

Mitotically-associated long non-coding RNA promotes cancer cell proliferation in hepatocellular carcinoma by downregulating miR-122a

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Abstract. Mitotically-associated long non-coding RNA (MANCR) is involved in malignant breast cancer. The present study analyzed the role of MANCR in hepatocellular carcinoma (HCC). MANCR was found to be upregulated in HCC and high expression level of MANCR in cancer tissues predicted poor survival of HCC patients. MicroRNA (miR)-122a was downregulated HCC and was inversely correlated with MANCR only in cancer tissues. MANCR overexpression resulted in downregulation of miR-122a, while miR-122a overexpression showed no obvious effects on MANCR expression. MANCR overexpression showed no significant effects on HCC cell proliferation but led to promoted cell migration and invasion. miR-122a overexpression led to inhibited migration and invasion of HCC cells and attenuated the effects of MANCR overexpression. Therefore, lncRNA MANCR may promote cancer cell proliferation in HCC by downregulating miR-122a.

Introduction

Incidence of hepatocellular carcinoma (HCC) ranks 5th place among all malignancies worldwide (1). Due to the high rate

of extrahepatic and intrahepatic metastases by the time of initial diagnosis, prognosis of HCC patients is usually poor and the recurrence rate is high, making HCC a major cause of cancer-related deaths (2). Therefore, how to prevent and treat cancer metastasis is still a major task in the treatment of cancers, such as HCC (3). Genetic factors are the major players in HCC and a large number of tumor suppressors and oncogenes are involved (4,5). However, the molecular mechanism of the pathogenesis of HCC remains unclear, leading to failures in clinical treatment and prevention.

Long (>200 nt) non-coding RNAs (lncRNAs) have no or limited potential for protein coding (6). LncRNAs play their roles through the regulation of downstream genes (6). Aberrant expression of lncRNAs in human body may lead to dysregulated expression of certain genes involved in human diseases (7), such as oncogenes and tumor suppressors in cancers (8,9). In human cancers including HCC, lncRNAs regulate downregulate tumor suppressors and oncogenes to promote or inhibit cancer development (8,9). Therefore, regulation of the expression of key lncRNA regulators may contribute to the recovery of cancer patients (10). It has been reported that novel mitotically-associated long non-coding RNA (MANCR) is involved in malignant breast cancer (11). In breast cancer, MANCR affects cell division and genomic stability to promote the progression of cancer. However, the involvement of MANCR in HCC is unknown. MicroRNA (miR)-122a is a key player in a number of types of diseases including HCC (12-16). miR-122a is downregulated in HCC and predicted poor survival (16). An inverse correlation between MANCR and miR-122a was observed from the transcriptome analysis data (data not shown). Therefore, miR-122a may have interactions with MANCR in HCC. The present study therefore investigated the involvement of MANCR in HCC and explored its interactions with miR-122a.

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Research subjects. This is a prospective study. From January 2010 to April 2013, the present study included 68 patients (39 males and 29 females, aged 38 to 69 years, mean age 48.4±5.7 years) who were diagnosed as HCC through

histopathological examinations in the Central Hospital of Wuhan. All the patients were enrolled according to strict inclusion and exclusion criteria. Inclusion criteria: i) First diagnosis; ii) no abnormal function of other organs observed. Exclusion criteria: i) Other clinical conditions other than HCC were observed; ii) any therapies received within 3 months after admission. Based on the staging criteria proposed by AJCC (17), there were 16, 14, 20 and 18 cases at stage I, II, III and IV, respectively. Among these patients, 39 were combined with liver fibrosis. All patients had signed informed consent before the study and the Ethics Committee of the Central Hospital of Wuhan had approved this study before admission.

Follow-up. All patients were followed up for 5 years after admission. Follow-up was performed either by outpatient visit or phone call once per 1-2 months. Patients who were lost during follow-up, who died of other diseases or accidents were excluded.

Specimen collection and cell lines. Liver biopsy was performed to collect HCC tissues and non-HCC tissues (within 2 cm around tumors) were collected from each patient. All specimens were confirmed by at least 3 experiences pathologists.

SNU-398 and SNU-182 human HCC cell lines (American Type Culture Collection) were used. The culture medium was RPMI 1640 medium (Sigma-Aldrich; Merck KGaA) with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA). Culture conditions were 5% CO₂ and 37°C.

RNA interaction prediction. The interaction between miR-122a and MANCR was predicted by using IntaRNA (version 2.0; <http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp>). The long sequence was MANCR and the short sequence was miR-122a. All other parameters were default.

RNA extractions and reverse transcription-quantitative (RT-qPCR). Expression of MANCR was detected according to the following steps: TRIzol reagent (Thermo Fisher Scientific, Inc.) was used to extract total RNAs from tissue specimens and *in vitro* cultivated cells, following by reverse transcription performed using Expand™ Reverse Transcriptase (Sigma-Aldrich; Merck KGaA) to synthesize cDNA. The reaction conditions were: 25°C for 5 min, 55°C for 20 min and 80°C for 10 min. After that, PCR SYBR® Green Quantitative RT-qPCR kit (Sigma-Aldrich; Merck KGaA) was used to prepare PCR reaction systems with cDNA as template and 18s rRNA as endogenous control. The reaction conditions were: 95°C for 1 min, followed by 40 cycles of 95°C for 10 sec and 55°C for 20 sec, and 72°C for 30 sec. Expression of miR-122a was detected through following steps: mirVana miRNA Isolation kit (Thermo Fisher Scientific) was used to extract miRNAs, following by reverse transcription performed using TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific., Inc.). The reaction conditions were: 25°C for 5 min, 52°C for 20 min and 80°C for 10 min. After that, Applied Biosystems™ TaqMan™ MicroRNA Assay (Thermo Fisher Scientific) was used to prepare PCR reaction systems with U6 as endogenous control. The primer sequences were: MANCR forward, 5'-CAATACCACAATTGCAATC-3'; MANCR reverse, 5'-CATGTTCTTCCTCATATGGA-3';

18S rRNA forward, 5'-GTAACCGTTGAACCCCATT-3'; 18S rRNA reverse, 5'-CCATCCAATCGGTAGTAGCG-3'; miR-122 forward, 5'-GCTCGACCTCTCTATGGGC-3'; and miR-122 reverse primer of and U6 primers were included in the kit. The 2^{-ΔΔCq} method (18) was used to perform all data normalizations.

Transient transfection. Scrambled negative control miRNA (5'-UCAGCUUGCGAGCAGUCGAAA-3') and MISSION® microRNA Mimic hsa-miR-122a (5'-UGGAGUGUGACA AUGGUGUUUG-3') were purchased from Sigma-Aldrich (Merck KGaA). MANCR-expression pcDNA3.1 vectors and empty vectors were provided by Sangon Biotech Co., Ltd. Transient transfections were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Doses of vectors and miRNAs were 10 and 40 nM, respectively. Two controls were included in this experiment. They are control (non-transfection) and negative control (empty vector or control miRNA transfection). Cells were harvested at 24 h after transfection to perform subsequent experiments.

Transwell migration and invasion assay. Cells were harvested at 24 h after transfection to prepare single cell suspensions (4x10⁴ cells/ml) using non-serum RPMI 1640 medium. The upper Transwell chamber was coated one day before invasion assay with Matrigel (cat. no. 356234; 300 µg/ml; EMD Millipore). Cell suspensions were added into the upper chamber, while RPMI 1640 medium (20% FBS) was added into the lower chamber. The plate was incubated for 2 h, followed by upper chamber membrane staining at room temperature with 0.5% crystal violet (Sigma-Aldrich, Merck KGaA) for 1.5 h. Invading and migrating cells were observed and counted under a light microscope at x40 magnification.

Statistical analysis. The mean values of 3 biological replicates were calculated. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc.) Paired t-test was used for comparisons between HCC and non-HCC tissues. Analysis of variance (one-way) and Tukey's test were used for multi-group comparisons. Linear regression was performed to analyze the correlations between MANCR and miR-122a. Based on Youden's index (19) and expression levels of MANCR in HCC tissues, patients were divided into high (n=31) and low (n=37) groups. Survival curves were plotted and compared using the Kaplan-Meier (K-M) method and log rank t test, respectively. A χ² test was used to analyze the correlations between patients' clinical data. P<0.05 was considered to indicate a statistically significant difference.

Results

MANCR is upregulated in HCC and predicted survival. RT-qPCR experiments were performed to investigate the differential expression of MANCR in HCC and non-HCC tissues. Comparison between HCC tissues and non-HCC tissues using a paired t-test showed that MANCR was significantly upregulated in HCC tissues compared with non-HCC (P<0.05; Fig. 1A). However, no significant differences in the expression levels of MANCR in HCC tissues were found among patients with different clinical stages (Fig. 1B). Based on Youden's index and

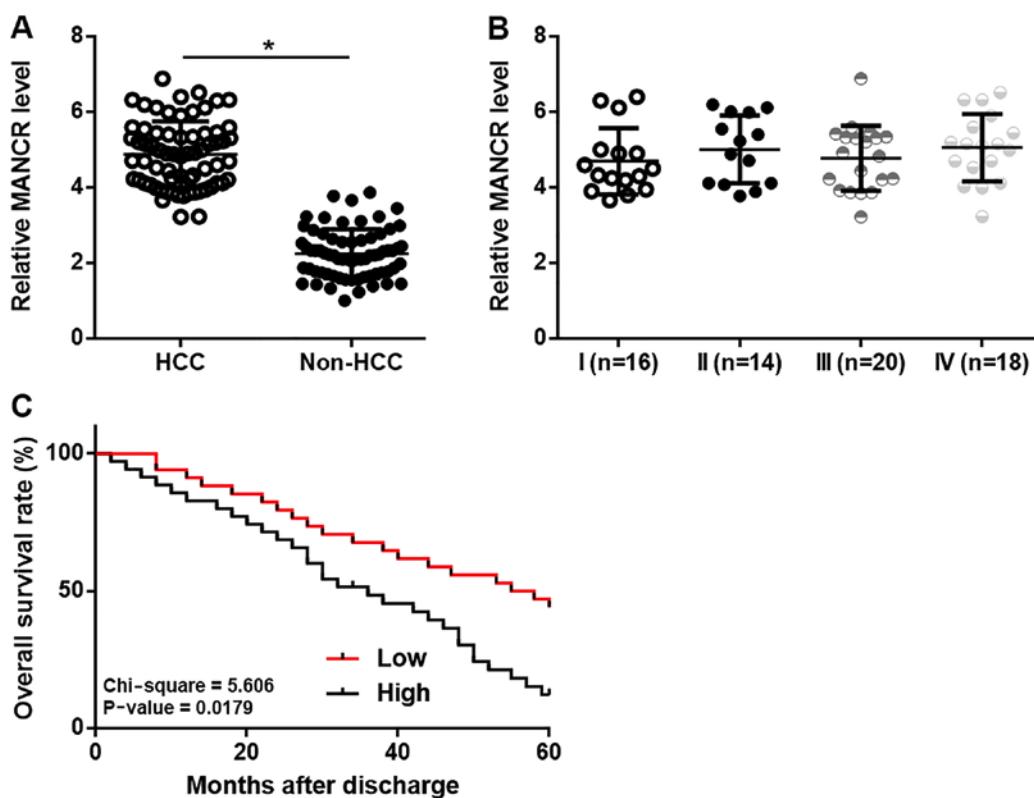


Figure 1. MANCR is upregulated in HCC and predicts survival. (A) Reverse transcription-quantitative PCR showed that MANCR was significantly upregulated in HCC tissues compared with non-HCC, while expression of (B) MANCR in HCC tissues was not significantly affected by clinical stages. (C) Survival curve analysis showed that overall survival rate of high MANCR level group was significantly decreased compared with low MANCR level group, $^*P<0.05$. MANCR, mitotically-associated long non-coding RNA; HCC, hepatocellular carcinoma.

expression levels of MANCR in HCC tissues, patients were divided into high ($n=31$) and low ($n=37$) MANCR level groups. Survival curves were plotted and compared using K-M method and log rank t test, respectively. It was observed that the overall survival rate of high MANCR level group was significantly decreased compared with low MANCR level group (Fig. 1C). The χ^2 test showed that MANCR is not associated with liver fibrosis, gender and age ($P>0.05$).

MiR-122a is inversely correlated with MANCR in HCC. RT-qPCR experiments were also performed to investigate the differential expression of miR-122a in HCC and non-HCC tissues. Comparison between HCC tissues and non-HCC tissues using a paired t test showed that miR-122a was significantly downregulated in HCC tissues compared with non-HCC ($P<0.05$; Fig. 2A). Linear regression was performed to analyze the correlations between MANCR and miR-122a. It was observed that miR-122a was inversely and significantly correlated with MANCR in HCC tissues ($P<0.01$; Fig. 2B), but not in non-HCC tissues (Fig. 2C).

MANCR overexpression leads to downregulation of miR-122a in HCC cells. To further investigate the interaction between MANCR and miR-122a in HCC, MANCR-expression vectors and miR-122a mimics were transfected into cells of both SNU-398 and SNU-182 cell lines, followed by the detection of MANCR and miR-122a expression by RT-qPCR. Comparing with the negative control (NC) and control (C) groups, MANCR and miR-122a were significantly overexpressed

in cells of both cell lines at 24 h after transfection ($P<0.05$; Fig. 3A). In addition, MANCR overexpression in cells of these 2 cell lines resulted in the significant downregulation of miR-122a ($P<0.05$; Fig. 3B), while miR-122a overexpression in cells of these two cell lines showed no obvious effects on MANCR expression (Fig. 3C).

MANCR overexpression promotes HCC cell migration and invasion through miR-122a. Transwell migration and invasion assays showed that, compared with the NC and C groups, MANCR overexpression showed no significant effects on HCC cell proliferation (data not shown), but led to significantly promoted cell migration ($P<0.05$; Fig. 4A) and invasion ($P<0.05$; Fig. 4B). miR-122a overexpression led to significantly inhibited migration ($P<0.05$; Fig. 4A) and invasion ($P<0.05$; Fig. 4B) of HCC cells and attenuated the effects of MANCR overexpression.

Discussion

It has been reported that MANCR participates in breast cancer (11). The present study first proved that MANCR may also be involved in HCC. The current study also showed that MANCR was upregulated in HCC and MANCR overexpression may promote HCC cell migration and invasion by downregulating miR-122a.

The function of miR-122a has been reported in several human diseases (12-15). It has been shown that miR-122a is likely involved in Warburg-like effects of hepatocyte deficient in mice (12). In another study, miR-122a was proved to

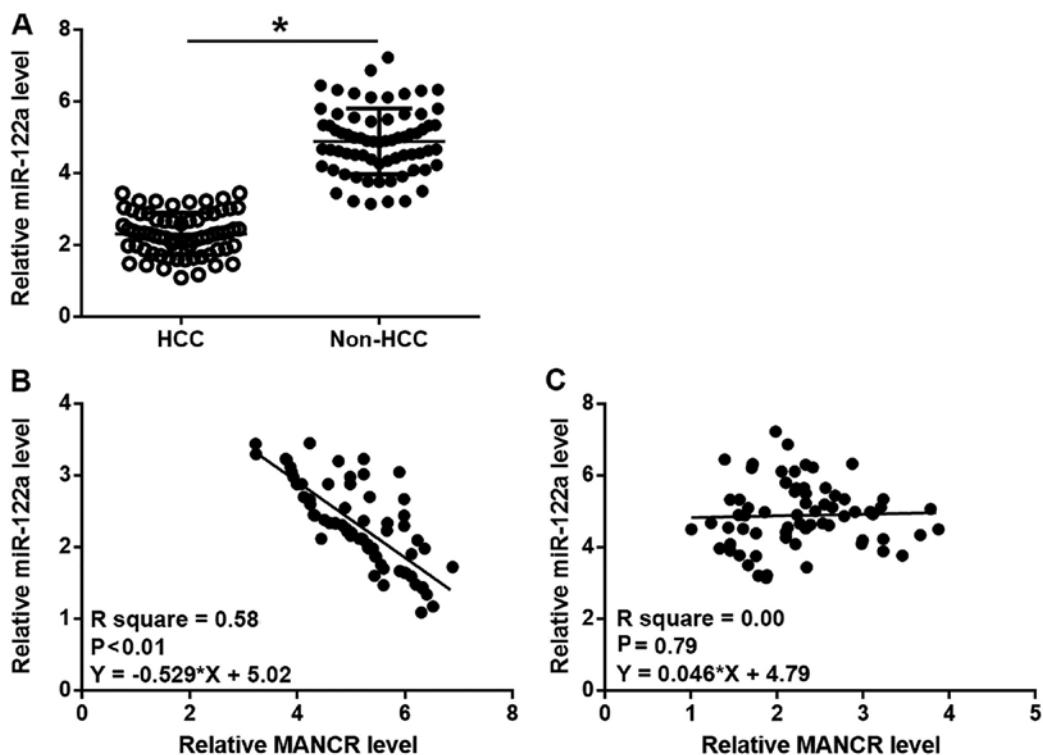


Figure 2. miR-122a is inversely correlated with MANCR in HCC. (A) Reverse transcription-quantitative PCR showed that miR-122a was significantly downregulated in HCC tissues than in non-HCC, $*P<0.05$. (B) Linear regression showed that miR-122a was inversely and significantly correlated with MANCR in HCC tissues, but not in (C) non-HCC tissues. Mitotically-associated long non-coding RNA; HCC, hepatocellular carcinoma; miR, microRNA.

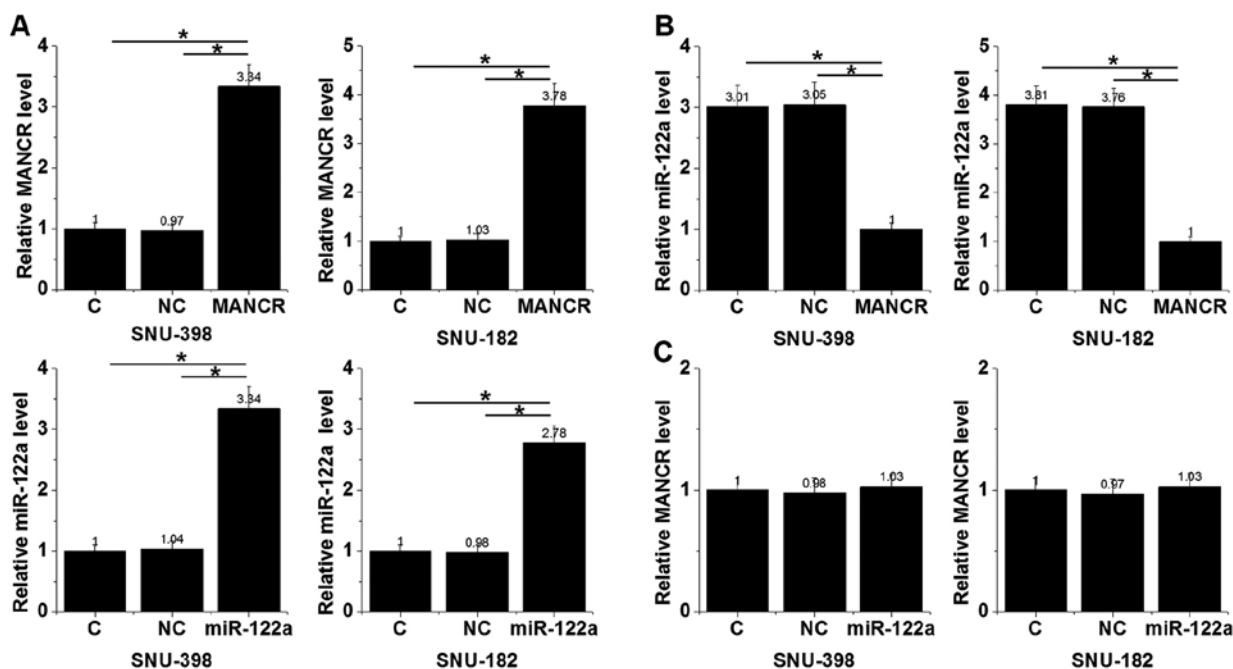


Figure 3. MANCR overexpression led to downregulation of miR-122a in HCC cells. (A) Compared with NC and C groups, MANCR and miR-122a were overexpressed in cells of both SNU-398 and SNU-182 cell lines at 24 h after transfection. (B) In addition, MANCR overexpression resulted in the downregulation of miR-122a, while (C) miR-122a overexpression showed no obvious effects on MANCR expression, $*P<0.05$. Mitotically-associated long non-coding RNA; HCC, hepatocellular carcinoma; miR, microRNA; NC, negative control; C, control.

participate in hepatitis C virus infection (13), which is related to a number of liver diseases (14), such as HCC (15). The present study showed that miR-122a was downregulated in HCC. The results of the current study are consistent with the

report of Zhang *et al* (16), which reported the downregulation of miR-122 in HCC and its prognostic values for HCC. It was also found that miR-122a overexpression led to the inhibited migration and invasion of HCC cells. Therefore, miR-122a

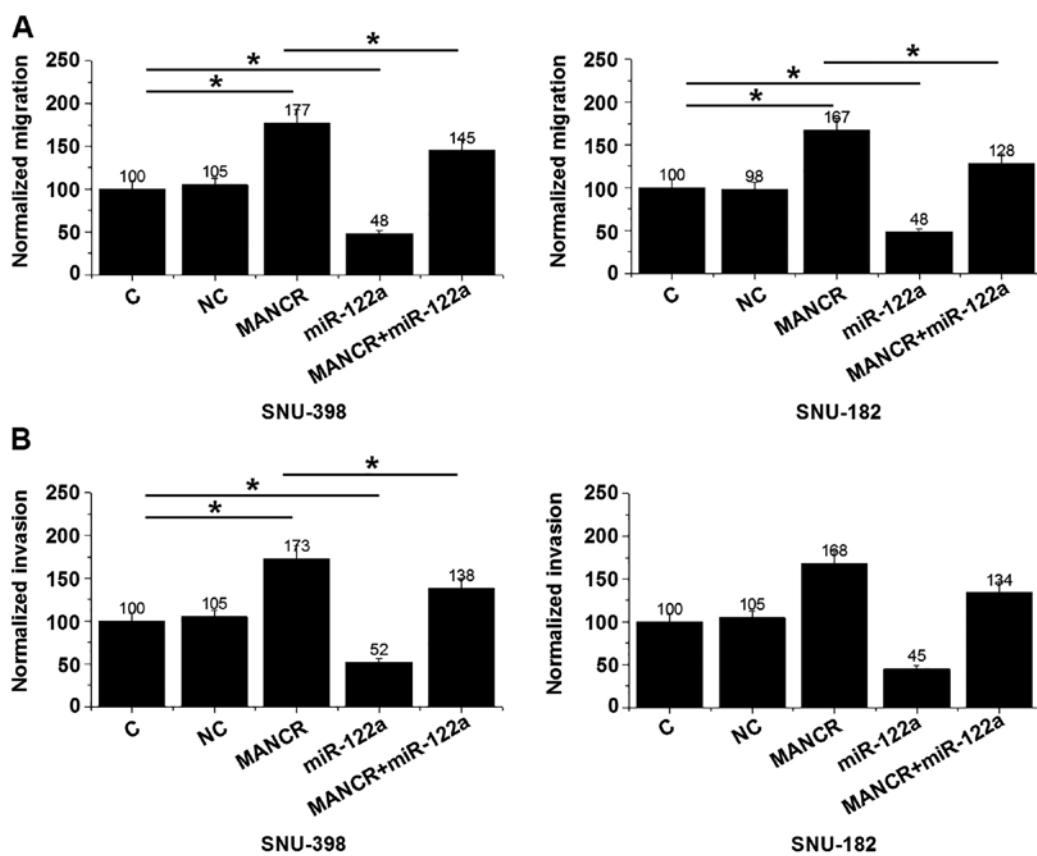


Figure 4. MANCR overexpression promoted HCC cell migration and invasion through miR-122a. MANCR overexpression showed no significant effects on HCC (A) cell migration and (B) invasion, but led to promoted cell proliferation. miR-122a overexpression led to inhibited proliferation of HCC cells and attenuated the effects of MANCR overexpression * $P<0.05$. Mitotically-associated long non-coding RNA; HCC, hepatocellular carcinoma; miR, microRNA; NC, negative control; C, control.

may play an oncogenic role in HCC. MANCR participates in breast cancer by affecting cell division and genomic stability (11), which are involved in all types of cancer (20), indicating the involvement of MANCR in other types of cancer, such as HCC. Interestingly, the present study showed that MANCR was upregulated in HCC tissues and overexpression of MANCR led to promoted migration and invasion of HCC cells, but showed no significant effects on cancer cell proliferation. Therefore, MANCR may play different roles in different types of cancer.

LncRNAs play their roles through the regulation of downstream genes at posttranscriptional and translational levels or through epigenetic pathways (6). Previous studies showed that lncRNAs may serve as miRNA sponges to downregulated tumor suppression and oncogenic miRNAs in cancer biology (21,22). The present study showed that MANCR can downregulate miR-122a in HCC cells. However, no promising target of miR-122a was found on the MANCR sequence. Therefore, lncRNAs may regulate the expression of miRNAs through other mechanisms. More studies are still needed.

In conclusion, MANCR was upregulated and miR-122a was downregulated in HCC. MANCR may downregulate miR-122a to participate in HCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ and HL were responsible for the experiments, analysis of the data, and the manuscript writing. JZ and SY performed the laboratory work and literature research. MM and XW performed the project management, research design and literature research. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of the Central Hospital of Wuhan. The study followed the tenets of the Declaration of Helsinki and informed written consent was obtained from all patients of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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