LncRNA MEG3 affects cell proliferation, apoptosis and migration by targeting miR-9-5p/MDK axis and activating PDK/AKT pathway in hepatocellular carcinoma

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Research

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Abstract

Background

Long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3) was supposed to be a tumor suppressor in various cancers. However, the role of MEG3 in hepatocellular carcinoma (HCC) and the related molecular mechanisms are not well illustrated. This study aimed to determine the biological function of MEG3 in regulating HCC cell proliferation, apoptosis and migration. Moreover, the interaction among MEG3, microRNA (miR)-9-5p and Midkine (MDK), and the activation of phosphoinositide-dependent kinase (PDK)/AKT pathway in HCC cells were examined.

Methods and Results

Expression of MEG3 in a series of liver cancer cell lines was detected by RT-qPCR. Luciferase reporter assay, RT-qPCR and western blot were used to determine the interaction among MEG3, miR-9-5p and MDK, and the activation of PDK/AKT pathway. Cell proliferation and apoptosis were evaluated by CCK8, flow cytometry analysis for cell cycle and apoptosis, and Caspase 3/9 activity. Cell migration was determined by wound healing assay and MMP1 expression. We found MEG3 was decreased in HCC cell lines compared with the normal liver cell line. MEG3 suppressed HCC cell proliferation and migration, and induced cell apoptosis. Further, we found MEG3 targets miR-9-5p/MDK axis and modulates PDK/AKT pathway in HCC.

Conclusion

Our findings demonstrated that lncRNA MEG3 affects HCC cell proliferation, apoptosis and migration through its targeting of miR-9-5p/MDK and regulating of PDK/AKT pathway. This study suggested MEG3/miR-9-5p/MDK axis as the potential therapeutic target in HCC.

Background

Hepatocellular carcinoma (HCC), a malignant tumor arising from the liver, accounts for 80% of primary liver cancer [1]. It is the third leading cause of cancer-related deaths globally [2], and the 5-year survival rate of patients with HCC is as low as 30% [3]. Further understanding of hepatocarcinogenesis is imperative, and novel strategies on the treatment of HCC need to be developed.

Long non-coding RNAs (lncRNAs), a class of non-coding transcripts with lengths exceeding 200 nucleotides, participate in a variety of physiological and pathological processes including tumorigenesis [4]. lncRNA maternally expressed gene 3 (MEG3) has been reported to be abnormally expressed in HCC tissues [5, 6], however, its role in HCC and the related mechanisms are not well illustrated. MicroRNAs (miRs) are small (~22nt) non-coding RNAs which also participate in various biological processes. lncRNAs and miRNAs could interact with each other to serve a wide spectrum of biological functions [7,

8]. MEG3 has been reported to serve as a competing endogenous RNA (ceRNA) for miR-9-5p in prostate cancer [9] and esophageal cancer [10] to mediate tumor progression.

Midkine (MDK) is a growth factor which initially found in retinoic acid-induced embryonic tumor cells. Currently, MDK is viewed as a multifaceted factor promoting cell growth, survival, metastasis, migration, and angiogenesis, in particular during tumorigenesis [11]. Phosphoinositide-dependent kinase 1 (PDK1) is a serine / threonine protein kinase which plays central roles in signals transduction [12]. Several studies have shown that PDK1 can promote cell proliferation by activating Akt/mTOR signaling pathway [13, 14]. A recent study by Lu et al reported that miR-9 could inhibit angiogenesis by targeting MDK and regulating PDK/AKT pathway in nasopharyngeal carcinoma [15].

In this study, we examined the effect of MEG3 on HCC cell proliferation, apoptosis and migration. Further, we determined the interaction among MEG3, miR-9-5p and MDK, and the activation of PDK/AKT pathway in HCC cells.

Materials And Methods

Cell culture

Human normal liver cell line L02 and the liver cancer cell lines (MHCC-97H, MHCC-97L, Hep3B and HepG2) were purchased from Bnbio Company (Beijing, China). The cells were maintained in RPMI 1640 medium (Nanjing KeyGen Biotech. Co. Ltd., Nanjing, Jiangsu, China) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), at 37 °C in a humidified atmosphere of 5% CO₂.

Transfection

MiR-9-5p mimic, miR-9-5p NC, lncRNA MEG3 overexpression vector and lncRNA MEG3 NC were purchased from General Biosystems, Inc.(Hefei, Anhui,China). Cell transfection was conducted using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the cells using the Ultrapure RNA Kit (CW Biotech, Beijing, China), and then reverse-transcribed into cDNA using a HiFiScriptcDNA Synthesis Kit (CW Biotech). qRT-PCR assays were performed using the UltraSYBR Mixture (CW Biotech). Data were analyzed using the 2^{-ΔΔCt} method.

Western Blot

Total proteins were extracted, and protein concentrations were quantified via the bicinchoninic acid (BCA) method (Thermo Scientific). A total of 50 µg of protein was separated in 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking overnight at 4 °C in the 5% BSA, the PVDF membranes were incubated with the primary antibodies, including Rabbit Anti AKT (1/500), Rabbit Anti Caspase-3 (1/5000), Rabbit Anti Caspase-9 (1/2000), Rabbit Anti MDK (1/1000), Rabbit Anti MMP-1 (1/1000), Rabbit Anti PDK1 (1/2000) (Abcam, Cambridge, UK), Rabbit Anti p-AKT (Bioss, 1/1000), Rabbit Anti p-PDK1 (1/1000; Affinity Biologicals, Ancaster, ON, Canada) and Mouse Monoclonal Anti-GAPDH (1/2000; ZSBO, Beijing, China) at 4 °C overnight. Horseradish peroxidase-labeled secondary antibodies, including Goat Anti-Mouse IgG(H + L) and Goat Anti-Rabbit IgG(H + L) (ZSBO), were used at 1:2000 dilution to incubate the membranes at room temperature for 2 h. Signals were detected with the SuperSignal® west pico chemiluminescent substrate (Thermo Fisher Scientific).

Luciferase Reporter Assay

The wild-type lncRNA MEG3 3'-UTR and MDK 3'-UTR containing the putative binding sites of miR-9-5p were amplified and inserted into the firefly luciferase reporter vector. Cells were co-transfected with miR-NC/miR-9-5p mimic and the wild-type 3'-UTR of MDK or lncRNA MEG3 using Lipofectamine 3000. 48 h following transfection, firefly and *Renilla* luciferase activities were detected by the Dual-Luciferase Reporter Assay System (Beyotime, Shanghai, China). The firefly luciferase activity was normalized to the *Renilla* luciferase activity for each individual analysis.

CCK8

Cells were seeded at 1×10^4 cells/well in the 96-well plates. Then, the cells were maintained in RPMI1640 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. Absorbance at 450 nm was measured using a Tecan Safire II Microplate Reader (Tecan, Männedorf, Switzerland).

Flow Cytometry (FCM) Analysis Of Cell Cycle

FCM analysis of cell cycle was performed using a Cell Cycle Staining Kit (MultiSciences(Lianke) Biotech Co., Ltd., Hangzhou, Jiangsu, China) according to the manufacturer's instructions. The cell suspension was centrifuged at 2500 rpm for 3 min, and the cells were then fixed in ethanol at 4 °C for 2 h. After washing with PBS, 1 ml of DNA staining solution was added to the tubes. The cells were incubated at room temperature in the dark for 30 min, and cell cycle was analyzed by NovoCyte 2060R Flow Cytometer (ACEA Biosciences, Inc., San Diego, CA, USA).

Flow Cytometry Analysis Of Cell Apoptosis

Cell apoptosis rate was determined by flow cytometry using an Annexin V-FITC/PI Apoptosis Kit (MultiSciences(Lianke) Biotech Co., Ltd.). Briefly, 1×10^6 cells were washed with cold PBS twice, and re-suspended in 300 μ l Binding Buffer. After incubation with 3 μ l Annexin V-FITC and 3 μ l PI-PE at room temperature for 10 min in the dark, the cells were mixed with 200 μ l Binding Buffer, and cell apoptosis rate was analyzed using a NovoCyte 2060R Flow Cytometer (ACEA Biosciences, Inc.).

Wound Healing Assay

Cells were grown to basically 100% confluence in 6-well plates after transfection. The confluent monolayers were scratched by using a 10- μ l pipette tip, and then washed with PBS for 3 times. Photographs were taken immediately or 24 h after wounding, and migration was quantified.

Statistical Analyses

Statistical analyses were performed using SPSS v19.0 (IBM, Armonk, NY, USA). Experimental data were presented as the mean $\pm$ SD, and the statistical significance was assessed by oneway analysis of variance. $P < 0.05$ was considered significant.

Results

Expression of lncRNA MEG3 in liver cancer cell lines

Expression of lncRNA MEG3 in normal liver cell line L02 and liver cancer cell lines (MHCC-97H, MHCC-97L, Hep3B and HepG2) were detected by RT-qPCR. As shown in Fig. 1, compared with L02 cell line, the expression of lncRNA MEG3 in MHCC-97H, MHCC-97L, Hep3B and HepG2 cell lines was significantly decreased. Among these liver cancer cell lines, MHCC-97L showed the lowest lncRNA MEG3 expression. We chose MHCC-97L cell line for subsequent experiments.

Effect of lncRNA MEG3 on HCC cell proliferation, apoptosis and migration

lncRNA MEG3 overexpression vector was transfected into the MHCC-97L cells to investigate the effect of lncRNA MEG3 on HCC cell proliferation, apoptosis and migration. As demonstrated in Fig. 2a, lncRNA MEG3 NC did not affect lncRNA MEG3 expression. Compared with the lncRNA MEG3 NC group, the relative expression of lncRNA MEG3 was significantly increased in the lncRNA MEG3 group ($P < 0.01$). The effect of lncRNA MEG3 on cell proliferation was examined by CCK8 and FCM. The results from CCK8 assay showed that cell viability in the lncRNA MEG3 NC group was decreased significantly when compared with that in the lncRNA MEG3 group ($P < 0.05$; Fig. 2b). FCM analysis of cell cycle showed that the fraction of S-phase cells was significantly higher and the fraction of G2-phase cells was significantly lower in the lncRNA MEG3 group than in the lncRNA MEG3 NC group ($P < 0.05$; Fig. 2c and 2d). FCM analysis of cell apoptosis revealed that compared with the cells transfected with lncRNA MEG3

NC, cell apoptosis rate was increased significantly in the cells transfected with lncRNA MEG3 overexpression vector ($P < 0.05$; Fig. 3a and 3c). Western blot analysis showed that the protein levels of Caspase-3 and Caspase-9 were upregulated significantly in lncRNA MEG3-transfected cells ($P < 0.05$; Fig. 3b). Western blot results (Fig. 4a) indicated that the expression level of MMP1 was evidently lower in the lncRNA MEG3-transfected cells than in the lncRNA MEG3 NC-transfected cells ($P < 0.05$). Wound healing assay was performed to examine the effect of lncRNA MEG3 on cell migration. The results demonstrated that compared with the lncRNA MEG3 NC group, cell migration was inhibited in the lncRNA MEG3 group (Fig. 4b and 4c). lncRNA MEG3 NC did not show any effects on cell proliferation, apoptosis or migration.

lncRNA MEG3 Targets miR-9-5p, And miR-9-5p Targets MDK

We did luciferase reporter assay to investigate whether lncRNA MEG3 targets miR-9-5p. As shown in Fig. 5a, compared with the lncRNA MEG3 + miR-9-5p mimic NC group, the luciferase activity was decreased significantly in the lncRNA MEG3 + miR-9-5p mimic group ($P < 0.05$). Further, we examined whether MDK was a direct target of miR-9-5p. It was revealed that the luciferase activity was decreased significantly in the MDK + miR-9-5p mimic group when compared with that in the MDK + miR-9-5p mimic NC group ($P < 0.05$).

Effect Of lncRNA MEG3 On miR-9-5p And MDK Expression

The effect of lncRNA MEG3 on miR-9-5p expression was determined by RT-qPCR analysis. As shown in Fig. 5b, compared with the lncRNA MEG3 + miR-9-5p mimic NC group, the relative expression of miR-9-5p was decreased significantly in the lncRNA MEG3 + miR-9-5p mimic group ($P < 0.05$). The effect of lncRNA MEG3 on MDK expression was determined by western blot analysis. We found the relative protein level of MDK was increased significantly in the lncRNA MEG3 + miR-9-5p mimic group when compared with that in the lncRNA MEG3 + miR-9-5p mimic NC group ($P < 0.05$).

Effect Of lncRNA MEG3 On PDK/AKT Signaling

In order to verify the effect of lncRNA MEG3 on PDK/AKT signaling, western blot assay was performed to examine p-PDK1 and p-AKT expression. Compared with the lncRNA MEG3 NC group, the protein expression of p-PDK1 and p-AKT was upregulated in the lncRNA MEG3 group (both $P < 0.05$; Fig. 5c).

Discussion

The pathogenesis of HCC is complex, and numerous studies have shown that lncRNAs are involved in the initiation, development and metastasis of HCC [16]. Some studies have shown that MEG3 is significantly

downregulated in HCC tissues [5, 6]. In agreement with the reports in HCC tissues, we found MEG3 expression is also decreased in a series of HCC cell lines. MHCC-97L cell line showed the lowest expression of MEG3, thus we chose this HCC cell line for the subsequent assays.

Numerous studies reported that restoring MEG3 expression in cancer cells could suppress cell proliferation, invasion as well as angiogenesis, and induce cell apoptosis [17, 18]. These studies indicated that MEG3 is one of the lncRNAs with tumor suppressor activity [19]. In this study, we transfected MEG3 overexpression vector into MHCC-97L cells to investigate the effect of MEG3 on cell proliferation, apoptosis and migration in HCC. Our results demonstrated that MEG3 inhibits HCC cells proliferation, and a large number of cells accumulated in S phase. Similarly, the result presented by He et al confirmed that MEG3 overexpression suppressed clone formation of hepatoma cells [20]. Caspase proteins play important roles in the process of apoptosis. Caspase-9 is an initiator of apoptosis, and Caspase-3 is the final executor of apoptosis [21]. MEG3 overexpression led to increased Caspase-3 and Caspase-9 expression, as well as increased cell apoptosis rate, suggesting the promotive role of MEG3 in cell apoptosis. We found MMP1 expression was inhibited by MEG3. MMP1 is involved in invasion and metastasis during the development of cancer, and high expression of MMP1 is associated with poor prognosis of HCC patients [22]. The wound healing assay further verified the inhibitory effect of MEG3 on cell migration. These results were consistent with the previous studies (5,16), and suggested that MEG3 may be a tumor suppressor in HCC.

lncRNAs could serve as a kind of ceRNA to regulate target genes through interacting with miRNAs [23, 24]. Recent studies have clarified that lncRNA-miRNA-mRNA form a novel regulatory network in various pathophysiological processes [25]. The role of MEG3 in cancers has been reported in several studies in which the interaction between lncRNAs, miR and mRNA, has been highlighted [26, 27]. In glioma, MEG3 inhibited the proliferation, migration and invasion of glioma cells by regulating miR-96-5p/MTSS1 [28]. In multiple myeloma, MEG3 inhibited tumor progression through regulating the miR-181a/HOXA11 axis [29]. Particularly, previous studies have suggested that MEG3/miR-9-5p is involved in regulating the progression of prostate cancer and esophageal cancer by targeting QKI-5 and FOXO1. Recently, a study by Liu et al found that MEG3 can serve as a ceRNA for miR-9-5p to mediate the development of HCC through upregulating SOX11 [30]. To further explore the molecular mechanisms underlying the role of MEG3 in HCC, we investigated whether MEG3 exerts its protective effects in HCC via regulating miR-9-5p/MDK axis. In this study, to verify the targeting relationship between MEG3, miR-9-5p and MDK, a dual luciferase reporter assay was performed. Consistent with the above reports [9, 30], we found MEG3 directly targets miR-9-5p. Subsequently, we demonstrated that MDK is a direct target of miR-9-5p. This finding was in agreement with a previous study reporting that exosomal miR-9 targets MDK in nasopharyngeal carcinoma [15]. Further, we found the expression of miR-9-5p is downregulated, and the expression of MDK is upregulated by MEG3, indicating that MEG3 could 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 HUVEC [15]. In this study, the results revealed that PDK/AKT pathway was activated by MEG3 in HCC. Altogether, these findings demonstrated that MEG3 targets the miR-9-5p/MDK axis and modulates PDK/AKT pathway in HCC.

Conclusions

This study demonstrated that MEG3 affects HCC cell proliferation, apoptosis and migration through its targeting of miR-9-5p/MDK and regulating of PDK/AKT pathway. We provided new insights into the molecular mechanisms of MEG3 in HCC, and suggested MEG3/miR-9-5p/MDK axis as the potential therapeutic target in HCC.

Abbreviations

HCC, hepatocellular carcinoma; lncRNA, long non-coding RNA; MEG3, maternally expressed gene 3; miR, microRNA; MDK, Midkine; PDK, phosphoinositide-dependent kinase.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

DZW and QQL designed the study, analyzed the data and prepared the manuscript. DZW, ZM and DYM conducted the experiments. All authors were substantially involved in the research, acquisition of data, analysis and manuscript preparation. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

All authors declare that they have no conflict of interest.

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Figures

Figure 1

Expression of LncRNA MEG3 in normal liver cell line L02 and liver cancer cell lines (MHCC-97H, MHCC-97L, Hep3B and HepG2). * $P < 0.05$ compared with L02. LncRNA MEG3, long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3).

Figure 2

Effect of lncRNA MEG3 on HCC cell proliferation.(a) The relative expression of lncRNA MEG3 was significantly increased in the cells transfected with the lncRNA MEG3 overexpression vector.* $P<0.01$ compared with the lncRNA MEG3 NC group.(b)Cell viability determined by CCK8 assay.* $P<0.05$ compared with the lncRNA MEG3 NC group.(c,d) Cell cycle determined by flow cytometry. * $P<0.05$ compared with the lncRNA MEG3 NC group.lncRNA MEG3, long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3); HCC, hepatocellular carcinoma.

Figure 3

Effect of lncRNA MEG3 on HCC cell apoptosis.(a,c)Cell apoptosis rate determined by flow cytometry.* $P<0.01$ compared with the LncRNA MEG3 NC group.(b)Relative protein expression of Caspase-3 and Caspase-9 determined by western blot.* $P<0.05$ compared with the LncRNA MEG3 NC group.LncRNA MEG3, long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3); HCC, hepatocellular carcinoma.

Figure 4

Effect of lncRNA MEG3 on HCC cell migration.(a)Relative protein expression of MMP1 determined by western blot.* $P < 0.05$ compared with the LncRNA MEG3 NC group.(b,c)Cell migration determined by wound healing assay.* $P < 0.05$ compared with the LncRNA MEG3 NC group. LncRNA MEG3, long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3); HCC,hepatocellular carcinoma.

Figure 5

The interaction among MEG3, miR-9-5p and MDK, and the effect of MEG3 on PDK/AKT pathway activation. (a) LncRNA MEG3 targets miR-9-5p (* $P < 0.05$ compared with the LncRNA MEG3+miR-9-5p mimic NC group), and miR-9-5p targets MDK (* $P < 0.05$ compared with the MDK+miR-9-5p mimic NC group), as determined by luciferase reporter assay. (b) Effect of LncRNA MEG3 on miR-9-5p and MDK expression. * $P < 0.05$ compared with the LncRNA MEG3 NC group. (c) Effect of LncRNA MEG3 on PDK/AKT

signaling.* $P < 0.05$ compared with the LncRNA MEG3 NC group. LncRNA MEG3, long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3); miR, microRNA; MDK, Midkine; PDK1, phosphoinositide-dependent kinase 1.