

## Original Article

# MicroRNA-21 involves in VEGFR-1 activation induced epithelial-mesenchymal transition via Src in hepatocellular carcinoma

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**Abstract:** Objective: This study aimed to investigate the regulatory mechanism of epithelial-mesenchymal transition (EMT) induced by vascular endothelial growth factor receptor-1 (VEGFR-1) activation. Methods: Liver cancer was introduced to nude mouse, and microRNA microarray assay was then used to screen the downstream targeting molecules. The targeting molecules were identified through gene knockdown, Western blot assay and cell migration and invasion tests. Results: Of 1908 mature microRNAs and 1255 pre-miRNAs tested in liver cancer of nude mouse, microRNA-21 (miR-21) was one with most significantly down-regulated expression. Silencing of miR-21 increased E-cadherin expression ( $P=0.032$ ) and decreased Vimentin expression ( $P=0.046$ ) in liver cancer MHCC97-H cells. The migratory and invasive abilities of liver cancer cells transfected with LNA-anti-miR-21 decreased significantly ( $P=0.027$ ). Conclusion: miRNA-21 involves in the EMT induced by VEGFR-1 activation via Src and contributes to the metastasis and invasion of liver cancer cells.

**Keywords:** Cancer, hepatocyte, miR-21, epithelial-mesenchymal transition, Src

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. In China, 370,000 patients die of HCC every year, accounting for 53% of patients died of liver cancer [1]. Our previous results showed Src mediated the epithelial-mesenchymal transition (EMT) induced by vascular endothelial growth factor receptor-1 (VEGFR-1) activation and contributed to the migration and invasion of HCC cells [2]. However, how Src mediates the EMT, what the downstream targeting molecules of Src are and whether there is crosstalk between Src kinase pathway and other pathways remain unclear. Illustrating above issues will provide theoretical evidence for clinically preventing from recurrence and metastasis of HCC.

Saracatinib is the first Src inhibitor and able to inhibit the Src signaling pathway in human tumors [3]. Saracatinib is a potent Src inhibitor in cell-free assays, and may also significantly inhibit the c-Yes, Fyn, Lyn, Blk, Fgr and Lck; but

has weak inhibition on Abl and EGFR. Saracatinib may also significantly inhibit other Src tyrosine kinase family members including c-Yes, Fyn, Lyn, Blk, Fgr, and Lck. It's been reported that saracatinib may significantly inhibit Src Y530F NIH 3T3 (an isoform of Src), impair the invasion of HT1080 cells and completely inhibit epidermal growth factor (EGF)-induced cell scattering in NBT-II bladder cancer cells [4]. Saracatinib significantly inhibits the migration of DU145 cells and PC3 cells in the Boyden chamber, through inhibiting Y419 phosphorylation. Saracatinib inhibits the growth of prostate cancer cells including PC3 cells, DU145 cells, CWR22Rv1 cells and LNCaP cells, while Saracatinib shows low activity in LAPC-4, PZ-HPV7 and RWPE-1 cells [5]. Saracatinib may arrest cells in G1/S phase, but not cause caspase 3 cleavage [6]. Saracatinib inhibits AKT and enhances the sensitivity of A549 cells and Calu-6 cells to radiotherapy [7]. Saracatinib inhibits the activity of osteoclasts, and the resorption and formation of the bone. Saracatinib also reversibly prevents osteoclast

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**Table 1.** Primers used in the present study

Name	Sequence
MicroRNA-21, forward primer	GGGTAGCTTATCAGACTGATGTT
U6 forward primer	CTCGCTTCGGCAGCACCA
U6 reverse primer	AACGCTTCACGAATTTGCGT

**Table 2.** Expression of miR21

Group	Relative value of miR-21
MHCC97-H Cells	538.41±96.72
LNA-anti-miR-21 Cells	19.32±8.56
LNA-sramble Cells	543.27±86.11

precursor migration [8]. However, it is unclear that the downstream molecules of Src in EMT induced by VEGFR-1 activation. Moreover, whether saracatinib also inhibits the migration and invasion of HCC cells is still unknown.

## Materials and methods

### Cell culture and antibodies

Human HCC MHCC97-H cells were purchased from the Institute of liver cancer, Fudan University (Shanghai, China) and grown under the recommended conditions. DMEM and fetal calf serum (FCS) were obtained from Invitrogen (California, USA) and HyClone (Utah Logan, USA), respectively. Src inhibitor saracatinib (AZD0530) was purchased from Selleck Chemical (Houston, USA). Lipofectamine 2000 was purchased from Invitrogen (California, USA), transwell chambers (1 cm<sup>2</sup>/8 mm pore size) were from Costar (Cambridge, USA), and Matrigel was from BD Biosciences (Bedford, USA). Antibodies against p-Src-Y416 were purchased from Cell Signalling Technology, Inc (Taizhou, China), and anti- $\alpha$ -catenin, E-cadherin, N-cadherin and Vimentin antibodies were from Zhongshanjinqiao (Wuhan, China). VEGF-B167 was purchased from SantaCruz Biotechnology (Delaware, USA). RNA kit was purchased from TaKaRa Bio (Dalian, China).

### Oligonucleotides

Primers used for the quantification of miR-21 (ID: 000397) were purchased from Applied Biosystems (Foster City, USA). Control miR/Scrambled (AM17110) was obtained from Ambion (Austin, USA).

### Establishment of HCC animal model in nude mice

MHCC97-H cells were subcutaneously injected into nude mice. One week later, the tumors were harvested and transplanted into the left liver lobe of nude mice. The paired box gene 5 (PAX5), a tumor suppressor in HCC, was transfected into HCC cells to validate whether this model was successfully established. Tumor growth was measured by bioluminescence imaging technology. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and histopathological examination were used to confirm the transplanted tumor from MHCC97-H cells.

### Preparation of RNA and protein extracts and immunoblotting

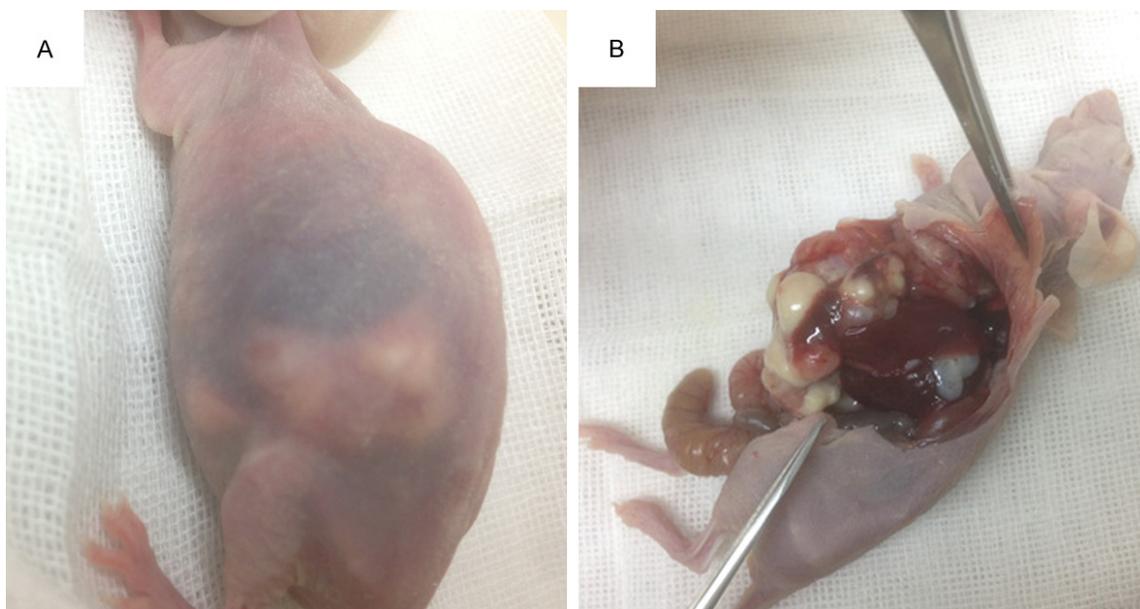
The extraction of RNA and protein, qRT-PCR and western blot assay were performed as described previously [9]. Mature miRNA-21 expression was determined by the Taqman miRNA assay (primers shown in **Table 1** and Results in **Table 2**), and normalized to U6-snrRNA with the  $2^{-\Delta\Delta Ct}$  method. PCRs were performed in triplicate.

### qRT-PCR

Sample preparation, RNA extraction, and high-density oligonucleotide array hybridization and scanning were performed as described previously [10]. Fluorescence intensities were quantified using the Affymetrix Microarray Suite 5.0 statistical algorithm with default parameters for the array type used in this study (Affymetrix HuFL).

### Gene microarray assay

RNA was extracted from MHCC97-H cells using the RNAeasy kit (Qiagen, CA). Primers and probes were obtained from Applied Biosystems Assays (Foster City, CA). All reactions were performed on a Gene Amp 5700 sequence detector (Applied Biosystems, Foster City, CA) under the conditions recommended by the manufacturer. Standard curves were created using the PCR products at concentrations ranging from 0.1 ng/uL to 0.0001 ng/uL. The absence of non-specific amplification was confirmed by agarose gel electrophoresis of PCR products. Standards and samples were detected in triplicate and data were averaged. The expression of target genes was normalized to that of



**Figure 1.** Balb/c nude mice with Hepatocellular carcinoma. A. Subcutaneous liver tumors were transplanted into the liver of Balb/c nude mice. These Balb/c nude mice were housed for 4 weeks, and the liver tumor was collected for histological examination. B. Macroscopic liver tumors after the abdominal cavity were opened.

$\beta$ -actin. The expressions of following genes were detected by qPCR: endothelial cell growth factor-1 (ECGF-1), adrenomedullin (ADM) and tumor necrosis factor receptor super family 14 (TNFSF14; Herpesvirus entry mediator [HVEM]). The primers and probes for q-PCR were purchased from the Applied Biosystems (Foster City, CA).

#### Cell migration and invasion assays

MHCC97-H cells were transfected with either LNA-anti-miR-21 or LN A-Scramble. Two days after transfection, cells were washed once with ice-cold PBS, trypsinized, and counted, and  $3 \times 10^5$  cells were seeded on transwell plates either coated with 10 mg matrigel/well (invasion assay) or uncoated (*in vitro* migration assay) in serum-free medium containing 0.1% bovine serum albumin (BSA). As a chemoattractant, 10% FBS was used in the lower chamber. After 14 h, the invaded cells were trypsinized and counted using the ATP-luminescence-based motility-invasion assay (Promega) as previously described [9].

#### Statistical analysis

Statistical analysis was performed using SPSS version 14.0 (SPSS14.0). The Wilcoxon Sign

Rank Test was used to compare the miR-21 expression in HCC cells. Correlations among continuous variables were analyzed with Spearman correlation analysis. A value of  $P < 0.05$  was considered statistically significant.

#### Results

##### Animal HCC model in nude mice

MHCC97-H cells were subcutaneously injected into nude mice, and tumor cells proliferated and formed metastatic tumors. A small part of tumor was excised and transplanted into the liver of nude mice. These nude mice with xenograft tumor were housed for 4 weeks, and the liver tumor was collected and processed for histological examination (**Figure 1**).

##### Saracatinib significantly down-regulated miRNA-21 expression in HCC

Our previous results [2] showed the c-Src mediated EMT and contributed to the VEGFR-1 activation induced migration and invasion in MHCC97-H cells. To further identify the downstream targeting molecules and signaling pathway of c-Src, c-Src inhibitor saracatinib was used to treat nude mice with HCC, and microR-

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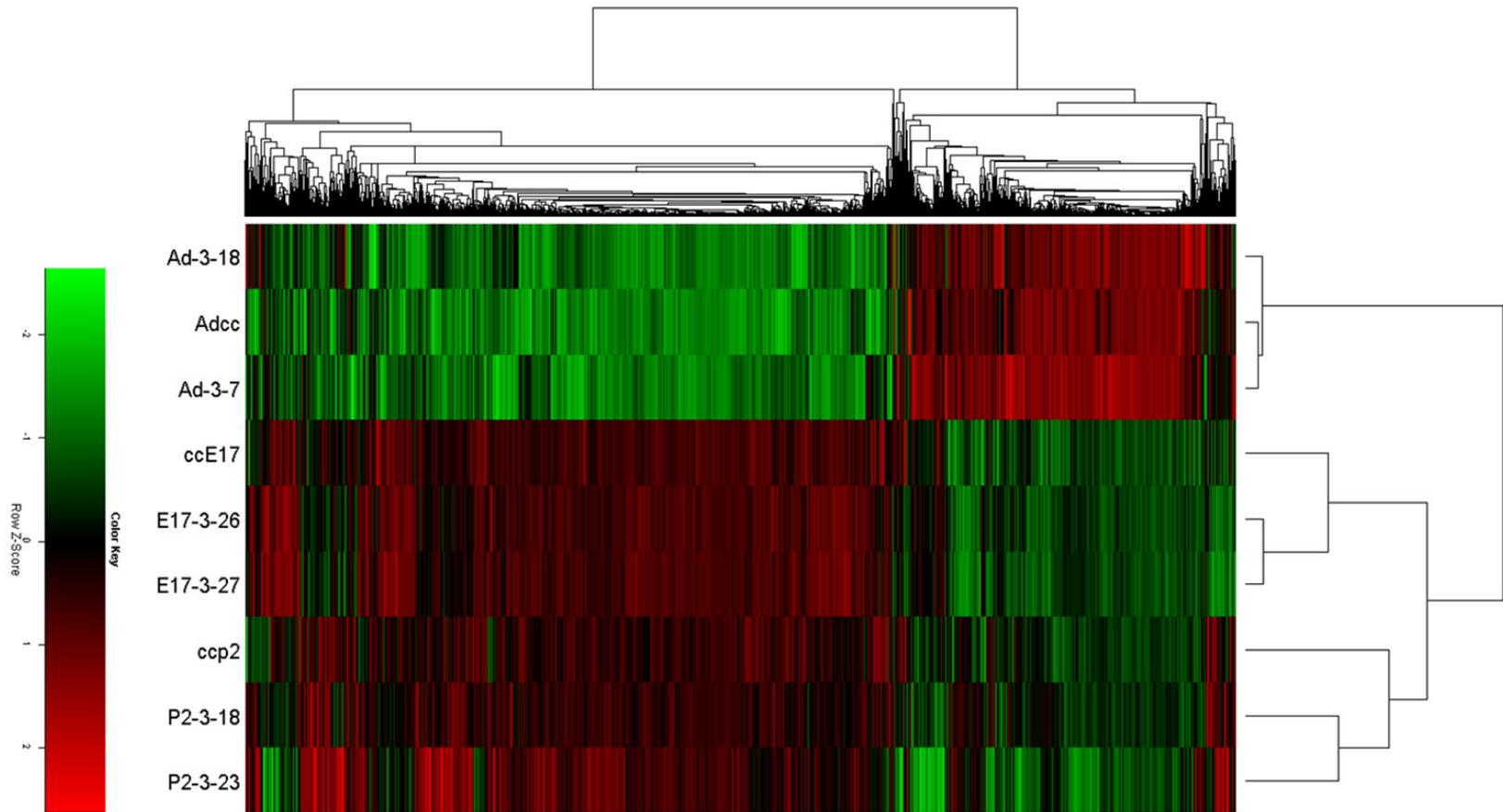
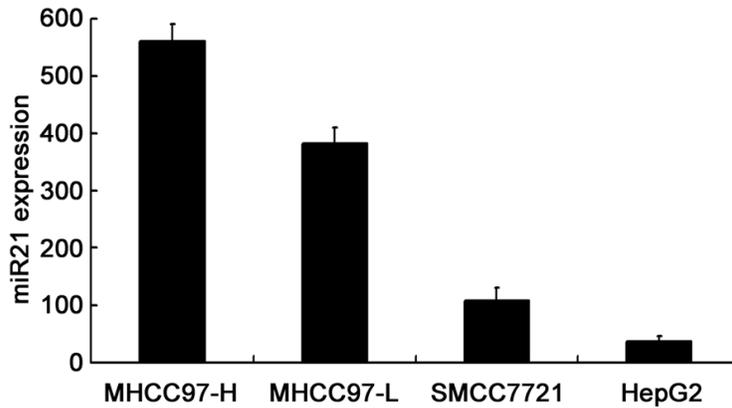


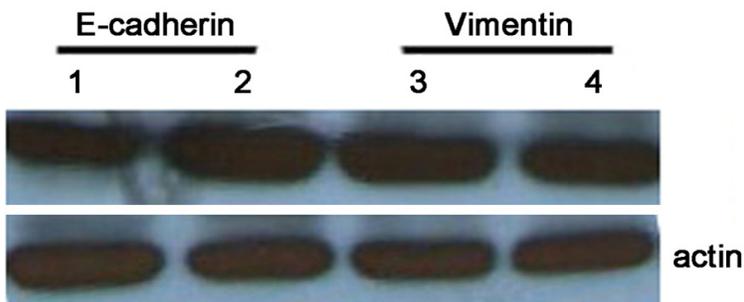
Figure 2. MicroRNAs microarray analysis.

**Table 3.** Effect of saracatinib at different concentrations on the miRNA-21 expression in nude mice

Group	MicroRNA-21 expression
CON	1
sara-0.5	0.895414095
sara-1	0.555537146
sara-2	0.389226037
sara-4	0.36270516
sara-8	0.29915001



**Figure 3.** MicroRNA-21 expression in HCC cells with different metastatic potentials. MHCC97-H cells had the most potent metastatic potential and the highest miR-21 expression. MHCC97-L cells had the second potent metastatic potential and the second highest miR-21 expression, followed by SMCC7721 cells and HepG2 cells.



**Figure 4.** Saracatinib up-regulated E-cadherin expression and down-regulated vimentin expression. Lane 1 and 3: LNA-sramble group (control group). Lane 2 and 4: LNA-anti-miR-21 group. The protein relative value of Lanes 1, 2, 3 and 4 were 1067.82±11.34, 1326.32±14.21, 1239.64±15.78 and 1152.43±12.67 respectively.

NA microarray was used to screen 1908 mature microRNAs and 1255 pre-microRNAs. Our results showed microRNA-21 was a microRNA with the most significant reduction in expression, which indicates miR-21 may be a downstream targeting molecule of Src (Figure 2 and Table 3).

*MiRNA-21 expression was the highest in MHCC97-H cells*

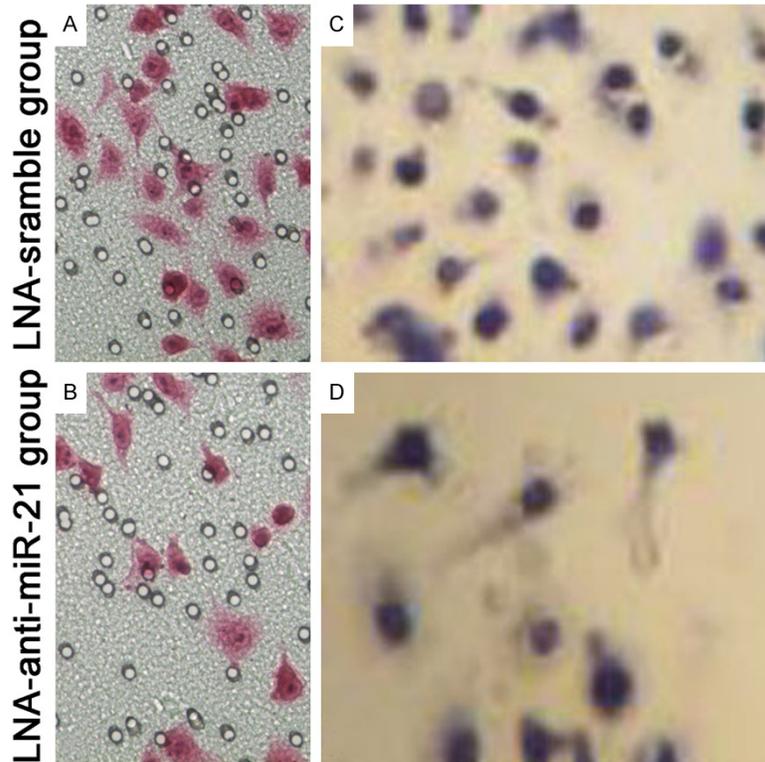
To further investigate whether miRNA-21 was a down-stream targeting molecule of Src, the miRNA-21 expression was detected in different types of HCC cells. Our previous results showed high expressions of VEGFR-1 and VEGF-B in MHCC97-H cells. In this study, the miRNA-21 expression was detected in different cell types: MHCC97-H cells, MHCC97-L cells, SMCC-7721 cells and HepG2 cells which have different migratory potentials. Results showed the miRNA-21 expression was the highest in MHCC97-H cells (Figure 3). Therefore, MHCC97-H cells were used in the following experiments.

*Silencing miR-21 up-regulated E-cadherin expression and down-regulated vimentin expression*

To further investigate whether microRNA-21 is involved in VEGFR-1 activation induced EMT, the locked nucleic acid anti miR-21 (LNA-anti-miR-21) was constructed to silence miR-21, and transfected into MHCC97-H cells. Exogenous VEGF-B was used to activate VEGFR-1. Results showed the expression of E-cadherin (an epithelial marker) in the LNA-anti-miR-21 group than in control group (P=0.032). However, the expression of vimentin (a mesenchymal marker) in LNA-anti-miR-21 group than in control group (P=0.046) (Figure 4).

*Silencing of miR-21 reduced migratory and invasive potentials of MHCC97-H cells*

After incubation of MHCC97-H cells with VEGF-B for 6 h, the number of migrating MHCC97-H cells increased twice in the improved Boyden chamber (Figure 5). However, after miR-21



**Figure 5.** Migration and invasion assay. A, B: Migration assay; C, D: Invasion assay.

**Table 4.** Migration and invasion assays of MHCC97-H cells after miR-21 silencing

	Migration assay			Invasion assay		
	$\bar{x} \pm S$	$\chi^2$	<i>P</i>	$\bar{x} \pm S$	$\chi^2$	<i>P</i>
LNA-anti-miR-21 treating group	14±2	8.17	0.003	8±2	13.58	0.001
LNA-scramble treating group	23±1			24±1		

silencing, the number of migrating MHCC97-H cells decreased significantly (**Figure 5**) ( $\chi^2=8.17$ ,  $P=0.003$ ). Similarly, the invasive ability of MHCC97-H cells decreased markedly after transfection with LNA-anti-miR-21 ( $\chi^2=13.58$ ,  $P=0.001$ ) (**Table 4**).

**Discussion**

Liver cancer is the third leading cause of cancer-related death, following gastric carcinoma and lung cancer [11]. Although great progresses have been achieved in the early diagnosis and treatment in recent years, the overall cure rate and survival rate are still very low. Various factors influence the overall cure rate, but the most important factors related to a poor prog-

nosis are tumor invasion, intra-hepatic dissemination and extra-hepatic metastasis of liver cancer. Therefore, to illustrate the molecular mechanism of invasion and metastasis of liver cancer is important to increase the overall cure rate and improve the prognosis of liver cancer. VEGFR-1 is a member of growth factor receptor family and mainly expressed on vascular endothelial cells. It contributes to physiological and pathological angiogenesis and lymphangiogenesis. Recent findings have shown the ectopic VEGFR-1 expression in many solid tumor cells, and the VEGFR-1 activation, specifically by its ligands, such as VEGF-B and placenta growth factor (PI-GF), may increase the invasive and metastatic potentials of tumor cells. This suggests that the ectopic expression of VEGFR-1 is related to the tumor progression independent of angiogenesis. Our previous results showed VEGFR-1 activation at a high level contributed to the invasion and metastasis of HCC cells through inducing the EMT [12].

Src involves in EMT through phosphorylating specific substrates. These substrates are the main members of cytoskeleton and molecules involved in adhesion between cells and between cells and substrates between cells and cells and between cells and substrates [13]. The targets of Src are proteins that are able to regulate the dynamics of cytoskeleton (such as actin), besides receptors on cells. In a recent study, results indicated Src up-regulated the expression of some microRNAs, and these microRNAs contributed to the invasion and metastasis of malignant tumors through inhibiting programmed cell death 4 (PDCD4), mitogen-activated protein kinase-kinase 3 (MAP2K3) and phosphatase and tensin homolog (PTEN) [10], which suggests Src contributes

to invasion and metastasis of malignant tumors through inhibiting tumor suppressors in a miRNA dependent manner. It has been reported that miR-21 contributes to the invasion and metastasis of HCC cells through its downstream molecules which may activate the matrix-degrading metalloproteinases (MMP-9) via Ca<sup>2+</sup>-dependent ERK [14]. It is well known that MMP-9 is the main hydrolytic enzyme of the extracellular matrix. Therefore, we speculate miR-21 involves in the EMT induced by VEGFR-1 activation.

In this present study, our results showed Src inhibitor--saracatinib down-regulated miR-21 expression in the nude mice with HCC, which was consistent with the results of Muppala et al. [10]. Further experiment *in vitro* showed miR-21 silencing reduced the E-cadherin expression and increased the vimentin expression following exogenous VEGF-B treatment in MHCC97-H cells, and miR-21 silencing reduced the migratory and invasive capacities of MHCC97-H cells, which suggest miR-21 involves in the EMT induced by VEGFR-1 activation in MHCC97-H cells. According to the structure of Src, Src may not bind to miRNAs of the known substrate in cells, but can activate the activator protein-1 (AP-1) [15]. It's well known that miR-21 may be regulated by AP-1 family members [16, 17]. AP-1 is a transcription factor and a heterodimer of c-Fos and c-Jun, and may bind to the 5'-TGAGTCA-3' element of DNA to regulate the expression of various genes [18].

In conclusion, miRNA-21 involves in the EMT induced by VEGFR-1 activation via Src and contributes to the metastasis and invasion of liver cancer cells. In our future studies, we will explore how Src affects the miR-21 expression and regulates the EMT induced by VEGFR-1 activation.

#### Disclosure of conflict of interest

None.

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