circFOXM1 contributes to sorafenib resistance of hepatocellular carcinoma cells by regulating MECP2 via miR-1324

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As one of the most common malignant tumors, hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths around the world. Emerging studies have indicated that circular RNAs (circRNAs), which play a crucial role in HCC pathogenesis and metastasis, are differentially expressed in HCC. However, the regulatory mechanisms of circRNA on sorafenib resistance of HCC are still unknown. In our study, we identified a novel circRNA, circFOXM1, using RNA sequencing (RNA-seq) that was increased in sorafenib-resistant HCC tissues. Functionally, circFOXM1 significantly inhibited HCC growth and enhanced sorafenib toxicity in vitro. Mechanistically, circFOXM1 acted as a sponge of microRNA (miR)-1324, which is a negative regulator of MECP2, indicating that circFOXM1 downregulation would regulate sorafenib resistance of HCC via releasing more free miR-1324 and suppressing MECP2 expression. Furthermore, miR-1324 overexpression was capable of reversing the circFOXM1-induced malignant phenotypes and elevated expression of MECP2 in HCC cells. circFOXM1 partially contributed to sorafenib resistance of HCC cells through upregulating MECP2 expression by sponging miR-1324.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common pathological type (75%–85%) of primary liver cancer and is the 4th leading cause of cancer-related death worldwide. However, the 5-year survival rate is approximately 18%, and the recurrence rate after surgery is as high as 65%–80%. Although a multi-kinase inhibitor, sorafenib, has exhibited prominent clinical efficacy in HCC patients, poor prognosis of HCC is closely associated with the development of acquired resistance. Sorafenib resistance is one of the most important factors that restrict the long-term survival of HCC patients. Therefore, novel targets for reducing sorafenib resistance in HCC need to be developed.

Circular RNA (circRNA) is a novel non-coding RNA (ncRNA) with a highly conserved and stable covalently closed structure. circRNAs are mostly derived from the gene’s exon region, with a small portion formed by intron cleavage. circRNA functions in HCC were recently revealed and include competing endogenous RNAs (ceRNAs) or microRNA (miRNA) sponges and interactions with RNA-binding proteins and translating proteins. Because studies on the regulatory role of circRNAs in sorafenib resistance of HCC have not been reported, there are still great prospects to investigate the functions of circRNAs and the regulatory mechanism of sorafenib resistance in HCC.

Therefore, the principal purpose of our study was to reveal the significant role of circFOXM1, which was markedly elevated in sorafenib-resistant HCC tissues and cell lines. circFOXM1 is an important mediator of resistance to sorafenib in HCC. Furthermore, we illustrated that circFOXM1 may act as a sponge of miR-1324 to upregulate the level of MECP2 and therefore promote drug resistance of HCC. Our study aimed to provide a potential prognostic biomarker for prognostic evaluation or a therapeutic target for the clinical treatment of HCC.

RESULTS

Profile of circRNAs in sorafenib-resistant HCC tissues

To investigate the expression profiles and regulatory effect of circRNAs, RNA-seq was performed in both sorafenib-resistant HCC cell lines (SR-HepG2 and SR-Huh7). By comparing the circRNA profiles between sorafenib-resistant and sorafenib-sensitive cells, a total of 269 most differentially expressing circRNAs (filtered by fold change

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[FC] > 1.5 and false discovery rate [FDR] < 0.05) were selected for overlap analysis. As a result, four circRNAs (hsa-circ-0025039, hsa-circ-0051240, hsa-circ-0002211, and hsa-circ-0003222) were identified (Figure 1A). Among them, one circRNA was downregulated, while three circRNAs were upregulated. We then experimentally validated the three most upregulated circRNAs expression levels by quantitative real-time PCR using sorafenib-resistant and sorafenib-sensitive HCC tissue samples. The quantitative real-time PCR results indicated hsa-circ-0025039 (chr12:2975558-2977920) showed higher fold change in the sorafenib-resistant HCC tissues than in the sorafenib-sensitive tissues (Figure 1B). By browsing the human reference genome (GRCh37/hg19), we knew that has_circ_0025039 was derived from the exon 4 and 5 of FOXM1 loci, and thus we named it circFOXM1 in this study. Furthermore, we found that knockdown of circFOXM1 reversed sorafenib resistance in both sorafenib-resistant HCC cell lines. (D) The qPCR results further confirmed that circFOXM1 was significantly increased in HCC tissues compared with ANT. (E) The qPCR results further confirmed that circFOXM1 was higher in sorafenib-resistant HCC tissues than in the sorafenib-sensitive tissues. (F) Patients with circFOXM1high cell expression had a significantly worse prognosis than those with circFOXM1low. All tests were performed at least three times. Data were expressed as mean ± SD. ***p < 0.001; **p < 0.01.

To investigate the clinical significance of circFOXM1 expression in sorafenib sensitivity of HCC patients, the expression of circFOXM1 expression in 56 tumor tissues (31 sorafenib-sensitive HCC tissues and 25 sorafenib-resistant HCC tissues) and paired adjacent normal tissues (ANTS) were analyzed. The medians of circFOXM1 expression in tumor and adjacent tissues were calculated, respectively, which showed a higher expression level in tumorous tissues than in adjacent nontumor tissues (Figure 1D), and its expression was higher in sorafenib-resistant HCC tissues than in the sorafenib-sensitive tissues (Figure 1E). Next, we explored the relationship between circFOXM1 expression and the clinicopathological characteristics of HCC patients, as listed in Table 1. The results showed that HCC patients with circFOXM1high cells had larger tumors (p = 0.031), microvascular invasion (p = 0.006), and more advanced tumor stage (p = 0.049). Then, we explored the prognostic implications of circFOXM1 expression. Importantly, our results showed that patients with circFOXM1high cell expression had a significantly worse prognosis than those with circFOXM1low. These results indicate that circFOXM1 likely participates in the progression of HCC.

circFOXM1 silencing weakened sorafenib resistance in sorafenib-resistant HCC cells

To explore the biological functions of circFOXM1 in HCC, we measured circFOXM1 expression in HCC cell lines and normal liver cell line L02. circFOXM1 was markedly upregulated in sorafenib-resistant HCC cell lines compared with that in their parental HCC cell lines (Figure S1A). To confirm whether SR-HepG2 and SR-Huh7 cells were...
resistant to sorafenib, Cell Counting Kit-8 (CCK-8) assay was performed to test IC50 of sorafenib. As shown in Figures 2A and 2B, IC50 values of sorafenib in SR-HepG2 and SR-Huh7 cells were significantly higher than those of their parental HepG2 and Huh7 cells (p < 0.01). Next, we designed two small hairpin RNA (shRNA) plasmids to target the unique back-splice junction. The back-splice junction-specific shRNAs (sh-circFOXM1#1 and sh-circFOXM1#2) had no effect on the level of FOXM1 mRNA in the SR-HepG2 and SR-Huh7 cells (Figure S1B). The quantitative real-time PCR analysis confirmed that circFOXM1 expression level was significantly downregulated in SR-HepG2 and SR-Huh7 cells by sh-circFOXM1#1 instead of sh-circFOXM1#2, so we chose shcircFOXM1#1 subsequently for the following experiments (Figure 2G; p < 0.01). Meanwhile, using the above-mentioned vector, we succeeded in overexpressing circFOXM1 in HepG2 and Huh7 cells. The quantitative real-time PCR assay indicated the relative abundance of circFOXM1 in HepG2 and Huh7 cells infected with circFOXM1 overexpression plasmid (Figure 2D; p < 0.01). Consistently, CCK-8 assay also implicated that knockdown of circFOXM1 rendered both SR-HepG2 and SR-Huh7 cells more sensitive to sorafenib-mediated cytotoxicity compared with the control group, as demonstrated by the decreased IC50 value of sorafenib following circFOXM1 downregulation (Figures 2E and 2F; p < 0.01). However, the opposite phenomenon was observed after overexpression of circFOXM1, and the IC50 values of sorafenib in SR-HepG2 and SR-Huh7 cells were signifi-

Table 1. Correlation between circFOXM1 expression and clinicopathologic characteristics of HCC patients

| Variable            | circFOXM1 |    | p value |
|---------------------|-----------|----|---------|
| Variable            | Low       | High |         |
| All cases           | 28        | 28  |         |
| Age, years, ≥ 50<50 | 16:12     | 14:14| 0.789   |
| Gender, male:female | 10:18     | 16:12| 0.179   |
| HBsAg, positive:negative | 19:9     | 14:14| 0.277   |
| Liver cirrhosis, with/without | 16:12 | 10:18| 0.179   |
| AFP, μg/L, >20≤ 20 | 14:14     | 16:12| 0.789   |
| Edmondson’s grade, III + IV/I + II | 9:19     | 18:10| 0.144*  |
| Tumor size, cm, >5: ≤ 5 | 8:20     | 17:11| 0.031*  |
| Microvascular invasion, present/absent | 10:18     | 21:7 | 0.006*  |
| Encapsulation, incomplete:complete | 14:14     | 16:12| 0.789   |
| TNM stage, II + III/I | 14:22     | 26:6 | 0.049*  |
| BCLC stage, B + C/A | 10:18     | 21:7 | 0.006*  |

* χ2 test was used to test the association between two categorical variables. AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen.

| Edmondson’s grade, III + IV/I + II | 9:19 | 18:10 | 0.014* |
|-----------------------------------|------|-------|---------|
| Tumor size, cm, >5: ≤ 5           | 8:20 | 17:11 | 0.031*  |
| Microvascular invasion, present/absent | 10:18 | 21:7  | 0.006*  |
| Encapsulation, incomplete:complete | 14:14 | 16:12 | 0.789   |
| TNM stage, II + III/I | 14:22 | 26:6 | 0.049* |
| BCLC stage, B + C/A | 10:18 | 21:7 | 0.006* |

Statistically significant.

circFOXM1 knockdown enhanced sorafenib-induced apoptosis in SR-HepG2 and SR-Huh7 cells with respect to the sh-NC group (Figure 3A; p < 0.01). However, cell apoptosis assays revealed that following overexpression of circFOXM1, the sorafenib-induced apoptosis of HepG2 and Huh7 cells was significantly decreased compared to the control group (Figure 3B; p < 0.01). The results of flow cytometry assays showed that circFOXM1 knockdown significantly increased the percent of cells in G0/G1 phase of SR-HepG2 and SR-Huh7 cells in the presence of sorafenib (3 μM) (Figures 3C and 3D; p < 0.01); however, overexpression of circFOXM1 significantly decreased the percent of cells in G0/G1 phase of HepG2 and Huh7 cells in the presence of sorafenib (3 μM) (Figures 3E and 3F; p < 0.01). Collectively, these results indicated that circFOXM1 silencing enhanced sorafenib-induced cytotoxicity in HCC cells.

Confirmation of subcellular localization of circFOXM1
We investigated the stability and localization of circFOXM1 in SR-HepG2 cells. Total RNAs from SR-HepG2 cells were isolated at the indicated time points after treatment with actinomycin D, an inhibitor of transcription. Analysis for stability of circFOXM1 and FOXM1 in SR-HepG2 cells treated with actinomycin D, an inhibitor of transcription, revealed that the half-life of the circFOXM1 transcript exceeded 24 h, which was more stable than FOXM1 (Figure 4A). According to the degradation effect of RNase R on linear RNA and the inhibitory effect of actinomycin D on RNA transcription, the degradation of linear FOXM1 was significantly faster than that of circFOXM1 in SR-HepG2 cells, indicating that the stability of circFOXM1 was increased because of its circular structure (Figure 4B). We then investigated the localization of circFOXM1. The quantitative real-time PCR of RNAs from nuclear and cytoplasmic fractions indicated that circFOXM1 was predominantly localized in the cytoplasm of SR-HepG2 cells (Figure 4C). Our results implied that circFOXM1 harbored a loop structure and was predominantly localized in the cytoplasm.

circFOXM1 serves as a sponge for multiple miRNAs
An increasing number of studies have reported that circRNAs act as miRNA sponges; therefore, we investigated whether circFOXM1 has the ability to bind to miRNAs. Through StarBase v3.0, we found that 12 miRNAs were predicated to be possible targets of circFOXM1. To verify the critical functional miRNAs that may interact with circFOXM1 in HCC cells, a circFOXM1-specific probe was used to perform RNA in vivo precipitation (RIP) in SR-HepG2 cells, which were then screened by the qRT-PCR for the potential miRNAs that had been predicted. Using RIP circFOXM1 pull-down experiments, we purified circFOXM1-associated RNAs and analyzed 12 candidate miRNAs in the complex. The results showed a specific enrichment of circFOXM1 and miR-1324 compared to the negative control (NC) probe, whereas the other miRNAs were slightly enriched or not enriched, indicating that miR-1324 is one of the critical circFOXM1-associated miRNAs in HCC cells (Figure 5A). Next, we performed RNA immunoprecipitation (RIP) with argonaute 2 (AGO2) antibody in SR-HepG2 cells. Our results showed that circFOXM1 and miR-1324, but not circANRIL (a circular RNA that reportedly does not bind to AGO2), were significantly enriched, as they were precipitated
by the AGO2 antibody (Figure 5B). These results indicated that circ-FOXM1 may act as a binding platform for AGO2 and miR-1324. To verify these results, we performed a luciferase assay using miR-1324 mimics co-transfected with luciferase reporters (which contained a wild-type [WT] or miR-1324-target mutant circFOXM1 sequence) into HEK293 T cells. Compared with the NC RNA, miR-1324 decreased the luciferase reporter activity significantly in the cells with the wild-type circFOXM1 sequence but not the cells with either the WT- or the miR-1324-target mutant circFOXM1 sequence (Figures 5C and 5D). Furthermore, using a pull-down assay with biotinylated miR-1324 mimics, we observed significant enrichment of circFOXM1 compared with the level in the NCs, while circANRIL was not enriched in the SR-HepG2 cells (Figure 5E). In addition, miR-1324 did not show significant changes after circFOXM1 was silenced, and circFOXM1 did not show significant changes after miR-1324 expression was upregulated (Figures 5F and 5G). These findings indicate that circFOXM1 and miR-1324 are likely not degraded by each other. All of the above experiments confirmed that circFOXM1 may function as a sponge for miR-1324 in HCC cells.

circFOXM1 positively regulated MECP2 expression by interacting with miR-1324 in HCC cells

Through overlapping the results of miRNA target prediction by miR-Walk, TargetScan, mirDIP, and miRDB, the 3'UTRs of 4 candidates (MECP2, ZNRF1, ETF1, and CPLX4) were considered as putative targets of miR-1324 (Figure 6A). However, we did not detect significant changes in the expression levels of these mRNAs in SR-HepG2 cells transfected with a miR-1324 mimic or in HepG2 cells transfected with a miR-1324 inhibitor, except for the expression of the MECP2 (Figures 6B and 6C; p < 0.01). To verify whether the 3'UTR of MECP2 mRNAs were targets of miR-1324 in the HCC cells, a pLG3 luciferase reporter gene assay was used. The WT 3'UTR sequence and the mutant (mu) 3'UTR sequence of MECP2 were cloned and placed into a pLG3 luciferase reporter vectors. The luciferase activity was significantly inhibited by the miR-1324 mimics in the HEK293 T cells transfected with the WT 3'UTR sequence. The luciferase activity was not changed by the miR-1324 mimics in the HEK293 T cells transfected with the mu 3'UTR sequence (Figure 6D; p < 0.01). Furthermore, we found that circFOXM1 knockdown triggered a substantial decline of MECP2 mRNA and protein level in SR-HepG2 cells. Moreover, circFOXM1 knockdown-mediated decrease of MECP2 expression was significantly recuperated following miR-1324 inhibitor (Figures 6E and 6F). All of these data led to the conclusion that circFOXM1 positively regulated MECP2 expression by interacting with miR-1324 in sorafenib-resistant HCC cells.

DISCUSSION

In the current study, we investigated the role of circFOXM1 on sorafenib resistance of HCC and demonstrated the regulatory mechanism of miR-1324/MECP2 signaling pathway. Our data suggested...
that circFOXM1 knockdown could increase the sorafenib sensitivity of HCC cells. circFOXM1 could serve as a molecular sponge of miR-1324, which weakens the inhibitory effect of miRNA on the downstream target gene MECP2. Furthermore, dual-luciferase reporter system and RIP assay verified the direct interaction of circFOXM1, miR-1324, and MECP2. These results indicated that silencing circFOXM1 could increase the sensitivity of HCC cells to sorafenib, thus suppressing tumor development.

For patients with advanced liver cancer, the emergence of sorafenib has brought new hope to their treatment. Acquired resistance, however, often happens within 6 months, and only 30% of HCC patients could benefit from sorafenib. Such high incidence of resistance has greatly limited its clinical application, while the underlying mechanisms of sorafenib resistance in HCC have not been well characterized. circRNAs are a large class of ncRNAs that are composed of special exonic sequences in the absence of a free 3 or 5 end.11 circRNAs act as tumor suppressors or oncogenes to participate in the development of a variety of tumors and are becoming novel diagnostic and prognostic biomarkers.10 They can also serve as ceRNAs through the combination of their complementary miRNA response elements (MREs) and the primary miRNAs, exerting positive or negative effects on the processing and expression of mature mRNAs, thus indirectly involved in various progress of physiological processes.11 circRNAs have been identified as diagnostic or predictive biomarkers of various diseases, especially cancers, in recent years by an increasing number of studies.12 However, the roles of circRNAs in drug resistance of HCC are still unclear.

To address this question, we initially detected the profile of circRNAs in both sorafenib-resistant HCC cell lines (SR-HepG2 and SR-Huh7) using RNA-seq and found that circFOXM1 expression was aberrantly upregulated in sorafenib-resistant HCC cells. Then, we found that circFOXM1 was upregulated in HCC tissues, and patients with high circFOXM1 expression were prone to have a higher incidence of tumor metastasis and poorer prognosis. In line with HCC tissues, circFOXM1 was prominently upregulated in two sorafenib-resistant HCC cell lines in comparison to their parental counterparts. In addition, in vitro loss/gain-of-function assays illustrated that circFOXM1 inhibited sorafenib sensitivity in HCC cells and facilitated HCC cell proliferation. These results demonstrated that circFOXM1 functions as an oncogene that plays an important role in the development of sorafenib resistance in HCC cells through promoting multiple malignant phenotypes.
It has been reported that circRNAs can absorb miRNA and overcome the original repression on the miRNA-targeted gene by functioning as a post-transcriptional regulator (miRNA sponge), which is deemed as ceRNA in the cytoplasm. Herein, we predicted and subsequently confirmed that miR-1324 could interact directly with circFOXM1 using luciferase reporter assay, RIP, and RNA pull-down assays. Some reports revealed that miR-1324 could inhibit cell proliferation, induce cell apoptosis, and reduce cell migration and invasion by targeting multiple oncogenes in laryngeal squamous cell carcinoma, papillary thyroid cancer, glioma, and non-small cell lung cancer. Our results showed that miR-1324 acts as a tumor suppressor in HCC, consistent with those of previous reports. The reciprocal regulation between circFOXM1 and miR-1324 was validated in our current study. Knockdown of circFOXM1 significantly affected miR-1324 expression. Moreover, our data demonstrate that the knockdown of circFOXM1 promoted sensitivity of HCC cells to sorafenib by upregulating miR-1324.

Next, we explored targets of miR-1324 and confirmed MECP2 as a functional target of miR-1324 in HCC. MECP2 is known to prompt oncogenic and metastatic programs in addition to its proliferative and apoptotic functions. Li et al. found that MeCP2 was expressed significantly higher in HCC tissues compared with cirrhosis and non-cirrhosis tissues. MeCP2 could be a novel risk marker to predict HCC development in CHB patients with profound viral suppression under NA therapy. MeCP2 measurement may serve as a useful strategy for risk stratification in terms of follow-up interval and HCC surveillance. We found that circFOX1 could promote MEC2 expression by competitively sponging miR-1324, uncovering the ceRNA network of circFOX1/miR-1324/MECP2 in HCC.

In conclusion, our study on the oncogenic role of circFOX1 in sorafenib resistance of HCC showed that circFOX1 knockdown in sorafenib-resistant HCC cells could increase their sensitivity to sorafenib treatment both in vitro and in vivo, possibly by regulating the miR-1324/MECP2 axis as a ceRNA. This study elucidated a new mechanism for development of HCC and indicated a novel target for treatment of HCC.

MATERIALS AND METHODS
 Patients and tissues
 Pairs of fresh HCC tissues and ANTs were collected from 56 HCC patients at the Department of Hepato-Biliary Surgery, Dongguan People’s Hospital, Southern Medical University between 2014 and 2019. Tumor specimens and corresponding ANTs were collected and stored in liquid nitrogen until use. After completion of 2 cycles of sorafenib-based adjuvant target therapy, patients were divided into sorafenib-sensitive (n = 31) and sorafenib-resistant groups (n = 25). All tumor specimens were obtained by surgical resection prior to undergoing target therapy. The study was approved by the Ethics Committee of Southern Medical University, and written informed consent was obtained from each patient prior to surgery.

Cell culture and reagents
 HCC cell lines HepG2, Huh-7, and the normal human liver cell line LO2 were purchased from the Chinese Academy of Sciences Cell Bank Type Culture Collection. The cells were cultured with DMEM and RPMI-1640 (Gibco, Carlsbad, CA, USA) together with 10% fetal bovine serum (Gibco) at 37°C in an atmosphere containing 5% CO2. Sorafenib (BAY 43-9006) was contributed by MedChem Express. Sorafenib was dissolved in DMSO with a final concentration of DMSO <0.1%. To generate sorafenib-resistant hepatoma cells, HepG2 and Huh-7 cells were cultured with 1 mmol/L sorafenib. The concentration was slowly increased by 0.5 mmol/L per month (up to 5 mmol/L). After more than 10 months, two sorafenib-resistant cell lines were obtained and named sorafenib-resistant HepG2 (SR-HepG2) and sorafenib-resistant Huh7 (SR-Huh7).

Analyzing the circRNA expression profile
 Total RNA from parental sorafenib-resistant and sorafenib-sensitive HCC cells was extracted with TRIzol reagents (Invitrogen, Carlsbad, CA, USA), as per the manufacturer’s protocol. The rRNA was
removed from approximately 2 μg total RNA from each sample using the Epicenter Ribo-Zero rRNA Removal Kit (Illumina, USA), followed by RNase R treatment (Epicenter Technologies, Madison, WI, USA). Subsequently, strand-specific RNA-seq libraries were prepared using the NEBNext Ultra RNA Library Prep Kit from Illumina (New England Biolabs, Beverly, MA, USA), and they were subjected to deep sequencing with an Illumina HiSeq 3000 at Ribobio (Guangzhou, China).

Identification and quantification of circRNAs
The RNA-seq FASTQ reads were first mapped to a human reference genome (GRCh37/hg19) using TopHat2.50. The unmapped reads were then used to identify circRNAs as previously described. Differential expression analysis of circRNAs was executed using R software package DEGseq. Only the circRNAs that were differently expressed with a q value < 0.05 were chosen for further analysis. The FC was log2 transformed, and we used a log2 (FC) > 1.5 (or < −1.5) and a q value < 0.05 to sort the differently expressed circRNAs. Subsequently, to generate an overview of circRNA expression profiles between the two groups, hierarchical clustering analysis was performed.

RNA preparation and quantitative real-time PCR
Total RNA extraction and quantification, RNA purification, and cDNA synthesis were performed. 2 μg total RNA was incubated for 15 min at 37°C with or without 3 U/μg RNase R (Epicenter Technologies, WI, USA) for RNase R treatment. Quantitative real-time PCR was performed with PowerUp SYBR Green Master Mix (Thermo Fisher, MA, USA) and the Applied Biosystems StepOnePlus Real-
Time PCR Detection System (Life Technologies, CA, USA) to detect RNA expression. To calculate the relative gene expression, the $2^{-\Delta\Delta CT}$ method normalized to GAPDH was used, and the FC of gene expression was calculated by the $2^{-\Delta\Delta CT}$ method. Bulge-loop miRNA quantitative real-time PCR Primer Sets (one RT primer and a pair of qPCR primers for each set) specific for miR-1324 were designed by RiboBio (Guangzhou, China). The relative expression of miR-1324 was normalized to human U6 snRNA.

Cell transfection
Knegged down or overexpressed circFOXM1 transfection experiment shRNAs targeting the junction region of the circFOXM1 sequence and circFOXM1-overexpressing lentivirus were synthesized by Hanbio Company (Shanghai, China). HCC cell lines were transfected with circFOXM1 shRNA or the circFOXM1-overexpressing lentivirus according to the manufacturer’s instructions. The miRNA mimics, inhibitors, and small interfering RNAs (siRNAs) were obtained from GenePharma (Shanghai, China). For transient transfection, cells were transfected with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. For stable cell line establishment, the lentiviral vector was introduced into HEK293T cells by transient transfection. After 6 h, the cell culture medium was replaced, and viral supernatants were collected 48 h later. The supernatant was then collected and filtered through a 0.22-µm filter. Cells were infected at approximately 70% confluence in complete medium supplemented with 8 µg/mL polybrene (Sigma), followed by selection with puromycin at 0.5 µg/mL (Sigma). The overexpression efficiency was determined by quantitative real-time PCR.

Cell proliferation assay
CCK-8 assay (Dojindo, Japan) was used as previously described. Briefly, transfected cells were seeded into 96-well plates (3,000 cells/well) and incubated overnight. Cells were then treated with sorafenib at various concentrations for 24 h. To test the cell proliferation, 10 µL of CCK-8 reagent was added to each well and incubated for 2 h at 37°C. Then, the absorption was evaluated by a microplate reader at 450 nm (Tecan, Switzerland).

Cell cycle and apoptosis assay
Cells were seeded into 6-well plates and treated with sorafenib for 24 h. $3 \times 10^5$ treated cells were seeded into 6-well plates and cultured for 48 h at 37°C to assess the cell cycle and apoptosis. The cells for cell cycle analysis were digested using trypsin (Hyclone), washed twice...
with phosphate-buffered saline (PBS), and fixed in 70% ethanol overnight at 4°C. Then the cells were centrifuged at 500 × g for 5 min, washed twice with cold PBS, and centrifuged. Cell cycle analysis was performed through fluorescence-activated cell sorting flow cytometry (Beckman Coulter, Palo Alto, CA, USA) after treating the cells with RNase A (0.1 mg/mL) and propidium iodide (PI, 0.05 mg/mL) purchased from 4A Biotech (Beijing, China) for 30 min at 37°C. Following the instructions of the manufacturer, cells were harvested and were stained with Annexin V-FITC/PI (KeyGEN Biotech, Nanjing, China) for the analysis of apoptosis. Then the cells were acquired by flow cytometry (FACScan, BD Biosciences, USA) and analyzed by FlowJo 7.6.1.

**Actinomycin D and RNase R treatment**

Transcription was inhibited by adding actinomycin D (2 mg/mL) or DMSO (Sigma-Aldrich, St. Louis, MO, USA) as a control to the culture medium. Total RNA (5 μg) was incubated with or without 3 U/μg RNase R (EpigenTech Technologies) at 37°C for 30 min, and the resulting RNA was purified using an RNeasy MinElute Cleaning Kit (QIAGEN, Germany). After the treatment above, RNA was transcribed into cDNA, and the expression levels of GAPDH and circ-FOXM1 were determined by quantitative real-time PCR.

**Cell fractionation assay**

Cytoplasmic and nuclear RNA were acquired using a Cytoplasmic and Nuclear RNA Purification Kit (Invitrogen, CA, USA). Briefly, the cells were harvested and incubated for 10 min with lysis solution on ice, then centrifuged for 3 min at 12,000 × g. The supernatant was collected for cytoplasmic RNA, and the nuclear pellet was used for nuclear RNA extraction. GAPDH was used as the cytoplasmic endogenous control and U6 small nuclear RNA as the nuclear endogenous control.

**In vivo circRNA precipitation, RIP, and luciferase reporter assays**

Biotin-labeled circFOXM1 and NC probes were synthesized by the GeneChem Company. In brief, cells were washed with cold PBS, fixed with 1% formaldehyde, lysed in co-immunoprecipitation (coIP) buffer, sonicated, and centrifuged. Then, the supernatant was collected using an RNase R (Epicenter Technologies) at 37°C for 30 min, and the result- ing RNA was purified using an RNeasy MinElute Cleaning Kit (Qiagen, Germany). After the treatment above, RNA was transcribed into cDNA, and the expression levels of GAPDH and circ-FOXM1 were determined by quantitative real-time PCR.

For the luciferase reporter assay, cells (5 × 10^3) were seeded into 96-well plates and co-transfected with corresponding plasmids and microRNA mimics or inhibitors using the Lipofectamine 2000 transfection reagent. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) after 48 h of incubation following the manufacturer’s instructions. Independent experiments were performed in triplicate. Relative luciferase activity was normalized to the Renilla luciferase internal control.

**Western blot assay**

The lysates from cells were collected by RIPA buffers (Beyotime Biotechnology, Shanghai, China) and boiled for 5 min at 100°C. Then, the proteins were transferred to polyvinylidene fluoride (PVDF) membrane and blocked by non-fat dried milk. The membrane was incubated with primary antibodies at 4°C overnight. On the following day, the membrane was washed strictly and probed with horseradish peroxidase (HRP)-conjugated secondary antibody, followed by visualization with ECL Plus chemiluminescence reagent (Beyotime Biotechnology).

**Statistical analysis**

Variables were expressed in mean ± standard deviation. To measure the difference between two groups, Student’s t test was performed. One-way ANOVA was employed to measure differences between more than two groups. p values <0.05 were considered statistically significant.

**Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article and Supplemental information.

**Consent for publication**

We have received consent from individual patients who have participated in this study. The consent forms will be provided upon request.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.12.019.

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**AUTHOR CONTRIBUTIONS**

H.W. and Z.W. performed primer design and experiments. L.C. and Y.Y. contributed flow cytometry assay and animal experiments. X.C. and L.Y. collected and classified the human tissue samples. W.F. and L.L. contributed to quantitative real-time PCR. Y.Y. analyzed the data. H.W. wrote the paper. All authors read and approved the final manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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