MicroRNA-20a-5p regulates the epithelial-mesenchymal transition of human hepatocellular carcinoma by targeting RUNX3

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Abstract
Background: MicroRNA-20a (miR-20a) is dysregulated in many types of malignancies, including human hepatocellular carcinoma (HCC), but its expression level and functional significance in HCC are still disputed. We aimed to study the role of miR-20a-5p in HCC and its downstream molecular mechanisms.

Methods: We used real-time polymerase chain reaction to detect the expression of miR-20a-5p and runt-related transcription factor 3 (RUNX3) in HCC and paraneoplastic tissue, transfected Huh7 and highly metastatic human hepatocellular carcinoma (MHCC97H) cells. A live cell workstation was used to observe the proliferation and migration of transfected cells. The invasiveness of transfected cells was verified by Transwell assay. Cell apoptosis was detected by flow cytometry. The expression levels of proteins after transfection were measured using simple western immunoblot measurements. Gene expression profiles between HCC and normal samples were obtained from The Cancer Genome Atlas. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment results were processed by the database for annotation, visualization and integrated discovery. Potential target genes of miR-20a-5p were predicted to further investigate how miR-20a-5p regulates epithelial-mesenchymal transition (EMT) in HCC.

Results: MiR-20a-5p was significantly highly expressed in HCC tissues, and overexpression of miR-20a-5p significantly promoted HCC cell proliferation, migration, and invasion and inhibited apoptosis in vitro. The protein expression of E-cadherin was decreased and that of vimentin was increased after overexpression of miR-20a-5p in HCC cells. We discovered the intersection of genes from miRDB, miR TarBase, and TargetScan, obtained 397 target genes and finally focused on RUNX3. RUNX3 was not only reduced in HCC specimens but also drastically reduced in HCC cells overexpressing miR-20a-5p. RUNX3 expression decreased with elevated miR-20a-5p, which activated downstream EMT signaling and promoted cell proliferation, migration, and invasion.

Conclusions: Since RUNX3 is involved in EMT in HCC, as proven by previous research, our findings provide further evidence for a novel regulatory pathway comprising the miR-20a/RUNX3/EMT axis that upregulates EMT signaling and enhances the migration of HCC cells.

Keywords: Epithelial-mesenchymal transition; Hepatocellular carcinoma; miR-20a-5p; RUNX3

Introduction
Primary liver cancer is the sixth most common cancer in the world, and 80% of liver cancer is hepatocellular carcinoma (HCC), which is the second leading cause of cancer-related death globally. Although the majority of cases occur in developing countries, the incidence has recently increased in developed countries. In recent years, studies on the molecular mechanism of HCC have provided more indications for its pathogenesis, diagnosis, and prognosis. Many microRNAs (miRNAs) associated with HCC, including miR-21, miR-221, and miR-224, have increased expression in HCC, while others, such as miR-122a, miR-199a, and miR-223, have decreased expression in HCC. Many miRNAs could play important roles in the tumor microenvironment during HCC development. MiRNAs can also regulate multiple target genes and signaling pathways in HCC, and miRNA-targeted cancer treatment is an attractive approach for the prevention of HCC development. Various miRNAs, including miR-32a-5p, miR-122, miR-221, and miR-1468, have potential as prognostic biomarkers for HCC, and upregulation of these miRNAs indicates poor prognosis in HCC patients. MiR-20a has significantly higher expression in ovarian cancer, lung cancer, colorectal cancer, cervical cancer, gastric cancer, and head and neck cancer according to The
Recently, it was proven that overexpression of miR-20a-5p in cancer genome.nih.gov/ statistics, and it exhibits potential for clinical application as a novel diagnostic biomarker and therapeutic target. In liver cancer, some studies reported that miR-20a was expressed at low levels in HCC tissues and cell lines. While another found that 52% of miR-20a was highly expressed in 39 pairs of HCC specimens. Similar findings have been reported in breast cancer[18,19] and these conflicting results prompted us to further study the TCGA database and HCC specimen analysis.

Recently, it was proven that overexpression of miR-20a-5p promoted the development of liver cancer[17] breast cancer[19] lung cancer[20] and cervical cancer[21] by targeting RUNX3. In addition, it was reported that downregulation of RUNX3 promoted epithelial-mesenchymal transition (EMT) in HCC. However, it is still unclear whether and how miR-20a-5p affects the EMT process in HCC. In the present study, we investigated the expression of miR-20a-5p and RUNX3 in HCC cells and tissues and found that miR-20a-5p affected the EMT process by regulating the expression of RUNX3 in HCC cells. We identified the miR-20a/RUNX3/EMT regulatory axis, providing strong evidence for molecular targeted therapy of liver cancer.

Methods
Ethical approval
All patients or patients’ families signed the written informed consent, and the procedure of tissue collection was approved by the Ethics Review Committee of the Affiliated Hospital of Inner Mongolia Medical University (No. WZ2021043).

Tissue specimens
Six tumor tissue specimens along with six adjacent tissues were obtained from HCC patients from the Affiliated Hospital of Inner Mongolia Medical University. After resection of the tumor and adjacent tissues, the specimens were immediately frozen in liquid nitrogen and stored at −80°C. All experiments strictly adhered to the code of ethics of the World Medical Association and were conducted following the guidelines of Inner Mongolia Medical University.

Cell culture and transfection
The human HCC cell line MHCC97H was cultured in Dulbecco’s Modified Eagle’s Medium replenished with 10% fetal bovine serum (FBS, Gibco, Life Technologies Limited, Paisley, UK). The human HCC cell line Human hepatocellular carcinoma-7 was cultured in Modified Eagle’s Medium containing 10% FBS. All cells were cultured at 37°C in a humidified 5% CO2 incubator. The synthesized miR-20a-5p mimics and the random negative control RNAs (control mimic and inhibitor) were obtained from GenePharma (Shanghai, China). When the cells in the plate reached a fusion degree of 70% to 80% per well, the overexpression of miR-20a was performed by transfecting a miR-20a mimic with a synthesized double-stranded RNA oligonucleotide imitating the miR-20a precursor. The plasmids were transfected into the cells by an electroporation system (Bio-Rad, USA) based on the protocols.

Quantitative real-time (qRT) polymerase chain reaction (PCR)
TRIZOL reagent (Invitrogen, CA, USA) was used to isolate total RNA from frozen tumor tissues and cells. We used the (cDNA) Synthesis Kit (Takara, Tokyo, Japan) for the synthesis of cDNA. The qRT-PCR for miRNAs and (mRNA) was conducted with specific primers following the instructions of the manufacturer (Life Technologies, Carlsbad, CA, USA). qPCR results of miRNA and mRNA were expressed corresponding to Rnu6, gene ID 19862 snRNA or Glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) mRNA, respectively.

Cell proliferation assay
The cells after transient transfection were seeded onto 96-well plates at 1 × 104 cells/well. After 0, 6, 12, 18, 24, 36, and 72 h of culture, the cells were captured in a living cell workstation (IncuCyte Zoom, Essen BioScience, USA).

Cell apoptosis assay
The Annexin V-fluorescein isothiocyanate (FITC)/PI staining kit (Becton, Dickinson and Company [BD] Biosciences, San Diego, CA, USA) was used for cell apoptosis. MHCC97H and Human hepatocellular carcinoma-7 cells were seeded in 6-well plates with a density of 104 cells/mL. After transfecting for 24 h, Annexin V-FITC was used to label the cells for 20 min in the darkness. In total, 50 μg/mL of PI was added and further incubated for 30 min. The situation of cell apoptosis was detected to calculate the percentage of cell death based on flow cytometry with the FACScan flow cytometer (BD Biosciences). All tests were performed in triplicate.

Wound healing assay
The transfected cells were cultured on 96-well plates forming a single cell layer, and allowed to grow to 100% confluence. A straight wound line was made in the middle of the cell layer. The closure of the wound was observed by taking pictures at different time points by the IncuCyte (IncuCyte Zoom, Essen BioScience) for 24 h.

Cell invasion assay
Matrigel (Fisher Scientific, USA) invasion chambers (8.0 μm pore size, BD Biosciences, Franklin Lakes, NJ, USA) were used to investigate the invasion capability of transfected cells. Relative cells (5 × 105 cells/mL) were seeded in a serum-free medium into the upper chamber, and the bottom chamber added 10% FBS as chemo-attractant inducing cells invading toward the bottom chamber. After 24 h, the cells invaded through the membrane and adhered to the underside of the membrane were stained by 0.05% crystal violet and counted under a microscope (Olympus, USA). The number of cells that migrated through the transwell pores was quantified using the Image J (NIH, USA) procedure. The reported data represent the average of three independent experiments performed in triplicate. Each experiment was repeated three times.
Western blot analysis

Cells were seeded in 6-well plates with a density of $4 \times 10^5$ cells/well and were treated with miR-20a-5p mimics and inhibitor for 48 h. Followed by treatment, cells were lysed with radioimmunoprecipitation assay (RIPA) Lysis buffer (Solarbio, Beijing, China) and the protein concentration was quantified using the bicinchoninic acid (BCA) protein assay kit (Applygen Technologies, China). The simple western immunoblots were performed on a Wes (Protein Simple Santa Clara, San Jose, CA, USA) using the Size Separation Master Kit with Split Buffer (12–230 D) according to the manufacturer’s standard instruction. The antibodies used included the following: anti-RUNX3 (ab40278, abcam, UK), anti-Vimentin (10366-1-AP, Proteintech, USA), anti-E-cadherin (20874-1-AP, Proteintech, USA), and anti-GAPDH (10494-1-AP, Proteintech, USA).

Results

miR-20a-5p is upregulated in HCC tissues

miR-20a is among the most frequently altered miRNAs in human cancers. To investigate the expression of miR-20a-5p in HCC, we collected data on miR-20a-5p expression in a large number of HCC patients from the TCGA database. Through TCGA analysis, we found miR-20a-5p was significantly increased in HCC tissues compared with normal liver tissues ($P < 0.001$) [Figure 1A]. miR-20a-5p expression was further measured in six pairs of tumors and adjacent normal tissues of patients with primary HCC in our hospital and was significantly increased in HCC tissues compared with adjacent normal tissues ($P < 0.05$) [Figure 1B].

Overexpression of miR-20a-5p promotes the proliferation and inhibits apoptosis of HCC cells

To investigate the effect of miR-20a-5p on cell proliferation and apoptosis, we overexpressed both miR-20a-5p mimics and a miR-20a-5p inhibitor in Huh7 and MHCC97H cells. The efficiency of transfection was confirmed by qRT-PCR. At 48 h posttransfection with miR-20a-5p mimics, the expression of miR-20a-5p was increased ~3- to 5-fold in the two HCC cell lines ($P < 0.01$). The expression of miR-20a-5p was reduced after transfection with the inhibitor [Figure 2A]. The data from a live cell workstation showed a significant increase in proliferation in cells after transfection with miR-20a-5p mimics ($P < 0.001$) [Figure 2B]. After Annexin V-FITC/PI staining, flow cytometry analysis showed that transfection of miR-20a-5p mimics resulted in a significant reduction in the apoptotic rate ($P < 0.01$), while the opposite effect was observed upon treatment with the miR-20a-5p inhibitor [Figure 2C].

Overexpression of miR-20a-5p promotes the migration and invasion of HCC cells

The wound healing assay showed that miR-20a-5p overexpression also enhanced Huh7 cell ($P < 0.01$) and MHCC...
-97H cell ($P < 0.05$) migration ability in vitro [Figure 3A]. Accordingly, treatment with the miR-20a-5p inhibitor caused the opposite effects. The Transwell assay results showed a significant increase in the number of invasive cells after transfection with miR-20a-5p mimics ($P < 0.01$), which was greatly reduced by the miR-20a-5p inhibitor [Figure 3B]. These data indicated that HCC cell migration and invasion could be modulated by the expression of miR-20a-5p.
MiR-20a-5p promotes EMT of HCC cells

The invasion and migration of tumors involve many biological processes (BP), among which EMT plays a very important role.\(^{[23]}\) To determine how miR-20a-5p promotes the migration and invasion of HCC cells, we determined whether miR-20a-5p could regulate the EMT process. The results revealed that the expression of E-cadherin, a growth and invasion suppressor of the EMT pathway, was significantly decreased after overexpression of miR-20a-5p \((P < 0.001)\). In addition, the expression of Vimentin, a marker correlated with the EMT phenotype, was increased markedly upon miR-20a-5p overexpression \((P < 0.01)\). The inhibition of miR-20a-5p expression showed the opposite result [Figure 4].

The miR-20a/RUNX3/EMT axis was established via enrichment analysis and validated

It was proven that miR-20a-5p could regulate the EMT process and then promote the migration and invasion of HCC cells. To investigate the miR-20a-5p target that

Figure 3: Overexpression of miR-20a-5p promotes the migration and invasion of HCC cells. (A) The effect of transfection of NC, miR-20a-5p mimics and inhibitor on cell migration was observed by wound healing assays. The wound healing was accelerated after transfection with miR-20a-5p mimics. (B) Transwell assays revealed cell invasion after transfection with NC, miR-20a-5p mimics and the inhibitor. The cells were stained by 0.05% crystal violet. The number of invasive cells after transfection with miR-20a-5p mimics was significantly increased. \(* P < 0.05, \dagger P < 0.01\) vs. NC. HCC: Hepatocellular carcinoma; NC: Negative control.
regulates the EMT process, miRDB, miRTarBase, and TargetScan were used to predict potential mRNAs that could be targeted by miR-20a-5p. After the intersection of the three databases, we obtained 397 target genes, except for the enrichment of “the positive regulation of epithelial to mesenchymal transition,” and we finally found that RUNX3 was one of the common predicted target genes [Figure 5A and 5B]. Compared with the adjacent normal
tissues, the expression of RUNX3 mRNA significantly decreased in HCC specimens with high miR-20a-5p expression ($P < 0.05$) [Figure 5C]. In addition, the enriched pathways of the 397 target genes by KEGG analysis were associated with “cell migration” and “cell adhesion” [Figure 5D].

Further analysis was carried out to determine whether miR-20a-5p can regulate the target gene RUNX3 in vitro. The qRT-PCR result showed that overexpression of miR-20a-5p led to decreased levels of RUNX3 mRNA [Figure 6A]. Accordingly, transfection of the miR-20a-5p inhibitor increased the level of RUNX3 protein [Figure 6B]. RUNX3 expression decreased with increasing miR-20a-5p, which in turn promoted cell proliferation, migration, and invasion and further activated downstream EMT signaling. These changes contributed to the proliferation and migration of HCC cells, ultimately increasing tumor formation and metastasis [Figure 7].

Discussion

miRNAs affect HCC tumor growth and metastasis at multiple stages of tumor development, and dysregulation of miRNAs has been related to poor outcome of patients with HCC. To date, 55 upregulated miRNAs, including miR-17–92 polycistron and miR-21, as well as 96 downregulated miRNAs, including miR-29 and miR-122, have been found in liver cancer. \cite{24} For miR-20a-5p, the different expression trends have made it an ongoing hot topic in HCC research. Some studies have suggested that the levels of miR-20a-5p are elevated in HCC cell lines and tissues,\cite{13,23} while others have concluded that miR-20a-5p expression is lower in primary HCC than that in normal liver tissue.\cite{26} We used primary HCC tissue to verify that the expression of miR-20a-5p was higher than that in adjacent normal tissue. Moreover, we found that overexpression of miR-20a-5p significantly promoted cell proliferation, migration, and invasion and inhibited apoptosis.

To identify the target genes of miR-20a-5p and their functions, we first predicted the target genes of miR-20a-5p using public databases: miRTarBase, miRDB, and TargetScan. A total of 397 target genes of miR-20a-5p were obtained after the intersection of the above three databases, and the DAVID website was used for KEGG
pathway and GO functional enrichment analyses. The KEGG analysis results showed that a portion of the enriched pathways was associated with “cell migration” and “cell adhesion”. The BP in GO functional analysis was enriched in the “positive regulation of epithelial to mesenchymal transition”. Overexpression of miR-20a-5p could inhibit the expression of E-cadherin and stimulate the expression of Vimentin, an invasion suppressor and promoter of the EMT pathway, respectively.

To further investigate the miR-20a-5p target involved in regulating EMT, we also examined the intersection of the three predicted results by Venn diagram and found that RUNX3 was one of the common predicted target genes. Previous research proved that miR-20a-5p directly targets RUNX3 by using a luciferase assay.[17] Here, by performing western blot experiments, we further proved that miR-20a-5p can directly downregulate the protein level of RUNX3. RUNX3 is frequently lost in heterozygosity in HCC,[27] and homozygous deletions are found in 35% to 40% of HCC cases, resulting in the absence of RUNX3 expression.[28,29] This clinical evidence indicates that loss of RUNX3 causes the development and progression of HCC.

The role of RUNX3 in HCC EMT has been proven in previous research. For example, Tanaka[22] proved that E-cadherin expression was induced by exogenous RUNX3 protein in low-EMT cells, and Gou[30] further found that RUNX3 increased E-cadherin expression by repressing microRNA-186. In this study, we examined the expression of RUNX3 and classic EMT proteins simultaneously by up- or downregulation of miR-20a expression. An increase in miR-20a-5p expression was followed by a decrease in RUNX3 protein expression, a significant decrease in E-cadherin expression, and an increase in Vimentin expression. When miR-20a-5p expression was inhibited, the opposite result was obtained. RUNX3 expression decreased with elevated miR-20a-5p, which activated downstream EMT signaling and further promoted cell proliferation, migration, and invasion.

Since EMT plays an important role in tumor invasion and metastasis, we surmised that RUNX3 may prevent HCC cell metastasis by modulating EMT. Studies have shown that the expression levels of E-cadherin were increased and those of Snail and N-cadherin were decreased in the RUNX3-overexpressing group compared with the vector control group.[31] RUNX3 inhibits the expression of claudin, another cell–cell junction protein involved in the development of EMT. As both E-cadherin and claudin are negatively regulated in EMT, RUNX3 could be an EMT regulator.[32] In addition, RUNX3 controls Notch signaling, which is closely linked to cancer stem cells.[33] Ectopic RUNX3 expression deactivated Notch signaling by decreasing jagged-1 expression in HCC.[34] These are the possible molecular mechanisms by which RUNX3 promotes the EMT signaling pathway and migration in HCC cells.

In conclusion, our findings provide further evidence for a novel regulatory pathway comprising the miR-20a/RUNX3/EMT axis in HCC. miR-20a-5p can promote EMT and then enhance migration by targeting RUNX3. We believe that these findings significantly improve our understanding of HCC metastasis. These results suggest that miR-20a-5p may be an effective target for HCC and is worth investigating through in vivo experiments.
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Conflicts of interest
None.

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