Drug Resistance Updates 71 (2023) 101015

Contents lists available at ScienceDirect

Drug Resistance Updates

journal homepage: www.elsevier.com/locate/drup

CRISPR/Cas9 screens unravel miR-3689a-3p regulating sorafenib resistance in hepatocellular carcinoma via suppressing CCS/SOD1-dependent mitochondrial oxidative stress

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ARTICLE INFO

Keywords:
In vivo CRISPR/Cas9 screen
Sorafenib
MiR-3689a-3p
Drug resistance of hepatocellular carcinoma
CCS

ABSTRACT

Aims: Therapeutic outcome of sorafenib in hepatocellular carcinoma (HCC) is undermined by the development of drug resistance. This study aimed to identify the critical microRNA (miRNA) which is responsible for sorafenib resistance at the genomic level.

Methods: CRISPR/Cas9 screen followed by gain- and loss-of-function assays both in vitro and in vivo were applied to identify the role of miR-3689a-3p in mediating sorafenib response in HCC. The upstream and downstream molecules of miR-3689a-3p and their mechanism of action were investigated.

Results: CRISPR/Cas9 screening identified miR-3689a-3p was the most up-regulated miRNA in sorafenib sensitive HCC. Knockdown of miR-3689a-3p significantly increased sorafenib resistance, while its overexpression sensitized HCC response to sorafenib treatment. Proteomic analysis revealed that the effect of miR-3689a-3p was related to the copper-dependent mitochondrial superoxide dismutase type 1 (SOD1) activity. Mechanistically, miR-3689a-3p targeted the 3′UTR of the intracellular copper chaperone for superoxide dismutase (CCS) and suppressed its expression. As a result, miR-3689a-3p disrupted the intracellular copper trafficking and reduced SOD1-mediated scavenge of mitochondrial oxidative stress that eventually caused HCC cell death in response to sorafenib treatment. CCS overexpression blunted sorafenib response in HCC. Clinically, miR-3689a-3p was down-regulated in HCC and predicted favorable prognosis for HCC patients.

Conclusion: Our findings provide comprehensive evidence for miR-3689a-3p as a positive regulator and potential druggable target for improving sorafenib treatment in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, ranking as second leading cause in cancer-related mortality worldwide (Qi et al., 2023). Unfortunately, many patients with HCC are diagnosed at advanced stages, making treatment more difficult (Vogel et al., 2022). As a result, these patients often undergo various systemic therapies. Sorafenib, a tyrosine kinase inhibitor (TKI), was the first drug of its kind to receive approval from the U.S. Food and Drug Administration (FDA) for the first-line systemic treatment of advanced HCC patients (Bai et al., 2021; Huang et al., 2022). Tyrosine kinase inhibitors work by blocking the activity of enzymes called tyrosine kinases, which are essential for the growth and division of cancer cells. By inhibiting these enzymes, sorafenib can slow down the progression of HCC. However, the clinical effectiveness of sorafenib is often limited due to the development of drug resistance in patients (Tang et al., 2020; Xia et al., 2020). This means that, over time, the cancer cells become less responsive to the drug, and the treatment becomes less effective. This has been a significant challenge in the management of advanced HCC. In the past decade, researchers have developed additional systemic therapies for HCC, but the progress in improving patient outcomes has been limited (Llovet et al., 2018). A better understanding

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https://doi.org/10.1016/j.drup.2023.101015
Received 12 August 2023; Received in revised form 23 October 2023; Accepted 24 October 2023
Available online 29 October 2023

of the mechanisms behind sorafenib treatment resistance is crucial to advance the development of more effective therapies for HCC patients. Currently, the mechanism of sorafenib treatment remains a major topic of research in the field of HCC (Ladd et al., 2023; Llovet et al., 2018; Wei et al., 2019a). Gaining deeper insights into how this drug works, as well as the factors contributing to drug resistance, could potentially pave the way to new therapeutic approaches, ultimately improving the prognosis and quality of life of patients with advanced HCC.

MicroRNAs (miRNAs) are a group of small, noncoding RNA molecules, approximately 22 nucleotides long, that are widely distributed in organisms. They play a crucial role in gene silencing, which helps maintain homeostasis under normal physiological conditions by buffering fluctuations in gene expression (Wang et al., 2022). In the context of hepatocellular carcinoma (HCC) and sorafenib treatment, miRNAs have been found to be dysregulated, and their expression levels are altered compared to normal conditions (Wei et al., 2019b) (Liu et al., 2023). Previous studies, including those conducted by our team and others, have identified several miRNAs that are dysregulated in sorafenib-treated HCC (Bergamini et al., 2023; Dong et al., 2022; Hu et al., 2023; Lu et al., 2022). These miRNAs can function either as oncogenic miRNAs or tumor suppressors, and they can influence how cancer cells respond to sorafenib treatment. For instance, miR-23a-3p, miR-494, miR-1274a, miR-23b-3p, miR-486–3p, miR-122, miR-125b-5p, and miR-19a-3p were found to be upregulated upon sorafenib treatment, and these miRNAs promoted sorafenib resistance in HCC cells (Bergamini et al., 2023; Hirao et al., 2021; Ji et al., 2020; Jiang et al., 2018; Kaur et al., 2022; Lu et al., 2022; Turato et al., 2019; Zhou et al., 2011). In contrast, downregulated miRNAs, such as miR-34a, miR-106b-5p, miR-4277, and miR-545 were found to enhance sorafenib sensitivity, making HCC cells more responsive to the treatment (Enkhnaran et al., 2022; He et al., 2021; Lu et al., 2020; Shen et al., 2023). These dysregulated miRNAs hold potentials as promising biomarkers for predicting response to sorafenib treatment in HCC patients, as well as possible druggable targets for developing more effective therapeutic strategies. By targeting these specific miRNAs, it may be possible to improve the efficacy of sorafenib treatment and overcome the issue of drug resistance in HCC patients.

The CRISPR/Cas9 system was originally discovered as an immune defense mechanism in bacteria, which protects them from viral DNA invasion (Abavisani et al., 2023; Barrangou et al., 2007). In recent years, this system has been adapted and engineered into a powerful tool for gene editing in mammalian cells and animal models (Wu et al., 2022). CRISPR/Cas9 libraries consist of a pool of single guide RNAs (sgRNAs) that target thousands of genes, enabling large-scale gene knockout experiments. CRISPR-based screens have been widely used to identify key genes involved in cancer development and response to therapeutic treatments, including sorafenib-treated HCC. In these screens, HCC cells modified with CRISPR libraries are subjected to sorafenib treatment at various concentrations or durations (Wei et al., 2019a). Researchers then use either negative or positive selection strategies to identify genes that are associated with sorafenib resistance or those that could be potential druggable targets. Through these CRISPR-based screens, several critical genes have been identified as essential for the response of HCC cells to sorafenib treatment. Examples of these genes include KEAP1, PSTK, NMDAR, MTX1, LRP8, PHGDH, and SGOL1 (Chen et al., 2022; Huang et al., 2022; Li et al., 2021; Sun et al., 2018; Wei et al., 2019a; Xia et al., 2020; Xu et al., 2021). The identification of these genes provides valuable insights into the molecular mechanisms underlying sorafenib resistance in HCC and can potentially guide the development of novel therapeutic strategies to overcome drug resistance and improve patient outcomes.

In our study, we utilized the GeCKO v2 library knockout screening method in a xenograft mouse model, which is more accurate compared to traditional monolayer cell cultures. By comparing sorafenib-treated HCC tumors with their respective controls, we discovered that miR-3689a-3p plays a crucial role in determining sorafenib sensitivity in HCC. Our in vitro and in vivo experiments demonstrated that miR-3689a-3p interferes with the SOD1-mediated antioxidant response by inhibiting the CCS-dependent copper incorporation process, thereby enhancing sorafenib-induced oxidative damage in HCC cells. These findings suggest that miR-3689a-3p has the potential to serve as a promising druggable target for improving sorafenib sensitivity in HCC treatment.

2. Materials and methods

2.1. Human samples

A tissue microarray (TMA) chip containing 90 pairs of human hepatocellular carcinoma (HCC) samples, along with their adjacent normal liver tissues and associated clinicopathological information, was acquired from Shanghai OUTDO Biotech Co., Ltd., Shanghai, China (product code: LivH180Su08). The patients in this cohort included 9 females and 81 males. They had adopted a surgery and had a five-to-six-year follow-up. At the follow-up visit, 49 patients had been deceased and 53 patients present HCC relapse. Apart from that, the clinicopathological information had been recorded including age, stage, tumor size, tumor number, cirrhotic nodule, tumor capsule, cirrhosis, etc. The patient information is listed in table S1. Five pairs of TMA tissues were excluded for data analysis due to bad damage. A total of 85 pairs of tissues were included for data analysis.

2.2. In situ hybridization (ISH) combined immunofluorescence

The protocol of ISH in combination with immunofluorescence followed our previous publication (Lu et al., 2022). Briefly, the general rehydration steps were followed by a pre-hybridization step at 83 °C for 30 min. The miR-3689a-3p probe was denatured at 65 °C for 5 min and immediately chilled on ice for 5 min. Then the hybridization was performed in solution with 40 nM of miR-3689a-3p probe at 53 °C overnight. The slide was washed stringently with 1 × saline-sodium citrate buffer (SSC) at 53 °C for 10 min twice and 0.5x SSC at room temperature for 10 min. The staining steps followed the manual of Alexa Fluor™ 488 tyramide kit (Thermo Fisher B40932). It was processed with standard immunofluorescence protocol using Alexa Fluor™ 647 and Alexa Fluor™ 568 secondary antibodies and captured by the Akoya Vectra Polaris™ automated quantitative pathology imaging system.

2.3. Animal experiment

All animal protocols were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong (CULATR 5542–20).

2.3.1. In vivo CRISPR/Cas9 screen

The human GeCKOv2 CRISPR knockout pooled library consisting of Cas9 coding sequences and 65,383 sgRNAs which targets 19,050 coding genes and 1864 miRNAs, was a gift from Feng Zhang (Addgene #1000000049). The sgRNAs amplification and the lentivirus packaging followed the protocol from the protocol from Zhang's Lab (Joung et al., 2017). We transduced a half-library A (3 sgRNAs per gene and 4 sgRNAs per miRNA) into MHCC97L cells by lentivirus at a multiplicity of infection of 0.3. Then we subcutaneously transplanted 2×10^7 cells (over 100x coverage) into one side of the flanks of each NOD/SCID mouse. At 19-day after post-transplantation, mice were randomly divided into two groups: three mice orally adopted 25 mg/kg of sorafenib every other day, while the other three mice received vehicle (0.5% CMC-Na) as control. The body weight and tumor size were measured every three days once mice were given sorafenib treatment. After 19-day treatment, tumors in each group were collected and subject to genomic DNA (gDNA) isolation using the Zymo Research Quick-gDNA MidiPrep (D4075, Zymo Research) following the manufacturer's

protocol. The PCR products for indel analysis by using specific primer sets were subjected to the platform of next-generation sequencing (NGS) (Novogene).

2.3.2. Orthotopic implantation of HCC in mice

Luciferase-tagged MHCC97L cells (1×10^6) were subcutaneously injected into the right flank of NOD-SCID mice. When the tumor diameter reached 10 mm, as measured by a caliper, the mice were euthanized. The tumor was then harvested and cut into small cubes, approximately 1 mm^3 in size. A single tumor cube was implanted into the left lobe of the liver of a 5-week-old BALB/cAnN-nu mouse. Sorafenib (25 mg/kg) or its vehicle was orally given every other day. LCS-1 (10 mg/kg) and DC_AC50 (50 mg/kg) solution were sequentially dissolved in 5% DMSO, 10% corn oil, and 85% saline and given daily through IP. The growth of the orthotopic HCC model was monitored weekly by obtaining bioluminescence signals using the IVIS Spectrum system (PerkinElmer).

2.4. Cell culture, reagents and plasmids

2.4.1. Cells

The PLC/PRF/5 cell line was obtained from the American Type Culture Collection (ATCC) (VA, USA). The MHCC97L cell line with a luciferase tag was kindly provided by Prof. Man Kwan from the Department of Surgery at the University of Hong Kong. The 293FT cell line was gifted by Prof. Xinyuan Guan from the Department of Clinical Oncology at the University of Hong Kong. Both PLC/PRF/5 and MHCC97L cells were cultured in high-glucose DMEM (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The 293FT cells were grown in complete high-glucose DMEM medium containing 1 mM sodium pyruvate.

2.4.2. Reagents

Sorafenib (S-8502) was purchased from LC laboratories. LCS-1 (HY-115445) and DC_AC50 (HY-107636) were purchased from MeChemExpress. Fludarabine (NSC118218) was purchased from Selleckchem. Lipofectamine 3000 reagent (Invitrogen) for transient transfection was used according to the manufacturer's instructions.

2.4.3. Plasmids, miRNA mimics and RNA interference

The miR-3689–3p mimics and NC, Anti-miR-3689a-3p and Anti-NC, STAT1 siRNAs, were commercially obtained from GenePharma, China. The plasmids pLenti-III-miR-Off (3689a-KO) and its control (Blank), and LentimiRa-has-miR-3689a-3p (3689a-OE) and its control (Mock) were purchased from Applied Biological Materials (Canada). The pCDH-CMV-3xflag-CCS-EF1-puro plasmid (CCS-OE) and its control (Scramble), CCS shRNAs and the control were purchased from Guangzhou IGE Biotechnology LTD. Lentivirus packaging vectors: pRSV-Rev, pMDLg/pRRE were gifts from Didier Trono (Addgene plasmid #12251 and #12253; http://n2t.net/addgene:12253; RRID: Addgene_12,253) [24]. Lentivirus enveloping plasmid pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid #8454; http://n2t.net/addgene:8454; RRID: Addgene_8454) [25]. pGL3–23P639 luciferase reporter vector was a gift from Narry Kim (Addgene plasmids #51388; http://n2t.net/addgene:51388; RRID: Addgene_51,388) [26].

2.5. Statistical analysis

Statistical analyses were carried out using GraphPad Prism 7 software (CA, USA). For comparisons between two groups, two-tailed Student's t-test was employed for normal distribution data, while Mann-Whitney U test was used for non-normal distribution data. For multi-group comparisons, Kruskal-Wallis test was applied for non-parametric data, and one-way ANOVA with Tukey's multiple comparison test was used for parametric data. A p-value of less than 0.05 was deemed statistically significant.

Other Materials and Methods were presented in supplemental materials.

3. Results

3.1. CRISPR/Cas9 screens identified miR-3689a-3p as an essential gene for response to sorafenib in HCC

Resistance to sorafenib in HCC may develop from the intracellular stress as well as environmental stress in the tumor microenvironment. To comprehensively investigate the molecular basis by which microRNA enables HCC cells response to sorafenib in vivo, we developed an in vivo CRISPR/Cas9 screen platform by applying genome-scale CRISPR/Cas9 knockout library screening approach in xenograft mouse models following sorafenib treatment (Fig. 1A). It was observed that sorafenib-treated mice appeared significant reduction in both body weight and tumor size (Fig. 1B and Fig.S1A). The dissected tumors are much smaller in sorafenib-treated group (Fig. 1C), supporting the antitumor effect of sorafenib.

The high mappability of sgRNA read counts indicate good sample quality (Fig.S1B). Genes with absolute log2 fold change (log2FC) beyond 5 and $p < 0.05$ were considered differentially expressed genes (DEGs) (Fig.S1C). We assumed that cells carrying sgRNAs targeting sorafenib sensitive genes would be survived after sorafenib treatment, so that the corresponding sgRNAs would be enriched in deep sequencing of sgRNA. Total 1397 DEGs were enriched in sorafenib-treated tumors. Among a list of enriched DEGs, miR-3689a was identified the most positively selected gene by sorafenib treatment (log2FC=14.287, $P = 0.000132$) (Fig. 1D&1E). Due to the fact that no sufficient evidence determining which sequence (−3p or −5p, from the 3′ arm and the 5′ arm respectively) represents the predominant role of miR-3689a, we first examined the level of both miR-3689a-3p and miR-3689a-5p in HCC. To see whether the increased expression of miR-3689a could be induced by the direct exposure to sorafenib, we treated HCC cells with cytotoxic doses of sorafenib. Short-term exposure to sorafenib caused induction of miR-3689a-3p but not miR-3689a-5p in two HCC cell lines MHCC97L and PLC/PRF/5 (Fig. 1F&1 G). In the miRNA database of human cancer dataset, it was commonly observed that miR-3689a-3p was down-regulated but miR-3689a-5p was upregulated among different cancers, suggesting that miR-3689a-3p may express against tumor progression in cancers, including HCC (Fig.S1D). Taken together, we identified that miR-3689a-3p is the most up-regulated miRNAs in HCC upon sorafenib treatment.

3.2. miR-3689a-3p potentiated sorafenib response in HCC

To identify the role of miR-3689a-3p in mediating the cellular response of HCC cells to sorafenib treatment, we applied microRNA mimics or inhibitor to forcedly modify the expression of miR-3689–3a in HCC cells, which either transiently up-regulated or down-regulated the expression of targeted miRNAs respectively (Fig. 2 A). HCC cells with miR-3689a-3p overexpression showed lower IC50 value of sorafenib and effectively induced apoptosis and cell death in the presence of sorafenib, whereas HCC cells with miR-3689a-3p inhibition had higher IC50 value of sorafenib and significantly impeded cell apoptosis and total cell death upon sorafenib treatment (Fig. 2B&2 C). These results suggested that the miR-3689–3p expression is positively correlated with the sensitivity of HCC cells in response to sorafenib treatment.

Stable clones of MHCC97L cells with miR-3689a-3p overexpression or knockdown were established (Fig. 2D). The in vivo role of miR-3689a-3p in mediating the response of HCC cells to sorafenib treatment was then measured in an orthotopic liver cancer model from MHCC97L cells with or without changing miR-3689a-3p expression. It was observed that the growth of hepatic tumors with miR-3689a-3p overexpression (denoted as 3689a-OE) appeared remarkable slow and completely retarded by sorafenib treatment; while miR-3689a-3p knockdown

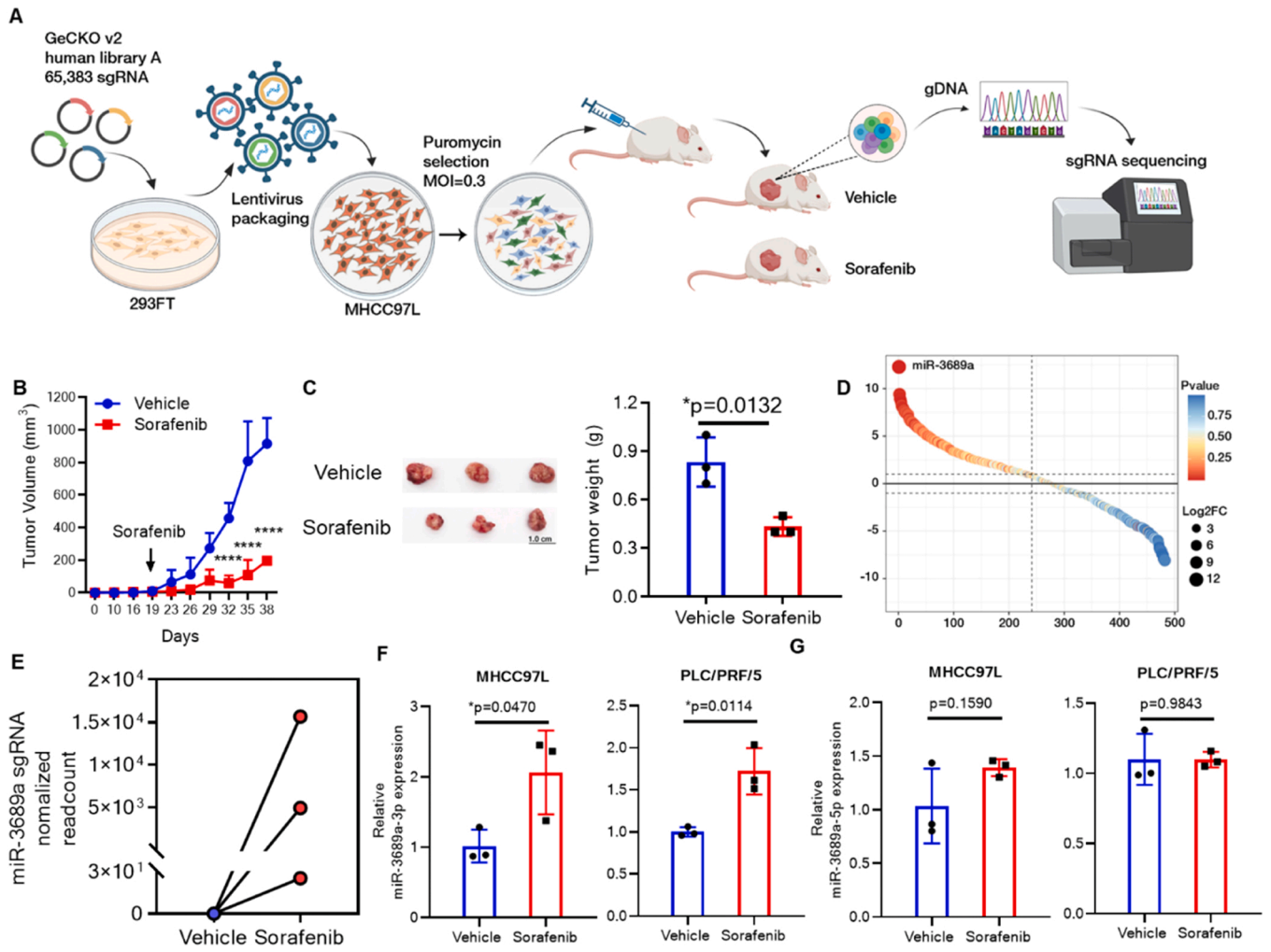

Fig. 1. In vivo CRISPR/Cas9 screening identified miR-3689a-3p as the most upregulated gene in HCC in response to sorafenib treatment. A. Design and flowchart of the in vivo CRISPR/Cas9 screening system. 15 mg/kg oral sorafenib treatment significantly suppressed tumor growth rate (**B**), and reduced tumor size (**C**). **D&E.** miR-3689a-3p was the most upregulated genes upon sorafenib treatment. **F.** Sorafenib treatment significantly induced miR-3689a-3p in MHCC97L and PLC/PRF/5 cells. **G.** Sorafenib did not induce miR-3689a-5p in HCC cells. *p < 0.05.

(denoted as 3689a-KD) group presented rapid growth of hepatic tumors even in the presence of sorafenib without altering body weight (Fig.S1E, Fig. 2E). Similar patterns to the hepatic luciferase signal were observed in the excised livers bearing HCC. (Fig. 2 F). The ki67 staining of tissue sections revealed a lower proportion of proliferative cells in both the 3689a-OE group and sorafenib-treated 3689a-OE group, while a higher proportion observed in the 3689a-KD group and sorafenib-treated 3689a-KD group in comparison to their respective control groups. (Fig. 2 G). As lung is the most common site for extrahepatic metastasis, luciferase signals in lung tissues could be indicator for metastatic HCC cells. It was found that few metastatic HCC cells existed in the lung of 3689a-OE group while more metastatic HCC cells observed in the lung of 3689a-KD group (Fig.S1F). These in vivo results demonstrated that miR-3689a-3p effectively potentiate anti-tumor effect of sorafenib through inducing more cell apoptosis and inhibiting cell proliferation and metastasis.

3.3. miR-3689a-3p was required for sorafenib-induced mitochondrial oxidative stress

To understand the cellular events involved in the regulation of miR-3689a-3p on sorafenib response in HCC cells, proteomics analysis was performed to investigate the changes of gene expression among 3689a-KD, blank, and sorafenib-treated groups. A total of 1135 DEGs among were obtained in these three groups (Fig. 3 A, S2A). We conducted GO annotation and Gene Set Enrichment Analysis (GSEA) for KEGG enrichment. It was found that mitochondrial reactive oxygen species (mtROS) might probably be the most essential pathway (Fig. 3B). Sorafenib treatment induced production of ROS, which was responsible for its cytotoxicity to HCC cells, as evidenced by a restoration of cell viability of sorafenib-treated HCC cells in the presence of ROS scavenger N-acetyl-L-cysteine (NAC, Fig.S2B). We then measured both cellular and mitochondrial ROS level. It was found that sorafenib intervention triggered ROS production, however, only significantly decreased mtROS production was observed in 3689a-KD cells upon sorafenib treatment compared to the control (Fig. 3 C, S2C). Given that increased mtROS could lead to increased mitochondrial mass and defective mitochondrial function, we stained cells with mitoTracker to observe the morphology of mitochondrion and measured intracellular ATP level. As the results shown, the enlargement of mitochondrion was observed in sorafenib-treated blank cells, but not in sorafenib-treated 3689a-KD cells (Fig. S2D). ATP production was relatively higher in 3689a-KD cells compared to their blank control even in the presence of sorafenib (Fig.S2E). These data suggested that miR-3689a-3p knockdown alleviated sorafenib-induced mtROS and subsequent mitochondrial dysfunction.

Antioxidant system is critical for redox balance to eliminate constant ROS production. The cellular enzymatic antioxidant system was mostly composed of three antioxidant enzymes, SOD, catalase (CAT), and

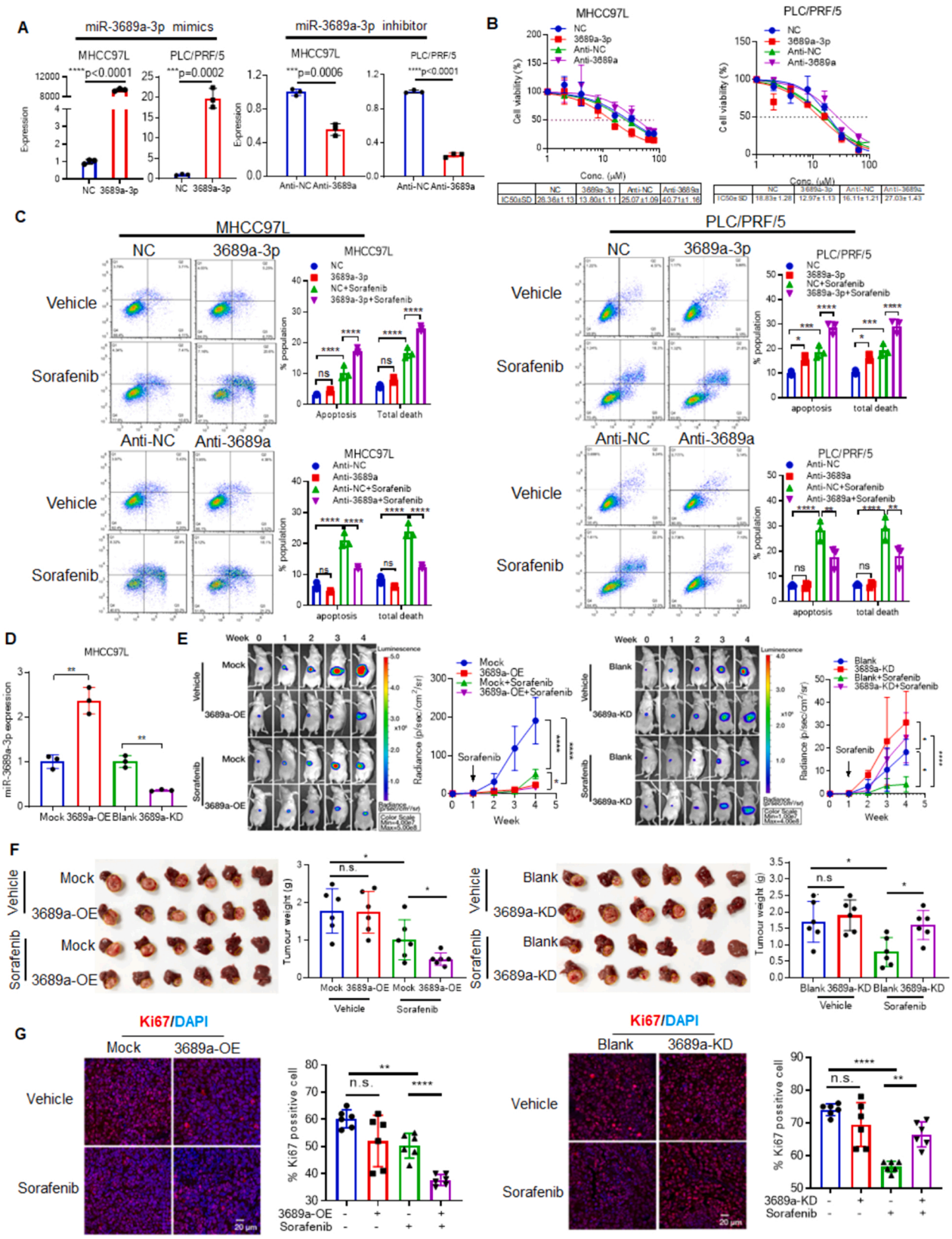

Fig. 2. miR-3689a-3p regulated sorafenib sensitivity in HCC. A. transfection of miR-3689a-3p mimics induced miR-3689a-3p expression while miR-3689a-3p inhibitor suppressed its expression in HCC cells. **B&C.** overexpression of miR-3689a-3p increased cell death and apoptosis of HCC cells upon sorafenib treatment, while knockdown of miR-3689a-3p restored cell viability and suppressed apoptosis of HCC cells in the presence of sorafenib. **D.** Stable overexpression (OE) and knockdown (Off) of miR-3689a-3p in MHCC97L. **E&F.** Overexpression of miR-3689a-3p sensitized the orthotopic HCC tumors to sorafenib treatment, while knockdown of miR-3689a-3p blunted the tumor inhibitory effect of sorafenib in vivo. G. Overexpression of miR-3689a-3p suppressed cell proliferation marker Ki67 expression in HCC tumors, while knockdown of miR-3689a-3p restored Ki67 expression. * *p < 0.01, * **p < 0.001, * *** *p < 0.0001.

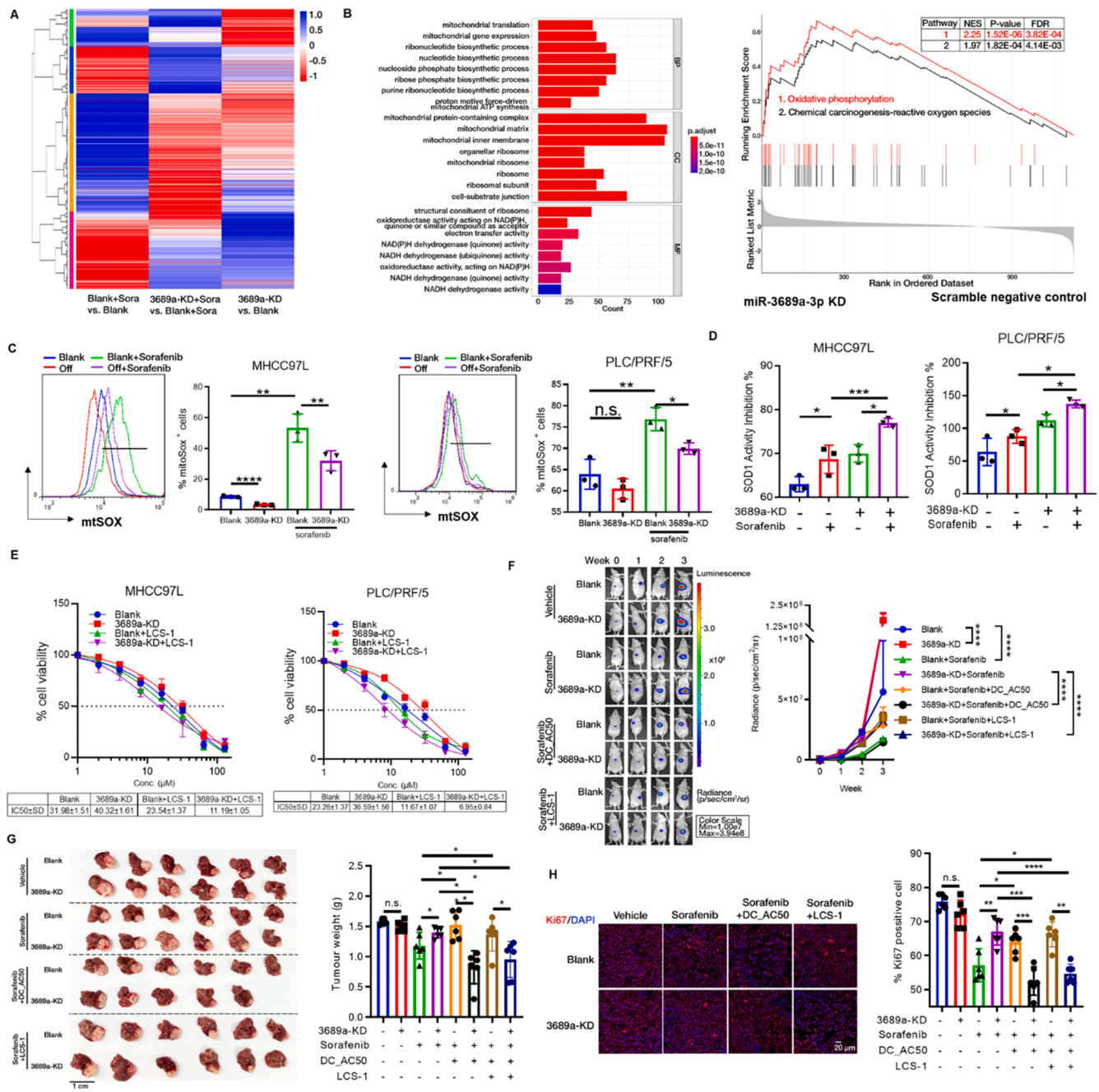

Fig. 3. miR-3689a-3p modulated the mitochondrial oxidative stress in HCC cells. A. Proteomic analysis of the downstream targets of miR-3689a-3p. **B.** Gene ontology (GO) and GSEA analysis revealed the involvement of mitochondria-related signaling molecules. **C.** miR-3689a-3p knockdown suppressed sorafenib-induced mitochondrial ROS production. **D.** miR-3689a-3p knockdown restored SOD1 activity in HCC cells exposed to sorafenib treatment. **E.** Treatment of SOD1 inhibitor LCS-1 induced cell viability in sorafenib-treated HCC cells with miR-3689a-3p knockdown. Mice with orthotopic liver tumor were subjected to treatment with vehicle, sorafenib, sorafenib+LCS-1 and sorafenib+DC_AC50. This setting allows sharing of control groups (vehicle and sorafenib-treated groups) to other intervention groups in order to save the use of experimental animals. **F&G.** Presence of LCS-1 and DC_AC50 sensitized sorafenib treatment in the in vivo tumor growth of HCC with miR-3689a-3p knockdown. **H.** Presence of LCS-1 and DC_AC50 suppressed Ki67 expression in HCC with miR-3689a-3p knockdown. *p < 0.05, * *p < 0.01, * **p < 0.001, * ** *p < 0.0001.

glutathione-peroxidase (GPx). Cellular activities assay revealed that changes of miR-3689a-3p in sorafenib-treated HCC cells had minimal effect on the GPx and CAT activities (Fig.S3A). Copper zinc superoxide dismutase 1 (SOD1) is a ubiquitous enzyme that catalyze the dismutation of O2-·to H_2O_2 (Mueller et al., 2022). The sufficient activation of SOD1 could efficiently fight against ROS, thus preventing cell damage from oxidative stress. Herein, SOD1 activity was measured. It was found that the inhibition on SOD1 activity was significantly higher in sorafenib-treated control cells, but greatly reduced in 3689a-KD cells, indicating higher SOD1 activity in 3689a-KD cells (Fig. 3D). When supplemented with LCS-1, a chemical inhibitor of SOD1 activity, 3689a-KD cells and the control counterparts produced relatively more mitochondrial superoxide and had less cell viability than those with sorafenib treatment alone (Fig. 3E, S3B). We also gave LCS-1 together with sorafenib to 3689a-KD- and its control-derived orthotopic HCC mouse model to see if SOD1 activity inhibition could lead to efficient anti-tumor effect in 3689a-KD group. After three-week treatment, retarded tumor growth, smaller tumor size and less metastatic sites in

lungs were observed in 3689a-KD group administrated with combination treatment compared to sorafenib treatment alone (Fig. 3 F, 3 G, S3C, S3D). Consistently, the tissue sections showed less proliferative cells in HCC tissues of 3689a-KD with combination treatments (Fig. 3 G). These results suggested that higher SOD1 activity in 3689a-KD cells might facilitate the removal of mtROS induced by sorafenib, and it was responsible for less effectiveness of sorafenib treatment in miR-3689a-3p knockdown HCC.

3.4. CCS was the target of miR-3689a-3p to suppress mitochondrial SOD1 activity

The unchanged expression level of SOD1 suggested that the regulation of miR-3689a-3p on SOD1 activity might probably be through mechanisms other than suppression of the miRNA-mediated SOD1 expression (Fig.S4A). It was known that the activation of SOD1 requires copper acquisition. We first examined whether supplement of copper could affect sorafenib-induced cytotoxicity to HCC cells. The MTT assays revealed that sorafenib-suppressed cell viability could be rescued in the presence of low concentrations, non-cytotoxic copper, suggesting the involvement of copper (Fig. 4A&S4B). The entry of copper is mainly through the copper transporter (CTR1), while the export of excess cellular copper is dependent on the ATPase (ATP7A and ATP7B) which deliver copper to the secretory pathway. To preclude the influence caused by copper transport, the expression of ATP7A, ATP7B, and SLC31A1 were detected, and the results indicated that only CCS was significantly changed in miR-3689a-3p-modified HCC cells (Fig. S4C). To see if CCS participated in the cytoprotective function of lower intracellular copper in sorafenib-treated HCC cells, we used its chemical inhibitor DC_AC50 to suppress its function and expression. It was found that CCS inhibition diminished cytoprotective effect in the presence of copper (Fig. 4B). These results indicated that CCS was responsible for the copper-dependent SOD1 activation in the treatment of sorafenib.

Due to the predominant role of CCS in controlling the SOD1 activity, we evaluated whether miR-3689a-3p targeted CCS. As the results shown, both mRNA and protein level of CCS was negatively regulated by miR-3689a-3p (Fig. 4C). Moreover, miR-3689a-3p showed high binding affinity with 3′UTR of CCS (Fig. 4D). We used CCS-3′UTR luciferase reporter activity assay to examine their binding possibility and observed significantly reduced luciferase activity in the co-transfection of miR-3689a-3p and CCS-3′UTR luciferase reporters (Fig. 4E). No changes in luciferase activity was observed upon CCS-3′UTR reporter co-transfected

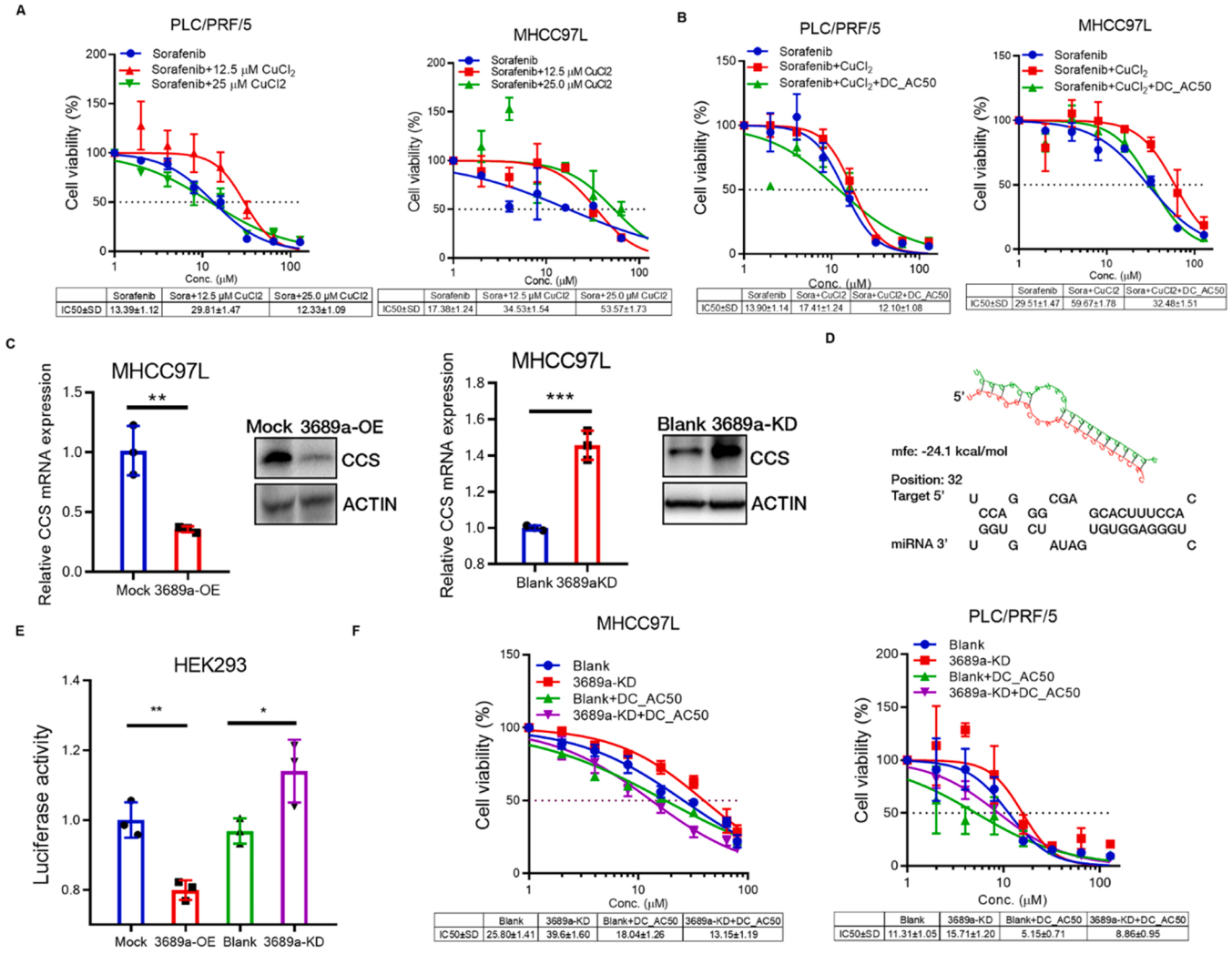

Fig. 4. miR-3689a-3p targets CCS to disrupt intracellular copper transport to mitochondrial SOD1. A. low dose supplementation of copper reduced cytotoxicity of sorafenib in HCC cells. **B.** Treatment of Atox1/CCS inhibitor restored the cytotoxic effect of sorafenib in HCC cells in the presence of low dose copper. **C.** Overexpression of miR-3689a-3p reduced CCS mRNA and protein expression, while miR-3689a-3p knockdown restored CCS. **D.** Prediction of binding between miR-3689a-3p and the 3′UTR region of CCS mRNA. **E.** Overexpression of miR-3689a-3p reduced luciferase expression of plasmid containing 3′UTR of CCS mRNA, while miR-3689a-3p knockdown restored it. **F.** Presence of DC_AC50 significantly increased cytotoxicity of sorafenib to HCC cells with miR-3689a-3p knockdown. *p < 0.05, * *p < 0.01, * **p < 0.001, * ** *p < 0.0001.

with plasmid expressing miR-23a, suggesting no off-target effect in our assay (Fig.S4D). These findings demonstrated that CCS might be the target of miR-3689a-3p. Then we co-treated DC_AC50 with sorafenib in 3689a-KD cells to see whether the 3689a-KD-mediated cytoprotective effect and suppression on mtROS in the presence of sorafenib could be restored after CCS inhibition. It was interesting to observed that CCS inhibition led to less cell viability in sorafenib-treated 3689a-KD cells (Fig. 4 F). The 3689a-KD-generated orthotopic HCC model co-treated with sorafenib and DC_AC50 was established to determine the in vivo effect of CCS inhibition. It was observed that remarkable reduction in tumor growth, tumor size and metastasis to lung in the 3689a-KD group administrated with sorafenib (Fig. 3 F, 3 G, S3C, S3D). Consistently, less proliferative cells were detected in sorafenib-treated 3689a-KD group (Fig. 3H). These data demonstrated that CCS was the target of miR-3689a-3p and responsible for activating SOD1 activity, thus facilitating antioxidant defense to sorafenib-induced oxidative damage to mitochondrion.

3.5. CCS expression determined the differential response of HCC cells to sorafenib treatment

The role of CCS in mediating sorafenib response in HCC cells was yet identified. In the publicly available human HCC dataset, CCS was significantly downregulated in tumor tissues compared to their normal counterparts (Fig. 5A). Survival analysis revealed that higher CCS did not predict better survival outcome in overall survival (OS) but showed trend for better disease-free survival (DFS) although without statistical significance (Fig.S5A, S5B). In the HCC patient's cohort with sorafenib administration, CCS expression was significantly higher in HCC tissues without response to sorafenib (non-responders) compared to responders (Fig. 5B). High CCS in sorafenib-treated patients might probably act as a risk factor that predict poor survival (Fig. 5C). We established CCS-overexpressed MHCC97L and PLC/PRF/5 cell lines (Fig. 5D) and compared the actions of CCS overexpression in terms of cell viability, cell apoptosis, and mtROS. Cells with CCS overexpression showed lower

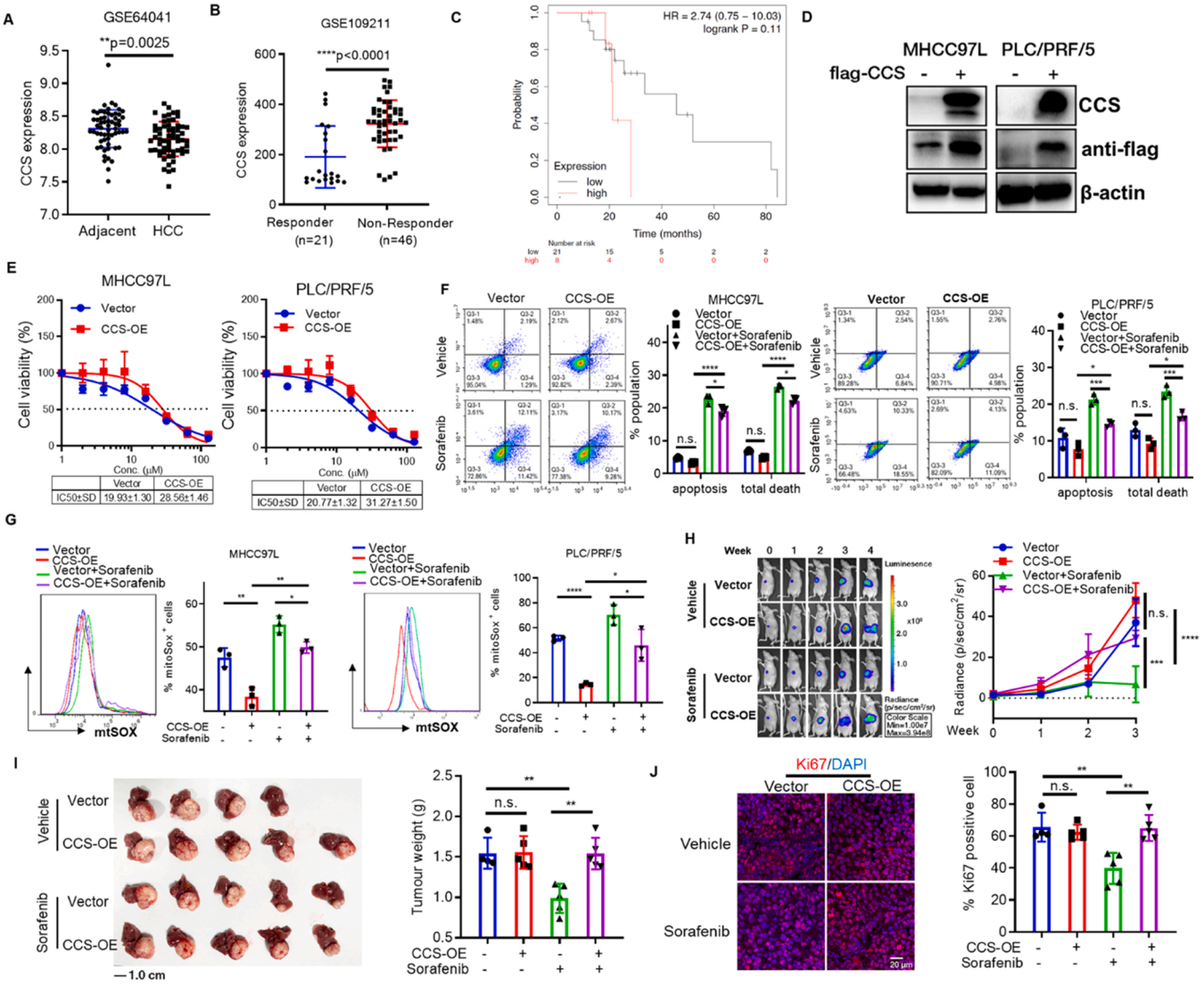

Fig. 5. CCS expression regulated sorafenib sensitivity in HCC cells. A. CCS was down-regulated in HCC tissue compared to its adjacent liver tissue. The cohort of GSE64041 dataset contains expression profile from 60 pairs of biopsies from HCC patients. **B.** CCS was overexpressed in HCC patients not responding to sorafenib treatment compared to that in responders. The expression profile of GSE109211 dataset was from formalin-fixed paraffin-embedded tissue blocks from 140 HCC patients in the STORM clinical trial (NCT00692770). Total 67 samples treated with sorafenib and 73 treated with placebo. Only 21 sorafenib-treated patients responded to sorafenib treatment. **C.** HCC patients with high expression of CCS showed dismal progression-free survival after sorafenib treatment. **D.** Overexpression of CCS in MHCC97L and PLC/PRF/5 cells. Overexpression of CCS (CCS-OE) reduced cell viability (**E**), apoptosis (**F**) and mitochondrial ROS production (**G**) of HCC cells exposed to sorafenib. **H&I.** Overexpression of CCS blunted sorafenib sensitivity in the orthotopic HCC of mice. **J.** Overexpression of CCS restored Ki67 expression in HCC tumors. *p < 0.05, * *p < 0.01, * **p < 0.001, * ** *p < 0.0001.

IC50 value of sorafenib (Fig. 5E), less apoptotic cell and mtROS-positive cells upon sorafenib treatment (Figs. 5F, 5G). The in vivo orthotopic HCC model generated from CCS-overexpressed MHCC97L cells (CCS-OE) appeared faster hepatic tumor growth, larger tumor size and more metastasis to lung tissues in the presence of sorafenib without disturbing body weight (Figs. 5H, 5I, S5C, S5D). In consistent with the in vitro results, more apoptotic cells were found in sorafenib-treated CCS-OE group, while less proliferative cells were observed in sorafenib-treated CCS-OE group (Fig. 5J). Taken together, these results indicated that CCS overexpression limited the anti-HCC effect of sorafenib.

3.6. Transcription activation of miR-3689a-3p by sorafenib required STAT1

To understand the upstream mechanism underlying sorafenib-induced miR-3689a-3p upregulation in HCC cells, we first detected the expression pattern of both primary form and precursor form of miR-3689a (denoted as pri-miR-3689a and pre-miR-3689a, respectively) in the treatment of sorafenib with different concentrations and durations. It was found that both pri-miR-3689a and pre-miR-3689a were increased by sorafenib in a dose- and time-dependent manner, suggesting that sorafenib could induce miR-3689a transcription (Figs. 6A, 6B). Based on the TF-miRNA network in TransmiR database, several TFs were identified to facilitate miR-3689a transcription. Among them, STAT1 was reported as a sensitive protein in redox reaction and displays multi-faced functions through transcriptional activation in the form of phosphorylated STAT1 (Fig. 6C). As the results shown, the addition of chemical STAT1 inhibitor Fludarabine could suppress the expression of phosphorylated STAT1 and both primary and mature forms of miR-3689a-3p induced by sorafenib (Fig. 6D, S6A). We also used STAT1 siRNA to suppress the endogenous STAT1 and observed reduced expression of miR-3689a in the presence of sorafenib (Fig. 6E, S6B, S6C). To further investigate the regulatory mechanism of phosphorylated STAT1 in sorafenib-induced transcription activity of miR-3689a, we

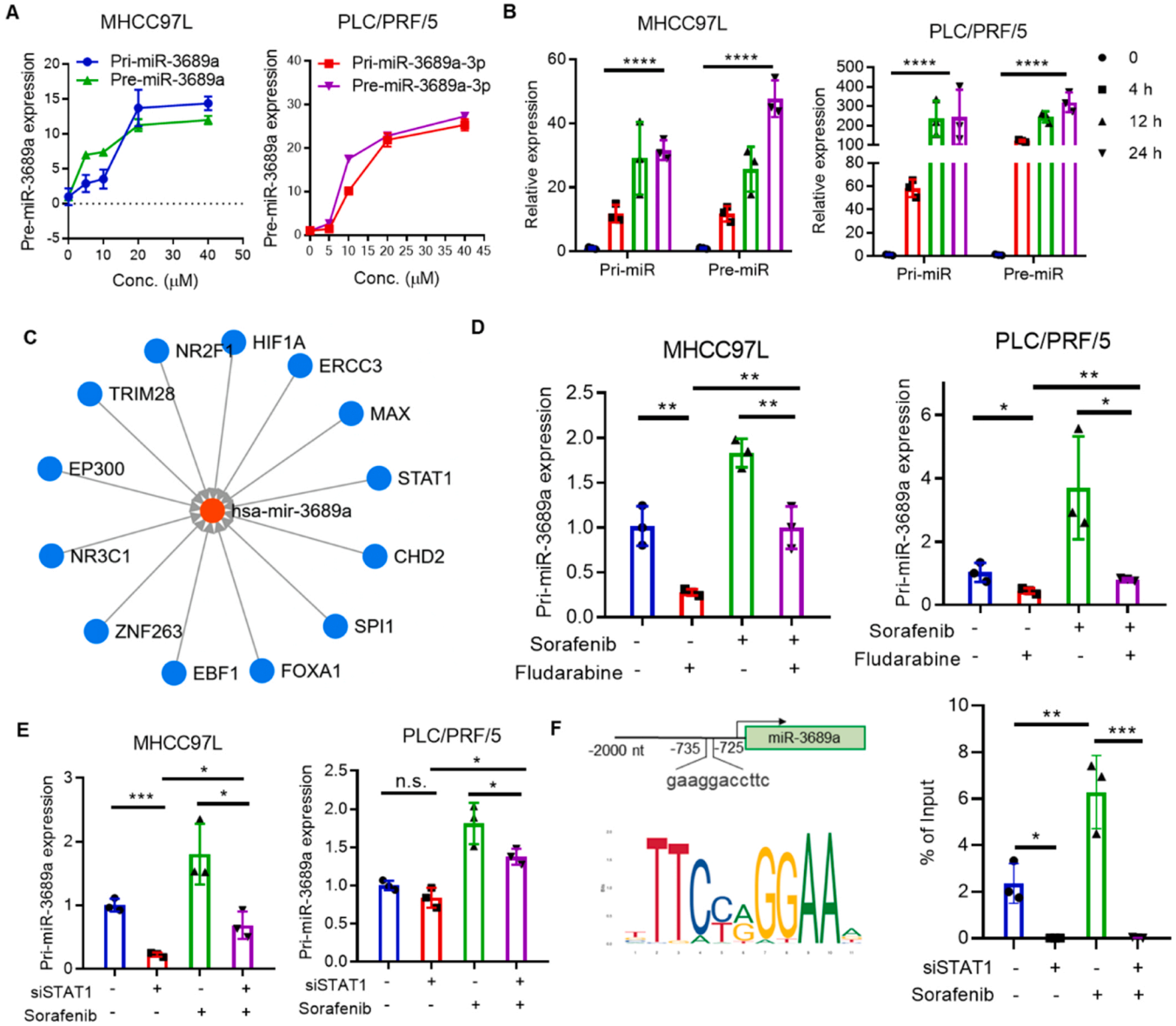

Fig. 6. STAT1 transcriptionally activated miR-3689a-3p in HCC cells exposed to sorafenib. Sorafenib treatment induced a dose-dependent **(A)** and a time-dependent **(B)** up-regulation of primary and precursor forms of miR-3689a-3p in HCC cells; **C.** Prediction of transcription factor that may bind to the promoter region of miR-3689a-3p. **D.** Sorafenib-induced expression of primary and precursor forms of miR-3689a-3p was suppressed by the presence of STAT1 inhibitor fludarabine in HCC cells. **E.** Sorafenib-induced expression of primary and precursor forms of miR-3689a-3p was suppressed by RNA interference to STAT1 in HCC cells. **F.** STAT1 binds to the specific sequence at the promoter region of miR-3689a-3p by ChIP assay. *p < 0.05, * *p < 0.01, * **p < 0.001, * ** *p < 0.0001.

browsed the potential binding sites of STAT1 on the promoter region of miR-3689a and designed specific primers to perform ChIP assay (Fig. S6D). It was found that the fragment of promoter in the range of − 725-− 735nt was highly enriched in the pulldown DNA products by phosphorylated STAT1 antibody in the presence of sorafenib, suggesting the binding relationship between phosphorylated STAT1 and the promoter of miR-3689a in sorafenib-treated HCC with binding motif (Fig. 6F).

3.7. The clinicopathological significance of miR-3689a-3p in HCC

In order to assess the importance of miR-3689a-3p in HCC, we applied in situ hybridization and multiplex immunofluorescence to examine its expression and related signaling molecules identified in this study (Fig.S7A, representative image in Fig. 7A, patient information in Supplementary Table S2. Our tissue assay, which included sections from 90 patients, showed that miR-3689a-3p expression was significantly decreased in HCC tumor tissues compared to non-tumor adjacent tissues (Fig. 7B). This was consistent with data reported from two other HCC patient cohorts, though statistical significance between groups were not obtained probably due to the unpaired nature of the groups (Fig.S7B). Furthermore, the expression of miR-3689a-3p in HCC tissues was negatively correlated with overall survival and progression-free survival of patients (Fig. 7C&7D). Interestingly, miR-3689a-3p expression was not associated with HCC staging or recurrence status (Fig.S7C&S7D). We also found a significant negative correlation between miR-3689a-3p expression and CCS in HCC tumors (Fig. 7E), while expression of miR-3689a-3p was positively correlated with phosphor-STAT1 in HCC tissues (Fig. 7F). Additionally, expression of miR-3689a-3p was negatively associated with the tumor size and AFP level in the serum (Fig. S7E&S7F). Univariate analysis using the Cox regression survival model revealed that better overall survival and recurrence-free survival were associated with high expression of miR-3689a-3p (Fig. 7G). In summary, our findings demonstrate the clinicopathological significance of miR-3689a-3p expression in HCC.

4. Discussion

The results of this study indicate that miR-3689a-3p modulates the supply of copper to mitochondrial SOD1 by targeting its specific copper transporter, which has significant implications in understanding the role of copper in HCC cell response to sorafenib. Over the past few decades, research has shown that copper plays a crucial role in the function of several oncogenic proteins, such as ATOX1 (Blockhuys et al., 2020; Jin et al., 2022), LOXs (Lelievre et al., 2020), and SPARC (Weaver et al., 2008), that contribute to the development, progression, and metastasis of various types of human cancers. Consequently, inhibiting copper transport has emerged as a potential therapeutic strategy for impeding tumor growth in different cancer models (Shanbhag et al., 2021). Previous studies have demonstrated that copper chelators, such as disulfiram and tetrathiomolybdate, can enhance the effectiveness of sorafenib by inhibiting ERK signaling (Xu et al., 2018; Zhang et al., 2022). However, recent findings related to cuproptosis, a unique form of cell death induced by copper emulsion, have added complexity to our understanding of copper's role in cancer (Xie et al., 2023). In fact, our study revealed that simply regulating intracellular copper concentration through supplementation or chelation did not directly correspond to changes in HCC cell response to sorafenib treatment. This finding suggests that adjusting cellular copper levels may not be an effective strategy for influencing HCC cell response to sorafenib. Instead, a more refined and accurate approach may involve modulating the cellular trafficking of copper, which could have a greater impact on HCC cell response to sorafenib. This highlights the need for further investigation into the specific mechanisms governing copper uptake, transport, and release in cancer cells. By obtaining a deeper understanding of these processes, it may be possible to develop targeted therapies that can more effectively manage the response of HCC cells, and potentially other cancer types, to treatments like sorafenib.

CCS is a gene that encodes a protein responsible for delivering copper to the antioxidant enzyme copper-zinc superoxide dismutase (SOD1) (Casareno et al., 1998). This process is essential for the proper functioning of SOD1, which protects cells from oxidative damage by converting harmful superoxide radicals into less toxic substances, such as hydrogen peroxide and molecular oxygen (Dong et al., 2016; Xie et al., 2023). The role of CCS in human cancer is not yet fully understood, but some studies have suggested that it may have both tumor-promoting and tumor-suppressive effects, depending on the context and the types of cancers. Increased CCS expression has been observed in some cancer types, such as breast (Li et al., 2019), lung (Wang et al., 2015), and ovarian (Kim et al., 2019) cancers. This overexpression of CCS may lead to increased SOD1 activity, which can protect cancer cells from oxidative stress, promote their survival, and contribute to tumor progression (Li et al., 2019). In this context, CCS may function as a tumor promoter by enhancing the antioxidant capacity of cancer cells and supporting their growth and proliferation (Li et al., 2019). The role of CCS in HCC was yet reported, however, from our study, we found that in the absence of sorafenib, modulation on CCS expression had minimal effect on the in vivo progression of HCC cells, as evidenced by the similar tumor size between the vector-expressing and CCS-expressing HCC cells. However, CCS expression was critical in the cellular response to sorafenib treatment. Overexpression of CCS significantly blunted the cytotoxic effect of sorafenib in vitro and tumor growth inhibition in vivo, which facilitated copper transportation into mitochondrial SOD1 and resulted in the scavenge of mitochondrial ROS and the maintenance of mitochondrial homeostasis. Interestingly, a recent study in breast cancer suggested that CCS can facilitate the accumulation of overall cellular ROS and therefore increase the ERK signaling activity, which in term facilitated tumor progression (Li et al., 2019). Another study found that CCS facilitated copper transport to MEK, the upstream regulator of ERK signaling, to promote kinase activation (Grasso et al., 2021). In our study, we did not observe a significant change on the overall ROS level or the ERK signaling activity after CCS inhibition by miR-3689a-3p, suggesting that the regulation of CCS on sorafenib response in HCC cells may be independent to overall ROS level or ERK signaling, but was related to its regulation on the mitochondrial ROS level mediated by mtSOD1. Our study provides a new insight in CCS-mediated cellular response to sorafenib treatment.

We found that elevation of mtROS, as a representative of mitochondrial oxidative stress, can potentiate HCC cells to sorafenib treatment (Xu et al., 2021). While moderate levels of mtROS are important for maintaining cellular homeostasis and signaling, and excessive mtROS production can lead to oxidative stress and cellular damage. High levels of mtROS can lead to DNA damage, which activates cell cycle checkpoint proteins, such as p53 (Chibaya et al., 2021), and halts cell cycle progression. Excessive mtROS can trigger apoptosis through both intrinsic and extrinsic pathways. The intrinsic pathway involves the release of pro-apoptotic factors, such as cytochrome c, from the mitochondria, leading to the activation of caspases and subsequent cell death (Zhou et al., 2018). mtROS can activate the AMP-activated protein kinase (AMPK) pathway, which inhibits the mechanistic target of rapamycin (mTOR) signaling and suppresses cell growth and proliferation (Enkhnaran et al., 2022). Additionally, mtROS can inhibit the phosphoinositide 3-kinase (PI3K)/Akt pathway, which is frequently hyperactivated in cancer cells and promotes cell survival and resistance to apoptosis (Su et al., 2019) (Huang et al., 2022). In addition, some recent studies also revealed that mtROS can trigger the activation of anti-tumor immunity by inducing maturation, differentiation and priming of innate and adaptive immune cell populations, therefore facilitated the elimination of cancer cells (Cheung and Vousden, 2022). However, it is also reported that mtROS serves to initiate and promote tumor progression, because mtROS can cause a certain level of DNA damage in the mitochondria, leading to mutations, deletions, and other genetic alterations

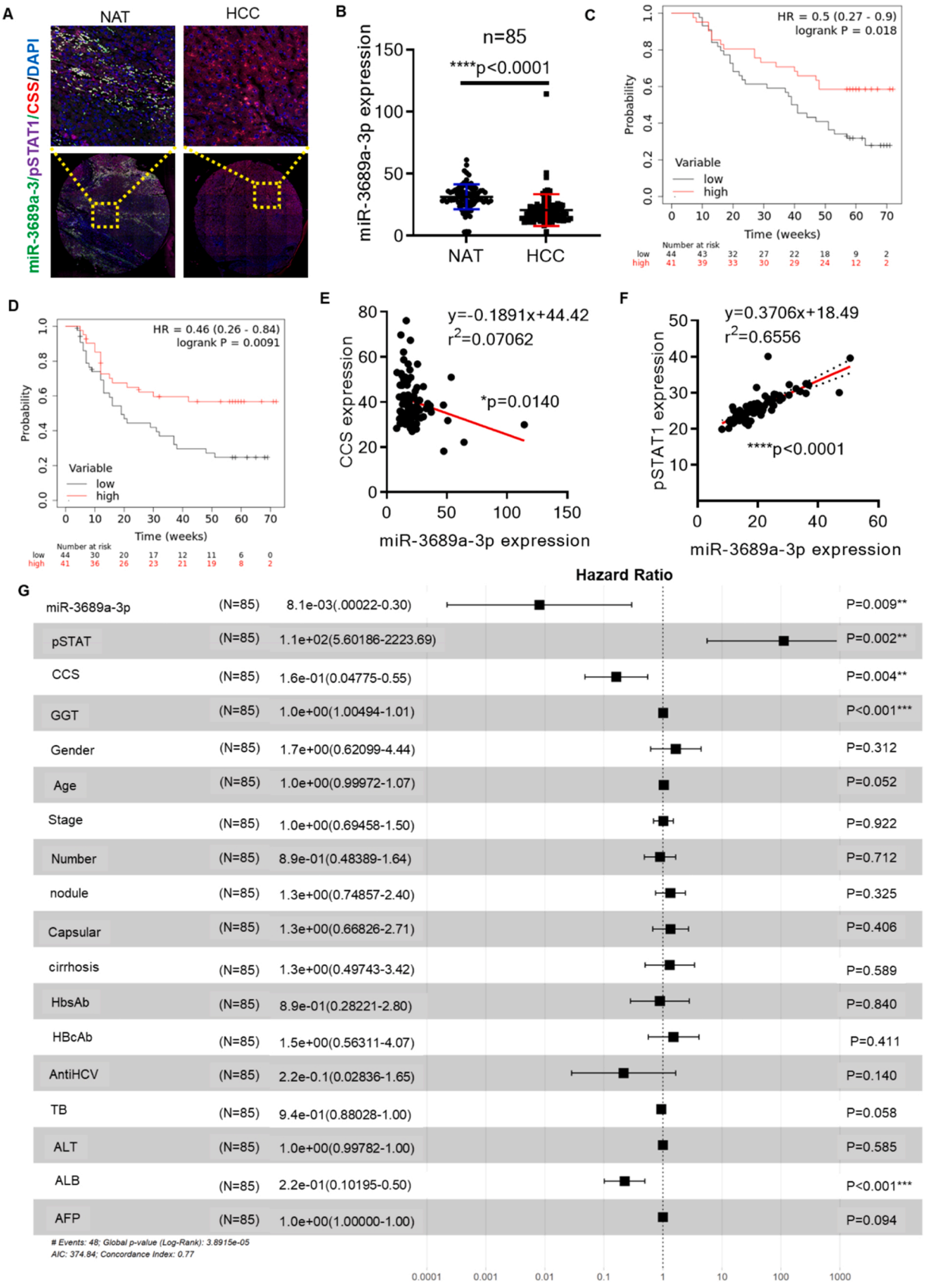

Fig. 7. The clinicopathological significance of miR-3689a-3p in HCC. A. Representative images of adjacent normal liver tissue and tumors on tissue microarray of human HCC specimens. **B.** miR-3689a-3p expression was significantly decreased in HCC tumor tissue compared to non-tumor adjacent tissues. **C&D.** the expression of miR-3689a-3p in HCC tissues was negatively correlated with overall survival and progression-free survival of patients. **E.** miR-3689a-3p expression was negatively associated with CCS in HCC tumors. **F.** miR-3689a-3p expression was positively correlated with phosphor-STAT1 in HCC tissues. **G.** Multivariate analysis revealed that miR-3689a-3p predicted poor prognosis of HCC patients. *p < 0.05, * *p < 0.01, * **p < 0.001, * *** *p < 0.0001.

that can drive oncogenesis and tumor progression (Gao et al., 2022; Gorrini et al., 2013). Certain level of mtROS can activate oncogenic signaling, induce epithelial-to-mesenchymal transition, and facilitate the expression of pro-angiogenic vascular endothelial growth factor (VEGF), therefore cause cancer cell proliferation, migration and angiogenesis (Liu et al., 2022). These studies suggested that the mtROS may play a dual role in cancers, probably depending on their concentration and cellular context. In our study, we found that sorafenib-induced mtROS production tended to be tumor suppressive. Although the detail mechanism that sorafenib induces mtROS needs further investigation, the excessive level of mtROS in our observation could be most likely due to the disruption of internal antioxidative system mitochondrial SOD1 as a negative endogenous mechanism to maintain mtROS level. The sorafenib-induced miR-3689a-3p promotes loss of mitochondrial SOD1 activity through inhibiting its copper transporter CCS, indicating that miR-3689a-3p is an important factor that is required for the sorafenib response in HCC cells, and maintaining the miR-3689a-3p level may promote HCC patient response to sorafenib treatment.

This study has its limitations. In our clinicopathological analysis, the HCC samples were collected from patients receiving curative resection, and sorafenib treatment was not a standard treatment for post-operative management for these patients. A previous network meta-analysis showed that sorafenib did not improve the therapeutic outcome of post-operative HCC patients (Ye et al., 2023). Because most of the patients receiving sorafenib treatment are with tumors clinically non-resectable, it is difficult to collect a cohort of samples specifically with sorafenib resistance. Indeed, there is very few open-data cohort reporting HCC samples from sorafenib-sensitive and sorafenib-resistant patients. However, as miR-3689a-3p was a recently discovered miRNA whose function has yet been explored, we used the HCC samples to identify whether the mechanisms we identified in our cell and animal study was relevant to human scenario. Furthermore, one of the tumor markers AFP that is used as an indicator of sorafenib efficacy was correlated with miR-3689a-3p, which indirectly suggested a potential correlation between miR-3689a-3p expression and sorafenib response in HCC. Further precise investigation to prove more direct evidence of miR-3689a-3p on sorafenib response in HCC patients warrants further study.

In summary, we used an in vivo CRISPR/Cas9 screening approach and identified miR-3689a-3p as an important epigenetic regulator of sorafenib treatment response in human HCC. Treatment of sorafenib in HCC cells significantly induced transcription activation of miR-3689a-3p in a STAT1-dependent manner, which binds to the promoter domain of primary miR-3689a-3p. The upregulation of miR-3689a-3p upon sorafenib treatment targets the 3′UTR region of CCS, the copper transporter for SOD1, leading to the disruption of intracellular copper trafficking, therefore suppresses the antioxidative capacity of mitochondrial SOD1. This results in the accumulation of mitochondrial ROS, inducing oxidative stress-associated cell apoptosis. Expression of miR-3689a-3p negatively correlates with the related molecules CCS and SOD1 while positively correlates with pSTAT1 in human HCC tissues and predicts a better overall and progression-free survival of the HCC patients. Our study sheds lights on an important epigenetic regulator and potential target for enhancing sorafenib response in HCC treatment.

Funding

This work is financially supported by the University Research Committee of The University of Hong Kong (Project Code: 104006600), the Research Grant Committee of Hong Kong (Project Code: 17119621), The Health and Medical Research Fund (Project Code: 19201591 and 15162961) and Innovation and Technology Fund (Project Code: PRP/028/22FX).

CRediT authorship contribution statement

NW conceived the idea, designed the study, analyzed the data and drafted the manuscript. YL conducted the experiments, analyzed the data and drafted the manuscript. YTC, JW, ZF, HY, QL, TX conducted the experiments, CZ, HYT, TKL, YF revised the manuscript.

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.

Data availability statement

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

Acknowledgements

The authors would like to express their appreciation to Mr. Keith Wong, Ms. Cindy Lee, Mr. Alex Shek in School of Chinese Medicine and Dr. Rakesh Sharma in the Proteomics and Metabolomics Core and Centre for PanorOmic Science (CPOS): Imaging and Flow Cytometry Core of Li Ka Shing Faculty of Medicine, The University of Hong Kong for their technical supports.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.drup.2023.101015.

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