

## Original Article

# MiR-200a inhibits cell proliferation and EMT by down-regulating the ASPH expression levels and affecting ERK and PI3K/Akt pathways in human hepatoma cells

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**Abstract:** The primary objective of this study was to investigate the role of miR-200a in cell proliferation and epithelial-mesenchymal transition (EMT) through regulating targeting aspartate- $\beta$ -hydroxylase (ASPH), which may further affect the activation of ERK/PI3K/Akt pathway. Liver cancer and adjacent tissues were collected from 72 cases of liver cancer patients with surgery in our hospital. In this study, the mRNA expression level of miR-200a was significantly decreased by real-time PCR (RT-PCR) detection. ASPH expressions, however, had an opposite tendency compared to that of miR-200a. We found a significantly negative correlation between miR-200a expressions and ASPH expressions. The survival rate of liver cancer patients with the low expressed ASPH was significantly higher than those with the high expressed ASPH. RT-PCR and Western blot results showed that low expressed miR-200a and high expressed ASPH were found in liver cancer cell lines. Further research discovered that miR-200a transfection could significantly decrease the relative luciferase activity when it was integrated with ASPH 3'-untranslated region (3'-UTR) in HepG2 cells. Cell Counting Kit (CCK-8) detection showed that treatment with miR-200a mimics reduced cell viability, while the over-expressed ASPH increased cell viability by regulating the c-myc mRNA (c-Myc) and Cyclin-D1 expressions. The EMT-related genes including E-Cadherin, N-Cadherin and Vimentin expressions were significantly increased, whereas the over-expressed ASPH exerted the opposite effects. In addition, extracellular signal regulated kinase (ERK), phosphoinositide-3-kinase (PI3K) and serine threonine kinase (AKT) were suppressed by miR-200a mimics. In conclusion, miR-200a inhibits cell proliferation and EMT in human hepatoma cells by targeting ASPH and affecting ERK and PI3K/Akt signaling pathways.

**Keywords:** MiR-200a, ASPH, cell proliferation, EMT, liver cancer

## Introduction

Primary hepatocellular carcinoma (HCC) is, clinically most prevalent primary liver cancer that seriously endangers human lives and health and ranks the 2nd highest cancer mortality rate [1]. Although treatments for liver cancer have been improved, the average five-year survival rate for liver cancer patients is only 5%-9% [1, 2]. The pathogenesis of liver cancer is a multi-step and multistage process which is a set of behavior habits, hereditary factors and environmental factors [3]. In this process, the malignant phenotype of liver cancer cells including intrahepatic metastasis and distant

spread gradually turns to be apparent through malignant proliferation and abnormal differentiation of liver cancer cells, which involves in cell-cycle regulation, invasion and migration of cancer cells [4, 5]. Therefore, research on liver cancer would focus on learning molecular mechanism and searching relative genes. Past researches mainly focused on some key genes and proteins in the initiation and progression of liver cancer [6, 7]. Recent years, with the advanced development of molecular biology, correlational research on tumor genesis and epigenetics has already become an important research content in tumor etiology, and especially microRNAs' (miRNAs) findings [8-10].

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MiRNAs-mediated regulation after transcription provides a new sight on the research of the pathogenesis of malignant tumors.

MiRNAs are common in various cells and are a family of small, endogenous non-coding RNAs, which exhibit specific tissue, high conservation and time sequence, and are involved in the regulation of multiple biological processes [11]. A large number of studies have confirmed that miRNAs can regulate cell proliferation, differentiation, apoptosis, migration and invasion through regulating transcription of a series of target genes [12-15]. Under some pathological conditions, a disorder of miRNAs expression can cause the abnormality of processes of cellular biology result in the occurrence of many diseases including cancer [16, 17]. miRNAs play an important role in many liver diseases (HCC, viral hepatitis, liver fibrosis, etc) [18-20]. MiR-200a, miR-200b, miR-200c, miR-141 and miR-429 belong to miR-200 family [21]. Current research on miR-200 family focused on tumor field. Many research showed that has-miR-200 can inhibit cellular transformation, proliferation, invasion, migration, tumor growth and metastasis in breast cancer, cervical cancer, ovarian cancer, liver cancer, colon cancer, etc [22]. Some studies found that miR-200a was down-regulated in liver cancer [23]. In addition, the occurrence and development of liver cancer can be inhibited by up-regulating miR-200a expressions [24]. Therefore, we speculated that miR-200a played an important role in liver cancer as a suppressant miRNA.

ASPH is a Type II transmembrane protein and belongs to the  $\alpha$ -ketoglutarate-dependent dioxygenase family [25], which can catalyze the hydroxylation of aspartyl and asparaginyl residues located in the epidermal growth factor (EGF)-like domain of various proteins. ASPH is expressed at high levels in many malignant neoplasms of different histogenesis, and at very low levels in most normal cells and tissues [26]. Correspondingly, some research showed that the over-expression of ASPH can promote cancer cell production, proliferation, invasion and migration in liver cancer, lung cancer, gastric cancer and so on that is considered to be a potential broad-spectrum tumor marker [27-29]. It suggested that ASPH may be an oncogene involved in the occurrence and development of liver cancer. In this study, we found that

ASPH is the target gene of miR-200a. If these two could be combined and researched, it would make great contribution to further learn the pathogenesis of liver cancer.

### Materials and methods

#### *Patients and tissue samples*

This research included a total of 72 samples of HCC patients, and all tissue specimens were obtained from surgical tumor resections in Zhejiang Provincial People's Hospital. The adjacent normal liver tissue specimens were also collected from these patients as negative control. Ethical approval for the study was provided by the ethics committee of the hospital. Written informed consent was obtained from the study subjects. Preoperative clinical and pathological follow-up data were completed by all patients.

#### *Cell lines and grouping*

Normal liver cell line (L02) and HCC cell lines (HepG2, SMMC7721, Bel-7402, PLC and LM3) were purchased from the Jining Real Co. (Shanghai, China). Cells were cultured in DMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) with the supplements of 10% heat-inactivated fetal bovine serum (Invitrogen Life Technologies), 100 $\times$  penicillin-streptomycin solution (Invitrogen Life Technologies), and incubated in the incubator (Thermo, Fisher Scientific Inc., Waltham, MA, USA) set at 37 $^{\circ}$ C, 100% humidity and 5% CO<sub>2</sub>.

HepG2 cells were further allocated into seven groups: control group (cells without any treatment), miR-200a mimics group (cells were transfected with miR-200a mimics), miR-200a inhibitors group (cells were transfected with miR-200a inhibitors), mimics NC group (cells were transfected with mimic control), inhibitors NC group (cells were transfected with inhibitor control), ASPH group (cells were transfected with pLKO.1-EGFP-ASPH plasmids), vector-NC group (cells were transfected with pLKO.1-EGFP-vector). All groups were incubated in a normoxia incubator containing 21% O<sub>2</sub> for 48 h.

#### *Dual-luciferase reporter assay*

The 3'-UTR fragment of ASPH containing the miR-200a binding site was amplified using PCR with the prime sequences shown in **Table 2** and

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**Table 1.** The sequence of miR-200a mimics and miR-200a inhibitors

Name	Sequence
miR-200a mimics	Sense: 5'-UAACACUGUCUGGUAACGAUGU-3' Anti-sense: 5'-AUCGUUACCAGACAGUGUUUU-3'
Mimics NC	Sense: 5'-UUCUCCGAACGUGUCACGUTT-3' Anti-sense: 5'-ACGUGACACGUUCGGAGAATT-3'
miR-200a inhibitors	5'-ACAUCGUUACCAGACAGUGUUA-3'
Inhibitors NC	5'-CAGUACUUUUGUGUAGUACAA-3'

**Table 2.** Relationship between ASPH and clinical data of HCC patients

Cancer staging	Male/Female	Age (<45/≥45)	ASPH expression (Lower/Higher)
TNM	I	10/12	11/13
	II	15/12	10/13
	III	13/10	12/14
P-values	0.710	0.999	0.034*

\* $P < 0.05$ , Chi-square test.

cloned into the luciferase vector (Promega, USA). Other sequences in the binding site were intentionally mutated using the GeneTailor Site-Directed Mutagenesis System (Invitrogen, USA), the mutated 3'-UTR was then cloned into the same vector. The two types of sequences were named as ASPH 3'-UTR and ASPH mutated 3'-UTR [30]. The sequence of miR200a mimics and miR-200a inhibitors were listed in **Table 1**.

HeGp2 cells ( $2 \times 10^5$  cells per well) were seeded in 24-well plates, one day prior to transfection, and co-transfected with miR-200a mimic or inhibitor (50 pmol; Santa Cruz Biotechnology, Inc), 500 ng luciferase reporter and 50 ng pRL-TK vector (Santa Cruz Biotechnology, Inc) together with ASPH 3'-UTR or ASPH mutated 3'-UTR or the control vector. After cells were incubated with DMEM for 24 h, the miR-200a mimics/inhibitors/control transfected HepG2 cells were analyzed by RT-PCR or identification and the luciferase assay and subsequent luminescence calculations were performed using the Dual-Glo luciferase reporter assay system (Promega). Three independent experiments were performed in triplicate.

### Construction and infection

Cells in logarithmic growth phase were seeded in a 6-well plate for culturing 24 h. The recombi-

nant pLKO.1-EGFP-vector and pLKO.1-EGFP-ASPH vector expressing ASPH plasmids (Sangon Biotech Inc. Shanghai, China) were transfected into cells according to the manufactures' directions of Invitrogen Lipofectamine™ LTX (Invitrogen, Shanghai, China). Next, 2 µg of pLKO.1-EGFP-vector or pLKO.1-EGFP-ASPH, 5 µL of Lipofectamine™ LTX and 250 µL Opti-MEM® were prepared well mixed and then incubated for 25 min at room temperature. 500 µL of mixture was added into 6-well plate with RPMI 1640 medium for cultured 48 h. The transfected cells were harvested and used for next experiment. The pLKO.1-EGFP-vector/pLKO.1-EGFP-ASPH transfected HepG2 cells were analyzed by RT-PCR and Western blot for identification.

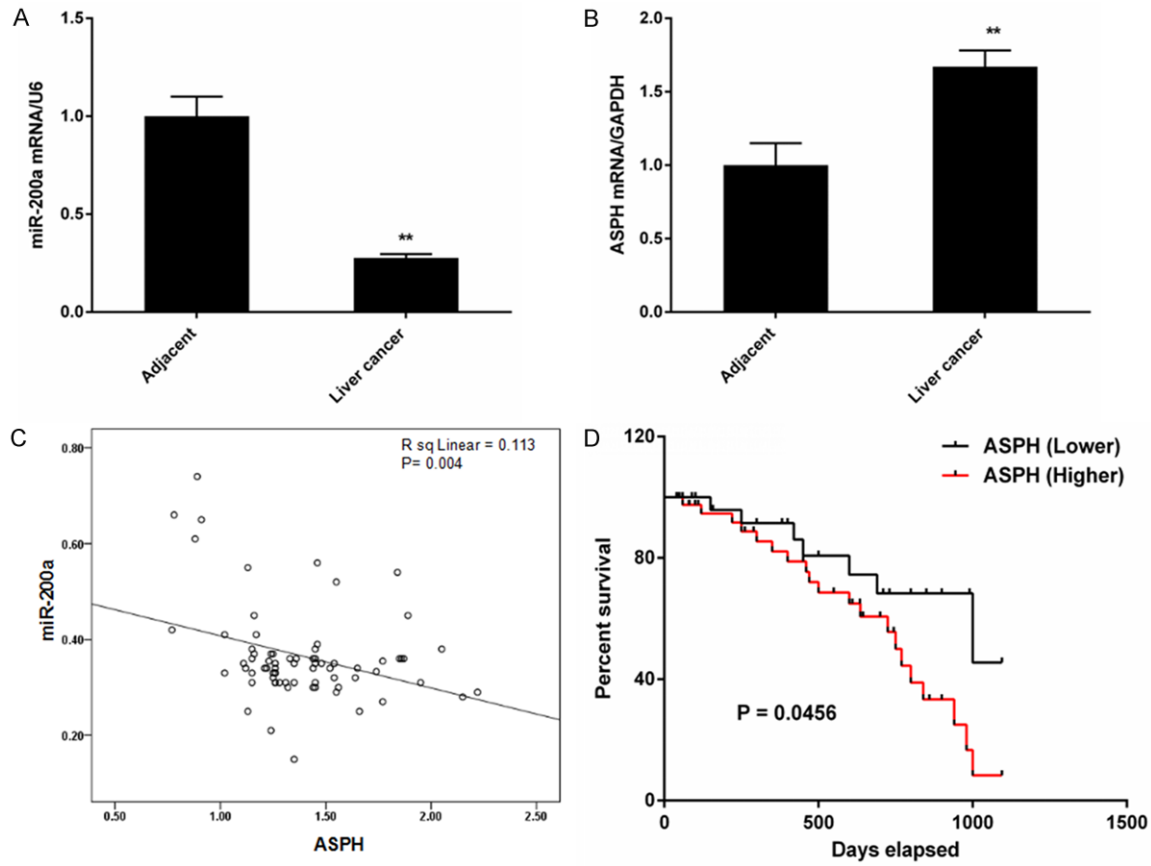
### CCK-8 assay

HeGp2 cells ( $1 \times 10^5$  cells per well) were seeded in 96-well plates for culturing 24 h, and the CCK-8 assay (Dojindo Molecular Technologies, Gaithersburg, USA) was used to detect HepG2 cell proliferation. The cells that were transfected with miR-200a mimics/mimics control/ASPH were cultured for another 12, 24 and 48 h. Subsequently, all cells were treated with 10 µl CCK-8 solution at 37°C for 4 h. The plate was then read at 450 nm to obtain the cell growth curve using iMark microplate absorbance reader (BioRad Laboratories, Inc., Hercules, CA, USA). All experiments were repeated in triplicate.

### RNA preparation and RT-PCR

Cultured cells were collect with Trizol (Life Technologies) and RNA was extracted according to the manufacture's protocol. 5 µg of RNA were reverse transcribed to cDNA with MMLV RT reagent kit (Thermo Fisher Scientific, Inc.) as described in the instruction manual. RT-PCR was performed using SYBR® Green 10× Supermix (Takara, Japan) in a 25 µl total volume and on Roche Light Cycler® 480 II System (Roche Diagnostics Ltd., Switzerland). The expression level of target genes was normalized to that of glyceraldehyde-3-phosphate

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**Figure 1.** MiR-200a and ASPH expressions in liver cancer tissues. Seventy-two liver cancer tissues and their adjacent normal ones were collected. A. The mRNA expression level of miR-200a in liver cancer group was lower than that of adjacent group through RT-PCR analysis. B. The mRNA expression level of ASPH in liver cancer group was higher than that of adjacent group through RT-PCR analysis. C. Linear correlation was applied to analyze the correlation between the miR-200a and ASPH in mRNA expression level. D. The survival rate of 72 liver cancer patients showed that higher ASPH expression of patients was significantly shorter than that of lower ASPH expression of patients. U6 and GAPDH were detected as the control of sample loading. Data are expressed as the mean  $\pm$  SD for three independent experiments. \*\* $P < 0.01$  versus adjacent normal tissue.

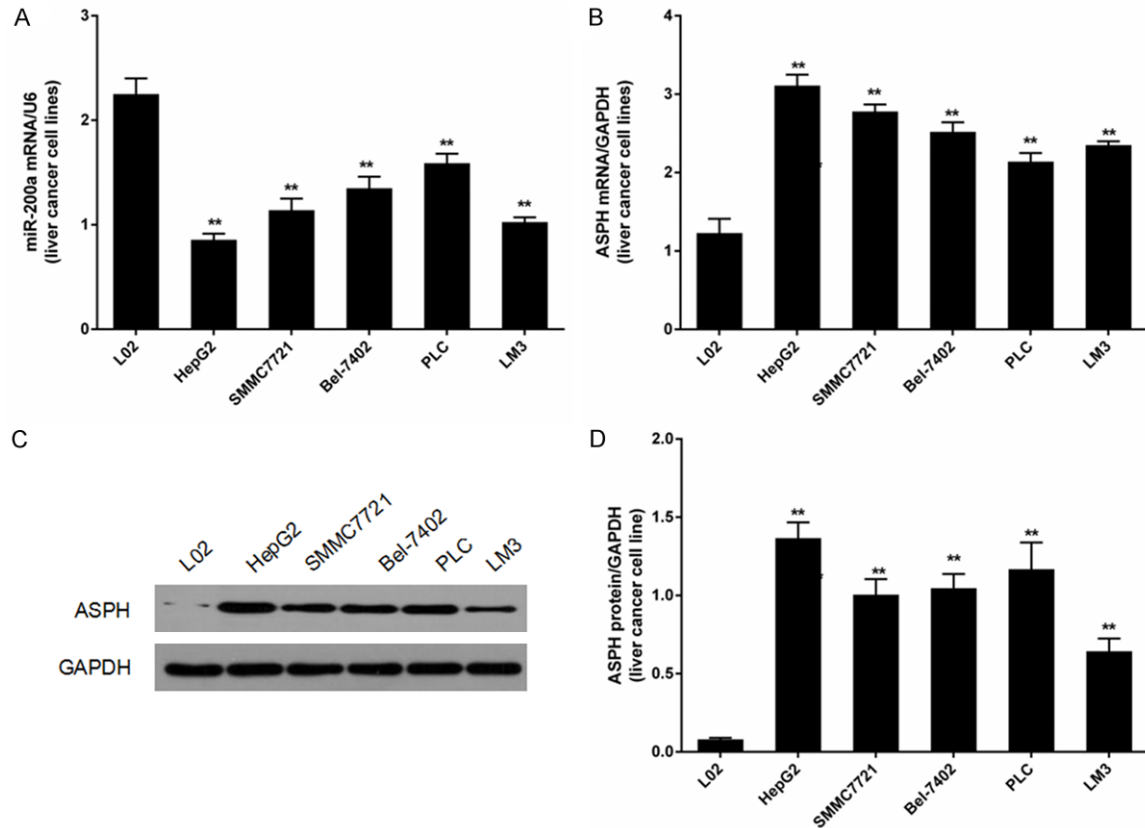
dehydrogenase (GAPDH) and was calculated by the method of  $2^{-\Delta\Delta CT}$  method. Primers used were: ASPH, forward: 5'-GTTACCACGTGGAAGAGAC-3' and reverse: 5'-GCTTGTTCCTCATAGACTTG-3'; Cyclin-D1, forward: 5'-TTTGTGTGTGTGCAGGGAC-3' and reverse: 5'-TTTCTTCTTGACTGGCAGC-3'; c-Myc, forward: 5'-ATTCTCTGCTCCTCGACG-3' and reverse: 5'-CTGTGAGGAGTTTGCTGTG-3'; E-cadherin, forward: 5'-ACAC-TGGTGTGCCCTCTGC-3' and reverse: 5'-AAGG-CTGCAGTGAGCTGTGA-3'; N-cadherin, forward: 5'-ATATTTCCATCCTGCGCGTG-3' and reverse: 5'-GTTTGGCCTGGCGTTCTTTA-3'; Vimentin, forward: 5'-GAGTCCACTGAGTACCGGAG-3' and reverse: 5'-ACGAGCCATTTCTCCTTCA-3'; U6, forward: 5'-CTCGCTTCGGCAGCAGCACA-3' and reverse: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH,

forward: 5'-CGGGAAACTGTGGCGTGATG-3' and reverse: 5'-ATGACCTTGCCACAGCCTT-3'.

### Protein preparation and Western blot

Total protein was extracted from cells using ProteoPrep® Total Extraction Sample Kit (Sigma). Cell lysis was centrifuged  $12,000\times g$  at  $4^{\circ}\text{C}$  for 10 min and the proteins in supernatant was separated by 10-15% SDS gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Shanghai, China). Protein expressions were analyzed by Western blot using various antibodies at the following dilutions: ASPH antibody (1:800), Cyclin-D1 antibody (1:1000), c-Myc antibody (1:1000), E-Cadherin antibody (1:600), N-Cadherin anti-

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**Figure 2.** MiR-200a and ASPH expressions in liver cancer cell lines (HepG2, SMMC7721, Bel-7402, PLC and LM3). A. The mRNA expression levels of miR-200a in liver cancer cell lines (HepG2, SMMC7721, Bel-7402, PLC and LM3) were lower than that of normal hepatocytes (LO2) through RT-PCR analysis. B. The mRNA expression levels of ASPH in liver cancer cell lines (HepG2, SMMC7721, Bel-7402, PLC and LM3) were lower higher that of normal hepatocytes (LO2) through RT-PCR analysis. C and D. The protein expression level of ASPH in liver cancer cell lines (HepG2, SMMC7721, Bel-7402, PLC and LM3) were lower higher that of normal hepatocytes (LO2) through Western blot analysis. U6 and GAPDH were detected as the control of sample loading. Data are expressed as the mean  $\pm$  SD for three independent experiments. \*\* $P < 0.01$  versus normal cell (LO2).

body (1:1000), Vimentin antibody (1:1000), ERK antibody (1:800), p-ERK antibody (1:500), PI3K antibody (1:800), p-PI3K antibody (1:400), Akt antibody (1:1000), p-Akt antibody (1:500) and GAPDH antibody (1:2000).

### Statistical analysis

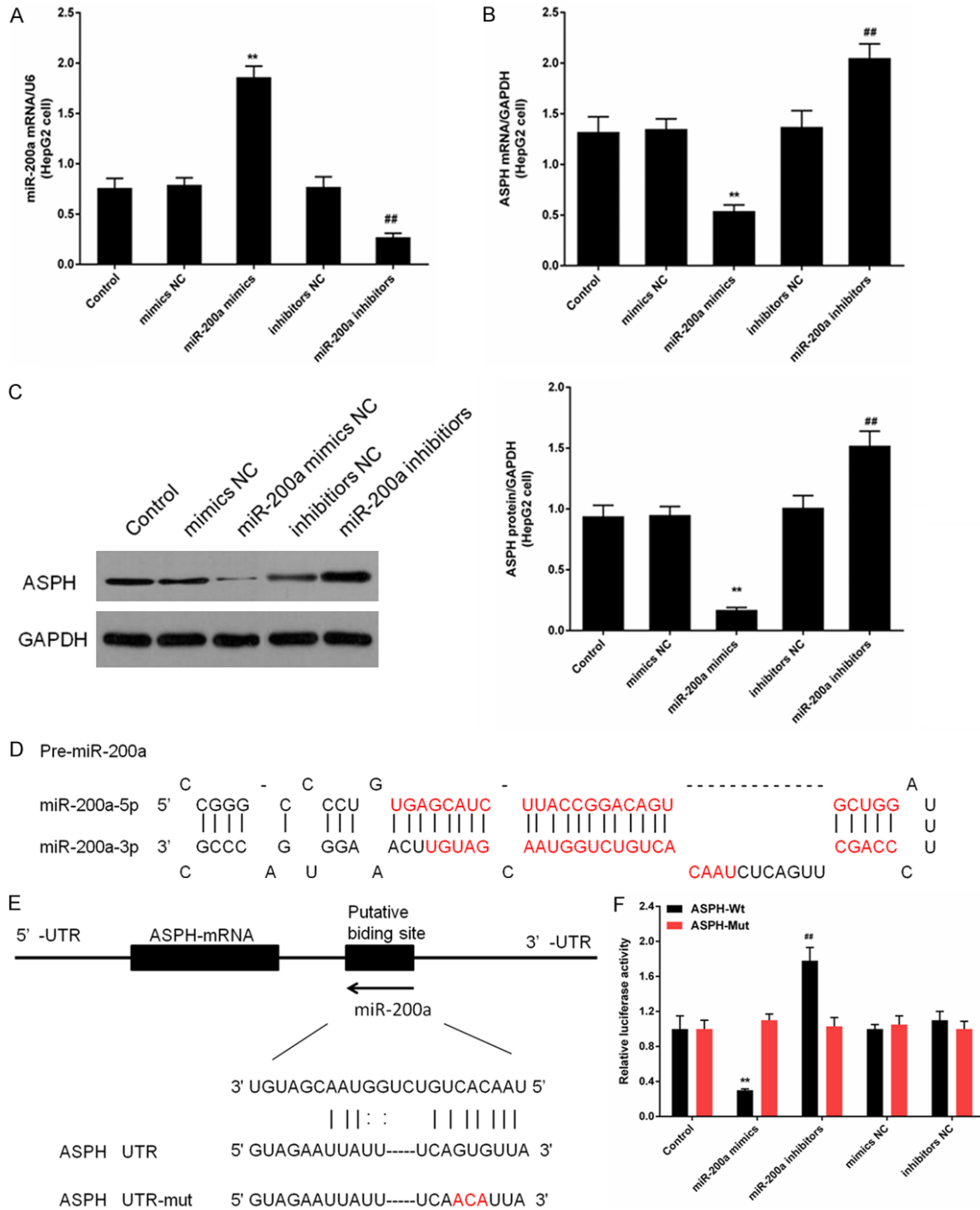
Statistical analyses were performed using SPSS software, version 22.0 (SPSS, Inc., Chicago, IL, USA). Each experiment was repeated three times, with all data presented as the mean  $\pm$  standard deviation. Student's t-test and one-way ANOVA were used in two or multiple groups for statistical significance. Spearman rank order was used to analyze the correlations between variables. The method of survival analysis was used for testing divided phase.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

### Expression of miR-200a and ASPH in HCC specimens and liver cancer cell lines

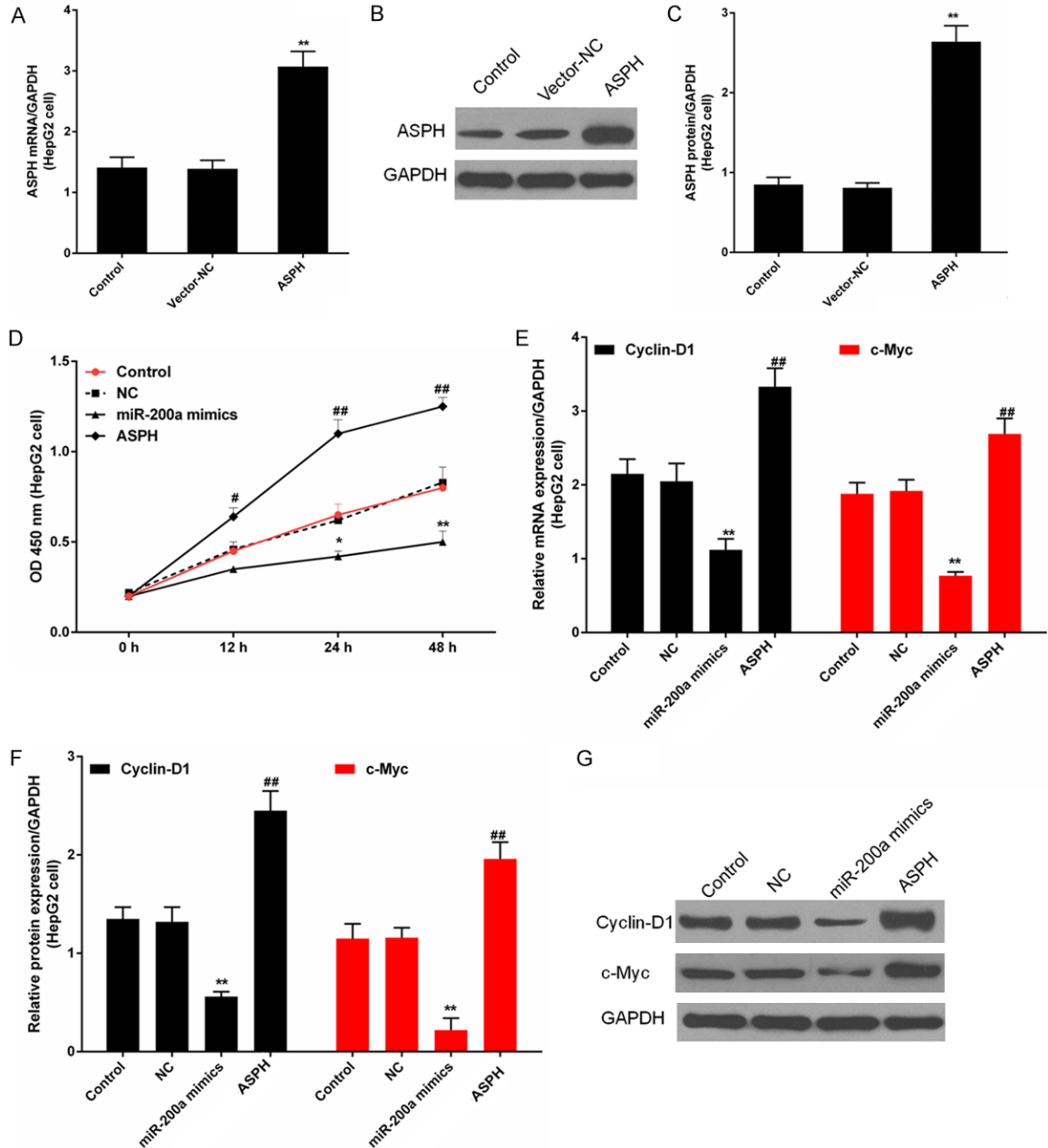
This research included a total of 72 patients, with an average age of  $48.23 \pm 15.66$  years old (Table 2). All HCC patients were selected from Zhejiang Provincial People's Hospital between March 2013 and Jun 2014. The expressions of miR-200a and ASPH were investigated in surgically resected RCC specimens and corresponding adjacent normal liver specimens. RT-PCR results showed that the mRNA expression of miR-200a was lower and that of ASPH was higher in liver tissues compared with adjacent normal liver tissues (Figure 1A and 1B). The correlation analysis showed that miR-200a expression was negatively related to ASPH expressions, which indicated miR-200a had

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**Figure 3.** Binding site prediction and results of luciferase report. A. Mimics NC, miR-200a mimics, inhibitors NC and miR-200a inhibitors were transfected into HepG2 cells, miR-200a mRNA was highly expressed in miR-200a mimics group and lowly expressed in miR-200a inhibitors group by RT-PCR analysis. B. Mimics NC, miR-200a mimics, inhibitors NC and miR-200a inhibitors were transfected into HepG2 cells, ASPH mRNA was lowly expressed in miR-200a mimics group and highly expressed in miR-200a inhibitors group by RT-PCR analysis. C. Mimics NC, miR-200a mimics, inhibitors NC and miR-200a inhibitors were transfected into HepG2 cells, ASPH protein was lowly expressed in miR-200a mimics group and highly expressed in miR-200a inhibitors group by Western blot analysis. U6 and GAPDH were detected as the control of sample loading. Data are expressed as the mean  $\pm$  SD for three independent experiments. \*\* $P < 0.01$  versus control and mimics NC, ## $P < 0.01$  versus control and inhibitors NC. D. The cervical-loop structures of pre-miR-200a. E. Putative targets predicted by microRNAdb. F. Relative luciferase activity resulted from binding of ASPH 3'-UTR reporter and miR-200a in HepG2 cell 48 h after transfection. \*\* $P < 0.01$ , ## $P < 0.01$ , t-test.

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**Figure 4.** Effect of miR-200a and ASPH on the cell viability. A. RT-PCR was used for detecting the transfection efficiency of ASPH over-expressed plasmid. B and C. Western blot was used for detecting the transfection efficiency of ASPH over-expressed plasmid. D. CCK-8 assay was used for detecting the cell viability after cell transfection with miR-200a mimics and ASPH over-expressed plasmid. E. The mRNA expression levels of Cyclin-D1 and c-Myc were decreased after cells transfection with miR-200a mimics and increased after cells transfection with ASPH over-expressed plasmid by RT-PCR analysis. F and G. The protein expression levels of Cyclin-D1 and c-Myc were decreased after cells transfection with miR-200a mimics and increased after cells transfection with ASPH over-expressed plasmid by Western blot analysis. GAPDH was also detected as the control of sample loading. Data are expressed as the mean  $\pm$  SD for three independent experiments. \*\* $P < 0.01$  and \* $P < 0.05$  versus control and NC groups. ## $P < 0.01$  and # $P < 0.05$  versus ASPH.

significance to ASPH (**Figure 1C**). Besides, survival analysis showed that the survival rate of HCC patients with lower expressed ASPH was significantly higher than those with higher expressed ASPH ( $P < 0.05$ ; **Figure 1D**).

Furthermore, the miR-200a and ASPH expressions were confirmed in liver cancer cell lines (HepG2, SMMC7721, Bel-7402, PLC and LM3) and normal liver cell L02 by RT-PCR and Western blot. In our results, the miR-200a with

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lower expression and the ASPH with higher expression were found in liver cancer cell lines, respectively. Compared with other cells, miR-200a expressions in HepG2 cell were the lowest and ASPH expressions in HepG2 cell were the highest (**Figure 2**).

### *Down-regulation of ASPH gene in HepG2 cell is mediated by miR-200a*

Based on the above analysis results and results of miR target prediction databases (miRBase, miRDB and microRNAdb), we found that miR-200a with predicted targeting ASPH had high target score that was 92. Therefore, HepG2 cell was transfected with miR-200a mimic/inhibitor/con to test the predicted effect. The transfection efficiency of miR-200a was confirmed by RT-PCR, ASPH expressions were detected by RT-PCR and Western blot. We found that the mRNA expression level of miR-200a was significantly increased in miR-200a mimics HepG2 cell, while dramatically decreased in miR-200a inhibitors HepG2 cell, compared to control, mimics NC and inhibitors NC groups (**Figure 3A**). The ASPH expressions in miR-200a mimics HepG2 cell was lower than that of control, mimics NC and inhibitors NC groups. In addition, the ASPH expressions in miR-200a inhibitors HepG2 cell had an opposite trend to miR-200a mimics HepG2 cell (**Figure 3B** and **3C**).

The target prediction databases indicated that one highly conserved miR-200a binding site was predicted in ASPH 3'-UTR (**Figure 3D** and **3E**). Direct interaction between miR-200a and ASPH was determined by the luciferase activity assay. miR-200a mimics transfection significantly decreased the relative luciferase activity of ASPH 3'-UTR, while miR-200a inhibitors transfection significantly increased the relative luciferase activity of ASPH 3'-UTR in HepG2 cell ( $P < 0.05$ ); there was no significant difference among the control group, mimics NC, inhibitors NC and ASPH mutation 3'-UTR group ( $P > 0.05$ ; **Figure 3F**).

### *Effect of miR-200a and ASPH on HepG2 cell proliferation*

Firstly, ASPH expressions were determined by RT-PCR and Western blot after transfecting plasmid which carried ASPH gene for 24 h in HepG2 cell. ASPH expressions in ASPH group were significantly higher than that of control

and vector-NC groups (**Figure 4A-C**). CCK-8 results showed that miR-200a mimics could obviously inhibit cell viability in a time dependent manner. When over expressed ASPH gene was transfected into cell, the cell viability was increased significantly in a time dependent manner (**Figure 4D**).

Cyclin-D1 and c-Myc, as cell cycle-related genes, can reflect cellular proliferation activity. In our results, the expression levels of cyclin-D1 and c-Myc were significantly down-regulated in cell with miR-200a mimics compared with control and mimics NC groups by RT-PCR and Western blot assay (**Figure 4E-G**). Moreover, the expression levels of cyclin-D1 and c-Myc in cell transfected with ASPH plasmids were significantly higher than that of control, mimics NC and miR-200a groups.

### *Effect of miR-200a and ASPH on EMT in HepG2 cell*

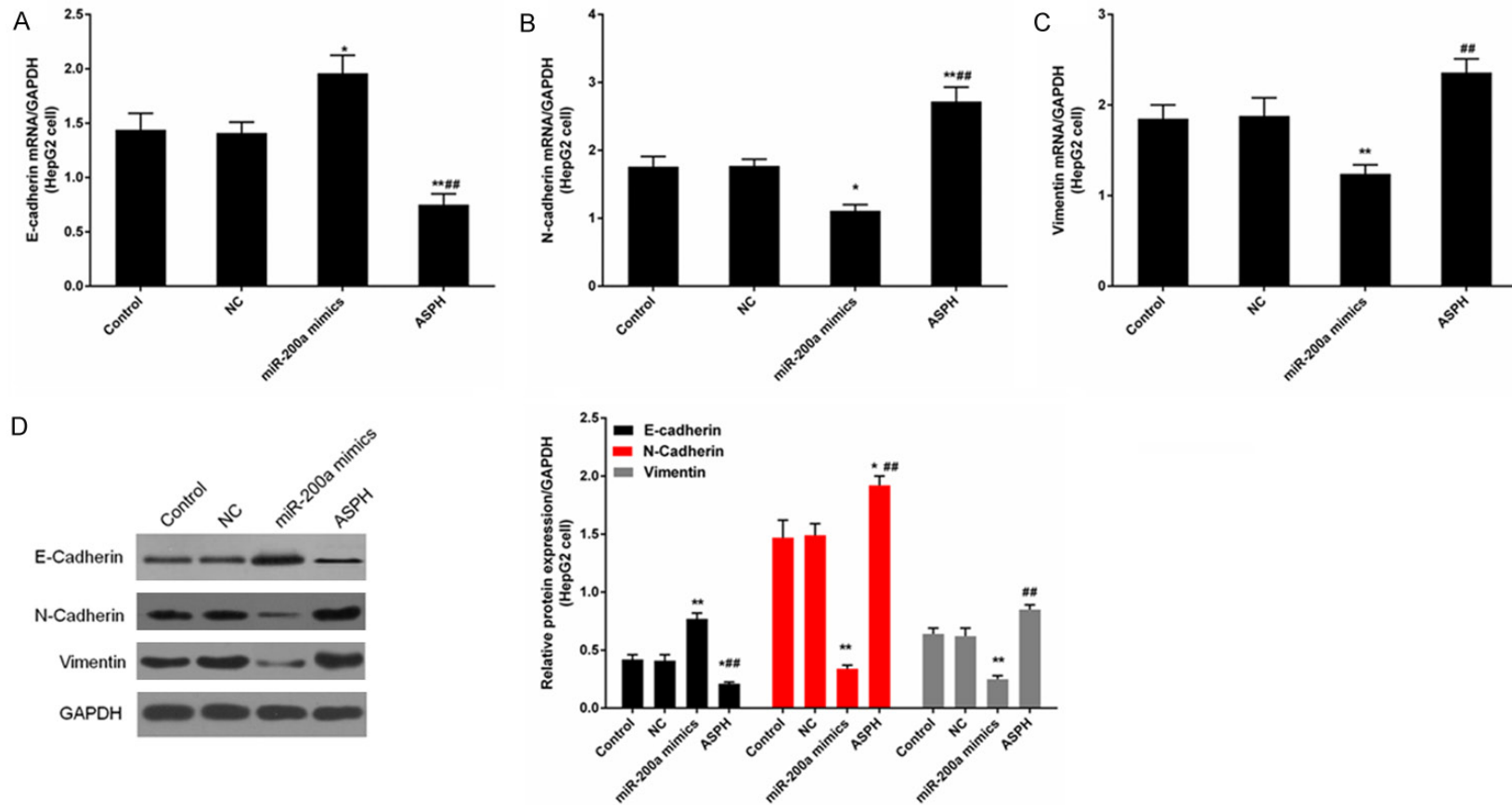
In this study, mRNA and protein expressions of E-Cadherin, N-Cadherin and Vimentin were detected by RT-PCR and Western blot. As shown in **Figure 5**, the E-Cadherin expressions in miR-200a mimics group were dramatically higher than that of control and mimics NC group, whereas E-Cadherin expressions in ASPH group were significantly lower than that of control, mimics NC and miR-200a groups. N-Cadherin expressions in miR-200a group were significantly lower than that of control and mimics NC groups, whereas N-Cadherin expressions in ASPH group were dramatically higher than that of control, mimics NC and miR-200a groups. Vimentin expressions in miR-200a group significantly lower than that of control and mimics NC, whereas dramatically higher than that of miR-200a group.

### *Effect of miR-200a and ASPH on ERK and PI3K/Akt signalling pathways*

The phosphorylated ERK, PI3K and Akt proteins were obviously detected by Western blot in HepG2 cell. Have cell transfected with miR-200a mimics, the phosphorylation levels of ERK, PI3K and Akt proteins were inhibited (**Figure 6A-C**). We further found that phosphorylation levels of ERK and Akt proteins in ASPH group were significantly higher than that of control, mimics NC and miR-200a mimics groups. In addition, the phosphorylation levels

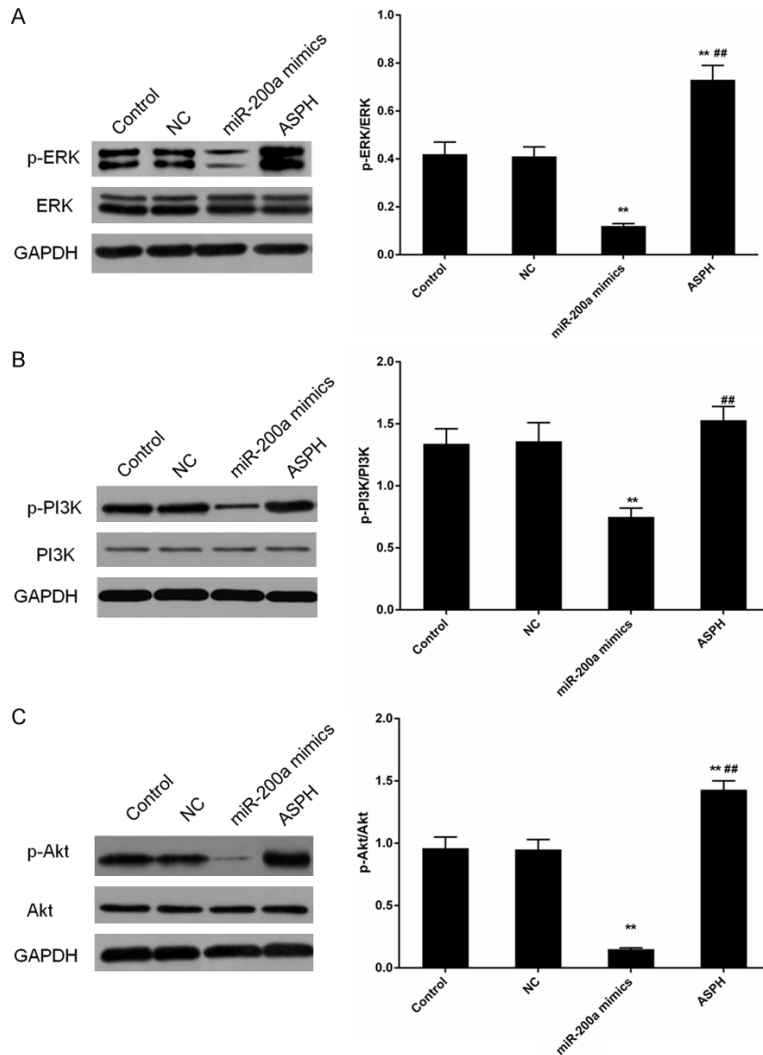


## Effect of miR-200a on HepG2 cell proliferation and EMT



**Figure 5.** Effect of miR-200a and ASPH on EMT. A. The mRNA level of E-cadherin was increased after cells transfection with miR-200a mimics and decreased after cells transfection with ASPH over-expressed plasmid by RT-PCR analysis. B. The mRNA level of N-cadherin was decreased after cells transfection with miR-200a mimics and increased after cells transfection with ASPH over-expressed plasmid by RT-PCR analysis. C. The mRNA level of Vimentin was decreased after cells transfection with miR-200a mimics and increased after cells transfection with ASPH over-expressed plasmid by RT-PCR analysis. D. The protein expressions of E-cadherin, N-cadherin and Vimentin were detected by Western blot analysis. GAPDH was also detected as the control of sample loading. Data are expressed as the mean  $\pm$  SD for three independent experiments. \*\* $P < 0.01$  and \* $P < 0.05$  versus control and NC groups. ## $P < 0.01$  and # $P < 0.05$  versus ASPH.

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**Figure 6.** Effect of miR-200a and ASPH on ERK and PI3K/Akt signaling pathways. A. The phosphorylation-level of ERK was decreased after cells transfection with miR-200a mimics and increased after cells transfection with ASPH over-expressed plasmid by Western blot analysis. B. The phosphorylation-level of PI3K was decreased after cells transfection with miR-200a mimics and increased after cells transfection with ASPH over-expressed plasmid by Western blot analysis. C. The phosphorylation-level of Akt was decreased after cells transfection with miR-200a mimics and increased after cells transfection with ASPH over-expressed plasmid by Western blot analysis. GAPDH was also detected as the control of sample loading. Data are expressed as the mean  $\pm$  SD for three independent experiments. \*\* $P < 0.01$  and \* $P < 0.05$  versus control and NC groups. ## $P < 0.01$  and # $P < 0.05$  versus ASPH.

of PI3K protein were significantly higher than that of miR-200a group ( $P < 0.05$ , **Figure 6**).

### Discussion

It reported that miR-200a and ASPH played a key role in tumorigenesis and liver cancer progression [12, 31]. Therefore, this study is impor-

tant- it might further clarify the molecular mechanism of liver cancer. The miR-200a expression was significantly decreased in liver cancer tissues and liver cancer cell lines (**Figures 1, 2**). ASPH expressions had an opposite tendency compared to miR-200a expressions (**Figures 1, 2**). In addition, there was no significant relationship in tumor staging between sex and age (**Table 2**). There were significant differences between ASPH expressions and tumor staging ( $P < 0.05$ , **Table 2**). Moreover, miR-200a expression was significantly negatively correlated to ASPH expressions. All HCC patients were followed-up for a mean of 1500 days, the survival rate of patients with ASPH-low was significantly higher than those with ASPH-high (**Figure 1**). These results indicated that there might be a relationship between miR-200a and ASPH in liver cancer. The functions of microRNA depend on the regulation of the target gene of microRNA [32]. The target gene of miR-200a was searched by computational analysis. The predicated results showed that ASPH is the target gene of miR-200a. Based on this screening and results of miR target prediction databases, it showed that miR-200a can regulate ASPH expressions under both physiologic and pathologic conditions. To confirm the findings, the miR-

200a and ASPH expressions were detected in HepG2 cell which was transfected miR-200a mimics and miR-200a inhibitors by RT-PCR and Western blot. We found that the miR-200a expressions in HepG2 cell which was transfected miR-200a mimics was significantly increased compared to control and mimics NC groups and the miR-200a expressions in HepG2 cell which

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was transfected miR-200a inhibitors was significantly decreased compared to control and inhibitors NC groups (**Figure 3A**). It showed that miR-200a mimics/inhibitors was successfully constructed and used for next experiment. In addition, ASPH expressions in HepG2 cell which was transfected miR-200a mimics was significantly decreased compared to control and mimics NC groups and ASPH expressions in HepG2 cell which was transfected miR-200a inhibitors was significantly increased compared to control and inhibitors NC groups (**Figure 3B** and **3C**). These results further indicated that high expressed miR-200a could inhibit ASPH expressions. According to computational analysis, we found ASPH 3'-UTR contained the binding site of miR-200a. The recombinant plasmid of ASPH-WT 3'-UTR and ASPH-Mut 3'-UTR and miR-200a mimics/inhibitors were co-transfected into HepG2 cell. The results showed that miR-200a mimics could significantly inhibit the relative luciferase activity of ASPH-WT 3'-UTR, but miR-200a mimics had no effect on the relative luciferase activity of ASPH-Mut 3'-UTR (**Figure 3**). Besides, the relative luciferase activity of ASPH-WT 3'-UTR in miR-200a inhibitors was significantly enhanced and the relative luciferase activity of ASPH-Mut 3'-UTR in miR-200a inhibitors was changed (**Figure 3**). From the above, it confirmed that miR-200a exerted an important effect on the regulation of its targeting ASPH.

In a follow-up experiment, ASPH highly expressed in HepG2 cells after cells were transfected with ASPH expression plasmid (**Figure 4A-C**). CCK-8 results showed that the cell proliferation was suppressed after cell were transfected with miR-200a mimics in a time dependent manner and was promoted after being transfected with ASPH expression plasmid (**Figure 4D**). miRNA inhibit cell proliferation only through regulating its target gene. miR-200a could inhibit the transcription and translation levels of ASPH. Therefore, we thought that cell proliferation was inhibited after miR-200a inhibited the target ASPH expressions. Some studies showed that many factors of tumorigenesis ultimately resulted in abnormality of cell proliferation. Cyclin-D1, as one of essential proteins in G1-phase of tumor cell, high expresses in cell with the high activity of proliferation [33]. C-Myc can promote the cell from G1-phase into S-phase and over expresses in tumors. The

high expression persistent of c-Myc can promote cell division, stimulate cell proliferation and selective conversion which results in the occurrence of liver cancer [34]. Cyclin-D1 and c-Myc genes are closely related to cell proliferation. In our results, cyclin-D1 and c-Myc expressions in miR-200a mimics group were significantly decreased and those in ASPH group were significantly increased by RT-PCR and Western blot. It showed that ASPH promoted cell proliferation by up-regulating cyclin-D1 and c-Myc expressions, and miR-200a inhibited the cyclin-D1 and c-Myc expressions through down-regulating ASPH expressions.

EMT is activated in tumor progression, infiltration and invasion, and plays an important role in tumor development, metastasis and dissemination [35]. For tumor cell, obtaining the invasiveness is key to activating EMT [36]. Studies showed that the interruption or reversal of EMT could inhibit the invasion and migration, and decrease the rate of metastasis [37]. Therefore, EMT has become a hotspot in tumor research. As many transcription factors and proteins play a key role in EMT, their expressions of upward or downward can reflect the process EMT. E-Cadherin acts as an epithelial marker lowly expressed in tumor, whereas N-Cadherin and vimentin as interstitial markers highly expressed in tumor [38]. The down-regulation of E-cadherin and the up-regulation of N-cadherin are the characteristic performances of EMT. Therefore, E-cadherin, N-cadherin and vimentin are considered as marker proteins of EMT. In our results, the results showed that E-cadherin expressions were significantly increased, whereas N-cadherin and vimentin expressions were significantly decreased in cell transfected with miR-200a mimics compared to control and mimics NC groups. In addition, E-cadherin, N-cadherin and vimentin expressions in ASPH group had an opposite tendency compared to miR-200a mimics group. It showed that ASPH promoted EMT and miR-200a inhibited EMT by down-regulating ASPH expression (**Figure 5**).

Many signaling pathways involve in cell proliferation and EMT. ERK can promote epithelium-like cells to differentiate into interstitial cell, which induce EMT to promote cell invasion [39, 40]. In addition, PI3K/Akt signaling pathway regulates EMT in liver cancer [41]. ERK and PI3K/Akt signaling pathways play an important

role in cell proliferation, growth, survival, invasion and migration and can regulate cooperatively the multitude cellular functions including cell cycle and apoptosis. It reported that TGF- $\beta$ 1 could involve in EMT through ERK and PI3K/Akt signaling pathways [42]. Therefore, ERK and PI3K/Akt signaling pathways regulate cell proliferation. In our results, having cell transfected miR-200a mimics group, the phosphorylation-levels of ERK, PI3K and Akt were significantly decreased compared with control and NC groups, after having cell transfected ASPH plasmid, the phosphorylation-levels of ERK, PI3K and Akt were significantly increased compared to miR-200a mimics group (**Figure 6**). It showed that ERK and PI3K/Akt pathways were activated in human hepatoma cells, ASPH might be an downstream targeting gene of ERK and PI3K/Akt pathways and could be activated. The over-expression of miR-200a could inhibit the activation of ERK and PI3K/Akt pathways.

Above all, miR-200a was lowly expressed in liver cancer tissues and cell lines, while the expression of ASPH had an opposite trend to that of miR-200a. And ASPH, as a miR-200a downstream targeting gene, could be activated by ERK and PI3K/Akt signaling pathways in liver cancer. miR-200a could inhibit the up-regulation of ASPH to suppress the cell proliferation and EMT by down-regulating the activation of ERK and PI3K/Akt signaling pathways.

#### Disclosure of conflict of interest

None.

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