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miR-663a inhibits hepatocellular carcinoma cell proliferation and invasion by targeting HMGA2

Weizhen Huang[a], Jun Li[a], Xiaohong Guo[a], Yingchu Zhao[b], Xia Yuan[a,*]

[a] Department of Medical Oncology, Huizhou Municipal Central Hospital of Guangdong Province, 41 North Eling Road, Huizhou, Guangdong Province 516000, PR China
[b] Department of Biochemistry, University of Missouri, 117 Schweitzer Hall, Columbia, MO 65211, USA

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ABSTRACT

Hepatocellular carcinoma (HCC) is a highly aggressive solid malignancy throughout the world. Dysregulation of miRNAs play essential roles in HCC progression via aberrant regulation of cell proliferation, apoptosis, as well as metastasis. miR-663a is a poorly investigated miRNA. Whether miR-663a regulates HCC development remains unknown. The aim of the study was to explore the role of miR-663a in HCC development. To determine the expression level of miR-663a in HCC, we analyzed the data from GSE21362 and TCGA. The results showed that miR-663a was significantly down-regulated in HCC tissue compared with adjacent non-tumor tissue. Gain of function and loss of function assays revealed that miR-663a distinctly inhibited cell proliferation, migration and invasion. Mechanistic investigations demonstrated that miR-663a modulated cell functions through targeting and suppressing high mobility group A2 (HMGA2). In addition, overexpression of HMGA2 remarkably attenuated the tumor repressive effect of miR-663a. Taken together, miR-663a inhibits HCC cell proliferation and motility by targeting HMGA2.

1. Introduction

Hepatocellular carcinoma (HCC) is a highly aggressive solid human malignancy worldwide [1–3]. Because of the highly malignant potential of HCC, HCC ranks as the second major cause of cancer-related death throughout the world [4]. The overall survival or recurrence-free survival time of HCC patients remains unsatisfactory despite of advance treatment strategies or various treatment options [5,6]. Metastasis and proliferation contribute to two essential hallmarks of HCC malignancy [7]. Therefore, a better understanding of the molecular mechanisms underlying the metastasis or proliferation of HCC is of great importance for identifying novel predictive biomarkers and improving therapeutic modalities [8,9].

MicroRNAs (miRNAs) are a cluster of highly conserved small noncoding RNAs with about 17–25 nucleotides in length [9–11]. It has been reported that miRNAs commonly function as post-transcriptional regulators [12]. They can negatively regulate target gene expression through binding directly to the 3′ untranslated region (3′-UTR) of target mRNAs in a sequence-specific manner, which results in degradation of mRNA or suppression of protein translation [13,14]. Accumulating evidence has demonstrated that deregulations of miRNAs played important roles in regulation of multiple biological processes like cell proliferation, apoptosis, and metastasis and so on [15]. Deregulation or dysfunction of miRNAs involve in the development of cancer progression and can serve as biomarkers for patients with caners [16].

Previous studies have demonstrated that miR-663a, as a member of the primate-specific miRNA family, played essential roles in different biological processes, including inflammatory responses, differentiation, viral infection, as well as tumorgenesis [17–21]. However, its potential role in tumor development remains quite contradictory. It is reported that miR-663a may function as an oncogene and promote tumor progression in lung cancer, breast cancer and nasopharyngeal [22–24]. MiR-663a may also function as a tumor suppressor in colorectal carcinoma, gastric cancer, or pancreatic cancer [21,25,26]. To date, the effects or mechanisms of miR-663a on HCC remain largely unknown.

Abbreviations: HCC, hepatocellular carcinoma; miRNA, microRNA; TCGA, the Caner Genome Atlas project; GEO, Gene Expression Omnibus; HMGA2, high mobility group A2; 3′-UTR, 3′ untranslated region; mRNA, message RNA; EMT, epithelial-to-mesenchymal transition.

* Corresponding author.
E-mail addresses: flyhuizhouzhongxin@163.com (W. Huang), fly_lijun@aliyun.com (J. Li), gxh1979@sina.com (X. Guo), yingchuzhao@163.com (Y. Zhao), yuanxia71@hotmail.com (X. Yuan).

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In the present study, we explored the expression of miR-663a in HCC through analyzing the data from two public datasets: Gene Expression Omnibus (GEO, accession number GSE21362) and the Caner Genome Atlas project (TCGA). Moreover, we investigated the biological functions of miR-663a in HCC cell lines. Additionally, the contributions of miR-663 to HCC malignancy and its underlying mechanisms were also explored in the study. Our results suggested that miR-663a, frequently down-regulated in HCC, may act as a tumor suppressor and serve as prognostic biomarker for HCC.

2. Material and methods

2.1. Cell culture

The HL-7702, HCC-LM3, SMMC-7721, SK-Hep-1, BEL-7402, MHCC-97H cell lines were all purchased from the Cell Bank of Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). HL-7702 is a primary human liver cell line which is used for normal control in most studies. HCC-LM3, SMMC-7721, BEL-7402, and MHCC-97H are HCC cell lines with advanced stage phenotype, while SK-Hep-1 is a lower malignant HCC cell line. All HCC cell lines above are capable of tumor formation. The cell lines were then maintained in Dubecco's modified Eagle's medium (DMEM, Gibco, Geand Island, NY) with 10% fetal bovine serum and incubated in a humidified incubator containing with 5% CO_2.

2.2. Expression datasets

To explore the expression level of miR-663a or HMGA2in HCC, we analyzed the Cancer Genome Atlas project (TCGA dataset, https://tcga-data.nci.nih.gov/tcga/) dataset. A total of 351 patients with miRNA and mRNA expression data were enrolled in the present study. Among these patients, 50 patients had HMGA2 expression data in non-tumor tissues, while 49 patients had miR-663a expression data in non-tumor tissues. We collected the miR-663a and HMGA2 expression data from each patient to compare the expression of miR-663a or HMGA2 in HCC tissues and non-tumor tissues.

In addition, a set of microarray data was downloaded from Gene Expression Omnibus (GEO, accession number GSE21362, platform: GPL10312, 3D-Gene Human miRNA Oligo chip v12-1.00) to assess the expression of miR-663a in HCC. In total, 78 patients with miRNA expression data were used to compare the expression of miR-663a in HCC tissues and adjacent non-tumor tissues.

2.3. RNA extraction, reverse transcription (RT) and real-time PCR

Total cellular RNA was isolated by Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacture's instruction. Then total cellular RNAs were reversed transcribed into cDNA using reverse transcription reagent kit (Takara Biotechnology, Dalian China). Real-time PCR was performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Kit (Takara Biotechnology, Dalian, China). Primer sequences used to amplify the indicated genes were as follows:

U6 forward: 5′-CTCGCTTCGGCAGCACA-3′

U6 reverse: 5′-AACGCTTCACGAATTTGCGT-3′

MiR-663a forward: 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCGGTCCC-3′

MiR-663a reverse: 5′-ACACTCCAGCTGGGAGGCGGGCGCCGCGG-3′

β-actin forward:5′-TCAAGATCATTGCTCCTCCTGA-3′

β-actin reverse: 5′-CTCGTCATACTCCTGCTTGCTG-3′

HMGA2 forward: 5′- TCCCTCTAA AGCAGCTCAAAA-3′

HMGA2 reverse: 5′- ACTTGTTGTGGC CATTTCCT-3′

Relative quantification was calculated as $2^{-\Delta\Delta Ct}$ and was used to calculate fold changes. Relative miR-663a or HMGA2 mRNA expression were normalized to U6 or β-actin respectively.

2.4. CCK-8 assay

CCK-8 assay were conducted to analyze the effect of miR-663a on HCC cell proliferation. After transfection with miR-663a mimics or inhibitor, 1×10^3 cells per well were incubated in 96-well plates. Every 24 h, 10 μl CCK-8 reagents (Dojindo, Kumamoto, Japan) were added to each plate. Then the mixture solutions were incubated for 2 h at 37 °C before assessing absorption under 450 nm light.

2.5. In vitro migration and invasion assays

In the migration assay, 1×10^5 cells were placed in the upper chambers (Corning) after resuspending in serum-free DMEM, and for the invasion assay, 1×10^5 cells were seeded in the upper chambers which were coated with matrigel (BD Bioscience). After 24 h (migration assay) or 48 h (invasion assay) of incubation, the nonmigrated cells on the upper surface of the membrane were removed, and the lower surface was fixed with 100% methanol before staining with 0.1% crystal violet. Cell numbers in five random microscopic fields in each replicate were counted and imaged using microscope (Olympus Corp).

2.6. Western blot

HCC cells were lysed on ice in RIPA buffer (Beyotime, China) with protease inhibitors and phosphatase inhibitors (Roche, Mannheim, Germany). Cell lysis buffer was centrifugated at 14,000*g* for 15 min before determining protein concentration using BCA protein assay kit (Beyotime, China). Quantified protein lysates were then separated on a 12% SDS polyacrylamide gel, electrotransferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, USA), blocked with 5% BSA for 1.5 h at room temperature, and immunoblotted with primary antibodies against β-actin (Santa Cruz Biotechnology, 1:1000) and HMGA2 (Abcam, 1:1000) overnight at 4 °C. After the incubation with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, USA), the signals of membranes were detected using ECL reaction kit (Bio-Rad, Hercules, USA).

2.7. Plasmid construction and transient transfection

To construct the HMGA2 3′-UTR wild type plasmid and HMGA2-3′UTR mutant plasmid for dual-luciferase reporter assays, wild type HMGA2 3′-UTR sequence and mutant HMGA2 3′-UTR sequence were cloned into psiCHECK 2.0 vector (Promega, Madison, WI).The following primers were used to amplify the sequences of the wild type HMGA2 3′-UTR sequence, HMGA2-3′-UTR-wt forward: 5′- CCGCTCGAGCTTGAATCTTGGGCAGGAACT-3′

HMGA2-3′-UTR-wt reverse: 5′- AAGGAAAAAAGCGGCCGCAGGTTAACAACTTTATTAC-3′. The mir-663a mimics, miR-663 inhibitor and negative control (NC) were purchased from GenePharma (GenePharma, Suzhou, China). Cells were seeded overnight and were transiently transfected with plasmids or miR-663a mimics or inhibitor using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 48 h