MiR-24-3p enhances cell growth in hepatocellular carcinoma by targeting metallothionein 1M

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Dysregulation of microRNAs has been demonstrated to contribute to malignant progression of cancers, including hepatocellular carcinoma (HCC). MiR-24-3p was previously reported to be significantly upregulated in HCC. However, the potential role and mechanism of action of miR-24-3p in the initiation and progression of HCC remain largely unknown. Quantitative reverse transcription polymerase chain reaction demonstrated that miR-24-3p was significantly upregulated in HCC tumor tissues compared with nontumor tissues. The cell viability, colony formation assay, and tumorigenicity assays in nude mice showed that miR-24-3p could enhance HCC cell growth in vitro and in vivo. Metallothionein 1M (MT1M) was verified as a miR-24-3p target gene by using dual-luciferase reporter assays, quantitative reverse transcription polymerase chain reaction, and Western blotting, which was involved in miR-24-3p regulated HCC cell growth. These results indicated that miR-24-3p plays an important role in the initiation and progression of HCC by targeting metallothionein 1M, and the miR-24-3p/metallothionein 1M pathway may contribute to the development of novel therapeutic strategies for HCC in the future.

KEYWORDS
cell growth, hepatocellular carcinoma, miR-24-3p, MT1M

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is a common tumor worldwide and the leading cause of cancer-related death.1 HCC tumorigenesis is a multifactorial and multistep process that involves activating oncogenes and inactivating tumor suppressor genes in different stages of HCC progression.2 Clarifying and investigating the roles of the genes involved in HCC development will contribute to our understanding of the mechanisms of tumorigenesis and application of efficient new targets for the early diagnosis and effective treatment of HCC.3

MicroRNAs (miRNAs) are endogenous noncoding 20 to 22 nucleotide RNAs that have been identified as post-transcriptional regulators of gene expression.4 The miRNAs mainly bind to the 3′ untranslated regions (UTRs) of target mRNAs, resulting in mRNA degradation or the blockade of mRNA translation. Increasing evidence showed that miRNAs have significant roles in diverse biological processes.5 Meanwhile, deregulation of miRNAs has been observed in a wide range of human diseases, including cancer.6 In human cancer, miRNAs can function as oncogenes or tumor suppressor genes during tumor development and progression.7

In this study, miR-24-3p was found frequently upregulated miRNAs in human HCC compared with adjacent nontumor tissues. Further investigation revealed that miR-24-3p enhances cell growth in HCC cell lines. Moreover, metallothionein 1M (MT1M) was characterized as a direct target of miR-24-3p.

2 | MATERIALS AND METHODS

2.1 | Tissue specimens and cell lines

Human HCC tumor tissues and adjacent nontumor tissues were obtained from patients undergoing primary surgical treatment of HCC in the Cancer Affiliated Hospital of Xinjiang Medical University, China. No patient had received preoperative irradiation or chemotherapy. The samples were frozen in liquid nitrogen and stored at −80°C until use. Written consent for tissue donation was obtained from the patients before tissue collection, and the protocol was approved by the Institutional Review Board of Xinjiang Medical University. The profiles of 58 patients with HCC are shown in Table 1.
HEK293T and human HCC cells lines HepG2 and Huh7 were maintained in Dulbecco’s modified eagle’s medium (HyClone, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 100 U of penicillin/ml, 100 mg of streptomycin/ml, and 10% fetal bovine serum (Gibco) at 37°C under a humidified atmosphere of 5% CO2.

2.2 | Quantitative real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed into cDNA by TaqMan microRNA assay kits (Applied Biosystems) according to the manufacturer’s protocol. The cDNA product was used for the following quantitative reverse transcription polymerase chain reaction (PCR) analysis. The PCR reaction was incubated at 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. PCR data were analyzed by SDS v2.3 software by using the 2−ΔΔCT method and presented as the fold-expression change in tumors compared with the nontumor tissues after normalization to the endogenous control.

2.3 | Transfection

The transfection was carried out by using FuGene HD transfection reagent (Roche, Indianapolis, IN, USA) following the manufacturer’s protocol. In brief, 5 × 10⁴ HEK293T cells in 24-well plate were transfected with indicated plasmid DNA or miRNA duplex (GenePharma, Shanghai, China) and collected 48 hours after transfection for assay.

2.4 | The construction of luciferase reporter plasmids and dual-luciferase reporter assay

The 3′-UTR of MT1M (Genbank accession AF348671.1) containing the putative miR-24-3p binding sequences (72-79 nt) was amplified with the primers 5′-TGTGGGAACAGCTCTTCTCC-3′ (forward) and 5′-AAGATTCAGCTCAAAATTGT-3′ (reverse). The PCR product was cloned into luciferase reporter vector pGL3 (Promega Corporation, Madison, WI, USA), termed as pGL3-MT1M-3′UTR. The plasmid of mutated seed region was generated by MutanBEST Kit (Takara Bio Inc., Shiga, JP) based on pGL3-MT1M-3′UTR plasmid, termed as pGL3-MT1M-3′UTR-mut. The plasmids were confirmed by sequencing analyses.

HEK293T cells seeded in 24-well plate in triplicate were cotransfected with pGL3-MT1M-3′UTR or pGL3-MT1M-3′UTR-mut and miR-24-3p mimic or nonrelative control RNA duplex (nucleocapsid [NC] duplex) by using FuGene HD transfection reagent. The pRL-TK (Promega Corporation, Madison, WI, USA) was also transfected as a normalization control. Cells were collected 48 hours after transfection, and luciferase activity was measured by using a dual-luciferase reporter assay kit (Promega Corporation) and recorded by multi-plate reader (Synergy 2, BioTek, Winooski, VT, USA).

2.5 | Cell viability and colony formation assay

Twenty four hours after transfection, 1000 transfected HepG2 and Huh7 cells were placed in a fresh 96-well plate in triplicate for 7 days. The cells were tested for growth per 24 hours by using Cell Titer-Blue cell viability assay (Promega Corporation, Madison, WI, USA), and the fluorescence value was recorded by multi-plate reader (Synergy 2, BioTek).

Twenty four hours after transfection, 2000 transfected HepG2 and Huh7 cells were placed in a fresh 6-well plate in triplicate for 2 weeks. Cell colonies were fixed with 20% methanol at −20°C for 20 minutes, then stained with 0.1% coomassie brilliant blue R250 at room temperature for 15 minutes. The colonies were counted by ELSpot Bioreader 5000 (BIO-SYS, Karben, GE).

2.6 | Tumorigenicity assays in nude mice

Male BALB/c nude mice (5-6 weeks of age) were obtained from Shanghai Experimental Animal Center (Shanghai, China). Animal experiment procedures were approved by the Animal Experiments Ethics Committee of Xinjiang Medical University. MiR-24-3p mimic or miR-24-3p inhibitor transfected HepG2 cells (1 × 10⁶) were suspended in PBS and then injected s.c. into the left side of the posterior flank of 6 BALB/c nude mice, respectively. NC transfected or nontransfected HepG2 cells were injected subcutaneously into the right side of the same 12 mice. Tumor volumes were calculated every week by using the formula for hemi-ellipsoids: V = length (cm) × width (cm) × height (cm) × 0.5236. After 5 weeks, the mice were sacrificed and the tumors were dissected and photographed.

2.7 | Western blot

Protein extracts were prepared from HCC tissues or cells by using a modified radioimmunoprecipitation assay buffer containing 1 mM of phenylmethylsulfonyl fluoride and a cocktail of protease and phosphatase inhibitors (Complete mini, Roche). The protein quantification was determined by BCA Protein Assay Kit (Thermo Scientific), and equal amounts of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gels). After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Immobilon P SQ, Millipore, Billerica, MA, USA). After blocking with 5% (w/v) nonfat dried skimmed milk (in phosphate buffered saline) for 30 minutes at room temperature, the membranes were

<table>
<thead>
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<th>Variable (n = 58)</th>
<th>Value</th>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Gender (male)</td>
<td>42 (72.4%)</td>
</tr>
<tr>
<td>Tumor size*(cm)</td>
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<td>Tumor grade</td>
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</tr>
<tr>
<td>Poorly differentiated (G4)</td>
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<td>TNM stage(b)</td>
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<tr>
<td>I</td>
<td>10</td>
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<td>II</td>
<td>28</td>
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<td>III</td>
<td>20</td>
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<td>IV</td>
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* Diameter of the biggest nodule.

bTNM, tumor-node-metastasis.
incubated with rabbit anti-MT1M antibody (1:1000 dilution, Epitomics, Inc., Burlingame, CA, USA) or mouse anti-glyceraldehyde 3-phosphate dehydrogenase antibody (1:5000 dilution, Epitomics, Inc.) at 4°C overnight. After 3 washes with phosphate buffered saline, the membranes were incubated with horseradish peroxidase conjugated secondary antibodies (Pierce) for 1 hour at room temperature. Finally, protein bands were developed by using Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA), visualized by the Gene Gnome HR Image Capture System (Syngene, Frederick, MD, USA), and analyzed by Gene tools software.

2.8 | Prediction of miRNAs targets

Three programs were used to predict the regulating miRNAs of MT1M, including the TargetScan (http://www.targetscan.org/), MiRanda (http://www.microrna.org/microrna/home.do), and PicTar (http://pictar.mdc-berlin.de/).

2.9 | Statistical analysis

Data are presented as mean ± SD. Comparisons were made by using a 2-tailed t-test or one-way ANOVA for experiments with more than 2 subgroups. Correlation analysis was made by using Spearman correlation coefficient. P < .01 was considered statistically significant.

3 | RESULTS

3.1 | MiR-24-3p correlates negatively with MT1M

To investigate the role of miR-24-3p in HCC tumorigenesis, firstly, the expression of miR-24-3p was measured in 58 HCC tumor tissues and adjacent nontumor tissues by quantitative reverse transcription PCR. MiR-24-3p showed significantly upregulated in HCC tissues compared with adjacent nontumor tissues (Figure 1A). MT1M was found to be downregulated in HCC tumor tissues compared with adjacent nontumor tissues (Figure 1B, upper panel). Moreover, a negative correlation was found between the downregulated MT1M protein and the upregulated miR-24-3p (r = -0.5918, P < .001; Figure 1B, lower panel).

3.2 | MiR-24-3p enhances cell growth

The downregulation of miR-24-3p in HCC tumor tissues compared with nontumor tissues indicated possible biological significance in HCC tumorigenesis. At first, the effect of miR-24-3p on cell growth was evaluated by transfection with, or not, miR-24-3p mimic or inhibitor in HepG2 and Huh7 cells. In HepG2 and Huh7 cells transfected with 20 nM of miR-24-3p mimic, the expression of miR-24-3p was increased 24- and 28-folds, respectively, but it was decreased 28- and 32-folds, respectively, in HepG2 and Huh7 cells transfected with 20 nM of miR-24-3p inhibitor (Figure 2A).

The viability of cells transfected with miR-24-3p mimic significantly increased, but the viability of cells with miR-24-3p inhibitor significantly decreased (Figure 2B) compared with that of NC duplex transfected or nontransfected cells, which indicated that miR-24-3p could enhance cell growth. To validate the effect of miR-24-3p on cell growth, the colony formation assay was performed. As showed in Figure 2C, HepG2 and Huh7 cells transfected with miR-24-3p mimic displayed much more and larger colonies (1238 or 1023 colonies), but cells transfected with miR-24-3p inhibitor displayed much fewer and smaller colonies (452 or 262 colonies), compared with the NC duplex transfected (783 or 682 colonies) or nontransfected control (736 or 664).

To confirm the above findings further, the tumorigenicity assay in vivo was used. During 5 weeks of miR-24-3p mimic or miR-24-3p inhibitor treatment, tumor volume curves revealed a significant increase in growth rates with miR-24-3p mimic treatment but a significant decrease in growth rates with miR-24-3p inhibitor treatment, whereas no significant differences in tumor growth rates were observed between the NC and control group (Figure 2D). These results indicated that introduction of miR-24-3p significantly enhances HCC tumorigene in xenograft nude mouse model.
3.3 | MT1M is a novel target of miR-24-3p

Metallothionein 1M was predicted as a potential target of miR-24-3p by TargetScan and miRanda but not PicTar. The 3′-UTR of MT1M mRNA contained a complementary site for the seed region of miR-24-3p (Figure 3A). In HEK293 cells cotransfected with the reporter plasmids and miR-24-3p mimic or NC duplex, the luciferase activity of the reporter that contained wild-type 3′-UTR was significantly suppressed by miR-24-3p mimic, but the luciferase activity of mutant reporter was unaffected (Figure 3B), indicating that miR-24-3p may suppress gene expression through miR-24-3p binding sequence at the 3′-UTR of MT1M. Furthermore, the expression of MT1M protein could be downregulated by miR-24-3p mimic transfection but upregulated by miR-24-3p inhibitor transfection in HepG2 cells (Figure 3C). Together, the above results suggested that miR-24-3p could regulate the expression of endogenous MT1M by directly targeting the 3′-UTR of MT1M mRNA, and human MT1M is a novel target of miR-24-3p.

3.4 | MT1M knockdown enhances cell growth

To identify whether inhibition of MT1M also resulted in HCC cell growth enhancing, just like miR-24-3p restoration, the effects of knockdown of MT1M on cell growth were examined. Firstly, HepG2 cells were transfected with, or not, MT1M siRNA or control siRNA. Forty eight hours after transfection, a dose-dependent knockdown of MT1M was observed in HepG2 cells (Figure 4A). In cell viability assay, in vitro knockdown of MT1M enhanced cell viability (Figure 4B). The similar data were obtained in Huh7 cells transfected with MT1M siRNA (data not shown). These results indicate that MT1M is most likely involved in the enhancement of cell growth by miR-24-3p.

4 | DISCUSSION

Compelling evidence demonstrates that miRNAs have important roles in HCC progression and directly contribute to the cell proliferation, avoidance of apoptosis, and metastasis of HCC. Identifying the biomarker proteins and their regulating miRNAs that are essential for HCC progression may provide promising therapeutic opportunities. Many miRNAs have been found to be dysregulated in HCC and function as oncogenes or tumor suppressors, depending on their targets in HCC tumorigenesis. Recently, studies have found that miR-24 acts as an oncogene in several tumors, including HCC. Liu YX et al. reported that miR-24 was upregulated in HCC tumor tissues; the high expression of miR-24 was significantly correlated with larger tumor size, higher microvesel density, and tumor dedifferentiation; and miR-24 overexpression progressed tumor cells proliferation, inhibited cell apoptosis, and developed the formation of AFB1-DNA adducts. Ma Y et al. reported that miR-24 was increased in HCC tissues and cell lines and miR-24 promotes the proliferation and invasion of HCC cells by targeting SOX7. In addition, serum miR-24-3p was
significantly upregulated in patients with HCC, significantly associated with vascular invasion in patients with HCC, and is an independent predictor of poor overall survival and disease-free survival in patients with hepatitis B virus-related HCC. However, to date, studies of the biological function and molecular mechanism of miR-24-3p in HCC remain limited. In this study, the upregulation of miR-24-3p was confirmed in HCC tumor tissues. We also compared the expression of miR-24-3p among the different stages of tumors, but no significance was found (data not shown). It is too early to say that there is no correlation between miR-24-3p and HCC tumor progression. The sample sizes in this study are relatively small; further analysis on large-scale prospective data would be advantageous. In addition, cross-sectional study and longer follow-up study would have been preferable, which helps to evaluate the role of miR-24-3p more accurately in HCC early diagnosis or survival prediction.

It is generally accepted that miRNAs exert their function through regulating the expression of their downstream target genes. In this study, we identified MT1M as a target of miR-24-3p in HCC. Members of the MT family are small cysteine-rich proteins that possess specific binding activity for metal ions, which are involved in metal detoxification and in the protection of cells against certain electrophilic carcinogens. Previously, other researchers have reported dramatic loss of MT expression in primary human HCC by immunohistochemistry methods. Recently, MT1M promoter methylation was reported as biomarkers for HCC or breast cancer. Majumder’s group reported that MT1 and MT2 double knockout accelerates hepatocarcinogenesis in mice exposed to the carcinogen diethylnitrosamine. Mao’s group reported that frequent downregulation of MT1M in human HCC may contribute to liver tumorigenesis by increasing cellular NF-κB activity. On the other
hand, MT1M had been reported to be regulated by several miRNAs, including miR-206,25 miR-506,26 miR-138,27 miR-124,28 miR-545,29 and miR-302.30 We identified MT1M as a target of miR-24-3p in HCC, which may provide new insights into the mechanisms underlying tumorigenesis.

In conclusion, we here provide evidence that low expression of miR-24-3p contributes to the cell growth in HCC by directly binding MT1M 3′-UTR. Therefore, miR-24-3p may function as a tumor suppressor in HCC and serve as a useful therapeutic agent for miRNA-based HCC therapy.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

REFERENCES