IncRNA MEG3 inhibits the growth of hepatocellular carcinoma cells by sponging miR-9-5p to upregulate SOX11

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Abstract

The long non-coding RNA (lncRNA) maternally expressed gene 3 (MEG3), a tumor suppressor, is critical for the carcinogenesis and progression of different cancers, including hepatocellular carcinoma (HCC). To date, the roles of IncRNA MEG3 in HCC are not well illustrated. Therefore, this study used western blot and qRT-PCR to evaluate the expression of MEG3, miR-9-5p, and Sex determining Region Y-related HMG-box 11 (SOX11) in HCC tissues and cell lines. RNA pull-down and luciferase reporter assay were used to evaluate these molecular interactions. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and flow cytometry detected the viability and apoptosis of HCC cells, respectively. The results showed that MEG3 and SOX11 were poorly expressed but miR-9-5p was highly expressed in HCC. The expression levels of these molecules suggested a negative correlation between MEG3 and miR-9-5p and a positive correlation with SOX11, confirmed by Pearson’s correlation analysis and biology experiments. Furthermore, MEG3 could combine with miR-9-5p, and SOX11 was a direct target of miR-9-5p. Moreover, MEG3 over-expression promoted cell apoptosis and growth inhibition in HCC cells through sponging miR-9-5p to up-regulate SOX11. Therefore, the interactions among MEG3, miR-9-5p, and SOX11 might offer a novel insight for understanding HCC pathogenesis and provide potential diagnostic markers and therapeutic targets for HCC.

Key words: Hepatocellular carcinoma; MEG3, miR-9-5p; SOX11; lncRNA

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths globally, accounting for approximately 90% of liver cancer (1,2). There are a variety of risk factors for HCC, mainly including obesity, smoking, alcohol consumption, liver cirrhosis, and chronic infection with hepatitis B and hepatitis C (3). Surgical resection, orthotopic liver transplantation, transarterial chemoembolization, radiofrequency ablation, and systemic chemotherapies are available treatments for HCC patients (4). Nevertheless, metastasis and recurrence still occur in varying degrees (4,5). New options to improve HCC patient survival rate are still badly needed, and exploring the molecular mechanisms of HCC may offer new insights into HCC treatment.

Numerous reports suggest that the gene regulatory networks are relevant for the development of cancer (6). Long non-coding RNAs (lncRNAs; containing >200 nucleotides) are known as key regulators for various cellular processes at transcriptional, posttranscriptional, and epigenetic levels (7,8). The regulation of lncRNA for biological processes has diverse molecular mechanisms such as RNA-RNA, RNA-protein, and RNA-DNA interactions (9). For instance, IncRNA maternally expressed gene 3 (MEG3) has been reported to function as a tumor suppressor in various cancers such as hemangioma (10), osteosarcoma (11), and thyroid carcinoma (12), and it can serve as a ceRNA for miR-9-5p to mediate the progression of esophageal cancer (13) and prostate cancer (14). Although IncRNA MEG3 has been demonstrated to be down-regulated in HCC (15,16), the roles of the interaction between MEG3 and miR-9-5p in HCC have not been reported.

Sex determining region Y-related HMG-box 11 (SOX11) is a type of structurally-related transcription factor and belongs to group C of SOX family (17,18). SOX11 is critical for regulating organ development, particularly in the nervous system (19,20). Accumulating evidence shows that SOX11 is involved in the biological processes of many human cancers (21), whereas the impact of SOX11 on the progression of different cancers is still controversial and
seems to be determined by cancer types (18,22,23). It has been reported that SOX11 is poorly expressed in HCC tissues (24) and yet, the roles of SOX11 on HCC are not well illustrated.

In the present study, we determined the expression of MEG3, miR-9-5p, and SOX11 in HCC tissues, and explored their interactions in HCC. Furthermore, we detected the effect of these molecules on HCC cell growth and apoptosis.

**Material and Methods**

**Human specimens**
A total of 30 pairs of hepatocellular carcinoma (HCC) tissues and the corresponding adjacent normal tissues were obtained from HCC patients undergoing surgery at the Affiliated Hospital of North Sichuan Medical College (China). None of the patients had received chemotherapy or radiotherapy prior to surgery. Before surgery, written informed consent was collected from HCC patients. This work was approved by the Affiliated Hospital of North Sichuan Medical College’s Ethics Committee. The clinical characteristics of the HCC patients are summarized in Table 1.

**Cell culture and transfection**
Human embryonic kidney cell line (293T) and Human HCC cell lines (SK-HEP-1 and Huh7) were purchased from Institute of Biochemistry and Cell Biology (China), and cultured in complete DMEM medium (Gibco, USA). The plasmid for mediating MEG3 overexpression (pcDNA-MEG3), the control plasmid (pcDNA-Vector), siRNAs for knockdown of MEG3 (MEG3 siRNA1 and MEG3 siRNA2), and the control siRNA (NC siRNA) were obtained from GenePharma (China). After adding to twelve-well plates, SK-HEP-1 and Huh7 cells were transfected with pcDNA-MEG3, pcDNA-Vector, MEG3 siRNA1, MEG3 siRNA2, or NC siRNA for 24–48 h using lipofectamine 3000 according to the manufacturer’s protocol (Invitrogen, USA). The transfected cells were harvested for the following experiments.

| Characteristics                  | Number of patients | High MEG3 expression (%) | Low MEG3 expression (%) | P value |
|----------------------------------|--------------------|--------------------------|-------------------------|---------|
| Gender                           |                    |                          |                         | 0.526   |
| Male                             | 23                 | 10 (43.48)               | 13 (56.52)              |         |
| Female                           | 7                  | 4 (57.14)                | 3 (42.86)               |         |
| Age (years)                      |                    |                          |                         | 0.654   |
| <55                              | 18                 | 9 (50.00)                | 9 (50.00)               |         |
| ≥55                              | 12                 | 5 (41.67)                | 7 (58.33)               |         |
| Tumor size (cm)                  |                    |                          |                         | 0.464   |
| <5                               | 15                 | 8 (53.33)                | 7 (46.67)               |         |
| ≥5                               | 15                 | 6 (40.00)                | 9 (60.00)               |         |
| Serum AFP (ng/mL)                |                    |                          |                         | 0.818   |
| <20                              | 7                  | 3 (42.86)                | 4 (57.14)               |         |
| ≥20                              | 23                 | 11 (47.83)               | 12 (52.17)              |         |
| HBsAg                            |                    |                          |                         | 0.873   |
| Negative                         | 9                  | 4 (44.44)                | 5 (55.56)               |         |
| Positive                         | 21                 | 10 (47.62)               | 11 (52.38)              |         |
| Liver cirrhosis                  |                    |                          |                         | 0.919   |
| Absence                          | 11                 | 5 (45.45)                | 6 (54.55)               |         |
| Presence                         | 19                 | 9 (47.37)                | 10 (52.63)              |         |
| Histological differentiation     |                    |                          |                         | 0.727   |
| Well                             | 4                  | 2 (50.00)                | 2 (50.00)               |         |
| Moderate                         | 13                 | 5 (38.46)                | 8 (61.54)               |         |
| Poor                             | 13                 | 7 (53.85)                | 6 (46.15)               |         |
| TNM stage                        |                    |                          |                         | 0.028   |
| I + II                           | 15                 | 10 (66.67)               | 5 (33.33)               |         |
| III + IV                         | 15                 | 4 (26.67)                | 11 (73.33)              |         |
| Metastasis                       |                    |                          |                         | <0.001  |
| No                               | 15                 | 12 (80.00)               | 3 (20.00)               |         |
| Yes                              | 15                 | 2 (13.33)                | 13 (86.67)              |         |

Data are reported as number and percentage. Chi-squared test was used to compare groups.
**Quantitative real-time PCR (qRT-PCR)**

Total RNA was isolated from each tumor sample and HCC cells by TRizol reagent (Invitrogen), and then reverse transcribed with the First Strand cDNA synthesis kit (New England Biolabs (Beijing) LTD., China). We performed amplifications with a SYBR Green PCR kit (Applied Biological Materials, Canada) according to the manufacturer’s instructions on Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, USA). The expression of RNA was normalized against GAPDH using the $2^{ΔΔCt}$ method. The PCR primers used are shown in Table 2. Three separate experiments were performed.

**Western blot**

The tissues and cell lines of HCC were lysed by an ice-cold RIPA lysis buffer (Beyotime, China) containing a protease inhibitor cocktail (Sigma-Aldrich, USA). The lysates were quantified using BCA methods and equally loaded on SDS-PAGE gels and electroblotted onto polyvinylidene fluoride (PVDF) membranes. After blocking the membranes with 5% skim milk powder, we incubated the membranes with the primary antibodies (1:1000 dilution) against SOX11 (Epitomics, USA), Bcl-2 (Cell Signaling Technology, USA), cleaved caspase-3 (Cell Signaling Technology), cleaved PARP (Cell Signaling Technology), and GAPDH (Cell Signaling Technology) overnight at 4°C. Next, we cultured the membranes with HRP-conjugated secondary antibodies for 2 h at 25°C. Finally, the membranes were visualized using ECL-PLUS/Kit (GE Healthcare, USA) and the protein was quantified using ImageJ software (National Institutes of Health, USA).

**RNA pull-down assay**

RNA pull-down assay was used to detect whether IncRNA MEG3 is relevant for miR-9-5p. In this assay, the biotin-labeled MEG3, as a probe, or random pull-down probe sequence, as negative control (NC), were reversely biotin-labeled MEG3, as a probe, or random pull-down probe. miR-9-5p, or mutant pull-down probe. GAPDH reverse AGGGGTCTACATGGCAACTG

**Results**

Expression of MEG3, miR-9-5p, and SOX11 in HCC tissues

To determine the expression of MEG3, miR-9-5p, and SOX11 in HCC tissues, we analyzed their expressions using qRT-PCR. The results revealed that the expression

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**Table 2. Primer sequence.**

| Primer       | Sequence (5′-3′)                  |
|--------------|----------------------------------|
| MEG3 forward | CTGCCCATCTACACCTCAG              |
| MEG3 reverse | CTCTCCGCGCTCGCTGAGGGGTG          |
| miR-9-5p forward | GTGAGGCTTCGAGGT                  |
| miR-9-5p reverse | GCGCTCTTTGGTATCTAGC              |
| SOX11 forward | GGTGGAGGTTGAGTTGAGTTG            |
| SOX11 reverse | GTCGCGCCGTCGACGTGAGT             |
| GAPDH forward | CAAGAATTGTGGCTACAGGA             |
| GAPDH reverse | AGGAGTCTACATGGCAACTG             |
levels of MEG3 and SOX11 were down-regulated but miR-9-5p was highly expressed in HCC tissues compared to the corresponding adjacent normal tissues (Figure 1A). SOX11 was poorly expressed in HCC tissues compared to the adjacent normal tissues, confirmed by western blot (Figure 1B). Furthermore, Pearson’s correlation analysis indicated that lncRNA MEG3 had a negative correlation with miR-9-5p and displayed a positive correlation with SOX11 in HCC tissues. There was a negative correlation between SOX11 and miR-9-5p (Figure 1C).

MEG3 served as a sponge for miR-9-5p in HCC cells
The correlation between MEG3 and miR-9-5p was further explored in HCC cells. Their interaction was firstly determined by RNA pull-down assay and qRT-PCR. Compared with the control RNA, a significant enrichment of miR-9-5p in the MEG3 pulled-down pellet was observed (Figure 2A). Thus, MEG3 could directly combine with miR-9-5p.

As shown in Figure 2B and C, the expression of MEG3 was significantly up-regulated and down-regulated after

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Figure 1. A, qRT-PCR detected the expression of MEG3, SOX11, and miR-9-5p in hepatocellular carcinoma tissues (HCC). B, Western blot was utilized to measure the protein expression of SOX11 in five random HCC tissues. C, Interactions among SOX11, miR-9-5p, and MEG3 were assessed by Pearson’s correlation analysis. Data are reported as means ± SD. **P<0.05, ***P<0.01, vs adjacent tissue (A, control group) (Student’s t-test). T: Tumor tissue.
Figure 2. A, qRT-PCR detected the expression of MEG3 in hepatocellular carcinoma (HCC) cells transfected with pcDNA-MEG3 (MEG3) or control vector. B, RNA pull-down assay was used to determine the relative miR-9-5p enrichment in HCC cells, and the mRNA level was measured using qRT-PCR. C, qRT-PCR determined MEG3 expression in HCC cells transfected with MEG3 siRNA1, MEG3 siRNA2, or control siRNA (NC siRNA). D, Relative expression of mi-9-5p was assessed using qRT-PCR after HCC cells transfection with pcDNA-MEG3 (MEG3), control vector, MEG3 siRNA1, MEG3 siRNA2, or control siRNA (NC siRNA). Data are reported as means ± SD. **P<0.05, ***P<0.01 vs control group (ANOVA).
HCC cells transfected with pcDNA-MEG3 (MEG3) and MEG3 siRNAs (MEG3 siRNA1 or MEG3 siRNA2), respectively.

The interaction between MEG3 and miR-9-5p was further assessed by qRT-PCR. Compared with the control group, miR-9-5p expression in HCC cells was decreased by the transfection of pcDNA-MEG3, while miR-9-5p expression in HCC cells was enhanced after MEG3 siRNAs transfection (Figure 2D). Therefore, MEG3 served as a sponge for miR-9-5p in HCC cells.

Relationship among MEG3, miR-9-5p, and SOX11 in HCC cells

StarBase (http://starbase.sysu.edu.cn/starbase2/index.php) and mirBase software (http://www.mirbase.org) were used to predict the targeting relationship between SOX11 and miR-9-5p (Figure 3A). As shown in Figure 3B, miR-9-5p expression in 293T cells was significantly up-regulated after miR-9-5p mimic transfection, and thus the miR-9-5p mimic transfection was effective. Additionally, the luciferase reporter assay suggested that HCC cell lines (huh7 and SK-HEP-1) co-transfected with miR-9-5p mimics and SOX11-WT showed a weakened luciferase activity in comparison to the control group (miR control mimics + SOX11-WT) (Figure 3C). Furthermore, western blot indicated that SOX11 expression was decreased by miR-9-5p mimic transfection (Figure 3D). Data suggested that SOX11 was a direct target of miR-9-5p. In addition, the negative correlation between SOX11 and miR-9-5p was consistent with results shown in Figure 1C.

The relationship between MEG3 and SOX11 in HCC cells was further validated by western blot. As shown in Figure 3E, pcDNA-MEG3 greatly increased the expression of SOX11, whereas MEG3 siRNA decreased SOX11 expression in comparison to the control group. Hence, MEG3 could enhance the expression of SOX11 and a positive correlation between MEG3 and SOX11 was consistent with results shown in Figure 1C.

MEG3 regulated the growth of HCC cells via mediating miR-9-5p

To detect the role of MEG3 on HCC cells, MTT, flow cytometry, and western blot were used in this study. MTT assay revealed that MEG3 over-expression induced by pcDNA-MEG3 transfection considerably inhibited the viability of HCC cells, while the cell viability was reversed by the introduction of miR-9-5p mimics (Figure 4A). Thus, miR-9-5p mimics could reverse the inhibition of HCC cell viability induced by MEG3 over-expression.

Flow cytometry analysis showed MEG3 over-expression (huh7 cell apoptotic rate, 31.15%; SK-HEP-1 cell apoptotic rate, 33.47%) significantly induced the apoptosis of HCC cell compared to MEG3 plasmid combined with miR-9-5p mimics transfection group (huh7 cell, 18.69%; SK-HEP-1cell, 22.56%) and the control group (pcDNA + miR mimics) (huh7 cell, 18.19%; SK-HEP-1cell, 21.89%). In addition, western blot demonstrated that the low expression of the anti-apoptosis protein Bcl-2 induced by MEG3 over-expression plasmid transfection was greatly changed by the transfection of miR-9-5p (Figure 4C). Furthermore, the expression of pro-apoptosis proteins cleaved PARP and cleaved caspase-3 was up-regulated by MEG3 over-expression plasmid transfection, whereas co-transfection with pcDNA-MEG3 and miR-9-5p considerably decreased their expression (Figure 4C). Collectively, MEG3 over-expression promoted the apoptosis of HCC cells, but this situation could be changed by miR-9-5p mimics introduction.

Additionally, the high expression of SOX11 in MEG3 over-expression group was remarkably decreased by the introduction of miR-9-5p mimics (Figure 4C). Therefore, MEG3 over-expression inhibited the viability of HCC cells, induced cell apoptosis, and enhanced SOX11 expression by mediating miR-9-5p.

MEG3 regulated the growth of HCC cells via sponging miR-9-5p to regulate SOX11 expression

On the one hand, it has been reported that SOX11 overexpression promotes apoptosis and growth inhibition in HCC cell (24); on the other, a negative correlation between MEG3 and miR-9-5p and a positive correlation with SOX11 have been confirmed. We speculated that the inhibition of MEG3 over-expression on HCC cells might be through sponging miR-9-5p to regulate SOX11 expression. In Figure 5A and B, the transfection of SOX11 siRNA (siSOX11) in HCC cells was effective, which was validated by qRT-PCR and western blot. As shown in Figure 5C, MEG3 over-expression inhibited cell viability and yet, siSOX11 introduction reversed the situation. Thus, SOX11 knockdown could improve the viability in HCC cells transfected with pcDNA-MEG3.

In addition, the apoptotic rates of Huh7 cells transfected with control plasmid, pcDNA-MEG3, or combined with siSOX11 were 23.6, 40.5, and 25.7%, respectively; the apoptotic rates of SK-HEP-1 cells transfected with control plasmid, pcDNA-MEG3, or combined with siSOX11 were 23.5, 39.2 and 24.9%, respectively (Figure 5D). Western blot further confirmed that the low expression of Bcl-2 and the high expression of SOX11, cleaved PARP, and cleaved caspase-3 induced by pcDNA-MEG3 transfection were changed via the introduction of siSOX11 (Figure 5E). Thus, MEG3 over-expression induced the apoptosis of HCC cells, which could be changed by knockdown of SOX11.

Collectively, MEG3 promoted apoptosis and growth inhibition in HCC cell through sponging miR-9-5p to up-regulate SOX11 expression.
Discussion

This study observed the low expression of MEG3 and SOX11 and the high expression of miR-9-5p in HCC. A negative correlation between MEG3 and miR-9-5p and a positive correlation with SOX11 were demonstrated. MEG3 acted as a sponge of miR-9-5p, and SOX11 was a direct target of miR-9-5p. Moreover, MEG3 regulated the growth of HCC cells via sponging miR-9-5p to regulate SOX11 expression.

The molecular mechanism of HCC pathology needs to be explored, as HCC possesses a high mortality rate and lacks effective therapies (1,2). lncRNAs have been reported to be relevant with many cancer-related molecular mechanisms (25). lncRNA regulatory networks (e.g. RNA-RNA, RNA-protein, and RNA-DNA interactions) are critical for cellular processes (9). In the present study, we selected tumor suppressor lncRNA MEG3 and its related molecules (miR-9-5p and SOX11) to explore the effects of those interactions on HCC. We measured the expression of MEG3, miR-9-5p, and SOX11 in HCC tissues. Compared to the normal tissues, the results showed that MEG3 and SOX11 were poorly expressed but miR-9-5p was highly expressed in HCC tissues, which is consistent with previous studies (24,26–28). In addition, their expression levels indicated a negative correlation between MEG3 and miR-9-5p and a positive correlation with SOX11.

Several studies suggest that lncRNAs can interact with miRNAs and act as miRNAs sponges to regulate the biological process of cancer (29). lncRNA MEG3 has demonstrated to suppress cell proliferation and induce cell apoptosis, leading to inhibition of tumorigenesis via different regulatory mechanisms (10). MEG3 serves as a ceRNA by sponging miR-183 to regulate the cell growth of pancreatic neuroendocrine tumor (30). Additionally, the interaction between MEG3 and miR-9-5p has been found in prostate cancer, and MEG suppresses prostate cancer progression via mediating miR-9-5p (14). Consistent with previous reports, our study revealed that MEG3 could combine with miR-9-5p and serve as a sponge for
miR-9-5p. MEG3 negatively mediated miR-9-5p expression in HCC cells.

miRNAs regulate target gene expression to mediate almost all key biological processes, e.g., cell differentiation, apoptosis, and proliferation (31). Recently, studies have shown that miR-9-5p is relevant to the progression of several cancers (32). Li et al. (33) have found that miR-9-5p can promote lung cancer cell proliferation and migration via suppressing the target gene TGFBR2 expression. Wang et al. have suggested that miR-9-5p mediates GOT1 to suppress cell proliferation, invasion, and glutamine metabolism in pancreatic cancer (34). In this study, the results showed that miR-9-5p was highly expressed in HCC tissues and cells. Moreover, we used StarBase and mirBase software to predict that SOX11 was a direct target of miR-9-5p. The targeting relationship between SOX11 and miR-9-5p was further confirmed by luciferase reporter assay, and this relationship was found for the first time. Furthermore, the data demonstrated that miR-9-5p could negatively mediate SOX11 expression in HCC cells.

In conclusion, this study found that MEG3 was poorly expressed in HCC tissues and cells. Moreover, MEG3 over-expression could regulate the miR-9-5p/SOX11 axis to inhibit cell growth and induce apoptosis in HCC. Therefore, our findings might provide novel molecular mechanisms for understanding the tumorigenesis of HCC and offer potential diagnostic markers and therapeutic targets for HCC.

Figure 4. MEG3 regulated the growth of hepatocellular carcinoma (HCC) cells via mediating miR-9-5p. HCC cells were transfected with pcDNA-MEG3 (MEG3) + control mimics (miR mimics), pcDNA-MEG3 (MEG3) + miR-9-5p mimics, or control vector + control mimics (miR mimics), and then (A) the transfected cell viability was assessed using MTT. B. The transfected cell apoptosis was measured using flow cytometry. C. The protein expression levels of BCL-2, cleaved PARP, cleaved caspase-3, and SOX11 were measured by western blot. Data are reported as means ± SD. **P < 0.05, ***P < 0.01 (ANOVA or t-test).
Figure 5. MEG3 regulated the growth of hepatocellular carcinoma (HCC) cells through sponging miR-9-5p to regulate SOX11 expression. **A**, mRNA and **B** protein expression of SOX11 were assessed using qRT-PCR and western blot, respectively. HCC cells were then transfected with pcDNA-MEG3 (MEG3) + siRNA control (NC), pcDNA-MEG3 (MEG3) + siSOX11, or control vector + siRNA control (NC). **C**, Viability of HCC cells was detected by MTT. **D**, Apoptosis rate of HCC cells was determined using flow cytometry. **E**, Protein expression levels of BCL-2, cleaved PARP, cleaved caspase-3, and SOX11 were measured using western blot. Data are reported as means ± SD. ***P < 0.01 (ANOVA or t-test).
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