The TargetScan revealed IL2 to be the potential target of miR-520b (**Figure 7A**). Next, dual luciferase assay also confirmed the interaction between miR-520b and IL2 (**Figure 7B**). We also examined the expression of IL2 in the all liver cancer cell lines and the qRT-PCR revealed IL2 to be aberrantly upregulated in all the cell lines (**Figure 7C**). However, miR-520b overexpression could cause decline in IL2 expression in the HepG2 cells (**Figure 7D**). We also sought to ascertain if silencing of IL2 caused similar effects on HepG2 cells. We found that IL2 silencing caused a remarkable decrease in the proliferation of HepG2 cells (**Figure 7E and 7F**). The impact of IL2 overexpression was also investigated on the proliferation of the HepG2

cells overexpressing miR-520b. We observed that overexpression of IL2 in the HepG2 cells overexpressing miR-520b promoted their proliferation thereby avoiding the growth inhibitory effects of miR-520b (**Figure 7G**).

Discussion

Liver cancer is the fifth most common type of cancer and causes tremendous human mortality [11]. As such there is pressing need to identify efficient therapeutic targets for the treatment of liver cancer. MicroRNAs (miRs) have shown great promise as therapeutic targets for treatment of cancer [12]. Since, the discovery of first miR in early 1990s, several miRs have been reported to play vital roles in different cellular processes, such as proliferation, cell death, metastasis via modulation of post-gene expression post-transcriptionally [13]. The miRs have been shown to act as tumor suppressors as well as oncomiRs and many of the miR-based therapies are being evaluated for cancer treatment [14]. The miR-520b has been found to act as tumor suppressor and its therapeutic implications have not been explored in liver cancer. Herein, we determined therapeutic potential of miR-520b in liver cancer. We found that miR-520b expression was considerably suppressed in liver cancer. These findings are also complimented by previous studies wherein miR-520b has been found to be downregulated colorectal cancer [15]. Next, miR-520b was overexpressed in liver cancer and we found that miR-520b overexpression inhibited the proliferation of SNU-182 and HepG2 cells by inducing apoptosis which was accompanied by depletion of Bcl-2 and upsurge of Bax and caspase-3. Studies carried out earlier have reported that miR-520b inhibits the growth of human glioblastoma and hepatoma cells [16, 6]. The effects of miR-520b overexpression were also examined on the Gemcitabine sensitivity and we found that miR-520b enhances the Gemcitabine sensitivity of the liver cancer cells. Earlier studies have also shown that miR-520b plays a role in the enhancement of the doxorubicin sensitivity of hepatocellular carcinoma cells [17]. The wound heal and transwell assays showed that miR-520b overexpression suppressed the migration and invasion of the SNU-182 and HepG2 liver cancer cells. These results are in agreement with a previous study wherein miR-520b has been

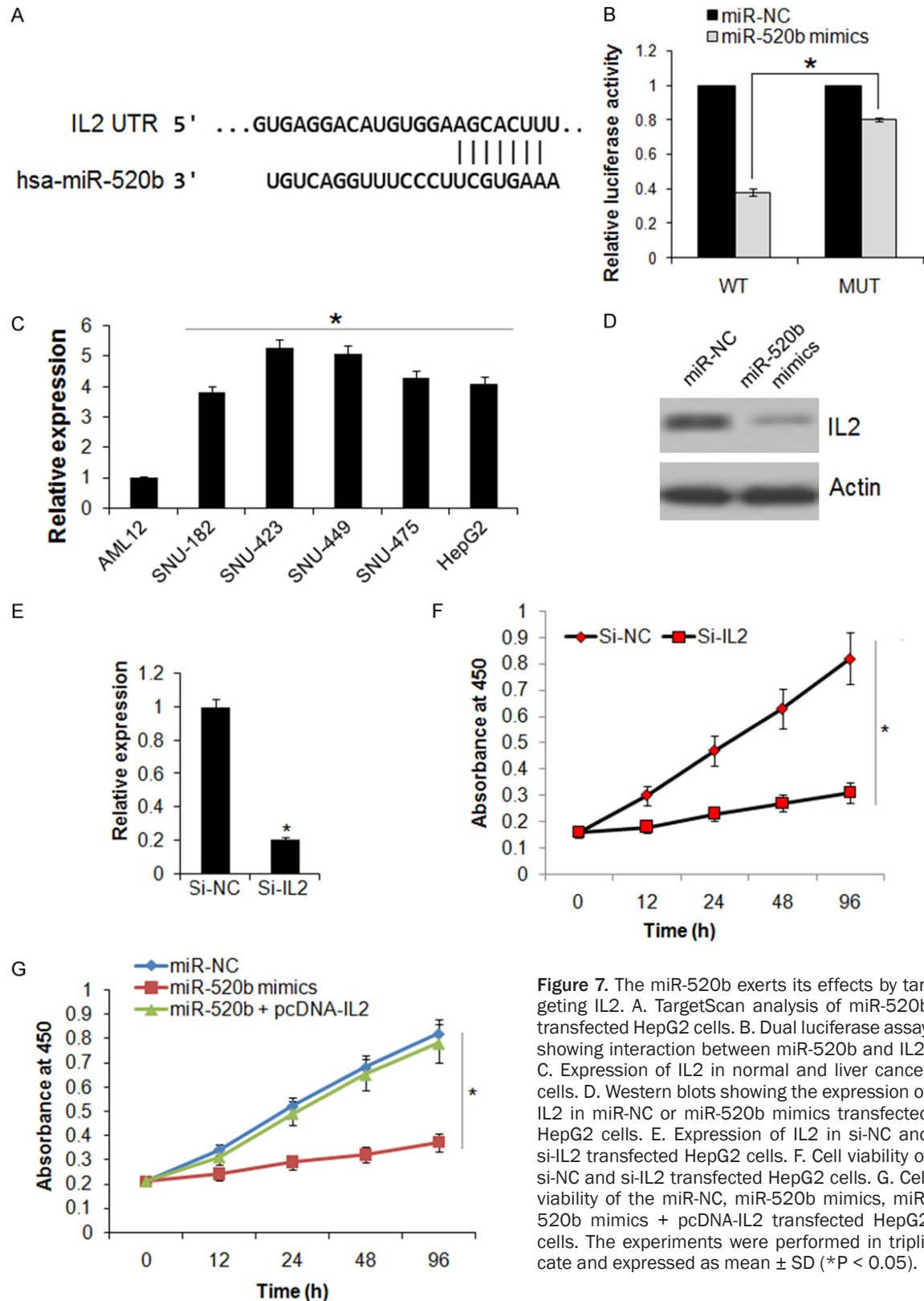


Figure 7. The miR-520b exerts its effects by targeting IL2. A. TargetScan analysis of miR-520b transfected HepG2 cells. B. Dual luciferase assay showing interaction between miR-520b and IL2. C. Expression of IL2 in normal and liver cancer cells. D. Western blots showing the expression of IL2 in miR-NC or miR-520b mimics transfected HepG2 cells. E. Expression of IL2 in si-NC and si-IL2 transfected HepG2 cells. F. Cell viability of si-NC and si-IL2 transfected HepG2 cells. G. Cell viability of the miR-NC, miR-520b mimics, miR-520b mimics + pcDNA-IL2 transfected HepG2 cells. The experiments were performed in triplicate and expressed as mean $\pm$ SD (* P < 0.05).

reported to target CHAF1A to suppress the migration and invasion of the non-small cell

lung cancer [18]. The miRs have been reported to exert its effects by targeting different genes

[17]. Herein we found that miR-520b targets IL2 in liver cancer and suppression of IL2 could inhibit the proliferation of the liver cancer cells. However, IL2 overexpression could reverse the tumor-suppressive effects of miR-520b.

Conclusion

The findings of the present study indicates that miR-520b is overexpressed in liver cancer and targets IL2 to controls the proliferation and metastasis of the liver cancer cells. Taken together, miR-520b may be utilised as therapeutic target for liver cancer treatment.

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Disclosure of conflict of interest

None.

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