Long Noncoding RNA MALAT1 Contributes to Sorafenib Resistance by Targeting miR-140-5p/Aurora-A Signaling in Hepatocellular Carcinoma
Lei Fan, Xiang Huang, Jing Chen, Kai Zhang, Yan-hong Gu, Jing Sun, and Shi-yun Cui

ABSTRACT
Long noncoding RNAs (IncRNA) have been found to play critical roles in tumorigenesis and the development of various cancers, including hepatocellular carcinoma (HCC). Metastasis-associated lung adenocarcinoma transcript-1 (MALAT1) has been identified as an oncogene and prognostic biomarker in HCC. Here, we demonstrated that MALAT1 expression was obviously high in sorafenib-resistant HCC cells. Furthermore, knockdown of MALAT1 increased sorafenib sensitivity in nonresponsive HCC cells, whereas forced expression of MALAT1 conferred sorafenib resistance to responsive HCC cells in vitro. In addition, loss/gain-of-function assays revealed that MALAT1 promoted cell proliferation, migration, and epithelial-mesenchymal transition in HCC cells. Mechanistically, MALAT1 regulated Aurora-A expression by sponging miR-140-5p, thus promoting sorafenib resistance in HCC cells. Moreover, MALAT1 inhibition enhanced the antitumor efficacy of sorafenib in vivo. Clinically, we found that MALAT1 expression was negatively correlated with miR-140-5p expression but positively correlated with Aurora-A expression in patients with HCC and that upregulated MALAT1 was closely correlated with poor survival outcomes in patients with HCC. These findings indicated that MALAT1 may be a novel target for prognosis prediction and therapeutic strategies in patients with HCC treated with sorafenib.

Introduction
Hepatocellular carcinoma (HCC) is the most frequent histologic type of primary liver cancer and the third leading cause of cancer-associated death worldwide (1). The treatment for patients with HCC is typically surgical resection and chemotherapy (2). Sorafenib (Nexavar), a multikinase inhibitor, is the first-line treatment option for advanced HCC, thus bringing hope to these patients combatting inoperable HCC (3). However, the limited clinical response caused by drug resistance still impairs the survival of patients with HCC. Therefore, developing an optimal strategy to overcome sorafenib resistance by probing the underlying molecular mechanisms needs to be urgently considered for the treatment of HCC.

Long non-coding RNAs (IncRNA) have a length of more than 200 nucleotides (nt) but no or limited protein-coding capacity (4). Recently, increasing evidence has shown that IncRNAs regulate the expression level of genes participating in various biological processes, such as cell proliferation, differentiation, apoptosis, invasion, and migration; thus, IncRNAs play potential carcinogenic or tumor suppressing roles in the development of multiple cancers (5, 6). Metastasis-associated lung adenocarcinoma transcript-1 (MALAT1) is a large and rarely spliced noncoding RNA that is dysregulated and functions as an oncogene in various cancers, including HCC (7–11). Recently, MALAT1 has also been found to be involved in the chemoresistance of several cancers. For example, MALAT1 knockdown is reported to reverse chemoresistance to temozolomide in glioblastoma, partially by promoting microRNA-101 expression (12). MALAT1 has also been demonstrated to regulate autophagy-associated chemoresistance via miR-23b-3p sequestration in gastric cancer (13, 14). In addition, emerging reports have revealed that IncRNAs play critical roles in sorafenib resistance (15, 16). For instance, IncRNA TUC338 is functionally involved in sorafenib-sensitized hepatocarcinoma cells by targeting RASAL1 (17). However, whether MALAT1 contributes to sorafenib resistance is still unknown.

In this study, we investigated the role of MALAT1 and its potential mechanisms involved in sorafenib resistance in HCC cells.

Materials and methods
Samples
A total of 56 pairs of HCC and corresponding nontumor liver tissues were obtained from the First Affiliated Hospital of Nanjing Medical University from 2012 to 2013. The diagnosis of hepatocellular carcinoma was histopathologically determined by three pathologists. All excised specimens were snap-frozen in liquid nitrogen and then stored at −80°C. The protocols of this study were in accordance with the Declaration of Helsinki. Each participant signed an informed consent form. The local ethics committee and institutional review board approved the collection and use of these samples.

Cell culture and reagents
Human HCC cell lines (HepG2, SMMC-7721) were obtained from the cell bank of the type culture collection at the Chinese Academy of...
Sciences on May 7, 2018. The cells were grown in RPMI1640 or DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin and maintained in a 5% CO2 incubator at 37°C. Sorafenib (BAY 43-9006) was contributed by MedChem Express. Cells used in this study was not contaminated by mycoplasma. Cells were proved to be usable by STR authentication on June 13, 2018. Sorafenib was dissolved in DMSO with a final concentration of DMSO lower than 0.1%. To generate sorafenib-resistant hepatoma cells, HepG2 and SMMC-7721 cells were cultured with 1 μM/λ sorafenib. The concentration was slowly increased by 0.5 μM/L per month (up to 5 μM/L). After more than 10 months, two sorafenib-resistant cell lines were obtained and named HepG2 R and SMMC R. The ICso values of the cells for sorafenib were determined by MTT assay.

Cell transfection

Small-interacting RNAs (saRNA) can increase the expression of endogenous genes by interacting with their promoter or GC-rich region. We designed and identified an saRNA that could promote MALAT1 expression using the methods described in a previous report (18). shRNA targeting MALAT1 (shMALAT1), shRNA targeting Aurora-A (shAurora-A) and saRNA used to overexpress MALAT1 (MALAT1-saRNA) were designed and constructed by GenePharma Co., Ltd., whereas the corresponding vectors were used as negative controls (shCtrl and dsRNA-Ctrl). shMALAT1, shAurora-A, and shCtrl sequences were inserted into the pSilencer4.1-CMVneo vector. The recombinant plasmids were termed pSil/shMALAT1, pSil/shAurora-A, and pSil/shCtrl sequences were inserted into the BglII and HindIII enzyme sites of the pSilencer4.1-CMVneo vector. The recombinant plasmids were termed pSil/shMALAT1, pSil/shAurora-A, and pSil/shCtrl, respectively, and all the constructed plasmids were confirmed by digest analysis with restriction endonuclease and DNA sequencing. The plasmid vector (pMD18/Auro) overexpressing Aurora-A was purchased from Sino Biological Inc. After being cultured in six-well plates overnight, HepG2 and SMMC-7721 cells were transfected with MALAT1-saRNA or dsRNA-Ctrl, whereas HepG2 R and SMMC R cells were transfected with miR-140-5p mimics or inhibitor with Lipo2000 (11668019; Life Technology) according to the manufacturer’s instructions. Sequences for miR-140-5p mimics, inhibitors, or their corresponding controls (NC mimics and NC inhibitors) were listed as follows: miR-140-5p mimics: CAACCACUUUACCCUAGGUGAU; NC mimics: UUCUCGGAACGGUUCAGGUU; miR-140-5p inhibitors: CUACCAUGGUAACCAUGU; NC inhibitors: CAGUACUUUGUUGAUAGCAAA. After 48 hours of transfection, the cells were collected and maintained for further use. For stable transfection, HepG2 R and SMMC R cells (at a density of 2 × 10^5 cells/well) transfected with shMALAT1, shCtrl vector, shAurora-A, or Aurora-A were stably selected with G418 (400 mg/mL) 48 hours after transfection, and individual clones were isolated and maintained in medium containing G418 (100 mg/mL).

Microarray analysis

RNA isolated from HepG2 R (sorafenib-resistant cell line) and HepG2-S (sorafenib-sensitive cell line) was used for microarray analysis performed by Genechem. Human GeneChip PrimeView (Affymetrix) was used for microarray detection according to the manufacturer’s protocols. Quantile normalization and subsequent data processing were performed using Agilent Gene Spring Software 11.5 (Agilent Technologies).

RT-qPCR

Total RNA was extracted with TRizol reagent (Invitrogen) and used to synthesize cDNA with M-MLV reverse transcriptase (Promega) and oligo (dT) 15 following the manufacturer’s protocols. Then, the cDNA samples (1 μL) were used for PCR amplification. A real-time PCR system (StepOne, Applied Biosystems) was used for RT-qPCR analysis, and the expression levels of genes were normalized to GAPDH using the 2^ΔΔCt method. The sequences of primers are listed here: MALAT1 (forward: AAAGCAAGGTCTCCCACAGAG and reverse: GGTCTGTCAGTATCAAAAAAGCA); miR-140-5p (forward: 5'-TGGCCGGAAGTTTACCCATG-3' and reverse: 5'-CCAGTGCAAGGTCGGAAGGTT-3'); GAPDH (forward: 5'-GCCCATATCAGCACAATCC-3' and reverse: 5'-AGGCCACATGCTCACAGACAC-3'); U6 (forward: 5'-TCCGGGTCTCCTGCTCCGCAAC-3' and reverse: 5'-CCAGTGCAAGGTCGGAAGGTT-3').

Western blotting

Total protein was extracted using RIPA buffer with protease inhibitors (Thermo Fisher Scientific), separated on 12% SDS-PAGE gels and then transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore). Afterward, the membranes were incubated with primary antibodies overnight, washed, and then incubated with secondary antibody for 2 hours. Next, the bands were visualized with Immobilon Western reagent (Millipore) and quantified using densitometry image analysis software (Image Master VDS; Pharmacia Biotech Inc.). GAPDH was used for the normalization of gene expression.

The primary antibodies are listed here: E-cadherin (ab1416), Vimentin (ab8978), N-cadherin (ab18203), Aurora A (ab1287), and GAPDH (ab8245; all from Abcam).

MTT assay

Cells (5 × 10^5 cells/well) were seeded into 96-well plates and incubated with different concentrations of sorafenib for 48 hours. Then, MTT solution (Sigma-Aldrich) was added to each well for an additional 4-hour incubation. Then, 150 μL of DMSO (Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan crystals, and the absorbance at 490 nm was detected using a microplate reader (Molecular Devices LLC). Each group had six duplicates, and the experiments were performed at least three times. Finally, the ICso values for sorafenib were calculated.

Colony formation assay

Stably transfected cells (1,000 cells/well) were seeded into 12-well plates, and the medium was replaced every 3 days. Two weeks later, the colonies containing more than 50 cells were counted, and the colony formation rate was calculated as follows: colony formation rate (%) = (number of colonies/number of seeded cells) × 100%. These assays were performed in triplicate.

Cell-cycle assay

First, cells were cultured for 48 hours and then trypsinized, washed, and fixed in 70% ethanol at 4°C overnight. Next, the cells were stained with propidium iodide (PI; Sigma) containing RNase A (Sigma) for 30 minutes at 4°C in the dark. Finally, a FACScan flow cytometer (Becton-Dickinson) was used to analyze the samples.

Annexin-V FITC/PI double-staining assay

The cell apoptosis rate was determined by Annexin-V FITC/PI analysis. First, the centrifuged cells collected from different groups were rinsed twice with ice-cold PBS. Then, the cells were incubated with 300 μL binding buffer, 5 μL PI, and 5 μL Annexin V-FITC in the dark at 4°C for 15 minutes. Finally, cell apoptosis was analyzed by flow cytometry (CytomicsTM FC 500).
Wound-healing assay
HCC cells were plated in six-well plates at a density of 5 × 10⁶ cells/well. After serum starvation for 24 hours, the cells were cultured in medium with 10% FBS. Wounds were made across the diameter of the well using a pipette tip. At 36 hours after scraping, wound closure was visualized under a microscope. This experiment was performed in triplicate.

Transwell assays
Transwell assays were carried out with poly-carbonate transwell filters (Corning, 8 μm). First, 100 μL medium containing 2 × 10⁵ cells was added to the upper chamber, whereas the bottom chamber was filled with culture medium with 20% FBS. After incubation for 24 hours at 37°C, cells that migrated to the bottom of the chamber were fixed and stained, whereas the remaining cells in the upper chamber were removed using cotton swabs. Finally, the stained cells were photographed and counted (cells in ×100 magnification). The experiments were carried out at least three times.

Immunofluorescence staining
HepG2 R and SMMC R cells, as well as their parental cells, were grown on glass cover slips for 2 days. Then, the slides were fixed with 4% paraformaldehyde, permeabilized using 0.1% Triton X-100, incubated with anti-E-cadherin or anti-Vimentin antibody, and finally incubated with secondary IgG antibodies conjugated with Alexa Fluor-488. DAPI was utilized to stain the nuclei.

Luciferase reporter assays
The wild-type (containing miR-140-5p binding sites) or mutant-type (with the binding sites mutated) sequences of both MALAT1 full length and Aurora-A 3’UTR region or were subcloned into the pmirGLO vector (GeneChem) carrying, and these sequences were constructed in the front of the reporter firefly luciferase reporter gene that was already carried into these vectors. In the meantime, HCC cells (2 × 10⁶ cells/well) were seeded in 24-well plates. Afterwards, the plasmids with wild-type sequences (MALAT1-WT or Aurora-A-WT) or those with mutated sequences (Aurora-A-WT or Aurora-A-Mut) were cotransfected into HCC cells together with miR-140-5p mimics or miR-NC using Lipofectamine 2000 transfection reagent (Life Technology). Twenty-four hours later, the cells were lysed by 1× passive lysis buffer (Promega) and the luciferase activities were determined using the Dual-Luciferase Reporter Assay System Kit (Promega) according to the manufacturer’s instructions. Relative luciferase activity was assessed by normalizing to Renilla luciferase (internal control). Experiments were repeated at least three times.

RNA pull-down assay
An RNA pull-down was performed as described previously. Briefly, biotinylated miRNAs (Bio-NC, Bio-miR-140-5p–WT, and Bio-miR-140-5p–Mut) were incubated with 1 mg cell extracts from different groups overnight; streptavidin beads were added and subsequently washed. The associated RNAs and proteins were detected by RT-qPCR and Western blotting, respectively.

RNA immunoprecipitation
RNA immunoprecipitation (RIP) experiments were conducted with a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer’s protocols. The antibodies used for the RIP assays included Ago2 (#2897; Cell Signaling Technology) and anti-Aurora A (ab1287; Abcam). Coprecipitated RNAs were analyzed by RT-PCR.

In vivo experiments
Male nude mice (n = 36, 4–6 weeks, 18–20 g) purchased from the Model Animal Research Center of Nanjing University were housed in a specific pathogen free (SPF) room with a 12 hours on-off light cycle and an adequate supply of food and water; 20 mice were used for the subcutaneous xenograft mouse model, and 16 mice were used for the in vivo metastasis model. To establish the xenograft mouse model, HepG2 R cells transfected with shMALAT1 or shCtrl were suspended in PBS at a final concentration of 2 × 10⁷ cells/mL, and 200 μL of the cell mixture was injected subcutaneously into the left backs of the nude mice. After injection, tumor volumes were assessed every 5 days and calculated as follows: \(V = 0.5 \times D \times D \times H\) (\(V\) represents volume, \(D\) indicates the longitudinal diameter, and \(H\) represents the transverse diameter). After the volume of xenografts >50 mm³, the mice (shMALAT1 injection or shCtrl transfection groups) received sorafenib (30 mg/kg) intraperitoneally or normal saline (NS; control) half-and-half every 3 days for 4 consecutive weeks. Then, all mice were euthanized, and the tumors were excised and used for real-time PCR and Western blot analysis. To establish the in vivo metastasis model, HepG2 R cells transfected with shMALAT1 or shCtrl were suspended in PBS at a final concentration of 2 × 10⁶ cells/mL, and 200 μL of this cell mixture was injected into the tail veins of the nude mice. After 2 weeks, three mice were sacrificed to determine the development of the liver metastatic nodules. The rest of mice (shMALAT1 injection and shCtrl transfection groups) received sorafenib (30 mg/kg) intraperitoneally or normal saline (NS; control) half-and-half every 3 days for 8 consecutive weeks. Then, all mice were euthanized, and malignant liver nodules ≥1 mm in diameter were measured by two independent investigators. All procedures of the in vivo experiments were approved and carried out strictly according to the guide for the care and use of laboratory animals of Nanjing Medical University and the Research Institute Animal Care and Use Committee.

IHC
HCC staining was performed as described (19). Briefly, paraffin-embedded sections were deparaffinized, rinsed, blocked, washed again, and then incubated with primary and secondary antibodies. Afterward, the sections were stained using DAB and photographed under a digitalized microscope (Nikon). The primary antibodies used included Ki67 (ab15580; Abcam) and proliferating cell nuclear antigen (PCNA; ab18197; Abcam).

TUNEL assay
To assess cell apoptosis in the tumors, in vitro terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was conducted on the paraffin-embedded sections using TACS-XL (TrevenGen Inc.) according to the kit instructions.

Statistical analysis
The results from three independent experiments are shown as the mean ± SEM. Significance analysis of the data with a normal distribution was evaluated using two-tailed Student t test or one-way ANOVA, as appropriate. The correlation between MALAT1 expression and the overall survival of patients with HCC was analyzed with the Kaplan–Meier method. Spearman correlation analysis was used to analyze the expression associations among genes. P values of <0.05 were considered to have statistical significance.
Results

Differentially expressed lncRNAs in sorafenib-sensitive and sorafenib-resistant HCC cells

To explore the potential lncRNAs functioning in sorafenib resistance in HCC, we first established sorafenib-resistant HCC cells by treating them with sorafenib at different concentrations. As shown in Fig. 1A, the sorafenib-resistant HCC cells (HepG2 R and SMMC R) had morphologic spindle shapes, whereas the sorafenib-sensitive cells (HepG2 S and SMMC S) had rounded or cobblestone-like shapes. In addition, the IC50 was higher for sorafenib-resistant cells than for sorafenib-sensitive cells (Fig. 1B). Subsequently, a microarray analysis was performed to identify the dysregulated lncRNAs in sorafenib-sensitive and sorafenib-resistant HCC cells (Supplementary Table S1). The results showed that 293 lncRNAs were upregulated, and 207 were downregulated in sorafenib-resistant HCC cells in comparison with the lncRNAs in sorafenib-sensitive cells; among these lncRNAs, MALAT1 was significantly overexpressed (Fig. 1C). MALAT1 has been widely reported in human cancers due to its oncogenic property (20–23). Thus, we chose it for further analysis. qRT-PCR examination further demonstrated the upregulation of MALAT1 in sorafenib-resistant cells (Fig. 1D). These data showed the potential involvement of MALAT1 in the sorafenib resistance of HCC.

MALAT1 modulated the sensitivity of HCC cells to sorafenib and facilitated HCC progression

To determine whether MALAT1-mediated sorafenib resistance in HCC cells, loss- and gain-of-function assays were conducted. As displayed in Fig. 2A, MALAT1 expression was downregulated in HepG2 R and SMMC R cells transfected with MALAT1-specific shRNA (sh-MALAT1). In addition, MALAT1 was overexpressed in HepG2 S and SMMC S cells transfected with MALAT1-saRNA. The IC50 value of HepG2 R and SMMC R cells was decreased by the knockdown of MALAT1, while that of HepG2 S and SMMC S cells was increased by the overexpression of MALAT1 (Fig. 2B; Supplementary Fig. S1A). Moreover, MALAT1 inhibition led to suppressed cell proliferation, enhanced apoptosis, G2–M cell-cycle arrest, and inhibited cell migration in HepG2 R and SMMC R cells. In contrast, ectopic expression of MALAT1 promoted cell proliferation, reduced the apoptosis rate, accelerated cell-cycle progression, and enhanced cell migration in parental sorafenib-sensitive cells (Fig. 2C–G; Supplementary Figs. S1B–S1F). Furthermore, MALAT1 knockdown resulted in the upregulation of the epithelial marker E-cadherin and the downregulation of the mesenchymal markers N-cadherin and Vimentin. However, overexpression of MALAT1 led to the opposite results (Fig. 2H–I; Supplementary Figs. S1G–S1H). On the basis of the above findings, we confirmed the role of MALAT1 in regulating sorafenib resistance and multiple biological processes of HCC.

MALAT1 interacted with miR-140-5p to regulate the expression of Aurora-A

LncRNAs can exert their effects by interacting with miRNAs, thus modulating the mRNA levels of certain genes (24, 25). By using starBase 2.0 (http://starbase.sysu.edu.cn/) and DIANA (http://carolina.imis.athena-innovation.gr/diana_tools/web/index).

Figure 1.

Differentially expressed lncRNAs in sorafenib-sensitive and sorafenib-resistant HCC cells. A, Sorafenib-resistant HCC cells (HepG2 R and SMMC R) were established from parental HepG2 and SMMC-7721 cells by gradually increasing the dose of sorafenib. Then, the stable sorafenib-resistant cells were used for the following assays. A, Images of sorafenib-resistant cells and the corresponding parental cells were taken under a light microscope. B, MTT curve of HepG2 R and SMMC R cells showed the cell viability in response to different dose of sorafenib. C, Heat map of lncRNA expression profiles of sorafenib-sensitive and sorafenib-resistant HCC cells. D, RT-qPCR results of MALAT1 expression in HepG2 R and SMMC R cells and their parental cells (*, P < 0.05; **, P < 0.01).
Figure 2. MALAT1 modulated the sensitivity of HCC cells to sorafenib and facilitated HCC progression. A pairs of HCC cells were used for this experiment: HepG2 R cells transfected with shMALAT1 or shCtrl; HepG2 S cells transfected with MALAT1-saRNA or dsRNA-Ctrl. A, RT-qPCR results of MALAT1 expression in four groups of cells, including HepG2 R, HepG2 S, SMMC R, and SMMC S cells. B, An MTT assay was used to evaluate the IC50 of the above cells. C-I, The effects of MALAT1 on HCC cell proliferation, apoptosis, migration, and EMT were assessed by using a colony formation assay (C), flow cytometry analysis for apoptosis (D) or cell-cycle distribution (E), wound healing assay (F), Transwell assay (G), Western blotting (H), and IF staining (I; *; P < 0.05; **; P < 0.01).
Figure 3.
MALAT1 interacted with miR-140-5p to regulate the expression of Aurora-A. 

A, Putative targets of miR-140-5p were predicted from starBase v2.0 and DIANA. 

B, The prediction for miR-140-5p binding sites on MALAT1 transcript. 

C, Luciferase reporter assay was conducted to confirm the binding relation between miR-140-5p and MALAT1. 

D, RNA pull-down assay was performed to determine the interaction between miR-140-5p and MALAT1. 

E-H, The interaction between miR-140-5p and MALAT1 was further verified using Ago2-RIP assay. 

I, The putative binding sites between miR-140-5p and Aurora-A are presented. 

J, A luciferase reporter assay was performed to validate the interaction between miR-140-5p and Aurora-A. 

K, The expression level of MALAT1 was evaluated in resistant cells after transfection with miR-NC or miR-140-5p mimics. 

L, MALAT1 expression was assessed in sensitive cells after miR-140-5p was downregulated (*, P < 0.05; **, P < 0.01).
Figure 4.
miR-140-5p is involved in MALAT1-induced sorafenib resistance in HCC cells. A, The expression level of miR-140-5p was determined by RT-qPCR analysis in cells transfected with shMALAT1 and MALAT1-saRNA. B–H, The roles of miR-140-5p in MALAT1-mediated sorafenib resistance in HCC cells were determined by MTT assay (B), colony formation assay (C), flow cytometry analysis for apoptosis (D) or cell-cycle distribution (E), wound healing assay (F), Transwell assay (G), and Western blotting (H; **, P < 0.01; ***, P < 0.001).
Among which, miR-140-5p has been reported to enhance the chemosensitivity in osteosarcoma (26, 27). Thus, we chose miR-140-5p for further analysis. We found that miR-140-5p contained complementary sequences to the MALAT1 transcript (Fig. 3B). Then, a luciferase reporter assay suggested that the luciferase activity of wild-type MALAT1 (MALAT1-WT), but not that of mutant type MALAT1 (MALAT1-Mut), was decreased by miR-140-5p mimics and not by miR-NC (Fig. 3C). Subsequently, we illustrated that MALAT1 expression was obvious in harvested pellets pulled down by Bio-miR-140-5p-WT (Fig. 3D). Furthermore, an RIP assay confirmed that both MALAT1 and miR-140-5p were enriched in Ago2 immunoprecipitates (Fig. 3E–3H). Next, we probed for an effective downstream factor of miR-140-5p and found that Aurora-A was a target of miR-140-5p by using the starBase tool. Previous studies have reported that Aurora-A is a tumor facilitator in HCC (28, 29). Here, we predicted the binding sites between miR-140-5p and Aurora-A (Fig. 3I). Similarly, the luciferase activity of Aurora-A-WT was decreased by the miR-140-5p mimics (Fig. 3J). Moreover, we also examined the MALAT1 expression in miR-140-5p-upregulated resistant cells or miR-140-5p-downregulated sensitive cells. As a result, MALAT1 expression was not significantly changed by either miR-140-5p mimics or miR-140-5p inhibitors (Fig. 3K–L). Collectively, the data indicate that miR-140-5p could interact directly with both MALAT1 and Aurora-A in HCC cells.

miR-140-5p/Aurora-A signaling is involved in MALAT1-mediated sorafenib resistance and the biological processes of HCC cells

Next, we examined whether MALAT1 promoted sorafenib resistance in HCC cells through miR-140-5p/Aurora-A signaling. As shown in Fig. 4A, the level of miR-140-5p was increased by silencing MALAT1 but decreased by overexpressing MALAT1. In contrast, the mRNA and protein levels of Aurora-A were downregulated by silencing MALAT1 but upregulated by overexpressing MALAT1 (Supplementary Figs. S2A–S2B). Next, we found that miR-140-5p mimics...
could reduce the IC50 value, attenuate the proliferative and migratory abilities, enhance the apoptosis rate, and reverse epithelial–mesenchymal transition (EMT) progression, whereas miR-140-5p inhibitors restored the effects of MALAT1 knockdown on the sorafenib sensitivity and biological processes of sorafenib-resistant HCC cells (Fig. 4B–H). Similarly, knockdown of Aurora-A also decreased the IC50 value, inhibited cell proliferation, migration, and EMT formation but increased the apoptosis rate; in contrast, overexpression of Aurora-A restored the effects of MALAT1 knockdown (Supplementary Figs. S2C–S2I). In summary, MALAT1 facilitated sorafenib resistance and progression in HCC cells by modulating the miR-140-5p/Aurora-A axis.

**Inhibition of MALAT1 enhanced the antitumor efficacy of sorafenib in vivo**

To further investigate the function of MALAT1 in the antitumor efficacy of sorafenib, HepG2 R cells transfected with shMALAT1 or shCtrl were injected into nude mice; then, the mice were treated with PBS or sorafenib as indicated. We established both a mouse subcutaneous xenograft model and an in vivo metastasis model. First, we discovered that tumor volumes were smaller in the MALAT1-silenced group than in the control group, and cotreatment with sorafenib led to much smaller tumors, indicating that MALAT1 knockdown increased the sensitivity to sorafenib treatment (Fig. 5A). The expression level of MALAT1 was assessed in different tumor tissues. As presented in Fig. 5B, the level of MALAT1 was more reduced by shMALAT1 in tissues treated with sorafenib than that in control group. In addition, silencing MALAT1 caused the greatest downregulation of Ki67 and PCNA expression in mice treated with sorafenib (Fig. 5C). In addition, the apoptosis rate was markedly increased after silencing MALAT1 and further increased after treatment with sorafenib (Fig. 5D). In the in vivo metastasis model, HepG2 R cells transfected with shMALAT1 or shCtrl were injected into mice via their tail veins. The livers of different treated mouse were resected and subjected to

**Figure 6.**

The clinical significance of MALAT1 expression in patients with HCC. **A,** Expression of MALAT1, miR-140-5p, and Aurora-A in paired HCC tissues and normal tissues. **B,** Spearman correlation curve of the relationships among MALAT1, miR-140-5p, and Aurora-A expression in HCC tissues. **C,** Overall survival curves determined by Kaplan–Meier analysis showed the association between the MALAT1 level and overall survival of 56 patients with HCC (**, P < 0.05; ***, P < 0.01).
hematoxylin and eosin staining. The liver weight was calculated in each group. The number of liver nodules was reduced by MALAT1 silencing in mice treated with sorafenib compared with that in control mice receiving the same treatment (Fig. 5E). Together, the data indicate that silencing MALAT1 facilitated the antitumor efficacy of sorafenib in vivo.

**MALAT1 expression was negatively correlated with miR-140-5p expression but positively correlated with Aurora-A expression in patients with HCC**

To further confirm the relationships among MALAT1, miR-140-5p, and Aurora-A, we detected their levels in 56 pairs of HCC and corresponding nontumor liver tissues using qRTPCR. As shown in Fig. 6A, MALAT1 and Aurora-A were highly upregulated in HCC tissues, whereas miR-140-5p was significantly downregulated in HCC tissues. Spearman correlation analysis revealed an inverse correlation between MALAT1 expression and miR-140-5p expression as well as between miR-140-5p and Aurora-A, whereas the expression of MALAT1 in HCC tissues was positively correlated with that of Aurora-A (Fig. 6B). According to their mean MALAT1 expression value, the HCC samples were divided into two groups: a MALAT1 high expression group and a MALAT1 low expression group. MALAT1 expression was found to be correlated with several clinicopathologic features, including TNM stage and distant metastasis (Table 1). MALAT1 expression and distant metastasis were two independent prognostic factors (Table 2). Furthermore, we found that higher MALAT1 levels in HCC patients resulted in poorer outcomes (Fig. 6C). These data showed the prognostic potential of MALAT1 for patients with HCC.

**Discussion**

In this study, we found that MALAT1 was involved in promoting sorafenib resistance in HCC cells and that the upregulated MALAT1 expression was strongly associated with downregulated miR-140-5p expression, increased Aurora-A expression and poor outcomes of HCC patients. In addition, we elucidated that MALAT1 interacted with miR-140-5p as a miRNA sponge and that miR-140-5p/Aurora-A signaling plays a vital role in MALAT1-mediated sorafenib resistance in HCC cells.

Currently, sorafenib resistance is one of the primary obstacles of chemotherapy for metastatic liver cancer (30, 31). Several mechanisms are involved, including the PI3K/Akt and JAK-STAT pathways, hypoxia-inducible pathways, and EMT (32). Recently, several lncRNAs have been reported to be associated with the tumorigenesis and progression of HCC (33–35). In addition, several lncRNAs have been shown to participate in sorafenib resistance in cancers, including HCC (15, 16). For example, lncRNA GAS5 sensitizes renal cell carcinoma to sorafenib via the miR-21/SOX5 pathway (36). lncRNA TUC338 plays a functional role in sorafenib-sensitized hepatocarcinoma cells by targeting RASAL1 (17). In our study, we performed an lncRNA microarray to compare the lncRNA expression pattern between sorafenib-sensitive and sorafenib-resistant HCC cells and found that MALAT1 was one of the most upregulated lncRNAs in sorafenib-resistant HCC cells. MALAT1 is a lncRNA that has been revealed to be highly expressed and serves as an oncogene in multiple cancers (37–39), including HCC (40). In addition, MALAT1 has also been found to play a key role in chemotherapy resistance in several cancers. For example, MALAT1 regulates autophagy-associated chemoresistance via miR-23b-3p sequestration in gastric cancer (13). It is also associated with a poor response to oxaliplatin-based chemotherapy in patients with colorectal cancer and promotes chemoresistance through EZH2 (41). Knockdown of MALAT1 enhances the chemosensitivity of ovarian cancer cells to cisplatin through inhibiting the Notch1 signaling pathway (42). Herein, we found that MALAT1 was upregulated in HCC tissues, and patients with high MALAT1 expression levels tended to have a higher incidence of tumor metastasis and poorer outcomes. Multivariate analysis indicated that MALAT1 was involved in promoting sorafenib resistance in HCC cells and that the upregulated MALAT1 expression was strongly associated with downregulated miR-140-5p expression, increased Aurora-A expression and poor outcomes of HCC patients. In addition, we elucidated that MALAT1 interacted with miR-140-5p as a miRNA sponge and that miR-140-5p/Aurora-A signaling plays a vital role in MALAT1-mediated sorafenib resistance in HCC cells.

Table 1. Correlation between the expression of MALAT1 and clinical features of patients with HCC (n = 56).

| Variable            | MALAT1 expression | P value |
|---------------------|-------------------|---------|
|                     | Low    | High   |         |
| Age                 |        |        |         |
| ≤60                 | 17     | 19     | 0.781   |
| >60                 | 11     | 9      |         |
| Gender              |        |        |         |
| Male                | 8      | 10     | 0.775   |
| Female              | 20     | 18     |         |
| Serum AFP (µg/L)    |        |        |         |
| <800                | 18     | 16     | 0.785   |
| ≥800                | 10     | 12     |         |
| Tumor size (cm)     |        |        |         |
| <5                  | 8      | 10     | 0.775   |
| ≥5                  | 20     | 18     |         |
| TNM stage           |        |        |         |
| I–II                | 17     | 8      | 0.031*  |
| III–IV              | 11     | 20     |         |
| Distant metastasis  |        |        |         |
| No                  | 19     | 9      | 0.015*  |
| Yes                 | 9      | 19     |         |

Note: Low/high by the sample mean. Pearson χ² test. *P < 0.05 was considered statistically significant.

Table 2. Multivariate analysis of prognostic parameters in patients with HCC by Cox regression analysis.

| Variable          | Category | HR (95%) | P value |
|-------------------|----------|----------|---------|
| Age               | ≤60      | 0.403-1.523 | 0.472   |
|                   | >60      |          |         |
| Gender            | Male     | 0.48-1.819 | 0.841   |
|                   | Female   |          |         |
| Serum AFP (µg/L)  | <800     | 0.628-2.226 | 0.604   |
|                   | ≥800     |          |         |
| Tumor size (cm)   | <5       | 0.48-1.796 | 0.825   |
|                   | ≥5       |          |         |
| TNM stage         | I–II     | 0.786-0.426 | 0.442   |
|                   | III–IV   |          |         |
| Distant metastasis| No       | 1.28-3.733 | 0.019*  |
|                   | Yes      |          |         |
| MALAT1 expression | Low      | 1.968-3.584 | 0.027*  |
|                   | High     |          |         |

Note: Proportional hazards method analysis showed a positive, independent prognostic importance of MALAT1 expression (P = 0.027), in addition to the independent prognostic impact of Distant Metastasis (P = 0.019*).

* P < 0.05 was considered statistically significant.
Overexpression of Aurora-A was correlated with poor outcomes and is reported to be overexpressed in many human cancers (54). Serine/threonine protein kinases involved in the regulation of mitosis, Aurora-A is a member of the Aurora kinase family, which is a group of gene because it has been proven to have effects similar to MALAT1 on 5p axis, we used starBase 2.0 to predict a series of possible target.

In decreasing evidence has indicated that lncRNAs regulate mRNA expression through acting as miRNA sponges; in this way, they can control human cancer progression (24, 43). For instance, SNHG16 drives the proliferation, migration, and invasion of hemangioma endothelial cells through modulating the miR-520d-3p/STAT3 axis (44). In addition, some reports revealed that lncRNAs can overcome sorafenib resistance in HCC by targeting miRNAs (16, 45). Here, we tried to determine whether MALAT1 exerted its effects by regulating miRNAs. We predicted and subsequently confirmed that miR-140-5p could interact directly with MALAT1 using luciferase reporter assay, RIP and RNA pull-down assays. miR-140-5p acts as a tumor suppressor in various cancers, including lung cancer, gastric cancer, colorectal cancer, and breast cancer (46–49). miR-140-5p was also reported to suppress tumor growth and metastasis by targeting transforming growth factor β receptor 1 (TGFBRI) and fibroblast growth factor 9 (FGF9) in hepatocellular carcinoma (50). By using starBase 2.0 and DIANA, 48 miRNAs were predicted and selected due to their relative high binding possibility. Among which, miR-140-5p has been reported to enhance the chemosensitivity in osteosarcoma (26, 27). In a recent report, MALAT1 served as a ceRNA of HDAC4 via decaying miR-140-5p in osteosarcoma cells (51). Therefore, we further detected whether MALAT1 regulated miR-140-5p to affect sorafenib resistance in HCC. The reciprocal regulation between MALAT1 and miR-140-5p was validated in our current study. Our findings were consistent with those of previous reports. However, the role of miR-140-5p in MALAT1-mediated sorafenib resistance in HCC has not yet been fully understood.

LncRNAs are known as miRNAs sponges to upregulate miRNAs. To further explore the downstream mechanisms of MALAT1/miR-140-5p axis, we used starBase 2.0 to predict a series of possible target miRNAs. Among them, Aurora-A was chosen as a candidate target gene because it has been proven to have effects similar to MALAT1 on the growth and metastasis of HCC cells (52) and other cancer cells (53). Aurora-A is a member of the Aurora kinase family, which is a group of serine/threonine protein kinases involved in the regulation of mitosis, and is reported to be overexpressed in many human cancers (54). Overexpression of Aurora-A was correlated with poor outcomes in patients with HCC (28). Aurora-A plays an important role in the chemoresistance of cancer cells (55, 56). In our previous studies, we also demonstrated that an Aurora-A inhibitor (MLN8237) could enhance the antitumor activity of sorafenib (57). Therefore, Aurora-A represents a promising prognostic biomarker and potential therapeutic target in HCC. However, the underlying mechanisms of Aurora-A overexpression in HCC are not fully understood. We identified some key regulators responsible for Aurora-A overexpression in HCC, including hypoaxia-inducible factor 1a and miRNA-129-3p, which regulate Aurora-A expression at the transcriptional and posttranscriptional levels, respectively (58, 59). In this study, we confirmed that Aurora-A was also a direct target gene of miR-140-5p using a luciferase reporter assay. Aurora-A knockdown could mimic the effects of miR-140-5p upregulation, whereas Aurora-A upregulation could partially reverse the effects of miR-140-5p upregulation. In addition, Aurora-A expression was negatively correlated with miR-140-5p expression but positively correlated with MALAT1 expression in patients with HCC, which provided strong support for their relationships. Thus, these results showed that MALAT1 might exert its functions mainly through regulating the miR-140-5p/Aurora-A axis.

In conclusion, our study suggested that the MALAT1/miR-140-5p/Aurora-A axis might play a key role in sorafenib resistance in HCC cells and that MALAT1 knockdown in sorafenib-resistant HCC cells could increase their sensitivity to sorafenib treatment both in vitro and in vivo; therefore, MALAT1 could be an effective therapeutic target for overcoming sorafenib resistance in HCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y.-h. Gu, J. Sun, S.-y. Cui
Development of methodology: L. Fan, J. Chen, Y.-h. Gu, S.-y. Cui
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Fan, X. Huang, K. Zhang, J. Sun, S.-y. Cui
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Fan, J. Chen, K. Zhang, J. Sun, S.-y. Cui
Writing, review, and/or revision of the manuscript: L. Fan, J. Chen, J. Sun, S.-y. Cui
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Fan, J. Sun, S.-y. Cui
Study supervision: S.-y. Cui

Acknowledgments
This study was supported by a grant from the National Natural Science Foundation of China (NSFC 81502611, 81201597,81302109, 81572389, and 81771944). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 3, 2019; revised September 9, 2019, accepted March 11, 2020; published first March 27, 2020.

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