

MiRNA-15a-3p inhibits the metastasis of hepatocellular carcinoma by interacting with HMOX1

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Abstract. – OBJECTIVE: To uncover the relationship between microRNA-15a-3p (miRNA-15a-3p) level and clinical features of hepatocellular carcinoma (HCC), and to explore the influence of miRNA-15a-3p on metastasis of HCC cells.

PATIENTS AND METHODS: HCC and paracancerous tissues were surgically resected from 44 HCC patients. Their clinical data and follow-up files were recorded. Differential expressions of miRNA-15a-3p in HCC samples were determined. The relationship between miRNA-15a-3p level and clinical features of HCC patients was analyzed. Changes in proliferative, migratory and invasive potentials in Huh7 and HepG2 cells overexpressing miRNA-15a-3p were examined. The downstream gene of miRNA-15a-3p and its involvement in HCC development were finally explored.

RESULTS: MiRNA-15a-3p was downregulated in HCC tissues. High metastasis rate and poor prognosis were observed in HCC patients expressing a low level of miRNA-15a-3p. Overexpression of miRNA-15a-3p attenuated proliferative, migratory and invasive potentials in HCC. Protein levels of HMOX1, CD31, c-Myc, MMP-2 and MMP-9 were downregulated in HCC cells after overexpression of miRNA-15a-3p. HMOX1 was the downstream gene of miRNA-15a-3p, which was upregulated in HCC samples. Highly expressed HMOX1 was unfavorable to the prognosis in HCC. Overexpression of HMOX1 abolished the regulatory effects of miRNA-15a-3p on HCC cell phenotypes.

CONCLUSIONS: MiRNA-15a-3p is closely linked to lymphatic metastasis, distant metastasis and poor prognosis in HCC. It inhibits the malignant development of HCC by interacting with HMOX1.

Key Words:

MiRNA-15a-3p, HMOX1, Hepatocellular carcinoma (HCC), Malignant development.

Introduction

Hepatocellular carcinoma (HCC) is a prevalent cancer in the world. Its pathogenesis in-

volves inactivated tumor suppressors and/or activated oncogenes¹⁻³. It is generally believed that viral hepatitis, alcoholism, and non-alcoholic fatty steatosis are the main causes of HCC⁴⁻⁶. Each year, there are 782,000 newly onsets of HCC globally, and 83% occur in developing countries⁷⁻⁹. The number of HCC cases in China accounts for 50% of the total cases. Due to the insidious onset, a great number of HCC patients are diagnosed in the middle or advanced stage. Conventional imaging examination and detection of serum alpha-fetoprotein (AFP) have limited diagnostic capabilities for HCC in the early stage or those with small foci. The prognosis in HCC patients is relatively poor^{9,10}. It is urgent to develop effective diagnostic and therapeutic strategies of HCC^{1,9,10}. In recent years, miRNAs have become a research hotspot in the field of molecular biology. Abnormally expressed miRNAs display good application prospects in the diagnosis, treatment, and prognosis of tumor diseases^{11,12}.

MicroRNAs (miRNAs) are highly conserved non-coding RNAs with 18-25 nucleotides long. They are widely expressed in cells, tissues and the circulating system^{13,14}. By recognizing and binding 3'UTR of target genes, miRNAs are responsible for negatively regulating target gene expressions^{15,16}. A miRNA can regulate multiple target genes, and a target gene can be regulated by multiple miRNAs, thus forming a complicated network^{16,17}. Mature miRNAs are involved in cell growth, hormone secretion and other life activities¹⁸. Upregulated miRNAs in HCC samples are believed as oncogenes, on the contrary, some miRNAs are downregulated in HCC samples as tumor suppressors^{11,12}.

MiRNA-15a-3p is reported to be downregulated in many types of malignant cancers, which contributes to inhibit cancer development^{19,20}. Our

preliminary work has searched differentially expressed miRNAs in HCC profiling and miRNA-15a-3p is selected. In this paper, we mainly explored the expression pattern and clinical significance of miRNA-15a-3p in the development of HCC.

Patients and Methods

HCC Samples

A total of 44 paired HCC and paracancerous tissues were surgically resected, and they were pathologically confirmed and stored at -80°C . The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). All patients did not receive any treatment, such as radiotherapy and chemotherapy before the surgery. Clinical data and follow-up data of included HCC patients were recorded. This study obtained the approval by the Ethics Committee of People's Hospital of Ningxiang City and it was conducted after informed consent of each subject.

Cell Lines and Reagents

HCC cell lines (Bel-7402, HepG2, MHCC88H, SMMC-7221, Huh7, Hep3B) and a normal hepatocyte cell line (LO2) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin in a 5% CO_2 incubator at 37°C . Cell passage was conducted when cells were reached 80-90% confluence.

Transfection

Cells were cultured to 50-70% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, cells were collected for the following use.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

Transwell Migration and Invasion Assay

200 μL of suspension (5.0×10^5 /mL) was applied in the upper side of transwell chamber (Millipore, Billerica, MA, USA) and inserted in a 24-well plate with 700 μL of medium containing 10% FBS in the bottom. After 48 h of incubation, the cells in the bottom were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope. Migratory cell number was counted in 5 randomly selected fields per sample (magnification 40 \times). Transwell invasion assay was similarly conducted except for pre-coating diluted Matrigel in the bottom of the chamber.

Quantitative Real Time-PCR (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR[®] Premix Ex Taq[™] (TaKaRa, Otsu, Shiga, Japan). Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta\text{Ct}}$ and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6. MiRNA-15a-3p: forward: 5'-GGGGCAGGCCATATTGTG-3', reverse: 5'-TGCCTGTCGTGGAGTC-3'; U6: forward: 5'-AAAGCAAATCATCGGACGACC-3', reverse: 5'-GTACAACACATTGTTTCTCGGA-3'; HMOX1: forward: 5'-CTCCTCTCGAGCGTCCTCAG-3', reverse: 5'-AAATCCTGGGGCATGCTGTC-3'; GAPDH: forward: 5'-TGTGGGCATCAATGGATTTGG-3', reverse: 5'-ACACATGTATTCCGGGTCAAT-3'.

Western Blot

Cells were lysed in cell lysis buffer for 30 min on ice and centrifuged at 14,000 \times g at 4°C for 15 min. The total protein concentration was calculated by the BCA Protein Assay kit (Pierce, Rockford, IL, USA). Proteins were separated by 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. The membranes reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted. The primary antibody and the anti-mouse and anti-rabbit secondary antibodies were obtained from Cell Signaling Technology, (Danvers, MA, USA).

Dual-Luciferase Reporter Assay

Cells were pre-inoculated in a 24-well plate. They were co-transfected with NC mimic/miRNA-15a-3p mimic and pmirGLO-HMOX1-WT/pmirGLO-HMOX1-MUT/pmirGLO, respectively. After 48 h cell culture, they were lysed for measuring the Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean \pm standard deviation (SD). Differences between groups were analyzed by the *t*-test. Chi-square test was conducted for analyzing the relationship between miRNA-15a-3p level and clinical data of HCC patients. Kaplan-Meier curves were depicted for survival analysis in HCC patients. $p < 0.05$ was considered as statistically significant.

Results

Downregulated MiRNA-15a-3p in HCC Samples

Compared with paracancerous tissues, miRNA-15a-3p was downregulated in HCC tissues (Figure 1A). Similarly, miRNA-15a-3p was lowly expressed in HCC cell lines, especially Huh7 and HepG2 cell lines (Figure 1B). These two cell lines were selected in the following experiments.

Clinical data and follow-up files of included HCC patients were analyzed. It is shown that miRNA-15a-3p level was negatively correlated to rates of lymphatic metastasis and distant metastasis in HCC patients (Table I). However, its level was unrelated to other clinical features of HCC. Kaplan-Meier curves illustrated a poor prognosis in HCC patients expressing low level of miRNA-15a-3p (Figure 1C).

Overexpression of MiRNA-15a-3p Inhibited Proliferative and Metastatic Potentials in HCC

Transfection efficacy of miRNA-15a-3p mimic was verified in Huh7 and HepG2 cells (Figure 2A). After transfection of miRNA-15a-3p mimic, the viability in HCC cells markedly decreased (Figure 2B). Transwell assay uncovered lower migratory and invasive cell numbers in HCC cells overexpressing miRNA-15a-3p than those of controls (Figure 2C).

Overexpression of MiRNA-15a-3p Downregulated HMOX1 in HCC

The protein levels of key genes in the MMP-9 signaling influenced by miRNA-15a-3p were determined. Overexpression of miRNA-15a-3p remarkably downregulated protein levels of HMOX1, CD31, c-Myc, MMP-2, and MMP-9 in HCC cells (Figure 3A). Based on the predicted binding sequences in the 3'UTR of miRNA-15a-3p and HMOX1, we constructed wild-type and mutant-type HMOX1 vectors. Overexpression of miRNA-15a-3p was able to decrease Luciferase activity in wild-type HMOX1 vector, while that of mutant-type one was unchangeable (Figure

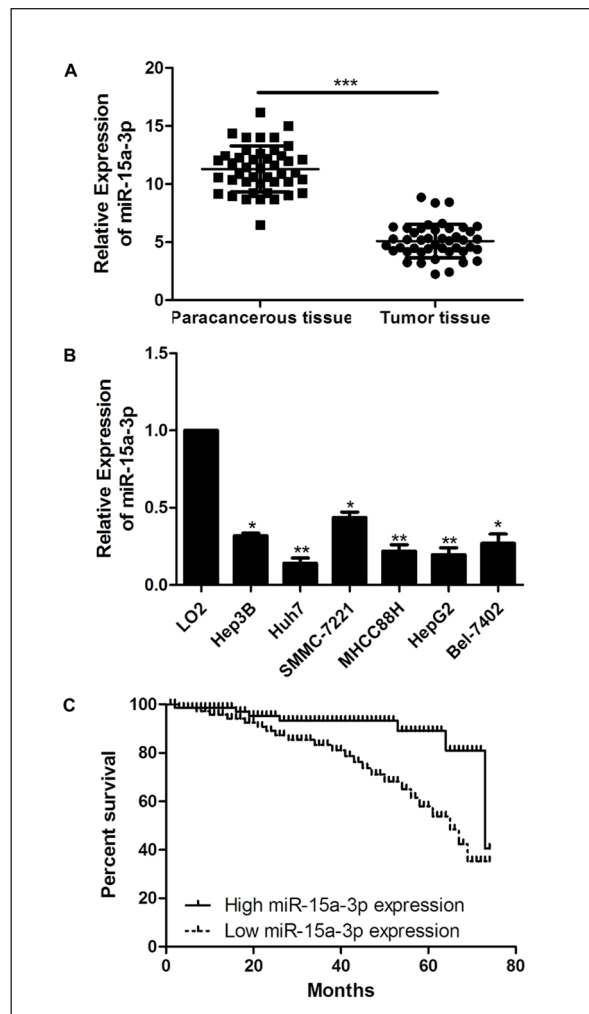


Figure 1. Downregulated miRNA-15a-3p in HCC samples. **A**, MiRNA-15a-3p levels in HCC tissues and paracancerous tissues. **B**, MiRNA-15a-3p levels in HCC cell lines. **C**, Overall survival in HCC patients based on miRNA-15a-3p level. Data were expressed as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

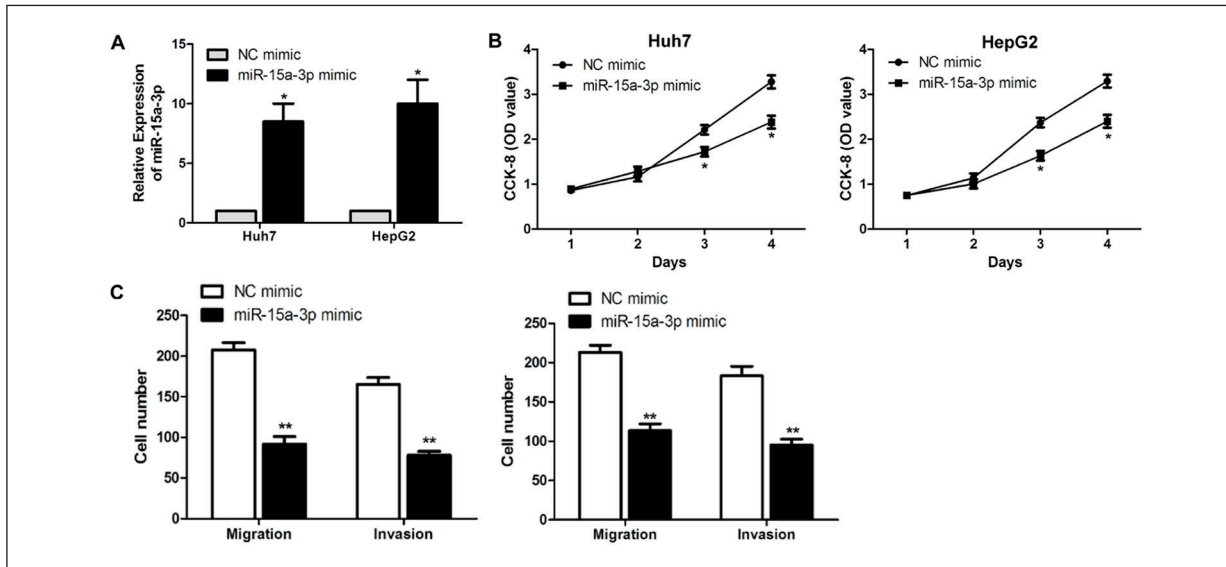


Figure 2. Overexpression of miRNA-15a-3p inhibited proliferative and metastatic potentials in HCC. **A**, Transfection efficacy of miRNA-15a-3p mimic in Huh7 and HepG2 cells. **B**, Viability in Huh7 and HepG2 cells transfected with NC mimic or miRNA-15a-3p mimic. **C**, Migration and invasion in Huh7 and HepG2 cells transfected with NC mimic or miRNA-15a-3p mimic (magnification: 40×). Data were expressed as mean±SD. * $p < 0.05$, ** $p < 0.01$.

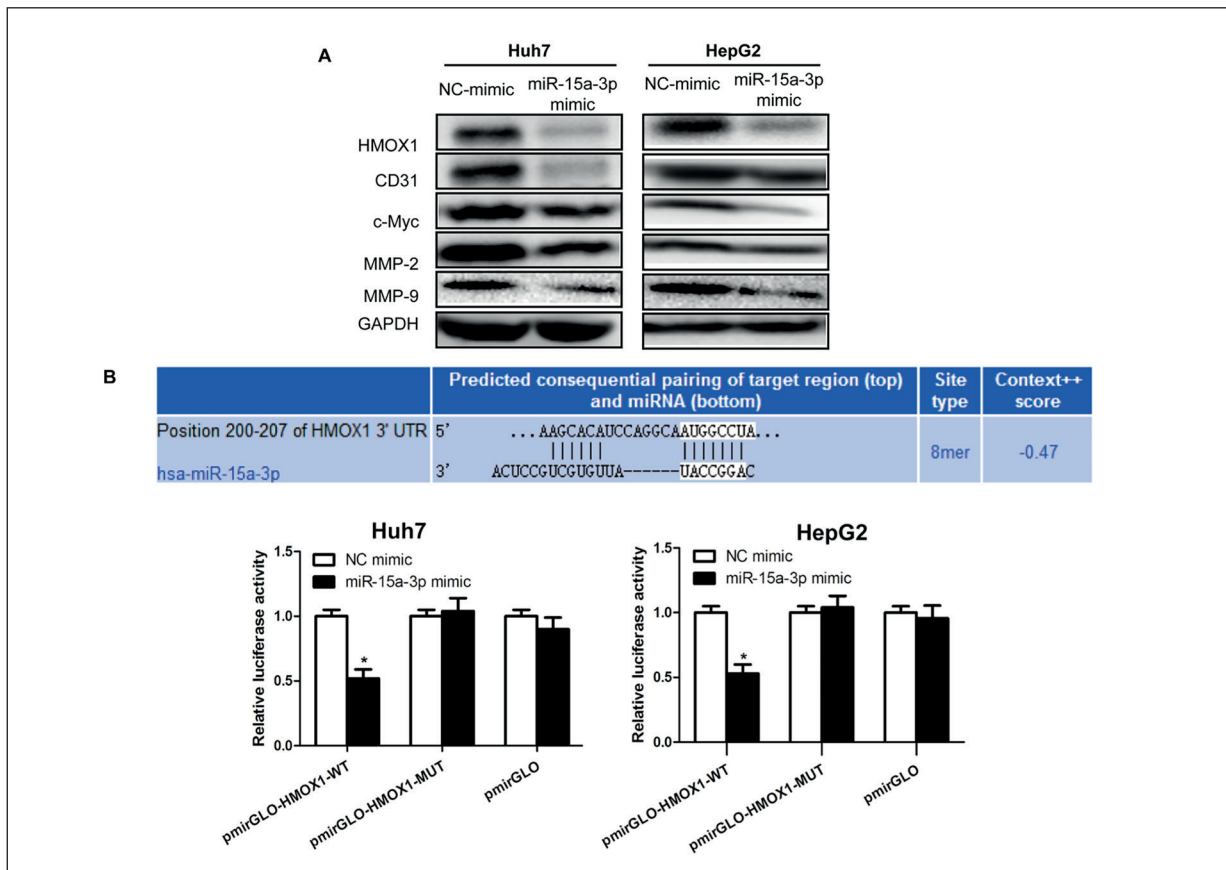


Figure 3. Overexpression of miRNA-15a-3p downregulated HMOX1 in HCC. **A**, Protein levels of HMOX1, CD31, c-Myc, MMP-2 and MMP-9 in Huh7 and HepG2 cells transfected with NC mimic or miRNA-15a-3p mimic. **B**, Luciferase activity in co-transfected Huh7 and HepG2 cells. Data were expressed as mean±SD. * $p < 0.05$.

3B). Hence, it is confirmed that HMOX1 was the downstream target of miRNA-15a-3p, which was negatively regulated by it.

HMOX1 Was Involved in HCC Development Regulated by MiRNA-15a-3p

Contrary to miRNA-15a-3p, HMOX1 was up-regulated in HCC tissues (Figure 4A). Highly expressed HMOX1 was an unfavorable factor for prognosis in HCC patients (Figure 4B). Notably, lower miRNA-15a-3p level was found in HCC cells co-overexpressing miRNA-15a-3p and HMOX1 than those solely overexpressing miR-

NA-15a-3p (Figure 4C). HMOX1 was downregulated in HCC cells overexpressing miRNA-15a-3p (Figure 4D). The inhibitory effect of miRNA-15a-3p on invasive potential in HCC was abolished by co-overexpression of miRNA-15a-3p and HMOX1 (Figure 4E).

Discussion

HCC cases account for more than 90% of primary liver cancer cases. Each year, over 600,000 people die of HCC globally. China is the typical high-risk region of HCC and 50%

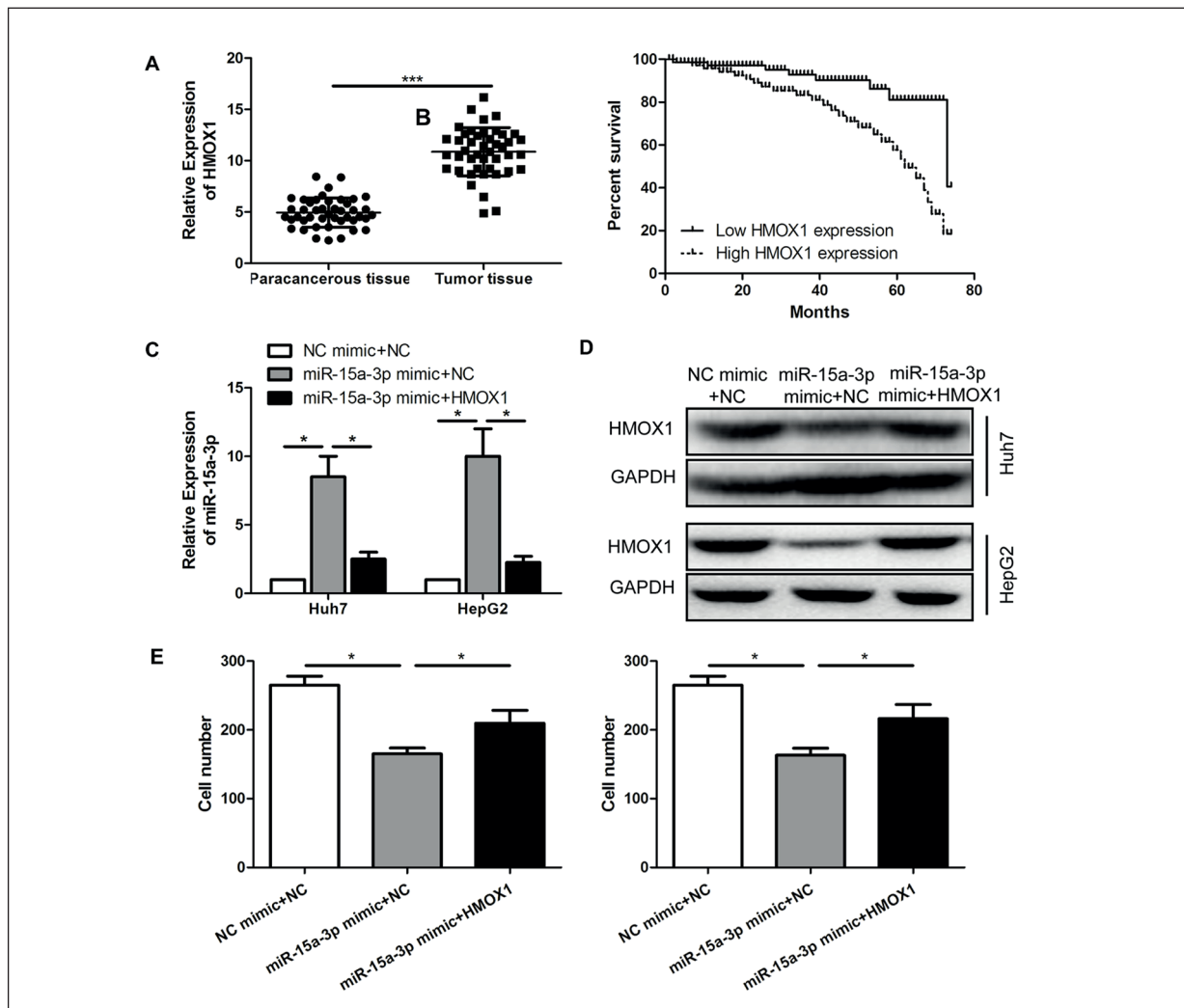


Figure 4. HMOX1 was involved in HCC development regulated by miRNA-15a-3p. **A**, HMOX1 levels in HCC tissues and paracancerous tissues. **B**, Overall survival in HCC patients based on HMOX1 level. **C**, MiRNA-15a-3p level in Huh7 and HepG2 cells influenced by both miRNA-15a-3p and HMOX1. **D**, Protein level of HMOX1 in Huh7 and HepG2 cells influenced by both miRNA-15a-3p and HMOX1. **E**, Invasion in Huh7 and HepG2 cells influenced by both miRNA-15a-3p and HMOX1 (magnification: 40×). Data were expressed as mean±SD. ***p* < 0.01, ****p* < 0.001.

HCC cases occur in our country¹⁻⁴. A great number of HCC patients lose the optimal surgical opportunity because of the insidious onset and malignant infiltration. Timely diagnosis and active treatment of HCC can significantly prolong the survival⁵⁻⁷. Imaging examination and tumor marker detection are the commonly used methods to screen and diagnose HCC. The latter method is of significance in early diagnosis, determination of prognosis, and recurrence monitoring of cancer⁸⁻¹⁰. Serum level of AFP is the most common tumor marker of HCC. However, false positive rate and relatively low sensitivity and specificity of AFP restrict its application in diagnosing HCC^{7,8}. It is important to explore an early non-invasive diagnostic technique and specific treatment based on the gene level for HCC.

Pri-miRNAs in the nuclei are transported to the cytoplasm after a series of complicated processing, and thus mature miRNAs are produced¹³⁻¹⁵. By recognizing and binding target genes, miRNAs exert vital regulatory effects on disease progression^{16,17}. Abnormally expressed miRNAs in the body are involved in malignant formation of cells, tumor microenvironment adaption, acclimation of tumor-associated immune cells, and tumor stem cell behaviors^{11,12,18}. They are promising diagnostic and prognostic indicators, and treatment targets of cancer diseases. Previous studies^{19,20} have reported the inhibitory effects of miRNA-15a-3p on lung cancer and prostate cancer. Our findings showed that miRNA-15a-3p was downregulated in HCC samples. MiRNA-15a-3p level was closely linked to metastasis and overall survival in HCC patients. Overexpression of miRNA-15a-3p inhibited proliferative, migratory, and invasive potentials in HCC cells. In addition, miRNA-15a-3p was able to downregulate HMOX1, CD31, c-Myc, MMP-2, and MMP-9 in HCC.

MiRNAs interact with target genes, thus forming a complicated network¹⁵⁻¹⁷. Bioinformatics analysis showed the binding sequences in the 3'UTR of miRNA-15a-3p and HMOX1. Furthermore, Dual-Luciferase reporter assay confirmed that HMOX1 was the target gene of miRNA-15a-3p. HMOX1 was highly expressed in HCC samples, which was negatively regulated by miRNA-15a-3p. Moreover, the overexpression of HMOX1 could abolish the inhibitory effects of miRNA-15a-3p on HCC cell phenotypes. The novelty of this study was that we firstly reported the anticancer role of miRNA-15a-3p in HCC and un-

cover the possible mechanism in the development of HCC, which could provide a new therapeutic target for HCC.

Conclusions

To sum up, this study showed that miRNA-15a-3p is closely linked to lymphatic metastasis, distant metastasis, and poor prognosis in HCC. It inhibits the malignant development of HCC by interacting with HMOX1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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