

Long noncoding RNA MAFG-AS1 promotes proliferation, migration and invasion of hepatocellular carcinoma cells through downregulation of miR-6852

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Abstract. Long noncoding RNAs (lncRNAs) have been shown to participate in the development and progression of a number of different types of cancer, including hepatocellular carcinoma (HCC). A recent report has indicated that lncRNA MAFG-antisense 1 (AS1) promotes colorectal cancer. However, the role of MAFG-AS1 in other types of cancer remains unclear. The aim of the present study was to examine the effect of lncRNA MAFG-AS1 in HCC. Based on The Cancer Genome Atlas database and reverse transcription-quantitative PCR results, it was determined that lncRNA MAFG-AS1 expression was increased in HCC tissues and cell lines. Following knockdown of lncRNA MAFG-AS1, a Cell Counting Kit-8 assay and Transwell assay demonstrated that the proliferation, migration and invasion of HCC cell lines were significantly inhibited. It was additionally demonstrated that there was a negative regulatory association between lncRNA MAFG-AS1 and miR-6852. Inhibition of miR-6852 increased proliferation, migration and invasion of HCC cell lines. lncRNA MAFG-AS1 promoted HCC development by dampening miR-6852 function and may thus be a novel target for treating patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) refers to malignant tumors of the liver, including primary and metastatic HCC (1). In recent years, the incidence of HCC has increased on a global scale, ranking as the fifth most common malignant tumors (2).

The number of cases of HCC in China accounted for ~55% of the world's total occurrence and presented with a high-risk of mortality (3). At present, surgical treatments, including liver resection and transplantation, are the primary means of treating patients with HCC (4). However, existing treatment techniques frequently fail to achieve favorable results due to a high incidence of metastasis and recurrence (5). Therefore, the underlying biological mechanisms need to be determined to develop improved therapeutic options.

At present, targeted therapy has been widely researched. In April 2018, antisense nucleic acid drug 'CT102 for injection' entered clinical trials in China and provided a new gene-targeted therapy for patients with HCC (6). Long noncoding RNAs (lncRNAs) are a group of noncoding RNAs over 200 nucleotides in length with little or no protein-coding ability (7). Various lncRNAs have been demonstrated to participate in the pathogenesis of HCC (7,8). In addition, lncRNA overexpression in HCC was closely related with other types of cancer, including colorectal cancer, gastric cancer, colorectal cancer and osteosarcoma (9). Xu *et al* (10) showed that lncSHRG promotes HCC progression through the transcription cofactor HES-6 pathway. Huang *et al* (11) found that lncAKHE contributes to HCC migration and invasion by activating the NOTCH signaling pathway. Upregulation of histone H2B ubiquitin ligase complex expression resulted in an interaction with miR-372, augmenting the cAMP-dependent protein kinase pathway and thus, promoting hepatocellular carcinoma (8). Furthermore, lncRNA metallothionein 1D, pseudogene was found to inhibit the expression of forkhead box (Fox)A1 in HCC cells by negatively regulating transcriptional coactivator YAPI and runt-related transcription factor 2 (12). Various lncRNAs can regulate several biological processes in tumor cells, including apoptosis, proliferation, migration and invasion (12). Therefore, it is important to investigate and understand the functions of the numerous dysregulated lncRNAs in cancer.

lncRNA MAFG-antisense 1 (AS1) has been screened as a novel target for treating patients with cancer (13). Zhang *et al* (13) confirmed that lncRNA MAFG-AS1 influenced the proliferation of osteosarcoma cells and also regulated the expression level of various downstream proteins.

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Cui *et al* (14) showed that MAFG-AS1 promotes colorectal cancer development. Additionally, Jia *et al* (15) determined that MAFG-AS1 promotes metastasis of lung cancer (15). However, the molecular mechanism of lncRNA MAFG-AS1 in other types of cancer is still unknown. In the present study, the role of lncRNA MAFG-AS1 in HCC was explored, and potential regulatory factors and associated mechanisms were examined. lncRNA MAFG-AS1 may be a potentially novel target for treating patients with HCC.

Materials and methods

The Cancer Genome Atlas (TCGA) dataset analysis and cell culture. Two sets of HCC data were downloaded from the The Cancer Genome Atlas (TCGA) database (tcga-data.nci.nih.gov/tcga) with one set containing 40 HCC samples and 25 controls, and the other containing 200 tumor samples and 50 controls. The human HCC cells (Hep3B and Huh7) and a human liver cell line (L02) were purchased from Shanghai Institute of Biochemistry and Cell Biology, and cultured using DMEM medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) with 100 U/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.) and streptomycin (100 µg/ml; Invitrogen; Thermo Fisher Scientific, Inc.) in an incubator with 5% CO₂ at 37°C.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was used to detect the expression level and distribution of MAFG-AS1. The cultured cells were digested and centrifuged at 4°C for 2 min at 3,000 x g. Total RNA was extracted using TRIzol[®] reagent according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified using NanoDrop 2000 (Thermo Fisher Scientific, Inc.). A total 1 mg RNA was used for cDNA synthesis using PrimeScript RT Master mix system (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. The reaction was performed with incubation at 42°C for 1 h, and the enzyme was subsequently inactivated by incubation at 85°C for 5 min. The total reaction volume was 20 µl and the mixture was prepared as previously described (16). For qPCR, the PCR solution contained 1 ml cDNA, 1 ml primers and 10 ml SYBR Green and 5 ml RT-qPCR Master mix (all from Invitrogen; Thermo Fisher Scientific, Inc.). The final volume was adjusted to 20 µl using RNase-free water. The amplification was carried out in an ABI FAST 7500 system (Applied Biosystems; Thermo Fisher Scientific Inc.). The relative expression level of each gene was normalized to U6 and calculated using the 2^{-ΔΔC_q} method (17). The thermocycling conditions were as follows: initial denaturation step of 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 2 min with a final extension step at 72°C for 10 min. Primers for PCR amplification were: MAFG-AS1 forward, 5'-ATGACGACCCCAATAA GGA-3' and reverse, 5'-CACCGACATGGTTACCAGC-3'; miR-6852 forward, 5'-AACGAGACGACGACAGAC-3' and reverse, 5'-CCCTGGGGTTCTGAGGACATG-3'; U6 forward, 5'-AACGAGACGACGACAGAC-3' and reverse, 5'-GCAAAT TCGTGAAGCGTTCCATA-3'.

Transient transfection. The siRNA (50 nM) targeting MAFG-AS1 (5'-GGGCAAUCCAACCAAGAAAC-3'),

negative control siRNA (50 nM; 5'-AAUUCUCCGAAC GUGUCACGU-3'), miR-6852 mimics (5'-CCCUGGGGU UCUGAGGACAUG-3'; 50 nM), miR-6852 inhibitors (5'-CAUGUCCUCAGAACCCAGGG-3'; 50 nM), inhibitor negative controls (5'-GCGUAACUAAUACAUCGGAUUCGU-3'; 50 nM) and mimic negative controls (5'-ACAUCU GCGUAAGAUUCGAGUCUA-3'; 50 nM) were obtained from Guangzhou RiboBio Co., Ltd. For MAFG-AS1 overexpression, the sequence of MAFG-AS1 was inserted into a pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). The cultured Hep3B and Huh7 cells were transfected using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following 48 h of incubation, the transfection efficiency was analyzed by RT-qPCR.

Cell Counting Kit-8 (CCK8) assay. CCK8 assay was used to analyze the proliferation of HCC cell lines. The cells transfected for 48 h were plated at a density of 2x10³ cells per well in a 24-well plate, and three replicate wells were used for each condition. The cells were cultured at 37°C in a 5% CO₂ incubator. Cell proliferation assays were performed at 0, 24, 48 and 72 h. A total of 10 ml CCK8 reagent was added to each well, incubated for 4-h at room temperature and the absorbance was measured at 450 nm (A) using a microplate reader.

Transwell migration and invasion assays. As described previously (18), 2x10⁴ cells per well were cultured in the upper Transwell chamber of a 24-well plate (18). Matrigel (BD Biosciences) was used to coat the upper side of the membrane at 37°C for 30 min. A total of 2x10⁴ cells cultured in the upper chamber were placed in 200 µl FBS-free DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) and 600 µl DMEM with 10% FBS was placed in the lower chamber. After a 17-h incubation, the cells were fixed in 70% methanol at room temperature for 30 min and subsequently stained with 0.1% crystal violet at room temperature for 30 min. The cells on the lower chamber were counted using light microscopy to calculate the invasion at x200 magnification. Five random fields of views were counted.

The experimental procedures of the migration assay were the same as invasion assay except that Matrigel was not used.

Luciferase reporter assay. Bioinformatics analysis and luciferase reporter assay were used to screen binding sites and confirm the target of miR-6852, respectively. By using the miRDB tool (<http://mirdb.org/miRDB/index.html>), miR-6852 was identified as the most likely candidate target of MAFG-AS1. The 3' end of MAFG-AS1 containing the miR-6852 binding site was cloned into a luciferase reporter vector. A MAFG-AS1 3' UTR wild type (WT) plasmid (MAFG-AS1 3' UTR-WT) was constructed based on the 3'-end primer sequence of MAFG-AS1. Using the MAFG-AS1 3' UTR-WT plasmid, a binding site was mutated to construct a MAFG-AS1 3' UTR mutant (MUT) plasmid (MAFG-AS12 3' UTR-MUT). The construction and sequencing of the plasmid was performed by Sangon Bioengineering Co., Ltd. The constructed luciferase reporter plasmids pmirGLO-MAFG-AS1-WT, pmirGLO-MAFG-AS1-MUT and miR-6852 mimics, mimics negative control were co-transfected into Hep3B and Huh7

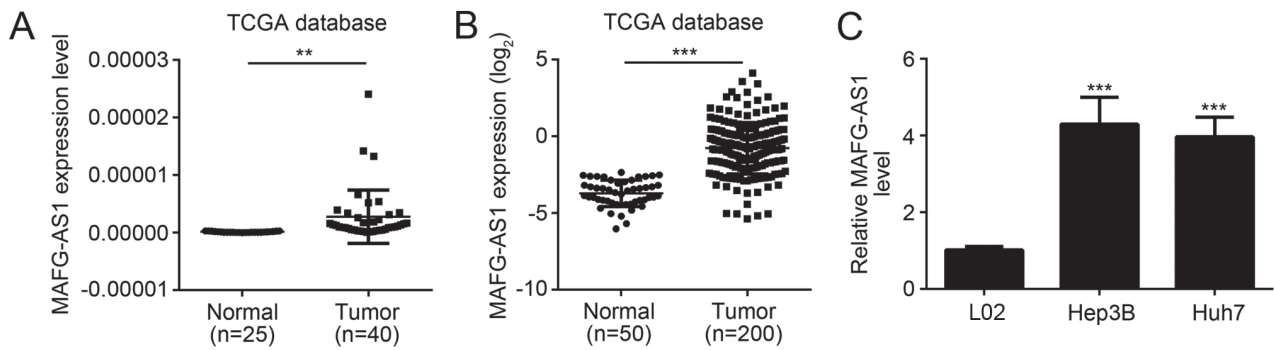


Figure 1. MAFG-AS1 expression is higher in HCC tissues and cell lines. (A) Based on the TCGA database, MAFG-AS1 expression in HCC tissues was significantly higher compared with normal tissues. ** $P < 0.01$ with comparisons indicated by lines. (B) Based on the other set of data in the TCGA database, MAFG-AS1 expression in HCC tissues was significantly higher compared with normal tissues. *** $P < 0.001$ comparisons indicated by lines. (C) Reverse transcription-quantitative PCR results revealed that MAFG-AS1 expression was significantly higher in HCC cell lines compared with the control cell line. *** $P < 0.001$ vs. L02. HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; AS, antisense.

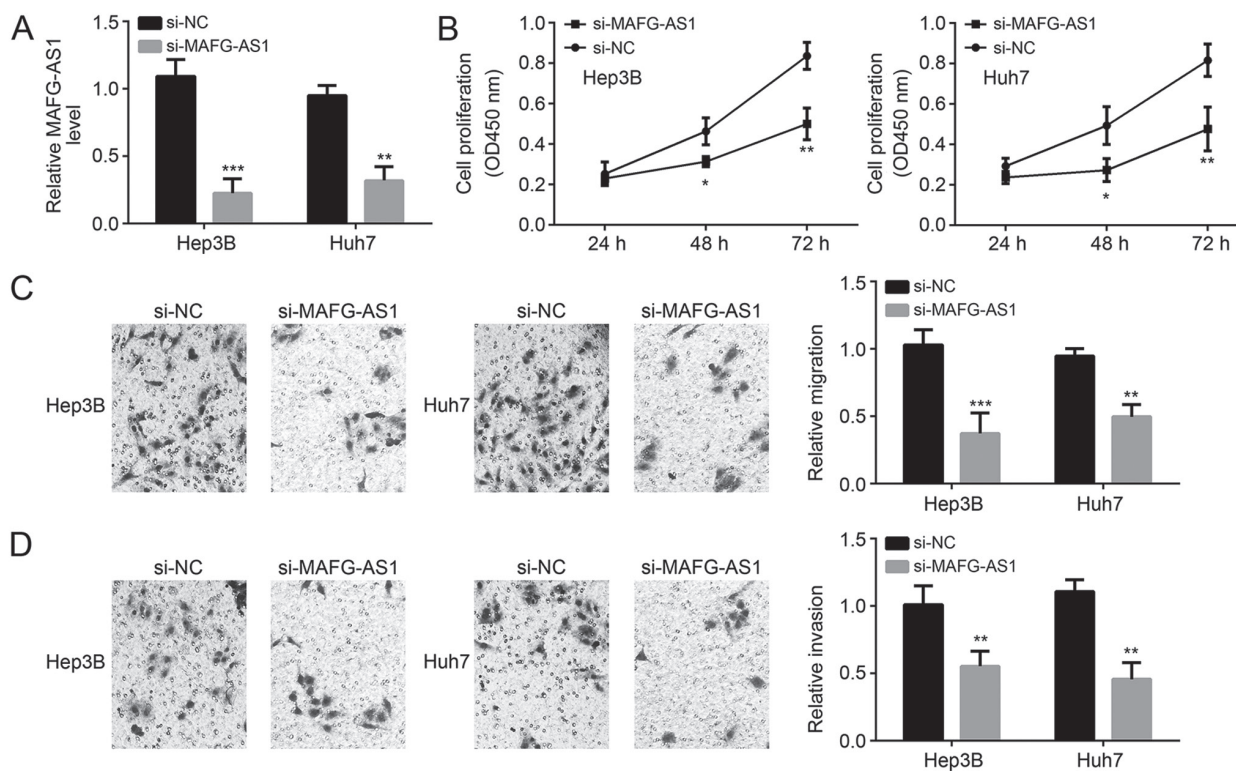


Figure 2. MAFG-AS1 promotes proliferation, migration and invasion of HCC cell lines. (A) MAFG-AS1 was successfully knocked down in HCC cell lines using siRNA. ** $P < 0.01$, *** $P < 0.001$. (B) Cell Counting Kit-8 experiment results showed that MAFG-AS1 knockdown decreased proliferation of HCC cells. * $P < 0.05$, ** $P < 0.01$. Transwell assays confirmed that MAFG-AS1 knockdown inhibited (C) migration and (D) invasion of HCC cell lines (magnification, $\times 100$). ** $P < 0.01$ and *** $P < 0.001$ vs. si-NC. HCC, hepatocellular carcinoma; AS, antisense; si, small interfering; NC, negative control; OD, optical density.

cell lines using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, the luciferase activity was measured using a dual luciferase activity assay kit (Promega Corporation) and results were normalized to *Renilla* luciferase activity.

Western blotting. HCC cells were lysed using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) and the total protein lysates were obtained. Protein concentration was determined using a bicinchoninic acid assay. Total protein (20 μ g) was separated using SDS-PAGE on a 4-20% gel and then transferred to polyvinylidene difluoride

membranes. Membranes were subsequently blocked in 5% non-fat milk/Tris-buffered saline containing 0.1% Tween-20 at 25°C for 1 h and then incubated at 4°C overnight with the primary antibodies. The following primary antibodies were used: Anti-PI3K (1:2,000; cat. no. 4292), anti-p-PI3K (1:2,000; cat. no. 4228), anti-p-STAT3 (1:2,000; cat. no. 9145), anti-STAT3 (1:2,000; cat. no. 12640), anti-MYC (1:2,000; cat. no. 5605) and GAPDH (1:2,000; cat. no. 5174) all from Cell Signaling Technology, Inc. Subsequently, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 2 h at 25°C. Signals were

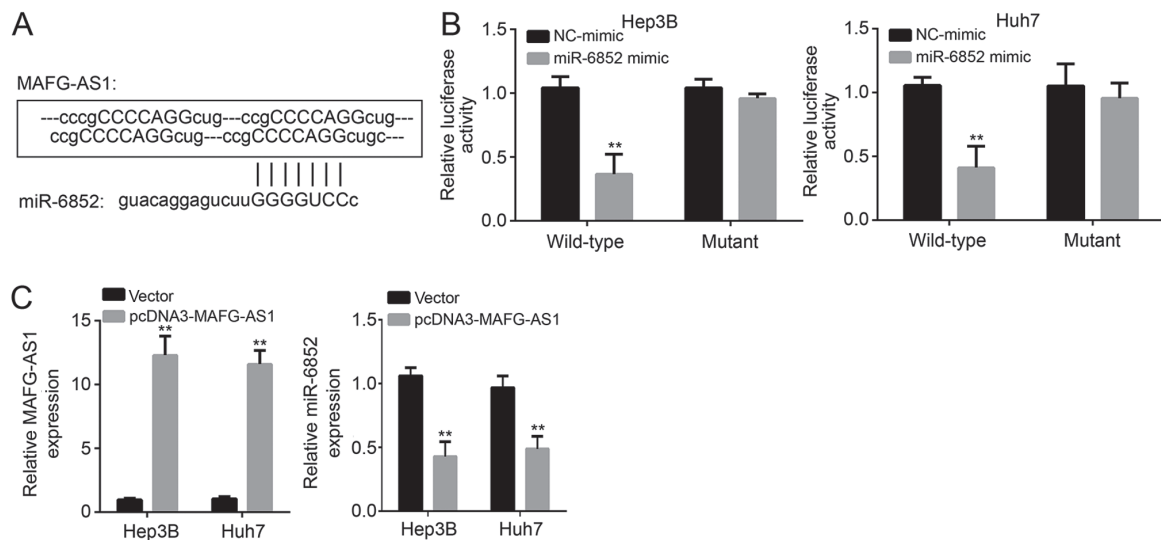


Figure 3. MAFG-AS1 targets and negatively regulates miR-6852. (A) Bioinformatics analysis showed that MAFG-AS1 has four potential binding sites for miR-6852. (B) Luciferase reporter assay revealed that overexpression of miR-6852 inhibited the activity of the wild-type MAFG-AS1 reporter in the hepatocellular carcinoma cell lines. ** $P < 0.01$ vs. NC-mimic. (C) Reverse transcription-quantitative PCR analysis revealed that overexpression of MAFG-AS1 decreased the expression levels of miR-6852. ** $P < 0.01$ vs. vector. AS, antisense; miR, microRNA; NC, negative control.

visualized using the Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). Densitometry analysis was performed using ImageJ version 1.41 (National Institutes of Health).

Statistical analysis. All data were analyzed using SPSS 18.0 statistical software (SPSS, Inc.). All data were expressed as the mean \pm standard deviation of at least three repeats. A Student's *t*-test was performed for comparison between two groups. A one-way ANOVA followed by Tukey's post-hoc test was performed for comparisons between multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of MAFG-AS1 in HCC tissues and cell lines. Based on two sets of data obtained from TCGA database, the expression of MAFG-AS1 in HCC tissues was significantly increased compared with the normal controls ($P < 0.01$; Fig. 1A and B). Similarly, the expression of MAFG-AS1 in Hep3B and Huh7 cell lines were significantly higher compared with the L02 cell line ($P < 0.01$; Fig. 1C). MAFG-AS1 expression was increased in both HCC tissues and cell lines compared with their respective controls.

MAFG-AS1 knockdown inhibits proliferation, invasion and migration of HCC cells. The relative MAFG-AS1 mRNA expression levels were significantly decreased following MAFG-AS1 knockdown in both cell lines confirming transfection was successful (Fig. 2A). Following MAFG-AS1 knockdown, the proliferation, invasion and migration of both Hep3B and Huh7 cell lines were significantly inhibited (Fig. 2B-D) suggesting that MAFG-AS1 was a critical gene in pathogenesis of HCC.

Cellular localization and target of MAFG-AS1. A previous study demonstrated that MAFG-AS1 was primarily localized

in the cytoplasm and inhibits miRNAs in colorectal cancer (14). Therefore, it was hypothesized that MAFG-AS1 may utilize a similar mechanism in HCC. Bioinformatics analysis was used to identify potential binding targets. The analysis indicated that miR-6852 ranked top among all the potential targets. MAFG-AS1 has four potential binding sites with miR-6852 (Fig. 3A). A luciferase reporter assay revealed that upregulation of miR-6852 decreased the luciferase activity of the wild-type MAFG-AS1 reporter in both HCC cell lines ($P < 0.01$; Fig. 3B). Upregulation of MAFG-AS1 decreased the expression levels of miR-6852 ($P < 0.01$; Fig. 3C). The above results demonstrate that MAFG-AS1 targets and negatively regulates miR-6852.

Inhibiting miR-6852 decreases proliferation, migration and invasion of HCC cells. miR-6852 was knocked-down using miR-6852 inhibitors in Hep3B and Huh7 cells (Fig. 4A). In the si-MAFG-AS1 and miR-6852 mimics groups, proliferation, migration and invasion of HCC cell lines were significantly decreased ($P < 0.01$; Fig. 4B). However, inhibiting miR-6852 restored proliferation, migration and invasion of the HCC cell lines ($P < 0.01$; Fig. 4C and D). Taken together, these results demonstrated that MAFG-AS1 may promote HCC progression through inhibition of miR-6852. The effect of MAFG-AS1 on expression of members of the STAT3, Wnt/ β -catenin and PI3K/AKT signaling pathways were determined, which are related to proliferation, migration and invasion of HCC (19). Western blotting showed that MAFG-AS1 silencing only suppressed the activation of the PI3K/AKT signaling pathway (Fig. 5).

Discussion

Numerous lncRNAs are aberrantly expressed in HCC, regulating various miRNAs and genes, and modulating a variety of biological processes (20-22). Therefore, it is possible that one or more of these lncRNAs may serve as a potential therapeutic target for treating patients with HCC. In the present study,

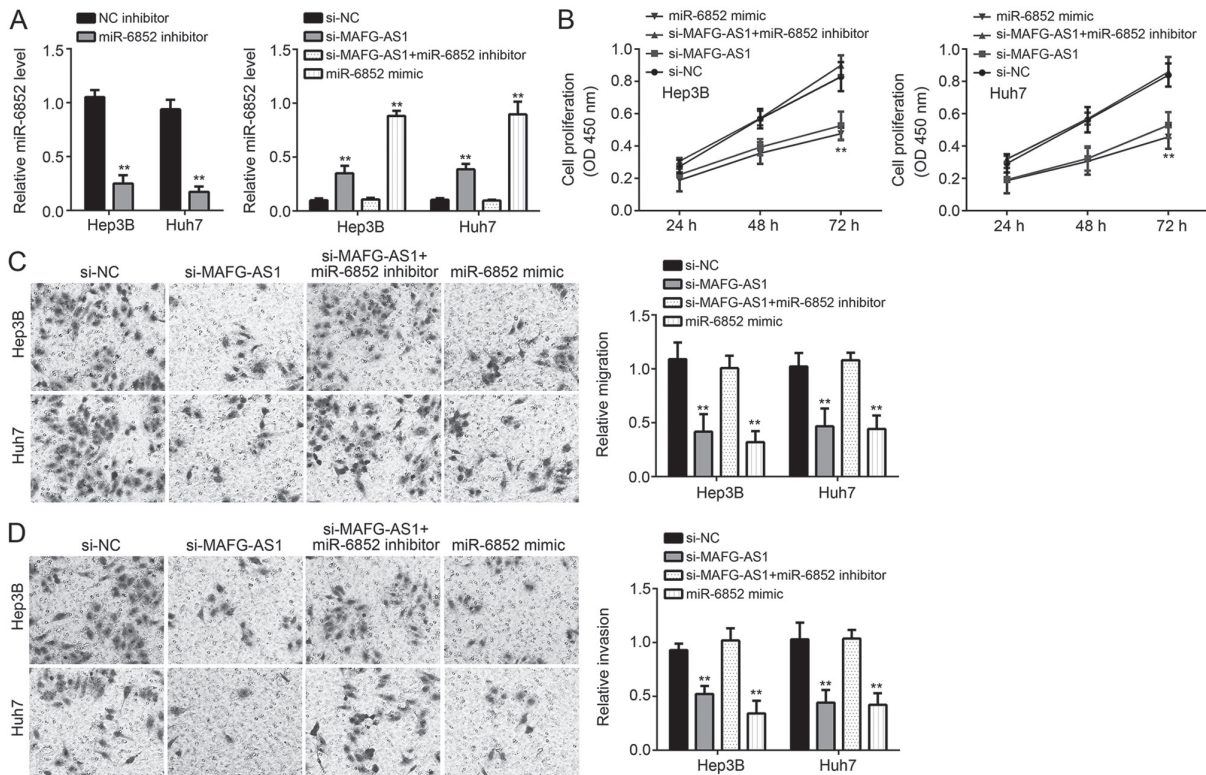


Figure 4. MAFG-AS1 knockdown inhibits cell proliferation, migration and invasion. (A) Reverse transcription-quantitative PCR analysis was used to determine the expression of miR-6852 in cells transfected with a miR-6852 inhibitor. **P<0.01 vs. NC inhibitor. (B) Cell proliferation was analyzed using a Cell Counting Kit-8 assay. **P<0.01 vs. si-NC. Transwell assays were used to determine (C) migration and (D) invasion (magnification, x100) in the hepatocellular carcinoma cell lines transfected with a miR-6852 inhibitor, siRNA against MAFG-AS1 or both. **P<0.01 vs. si-NC. AS, antisense; miR, microRNA; si, small interfering; NC, negative control; OD, optical density.

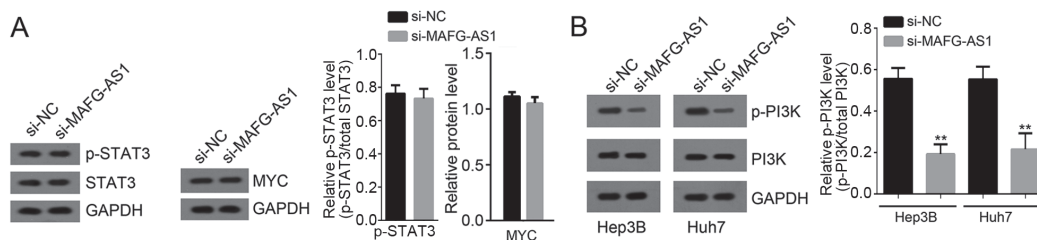


Figure 5. MAFG-AS1 silencing suppresses the activation of the PI3K/AKT signaling pathway. (A) Western blotting results and quantitative analysis of expression of p-STAT3 and MYC (a target of Wnt/ β -catenin pathway) in Hep3B cells. (B) Western blotting analysis and quantitative analysis of the expression levels of p-PI3K in Hep3B and Huh7 cells. **P<0.01 vs. si-NC. AS, antisense; p-, phosphorylated; si, small interfering; NC, negative control.

the functional effects and potential underlying mechanism of lncRNA MAFG-AS1 in HCC was examined. Based on the TCGA database and RT-qPCR results, lncRNA MAFG-AS1 was highly expressed in HCC tissues and cell lines. After knockdown of lncRNA MAFG-AS1, the proliferation, migration and invasion of HCC cell lines were significantly decreased. Interestingly, lncRNA MAFG-AS1 and miR-6852 were demonstrated to exhibit a reciprocally negative regulatory association with each other. By inhibiting the expression of miR-6852, MAFG-AS1 promoted proliferation, migration and invasion of HCC cells.

Zhang *et al* (23) processed a regulatory network analysis of lncRNAs in colorectal cancer and showed that lncRNA MAFG-AS1 was upregulated in this disease (23). In addition, high-throughput data analysis and *in vitro* experiments confirmed that lncRNA MAFG-AS1 was highly expressed and

affects the proliferation of osteosarcoma cells (13). Similarly, lncRNA MAFG-AS1 expression was also upregulated in HCC tissues and primarily distributed in the cytoplasm of HCC cells in the present study. After knockdown of lncRNA MAFG-AS1, the proliferation, migration and invasion of HCC cell lines were significantly inhibited. Therefore, lncRNA MAFG-AS1 may serve a critical role in pathogenesis of HCC.

lncRNA MAFG-AS1 was found to negatively regulate the expression of miR-6852 through four potential binding sites, and further influence the proliferation, migration and invasion of HCC cell lines. A luciferase reporter assay demonstrated that overexpression of miR-6852 inhibited the activity of the wild-type MAFG-AS1 reporter. As shown in previous studies, abnormal expression of miR-6852 could regulate the expression of FoxM1, and thus influence processes of cell cycle arrest and necrosis in cervical cancer cells (24). Kopanja *et al* (25)

found that upregulation of FoxM1 was associated with a poor prognosis in patients with HCC possibly by participating in the Ras signaling pathway. In colorectal cancer, miR-6852 was confirmed to target lncRNA transcription factor 7 (TCF7), and suppress tumor metastasis and growth (26). Furthermore, miR-6852 modulated cell invasion and proliferation by regulating the expression of FoxJ1 (27). lncRNA TCF7 could activate the Wnt signaling pathway and promote self-renewal of HCC cells (28). In the present study, miR-6852 was inhibited by MAFG-AS1, and was a critical factor for the proliferation, migration and invasion of HCC cell lines. Notably, MAFG-AS1 was also reported to prevent binding of miR-339-5p from MMP15 in non-small cell lung cancer (15). Whether MAFG-AS1 similarly suppressed miR-339-5p to upregulate MMP15 in HCC cells remains to be determined.

In the present study, the TCGA database was used to show the expression of lncRNA MAFG-AS1 in HCC tissues. TCGA was jointly developed by the National Cancer Institute and the National Human Genome Research Institute in 2006, and a total 36 types of cancer were examined (29). Large-scale sequencing-based genomic analysis technology was used to aid in understanding the molecular mechanisms of cancer (30). TCGA combined with other bioinformatics platforms may improve our understanding of the molecular basis of cancer and improve diagnosis, treatment and prevention (31). In the present study, results from the TCGA database analysis confirmed that lncRNA MAFG-AS1 was highly expressed in HCC tissues, it was also consistent with the results of the *in vitro* experiments.

In conclusion, lncRNA MAFG-AS1 was overexpressed in patients with HCC. Furthermore, overexpression of lncRNA MAFG-AS1 downregulated the expression of miR-6852 and thus increased the proliferation, migration and invasion of HCC cells. These results may improve our understanding of the molecular mechanisms underlying development and prognosis of HCC, and lncRNA MAFG-AS1 may be a valuable therapeutic target for treating patients with HCC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the author on reasonable request.

Authors' contributions

HOY contributed to the conception and design of the present study. In addition, HOY analyzed and interpreted the results and wrote the manuscript. LZ, ZX and SM performed the experiments. 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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