Long non-coding RNA maternally expressed gene 3 affects cell proliferation, apoptosis and migration by targeting the microRNA-9-5p/midkine axis and activating the phosphoinositide-dependent kinase/AKT pathway in hepatocellular carcinoma

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Received July 23, 2020; Accepted December 2, 2020

DOI: 10.3892/ol.2021.12606

Abstract. Long non-coding RNA (IncRNA) maternally expressed gene 3 (MEG3) is a tumor suppressor in several cancers, such as glioma, prostate cancer and esophageal cancer. However, the role of MEG3 in hepatocellular carcinoma (HCC) and the related molecular mechanisms are not well understood. The present study aimed to determine the biological function of MEG3 in regulating HCC cell viability, apoptosis and migration. In addition, the interaction between MEG3, microRNA (miR)-9-5p and Midkine (MDK), and the activation of the phosphoinositide-dependent kinase (PDK)/AKT pathway in HCC cell line MHCC-97L were examined. Luciferase reporter assays, reverse transcription-quantitative PCR and western blotting were used to determine the interaction between MEG3, miR-9-5p and MDK, and the activation of the PDK/AKT pathway. Cell viability was determined by the CCK8 assay and the cell cycle analysis using flow cytometry analysis. Cell apoptosis was examined by flow cytometry analysis and caspase 3/9 activity. Wound healing assays and western blotting were used to investigate cell migration. The present study demonstrated that MEG3 suppressed HCC cell viability and migration, and induced cell apoptosis. In addition, it was also found that MEG3 targets the miR-9-5p/MDK axis and modulates the PDK/AKT pathway in HCC. In conclusion, the findings of the present study demonstrated that IncRNA MEG3 affects HCC cell viability, apoptosis and migration through its targeting of miR-9-5p/MDK and regulation of the PDK/AKT pathway. The MEG3/miR-9-5p/MDK axis may be a potential therapeutic target in HCC.

Introduction

Hepatocellular carcinoma (HCC), a malignant tumor arising from the liver, accounts for 80% of primary liver cancer (1), and is the third leading cause of cancer-related deaths globally (2) with a mortality of 0.598 million according to the report released by WHO International Agency in 2018 (3). The 5-year survival rate of patients with HCC is as low as 30% (4). It is imperative to improve understanding of hepatocarcinogenesis and to develop novel strategies for the treatment of HCC.

Long non-coding RNAs (IncRNAs), are a class of non-coding transcripts with lengths exceeding 200 nucleotides, which participate in a variety of physiological and pathological processes, including embryogenesis, tumorigenesis and some cardiovascular diseases (5). IncRNA maternally expressed gene 3 (MEG3) has been reported to be abnormally expressed in HCC tissues (6,7), however, its role in HCC and the related mechanisms are not well understood. MicroRNAs (miRs) are small (~22 nucleotide) non-coding RNAs which also participate in various biological processes, such as development, growth, organogenesis and angiogenesis (8,9). IncRNAs and miRNAs can interact with each other to serve a wide spectrum of biological functions, such as neovascularization, angiogenesis and vasculogenic mimicry (10,11). miR-9-5p (GenBank: LM379059.1) is a well-known microRNA which serves important roles in the growth, angiogenesis, radio-resistance
and metastasis of various types of cancers, such as colorectal cancer, acute myeloid leukemia, and gastric cancer (12-14). MEG3 has been reported to serve as a competing endogenous RNA (ceRNA) for miR-9-5p in prostate cancer (15) and esophageal cancer (16) to mediate tumor progression.

Midkine (MDK) was initially found as a growth factor in retinoic acid-induced embryonic tumor cells (17). Currently, MDK is viewed as a multifaceted factor promoting cell growth, survival, metastasis, migration, and angiogenesis, in particular during tumorogenesis in cancer (18). Phosphoinositide-dependent kinase 1 (PDK1) is a serine/threonine protein kinase which serves central roles in signal transduction (19). Several studies have demonstrated that PDK1 can promote cell proliferation by activating the Akt/mTOR signaling pathway (20,21). A recent study by Lu et al (22) reported that miR-9-5p can inhibit angiogenesis by targeting MDK and regulating the PDK/AKT pathway in nasopharyngeal carcinoma.

The present study aimed to investigate the effect of MEG3 on HCC cell viability, apoptosis and migration. In addition, the interaction between MEG3, miR-9-5p and MDK, and the activation of PDK/AKT pathway in HCC cells was also assessed. The present study provides new insights into the molecular mechanisms of MEG3 in HCC and suggests a novel therapeutic target for HCC.

Materials and methods

Cell culture. MHCC-97L cell line (cat. no. BNCC337741) was purchased from Beijing Beina Chuanglian Institute of Biotechnology. The cells were maintained in RPMI-1640 medium (Nanjing KeyGen Biotech. Co. Ltd.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific Inc.) and 100 U/ml penicillin G sodium and 0.1 mol/ml streptomycin sulfate, at 37°C for 48 h in a humidified atmosphere of 5% CO2.

Transfection. miR-9-5p mimic (3.75 µl, 25 nM), miR-9-5p negative control (NC; 3.75 µl, 25 nM), IncRNA MEG3 overexpression vector (2.5 µg, 2.5 µg/ml) and IncRNA MEG3 NC (2.5 µg, 2.5 µg/ml) were purchased from General Biosystems, Inc. The sequences of miR-9-5p mimic and the corresponding NC were: miR-9-5p mimic sense, 5'-UCU UUG GUU AUC UAGG UUG UGA-3'; antisense, 5'-UCU ACU CUU UCU AGG AGG UUG-3'. Cells were co-transfected with the miR-NC and the wild-type 3'-UTR of MDK or lncRNA MEG3 and MDK 3'-UTR containing the putative binding sites of miR-9-5p and the corresponding NC were: miR-9‑5p mimic sense, 5'‑UCU  UUG GUU AUC UAGG UUG UGA‑3'; antisense, 5'‑UCU  ACU CUU UCU AGG AGG UUG‑3'; NC: Sense, 5'‑UCA CAA CCU  CCU AGA ACC ACA‑3'; antisense, 5'‑UCU ACU CUU UCU AGG AGG UUG‑3'; NC were: miR‑9‑5p mimic sense, 5'‑UCU  UUG GUU AUC UAGG UUG UGA‑3'; antisense, 5'‑UCU  ACU CUU UCU AGG AGG UUG‑3'; NC: Sense, 5'‑UCA CAA ACCUCUAGAGAGU AGA‑3'; antisense, 5'‑UCU ACU CUU UCU AGGAGGUUG UGA‑3'. Cells were co-transfected with the miR-NC/miR-9-5p mimic and the wild-type 3'-UTR of MDK or IncRNA MEG3 using Lipofectamine 3000® (Invitrogen; Thermo Fisher Scientific Inc.). Firefly and Renilla luciferase activities were detected 48 h later by the Dual‑Luciferase Reporter Assay System (Beyotime Institute of Biotechnology) using the Dual Luciferase Reporter Assay System (Beyotime).
Gene Assay kit (Beyotime Institute of Biotechnology). The firefly luciferase activity was normalized to the Renilla luciferase activity for each individual analysis.

**CCK8 assay.** Cells were seeded at 1x10^4 cells/well in 96-well plates and maintained in RPMI-1640 medium at 37°C in an atmosphere of 5% CO₂ for 48 h. Cell viability was measured using a CCK8 Kit (Nanjing KeyGen Biotech. Co. Ltd.) according to the manufacturer's instructions. Cells were incubated with the CCK8 reagent (10 µl) at 37°C for 1 h. Absorbance at 450 nm was measured using a Tecan Safire II Microplate Reader (Tecan Group Ltd.).

**Cell cycle analysis using flow cytometry (FCM).** FCM analysis of the cell cycle of MHCC‑97L cells was performed using a Cell Cycle Staining kit [MultiSciences (Lianke) Biotech Co., Ltd.] according to the manufacturer's instructions. The cell suspension was centrifuged at 978 x g for 3 min, and the cells were subsequently fixed in ethanol at 4°C for 2 h. After washing with PBS for 3 times, 1 ml of DNA staining solution (PI containing RNase A) was added to the tubes. The cells were incubated at room temperature in the dark for 30 min, and the cell cycle was analyzed using the NovoCyte 2060R flow cytomter (ACEA Biosciences, Inc.) with NovoExpress software v.1.2.5 (ACEA Biosciences, Inc.).

**LNC analysis of cell apoptosis.** Apoptosis rate of MHCC‑97L cells was determined by FCM using an Annexin V‑FITC/propidium iodide PI Apoptosis kit [MultiSciences (Lianke) Biotech Co., Ltd.]. Briefly, 1x10^5 cells were washed with cold PBS twice, and re-suspended in 300 µl binding buffer. After incubation with 3 µl Annexin V‑FITC and 3 µl PI‑PE at room temperature for 10 min in the dark, the cells were mixed with 200 µl binding buffer, and cell apoptosis rate was analyzed using a NovoCyte 2060R flow cytometer (ACEA Biosciences, Inc.) with NovoExpress software v.1.2.5 (ACEA Biosciences, Inc.). Early and late apoptosis were both analyzed.

**Wound healing assay.** MHCC‑97L cells were grown in FBS free medium to ~100% confluence in 6-well plates following transfection. The confluent monolayers were scratched by using a 10 µl pipette tip, and then washed with PBS 3 times. Images were taken by an inverted fluorescence microscope (MF53; Guangzhou Mingmei Photoelectric Co., Ltd.) immediately or 24 h after wounding and migration was quantified. Migration rate was calculated as the migration distance divided by the migration time.

**Statistical analyses.** Statistical analyses were performed using SPSS v.19.0 (IBM, Corp.). All experiments were performed at least 3 times. Experimental data were presented as the mean ± SD, and the statistical significance was assessed by one-way analysis of variance followed by the post hoc least significant difference (LSD) test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Effect of IncRNA MEG3 on HCC cell viability, apoptosis and migration. In preliminary experiments, the expression of IncRNA MEG3 was examined in 5 HCC cell lines and it was found that MHCC‑97L had the lowest IncRNA MEG3 expression (data not shown). Therefore, the MHCC‑97L cell line was chosen for subsequent gain-off-function experiments. IncRNA MEG3 overexpression vector was transfected into MHCC‑97L cells to investigate the effect of IncRNA MEG3 on HCC cell viability, apoptosis and migration. As demonstrated in Fig. 1A, IncRNA MEG3 NC did not affect IncRNA MEG3 expression. Compared with the IncRNA MEG3 NC group, IncRNA MEG3 was significantly increased in the IncRNA MEG3 group (P<0.01; Fig. 1A). The effect of IncRNA MEG3 on cell viability was examined by the CCK8 assay and FCM. The results from CCK8 assay demonstrated that cell viability in the IncRNA MEG3 group was significantly decreased when compared with that in the IncRNA MEG3 NC group (P<0.01; Fig. 1B). FCM analysis of cell cycle demonstrated that the fraction of S-phase cells was significantly higher and the fraction of G2-phase cells was significantly lower in the IncRNA MEG3 group compared with the IncRNA MEG3 NC group (P<0.01; Fig. 1C and D). FCM analysis of cell apoptosis revealed that compared with the cells transfected with IncRNA MEG3 NC, cell apoptosis rate was significantly increased in the cells transfected with IncRNA MEG3 overexpression vector (P<0.01; Fig. 2). Wound healing assays were performed to examine the effect of IncRNA MEG3 on cell migration. The results demonstrated that compared with the IncRNA MEG3 NC group, cell migration was inhibited in the IncRNA MEG3 group (P<0.01; Fig. 3). Compared with the control group, IncRNA MEG3 NC did not show any effects on cell viability, apoptosis or migration (Figs. 1-3).

**IncRNA MEG3 targets miR-9-5p and miR-9-5p targets MDK.** Luciferase reporter assays were used to investigate whether IncRNA MEG3 targets miR-9-5p. As shown in Fig. 4A, compared with the IncRNA MEG3+miR-9-5p mimic NC group, the luciferase activity was significantly decreased in the IncRNA MEG3+miR-9-5p mimic group (P<0.01). In addition, whether MDK was a direct target of miR-9-5p when compared with that in the MDK + miR-9-5p mimic NC group (P<0.01; Fig. 4B).

Effect of IncRNA MEG3 on the expression of miR-9-5p, MDK, apoptosis and migration-related proteins and PDK/AKT signaling. IncRNA MEG3 effect on miR-9-5p expression was determined by RT-qPCR analysis. As shown in Fig. 5A, compared with the IncRNA MEG3 NC group, miR-9-5p expression was significantly decreased in the IncRNA MEG3 group (P<0.01). The effect of IncRNA MEG3 on MDK expression was determined by western blotting and it was revealed that the relative protein level of MDK was significantly increased in the IncRNA MEG3 group when compared with that in the IncRNA MEG3 NC group (P<0.01; Fig. 5B and C). Western blotting also showed that the protein levels of caspase-3 and 9 were significantly upregulated in IncRNA MEG3-transfected cells compared with IncRNA MEG3 NC-transfected cells (P<0.01; Fig. 5B and C). Western blotting indicated that the expression level of MMP1 was significantly lower in the IncRNA MEG3-transfected cells compared with the IncRNA
Figure 1. Effect of lncRNA MEG3 on HCC cell viability. (A) Expression of lncRNA MEG3 in the cells transfected with the lncRNA MEG3 overexpression vector determined by RT-qPCR (n=5). (B) Cell viability determined by CCK8 assay. n=5. (C and D) Cell cycle analysis determined by flow cytometry. Cells in the control group were untransfected cells (n=3). *P<0.01 compared with the lncRNA MEG3 NC group. lncRNA, long non-coding RNA; MEG3, maternally expressed gene 3 (MEG3); HCC, hepatocellular carcinoma; NC, negative control; PI, propidium iodide; RT-q, reverse transcription-quantitative.

Figure 2. Effect of lncRNA MEG3 on HCC cell apoptosis determined by flow cytometry. Cells in the control group were untransfected cells (n=3). *P<0.01 compared with the lncRNA MEG3 NC group. lncRNA, long non-coding RNA; MEG3, maternally expressed gene 3 (MEG3); HCC, hepatocellular carcinoma; NC, negative control; PI, propidium iodide.

Figure 3. Effect of lncRNA MEG3 on HCC cell migration determined by wound healing assay. Cells in the control group were untransfected cells (n=3). *P<0.01 compared with the lncRNA MEG3 NC group. lncRNA, long non-coding RNA; MEG3, maternally expressed gene 3 (MEG3); HCC, hepatocellular carcinoma; NC, negative control.
Figure 4. Interaction between lncRNA MEG3, miR-9-5p and MDK determined by the dual luciferase reporter assay. (A) lncRNA MEG3 targets miR-9-5p. *P<0.01 compared with the lncRNA MEG3+miR-9-5p mimic NC group. (B) miR-9-5p targets MDK (n=3). *P<0.01 compared with the MDK+miR-9-5p mimic NC group. lncRNA, long non-coding RNA; MEG3, maternally expressed gene 3 (MEG3); NC, negative control; miR, microRNA; MDK, Midkine.

Figure 5. Effect of lncRNA MEG3 on the expression of miR-9-5p, MDK, apoptosis and migration-related proteins, and PDK/AKT pathway activation. (A) Effect of lncRNA MEG3 on miR-9-5p expression. (B) Western blot images. (C) Relative protein expression of MDK, caspase-3, caspase-9 and MMP1 determined by western blotting. (D) Effect of lncRNA MEG3 on PDK/AKT signaling. Cells in the control group were untransfected cells (n=3). *P<0.01 compared with the lncRNA MEG3 NC group. lncRNA, long non-coding RNA; MEG3, maternally expressed gene 3 (MEG3); HCC, hepatocellular carcinoma; NC, negative control; miR, microRNA; MDK, Midkine; PDK1, phosphoinositide-dependent kinase 1; p, phosphorylated; MMP-1, matrix metalloproteinase 1.
MEG3 NC-transfected cells (P<0.01; Fig. 5B and C). In order to verify the effect of IncRNA MEG3 on PDK/AKT signaling, western blotting was performed to examine p-PDK1 and p-AKT expression. Compared with the IncRNA MEG3 NC group, the protein expression of p-PDK1 and p-AKT was upregulated in the IncRNA MEG3 group (both P<0.01; Fig. 5B and D).

Discussion

The pathogenesis of HCC is complex and numerous studies have demonstrated that IncRNAs are involved in the initiation, development and metastasis of HCC (24,25). Some studies have shown that MEG3 is significantly downregulated in HCC tissues compared with carcinoma-adjacent tissues (6,7). Restoring MEG3 expression in cancer cells can suppress cell proliferation, invasion as well as angiogenesis, and induce cell apoptosis (26,27). These studies indicated that MEG3 is one of the IncRNAs with tumor suppressor activity (28). In the present study, the MEG3 overexpression vector was transfected into MHCC-97L cells to investigate the effect of MEG3 on cell viability, apoptosis and migration in HCC. The findings of the present study demonstrated that MEG3 inhibited HCC cell viability and a large number of cells accumulated in the S phase. Similarly, He et al (29) confirmed that MEG3 overexpression suppressed clone formation of hepatoma cells. Caspase proteins serve important roles in the process of apoptosis (30). Caspase-9 is an initiator of apoptosis, and caspase-3 is the final executor of apoptosis (30). In the present study, compared with IncRNA MEG3 negative control, MEG3 overexpression led to increased caspase-3 and caspase-9 expression, as well as an increased cell apoptosis rate indicating that MEG3 promotes cell apoptosis. In addition, in the present study MMP1 expression was inhibited by MEG3 in MHCC-97L cells. MMP1 is involved in invasion and metastasis during the development of cancer, and high expression of MMP1 is associated with poor prognosis of patients with HCC (31). The wound healing assay results of the present study further verified the inhibitory effect of MEG3 on cell migration. These results of the present study were consistent with the previous studies (6,24), and suggested that MEG3 may be a tumor suppressor in HCC.

IncRNAs can serve as a kind of ceRNA to regulate target genes through interacting with miRNAs (32,33). Recent studies have clarified that IncRNA-miRNA-mRNA form a novel regulatory network in various pathophysiological processes, such as differentiation, growth, necrosis and autophagy (34,35). The role of MEG3 in cancers has been reported in several studies, in which the interaction between IncRNAs, miRNA and mRNA has been highlighted (36,37). In glioma, MEG3 inhibited the proliferation, migration and invasion of glioma cells by regulating miR-96-5p/MTSS I-BAR Domain Containing 1 (38). In multiple myeloma, MEG3 inhibited tumor progression through regulating the miR-181a/Homeobox A11 axis (39). Particularly, previous studies have suggested that MEG3/miR-9-5p is involved in regulating the progression of prostate cancer and esophageal cancer by targeting Quaking 5 and Forkhead Box O1 (15,16). Recently, a study by Liu et al (40) reported that MEG3 can serve as a ceRNA for miR-9-5p to mediate the development of HCC through upregulating SOX11. To further explore the molecular mechanisms underlying the role of MEG3 in HCC, the present study investigated whether MEG3 exerts its protective effects on HCC via regulating miR-9-5p/MDK axis. In the present study, to verify the targetting relationship between MEG3, miR-9-5p and MDK, a dual luciferase reporter assay was performed. Consistent with previous studies (15,40), MEG3 directly targeted miR-9-5p in the present study. Subsequently, the present study demonstrated that MDK is a direct target of miR-9-5p. This finding was in agreement with a previous study which demonstrated that exosomal miR-9 targets MDK in nasopharyngeal carcinoma (22). In addition, the present study revealed that the expression of miR-9-5p was downregulated, and the expression of MDK was upregulated by MEG3, indicating that MEG3 can regulate the miR-9-5p/MDK axis in HCC. The PDK/AKT signaling pathway has been suggested to be linked with MDK in angiogenesis, and MDK knockdown led to the inhibition of PDK1 and AKT in HUVECs (22). In the present study, the results revealed that the PDK/AKT pathway was activated by MEG3 in HCC. Altogether, the findings of the present study demonstrated that MEG3 targets the miR-9-5p/MDK axis and modulates PDK/AKT pathway in HCC.

In the present study, the MHCC-97L cell line was chosen for gain-off-function experiments. Indeed, using at least one other HCC cell line would make the present study more convincing. It is a limitation of the present study that only 1 cell line was used. IncRNA and miRNA may also have negative feedback regulation. Another limitation of the present study is that it only demonstrated the effect of IncRNA MEG3 overexpression on miR-9-5p, without reverse validation. The MHCC-97L cell line is representative of HCC cell lines in previous studies (41,42). Li et al (41) only selected the MHCC-97L cell line to investigate the effects of miR-34a-5p on proliferation and chemical resistance of HCC cells (41). To examine the role of TIMP Metallopeptidase Inhibitor 2 (TIMP2) in HCC metastasis, Kai et al (42) chose the MHCC-97L cell line for research and found that the ectopic expression of the TIMP2 open reading frame in highly metastatic HCC cell line MHCC-97L significantly reduced the progress of HCC. Future studies should continue investigating the effect of IncRNA MEG3 on HCC, and to further explore the related mechanisms in different HCC cell lines.

In conclusion, the present study demonstrated that MEG3 affects HCC cell viability, apoptosis and migration through its targeting of miR-9-5p/MDK and regulation of the PDK/AKT pathway. The present study provided new insights into the molecular mechanisms of MEG3 in HCC, and suggested that the MEG3/miR-9-5p/MDK axis is a potential therapeutic target in HCC.

Acknowledgements

Not applicable.

Funding

This study was supported by the First-Class Discipline Construction Project in Guizhou Province [Chinese Pharmacy; grant no. (2017)008], Science and Technology Plan Project of
Guizhou Province [grant no. (2017)1073], and Department of Science and Technology Qian Ke He Platform Talent of Guizhou [grant no. (2017) 5655].

Availability of data and materials

The data sets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DW and QL designed the study, analyzed the data and prepared the manuscript. DW, ZM and DM conducted the experiments. All authors were substantially involved in the research, acquisition of data, analysis and manuscript preparation. DW and QL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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