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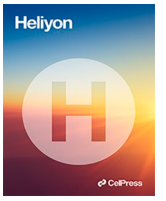

Research article

LINC00540 promotes sorafenib resistance and functions as a ceRNA for miR-4677-3p to regulate AKR1C2 in hepatocellular carcinoma

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ABSTRACT

Sorafenib resistance is one of the main causes of poor prognosis in patients with advanced hepatocellular carcinoma (HCC). Long noncoding RNAs (lncRNAs) function as suppressors or oncogenic factors during tumor progression and drug resistance. Here, to identify therapeutic targets for HCC, the biological mechanisms of abnormally expressed lncRNAs were examined in sorafenib-resistant HCC cells. Specifically, we established sorafenib-resistant HCC cell lines (Huh7-S and SMMC7721-S), which displayed an epithelial-mesenchymal transition (EMT) phenotype. Transcriptome sequencing (RNA-Seq) was performed to established differential lncRNA expression profiles for sorafenib-resistant cells. Through this analysis, we identified LINC00540 as significantly up-regulated in sorafenib-resistant cells and a candidate lncRNA for further mechanistic investigation. Functionally, LINC00540 knockdown promoted sorafenib sensitivity and suppressed migration, invasion, EMT and the activation of PI3K/AKT signaling pathway in sorafenib-resistant HCC cells, whereas overexpression of LINC00540 resulted in the opposite effects in parental cells. LINC00540 functions as a competing endogenous RNA (ceRNA) by competitively binding to miR-4677-3p , thereby promoting AKR1C2 expression. This is the first study that demonstrates a role for LINC00540 in enhancing sorafenib resistance, migration and invasion of HCC cells through the LINC00540/miR-4677-3p/AKR1C2 axis, suggesting that LINC00540 may represent a potential therapeutic target and prognosis biomarker for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death in the world and has a high incidence in patients with chronic liver disease [1,2]. The main causes of HCC include viral hepatitis (hepatitis B and C), alcoholism, and non-alcoholic fatty liver disease [3,4]. Recent advancements in hepatocellular carcinoma (HCC) treatment have introduced Lenvatinib and Atezolizumab plus

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List of abbreviations

Abbreviation	*Full name*
HCC	hepatocellular carcinoma
lncRNAs	long noncoding RNAs
EMT	epithelial-mesenchymal transition
RNA-Seq	Transcriptome sequencing
qRT-PCR	quantitative real-time polymerase chain reaction
CCK-8	cell counting kit-8
shRNA	short hairpin RNA
ceRNA	competing endogenous RNA
FISH	Fluorescence in situ hybridization
PVDF	polyvinylidene fluoride
ECL	enhanced chemiluminescence
IC_{50}	half-maximal inhibitory concentration
DE	differentially expressed

Bevacizumab as first-line options, showing promising outcomes in overall survival and progression-free survival when compared to Sorafenib [5,6]. However, these newer drugs have safety and cost-effectiveness concerns, with Lenvatinib exhibiting better efficacy but worse safety [7], while Atezolizumab plus Bevacizumab may not be cost-effective [8]. As a result, Sorafenib continues to be a significant first-line treatment for HCC. Sorafenib is a tyrosine kinase inhibitor that is used for patients with advanced liver cancer who are not eligible for surgery [9]. However, acquired sorafenib resistance frequently occurs and results in poor prognosis and outcome. Thus, chemoresistance is a significant problem in HCC.

Long noncoding RNAs (lncRNAs) are RNA molecules with a length exceeding 200 nucleotides that lack protein coding potential [10]. Numerous studies have established their role as regulators of transcription, mRNA processing, and nuclear domain organization [11,12]. Moreover, dysregulated lncRNAs play a significant role in the development of cancer, acting as either carcinostatic or oncogenic factors that influence tumor occurrence, metastasis, and drug resistance [13–15]. Mechanisms involving dysregulated lncRNAs have been extensively reported in HCC. For instance, the lncRNA CASC2 is expressed in HCC and exhibits an anti-metastatic effect by targeting miR-367/F-box and WD repeat domain containing 7 (FBXW7) axis [16]. MiRNAs and lncRNAs play a crucial role in the development of chemotherapeutic drug resistance in liver cancer by regulating cell proliferation, cell death, the cell cycle, and the epithelial-mesenchymal transition (EMT) [17]. In addition, lncRNAs exhibit significant potential in cancer diagnosis, prognosis, and treatment [18].

During EMT, cells transition between epithelial and mesenchymal states in a highly plastic and dynamic manner [19]. The adhesion molecules expressed by cells are changed, which cause them to adopt an increased migration and invasion behavior [20]. Recent studies have shown that the EMT not only increases metastasis, but also promotes drug resistance in various tumor types [21,22]. For example, Lgr5 is upregulated in HCC and acts as a tumor promoter to increase cell migration, induce EMT, and increase resistance to Doxorubicin [23]. Ataxia telangiectasia mutated promotes EMT and enhances cell invasion and tumor metastasis in cisplatin-resistant cells [24].

In the present study, by establishing sorafenib-resistant sublines (Huh7-S and SMMC7721-S) derived from parental HCC cells (Huh7 and SMMC7721), we obtained cells with an EMT phenotype. Whole transcriptome resequencing was performed on the parental and sorafenib-resistant cell lines, and differentially expressed lncRNA profiles were obtained by high-throughput microarray and intersection analysis. LINC00540, located at chromosome 13 (chr13: 22040975–22276524), was identified and overexpressed the sorafenib-resistant HCC sublines. Furthermore, we examined putative mechanisms of sorafenib resistance in HCC cells associated with LINC00540 function.

2. Materials and methods

2.1. Cell culture

Human HCC cell lines (Huh7, SMMC-7721) were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Huh7 and SMMC7721 cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS), 0.1 mg/mL streptomycin and 100 U/mL penicillin and maintained in a 5% CO_2 incubator at 37 °C.

Generation of sorafenib-resistant cells.

Huh7 and SMMC7721 cells were seeded into 25 cm^2 cell culture flasks. Firstly, when the cell density reached 80%, the cells were cultured in complete medium supplemented with 4.3 μM sorafenib (Selleck, USA) for 48 h. Subsequently, the cells were cultured in sorafenib-free complete medium for a specific duration and then exposed to a medium containing 8 μM sorafenib. After observing approximately 50% cell death, the medium was replaced with sorafenib-free complete medium. Upon steady growth and initiation of proliferation, the cells were cultured in complete medium containing 12 μM sorafenib, and sorafenib was removed after observing approximately 50% cell death. This cyclic culture process was repeated for approximately eight months, following the aforementioned

Table 1
Probe sequences for RNA FISH.

Gene	Sequence (5′–3′)
LINC00540-203	probe1: GAGGGCTGGAAGAGCCAAACCACA probe2: CGGCCCAGGTGCTCCTCCGACT probe3: CCTCCATTCGCAGTCACTTTAGAACCA
18s	CTGCC + TTCCT + TGGATGTGG + TAGCCGT + TTC

Table 2
Primer sequence for qRT-PCR.

Gene	Forward Primer/5′–3′	Reverse Primer/5′–3′
LINC00540	CCAGCCCTCTTAACAACGGT	TGCGTTTCAGCCATGAGAGT
MSTRG.11029.1	GCCAGCAGCCTCAGTCAAGAAC	CAAGCCTCCTCAAGATTGGGTCAC
MSTRG.13178.2	GGAGACCTGGGAGAACTGGGATG	TGTGCTGTGCTATGCTGCTGAAC
MSTRG.45015.1	TCCGCACAGCCCAGAAGACC	GCAGAGCCACAGGATGAGAACAC
MSTRG.23232.8	GATGAAACTGCGAACGAA	TCAAATCCACCTCACCCT
MSTRG.40443.1	AAAGTGCTGGGATTTACAGGTGTGAG	GTTGGCTTCATTCTATGTGGTTGCTTC
AKR1C2	CCTAAAAGTAAAGCTCTAGAGGCCGT	GAAAATGAATAAGATAGAGGTCAACATAG
GAPDH	GTCAAGGCTGAGAACGGGAA	AAA TGAGCCCCAGCCTTCTC
hsa-miR-4677-3p	TCAGTTCCCAGCAAAGGGAC	TTGGATGCGTCATCGCTTCT
U6	GCTTCGGCAGCACATATACTAAAAT	TACTGTGCGTTTAAGCACTTCGC

steps. Ultimately, the cells acquired stable resistance to sorafenib and were designated as Huh7-S and SMMC7721-S cells.

2.2. CCK-8 assay

Parental (Huh7 and SMMC7721) and sorafenib-resistant cells (Huh7-S and SMMC7721-S) were seeded into 96-well plates at a density of 1×10^4 cells/well and sorafenib was added at concentrations ranging from 0 to 51.6 μM. After 24–48 h, Cell Counting Kit-8 (CCK-8) reagent was added for 2 h and the absorbance of the cells was measured at 450 nm. The inhibitory concentration of sorafenib was determined. In other experiments, transfected cells were cultured with different concentrations of sorafenib after cell adherence for more than 24 h.

2.3. RNA isolation and identification

The extraction of total RNA from the cell lines was done using TRIzol reagent (Invitrogen) following protocols provided by the manufacturer. The concentration of the extracted RNA was determined using a Nanodrop2000 spectrophotometer (Nanodrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA).

2.4. High-throughput microarray and intersection analysis

Total RNAs from sorafenib-resistant and parental cell lines were used for microarray analysis in triplicate. The Human LncRNAs Array kit (v2.0, 8 × 60 K, ArrayStar, USA) was used according to the manufacturer's instructions. Differentially expressed lncRNAs were identified and intersection analysis was performed using BMKCloud (www.biocloud.net).

2.5. FISH (Fluorescence in situ hybridization)

A FISH kit from GenePharma (Shanghai, China) was used according to the manufacturer's protocol. Sorafenib-resistant cells were seeded into the small culture dishes and fixed in 4% paraformaldehyde. After permeabilization, the hybridization was carried out using

Table 3
The shRNA and small RNAs targeting sequences.

Gene	sequence/5′–3′
LINC00540-sh1	GGATTCAACAGAGTTGGATTG
LINC00540-sh2	GGGAGTATGAAAGTATGAAGA
LINC00540-sh3	GCATCAAGTGCAAATCCAAAG
AKR1C2-siRNA	sense: GGACUAUGUUGACCUCUAUTT; antisense: AUAGAGGUCAACAUAGUCCTT
hsa-miR-4677-3p mimics	S: UCUGUGAGACCAAAGAACUACU AS: UAGUUCUUUGGUCUCACAGAUU
hsa-miR-4677-3p inhibitor	AGUAGUUCUUUGGUCUCACAGA

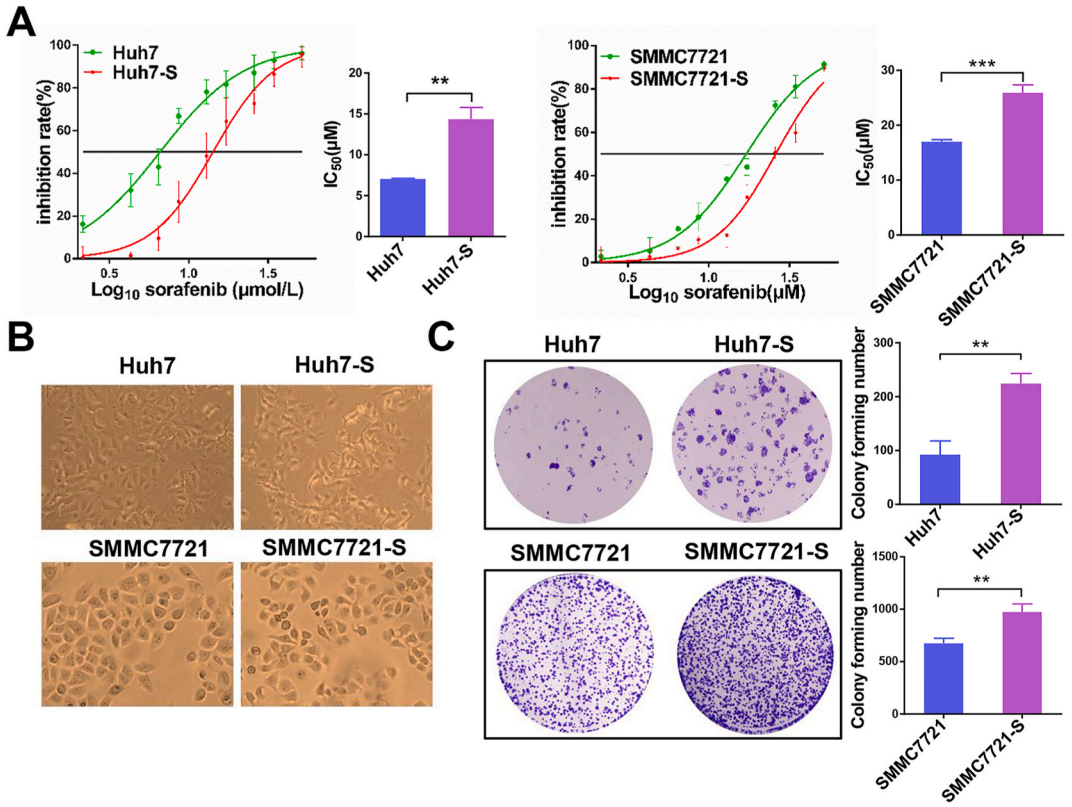

Fig. 1. Generation of sorafenib-resistant HCC cells. (A) A CCK8 cytotoxicity assay showed that the sorafenib IC_{50} was higher in sorafenib-resistant cells compared to parental cells; (B) Morphological images of Huh7 (100 ×) and SMMC7721 (150 ×) cells demonstrated the EMT-like phenotype observed in sorafenib-resistant cells; (C) Sorafenib-resistant cells exhibited enhanced colony growth ability compared to their parental counterparts at a concentration of 4 μM sorafenib. Error bars represent standard deviation (n = 3). N.S. = $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

specific LINC00540 cDNA probes (0.7 μg/mL) and 18s probes (1.5 μM) overnight in a humidified chamber. Images were acquired using a Leica TCS SP2 AOBS confocal microscope (Leica Microsystems, Mannheim, Germany). The probe sequences are provided in Table 1.

2.6. Quantitative RT-PCR

Total RNA was extracted from HCC cells using TRIzol reagent (Invitrogen) and reverse-transcribed to cDNA using the TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR kit (TransGen Biotech, Beijing, China). Real-time PCR was performed using PerfectStart™ Green qPCR SuperMix on VIIA7. The relative quantitation ($2^{-\Delta\Delta Ct}$) method was used to analyze the data. The sequences of the primers are listed in Table 2.

2.7. Cell transfection

For the knockdown and overexpression experiments, short hairpin RNAs (shRNAs), siRNAs, overexpression plasmids, hsa-miR-4677-3p inhibitor and hsa-miR-4677-3p mimics were purchased from Gene Pharma Co., Ltd. (Shanghai, China). The shRNA plasmid targeting LINC00540 (shLINC00540), siRNAs targeting AKR1C2, and pEX-3 was used to overexpress LINC00540. Transfections were performed in Huh7, Huh7-S, SMMC7721, and SMMC7721-S cells using TransIntro™ EL Transfection Reagent (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The targeting sequences are listed in Table 3.

2.8. Flow cytometry

Transfected cells were collected, washed, resuspended, and stained with the YF 647A-Annexin V/PI (Bioscience Biotechnology Co., Ltd) kit. Finally, apoptosis was analyzed by a Calibur flow cytometer equipped with CellQuest software (BD Biosciences).

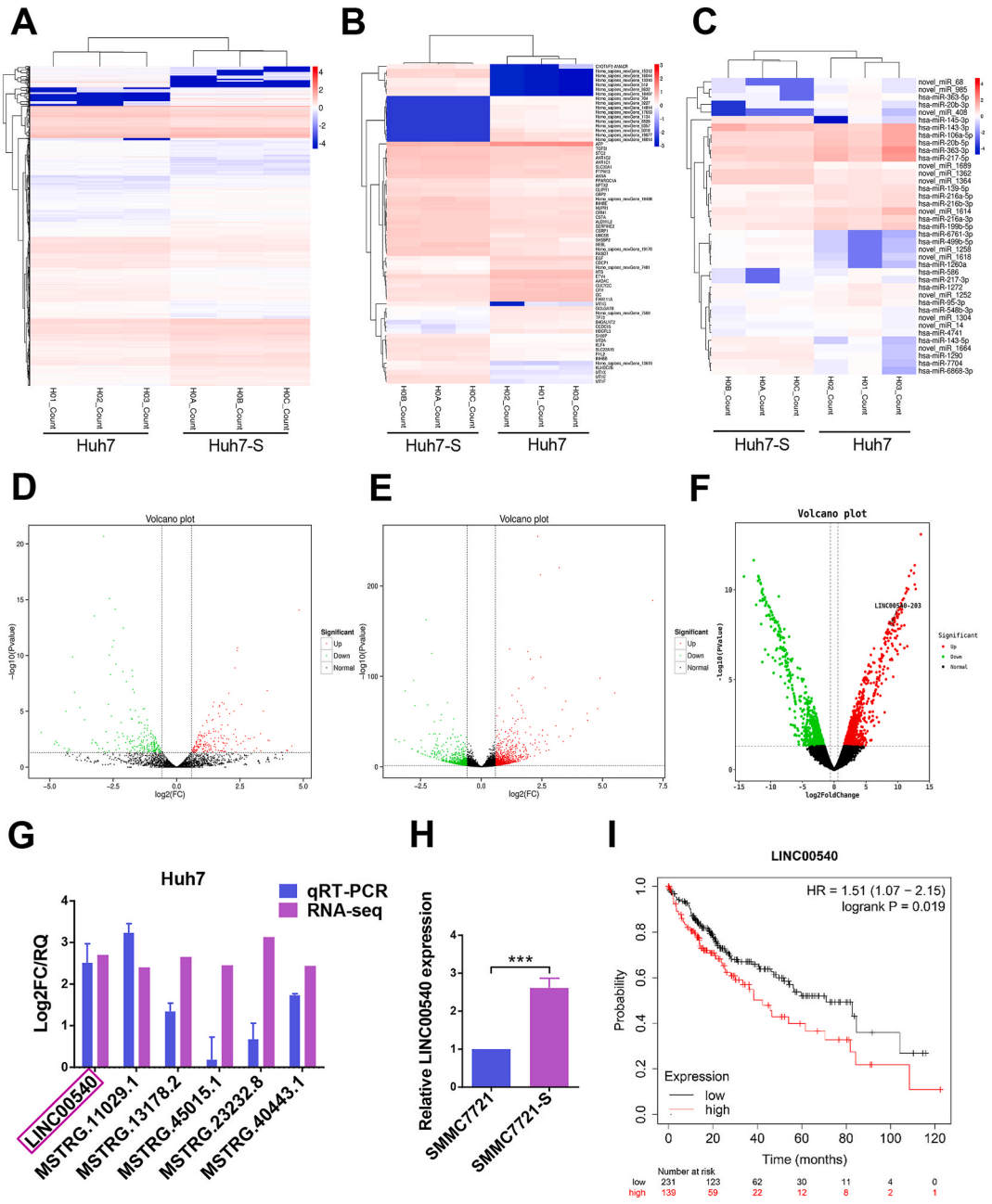

Fig. 2. Identification of DEGs between sorafenib-resistant and parental HCC cells. (A, B) Heat map illustrating the expression profiles of DEGs obtained from Huh7-S and Huh7 cells through whole transcriptome sequencing; (C) Heat map displaying the expression profiles of DEmiRNA obtained from Huh7-S and Huh7 cells through whole transcriptome sequencing; (D, E) Volcano plot showing the expression profiles of DEmiRNAs and DEmRNAs in sorafenib-resistant and parental cells. (F) Volcano plot displaying the expression profiles of DElncRNA in sorafenib-resistant and parental cells; (G, H) qRT-PCR analysis confirmed the overexpression of LINC00540 in sorafenib-resistant cells. (I) Kaplan–Meier curve demonstrating the correlation between high LINC00540 levels and reduced survival in HCC patients. Error bars represent standard deviation (n = 3). N.S. = $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

2.9. Cell migration and invasion assays

Transfected cells (5×10^3) were added to the upper compartment of a 24-well Transwell Boyden chamber (Corning, NY). The cells were resuspended in 100 μL of complete medium containing 10% FBS, while the lower compartment was filled with 0.6 mL of complete medium containing 20% FBS. After incubating for 72 h, cells in the upper chamber were removed using cotton swabs, and the remaining cells were fixed with 4% paraformaldehyde for approximately 30 min. Subsequently, the cells were stained with 0.1%

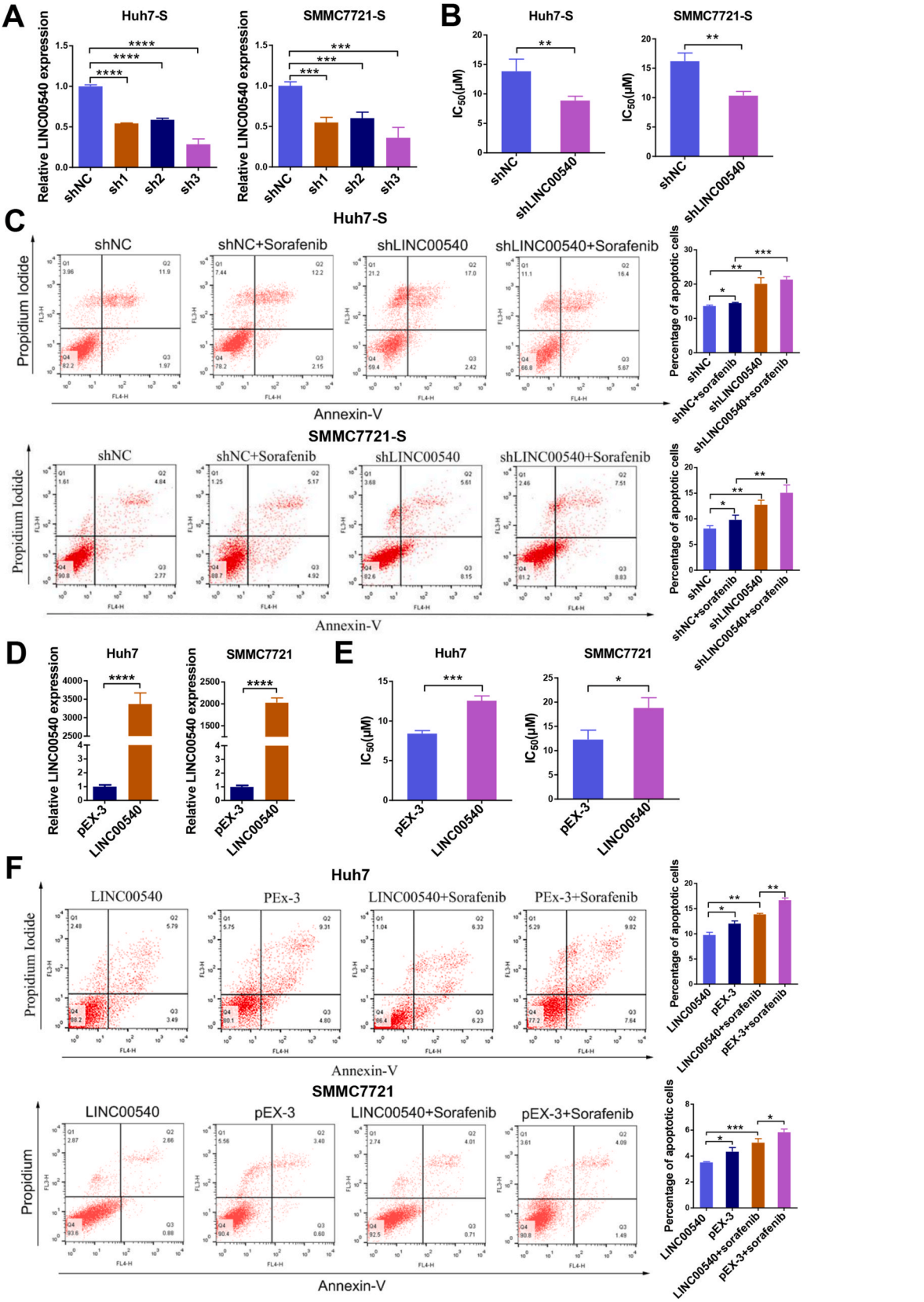

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Fig. 3. LINC00540 stimulates sorafenib resistance in HCC cells. (A) qRT-PCR results demonstrated the significant silencing effects of shRNA on Huh7-S and SMMC7721-S cells, with shRNA3 exhibiting the highest efficacy; (B) Downregulation of LINC00540 increased the sensitivity of Huh7-S and SMMC7721-S cells to sorafenib; (C) Knockdown of LINC00540 promoted apoptosis in sorafenib-resistant cells; (D) qRT-PCR confirmed the overexpression of LINC00540 in Huh7 and SMMC7721 cells; (E) Upregulation of LINC00540 reduced the sensitivity of Huh7 and SMMC7721 cells to sorafenib; (F) Overexpression of LINC00540 inhibited apoptosis in parental cells. Error bars represent standard deviation (n = 3). N.S. = $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

crystal violet for 10 min. The number of migrating cells was counted in five random microscopic fields using ImageJ software. For cell invasion assays, 0.05 mL of Matrigel (50 μg/mL, BD Biosciences, USA) was added to the plate surface and the cells were incubated for approximately 72 h at 37 °C. The plates were then processed as described above.

2.10. Colony formation assay

Stably transfected cells (2000 cells/well) were seeded into 6-well plates, and the medium was replaced every 48 h. After 14 days, the colonies were fixed with methanol and stained with crystal violet (Sigma). Visible colonies were then photographed, and the colony count was determined using ImageJ software (NIH, Bethesda, MD).

2.11. Wound healing assay

Transfected cells (3×10^6) were scratched using a 200-μl pipette tip in 6-well plates and cultured in serum-free medium. Cell images were captured at 0, 24, and 48 h using a microscope (Olympus, Tokyo, Japan).

2.12. Western blot analysis

Total protein was extracted from HCC cells using RIPA buffer supplemented with proteinase inhibitors (Thermo Fisher Scientific). The protein samples were separated on SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore). Subsequently, the membranes were incubated overnight with human primary antibodies against AKR1C2 (Abcam, ab194429), N-cadherin (Abcam, ab18203), vimentin (Abcam, ab8978), E-cadherin (Abcam, ab1416), β-Tubulin (Abcam, ab6046), and GAPDH (Zen Bio, 380626). After washing, the membranes were further incubated with the appropriate secondary antibody for 2 h. The expression levels of the target proteins were visualized using an enhanced chemiluminescence (ECL) kit (Beyotime, China).

2.13. Luciferase reporter assay

For the analysis of LINC00540 and miRNA-4677-3p interactions, sorafenib-resistant cells were seeded into 24-well plates (6×10^4 cells/well). The cells were co-transfected with LINC00540-WT-pesicheck2, LINC00540-MUT-pesicheck2 (FenghBio, Changsha, China), or miRNA-4677-3p mimics (or Control) (GenePharma, Co., Ltd, China) using TransIntroTM EL Transfection Reagent (TransGen Biotech, Beijing, China). Similarly, for the analysis of AKR1C2 and miRNA-4677-3p interactions, cells were co-transfected with AKR1C2-WT-pesicheck2 (or MUT) (FenghBio) and miRNA-4677-3p mimics (or Control) (GenePharma, China) using TransIntroTM EL Transfection Reagent. Luciferase activity was measured after 48 h of co-transfection using the TransDetect Double-Luciferase Reporter Assay kit (Transgen Biotech).

2.14. Analysis of public database sources

The subcellular localization of LINC00540 was predicted using the lncLocator website (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/). Potential miRNAs interacting with AKR1C2 and LINC00540 were predicted using online databases such as miRbase (https://www.mirbase.org/), miRDB (http://mirdb.org/), and TargetScan (http://www.targetscan.org/vert_80/).

2.15. Statistical analysis

Statistical analysis was performed using SPSS software (version 18.0, Chicago, USA) and GraphPad Prism 6.01 software (San Diego, CA, USA). The comparisons between the two groups were assessed using two-tailed Student *t*-test. The overall survival of patients with HCC were analyzed using Kaplan–Meier curves. All data are presented as mean ± SD from a minimum of three independent experiments. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Generation of sorafenib-resistant HCC cells

To establish sorafenib-resistant HCC cell lines, Huh7 and SMMC7721 cells were cultured in medium supplemented with gradually increasing concentrations of sorafenib for approximately 8 months. As illustrated in Fig. 1A, the sorafenib-resistant HCC cells (Huh7-S

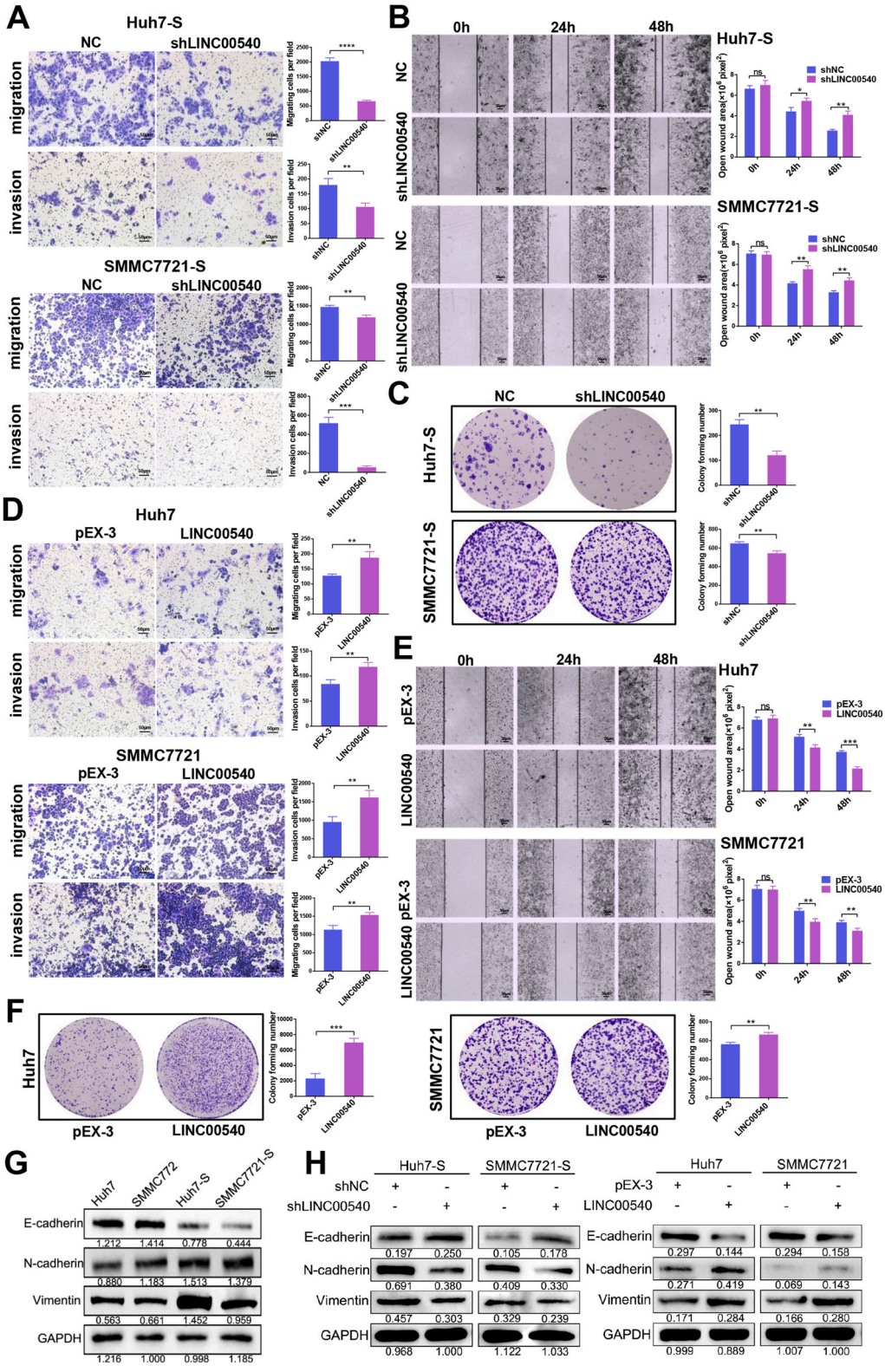

Fig. 4. LINC00540 enhances the migration, invasion, and EMT of HCC cells. (A, B) Transfection with shRNA3 significantly reduced the migration and invasion capabilities of Huh7-S and SMMC7721-S cells; (C) Colony-formation assay confirmed that the proliferation of Huh7-S and SMMC7721-S cells was repressed after transfection with the shRNA3 overexpression plasmid of LINC00540 for two weeks; (D, E) Migration and invasion ability of Huh7 and SMMC7721 cells were increased after transfection with the LINC00540 overexpression plasmid; (F) Colony-formation assay confirmed

that the proliferation of Huh7 and SMMC7721 cells was increased after transfection with the LINC00540 overexpression plasmid for two weeks; (G) Western blot analysis revealed downregulation of E-cadherin and upregulation of N-cadherin and vimentin proteins in sorafenib-resistant cells, indicating the occurrence of EMT; (H) Western blot analysis was conducted to determine the protein levels of E-cadherin, N-cadherin, and vimentin in HCC cells following LINC00540 knockdown and overexpression. Error bars represent standard deviation (n = 3). N.S. = $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

and SMMC7721-S) exhibited a significantly increased half-maximal inhibitory concentration (IC_{50}) compared to the parental cells. Fig. 1B shows the obvious morphological differences observed between the sorafenib-resistant cells and their parental counterparts. Furthermore, the colony growth of sorafenib-resistant cells was enhanced compared to the sensitive parental cell lines when treated with 4 μM sorafenib (Fig. 1C). Thus, the sorafenib-resistant HCC sublines were successfully established.

3.2. Screening for differentially expressed lncRNAs, mRNAs, and miRNAs

Whole transcriptome sequencing (RNA-seq) was performed to establish DEG expression profiles in Huh7-S and Huh7 cells. The screening criteria were defined as an absolute value of $\log_2FC > 2$ and $P < 0.05$. Compared with Huh7 cells, 578 lncRNAs (311 upregulated and 267 downregulated), 70 mRNAs (43 upregulated and 27 downregulated) and 39 miRNAs (15 upregulated and 24 downregulated) showed differentially expressed (DE) in Huh7-S cells (Fig. 2A–C). Fig. 2D–F depicts the volcano plots of the DEmiRNAs, DEmRNAs, and DElncRNAs expression profiles, respectively. LINC00540, MSTRG.11029.1, MSTRG.13178.2, MSTRG.45015.1, MSTRG.23232.8, MSTRG.40443.1 as rather greater difference lncRNAs were obtained after screening by various standards. The expression of six candidate lncRNAs was confirmed by qRT-PCR analysis (Fig. 2G). Among them, LINC00540 exhibited the most significant DE lncRNA in Huh7-S cells and showed high expression in SMMC7721-S cells (Fig. 2H). Furthermore, Kaplan–Meier curve (https://kmplot.com/analysis) and TCGA clinical data analysis demonstrated a significant negative correlation between the expression level of LINC00540 and overall survival in LIHC patients (Fig. 2I; Supplementary Figs. 1A and 1B are included in supplementary file 2). Consequently, we focused on LINC00540 for subsequent experiments. LINC00540, located on chromosome 13 (chr13: 22040975–22276524), is an intergenic lncRNA with a length of 2412 bp. Preliminary prediction using the lncLocator website indicated that LINC00540 is mainly located in the cytoplasm (Supplementary Fig. 2 is included in supplementary file 2).

LINC00540 promoted sorafenib resistance of HCC cells.

Since LINC00540 was upregulated in sorafenib-resistant cells, we conducted knockdown experiments to examine its biological function in Huh7-S and SMMC7721-S cells (Fig. 3A). As shown in Fig. 3B, LINC00540 knockdown significantly decreased the IC_{50} values of sorafenib. Furthermore, flow cytometry experiments indicated an increased apoptosis rate (Fig. 3C). LINC00540 was overexpressed in the parental cell lines (Huh7 and SMMC7721) which was verified by qRT-PCR (Fig. 3D). The CCK-8 assay revealed that the IC_{50} value for sorafenib was significantly increased following transfection with LINC00540 overexpression plasmids in Huh7 and SMMC7721 cells (Fig. 3E). Additionally, flow cytometry experiment showed that the 12.9 μM sorafenib-induced apoptosis was partly rescued by LINC00540 overexpression in Huh7-S and SMMC7721-S cells (Fig. 3F). These findings collectively suggest that LINC00540 contributes to sorafenib resistance in HCC cells.

3.3. LINC00540 enhanced the migration, invasion, and EMT of HCC cells

Sorafenib-resistant cells exhibited an EMT-like phenotype, leading us to speculate that LINC00540 might influence the migration and invasion of HCC cells. A cell migration and invasion assay (Fig. 4A) and a wound healing assay (Fig. 4B) showed that the knocking down LINC00540 inhibited the migration and invasion of sorafenib-resistant cells. Similarly, a colony forming assay revealed that downregulation of LINC00540 inhibited colony growth (Fig. 4C). Conversely, overexpression of LINC00540 promoted migration, invasion and colony growth in the parental cells (Fig. 4D–F).

In addition, Huh7-S and SMMC7721-S cells exhibited an irregular and elongated EMT-like phenotype. Western blot analysis showed a significant reduction in the expression of the epithelial marker E-cadherin, while the levels of the mesenchymal markers, N-cadherin and vimentin, were elevated (Fig. 4G; The original images of blots in Fig. 4G are attached in supplementary file 1). Subsequently, we investigated the association between LINC00540 and the occurrence of EMT in HCC cells. A Western blot assay revealed that downregulation of LINC00540 increased the expression of E-cadherin and repressed the expression of the mesenchymal markers, N-cadherin and vimentin (Fig. 4H; The original images of blots in Fig. 4H are attached in supplementary file 1). Conversely, upregulation of LINC00540 reduced E-cadherin expression and increased the expression of N-cadherin and vimentin. These results indicate that LINC00540 enhances the migration, invasion, and the EMT process in HCC cells.

3.4. LINC00540 functions as a ceRNA for miR-4677-3p to upregulate AKR1C2

To further investigate the potential mechanism of LINC00540, we examined its subcellular localization. The lncLocator website predicted that LINC00540 is mainly located in the cytoplasm (Supplementary Fig. 2). An RNA FISH assay revealed that the LINC00540 transcript was predominantly located in the cytosol of Huh7-S cells (Fig. 5A). Based on this, we hypothesized that LINC00540 functions as a competing endogenous RNA (ceRNA) to regulate downstream proteins. Analysis of potential lncRNA target genes revealed 34 DE mRNAs targeting LINC00540 (Fig. 5B). Among these, AKR1C2 was highly expressed in sorafenib-resistant cells (Fig. 5C) and demonstrated high expression in liver cancer tissues according to TCGA clinical data analysis (Fig. 5D). Furthermore, elevated levels of

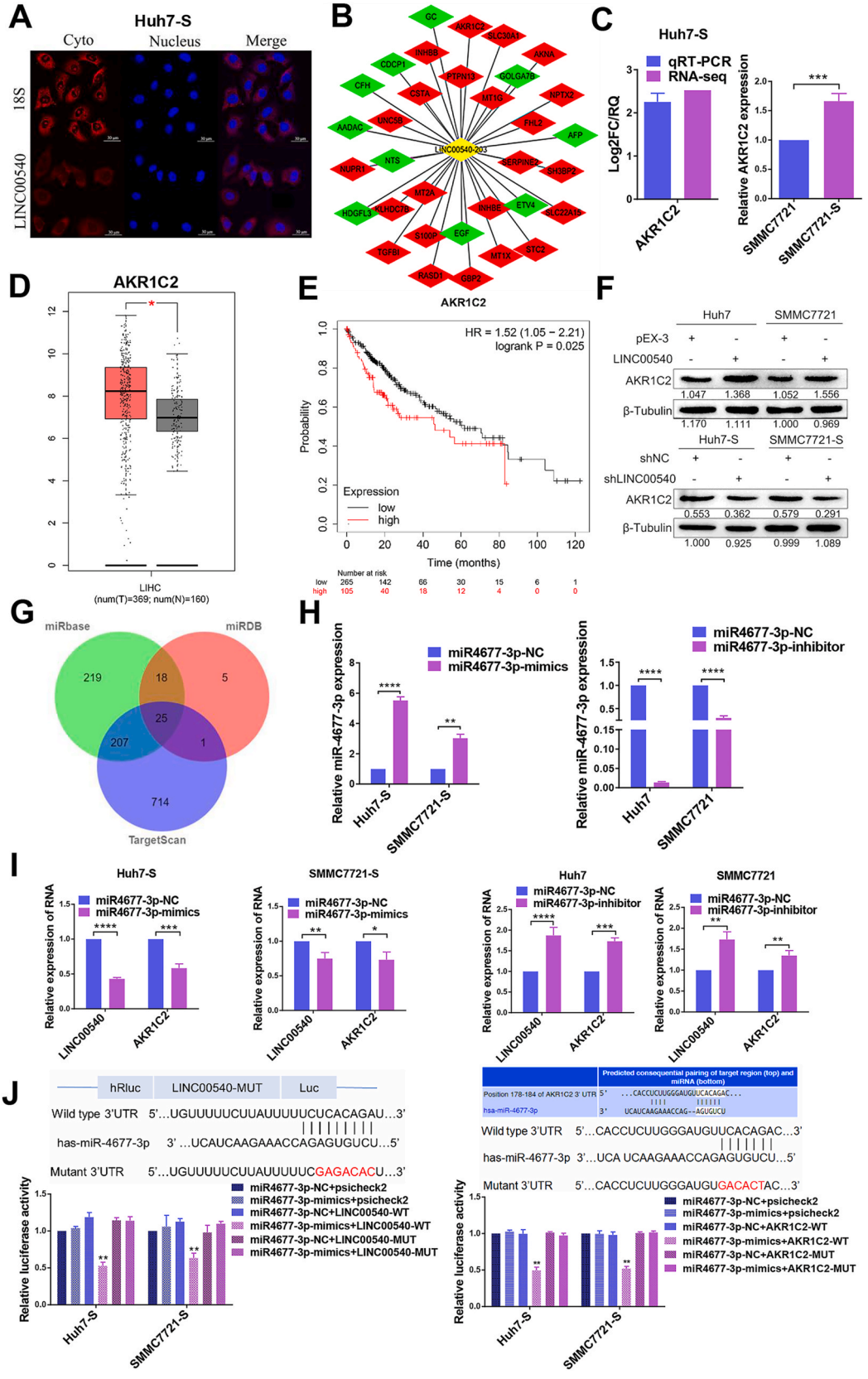

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Fig. 5. LINC00540 functions as a ceRNA for miR-4677-3p to upregulate AKR1C2. (A) RNA FISH assay confirmed LINC00540 was primarily located in the cytosol of Huh7-S cells; (B) Co-targeting analysis of LINC00540-mRNAs and the upregulated genes are indicated by red, and the downregulated genes are indicated by green; (C) AKR1C2 was significantly overexpressed in sorafenib-resistant cells by qRT-PCR analysis; (D) GEPIA data analysis for AKR1C2 of LIHC samples showing high expression; (E) Kaplan–Meier curve showing an association of high AKR1C2 levels with low survival in HCC patients; (F) qRT-PCR and Western blot assays confirming AKR1C2 was downregulated in LINC00540 knockdown cells, whereas the opposite occurred in LINC00540 overexpressing cells; (G) TargetScan, miRbase and miRDB databases consistently predicted 25 miRNAs targeting LINC00540; (H) The efficiency of knockdown or overexpression miR-4677-3p was verified using mimics and an inhibitor by qRT-PCR; (I) qRT-PCR assay showing LINC00540 and AKR1C2 downregulation following transfection with miR-4677-3p mimics in sorafenib-resistant cells, whereas the opposite result was observed following transfection with the miR-4677-3p inhibitor in the parental cells; (J) Prediction of miR-4677-3p binding sites on LINC00540 and AKR1C2 transcripts, and the interaction of miR-4677-3p with LINC00540 and AKR1C2 was verified in sorafenib-resistant cells. Error bars represent standard deviation (n = 3). N.S. = $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

AKR1C2 in HCC patients were associated with poorer outcomes (Fig. 5E). Previous studies have also indicated the close relationship between AKR1C2 and drug sensitivity in tumor cells [25–27]. Repression of LINC00540 using shRNA resulted in decreased AKR1C2 expression, while overexpression of LINC00540 yielded the opposite effect (Fig. 5F; The original images of blots in Fig. 5F are attached in supplementary file 1).

Next, we predicted 25 common miRNAs that may target LINC00540 and miRNAs that interact with AKR1C2 using miRbase, miRDB, and TargetScan databases (Fig. 5G). As a result, miR-4677-3p was selected due to its relatively high binding potential and as a common target of LINC00540 and AKR1C2. Furthermore, we observed high expression of miR-4677-3p through miRNA sequencing data analysis. Sorafenib-resistant cells (Huh7-S and SMMC7721-S) and parental cells (Huh7 and SMMC7721) were selected for knockdown or overexpression of miR-4677-3p using mimics and an inhibitor, respectively (Fig. 5H). As expected, the expression of LINC00540 and AKR1C2 were downregulated following transfection with miR-4677-3p mimics in sorafenib-resistant cell lines, whereas transfection with an miR-4677-3p inhibitor resulted in the opposite effect in the parental cell lines (Fig. 5I). Subsequently, we identified the potential miR-4677-3p binding sites in the 3′-UTR of LINC00540 and AKR1C2 through in silico analysis using TargetScan. We constructed luciferase reporter plasmids, including LINC00540-WT, LINC00540-MUT, AKR1C2-WT and AKR1C2-MUT, which contained miR-4677-3p binding sites. Overexpression of miR-4677-3p significantly repressed the luciferase activity of the plasmids carrying WT constructs (LINC00540-WT, AKR1C2-WT). In contrast, transfection with MUT plasmids or knockdown of miR-4677-3p did not inhibit luciferase activity (Fig. 5J). Together, these findings suggest that miR-4677-3p directly binds to LINC00540 and AKR1C2.

3.5. AKR1C2 expression was reversed by miR-4677-3p and promoted sorafenib resistance

To investigate the role of AKR1C2 as a target molecule in the LINC00540/miR-4677-3p signaling pathway, we conducted rescue experiments by transfecting miR-4677-3p mimics into LINC00540 overexpressing HCC cells. Our results demonstrated that LINC00540 overexpression upregulated AKR1C2 expression, while transfection with miR-4677-3p mimics downregulated AKR1C2 expression in Huh7 and SMMC7721 cells (Fig. 6A; The original images of blots in Fig. 6A are attached in supplementary file 1). Moreover, CCK-8 assays revealed that LINC00540 overexpression reduced the chemosensitivity of sorafenib, which was reversed by transfection with miR-4677-3p mimic in Huh7 and SMMC7721 cells (Fig. 6B). Subsequently, we used siRNA to knock down AKR1C2 expression (Fig. 6C), resulting in a significant decrease in the IC_{50} values of sorafenib in Huh7-S and SMMC7721-S cells (Fig. 6D), as well as an increased in apoptosis in sorafenib-resistant cells (Fig. 6E). Additionally, transfection with AKR1C2 siRNA suppressed cell invasion and migration in sorafenib-resistant cells (Fig. 6F). The knockdown effect of AKR1C2 at protein level was verified by Western blot analysis (Fig. 6G; The original images of blots in Fig. 6G are attached in supplementary file 1). Western blot analysis further demonstrated that AKR1C2 knockdown upregulated the expression of E-cadherin and downregulated the expression of N-cadherin and vimentin (Fig. 6H; The original images of blots in Fig. 6H are attached in supplementary file 1). These findings suggest that AKR1C2 serves as a downstream target gene of the LINC00540/miR-4677-3p axis, promoting sorafenib resistance, enhancing migration, invasion, and EMT, while suppressing apoptosis in HCC cells.

3.6. AKR1C2 as the target gene of LINC00540 promoted the activation of the PI3K/AKT pathway

KEGG enrichment analysis showed that the differentially expressed target genes of LINC00540 were mainly enriched in PI3K/AKT signaling pathway, which is implicated in the development of sorafenib resistance in HCC (Fig. 7A). Previous studies have demonstrated that AKR1C2 activates the PI3K/AKT pathway, promoting the proliferation of esophageal squamous cell carcinoma and prostate cancer cells [28,29]. Furthermore, many recent studies have shown that the PI3K/AKT pathway is involved in sorafenib resistance in hepatocellular carcinoma [30–32]. Western blot analysis further demonstrated a significant reduction in the phosphorylation levels of PI3K and AKT in LINC00540 knockdown HCC sorafenib-resistant cells, while the total protein levels of PI3K and AKT remained unchanged (Fig. 7B; The original images of blots in Fig. 7B are attached in supplementary file 1). Conversely, the levels of phosphorylated PI3K (p-PI3K) and p- AKT were significantly upregulated in the parental cells, as shown in Fig. 7C (The original images of blots in Fig. 7C are attached in supplementary file 1). Moreover, knockdown of AKR1C2 resulted in a reduction of PI3K and AKT phosphorylation levels, without significant changes in total protein expression levels of PI3K and AKT in Huh7-S and SMMC7721-S cells, compared with the control groups (Fig. 7D; The original images of blots in Fig. 7D are attached in supplementary

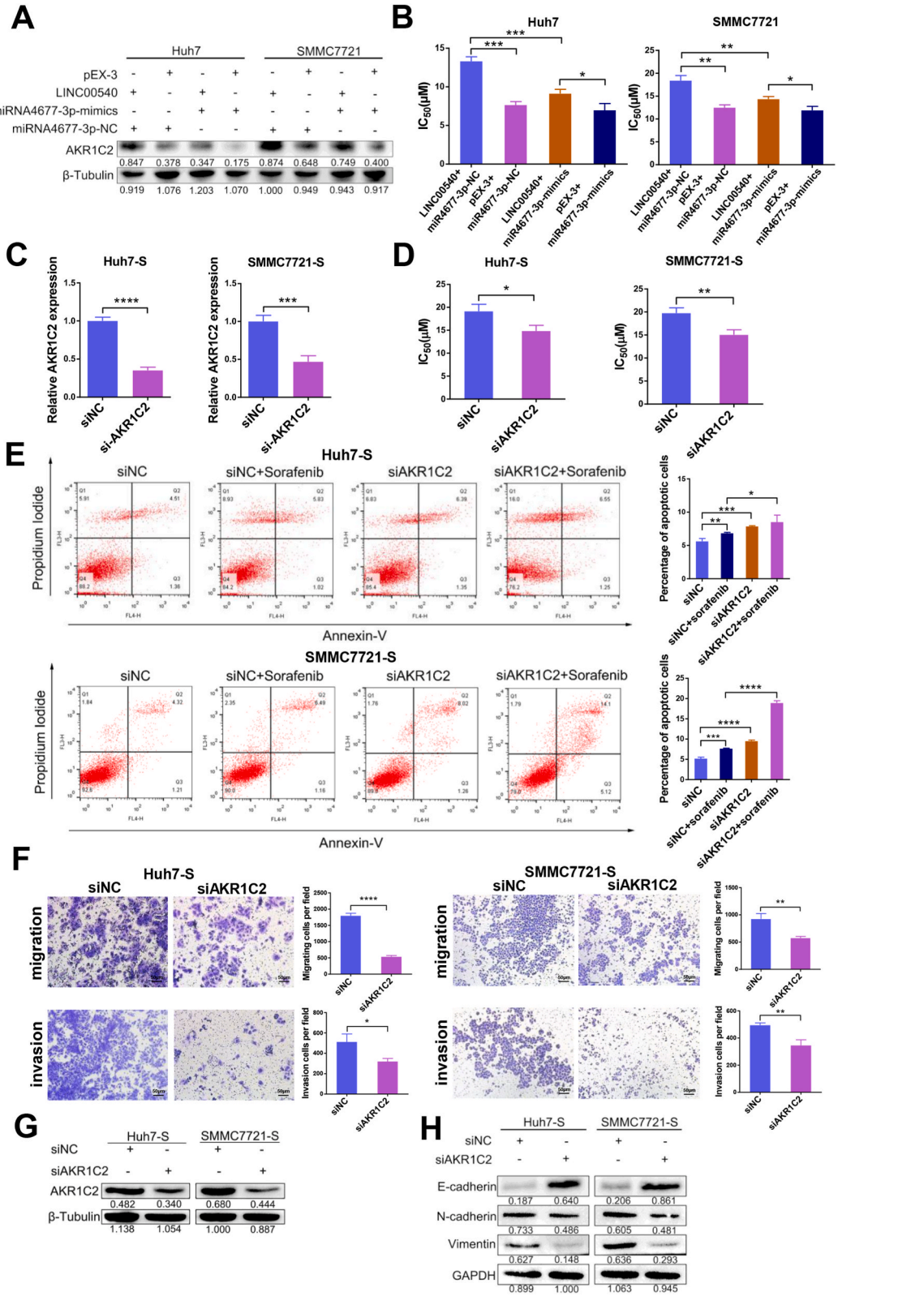

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Fig. 6. AKR1C2 expression was reversed by miR-4677-3p and promoted sorafenib resistance. (A) miR-4677-3p rescued the upregulation of AKR1C2 caused by LINC00540 overexpression in Huh7 and SMMC7721 cells; (B) miR-4677-3p reversed the sorafenib resistance mediated by LINC00540 overexpression; (C) AKR1C2 knockdown efficiency was confirmed using siRNA through qRT-PCR and Western blot assays in sorafenib-resistant cells; (D) AKR1C2 knockdown enhanced sorafenib sensitivity of Huh7-S and SMMC7721-S cells; (E) Knockdown of AKR1C2 promoted apoptosis in sorafenib-resistant cells; (F) Migration and invasion ability of Huh7-S and SMMC7721-S cells were repressed after siAKR1C2 transfection; (G) AKR1C2 protein expression was significantly reduced after siRNA treatment; (H) Western blot results demonstrated upregulation of E-cadherin and downregulation of N-cadherin and vimentin in sorafenib-resistant cells following AKR1C2 knockdown. Error bars represent standard deviation ($n = 3$). N.S. = $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

file 1). These findings indicate that LINC00540 promoted the activation of the PI3K/AKT pathway through its downstream target gene, AKR1C2.

4. Discussion

Sorafenib significantly improves survival in patients with advanced liver cancer. However, chemoresistance poses a major challenge in overcoming the poor efficacy of chemotherapy for HCC [33,34]. Sorafenib resistance can be classified into two types: primary resistance, which is inherent and mainly attributed to genetic heterogeneity prior to drug treatment [35]; and acquired resistance, which occurs under various conditions such as inhibition of sorafenib uptake, enhanced drug excretion, epigenetic changes, activation/inactivation of key pathways, disruption of DNA repair mechanisms, imbalance of cell cycle/apoptosis regulation, and changes in tumor microenvironment [34,36,37]. For instance, SETD1A enhances YAP activation to induce primary sorafenib resistance in HCC [38]. The PI3K/AKT and JAK-STAT signaling pathways, EMT process, and ATF2 signaling have also been implicated in sorafenib resistance [39–42]. In the sorafenib-resistant cell model, stemness EMT and lncRNA play a crucial role in driving sorafenib resistance at the single-cell level [43]. In the present study, we observed that LINC00540 can facilitate metastasis and sorafenib resistance in HCC cells by activating the EMT and PI3K/AKT signaling pathways. Additionally, we found that LINC00540 contributes to sorafenib resistance in HCC through the downstream target molecule AKR1C2, marking the first implication of this novel lncRNA in this process. We constructed sorafenib-resistant cells (Huh7-S and SMMC7721-S) and observed that the resistant sublines exhibited an epithelial-mesenchymal phenotype compared to the parental cells. Western blot analysis showed the promotion of the EMT process in Huh7-S and SMMC7721-S cells. This observation suggests that HCC cell proliferation may have been enhanced through the EMT pathway, potentially counteracting the deleterious effects of sorafenib.

LncRNAs play an increasingly critical role in the occurrence and development of tumors [44–46]. Moreover, they are involved in drug resistance and EMT pathways in various cancers. For example, Liu et al. identified MIR100HG as a potent EMT inducer in CRC, contributing to cetuximab resistance and metastasis through the activation of a MIR100HG/hnRNPA2B1/TCF7L2 feedback loop [47]. Growing research has demonstrated that lncRNAs modulate sorafenib sensitivity through various pathways and downstream target molecules. For instance, Chen et al. showed that LINC01234 promotes HCC progression and sorafenib resistance by modulating aspartic acid metabolic reprogramming [48]. Additionally, MALAT1 contributes to sorafenib resistance in HCC by regulating miR-140-5p/Aurora-A signaling [49]. Furthermore, lncRNA HEIH enhances sorafenib resistance in HCC by activating the PI3K/AKT pathway through miR-98-5p [50]. In this study, we aimed to identify lncRNAs associated with sorafenib resistance in HCC through transcriptional profiling. Our results revealed significant upregulation of LINC00540 in sorafenib-resistant HCC cells. Moreover, patients with high expression levels of LINC00540 exhibited poorer outcomes. Additional evidence indicated that downregulation of LINC00540 inhibited sorafenib resistance, migration, invasion, and EMT in HCC cells, whereas upregulation of LINC00540 had the opposite effect.

Subsequently, a subcellular localization assay revealed that the majority of LINC00540 is located in the cytosol. Thus, we hypothesized that LINC00540 functions as a miRNA sponge in HCC, suggesting that lncRNAs play a vital role in tumor progression through ceRNA mechanisms. Moreover, studies have shown that lncRNAs, acting as ceRNAs, are crucial in tumor development and drug resistance [51–53]. The analysis of lncRNAs target genes and subsequent Western blot assays confirmed AKR1C2 as a downstream target of LINC00540. By utilizing the miRbase, miRDB, and TargetScan databases, we identified miR-4677-3p, which targets both LINC00540 and AKR1C2. Recent studies have demonstrated the involvement of miR-4677-3p in multiple ceRNA mechanisms. For example, LINC02418 is involved in malignant lung adenocarcinoma through the miR-4677-3p/KNL1 axis [54], while GLIDR promotes glioma progression by regulating the miR-4677-3p/MAGI2 axis [55]. Western blot and qRT-PCR assays indicated that LINC00540 and AKR1C2 are direct target genes of miR-4677-3p in HCC cells. Additionally, a luciferase reporter assay demonstrated the direct interaction between miR-4677-3p and LINC00540, as well as AKR1C2. These findings suggest that LINC00540 acts as a ceRNA for miR-4677-3p, thereby upregulating AKR1C2. However, it should be noted that the current study does not exclude the possibility of LINC00540 affecting other miRNAs or associated genes in HCC, which warrants further investigation.

AKR1C2 is a member of the aldo-keto reductase superfamily, which metabolizes a wide range of substrates and serves as a potential drug target [56]. AKR1C2 reduces the activity of chemotherapeutic drugs through carbonyl reduction. As an oncogene, AKR1C2 is expressed in various cancers and contributes to tumor progression and chemoresistance [25,28,57]. For instance, AKR1C2 is regulated by AEG-1 and promotes cell migration, invasion, proliferation, and EMT in liver cancer [58,59]. Additionally, AKR1C2 plays a significant role in cancer growth and metastasis in triple-negative breast cancer [60]. Moreover, AKR1C2 as a targetable oncogene, promotes proliferation, migration and cisplatin resistance by activating the PI3K/AKT signaling pathway in esophageal squamous cell carcinoma [28]. Herein, we observed a significant upregulation of AKR1C2 in sorafenib-resistant cells compared to parental cells. Importantly, overexpression of LINC00540 led to increased sorafenib resistance and upregulation of AKR1C2, which could be reversed

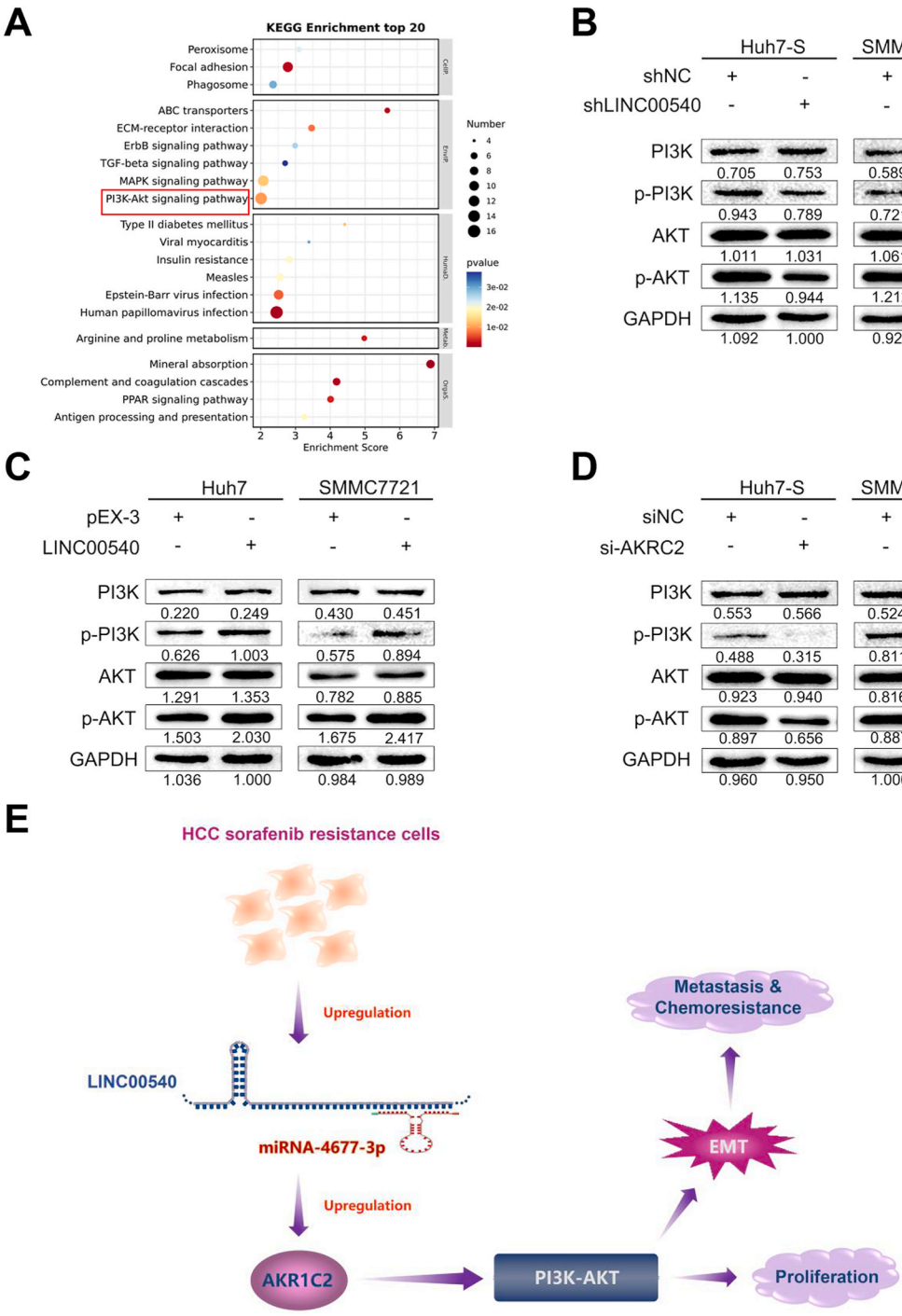

Fig. 7. AKR1C2 as the target gene of LINC00540 promoted the activation of the PI3K/AKT pathway. (A) KEGG analysis of the differentially expressed target genes of LINC00540 revealed the PI3K/AKT signaling pathway (highlighted in red); (B, C) Western blot results demonstrated the promotion of PI3K/AKT pathway activation by LINC00540; (D) AKR1C2 knockdown inhibited the activation of the PI3K/AKT pathway in sorafenib-resistant HCC cells; (E) Proposed model illustrating the involvement of the LINC00540/miR-4677-3p/AKR1C2 axis in sorafenib resistance in HCC cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

by transfection with miR-4677-3p mimics. These results suggest that AKR1C2 is a downstream target gene of the LINC00540/miR-4677-3p pathway. Loss-of-function assays demonstrated that siRNA-mediated knockdown of AKR1C2 reduced sorafenib resistance, migration, invasion, and promoted apoptosis in HCC cells, while repressing EMT. We observed upregulation of

AKR1C2 in HCC tissues, and patients with high AKR1C2 expression levels tended to have a poorer prognosis. To the best of our knowledge, the role of AKR1C2 in sorafenib resistance in HCC has not been reported. Prospectively, AKR1C2 serve as a promising marker for HCC therapy. Further exploration is required to fully elucidate the downstream regulatory mechanisms involving AKR1C2. KEGG pathway analysis revealed significant enrichment of the PI3K/AKT signaling pathway, which has been previously reported to be activated by AKR1C2. Furthermore, Liao et al. found that PI3K and AKT were activated by sorafenib in sorafenib-resistant HuH-7 cells [30]. Based on these findings, we hypothesize that LINC00540 may facilitate the activation of the PI3K/AKT pathway through AKR1C2. Subsequently, Western blot analysis revealed that silencing both LINC00540 and AKR1C2 reduced the levels of phosphorylated PI3K and AKT, while upregulation of LINC00540 increased the levels of phosphorylated PI3K and AKT. These results suggest that LINC00540 promotes the activation of PI3K/AKT pathway and EMT process through AKR1C2, thereby enhancing the progression and sorafenib resistance of HCC cells. Nevertheless, it is essential to acknowledge the limitations of this study. In our forthcoming research, we plan to delve deeper into the impact of LINC00540 on sorafenib resistance in an in vivo context, utilizing xenograft models. Moreover, the acquisition of clinical samples from HCC patients is imperative to assess LINC00540 expression levels and establish meaningful correlations with patients' clinical outcomes and responses to sorafenib treatment. These pivotal validations will serve to augment the comprehensiveness of our research findings and enhance their clinical significance.

In summary, we demonstrated that LINC00540, a significantly upregulated lncRNA, was associated with sorafenib resistance in HCC by establishing a transcriptome expression profile. Biochemical and cell biological experiments revealed that LINC00540 stimulates sorafenib resistance, metastasis, EMT and PI3K/AKT pathway in HCC cells. LINC00540 functions as a ceRNA for miR-129-5p and regulates the expression of AKR1C2 in HCC cells (Fig. 7E). Our findings suggest that LINC00540 may serve as a potential therapeutic target. Subsequent research will explore how targeting LINC00540 or the LINC00540/miR-4677-3p/AKR1C2 axis can be translated into clinical applications, offering new avenues for HCC treatment. Therefore, our study not only uncovers the critical role of LINC00540 in HCC but also paves the way for further investigations that have the potential to benefit HCC patients by improving prognosis and therapeutic options.

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Ethics statement

The original patient data for our study were obtained from public databases. The acquisition process strictly adheres to the database access policy. Therefore, Ethics approval and consent to participate are not applicable.

Data availability statement

The following information was supplied regarding data availability: Data is available at SRA using accession number SRP348321.

CRediT authorship contribution statement

Kaixuan Xu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Xinxin Wang:** Methodology, Formal analysis, Conceptualization. **Shuwei Hu:** Software, Methodology, Formal analysis, Data curation. **Jiaxuan Tang:** Visualization, Software, Data curation. **Shihui Liu:** Software, Resources, Project administration. **Hui Chen:** Writing – review & editing, Validation. **Xiaobin Zhang:** Writing – review & editing, Resources, Project administration. **Penggao Dai:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27322.

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