

## Original Article

# MicroRNA 424 regulates hepatocellular carcinoma cell migration via vascular endothelial growth factor A

Weijuan Yan<sup>1\*</sup>, Xiaoyong Xu<sup>2\*</sup>, Weiming Jiang<sup>2</sup>, Fengsheng Li<sup>1</sup>, Wei Li<sup>1</sup>, Kunming Zhou<sup>3</sup>, Jie Yang<sup>2</sup>, Sinian Wang<sup>4</sup>, Nan Yu<sup>4</sup>, Huijie Yu<sup>4</sup>, Qisheng Jiang<sup>1</sup>

<sup>1</sup>Department of Central Lab, General Hospital of Rocket Force of People's Liberation Army of China, Beijing, China; <sup>2</sup>Medical College of Soochow University, Industrial Park, Suzhou, Jiangsu, China; <sup>3</sup>Clinic Medical College of Jinzhou Medical University, Jinzhou, Jilin, China; <sup>4</sup>The General Hospital of The Second Artillery Corps of Chinese PLA, 16 Xijiekou Street, Xicheng District, Beijing, China. \*Co-first authors.

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**Abstract:** Recent findings have shown that microRNAs (miRNAs) are involved in the relapse and metastasis of hepatocellular carcinoma (HCC). However, the underlying mechanisms are not completely understood. Here, miR-424 expression was examined in 16 different HCC and adjacent, non-tumorous tissues from postoperative liver resections by stem-loop quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Then, HCC migration was tested in wound-healing assays following miR-424 overexpression or down-regulation. Next, functional miR-424 target genes were predicted using bioinformatics analysis and further verified in double-luciferase reporter assays. Finally, the mechanical role of miR-424 in migration was analyzed after miR-424 overexpression or down-regulation of miR-424 in HCC cells and parental cells. miR-424 expression was down-regulated in 12 out of 16 HCC tissues (75%). The wound-healing assay results indicated that miR-424 overexpression promoted HCC cell migration, while inhibiting miR-424 suppressed migration. Vascular endothelial growth factor A (VEGFA) was predicted to be an miR-424 target gene, which was confirmed in dual-luciferase assays. Moreover, VEGFA expression decreased in HCC cells after miR-424 overexpression and increased in HCC cells after miR-424 down-regulation, as determined by RT-PCR and western blot analysis. Importantly, VEGFA expression was reduced in HCC tissues where miR-424 expression was down-regulated compared with matching, adjacent noncancerous liver cancer tissues. Collectively, our results suggested that miR-424 regulates HCC cell migration by targeting VEGFA and is a potentially novel target for preventing the migration of HCC cells.

**Keywords:** Hepatocellular carcinoma, miR-424, migration, VEGFA

## Introduction

Liver cancer is one of the most common malignant neoplasms worldwide. It is the second and fifth most common cause of cancer-related death in males and females, respectively. Approximately 780,000 new cases and 740,000 deaths of liver cancer occurred globally in 2012 [1]. The death rate due to liver cancer is higher in less developed countries than in more developed countries. In China, the annual mortality rate ranked as the fifth in the most common malignant neoplasms [2]. Several types of liver cancer are known and hepatocellular carcinoma (HCC) is the most common form, accounting for approximately 75% of all liver cancers [3]. Although treatments for HCC patients (including surgical resection, chemotherapy, and radiotherapy) have improved [4-6], high relapse and metastases rates remain the main

causes of death. Thus, it is important to understand the cellular mechanism of HCC invasion and migration in order to develop antagonists against novel targets for HCC therapy.

MicroRNAs (miRNAs) are a class of endogenous, high conserved, single-stranded small non-coding RNAs that can regulate gene expression by pairing with the 3'-untranslated region (3'-UTR) of target mRNAs to inhibit translation or induce mRNA degradation [7]. These endogenous miRNAs may participate in genetic regulatory pathways affecting multiple biological processes such as cell differentiation, proliferation, and apoptosis [8, 9]. Emerging evidence has shown that multiple types of cancers exhibit aberrant miRNA expression [10-13], including HCC [14-16]. For example, miR-224, miR-30d, miR-10b, miR-9, and miR-17-5p were up-regulated in HCC, while let-7g, miR-23, miR-122,

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miR-101, miR-142-3p, miR-193b, miR-200, miR-424, miR-34a, miR-198, and miR-139 were down-regulated [17-31]. miRNAs can regulate the proliferation, apoptosis, invasion, and migration of HCC cells, suggesting that miRNAs are critical regulators of HCC development and progression. However, the underlying mechanisms whereby miRNAs regulate these processes remain largely unknown.

In the present study, we aimed to investigate the possible role and mechanism of miR-424 on the regulation of liver cells migration. We first verified that miR-424 expression level was down-regulated in HCC tissues compared with adjacent non-tumorous liver tissues; miR-424 had an inhibited effect on the HCC cells migration. We identified Vascular endothelial growth factor A (VEGFA) as a direct target gene of miR-424 by bioinformatics analysis and verified in dual-luciferase reporter assays. More importantly, miR-424 overexpression in HepG2 cells led to reduced VEGFA expression; VEGFA expression was up-regulated in HCC tissues whereby miR-424 was down-regulated. Collectively, our results suggested for the first time that miR-424 can inhibit HCC cell migration by targeting VEGFA.

### Materials and methods

#### *Patients and tissue samples*

HCC tissues and matched, adjacent non-tumorous liver tissues were obtained between 2013 and 2014 from 16 HCC patients who underwent hepatectomies at the General Hospital of Rocket Force of People's Liberation Army of China (Beijing, China). Patients provided informed consent, which was verified by a pathologist. Hard and firm tumor tissues were collected at the time of surgical resection and frozen immediately in liquid nitrogen. Patients enrolled in this study did not receive chemotherapy or radiotherapy before surgery. The mean age of the patients was  $51 \pm 3.6$  years (range: 35 to 72 years).

#### *Cell lines and cell culture conditions*

HepG2 and HEK 293T cells were maintained in the Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 100  $\mu$ g/ml streptomycin, and 100 U/ml penicillin (Gibco, USA).

Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

#### *Wound-healing assay*

HepG2 cells were seeded into 24-well plates ( $1 \times 10^5$  cells/well) for 16 h. Subsequently, a scratch was made with a sterile pipette tip in the central region of the confluent monolayer to create an artificial wound. Then, the cells were washed with serum-free medium, and an miR-424 mimic, miR-424 inhibitor, or a corresponding negative control (NC) was added in each well. A nonspecific miRNA mimic or inhibitor was used as a negative control, respectively. The cells were incubated in serum-free medium in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. The wounds were photographed (Olympus, Japan) at the indicated time points.

#### *miRNA transfections*

The miR-424 mimic, inhibitor, and their NCs were synthesized by GenePharma (Shanghai, China) and transfected into cells using final supernatant concentrations of 60, 60, 100, and 100 nM, respectively. miRNA transfections were performed using the INTERFERin lipid transfection reagent (Polyplus, French). Cells were harvested at the indicated time points for qPCR or western blot analysis.

#### *Total small RNA isolation and miR-424 quantification*

Total small RNAs were extracted from cells using the RNeasy Mini Kit (Qiagen, German), according to the manufacturer's protocol. Then, mRNA was reverse-transcribed using the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, USA). mRNA expression levels were verified by RT-PCR using the Real-Time Premix EX TaqMan Kit (Applied Biosystems, USA). The thermocycling conditions comprised an initial hold at 50°C for 2 min, a subsequent hold at 95°C for 5 min, followed by 40 cycles of 2-step PCR (95°C for 12 s and 60°C for 40 s). U6 small nuclear RNA was detected as an internal control and relative mRNA expression levels were calculated using the 2<sup>- $\Delta\Delta$ CT</sup> method. Each sample was tested in triplicate, and all experiments were repeated 3 times. Primers with the following sequences were used: miR-424 (forward, 5'-CAGCAGCAATTCATGT-3' and reverse, 5'-TGGTGTCTGGAG-

TCG-3') and U6 (forward, 5'-GCACCCGTCCAA-GAGAGTC-3' and reverse, 5'-GGTCCATCCGTA-CAGCCT-3').

### *Total RNA isolation and VEGFA quantification*

Total RNAs were isolated from cells using the RNeasy Mini Kit (Qiagen, German), according to the manufacturer's protocol. mRNAs were reverse transcribed with random hexamers using the All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopoeia, USA). mRNA expression was measured by RT-PCR using the Real-Time Premix EX TaqMan Kit (Takara, China). Ct values were normalized to that of  $\beta$ -actin, and relative mRNA expression levels were calculated using the  $2^{-\Delta\Delta CT}$  method. Each sample was tested in triplicate, and all experiments were repeated 3 times. The thermocycling conditions were comprised an initial hold at 50°C for 2 min and then 95°C for 5 min, followed by 40 cycles of 2-step PCR (95°C for 12 s and 60°C for 40 s). Primer and probes with the following sequences were used: VEGFA (forward, 5'-ACATCACCATGCAGATTATGC-3', reverse, 5'-GTCTTGCTCTATCTTTCTTTGGTC-3', probe, 5'-CACCAAGGCCAGCACATAGGAGAGA-3') and  $\beta$ -actin (forward, 5'-TCTGGCAACGGTGAAGGTG-ACA-3', reverse, 5'-CACCTCCCCTGTGTGGAC-TT-3', probe, 5'-AGCGAGCATCCCCCAAAGTTCA-CA-3').

### *Prediction of miR-424 target genes*

Target genes and 3'-UTR binding sites bound by the miR-424 seed region were predicted using the TargetScan (<http://www.targetscan.org/>), PicTar (<http://pictar.mdc-berlin.de/>), and MiRanda (<http://www.microrna.org/microrna/home.do>) databases. VEGFA, a molecule associated with metastasis, was identified as a candidate target mRNA of miR-424 by all 3 databases.

### *Vector construction and luciferase assay*

A 240-bp fragment of the VEGFA 3'-UTR containing a predicted binding site for human miR-424 and flanking sequences on each side was synthesized and then amplified by PCR. A second 240-bp fragment containing an altered binding-site sequence was also synthesized and amplified. The amplified products were cloned into the firefly luciferase reporter gene of the pEZX-MT01 vector, downstream of the Renilla luciferase reference gene (GeneCopo-

eia, USA). The 2 recombinant reporter-vector constructs were termed the WT (VEGFA-wild type) and MT (VEGFA-mutant) vectors, and were validated by sequencing. Each vector, along with 500 ng of the pEZX-MT01 vector and miR-424 mimics or mimic controls (60 nM), was transfected into 293 T cells using the Lipofectamine 3000 transfection reagent (Invitrogen, USA), according to the manufacturer's instructions. After a 36-h incubation, cells were harvested and the firefly and Renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA).

### *Western blot analysis*

Cells were harvested at the indicated time points and lysed with cell lysis buffer (Tiangen Biotech, China). Then, protein concentrations were measured using the BCA Protein Assay Kit (Thermo scientific, USA). Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, blocked in 5% nonfat milk (Bio-Rad, USA), incubated overnight at 4°C with primary antibodies and then with secondary antibodies for 1 h at room temperature, and visualized using ECL detection reagents (GE Healthcare, UK). Exposures were acquired using a ChemiDoc Imager (Bio-Rad, USA). The primary antibodies used included an anti-VEGFA antibody (Bioworld, rabbit, 1:500) and a horseradish peroxidase (HRP)-conjugated anti- $\beta$ -Actin antibody (Sigma, 1:10,000). The secondary antibodies used included an HRP-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech, 1:2,000) and an HRP-conjugated anti-mouse antibody (Amersham Pharmacia Biotech, 1:2,000).

### *Statistical analysis*

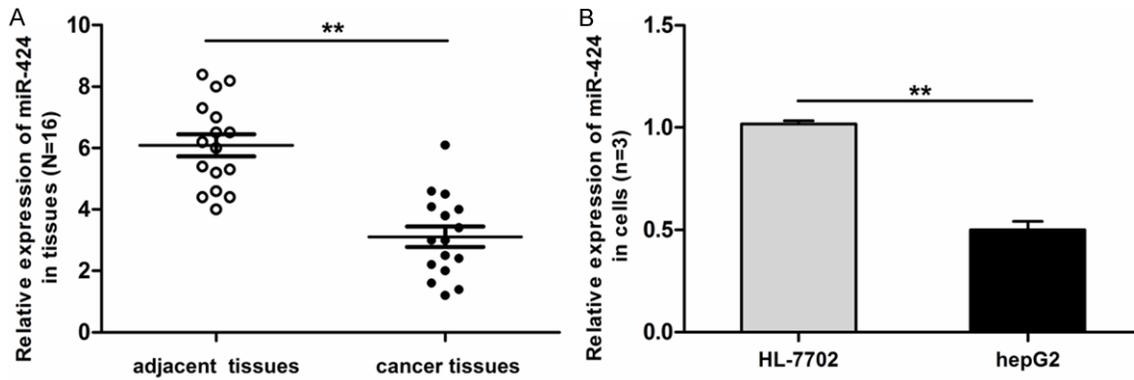
Statistical analyses were performed using SPSS software, version 20.0. All experiments were performed at least 3 times. Data are presented as the mean  $\pm$  SD. A 2-tailed Student's t-test was used to compare the means between 2 groups, and significance was accepted at  $P < 0.05$ .

## Results

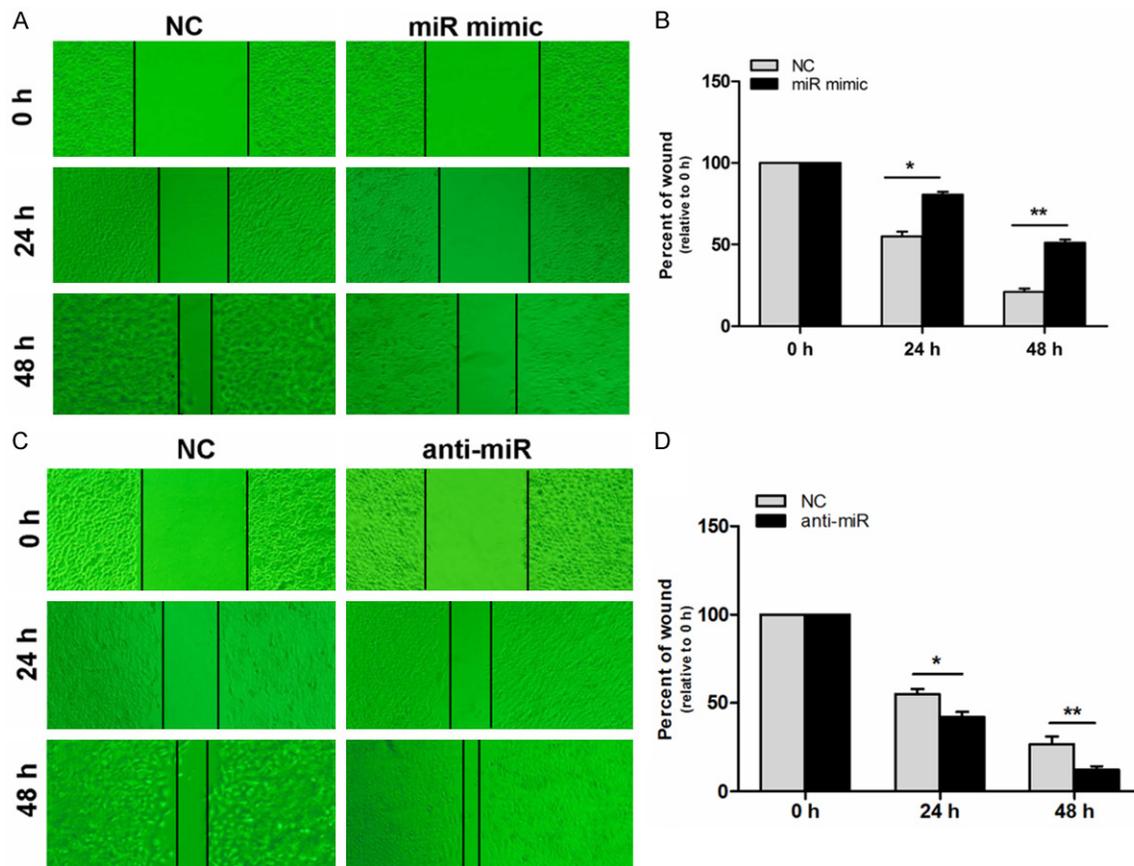
### *Down-regulation of miR-424 in HCC tissues*

In previous studies, it was shown that miR-424 expression was significantly reduced in HCC tis-

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**Figure 1.** Low miR-424 expression in HCC tissues and cell lines. A. Relative miR-424-expression levels were measured in HCC tissues and paired, adjacent non-cancerous tissues (n = 16) by stem-loop RT-PCR. B. Relative miR-424-expression levels were measured in the HepG2 HCC cell line and a normal human hepatocyte cell line (HL-7702) by stem-loop RT-PCR (n = 3, \*\*P < 0.01).

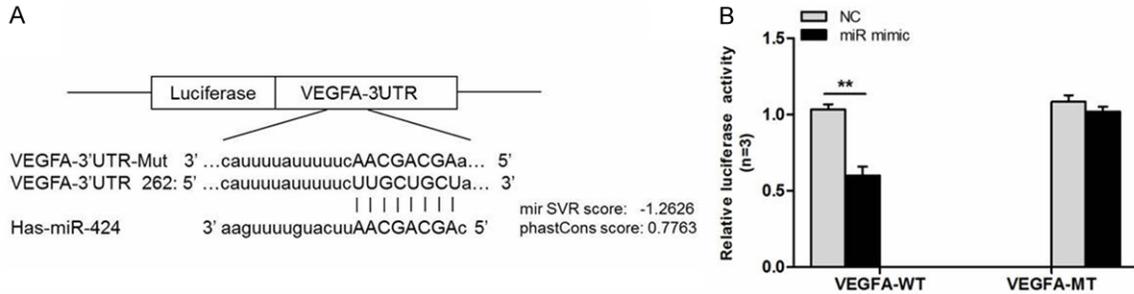


**Figure 2.** miR-424 suppressed HepG2 cell migration in vitro. A. Wound-healing assays were performed in HepG2 cells treated with either miR-424 mimics or NC. Representative images taken at 0, 24, or 48 h post-treatment are shown. B. Bar graph showing the percent of wound closure. C. Wound-healing assays were performed using HepG2 cells treated with miR-424 inhibitors or NC. Representative images photographed at 0, 24, or 48 h post-treatment are shown. D. Bar graph showing the percent of wound closure. \*P < 0.05, \*\*P < 0.01.

sues and To confirm these results, miR-424 expression was detected by stem-loop RT-PCR analysis with 16 paired samples of HCC and

HepG2 cells. As shown in **Figure 1A**, miR-424 expression strongly decreased in 12/16 (75%) clinical HCC tissues compared with the matched

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**Figure 3.** VEGFA is a direct target gene of miR-424. A. Diagram of the miR-424-binding sites in the 3'-UTR of VEGFA mRNA. B. HepG2 cells were co-transfected with miR-424 mimics/NC and a reporter plasmid containing WT or MT versions of the VEGFA 3'-UTR. Forty-eight hours later, luciferase activities were measured and normalized to firefly luciferase activity. \*\* $P < 0.01$ .

adjacent noncancerous liver tissues. Moreover, miR-424 expression in the HCC HepG2 cell line displayed a 0.5-fold decrease ( $P < 0.01$ ) relative to that in normal hepatocytes (HL-7792 cells) (**Figure 1B**). In general, miR-424 exhibited lower expression in HCC tissues and HepG2 cells.

### miR-424 inhibited HCC cell migration in vitro

miR-424 has been correlated with the metastasis of several different cancer types. Next, the effect of miR-424 on the migration ability of HepG2 cells was assessed by performing wound-healing assays. Because miR-424 expression was down-regulated in HepG2 cells, they were transfected with miR-424 mimics to evaluate the cell-migration activity. The NC mimic or inhibitor was used as a negative control for transfection. miR-424 overexpression significantly inhibited HepG2 cell migration (**Figure 2A, 2B**). Furthermore, synthetic miR-424 inhibitors or the NC was transfected into HepG2 cells. The results showed that miR-424 knockdown increased the migration of HepG2 cells (**Figure 2C, 2D**), suggesting that miR-424 normally inhibits HepG2 cell migration.

### Bioinformatics-based prediction that miR-424 targets the VEGFA 3'-UTR

miRNAs function through pairing with the 3'-UTR of target genes to down-regulate their expression, thereby participating in signaling pathways that modulate biological processes such as proliferation, apoptosis, and metastasis. Target genes of miR-424 were predicted using bioinformatics software programs. We first studied Wnt1, Wnt7, and VEGFA because of the predicted miR-424-binding sites in their

3'-UTRs and their known involvement in cell metastasis. More importantly, VEGFA has a low mirSVR score (-1.2626), which represents a high thermodynamic stability caused by the 3'-UTR, as well as a high PhastCons score (0.7763), which represents a high level of conservation in the 3'-UTR (**Figure 3A**). In addition, VEGFA is expressed aberrantly in various types of human cancers.

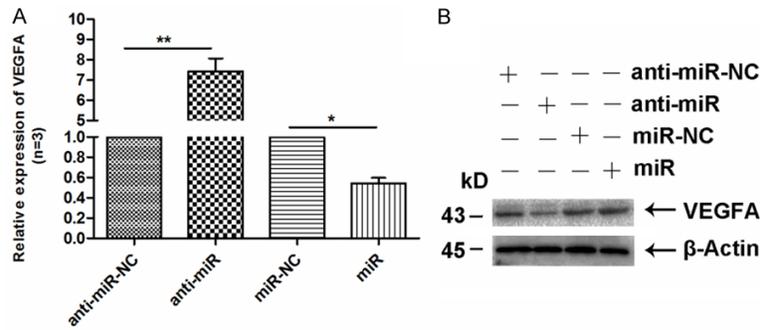
### VEGFA is a potential target of miR-424 in HCC cells

Dual-luciferase assays were performed to determine whether miR-424 can directly bind to the VEGFA 3'-UTR. The WT and MT luciferase reporter vectors containing the 3'-UTR and miR-424 mimic were transfected separately into HEK-293T cells. The results showed that WT 3'-UTR reporter vector and miR-424 mimic led to a significant decrease in the relative luciferase activity compared with the WT vector and NC (**Figure 3B**), while no significant decrease was observed with the MT vector and miR-424 mimic. These data suggested that miR-424 may repress VEGFA mRNA expression at the post-transcriptional level by directly targeting its 3'-UTR.

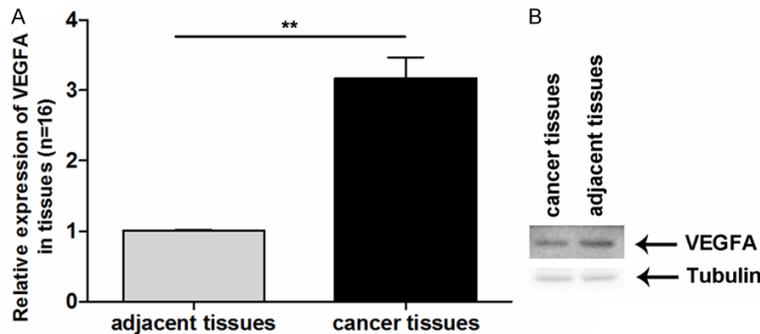
### miR-424 decreased VEGFA expression

To verify the effect of miR-424 on the regulation of endogenous VEGFA in HCC cells, miR-424 mimics/inhibitors and their corresponding negative controls were transfected into HepG2 cells, and VEGFA expression was subsequently detected by RT-PCR and western blotting. As shown in **Figure 4**, VEGFA expression significantly decreased in HepG2 cells transfected with the miR-424 mimics compared with the

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**Figure 4.** miR-424 suppressed the expression of VEGFA. A. HepG2 cells were transfected with miR-424 mimics, inhibitors, or the NC. After 48 h, the expression of VEGFA mRNA was tested by quantitative RT-PCR. B. The protein level of VEGFA was assessed after 72 h by western blotting analysis. \*\*P < 0.01.



**Figure 5.** VEGFA expression in cancer tissues was lower than that in paired adjacent non-cancerous tissues. A. VEGFA mRNA levels in cancer tissues and paired adjacent non-cancerous tissues were measured by quantitative RT-PCR. B. VEGFA protein expression detected by western blotting. \*\*P < 0.01.

NC, while increased VEGFA expression was observed when cells were transfected with the miR-424 inhibitors. These results suggested that miR-424 can regulate endogenous VEGFA expression in HepG2 cells.

### Up-regulation of VEGFA expression in HCC tissues

The above in vitro results indicated that miR-424 can modulate HepG2 cell migration by targeting VEGFA, which prompted us to further investigate VEGFA expression in HCC tissues. Thus, VEGFA expression was tested by RT-PCR and western blotting with 16 human HCC tissues, 12 of which showed down-regulated miR-424 expression relative to the matched, adjacent non-cancerous liver tissues. As predicted by our bioinformatics analysis, VEGFA expression was significantly higher in the HCC tissues

compared with adjacent non-cancerous liver tissues (Figure 5). Thus, these data further supported the hypothesis that miR-424 negatively regulates VEGFA.

### Discussion

Metastasis consists of multiple steps including the infiltration of local tumor cells into adjacent tissues, intravasation in blood or lymphatic vessels, survival in the vasculature, extravasation, and subsequent proliferation in remote and compatible regions leading to colonization [32, 33]. Aberrant activation of various genes, signaling pathways, cancer stem cells, and autophagy are reported to be involved in metastasis [34-37]. Despite these insights, the underlying mechanisms remain to be demonstrated. Dysregulated miRNA expression has been implicated in the invasion and migration of various human cancers, including HCC [23-25]. Data from previous studies showed that miR-424 expression was down-regulated in HCC tissues

and associated with the TNM stage and survival rate of HCC patients. To understand the potential mechanistic role of miR-424 in metastasis, the effect of miR-424 on the cell migration and its molecular mechanism were investigated. First, we confirmed that miR-424 expression was down regulated in HCC tissues and cells and that miR-424 overexpression blocks HCC cell migration. Furthermore, we predicted and demonstrated that VEGFA is an miR-424 target gene. More importantly, VEGFA expression decreased when miR-424 was overexpressed in HCC cells and increased when miR-424 was knocked down. Finally, we verified that VEGFA expression was up-regulated in tissues showing miR-424 down-regulation. Taken together, our data provide evidence, for the first time, that miR-424 inhibits HCC cell migration by targeting VEGFA.

miR-424 is located on chromosome X at q26.3, which has been associated with the genesis and development of cancers [19, 29]. In previous studies, differential miR-424 expression was observed with various cancer types. Up-regulated miR-424 has been observed in some cancers, such as pancreatic cancer, renal clear cell carcinoma, breast cancer, and tongue squamous cell carcinoma [38]. However, down-regulated miR-424 expression was observed in other studies of cervical cancer, breast cancer, leukemia, prostate cancer, and osteosarcoma [39-43]. Previously, it was reported that miR-424 was down-regulated in HCC tissues and cells and that its expression levels correlated with multiple nodules, TNM stage, and the survival rate of patients with HCC [21]. Consistent with these previous findings, we confirmed that miR-424 was down-regulated in 12/16 (75%) HCC tissues versus matched, adjacent non-cancerous liver tissues.

Wound-healing assays were performed to demonstrate the effect of miR-424 on HCC cell migration. Overexpression of miR-424 significantly blocked HCC cell migration and, conversely, miR-424 down-regulation promoted HCC cell migration, indicating that miR-424 inhibits HCC cell migration. The results were similar to those reported by Yu et al. [21].

Next, to explore the molecular mechanism whereby miR-424 blocked HCC cell migration, we conducted bioinformatics analysis, which revealed that miR-424 may directly target the 3'-UTR of VEGFA. This prediction was validated by performing dual-luciferase reporter gene assays. Next, we demonstrated that endogenous VEGFA expression (at both the mRNA and protein levels) decreased in HCC cells following miR-424 overexpression. It has been reported that VEGFA was up-regulated in many types of cancers and contributed to cancer development and progression [44-46]. VEGFA, a main member of the VEGF family, potently induces angiogenesis, which is crucial for the ability of solid tumors to acquire nutrients and undergo continuous growth and metastatic spread [47]. Silencing VEGFA mRNA expression with a small interfering RNA significantly suppressed colorectal carcinoma growth and metastasis [48]. Our results indicated that miR-424 suppresses the migration of HepG2 cells partially through down-regulating VEGFA expression. miRNAs can exert their functions through several target

genes; thus, miR-424 likely has several target mRNA molecules as well. Previous data showed that miR-424 inhibited HCC cell migration by targeting c-Myb, in contrast to our findings. Although the target mRNAs identified are different, the effects of miR-424 on HCC cell migration are the same. Finally, we verified that VEGFA expression was up-regulated in HCC tissues with down-regulated miR-424 expression.

In conclusion, the results of the current study revealed that miR-424 suppressed the migration of HepG2 cells via targeting VEGFA, indicating that miR-424 plays an inhibiting role in HCC cell metastasis. Therefore, miR-424 may be developed as a novel therapeutic target for HCC. However, further investigation of the clinical relevance of miR-424 in HCC patients should be conducted.

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Weijuan Yan, Department of Central Lab, General Hospital of Rocket Force of People's Liberation Army of China, 16 Xijiekou Street, Xicheng District, Beijing 100085, China. E-mail: missyan3611@163.com; Dr. Qisheng Jiang, Department of Hepatobiliary Surgery, General Hospital of Rocket Force of PLA, 16 Xijiekou Street, Xicheng District, Beijing 100088, China. E-mail: jqs598@sina.com

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