

## Original Article

# MicroRNA-9500 induces cell apoptosis of hepatoma through targeting AKT

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**Abstract:** Background: HCC is one of the most usual human carcinoma, while also the second commonest reason of cancer deaths among human. But its mechanism at post-transcriptional levels is not fully understood. The purpose of this study was to elucidate the post-transcriptional factors regulating the apoptosis process of HepG2 and Huh7. Methods: To confirm the predicted miR-9500 was matched with AKT-1, 3' UTR luciferase activity of AKT-1 was used to assess. Then, HepG2 and Huh7 were cultured in the presence or absence of miR-9500 mimics or inhibitors. The AKT signaling pathway involved in apoptosis process of HepG2 and Huh7 was investigated, respectively. Results: Our work indicated that miR-9500 over-expression induced reduction of luciferase activity on AKT-1 3'-UTR, as well as the protein expression of AKT-1 in HepG2 and Huh7 through luciferase system reporter gene analysis. Therefore, AKT-1 is direct targets of miR-9500. miR-9500 suppressed expression of AKT-1. Furthermore, miR-9500 substantially accentuated the apoptosis process of HepG2 and Huh7. But transfection with inhibitors of miR-9500, antagomiR-9500, overtly attenuated expression level of AKT-1 in HepG2 and Huh7. MiR-9500 exerted effects on cell proliferation or viability, and accentuated its caspase-dependent apoptosis of HepG2 and Huh7. Conclusions: We identified miR-9500, which specifically bind to AKT-1 mRNA 3'-UTR. MiR-9500 is a crucial mediator of regulating expression of AKT-1 at post-transcriptional levels, protecting against proliferation of hepatoma cells through inhibiting the AKT signaling pathway. Moreover, it exerts effects on controlling viability and cell apoptosis of HepG2 and Huh7. Our findings suggest that targeting miR-9500, being involved in pathogenesis of HCC, is a promising strategy for the treatment and prevention of HCC.

**Keywords:** MicroRNA, hepatocellular carcinoma (HCC), AKT, apoptosis

## Introduction

HCC is one of the most usual human carcinoma and ranked fifth, while it is also the second commonest reason of cancer deaths among human. HCC was almost come from inflammation process and liver cirrhosis induced by aflatoxins, ethanol, and chronic infection of hepatitis B or C [1]. The prognosis of HCC is so poor [2] that only tiny minority patients could be cured by surgical resection or liver transplantation. Consequently, the emergency necessity is effective medicines or therapeutic methods for HCC.

For ages, different genetic alterations were thought to the origin of hepatocarcinogenesis, and induce malignant transformation [3]. Contemporarily, development of cancer is no only

considered to result from genomic or genetic alterations, but also metabolism of lipid [4].

The miRNAs (MicroRNAs) are a group of tiny molecules and noncoding RNA, 22 to 25 nucleotides in length that function on regulation of gene expression at post-transcriptional level [5]. MicroRNAs (miRNAs) control gene expression by pairing with incompletely matching aim sites of the 3' untranslated regions (UTRs) of mRNA, and cause translational repression and/or mRNA destabilization, thereby down-regulating the expression of the targeted gene. The growing literatures hold up the vital function of miRNAs on expression regulation at post-transcriptional level. Moreover, the regulated expression of genes involved in numerous biological progressions, especially for the different pathogenesis disorders (including inflammatory

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disorders) [6]. A variety of miRNAs are regarded as symbolize of a new category of inflammation mediators, too [7-9].

To assess the role of miR-9500 in hepatoma cells, we first identify whether miR-9500 sequences is in the AKT-1 mRNA, and then evaluated the levels expression levels of miR-9500 in HepG2 and Huh7. Our work firstly demonstrated that AKT-1 is one of the targets of miR-9500 in HepG2 and Huh7. It revealed that miR-9500 could control survival of cell viability by regulating expression of AKT-1 and its pathway. We, therefore, investigated the effect of miR-9500 on regulating apoptosis of hepatoma cell lines. In particular, the cell behavior effects of miR-9500 have been associated with the regulation of AKT-1 and its signaling pathway.

## Materials and methods

### *Culture of cell*

HepG2 and Huh7 cell line was purchased from American type culture collection (ATCC). HepG2 and Huh7 were cultured with DMEM with 10% FBS. HepG2 and Huh7 cells were grown in a 5% CO<sub>2</sub> incubator at 37°C. HiPerFect Transfection Reagent (Qiagen) was used to transfect the HepG2 and Huh7 cells according to the manufacturer's instructions as previously described [10].

### *Reporter gene assays*

cDNAs encoding the entire 3' UTR of AKT-1 (300 kb) mRNAs were predicted and amplified from total RNA of HepG2 and Huh7 using Xho I and Not I linker/primers, and then cloned into the vector pGL4 (the luciferase reporter vector) included the gene could expressed firefly and renilla luciferase. AKT-1 3' UTRs was cloned in reverse orientation as controls lacking the miRNA target sequence [11]. Additionally, the complementary region to the seed region of miR-9500 sequence in position 955-961 and 1284-1290 of human AKT-1 3' UTR, TCTTGCC, was mixed up with CTGCCTT. These constructs were all identified with COS-7 cells (gift of Dr. Feng Liu, Chinese Academy of Sciences), which were transfected with the reporter construct and the indicated miRNA mimics or its negative control sequences by Lipofectamine 3000 (Invitrogen, Carlsbad, CA). The activity of Renilla luciferase was normalized with the correspond-

ing control of Dual-Glo Luciferase Assay System. The mutant construct of the AKT-1-3'-UTR was created with Site-Mutation kit (Promega, Madison, WI, USA). Then, either NC or miR-9500 and the plasmid were co-transfected into cells. The pGL4-control vector was used for normalization as a control. The analysis of luciferase activity of cells was detected with analyzer VICTOR (PerkinElmer, Foster City, CA, USA) by Dual-Luciferase Reporter Assay System kit of Promega. AKT-1 protein (active, AB79792) was purchased from Abcam company (MA, USA).

### *Extract total RNA and clone novel miRNA*

Total RNA of cells was obtained using method with TRIzol (Sigma, CA, USA), based on the protocol for manufacturer. Total RNAs were isolated with mirVana RNA isolation kit then get rid of the RNA smaller than 200 nt. The miR-9500 was cloned into the open code frame of vector with DynaExpress miRNA Cloning Kit based on its instructions for manufacturer, and then modified it.

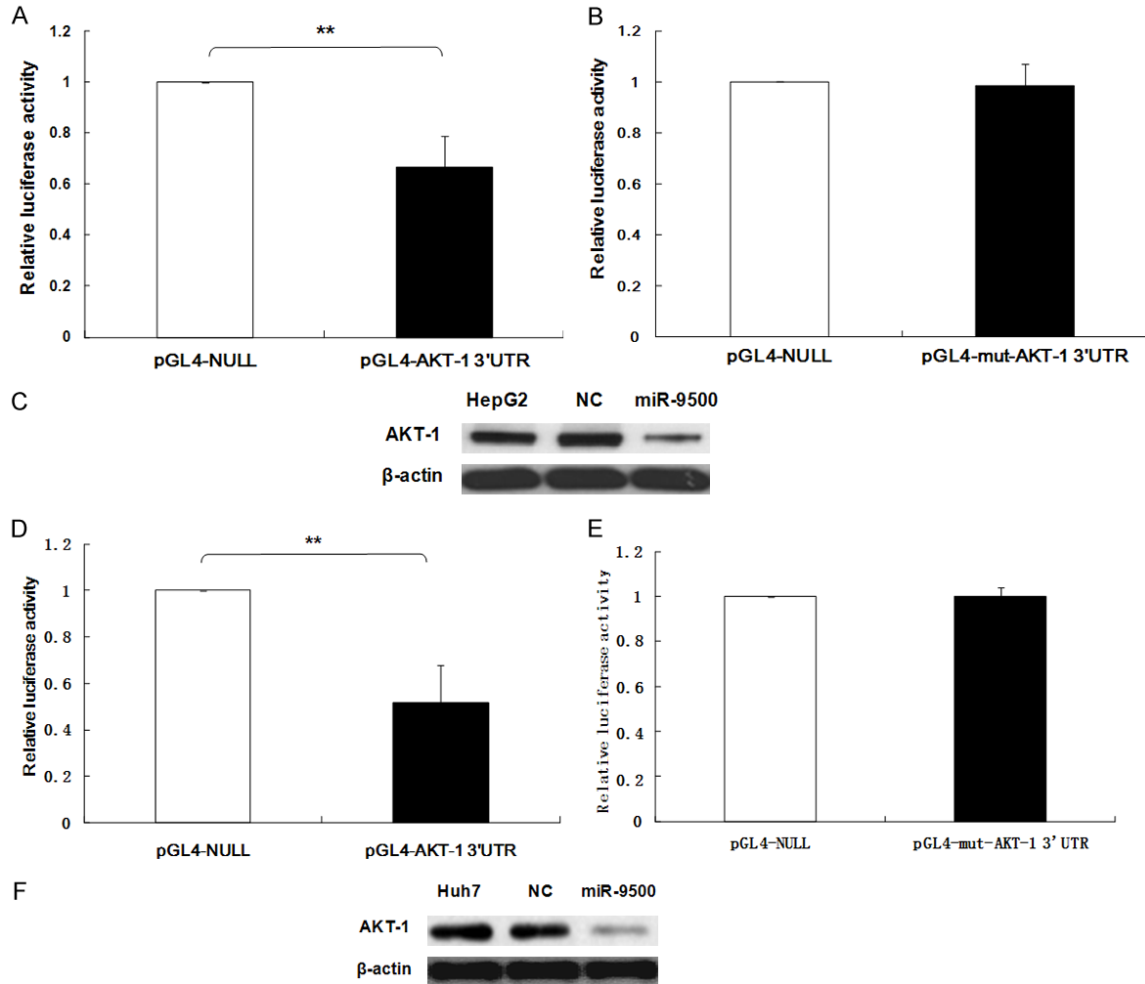
### *The mimics of miR-9500 and transfection*

Transfection with mimics of miR-9500 were performed using Lipofectamine 3000 (Sigma, CA, USA), according to the protocol of manufacturer. The mimics of miR-9500 were showed as follows: sense 5'-AAGGGAAGAUGGUGACCACUU-3' and antisense 5'-AAGUGGUCACCAUCU-UCCCUU-3'. The inhibitors of miR-9500 were showed as follow: 5'-GUGGUCACCAUCU-UCCCUU-3'. Moreover, the negative control (NC) was showed as follows: sense 5'-ACGUGACACGU-UCGGAGAAUU-3' and antisense 5'-AAUUCUCCGAACGUGUCACGU-3', which was not homologous with the genome sequences of human. The qRT-PCR was used to identify the dose effect of miR-9500.

### *The qRT-PCR analysis*

We had identified the expression pattern of miR-9500 in HepG2 and Huh7 according to the TaqMan miRNA assays with its specific primers. The 2<sup>-ΔΔCt</sup> method was used to analyze the data. The qRT-PCR was performed with SYBR Green kit (Sigma, USA) and the Real-Time PCR Detection System (Bio-Rad, Berkeley, CA, USA). The primer sequences were showed as follows: AKT-1 upstream 5'-TCCCAGGCCAAGTCCTT-3'

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**Figure 1.** MiR-9500 Inhibit Expression of AKT in hepatoma cell lines. In HepG2 and Huh7, to study if miRNAs regulate the expression of AKT-1, the miRNAs target AKT-1 were predicted at first using online software DIANA microT v4.0. The miR-9500 may be the potentially target on AKT-1. To further identify whether miR-9500 directly ties to the 3' UTRs of AKT-1 mRNAs, we conducted luciferase reporter assay. These results demonstrated that the relative 3' UTR luciferase activities of AKT-1 reduced remarkably in HepG2 transfected with miR-9500 compared to control group (A) ( $P=0.001$ ). But, the relative activities of 3' UTR mut-AKT-1 (mutation of AKT-1) luciferase were similar to miR-9500 treated HepG2 compared to control group (B) ( $P>0.05$ ). These data identified that mRNAs of AKT were the straight aim of miR-9500. To confirm if miR-9500 regulate the expression of AKT-1 in HepG2, we performed Western blot analyses (C). Moreover, we found that the relative activities of 3' UTR AKT-1 luciferase were obviously reduced in miR-9500 treated Huh7 compared to control group (D) ( $P=0.001$ ). But, the relative activities of 3' UTR mut-AKT-1 (mutation of AKT-1) luciferase were similar to miR-9500 treated Huh7 compared to control group (E) ( $P>0.05$ ). To identify whether the miRNAs regulate the expression of AKT-1 in Huh7, we performed Western blotting trials (F). The results indicated that, in HepG2, miR-9500 could suppress the AKT-1 expression (F). The data are presented as means  $\pm$  SD from three independent experiments. \* $P<0.05$ , \*\* $P<0.01$ .

and downstream 5'-AAGGACTTGGCCTCGGGA-3' and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) upstream 5'-CTCATGACCACAGTCCATGCC-3' and downstream 5'-GGCATG-GACTGTGGTCATGAG-3'.

### Western blotting

Cells were lysed, and then total proteins were extracted with RIPA lysis buffer. Total proteins

were analyzed with electrophoresis method using Sodium Dodecyl Sulfate-polyacrylamide (SDS-PAGE) gel, and then transferred to a polyvinylidene fluoride (PVDF) membrane (Roche, NJ, USA), which was blocked at room temperature with 5% skim milk and washed with TBST for three times, probed with primary antibodies: p-AKT, AKT-1 (1:3000 dilution, Cell Signaling, Danvers, MA, USA), PI3K (1:3000 dilu-

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tion, Santa Cruz, CA, USA), or  $\beta$ -actin (1:3000 dilution, Santa Cruz, CA, USA) overnight at 4°C. Then there were incubated at room temperature with proper secondary antibodies (1:5000 dilutions, Santa Cruz, CA, USA).

### *BrdU assays and mitotic index analysis*

Cells were synchronized through block method with double thymidine. After synchronizing for 16 hrs, cells were released, and then collected or fixed for assays. DNA synthesis or mitotic entry analysis were performed by BrdU labeling method. Cells were counted and cells with BrdU positive were scored with immunofluorescence microscope.

Mitotic events were determined with DNA staining and time-lapse videomicroscopy. After synchronizing, the real-time images of cells were obtained at each 10 min. Mitotic events in cells were detected through change of morphology. Mitotic cells were counted based on DNA condensation and nuclear morphology using DNA dye (Hoechst 33258).

### *Detection of apoptosis cells*

Using Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson, San Jose, CA, USA), the fluorescence-activated staining cells was sorting analyzed at 12, 24, 36 and 48 hrs after treated with inhibitors or mimics of miR-9500, respectively. These tests were performed with ACScan flow cytometer, the data analyse was done with Flowjo software.

### *Statistical analyses*

Data analysis was carried out using SPSS 18.0 software (Inc. Chicago, SPSS, IL). Statistical differences between groups were analyzed with two-tailed paired Student *t*-test. Data of qRT-PCR and luciferase reporter assays were expressed relative to the control in each experiment, and 95% confidence intervals were calculated.  $P < 0.05$  was considered with statistically significant.

## Results

### *MiR-9500 inhibit expression of AKT-1 in hepatoma cell lines*

To identify the miRNAs to target AKT-1 in HepG2 and Huh7, we first used the online software

(DIANA microT v4.0) to predict the miRNAs targeting AKT. MiR-9500, may be possibly to aimed the 3' UTRs of AKT-1 mRNAs. Then, we investigated whether miR-9500 straight ties to the 3' UTRs of AKT-1 mRNAs, and found that the relative activities of 3' UTR AKT-1 luciferase were obviously reduced in miR-9500 treated HepG2 compared to control group (**Figure 1A**) ( $P = 0.001$ ). But, the relative activities of 3' UTR mut-AKT-1 (mutation of AKT-1) luciferase were similar to miR-9500 treated HepG2 compared to control group (**Figure 1B**) ( $P > 0.05$ ). These data demonstrated that AKT-1 mRNAs straight object to miR-9500. To identify whether the miRNAs regulate the expression of AKT-1 in HepG2, we performed Western blotting trials (**Figure 1C**). The results indicated that, in HepG2, miR-9500 could suppress the AKT-1 expression (**Figure 1C**).

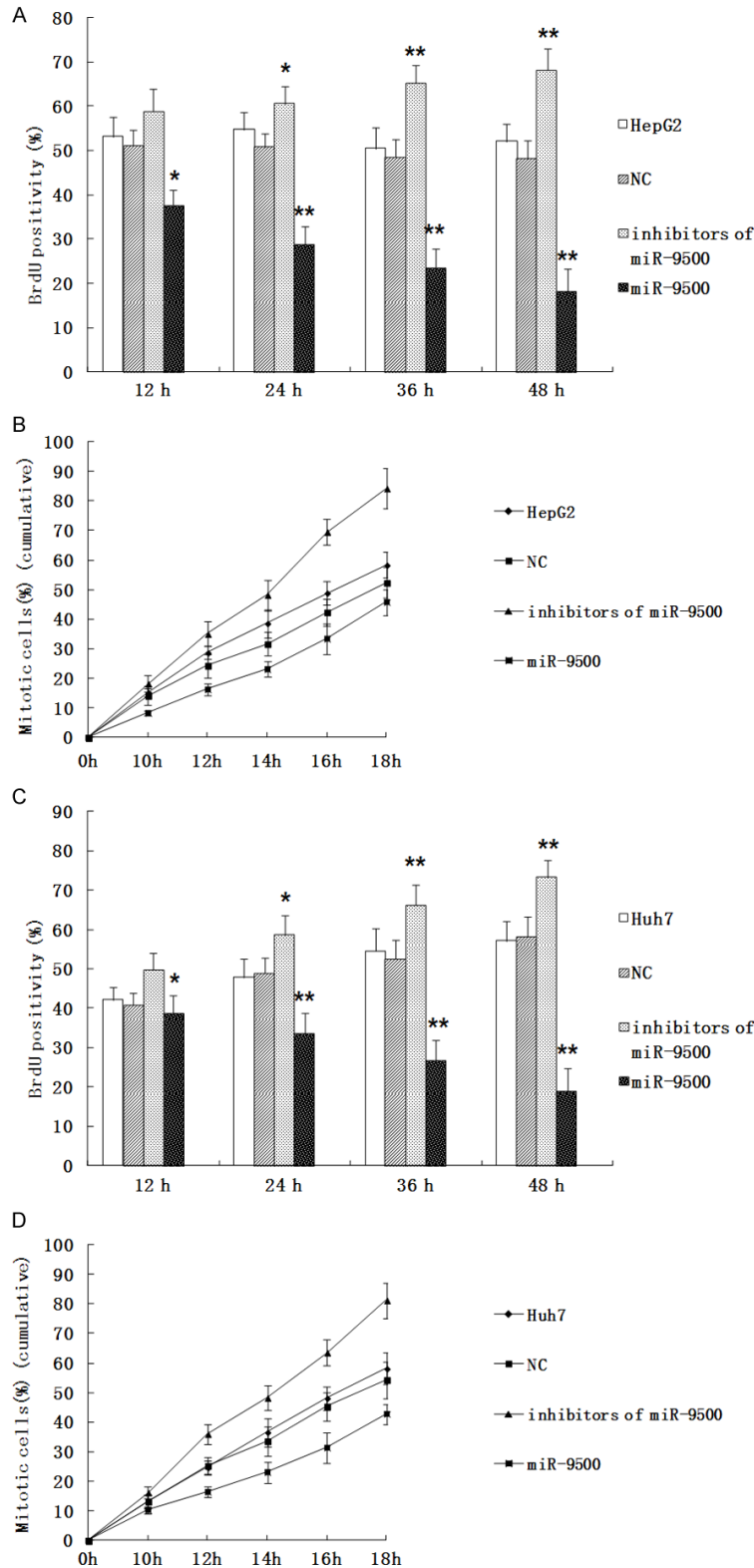
Moreover, we found that the relative activities of 3' UTR AKT-1 luciferase were obviously reduced in miR-9500 treated Huh7 compared to control group (**Figure 1D**) ( $P = 0.001$ ). But, the relative activities of 3' UTR mut-AKT-1 (mutation of AKT-1) luciferase were similar to miR-9500 treated Huh7 compared to control group (**Figure 1E**) ( $P > 0.05$ ). To identify whether the miRNAs regulate the expression of AKT-1 in Huh7, we performed Western blotting trials (**Figure 1F**). The results indicated that, in HepG2, miR-9500 could suppress the AKT-1 expression (**Figure 1F**).

### *MiR-9500 suppress mitosis entry and proliferation of hepatoma cells*

We further investigated the role of miR-9500 mimics and inhibitors on functions of host cells with BrdU. FCM (flow cytometry) was used to study the apoptosis progression induced by miR-9500 mimics and inhibitors. The results indicated that miR-9500 could significantly suppress proliferation (**Figure 2A**) and mitosis entry (**Figure 2B**) of HepG2 cells. Moreover, in HepG2 cells, inhibitors of miR-9500 could promote cell proliferation (**Figure 2A**) and mitosis entry (**Figure 2B**) in time dependent manner.

Moreover, miR-9500 could significantly suppress proliferation (**Figure 2C**) and mitosis entry (**Figure 2D**) of Huh7 cells. The inhibitors of miR-9500 could promote cell proliferation (**Figure 2C**) and mitosis entry (**Figure 2D**) in time dependent manner.

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**Figure 2.** MiR-9500 inhibited mitosis entry and proliferation of hepatoma cells. Moreover, the characterized effect induced by miR-9500 on host cells was investigated with BrdU to analyze cell viability progression. The results showed that miR-9500 could suppress proliferation (A) and mitosis entry (B) of HepG2 cells effectively. Moreover, in HepG2 cells, the inhibitors of

miR-9500 could promote its proliferation (A) and mitosis entry (B) in a time-dependent manner. Moreover, miR-9500 could significantly suppress proliferation (C) and mitosis entry (D) of Huh7 cells. The inhibitors of miR-9500 could promote cell proliferation (C) and mitosis entry (D) in time dependent manner. The data are presented as means  $\pm$  SD from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

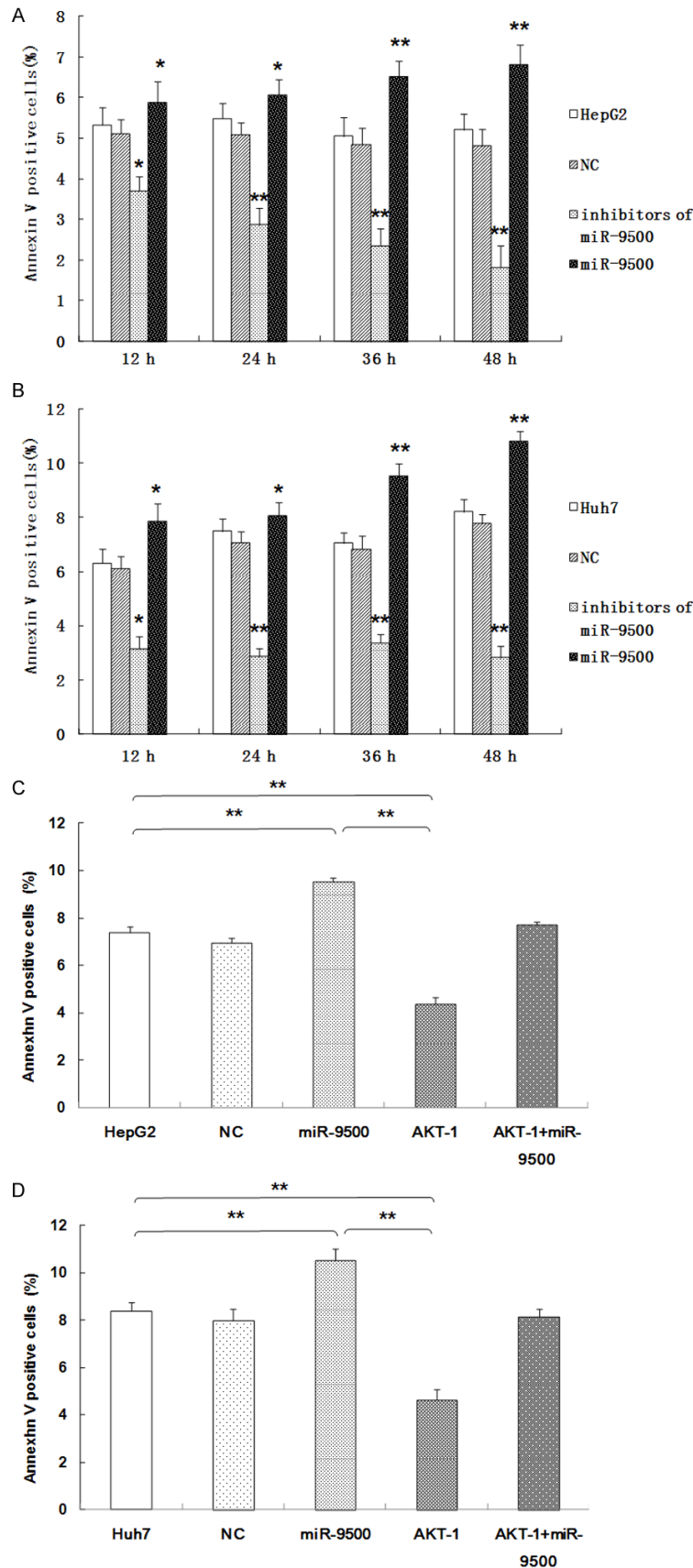
### *MiR-9500 promote apoptosis of HepG2*

The mimics of miR-9500 could induced obviously cell apoptosis of HepG2 in a time-dependent manner (Figure 3). Moreover, inhibitors of miR-9500 could repress apoptosis of HepG2 (Figure 3A) and Huh7 (Figure 3B), respectively. Both of those outcomes demonstrated miR-9500 suppress survival of hepatoma cells in time dependent manner.

### *AKT-1 rescue the apoptosis hepatoma cells induced by miR-9500*

It had been reported in prior that AKT-1 was the key regulate factor for cell proliferation [18], we further identified that through using of AKT-1 active protein in HepG2 and Huh7 cell model transfected by miR-9500. Moreover, the apoptosis cells of HepG2 and Huh7 were detected. These data confirmed that AKT-1 active protein could suppress significantly cell apoptosis of HepG2 (Figure 3C) and Huh7 (Figure 3D). Furthermore, the decreased of apoptosis hepatoma cells induced by miR-9500 may obviously suppress by AKT-1 active protein (Figure 3C and 3D).

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**Figure 3.** AKT-1 active protein rescue the apoptosis hepatoma cells induced by miR-9500. Furthermore, the characterized effect on cell apoptosis progression caused by miR-9500 was analyzed with flow cytometry (FCM). The results showed that the mimics of miR-9500 could induced a remarkably increase of apoptotic cells in a time-dependent manner. Moreover, inhibitors of miR-9500 could repress apoptosis of HepG2 (A) and Huh7 (B) in a time-dependent manner. It had been reported in prior that AKT-1 was the key regulate factor for cell proliferation [18], we further identified that through using of AKT-1 active protein in HepG2 and Huh7 cell model transfected by miR-9500. Moreover, the apoptosis cells of HepG2 and Huh7 were detected. These data confirmed that AKT-1 active protein could suppress significantly cell apoptosis of HepG2 (C) and Huh7 (D). Furthermore, the decreased of apoptosis hepatoma cells induced by miR-9500 may obviously suppress by AKT-1 active protein (C and D). The data are presented as means  $\pm$  SD from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

## Discussion

HCC is major reason of deaths in patients with cirrhosis, which primarily come from alcohol abuse or chronic viral hepatitis. HCC is almost come from inflammation process and liver cirrhosis induced by aflatoxins, ethanol, and chronic infection of hepatitis B or C. Moreover, it is the most usual human carcinoma and ranked fifth. While it is also an vital health problem and the second commonest reason of cancer deaths among human [12]. Although it is well known that several different mechanisms of HCC, it is still unclear about how miRNA modulate the pathogenesis process of HCC. In the present work, we

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ascertained the novel miRNA, miR-9500, and searched after its effects on pathogenesis process of HCC. A number of factors such as microRNAs play an important role in regulation of gene expression, which was involved in HCC [13], but the microRNAs-mediated regulation of AKT-1 activity during pathogenesis of HCC is poorly understood. As shown in the current study, our bioinformatics analysis by using online software (<http://targetscan.org/>) confirmed that 3'-UTR site of AKT-1 mRNA is complimentary to miR-9500. To further verify whether miR-9500 straight combine with 3' UTRs of AKT-1, a vector embracing the 3'-UTR of the AKT-1 gene has been constructed, including the sequence of it was from 529 to 535 nt. The results of luciferase reporter analysis indicated that the relative activity of AKT-1 3' UTR significantly decreased in HepG2 and Huh7 transfected with miR-9500. It confirmed that the expression of AKT-1 was modulated by miR-9500. Following over-expression of miR-9500 in HepG2 and Huh7 compared to NC, western blot analyses verified that protein expression levels of AKT-1 down-regulated in HepG2 and Huh7 induced by miR-9500. These results confirmed that miR-9500 could combine straight with AKT-1 3'-UTR and negatively regulates its mRNA and protein expression in HepG2 and Huh7, respectively. Therefore, this provide new therapy target for treatment of HCC.

We had found that miR-9500 inhibited proliferation of human hepatoma cell line HepG2. Moreover, AKT-1 was the key factor for cell proliferation, and the target genes of miRNA-9500. It confirmed that miR-9500 directly regulated mRNA expression of its target gene AKT-1. MiRNAs are nc-RNAs (non-codings RNAs) and growing held small RNAs influencing gene expression, mediate posttranscriptional gene silencing based on suppressing mRNAs translation of its target gene or degradation mRNAs according to mRNA sequence. The latest researches had demonstrated that miRNAs take part in variety of cellular progress, such as differentiation, development, proliferation and tumorigenesis [14].

In this present research work, we sought to obtain novel insight about the key role of miR-9500 in regulating proliferation and mitosis, apoptosis of human hepatoma cell lines HepG2 and Huh7. Our finding showed that miR-9500 could directly regulate the expression of

AKT-1. For confirming the ability of miR-9500 in interfering proliferation and mitosis, apoptosis of HepG2 and Huh7, mimics or inhibitors of miR-9500 were transfected into HepG2 and Huh7 cells, respectively. The results indicated that miR-9500 could significantly suppress proliferation and mitosis entry of HepG2 cells. Moreover, inhibitors of miR-9500 could promote cell proliferation and mitosis entry of HepG2 in a time-dependent manner. The mimics of miR-9500 could induced obviously cell apoptosis of HepG2 and Huh7 in a time-dependent manner. Moreover, inhibitors of miR-9500 could repress apoptosis of cells. Both of those outcomes demonstrated miR-9500 suppress survival of hepatoma cell lines HepG2 and Huh7 in a time-dependent manner.

It had been reported in prior that AKT-1 was the key regulate factor for cell proliferation [15], we further identified that through using of AKT-1 active protein in HepG2 and Huh7 cell model transfected by miR-9500. Moreover, the apoptosis cells of HepG2 and Huh7 were detected. These data confirmed that AKT-1 active protein could suppress significantly cell apoptosis of HepG2 and Huh7. Furthermore, the decreased of apoptosis hepatoma cells induced by miR-9500 may obviously suppress by AKT-1 active protein. The results indicated that AKT-1 rescue the apoptosis hepatoma cells induced by miR-9500.

In conclusion, our results indicated that miRNA-9500 induced down-expression of AKT-1 in HepG2 and Huh7, and then promoted cell apoptosis of hepatoma cell lines in time dependent manner. The miRNA-9500 may be a novel target on HCC. Moreover, these results demonstrated that miRNA-9500 could promote apoptosis of hepatoma cells associated with AKT-1 signaling pathway.

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### Disclosure of conflict of interest

None.

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