Inhibiting roles of FOXA2 in hepatocellular carcinoma cell migration and invasion by transcriptionally suppressing microRNA-103a-3p and activating the GREM2/LATS2 axis

Guangzhen Ma

the Second People's Hospital of Liaocheng

Jirong Chen

Suizhou Hospital Affiliated to Hubei Medical College

Tiantian Wei

Suizhou Hospital Affiliated to Hubei Medical College

Jia Wang

Suizhou Hospital Affiliated to Hubei Medical College

Wenshan Chen (✉ wenshan010501@163.com)

Suizhou Hospital Affiliated to Hubei Medical College <https://orcid.org/0000-0003-1221-325X>

Research

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Abstract

Background Forkhead box A2 (FOXA2) is a transcriptional activator for liver-specific genes. Hepatocellular carcinoma (HCC) is a prevalent fetal malignancy across the globe. This work focused on the role of FOXA2 in HCC cell migration and invasion and the involving molecules.

Methods FOXA2 expression in HCC tissues and cells was determined using RT-qPCR. Altered expression of FOXA2 was introduced to identify its role in HCC cell migration and invasion using Transwell assays. The potential target microRNA (miRNA) of FOXA2 was predicted via online prediction and validated through a ChIP assay, and the mRNA target of miRNA-103a-3p was predicted and confirmed through a luciferase assay. The roles of miR-103a-3p and GREM2 in HCC cell invasion and migration were determined, and the downstream molecules mediated by GREM2 were analyzed.

Results FOXA2 and GREM2 were poorly expressed while miR-103a-3p was abundant in HCC tissues and cells. Overexpression of FOXA2 or GREM2 suppressed migration and invasion of HepG2 and SK-HEP-1 cells, while up-regulation of miR-103a-3p led to reverse trends. FOXA2 transcriptionally suppressed miR-103a-3p to increase GREM2 expression, and silencing of GREM2 partially blocked the inhibitory effects of FOXA2 on cell migration and invasion. GREM2 increased LATS2 activity and YAP phosphorylation and degradation.

Conclusion This study evidenced that FOXA2 inhibits migration and invasion potentials of HCC cell lines through suppressing miR-103a-3p transcription. The following upregulation of GREM2 plays key roles in migration inhibition by promoting LATS2 activity and YAP phosphorylation. This study may offer new insights into HCC treatment.

Introduction

Though most of the risk factors such as obesity, excessive alcohol consumption, smoking, and hepatitis B and C virus (HBV and HCV) infection are potentially preventable, the incidence rate of liver cancer (LC) is increased most rapidly among human cancers by an annual 2–3% increase during 2007 to 2016 [1]. LC ranks the second highest causes of cancer death worldwide with hepatocellular carcinoma (HCC) representing the most common type [2]. China has a particularly high mortality and morbidity rate with estimated 466,100 new diagnoses and 422,100 deaths according to the cancer statistics in 2015, accounting for over half of all cases worldwide [3, 4]. In addition, due to the difficulty in early diagnosis, high malignancy with rapid progression, and the lacking of effective targeted drugs, the survival rate of LC is seriously low [5]. Though surgical resection, liver transplantation and ablation are promising curative strategies for HCC patients, these operations are only available for patients at early stages characterized by limited tumor in liver [6, 7]. Unfortunately, most patients are found at late stages with frequent malignant metastasis. Hence, developing novel ways in metastasis control may provide novel therapeutic options for HCC treatment.

Forkhead box A2 (FOXA2) is a member of the forkhead class of DNA-binding proteins that are transcriptional activators for the regulation of cell differentiation and metabolism [8, 9]. FOX proteins may influence multiple cellular processes that are correlated with the initiation, metastasis, and development of cancers by different regulation manners with diverse networks [10]. Interestingly, a previous study noted the silencing of FOXA2 by microRNA (miR)-92a could increase HCC cell proliferation and invasion [11]. However, the exact role of FOXA2 in HCC cell migration and invasion and the mechanisms remain largely unstudied. Transcription factors are capable of binding to promoter regions of downstream target genes [12] including miRNAs. miRNAs are the mostly studied non-coding RNAs that are often involved in FOXA-mediated events [13]. miRNAs comprise 17–25 nucleotides with a major role in gene degradation by binding to the target complementary mRNAs in a post-transcriptional level [14]. Aberrant expression of miRNAs has been broadly found in human malignancies, and they function either as oncogenes or tumor suppressors through the different post-transcriptional modifications in genes [15]. Unsurprisingly, a large number of miRNAs have been noted to play key roles in HCC pathogenesis by mediating cell malignancy and tumor metastasis, thus may serve as potential therapeutic approaches [16]. Here, our study validated that FOXA2 could bind to miR-103a-3p, which was found to directly target gremlin 2 (GREM2). miR-103a-3p has been noted to work as a tumor promoter in oral squamous cell carcinoma [17]. On the other hand, it was suggested to suppress invasion and proliferation of prostate cancer cells [18]. But its function in HCC has not been investigated yet. As for GREM2, it is suggested to be lowly expressed in HCC through the data on GEPIA (<http://gepia.cancer-pku.cn/>). Taken together, we hypothesized that FOXA2 could inhibit HCC metastasis through suppressing miR-103a-3p and the following up-regulation of GREM2, with cell migration and invasion experiments performed to validate the hypothesis.

Materials And Methods

Clinical tissue sample collection

From January 2018 to February 2019, 50 pairs of HCC tissues and the adjacent normal tissues (at least 3 cm away) were collected from HCC patients who treated in the Second People's Hospital of Liaocheng. The tissues were collected during surgery and instantly frozen at -80°C. There were 39 male (78%) and 11 female (22%) patients with an average age of 51 ± 11.3 years. All enrolled patients were diagnosed by pathological examination without surgery history or other malignancies.

Cell culture and treatment

Immortalized human liver cell line THLE-2 and HCC cell lines (HepG2 and SK-HEP-1) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Frederick, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), streptomycin (0.1 mg/mL) and penicillin (100 U/mL) at 37°C with 5% CO₂ with constant humidity.

miR-103a-3p mimic and small interfering RNAs (siRNAs) targeting GREM2 and LATS2 (siRNA-GREM2, siRNA-LATS2) were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). Meanwhile,

the sequences of FOXA2 and GREM2 were synthesized and cloned to the pcDNA3.1 vector (pcDNA-FOXA2, pcDNA-GREM2, Invitrogen). The empty pcDNA3.1 vector and negative control (NC) for siRNA (siRNA-NC) and miRNA control (NC) were constructed as well. The vectors were transfected into cells using Lipofectamine 3000 Reagent (Invitrogen).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA in cells and tissues was extracted using the TRIzol Reagent (Invitrogen). The RNA was reversely transcribed into cDNA utilizing a SuperScript RT kit (Invitrogen) as per the kit's protocol. Next, real time qPCR was conducted using SYBR Green (Applied Biosystems, Foster City, CA, USA) on a CFX96 System (Bio-Rad, Hercules, CA, USA) as per the manufacturer's protocols. U6 was served as the internal reference for miR-103a-3p, while GAPDH for other genes. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. Each procedure was performed 3 times. The primers are presented in Table 1.

Transwell assay

Invasion and migration of HCC cells was determined using Transwell assays. As for cell migration, 24 hours after transfection, a total of 2×10^4 cells were seeded into the apical chamber supplemented with FBS-free RPMI-1640, while each basolateral chamber was loaded with 600 μ L DMEM with 10% FBS. Then Transwells were placed in an incubator at 37°C for 24 hours. The cells in the apical chamber and on the upper surface were wiped out by cotton swabs. The migrated cells were fixed in 4% paraformaldehyde (Beyotime Biotechnology Co., Ltd., Shanghai, China) for 20 minutes and then stained with 0.5% crystal violet (Beyotime) for 15 minutes. The invasion of cells was determined in a similar manner with the apical chambers pre-coated with Matrigel (BD-Biosciences, CA, USA) before cell seeding. The number of invaded and migrated cells were observed and counted under an IX81 microscope (Olympus, Tokyo, Japan) with 5 fields randomly selected and the average value was calculated.

Dual luciferase reporter gene assay

Luciferase reporter plasmids were constructed with the ligation of oligonucleotides containing the wide type (WT) and mutant type (MT) binding sequences based on the putative target sites of GREM2 3'UTR and miR-103a and inserted into GV272 vectors (Genechem Co., Ltd., Shanghai, China). Well-constructed vectors were co-transfected with miR-103a mimic or mimic NC into HepG2 and SK-HEP-1 cells using the Lipofectamine 2000 (Invitrogen). Forty-eight hours later, the cells were lysed, and the luciferase activity was measured using a Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) as per the manufacturer's protocols. Three independent experiments were performed.

Chromatin immunoprecipitation (ChIP) assay

Cells were treated with 37% formaldehyde, collected and subjected to ultrasonication using a VCX750 (SONICS, USA) at a power of 25%. The cells were ultrasonicated 4.5 seconds each time for a total of 14 times at a 9-second interval. Thereafter, the cells were centrifuged at 10000 g at 4°C for 10 minutes to

discard the insolubles. Next, the antibody against FOXA2 (ab256493, Abcam, Cambridge, UK) and the control IgG antibody (ab6721, Abcam) were added to combine with the target protein-DNA compound. Next, Protein A was further administrated to precipitate with the antibody-target protein-DNA compound. The precipitate was successfully subjected to 1) low salt wash buffer-one wash; 2), high salt wash buffer-one wash; 3) LiCl wash buffer-one wash; 4) TE buffer-two washes. After that, the precipitate was eluted and decrosslinked. The enriched DNA sequences were purified for qPCR. The primer sequence used were: Forward: 5'-TCACGGACATTCCAGG-3'; Reverse: 5'-TCGCAGTATTGCGACG-3'.

Western blot analysis

Cells were lysed by Radio-Immunoprecipitation assay cell lysis buffer on ice and then centrifuged at 14,000 rpm for 20 minutes with the lysis buffer discarded to collect total protein. The protein concentration was determined using a bicinchoninic acid kit (Pierce, Rockford, IL, USA). Next, 30-50 µg total protein underwent 10% SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, Billerica, MA, USA). The membranes were blocked in 5% skimmed milk for an hour and then incubated with the primary antibodies at 4°C overnight and with the secondary antibody at 20°C for an hour. The next day, the membranes were cultured with horseradish peroxidase-labeled secondary goat anti-rabbit antibody (1:2000, ab6721, Abcam) for 1 hour. Then the immunoreactive protein level was determined using an enhanced chemiluminescence kit (NEN, Dreieich, Germany). The antibodies used were against large tumor suppressor 2 (LATS2, 1:100, ab110780, Abcam), p-Yes-associated protein (p-YAP, 1:1000, Ser127, Cell Signaling Technology, MA, USA), YAP (1:500, 13584-1-AP, Proteintech, Rosemont, USA), β-actin (1:2500, AF5003, Beyotime) and horseradish peroxidase-labeled secondary antibody (1:3000, ab6721, Abcam).

Statistical analysis

Measurement data were exhibited as mean ± standard derivation (SD). SPSS 21.0 (IBM Corp. Armonk, NY, USA) was applied for data analysis. Data were analyzed using the paired *t* test, Pearson's Correlation Analysis, one-way or two-way analysis of variance (ANOVA) and Tukey's multiple comparison test. The *p* value was obtained from two-tailed tests, and *p* < 0.05 was considered to present significant difference.

Results

FOXA2 is poorly expressed in HCC and inhibits HCC cell migration and invasion

FOXA2 is a transcriptional activator for liver-specific genes. Importantly, FOXA2 has been noted to be expressed at a lower level in HCC tissues than that in normal liver [19]. Here, our study measured the FOXA2 expression in 50 pairs of HCC tissues and the adjacent normal tissues using RT-qPCR, and the results suggested that the FOXA2 expression was much lower in tumor tissues than that in the paired normal ones (Fig. 1A). In terms of FOXA2 in cells, it was found that the HCC cell lines HepG2 and SK-HEP-

1 also presented lower levels of FOXA2 (Fig. 1B). Then, pcDNA-FOXA2 was administrated into HCC cell lines to artificially up-regulate FOXA2 expression in cells (Fig. 1C). Importantly, the invasion and migration abilities of cells were inhibited (Fig. 1D).

FOXA2 transcriptionally suppresses miR-103a-3p

FOXA2 is a transcription factor that can specifically bind to the upstream sequences of genes at the 5'end to suppress gene expression. miR-103a-3p has been suggested to play oncogenic roles in human cancers and is linked to worse prognosis of cancer patients [17]. Here, we determined miR-103a-3p expression in HCC and normal tissues, which suggested that the HCC tissues showed higher expression of miR-103a-3p (Fig. 2A). The similar trend was also found in HCC cell lines, where HepG2 and SK-HEP-1 cells presented increased miR-103a-3p expression as relative to THLE-2 cells (Fig. 2B). Then, miR-103a-3p mimic/inhibitor or the NC was transfected into cells, after which the miR-103a-3p expression in cells was correspondingly increased or decreased (Fig. 2C). Next, the Transwell assay results suggested that the migration and invasion of cells was increased following miR-103a-3p up-regulation, while decreased after miR-103a-3p down-regulation (Fig. 2D).

According to the prediction on the Bio-Information System Jasper (<http://jaspar.genereg.net/>), it was suggested that FOXA2 has binding sites with miR-103a-3p at the promoter region (Fig. 2E). The sequence with highest relative score was selected for ChIP assay, and the following RT-qPCR suggested that miR-103a-3p was enriched in the FOXA2 antibody rather than IgG, indicating that FOXA2 could bind with the promoter region of miR-103a-3p (Fig. 2F). In addition, the RT-qPCR results revealed an inverse correlation between miR-103a-3p and FOXA2 expression (Fig. 2G). Following pcDNA-FOXA2 administration, the miR-103a-3p expression was notably inhibited (Fig. 2H). The above experiments suggested miR-103a-3p promotes HCC cell migration and invasion, while FOXA2 transcriptionally suppresses miR-103a-3p expression.

miR-103a-3p directly targets GREM2

GREM2 was suggested as a poorly expressed gene in HCC according the prediction on GEPIA (Fig. 3A). Then, GREM2 was found as a putative target of miR-103a-3p in StarBase (Fig. 3B). We then supposed that miR-103a-3p could exert functions through binding with GREM2. First, RT-qPCR found that GREM2 was poorly expressed in HCC tissues as relative to the normal ones (Fig. 3C), and lower level of GREM2 was also found in HCC cell lines than that in THLE-2 cells (Fig. 3D). Then, pcDNA-GREM2 and siRNA-GREM2 were transfected into HCC cell lines, and then the GREM2 expression was successfully up- or down-regulated, accordingly (Fig. 3E). The Transwell assay results suggested that pcDNA-GREM2 notably inhibited the migration and invasion of HCC cell lines, while the invasion and migration potential of cells was promoted when they were treated with siRNA-GREM2 (Fig. 3F).

In addition, GREM2 presented a negative correlation with miR-103a-3p expression in HCC tumor tissues (Fig. 3G). After miR-103a-3p mimic transfection, it was found that the GREM2 expression was significantly decreased (Fig. 3H). To further validate the binding relationship between miR-103a-3p and

GREM2, a dual luciferase reporter gene assay was performed, which suggested that the relative reduced luciferase activity in cells co-transfected with miR-103a-3p mimic and GREM2-WT sequence, while the luciferase activity in cells subjected to other co-transfection showed little differences (Fig. 3I).

GREM2 activates LATS2 to promote YAP phosphorylation

The downstream effector YAP of the Hippo signaling pathway is a publicly accepted oncogene. The nuclear translocated YAP can bind with the transcription factor TEAD to promote the transcription of proliferation and invasion-related genes [20]. Here, we determined the expression of LATS2, a core kinase of the Hippo signaling pathway, and the phosphorylation of YAP in cells, to explore the potential mechanical molecules involved in the miR-103a-3p/GREM2-mediated events. The pcDNA-GREM2 and siRNA-GREM2 and the corresponding NC vectors were transfected into HCC cells, after which the western blot analysis results found that LATS2 expression and YAP phosphorylation were increased when GREM2 was up-regulated, though the total YAP expression was decreased. Accordingly, siRNA-GREM2 led to a significant increase in LATS2 expression and YAP phosphorylation, but a decline in YAP expression (Fig. 4). The phosphorylation of YAP blocks its potential in nuclear translocation, leading to a degradation in cytoplasm. These results indicated that GREM2 increases the LATS2 kinase expression to activate the Hippo signaling and to trigger YAP phosphorylation in HCC cells.

FOXA2 inhibits migration and invasion of HCC cells through the miR-103a-3p/GREM2/LATS2 axis

Then, pcDNA-FOXA2, pcDNA-FOXA2 + siRNA-LATS2 or the NC vectors were transfected into cells, and then it was found that the pcDNA-FOXA2 led to an increase in LATS2 expression and YAP phosphorylation but a decrease in YAP expression, but the siRNA-LATS2, accordingly, resulted in reversed trends in cells (Fig. 5A). In addition, a group of cells were co-transfected with pcDNA-FOXA2 and siRNA-LATS2. Then, it was found that the migration and invasion abilities of HCC cells inhibited by pcDNA-FOXA2 were increased following further administration of LATS2 (Fig. 5B). These findings inferred that the LATS2 is at least partially implicated in the functions of FOXA2.

Discussion

Owing to the frequent late diagnosis and lack of effective treatments, HCC patients at advanced stages presented very unsatisfactory prognosis, leaving the development of new therapeutic options of great urgency. FOXA factors were initially known as hepatocyte nuclear factor 3 and as a family of pioneer transcription factors for liver-specific genes [21, 22]. Studies have demonstrated the suppressing effects of FOXA2 in several human neoplastic diseases [23, 24]. The present study identified that by suppressing miR-103a-3p transcription, FOXA2 played inhibiting effects on HCC cell invasion and migration with the further involvement of GREM2 and LATS2 activation and the following YAP phosphorylation.

The FOXA subfamily participates in the differentiation and regulation of metabolic tissues including liver, pancreas and adipose tissues [8]. The initial finding of the study was that FOXA2 was expressed at low

levels in tissues from HCC patients as well as in HCC cell lines HepG2 and SK-HEP-1 as relative to the paired normal liver tissues or the normal cell line. This was partly in line with a previous report where researchers found a decrease in FOX2 expression in HCC cells and HepG2 cell line as compared to normal liver cells [19]. Importantly, this selective regulation in cellular gene by the FOXA pioneer factors may offer the chance for the specific suppression in HBV gene expression and the following resolution of chronic HBV infections that causes approximately one million of deaths each year around the world by liver cirrhosis and HCC [25, 26]. In addition, our study confirmed that FOXA2 was capable of inhibiting migration and invasion abilities of HCC cell lines. The FOXA factors may differ from each other in terms of tumorigenesis in different cancer types [10, 27, 28]. In particular, FOXA2 has been noted as a tumor suppressor in several cases. For example, unlike FOXA1 and FOXA3, FOXA2 was suggested to be significantly decreased in lung cancer cell lines and was positively linked to prognosis and better survival rate of lung cancer patients [29]. More relevantly, FOXA2 has been documented to inhibit matrix metalloproteinase-9 expression to suppress HCC metastasis [30]. FOXA2 was also suggested to fulfill key roles at enhancer regions of epithelial genes to maintain the enhancer structure and function, thus suppressing the epithelial-mesenchymal transition, a key process during invasion and metastasis [31, 32].

The preliminary finding above triggered us to explore the possible downstream molecules. FOX factors are capable of recognizing some specific patterns in DNA sequences and consequently bind to chromatin and promote the activities of other regulators [10]. Here, the present study identified that FOXA2 can bind to the promoter region of miR-103a-3p through integrated online prediction and ChIP assay. In addition, miR-103a-3p expression was found to be highly expressed in HCC tissues and cell lines, presenting an inverse correlation with FOXA2. Then, it was found that up-regulation of miR-103a-3p by miRNA mimic inhibited the migration and invasion potential of HCC cell lines. miR-103a-3p has been noted to be involved with cancer progression in several cancer types. For instance, it presented a high-expression profile in the tissues and plasma of colorectal cancer patients [33]. An *in vitro* study suggested miR-103a-3p promotes proliferation of gastric cancer cells by targeting ATF7 [34]. Likewise, inhibition of miR-103a-3p was found to inhibit the proliferation of oral squamous cell carcinoma [17]. Our finding here indicates the inhibiting potential of miR-103a-3p in HCC. Moreover, our study further identified that GREM2 as a putative target of miR-103a-3p. GREM2 was suggested to be lowly expressed in HCC according to the data on GEPIA bioinformation system. The role of GREM2 in cancers has hardly been investigated, though, it is a well-known antagonist of bone morphogenetic proteins (BMP) [35], which have been revealed to promote the progression of several human cancers [36–38]. Here, our study identified that GREM2 was decreased in HCC tissues and cells, with its artificial up-regulation inhibited cell migration and invasion.

Intriguingly, our study found that GREM2 increased the activity of LATS2 and the further phosphorylation of YAP, namely. LATS2 activation and the following YAP phosphorylation are typical signals of Hippo activation, whose dysfunction is involved in a large number of human diseases including cancer [39]. By phosphorylation, YAP is prevented from nuclear accumulation and consequently degraded in cytoplasm, while the non-phosphorylated YAP is localized in nuclear and that promotes cell proliferation and organ growth, which was inhibited by LATS1/2 [40]. Activation of the Hippo signaling has been found to inhibit

the malignant behaviors including growth, migration and invasion of HCC cell lines [41, 42]. The experimental findings that pcDNA-FOXA2 increased LATS2 expression and YAP phosphorylation, and siRNA-LATS2 recovered the migration and invasion abilities inhibited by pcDNA-FOXA2 evidenced that the LATS2 was involved in the FOXA2-mediated events.

Conclusion

To sum up, our study provided evidence that FOXA2 fulfills tumor suppressing roles in HCC by binding to the promoter region of miR-103a-3p to repress its transcription, after which GREM2 was up-regulated, which triggers LATS2 activation and YAP phosphorylation and degradation (Fig. 6). Although the paper was limited in exploring the migration and invasion in the cell perspective, we hope the current finding may offer new insights into HCC control, and we would like to further investigate the roles of above molecules in tumor metastasis in animals and to evaluate other cell behaviors such as proliferation and apoptosis in our future studies.

Abbreviations

ANOVA, analysis of variance; ChIP, chromatin immunoprecipitation; FBS, fetal bovine serum; FOXA2, Forkhead box A2; GREM2, gremlin 2; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LATS2, large tumor suppressor 2; LC, liver cancer; mean $\pm$ SD, mean $\pm$ standard derivation; miRNA, microRNA; MT, mutant type; NC, negative control; RT-qPCR, reverse transcription quantitative polymerase chain reaction; siRNA, small interfering RNAs; WT, wild type; YAP, Yes-associated protein.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

Guangzhen Ma and Jirong Chen conceived the study and participated in its design and coordination; Tiantian Wei and Jia Wang performed all experiments; Wenshan Chen and Guangzhen Ma analyzed and interpreted the data; The draft was improved through discussion and editing by all the authors who read and approved the final manuscript.

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Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The study was approved by the Clinical Ethical Committee of the Second People's Hospital of Liaocheng. Signed informed consent was acquired from each eligible participant.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1 Primer sequences in RT-qPCR

Gene	Primer sequence (5'-3')
FOXA2	F: GAGGCGACAGCGTTAGCA
	R: TACTCCATGGGACCCCTGTT
miR-103a-3p	F: ACTGTAAAGAAGCCGAGGGC
	R: CCCTATGTGTTTCTACTTTTTTGGT
GREM2	F: TCCCTCCCCTATCTGTGTGG
	R: ATGGAGGCTAGGGGTGGATT
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: TGGTGAAGACGCCAGTGGA

Note: RT-qPCR, reverse transcription quantitative polymerase chain reaction; FOXA2, forkhead box A2; GREM2, gremlin 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

Figures

Figure 1

FOXA2 is poorly expressed in HCC and inhibits HCC cell migration and invasion. A, FOXA2 expression in HCC tumor tissues and in the paired adjacent normal tissues determined by RT-qPCR (n = 50, paired t test, ***, $p < 0.001$); B, FOXA2 expression in THLE-2, HepG2, SK-HEP-1 cells determined by RT-qPCR (one-way ANOVA, ***, $p < 0.001$); C, transfection efficacy of pcDNA-FOXA2 determined by RT-qPCR (one-way ANOVA, ***, $p < 0.001$); D, migration and invasion abilities of cells determined by Transwell assays (one-way ANOVA, **, $p < 0.01$). Repetition = 3.

Figure 2

FOXA2 transcriptionally suppresses miR-103a-3p. A, miR-103a-3p expression in HCC tumor tissues and in the paired adjacent normal tissues determined by RT-qPCR ($n = 50$, paired t test, **, $p < 0.01$); B, miR-103a-3p expression in THLE-2, HepG2, SK-HEP-1 cells determined by RT-qPCR (one-way ANOVA, *, $p < 0.05$); C, miR-103a-3p expression in cells after miR-103a-3p mimic/inhibitor transfection determined by RT-qPCR; D, migration and invasion abilities of cells after miR-103a-3p mimic/inhibitor transfection measured by Transwell assays; E, putative binding sequence of miR-103a-3p and FOXA2 predicted on Jasper (<http://jaspar.genereg.net/>); F, miR-103a-3p expression in the precipitation of FOXA2-DNA compound in ChIP assay determined by RT-qPCR (one-way ANOVA, **, $p < 0.01$); G, correlation between miR-103a-3p expression and FOXA2 expression in 50 pairs of HCC tissues and the adjacent tissues analyzed by Pearson's Correlation Analysis ($r = -0.353$, * $p < 0.05$); H, miR-103a-3p expression in cells detected after pcNDA-FOXA2 transfection (one-way ANOVA, * $p < 0.05$).

Figure 3

miR-103a-3p directly targets GREM2. A, GREM2 expression in HCC predicted on GEPIA (<http://gepia.cancer-pku.cn/>); B, putative binding sites of miR-103a-3p and GREM2 predicted on Starbase; C, GREM2 expression in HCC tumor tissues and in the paired adjacent normal tissues determined by RT-qPCR (n = 50, paired t test, **, p < 0.01); D, GREM2 expression in THLE-2, HepG2, SK-HEP-1 cells determined by RT-qPCR (one-way ANOVA, **, p < 0.01); E, GREM2 expression in HCC cell lines after pcDNA-GREM2 or siRNA-GREM2 or the corresponding NC vector transfection determined by RT-qPCR (one-way ANOVA, **, p < 0.01); F, number of migrated and invaded HCC cells after pcDNA-GREM2 or siRNA-GREM2 transfection determined by Transwell assays (one-way ANOVA, *, p < 0.05); G, correlation between GREM2 expression and miR-103a-3p expression in 50 pairs of HCC tissues and the adjacent tissues analyzed by Pearson's Correlation Analysis (r = -0.427, **, p < 0.01); H, GREM2 expression in HCC cell lines after miR-103a-3p transfection determined by RT-qPCR (one-way ANOVA, *, p < 0.05); I, binding

relationship between miR-103a-3p and GREM2 validated through a dual luciferase reporter gene assay (two-way ANOVA, * $p < 0.05$); Repetition = 3.

Figure 4

GREM2 activates LATS2 to promote YAP phosphorylation. Expression of LATS2 and YAP and phosphorylation of YAP in HCC cells after pcDNA-GREM2/siRNA-GREM-2 transfection determined by western blot analysis (one-way ANOVA, *, $p < 0.05$); Repetition = 3.

Figure 5

FOXA2 inhibits migration and invasion of HCC cells through the miR-103a-3p/GREM2/LATS2 axis. A, protein levels of LATS2 and YAP and phosphorylation of YAP in each group of cells determined by western blot analysis (one-way, *, $p < 0.05$); B, migration and invasion abilities of cells transfected with pcDNA-FOXA2 and siRNA-LATS2 determined by Transwell assays (one-way ANOVA, *, $p < 0.05$).

Figure 6

A diagram for molecular mechanism. In the nuclear HCC cells, FOXA2 binds to the promoter region of miR-103a-3p to repress miR-103a-3p transcription. This triggers the following GREM2 up-regulation and LATS2 activation, leading to further YAP phosphorylation and degradation in cytoplasm, resulting in a decline in cell migration and invasion.