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miR-552 Regulates Liver Tumor-Initiating Cell Expansion and Sorafenib Resistance

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MicroRNAs (miRNAs) are involved in tumorigenesis, progression, recurrence, and drug resistance of hepatocellular carcinoma (HCC). However, few miRNAs have been identified and entered clinical practice. Herein, we report that microRNA (miR)-552 is upregulated in HCC tissues and has an important function in liver tumor-initiating cells (T-ICs). Functional studies revealed that a forced expression of miR-552 promotes liver T-IC self-renewal and tumorigenesis. Conversely, miR-552 knockdown inhibits liver T-IC self-renewal and tumorigenesis. Mechanistically, miR-552 downregulates phosphatase and tensin homolog (PTEN) via its mRNA 3′ UTR and activates protein kinase B (AKT) phosphorylation. Our clinical investigations elucidated the prognostic value of miR-552 in HCC patients. Furthermore, miR-552 expression determines the responses of hepatoma cells to sorafenib treatment. The analysis of patient cohorts and patient-derived xenografts (PDXs) further demonstrated that miR-552 may predict sorafenib benefits in HCC patients. In conclusion, our findings revealed the crucial role of the miR-552 in liver T-IC expansion and sorafenib response, rendering miR-552 an optimal target for the prevention and intervention in HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies in the world.[1] There are approximately 700,000 new HCC patients recorded every year, and more than half of these new cases are in China.[2] Most HCC patients are diagnosed at advanced stage and therefore lost the opportunity to be surgically treated. For those patients with advanced HCC, optimal therapeutic options are limited.[3] Currently, sorafenib is the most used targeted drug for advanced HCC, which provides limited survival benefits.[4] Therefore, it is urgent to explore the underlying mechanisms of HCC initiation and progression.

Several studies provided theoretical support on liver tumor-initiating cells (T-ICs) in liver cancer.[5] Liver T-ICs are a small part of liver cancer cells that are capable of extensive proliferation, self-renewal, and increased frequency of tumor formation.[6–8] Liver T-ICs can be identified by numerous surface markers, including cluster of differentiation 133 (CD133), epithelial cell adhesion molecule (EpCAM), CD24, and CD90.[9–12] It was reported that CD24 can promote liver T-IC self-renewal and tumor initiation via the signal transducer and activator of transcription 3 (STAT3)-mediated Nanog pathway. Tumors that harbor an abundant T-IC population or have high expression of stemness-related genes may signal a poor clinical outcome in HCC patients.[13] The understanding of how liver T-ICs regulate tumor initiation and progression is of key importance for future treatment strategies.

MicroRNAs (miRNAs) are a class of small noncoding RNA molecules that contain approximately 22 nucleotides, found in plants, animals, and some viruses, and with functions in the regulation of gene expression at both the transcriptional and translational levels.[14,15] miRNAs can regulate RNA silencing and post-transcriptional gene expression in general by binding to the 3′ UTR of target mRNAs.[16,17] Numerous studies found that miRNAs have important roles in the occurrence and development of various tumors, including liver, breast, lung, and bladder cancer.[18–20] miR-552 is a newly discovered miRNA, and its function and mechanism of action in biological processes and diseases are not completely understood. Previous studies found that miR-552 promotes colorectal cancer cell progression by directly targeting dachshund homologue 1 (DACH1) via the Wnt/β-catenin signaling pathway.[21] Moreover, miR-552 also enhances the metastatic capacity of colorectal cancer cells by targeting a disintegrin and metalloproteinase 28.[22] However, the function of miR-552 in liver T-ICs is unknown.

In the present study, we first found that the expression of miR-552 is upregulated in liver T-ICs and predicts poor prognosis in HCC

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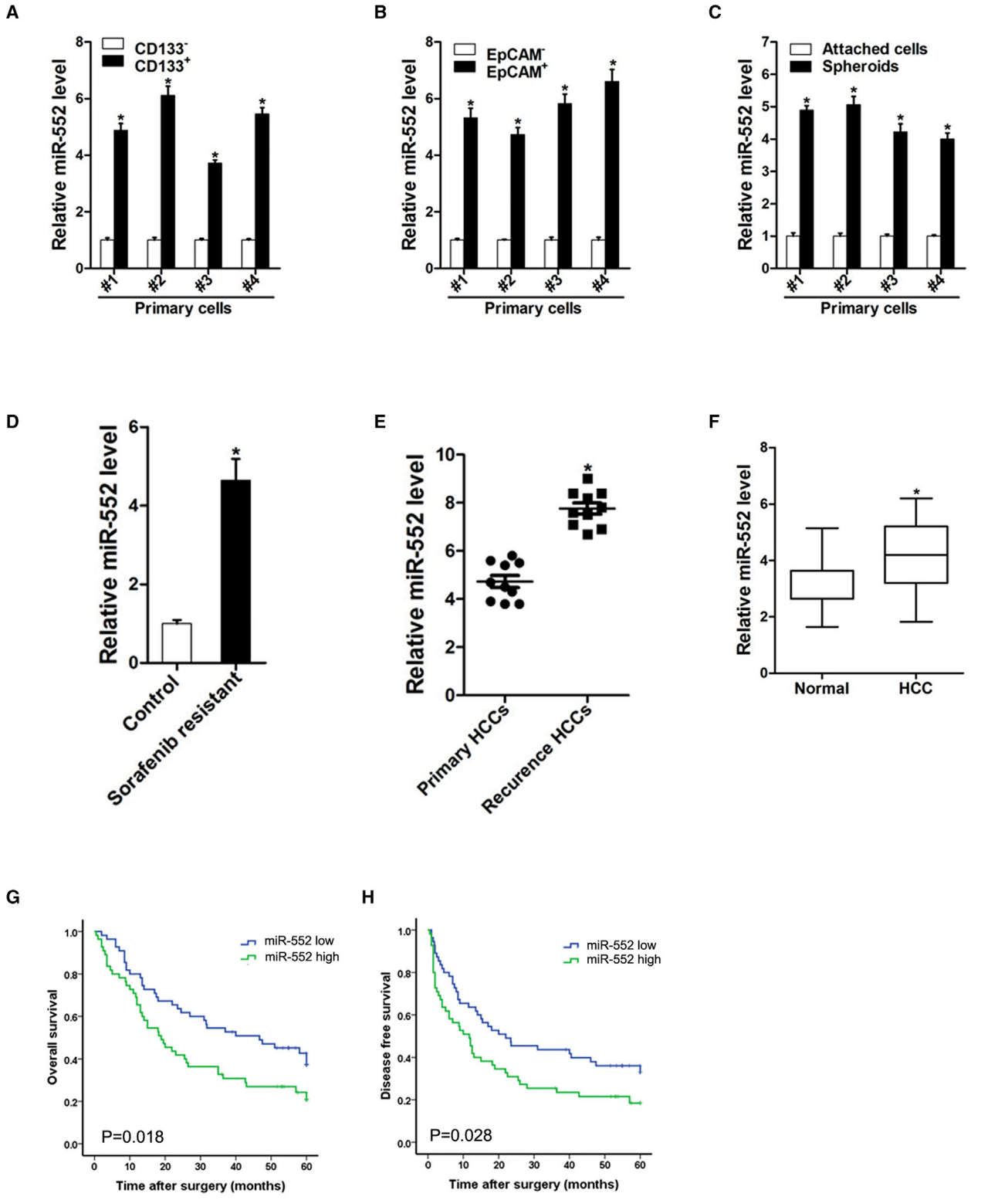

Figure 1. miR-552 Is Upregulated in Liver T-ICs and Predicts Poor Prognosis

(A) Real-time PCR analysis of miR-552 in MACS-sorted CD133$^+$ primary HCC cells relative to negative cells (n = 3). (B) Real-time PCR analysis of miR-552 in MACS-sorted EpCAM$^+$ primary HCC cells relative to negative cells (n = 3). (C) Real-time PCR analysis of miR-552 in primary HCC adherent and spheroids cells (n = 3). (D) Real-time PCR

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patients. Next, with the use of loss-of-function and gain-of-function analyses in liver T-ICs, we demonstrate that miR-552 can promote the self-renewal capacity and tumorigenicity of liver T-ICs. A further mechanism study reveals that miR-552 downregulates phosphatase and tensin homolog (PTEN) via its mRNA 3′ UTR and activates protein kinase B (AKT) phosphorylation. Our clinical investigations elucidated the prognostic value of miR-552 in HCC patients and also demonstrated that miR-552 may predict sorafenib benefits in HCC patients. In conclusion, our findings revealed the crucial role of the miR-552 in liver T-IC expansion and the sorafenib response, rendering miR-552 an optimal target for the prevention and intervention in HCC.

RESULTS

miR-552 Is Upregulated in Liver T-ICs and Predicts Poor Prognosis in HCC Patients

It is reported that CD133 and EpCAM are well-accepted liver T-IC markers.[9,11] As shown in Figures 1A and 1B, miR-552 expression was increased in $CD133^+$ and $EpCAM^+$ liver T-ICs that were sorted from primary HCC patients. Compared with the attached cells, miR-552 expression was also upregulated in HCC spheres derived from human primary HCC cells (Figure 1C). Several HCC cell lines showed similar results (Figures S1A–S1D). Increasing studies showed that liver T-ICs were associated with HCC chemoresistance and recurrence.[23,24] In comparison with control tumors, miR-552 expression was notably upregulated in the sorafenib-resistant HCC residual, indicating that miR-552 expression was associated with chemoresistance (Figure 1D). Consistently, we also observed that miR-552 expression was dramatically enhanced in recurrent HCC compared with the primary lesion (Figure 1E). These data indicated that miR-552 was preferentially upregulated in liver T-ICs.

To investigate the clinical significance of miR-552, we checked its expression in HCC tissues from patients' cohort 1. We observed a significant upregulation of miR-552 in HCCs from a cohort consisting of 110 patients (Figure 1F). More importantly, Kaplan-Meier analysis revealed that high miR-552 levels in HCCs correlated with worse disease-free survival (DFS) and overall survival (OS) of patients (Figures 1G and 1H).

miR-552 Is Required for the Maintenance of Liver T-ICs

To explore the role of miR-552 in liver T-ICs, miR-552 knockdown hepatoma cells were used (Figure 2A). We found that miR-552 interference reduced the expression of liver T-IC markers and stemness-associated genes in hepatoma cells (Figures 2B and 2C). Flow cytometry analysis revealed a decreased proportion of liver T-ICs in miR-552 knockdown hepatoma cells (Figure 2D). Consistently, the protein expression of EpCAM was also downregulated in miR-552 knockdown hepatoma cells (Figure 2E). Additionally, miR-552 interference hepatoma cells formed smaller and fewer spheroids than control cells (Figure 2F). Furthermore, the *in vitro*- and *in vivo*-limiting dilution assay revealed that suppression of miR-552 significantly reduced tumor incidence and T-IC frequency in hepatoma cells (Figures 2G and 2H).

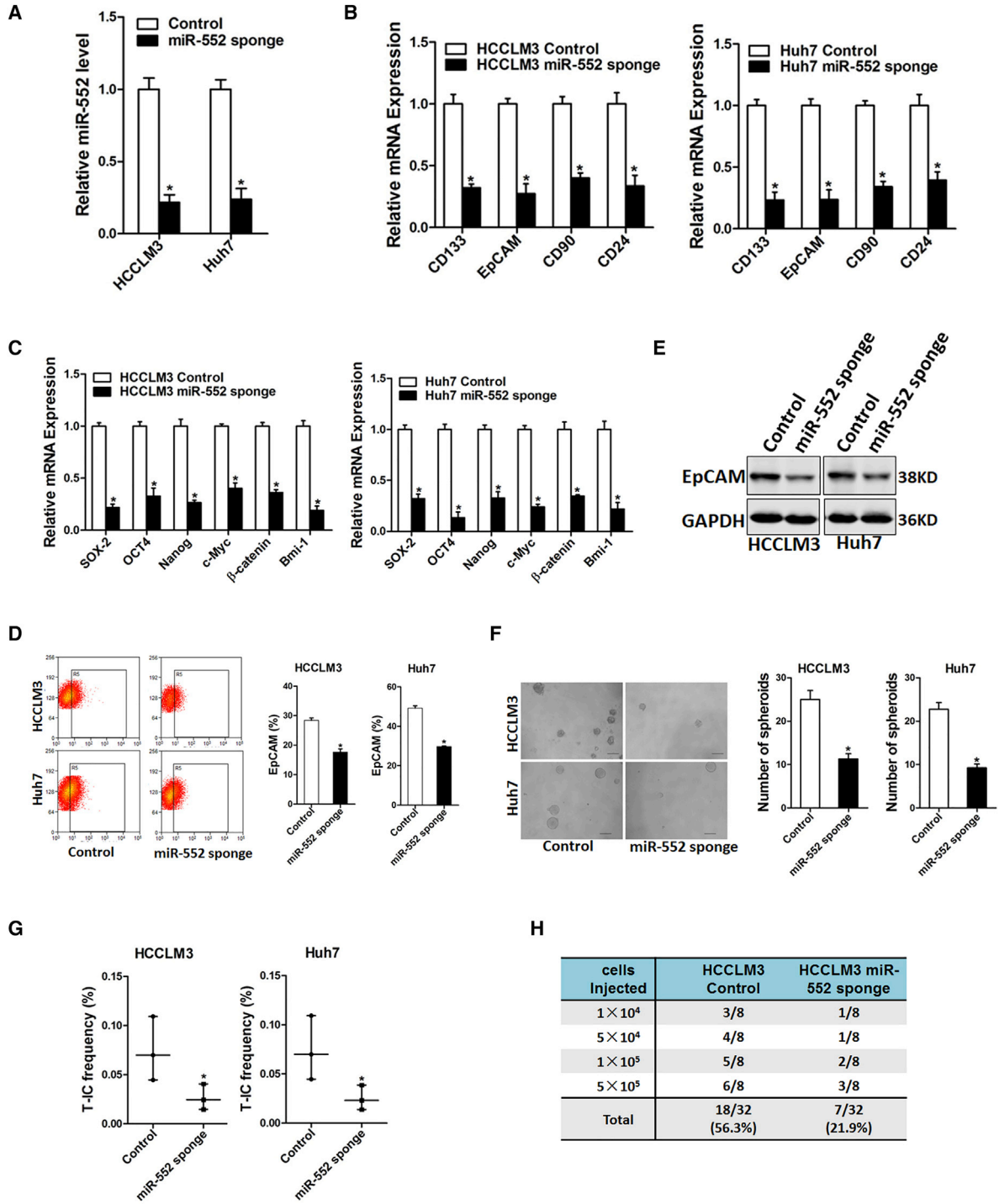
miR-552 Promotes Liver T-IC Expansion

To further explore the role of miR-552 in liver T-ICs, hepatoma cells stably overexpressing miR-552 were used (Figure 3A). As expected, the expression of liver T-IC markers and stemness-associated genes was dramatically increased in miR-552-overexpressing hepatoma cells (Figures 3B and 3C). Flow cytometric analysis revealed an increased proportion of liver T-ICs in miR-552-overexpressing hepatoma cells (Figure 3D). Consistently, EpCAM protein expression was also upregulated in miR-552-overexpressing hepatoma cells (Figure 3E). Additionally, miR-552-overexpressing hepatoma cells formed much more spheroids than control cells (Figure 3F). Moreover, the *in vitro*- and *in vivo*-limiting dilution assay revealed that overexpression of miR-552 significantly increased tumor incidence and T-IC frequency in hepatoma cells (Figures 3G and 3H).

PTEN Is a Direct Target of miR-552 in Liver T-ICs

To identify miR-552 target genes that may be involved in liver T-IC expansion, we investigated the expression of a series of important molecules that are involved in liver T-IC regulation and found that PTEN protein expression was downregulated in miR-552-overexpressing hepatoma cells (Figure 4A). Moreover, PTEN mRNA expression was also decreased in miR-552-overexpressing hepatoma cells (Figure 4B). Consistently, PTEN protein and mRNA expression were increased in miR-552 interference hepatoma cells (Figures S2A and S2B). Bioinformatics analysis suggested that PTEN mRNA harbors a putative miR-552 binding site in its 3′ UTR (Figure 4C). To further explore whether miR-552 directly regulates PTEN expression via interaction with its 3′ UTR, the wild-type (WT) or mutant PTEN 3′ UTR reporter plasmids were transfected into miR-552-overexpressing or -interference hepatoma cells. The mutation of the miR-552 binding site in the PTEN 3′ UTR diminished the distinct activation of PTEN 3′ UTR between miR-552-overexpressing (Figure 4D) or miR-552 knockdown cells (Figure S2C) and control cells. Consistently, there was a significant negative correlation among miR-552, EpCAM, CD133, and PTEN mRNA expression in HCC samples (Figure 4E and Figures S2D and S2E). To further confirm the role of PTEN in miR-552-mediated liver T-IC expansion, the PTEN-overexpressing virus was used (Figure S2F). PTEN overexpression completely abolished the enhancement of T-IC markers or stemness-associated gene expression, self-renewal,

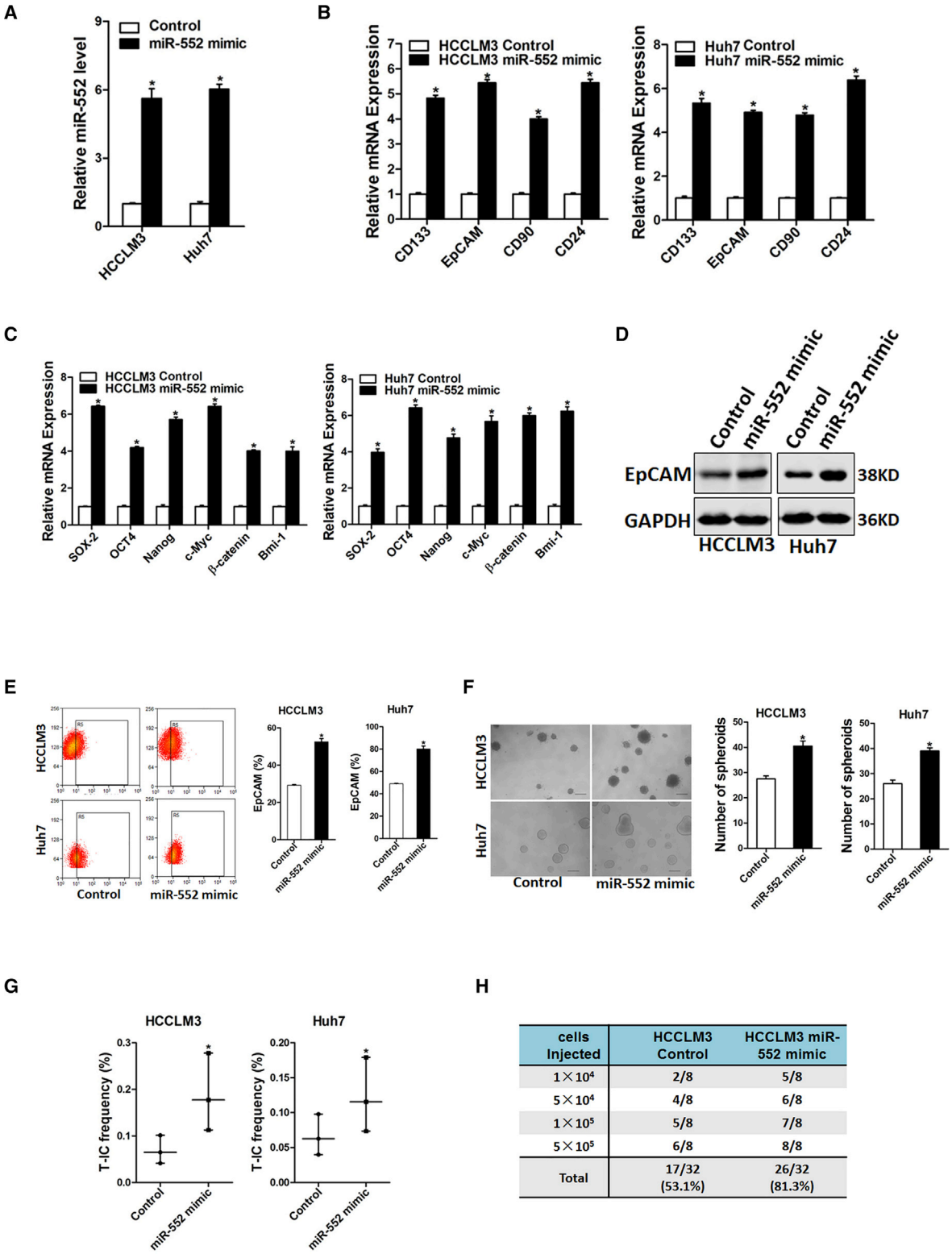
was performed to check the expression of miR-552 in a sorafenib-resistant HCC xenograft (n = 3). (E) Real-time PCR analysis of miR-552 in primary HCC tissues and recurrence HCC tissues (n = 10). (F) The expression of miR-552 in 110 pairs of HCC (cohort 1) and neighboring noncancerous tissues (normal) was checked by real-time PCR analysis. (G) The overall free survival time after surgery of the patients in cohort 1 was compared between the "miR-552 low" (n = 55) and "miR-552 high" (n = 55) groups. (H) The disease survival time after surgery of the patients in cohort 1 were compared between the "miR-552 low" (n = 55) and "miR-552 high" (n = 55) groups. Data are represented as mean ± SD; *$p < 0.05$; two-tailed Student's t test.



cells Injected	HCCLM3 Control	HCCLM3 miR-552 sponge
1×10^4	3/8	1/8
5×10^4	4/8	1/8
1×10^5	5/8	2/8
5×10^5	6/8	3/8
Total	18/32 (56.3%)	7/32 (21.9%)

Figure 2. miR-552 Is Required for the Maintenance of Liver T-ICs

(A) Hepatoma cells were infected with the miR-552 sponge virus, and the stable infectants were determined by real-time PCR (n = 3). (B) The expression of liver T-IC surface markers was checked in miR-552 sponge and control hepatoma cells (n = 3). (C) The expression of stemness-associated transcription genes was checked in miR-552 sponge and control hepatoma cells (n = 3). (D) Flow cytometric analysis of the proportion of $EpCAM^+$ cells in miR-552 knockdown and control hepatoma cells (n = 3). (E) The protein expression of EpCAM in miR-552 knockdown and control hepatoma cells was checked by western blot assay. (F) Spheres formation assay of miR-552 sponge and control hepatoma cells (n = 3). (G) The frequency of liver T-ICs in miR-552 sponge and control hepatoma cells was compared by *in vitro*-limiting dilution assay (n = 8). (H) The frequency of liver T-ICs in miR-552 sponge and control hepatoma cells was compared by *in vivo*-limiting dilution assay (n = 8). Data are represented as mean ± SD; *$p < 0.05$; two-tailed Student's t test.



H

cells Injected	HCCLM3 Control	HCCLM3 miR-552 mimic
1×10^4	2/8	5/8
5×10^4	4/8	6/8
1×10^5	5/8	7/8
5×10^5	6/8	8/8
Total	17/32 (53.1%)	26/32 (81.3%)

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and tumorigenesis, triggered by ectopic miR-552 expression in hepatoma cells (Figures 4F–4J).

miR-552 Promotes Liver T-IC Expansion through the PTEN/AKT Pathway

It was reported that the phosphatidylinositol 3-kinase (PI3K)/AKT pathway was regulated by PTEN in numerous tumors.[25,26] Therefore, we investigated whether AKT was also required for miR-552-mediated liver T-IC expansion. As expected, phosphorylation of AKT was increased in miR-552-overexpressing hepatoma cells and decreased in miR-552 knockdown hepatoma cells (Figures 5A and 5B). PTEN overexpression could abrogate the activation of AKT phosphorylation in miR-552-overexpressing hepatoma cells (Figure 5C). Moreover, the specific AKT inhibitor MK2206 diminished the enhancement of T-IC markers or stemness-associated genes expression, self-renewal, and tumorigenesis triggered by ectopic miR-552 expression in hepatoma cells (Figures 5E–5H). Taken together, the results demonstrate that miR-552 promotes liver T-IC expansion through the PTEN/AKT pathway.

miR-552 Predicts the Sorafenib Benefit in HCC Patients

Liver T-ICs were also closely correlated with HCC chemoresistance.[27] Thus, we next explored the correlation between the miR-552 expression and sorafenib response in HCC patients. miR-552 overexpression led to the resistance of hepatoma cells upon sorafenib-induced growth inhibition and cell apoptosis (Figures 6A–6C). Moreover, miR-552 knockdown sensitized hepatoma cells to sorafenib-induced growth inhibition and cell apoptosis (Figures 6D–6F). To assess the clinical significance of miR-552 in sorafenib therapy, we examined the expression of miR-552 in HCCs from a cohort consisting of postoperative patients who had received adjuvant sorafenib treatment (cohort 2). Kaplan-Meier analysis revealed that HCC patients with low miR-552 levels benefited in adjuvant sorafenib treatment (Figure 6G). We also investigated the expression of miR-552 in primary tumors from another cohort of patients who had received sorafenib after HCC recurrence (cohort 3). Kaplan-Meier analysis indicated that low miR-552 levels in the primary HCCs were significantly associated with prolonged overall survival in patients who were treated with sorafenib for recurrent tumors (Figure 6H). Furthermore, we found that the patient-derived xenografts (PDXs) derived from HCC tumors with high miR-552 levels were resistant to sorafenib treatment (Figure 6I). In contrast, sorafenib eliminated the growth of PDXs derived from the HCC tumors with low miR-552 levels compared with the vehicle controls (Figure 6J), further demonstrating that the miR-552 expression in HCC patients can serve as a reliable predictor for the sorafenib response.

DISCUSSION

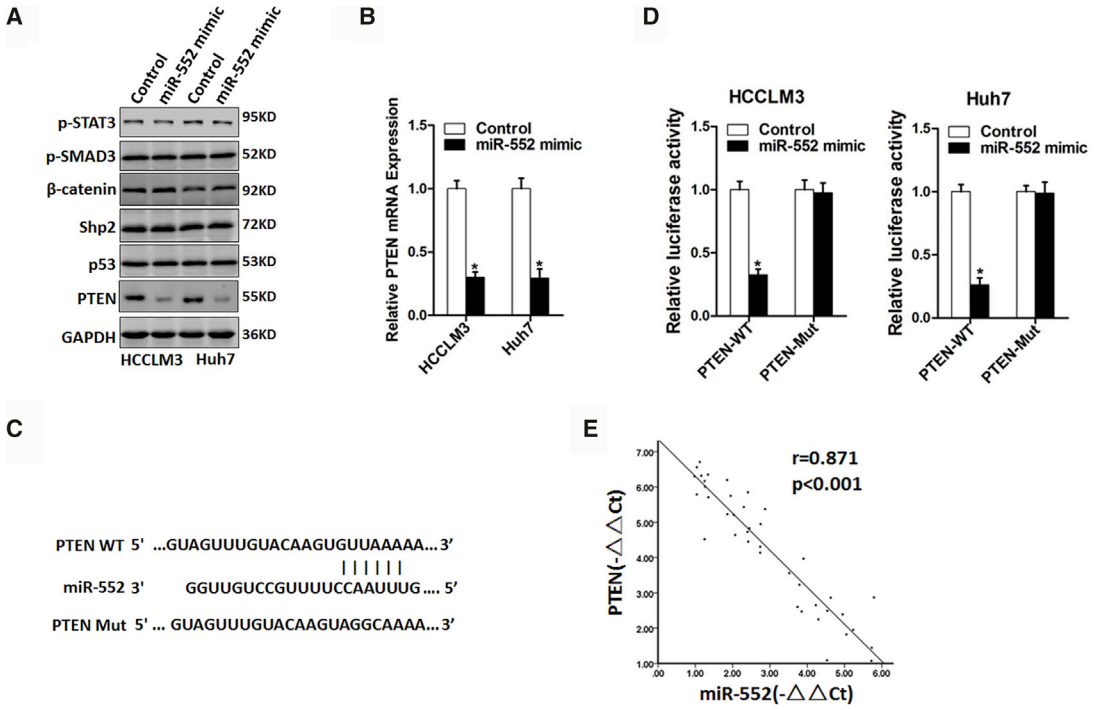
HCC is the sixth most common cancer in the world and comprises approximately 90% of human liver cancer. The incidence of HCC is increasing due to various factors, including hepatitis, alcoholic fatty liver, nonalcoholic fatty liver disease, and aflatoxin.[28] Hepatic resection and liver transplantation are always used for early HCC patients. However, most advanced HCC patients are not suitable for surgery, and the conventional transcatheter arterial chemoembolization (TACE) or the targeted agent sorafenib has limited survival benefits.[29] Therefore, more efforts are needed to clarify the development of HCC. In this study, we demonstrate, for first time, that miR-552 is highly expressed in liver T-ICs and promotes liver T-IC self-renewal and tumorigenesis. Our clinical data revealed that miR-552 can be used to predict a poor prognosis and sorafenib therapy response in HCC patients.

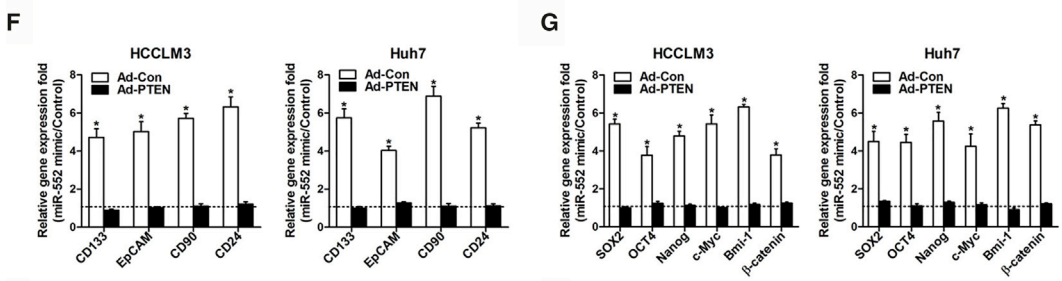
Previous studies have demonstrated that miRNAs have important functions in human cancers and can be used for the diagnosis and treatment of tumors. Disorders associated with miRNAs were first identified in chronic lymphoblastic leukemia.[30] miRNAs can also be used for cancer patients' prognosis. For instance, increased expression of miR-552 acts as a potential predictor biomarker for poor prognosis of colorectal cancer.[31] It was also reported that miR-552 promotes migration and invasion of osteosarcoma through targeting tissue inhibitors of metalloproteinase 2 (TIMP2).[32] However, the potential function of miR-552 in liver T-ICs has not been reported. Accumulating evidence showed that liver T-ICs contribute to HCC chemoresistance and postoperative recurrence. In this study, we found that miR-552 expression is significantly upregulated in chemoresistant xenografts and recurrent HCC tissues. Consistently, we also revealed that the miR-552 level is increasing in $CD133^+$ or $EpCAM^+$ liver T-ICs. Moreover, miR-552 can promote the self-renewal capacity and tumorigenicity of liver T-ICs.

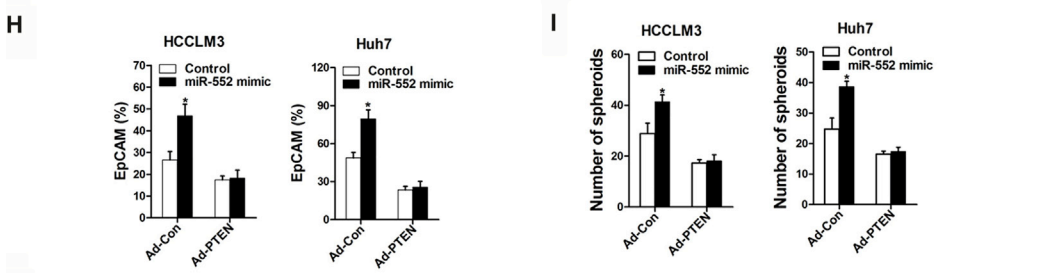
The PI3K/AKT signaling pathway is an important kinase cascade that plays a role in the regulation of cellular quiescence, proliferation, cancer, and longevity,[33] and its disruption can lead to tumorigenesis. The PI3K/AKT pathway has a natural inhibitor called PTEN, with the function to limit proliferation in cells, helping to prevent cancer.[34] PTEN negatively regulates the intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating the AKT signaling pathway.[35]

Figure 3. miR-552 Promotes the Expansion of Liver T-ICs

(A) Hepatoma cells were infected with miR-552 mimic virus, and the sable infectants were checked by real-time PCR (n = 3). (B) The expression of liver T-IC surface markers was checked in miR-552 mimic and control hepatoma cells (n = 3). (C) The expression of stemness-associated transcription genes was checked in miR-552 mimic and control hepatoma cells (n = 3). (D) Flow cytometric analysis of the proportion of $EpCAM^+$ cells in miR-552 overexpression and control hepatoma cells (n = 3). (E) The protein expression of EpCAM in miR-552 overexpression and control hepatoma cells was checked by western blot assay. (F) Spheres formation assay of miR-552 overexpression and control hepatoma cells (n = 3). (G) The frequency of liver T-ICs in miR-552 mimic and control hepatoma cells was compared by *in vitro*-limiting dilution assay (n = 8). (H) The frequency of liver T-ICs in miR-552 mimic and control hepatoma cells was compared by *in vivo*-limiting dilution assay (n = 8). Data are represented as mean ± SD; $^*p < 0.05$; two-tailed Student's t test.







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cells Injected	Control Ad-Con	miR-552 mimic Ad-Con	Control Ad-PTEN	miR-552 mimic Ad-PTEN
1×10^4	2/8	5/8	0/8	1/8
5×10^4	3/8	6/8	1/8	1/8
1×10^5	5/8	7/8	2/8	2/8
5×10^5	6/8	7/8	3/8	3/8
Total	16/32 (50%)	25/32 (78.1%)	6/32 (18.8%)	7/32 (21.9%)

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Previous studies have demonstrated that PTEN plays an essential role in liver cancer initiation and progression.[36] In addition, PTEN was reported to be involved in the regulation of cancer stemness.[37] We hereby revealed that miR-552 overexpression reduces PTEN mRNA and protein expression in liver T-ICs. Moreover, we found that miR-552 directly regulates PTEN expression via interaction with its 3′ UTR. Our data also showed that miR-552 downregulates PTEN and then activates AKT phosphorylation in liver T-ICs. The PTEN-overexpressing adenovirus or AKT inhibitor MK2206 further confirms that miR-552 via the PTEN/AKT pathway promotes liver T-IC expansion.

Sorafenib is a small inhibitor of several tyrosine protein kinases, including vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and Raf family kinases.[38] It is used for the treatment of primary kidney cancer and advanced primary liver cancer.[39] Sorafenib is the first US Food and Drug Administration (FDA)-approved targeted drug that was used for the treatment of advanced HCC patients.[40] However, only a small part of HCC patients who received sorafenib had a prolonged survival time. Therefore, it is important to find the right population of patients for sorafenib treatment. In this study, our finding revealed that miR-552 knockdown of liver cells is more sensitive to sorafenib treatment. The sorafenib cohort and PDX studies further indicated that a low miR-552 level in HCC patients can serve as a reliable predictor for a sorafenib response.

In conclusion, our findings provide insight into the miR-552/PTEN/AKT axis as a potential therapeutic target against liver T-ICs and a potential predictor for poor prognosis of HCC patients.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Patient-derived primary HCC cultures of tumor cells were obtained from fresh tumor specimens of HCC patients, as previously described. The human primary hepatoma cells were isolated by collagenase perfusion and centrifugation. Briefly, the liver cancer tissues were washed several times in precooled sterile PBS buffer containing double antibodies to remove blood and connective tissue; Gey's balanced salt solution (GBSS) mixed enzyme solution was used for digestion. The cells were centrifuged, and the supernatant was discarded. Cell viability and counting were performed using trypanosoma blue staining with cell filtrate and cultured in a bottle containing complete medium heavy suspension at 37°C and 5% CO_2 environment culture. During this process, the cell morphology was identified.

The HCC cell lines HCCLM3 and Huh7 were purchased from the Chinese Academy of Sciences (Shanghai, China) in 2017. HCCLM3 was subjected to hepatitis B virus (HBV) analysis and Huh7 to short tandem repeat (STR) analysis in July 2018 at Shanghai Vivacell Biosciences. Tests showed no problems with the cells. The HCC cells were cultured with Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine, and 25 μg/mL of gentamicin and maintained at 37°C in a 5% CO_2 incubator. The cultured cells were digested with 0.5% trypsin and moved to a new plate twice a week. miR-552 mimic or miR-552 sponge lentivirus and their control lentivirus were purchased from Shanghai GenePharma (Shanghai, China). The adenoviruses expressing PTEN and its control were obtained from Viagen (Shandong, China).

Patients and Samples

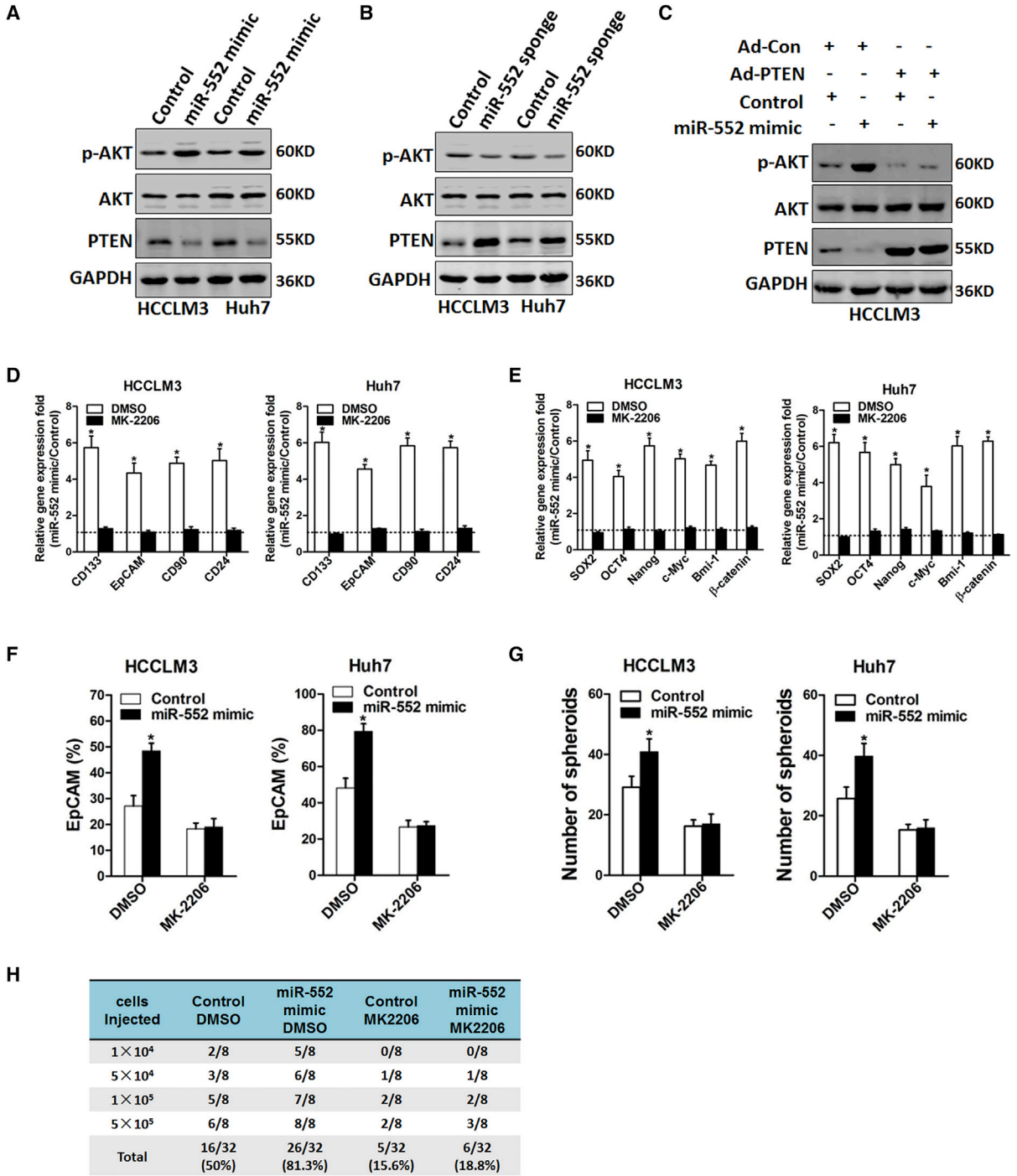
The HCC and corresponding peritumoral tissues were collected from surgical resections of patients without preoperative treatment at the General Hospital of Northern Theater Command. A total of 110 patients in cohort 1 were followed for 5 years, and recurrence-free survival (DFS) and OS analyses were performed using the Kaplan-Meier method. OS was defined as the interval between the dates of surgery and death. The recurrence was defined as the interval between the dates of surgery and recurrence; if recurrence was not diagnosed, then patients were censored on the date of death or on the last follow-up. Detailed clinicopathological features of the patients in cohort 1 are described in Table S1. Another group of 40 HCC specimens was used for analyzing the correlation between miR-552 and PTEN mRNA expression. Patient informed consent was obtained, and the procedure of human sample collection was approved by the Ethics Committee of the General Hospital of Northern Theater Command.

Flow Cytometry Analysis

For $CD133^+$ and $EpCAM^+$ cell sorting, primary HCC patients' cells and hepatoma cells were incubated with the primary anti-CD133

Figure 4. PTEN Is a Direct Target of miR-552 in Liver T-ICs

(A) The phosphorylation of STAT3, SMAD3, β-catenin, Shp2, p53, and PTEN in miR-552 mimic and control hepatoma cells was determined by western blot assay. (B) The mRNA expression of PTEN in miR-552 mimic and control hepatoma cells was checked by a real-time PCR assay (n = 3). (C) A potential target site for miR-552 in the 3′ UTR of human PTEN mRNA, as predicted by the program TargetScan. To disrupt the interaction between miR-552 and PTEN mRNA, the target site was mutated. (D) Luciferase reporter assays performed in miR-552 mimic and control cells transfected with wild-type or mutant PTEN 3′ UTR constructs (n = 3). (E) Spearman correlation analysis of the relationship between PTEN mRNA and miR-552 expression in 40 HCC specimens. (F) miR-552 mimic and control hepatoma cells were infected with the PTEN overexpression virus or control virus and subjected to a real-time PCR assay (n=3), the relative gene expression of CD133, EpCAM, CD90 and CD24 were analysis. (G) miR-552 mimic and control hepatoma cells were infected with the PTEN overexpression virus or control virus and subjected to a real-time PCR assay (n=3), the relative gene expression of SOX2, OCT4, Nanog, c-Myc, Bmi-1 and β-catenin were analysis. (H) miR-552 mimic and control hepatoma cells were infected with the PTEN-overexpressing virus or control virus, and the $EpCAM^+$ hepatoma cells were checked by the flow cytometric assay (n = 3). (I) miR-552 mimic and control hepatoma cells were infected with the PTEN-overexpressing virus or control virus and subjected to spheroid formation (n = 3). (J) miR-552 mimic and control hepatoma cells were infected with the PTEN-overexpressing virus or control virus and were then injected subcutaneously into NOD-SCID mice. Tumors were observed over 2 months; n = 8 for each group. Data are represented as mean ± SD; *$p < 0.05$; two-tailed Student's t test.



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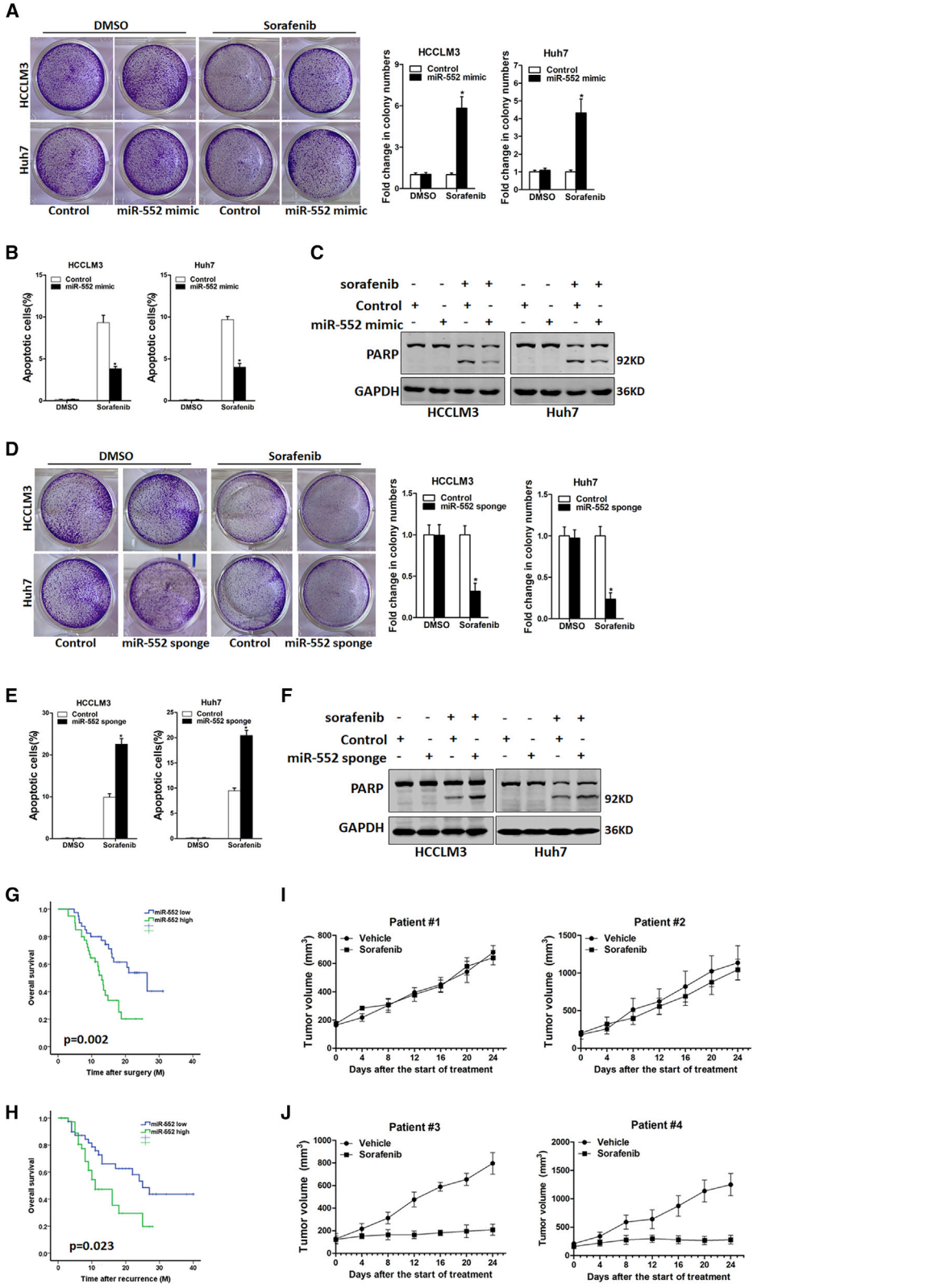
cells Injected	Control DMSO	miR-552 mimic DMSO	Control MK2206	miR-552 mimic MK2206
1×10^4	2/8	5/8	0/8	0/8
5×10^4	3/8	6/8	1/8	1/8
1×10^5	5/8	7/8	2/8	2/8
5×10^5	6/8	8/8	2/8	3/8
Total	16/32 (50%)	26/32 (81.3%)	5/32 (15.6%)	6/32 (18.8%)

Figure 5. miR-552 Promotes Liver T-IC Expansion via the PTEN/AKT Pathway

(A) The phosphorylation of AKT in miR-552 mimic and control hepatoma cells checked by western blot assay. (B) The phosphorylation of AKT in miR-552 sponge and control hepatoma cells checked by western blot assay. (C) miR-552 mimic and control hepatoma cells were infected with the PTEN-overexpressing virus or control virus and subjected to western blot assay. (D) miR-552 mimic and control hepatoma cells were treated with MK2206 (5 μM) or not and subjected to a real-time PCR assay (n=3), the relative gene expression of CD133, EpCAM, CD90 and CD24 were analysis. (E) miR-552 mimic and control hepatoma cells were treated with MK2206 (5 μM) or not and subjected to a real-time PCR assay (n=3), the relative gene expression of SOX2, OCT4, Nanog, c-Myc, Bmi-1 and β-catenin were analysis. (F) miR-552 mimic and control hepatoma cells were treated with MK2206 (5 μM) or not, and the $EpCAM^+$ hepatoma cells were checked by the flow cytometric assay (n = 3). (G) miR-552 mimic and control hepatoma cells were treated with MK2206 (5 μM) or not and subjected to spheroid formation (n = 3). (H) *In vivo*-limiting dilution assay of indicated hepatoma cells. Tumors were observed over 2 months; n = 8 for each group. Data are represented as mean ± SD; *$p < 0.05$; two-tailed Student's t test.



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(cat. no. 372806; BioLegend, San Diego, CA, USA) or anti-EpCAM (cat. no. ab8666; Abcam, USA) for 30 min at room temperature. The cells were then subjected to flow cytometry using a MoFlo XDP cell sorter from Beckman Coulter (Indianapolis, IN, USA), according to the manufacturer's instructions. The sorted cells from three independent experiments were subjected to real-time PCR assay.

miR-552 mimic or miR-552 sponge and control hepatoma cells were incubated with the primary anti-EpCAM for 30 min at room temperature. The flow cytometry analysis was performed using a MoFlo XDP from Beckman Coulter, according to the manufacturer's instructions.

Spheroid Formation Assay

miR-552 mimic or miR-552 sponge and their control hepatoma cells were cultured in a 96-well ultra-low attachment (300 cells per well) and cultured in DMEM/F12 (Gibco) media, supplemented with 1% FBS, 20 ng/mL basic fibroblast growth factor (bFGF), and 20 ng/mL epidermal growth factor (EGF) for 7 days. The total number of spheres was counted under the microscope (Olympus).

In Vitro-Limiting Dilution Assay

Various numbers of miR-552 mimic or miR-552 sponge and their control hepatoma cells (2, 4, 8, 16, 32, and 64 cells per well) were seeded into 96-well ultra-low attachment and cultured in DMEM/F12 (Gibco), supplemented with 1% FBS, 20 ng/mL bFGF, and 20 ng/mL EGF for 7 days. The cancer stem cell (CSC) proportions were analyzed using Poisson distribution statistics and the L-Calc Version 1.1 software program (Stem Cell Technologies, Vancouver, Canada), as previously described.[17]

In Vivo-Limiting Dilution Assay

For the *in vivo*-limiting dilution assay, hepatoma cells were mixed with Matrigel (Becton Dickinson) at a ratio of 1:1 and injected subcutaneously at indicated cell doses per non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mouse. After 8 months, tumor formation was evaluated.

Real-Time PCR

For detection of mature miR-552, total RNA was subjected to reverse transcription using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR analysis of miR-552 expression was carried out using TaqMan MicroRNA assay kits (Applied Biosystems). Results were normalized to U6 small nuclear RNA (snRNA) using the comparative threshold cycle (Ct) method.

The total RNA was extracted by using Trizol reagent (Invitrogen; 15596-018). Total cDNAs were synthesized by the ThermoScript RT-PCR system (Invitrogen; 11146-057). The total mRNA amount present in the cells was measured by RT-PCR using the ABI PRISM 7300 sequence detector (Applied Biosystems). PCR conditions included 1 cycle at 94°C for 5 min, followed by up to 40 cycles of 94°C for 15 s (denaturation), 60°C for 30 s (annealing), and 72°C for 30 s (extension). The sequences of primers used were listed in Table S4.

Western Blotting Assay

25 μg of proteins was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and incubated with the primary antibody overnight. The protein band, specifically bound to the primary antibody, was detected using an IRDye 800CW-conjugated secondary antibody and LI-COR imaging system (LI-COR Biosciences, Lincoln, NE, USA). The primary antibodies used were listed in Table S5.

Luciferase Reporter Assay

A 500-bp fragment of the PTEN 3′ UTR containing the conserved miR-552-binding sites was inserted into a luciferase reporter plasmid. The PTEN 3′ UTR mutant luciferase plasmid contained changes in potential miR-552-binding base sequence "GUUAAA" to "AGGCAA." Then, the 500-bp fragment of the PTEN mutant 3′ UTR fragment was inserted into a luciferase reporter plasmid. miR-552 mimic or miR-552 sponge and their control hepatoma cells were transfected with PTEN WT or PTEN mutant 3′ UTR plasmids. The luciferase activity was measured using a Synergy 2 Multidetection Microplate Reader (BioTek Instruments). The data were normalized for transfection efficiency by dividing firefly luciferase activity by Renilla luciferase activity.

Colony-Formation Assays

miR-552 mimic or miR-552 sponge and their control hepatoma cells were seeded into a 12-well plate and treated with sorafenib (2 μM) for 7 days. The cells were fixed with 10% neutral formalin for 4 h. Then, the cells were dyed with crystal violet (Beyotime).

Figure 6. miR-552 Is Associated with the Sensitivity of Sorafenib in HCC Patients

(A) miR-552 mimic and control hepatoma cells were treated with sorafenib (2 μM) for 7 days, and their colony formation was examined (n = 3). (B) miR-552 mimic and control hepatoma cells were treated with sorafenib (10 μM) for 48 h, and their apoptosis was checked by flow cytometry (n = 3). (C) miR-552 mimic and control hepatoma cells were treated with 10 μM sorafenib, as indicated, for 48 h. The protein of cleaved poly (ADP-ribose) polymerase (PARP) was determined by western blot. (D) miR-552 sponge and control hepatoma cells were treated with sorafenib (2 μM) for 7 days, and their colony formation was examined (n = 3). (E) miR-552 sponge and control hepatoma cells were treated with sorafenib (10 μM) for 48 h, and their apoptosis was checked by flow cytometry (n = 3). (F) miR-552 sponge and control hepatoma cells were treated with 10 μM sorafenib, as indicated, for 48 h. The protein of cleaved PARP was determined by western blot. (G) The overall survival of patients between miR-552-high (n = 40) or miR-552-low (n = 40) groups was evaluated by Kaplan-Meier analysis in HCC cohort 2. (H) The overall survival of patients between miR-552-high (n = 40) or miR-552-low (n = 40) groups was evaluated by Kaplan-Meier analysis in HCC cohort 3. (I) PDXs with high miR-552 levels in their primary tumors were treated with sorafenib (30 mg/kg body weight) or vehicle for 24 days (n = 6 for each group). The xenograft growth was monitored. (J) PDXs with low miR-552 levels in their primary tumors were treated with sorafenib (30 mg/kg body weight) or vehicle for 24 days (n = 6 for each group). The xenograft growth was monitored. Data are represented as mean ± SD; $p < 0.05$; two-tailed Student's t test.

Apoptosis Assay

miR-552 mimic or miR-552 sponge and their control hepatoma cells were treated with sorafenib (10 μM) for 48 h, followed by staining with Annexin V and 7-aminoactinomycin D (7-AAD) for 15 min at 48°C in the dark. Apoptotic cells were determined by an Annexin V FITC Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA) and detected by flow cytometry, according to the manufacturer's instructions.

Sorafenib Cohort

A total of 80 patients receiving adjuvant sorafenib therapy after surgery for primary HCC at the General Hospital of Northern Theater Command from 2010 to 2014 were included in cohort 2. Detailed clinicopathological features of these patients are described in Table S2. Another group of 80 patients who received sorafenib for the recurrent tumors at the General Hospital of Northern Theater Command from 2009 to 2015 was included in cohort 3. The detailed clinicopathological features and treatment of these patients are described in Table S3.

Statistical Analysis

GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses. Statistical analysis was carried out using t test or Bonferroni multiple comparisons test: $^*p < 0.05$. A p value of less than 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.12.043.

AUTHOR CONTRIBUTIONS

T.H., Y.Z., and X.Y. conducted all experiments and analyzed the data. L.H. and H.L. provided clinical samples. L.H. provided pathology evaluation, and H.L. analyzed clinical data. T.C. provided support with experimental techniques. T.H. wrote the manuscript, and Z.Z. contributed to the revision. H.L., T.C., and Z.Z. conceived the project and supervised all experiments.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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