Heliyon 9 (2023) e17295

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Inhibition of stress proteins TRIB3 and STC2 potentiates sorafenib sensitivity in hepatocellular carcinoma

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ARTICLE INFO

Keywords:
TRIB3
STC2
Sorafenib
GEO
Hepatocellular carcinoma (HCC)

ABSTRACT

Sorafenib resistance is one of the main obstacles to the treatment of advanced hepatocellular carcinoma (HCC). Stress proteins TRIB3 and STC2 confer cell resistance to a variety of stresses, including hypoxia, nutritional deprivation, and other perturbations, which induce endoplasmic reticulum stress. However, the role of TRIB3 and STC2 in sorafenib sensitivity to HCC remains unclear. In this study, our results indicated that the common differentially expressed genes (DEGs) in sorafenib-treated HCC cells obtained from the NCBI-GEO database (Huh7 and Hep3B cells; GSE96796) included TRIB3, STC2, HOXD1, C2orf82, ADM2, RRM2, and UNC93A. The most significantly upregulated DEGs were TRIB3 and STC2, which were both stress protein genes. Bioinformatic analysis in NCBI public databases indicated that TRIB3 and STC2 were highly expressed in HCC tissues and closely associated with poor prognoses in HCC patients. Further investigation showed that inhibition of TRIB3 or STC2 with siRNA could enhance the anti-cancer effect of sorafenib in HCC cell lines. In conclusion, our study showed that stress proteins TRIB3 and STC2 are closely associated with sorafenib resistance in HCC. The combination of TRIB3 or STC2 inhibition and sorafenib may be a promising therapeutic strategy for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant cancers, accounting for approximately 75%–85% of primary liver cancers [1]. Chronic hepatitis B and hepatitis C infection, alcohol consumption, aflatoxin exposure, non-alcoholic fatty liver disease (NAFLD), and diabetes are the major risk factors for HCC [2]. Surgical resection, liver transplantation, and percutaneous ablation have been demonstrated to be effective treatments for HCC, providing a high rate of complete responses at early stages, but limited benefits at advanced stages of HCC. Unfortunately, HCC is often diagnosed at an advanced stage, for which systemic medical treatment is indicated and the median survival rate is very poor [3].

HCC is highly refractory and insensitive to conventional cytotoxic chemotherapy, partly due to its tumor biology, including genetic and epigenetic changes, and pharmacokinetic properties [4]. In recent years, significant progress has been made in the systemic treatment of HCC, including antiangiogenic tyrosine kinase inhibitors and immunotherapy, which have been successfully applied in the clinic [5]. In addition, the combination of immunotherapy with the checkpoint inhibitor, the *anti*-PDL1 antibody atezolizumab,

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https://doi.org/10.1016/j.heliyon.2023.e17295
Received 16 February 2023; Received in revised form 10 June 2023; Accepted 13 June 2023
Available online 15 June 2023

and the humanized *anti*-VEGF monoclonal antibody bevacizumab has become a standard first-line treatment option for advanced HCC [6].

The orally-available multi-targeted tyrosine kinase inhibitor, sorafenib, is an antiangiogenic inhibitor that was the first systemic therapy approved in 2007 for advanced HCC. However, acquired drug resistance appears inevitable in patients with advanced HCC who still progress after the use of sorafenib, and the overall outcomes are often unsatisfactory [4]. Sorafenib-based combination therapy may be a viable approach to enhance the anticancer activity and improve outcomes of patients with HCC. Therefore, there is an urgent need to further elucidate the mechanism of sorafenib resistance and design more effective strategies for advanced HCC patients [7].

Stress proteins, including heat-shock proteins, RNA chaperones, and ER-associated stress proteins, comprise a family of proteins that play a crucial role in cellular protection, protein homeostasis, and cell survival in response to a diverse range of environmental and metabolic stresses [8]. Given that exposure to anticancer therapies can induce the expression of numerous stress proteins, it is unsurprising that these proteins have been implicated in resistance to various anticancer treatments [9].

In this study, we analyzed the common differentially expressed genes (DEGs) in sorafenib-treated Huh7 and Hep3B cells obtained from the NCBI-GEO database, and found that the expressions of stress protein genes Tribbles Pseudokinase 3 (TRIB3) and Stanniocalcin 2 (STC2) were significantly upregulated. Bioinformatic analysis of NCBI public databases revealed elevated levels of TRIB3 and STC2 in HCC tissues, and the upregulation of these genes demonstrated a significant correlation with unfavorable prognoses among HCC patients. Silencing TRIB3 or STC2 decreased cell proliferation and enhanced the anti-cancer effect of sorafenib in HCC cell lines. Our study indicated that stress proteins TRIB3 and STC2 are closely associated with sorafenib resistance in HCC. The combination of TRIB3 or STC2 inhibition and sorafenib may be a promising therapeutic strategy for HCC.

2. Materials and Methods

2.1. Cell culture and reagents

Human HCC cancer cell lines Huh7 and Hep3B were originally obtained from ATCC and preserved in our laboratory. All the cells were authenticated using AmpFlSTR Identifiler PCR assays in 2022 b y TSINGKE Biological Technology Company. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Gibco, USA) in a 5% CO2 atmosphere at 37 °C.

2.2. Transfection

SiRNAs targeting *TRIB3* and *STC2*, and negative control siRNA were synthesized by GenePharma. Following the manufacturer's instructions, we transfected $1 \times 10\hat{}3$ cells per well with the indicated constructs using Lipofectamine 2000 (Invitrogen). A 72-h harvest of the transfected cells was performed for further analysis. The siRNA sense strand sequence was as follows:

TRIB3: 5′-CTTCGTCCAGCCCCAGTCC-3';

STC2:5′-GUGGAGAUGAUCCAUUUCATT-3'.

2.3. Western blotting analysis

Assays for protein fractionation were performed using SDS polyacrylamide gel electrophoresis and immunoblotting. The study used *anti*-TRIB3 and *anti*-STC2 antibodies from Abcam (UK), and *anti*-GAPDH antibodies from Zhongshan Golden Bridge Biotechnology (China).

2.4. CCK-8

Cells were cultured and processed in 3–6 multiple wells per experimental group. We added 10 L of culture medium containing 10% CCK-8 to every well, mixed it with shock, and incubated it for 2–4 h at 37 °C. OD value was measured by microplate reader, and finally statistical analysis was performed. The above experiment was repeated three times.

2.5. Colony formation assays

Cells in exponential growth phase were digested and resuspended to make cell suspension. Blow cells repeatedly to fully disperse. Cells were counted and diluted to appropriate cell concentration. The cell suspension was inoculated with 5000 cells in a 5-cm dish to make the cells evenly dispersed. Place the culture dish in the cell incubator for 1 week. The dishes were immersed in PBS for 3 times, left to dry at room temperature, and fixed with 4% paraformaldehyde for 15 min. Finally, the dye was stained with crystal violet for 10 min, washed with PBS, dried, and photographed.

2.6. Identification of differentially expressed

We downloaded gene expression profiles from the Gene Expression Omnibus database for genes from public microarray data GSE96796. A series of R BIOCONDUCTOR packages was used to analyze the dataset, raw datasets were normalized, and DEGs were screened out with LIMMA via *P*-value >0.05 and $|\log2 \text{ (fold change)}|>1$ cut-off.

2.7. pathway enrichment analysis

KEGG pathway analysis of the obtained up-DEGs was carried out using the package clusterProfilerKEGG. $P < 0.05$ was considered as statistically significant.

2.8. Protein-protein interaction network construction

We constructed a protein-protein interaction (PPI) network of the DEGs using the Search Tool for Retrieval of Interacting Genes (STRING) database to interpret the underlying molecular mechanisms. The PPI was visualized with an interaction score not exceeding 0.4 (medium confidence score). The hub genes were then chosen based on their degree of connection by CYTOSCAPE software.

2.9. Statistical analysis

By using GraphPad Prism 5, we compared groups using Student's *t*-test (two-tailed) and one-way ANOVA followed by Tukey post hoc tests. Sample size was not predetermined statistically; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ were calculated based on the level of statistical significance. The experiments were all replicated at least three times.

3. Results

3.1. DEGs in sorafenib-treated HCC cells obtained from the NCBI-GEO database

The analysis flow of this study is shown in Fig. 1. Retrieval of gene expression data from GSE96796 via the Gene Expression Omnibus database facilitated examination of the molecular mechanisms governing resistance to sorafenib within hepatocellular carcinoma. The GSE96796 database contained two groups: control or 3 μM sorafenib treatment for 24 h in Hep3B and Huh7 cell lines. We identified 105 and 27 genes that were differentially expressed between the untreated and sorafenib-treated Hep3B and Huh7 cells, respectively (>1.5-fold, $p < 0.05$, Fig. 2A). The common DEGs between Hep3B and Huh7 cells were identified by Venn diagram analysis, which included the upregulated genes TRIB3, STC2, C2orf82, ADM2, UNC93A and the downregulated genes HOXD1, RRM2 (Fig. 2B, Table 1).

3.2. Sorafenib treatment significantly upregulates the expression of TRIB3 and STC2 in HCC cells by identification DEGs

By analyzing the DEGs in sorafenib-treated Hep3B and Huh7 cells, we found that TRIB3 and STC2 were both among the top 10 highly upregulated genes (Supplementary Figure 1A). Gene ontology analysis was applied to uncover the functions of the DEGs. Gene set pathway enrichment analysis revealed that a significant proportion of DEGs enriched in insulin resistance were found in up-regulated genes (Supplementary Figure 1B). Cytoscape software was used to perform module analysis of the STRING database and identify gene interactions. TRIB3 and STC2 were also identified as significant hubs of the network (Fig. 3). These results indicated that sorafenib treatment might significantly upregulate the expression of TRIB3 and STC2 in HCC cells.

3.3. The expression patterns of the common DEGs and survival analysis in HCC

To identify the potential genes that mediate sorafenib resistance in HCC, the expression patterns of the common DEGs in HCC were

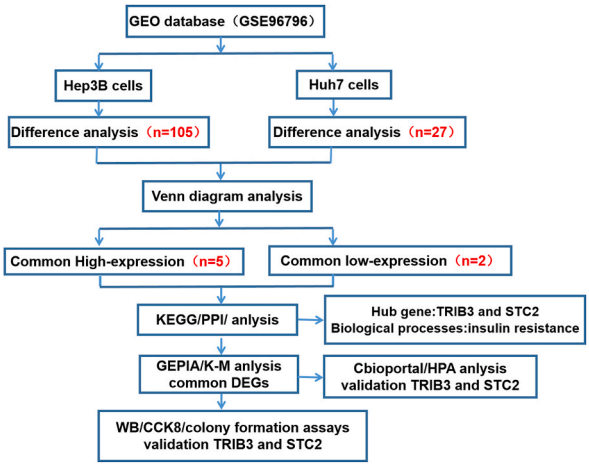

Fig. 1. The flow chart of this study.

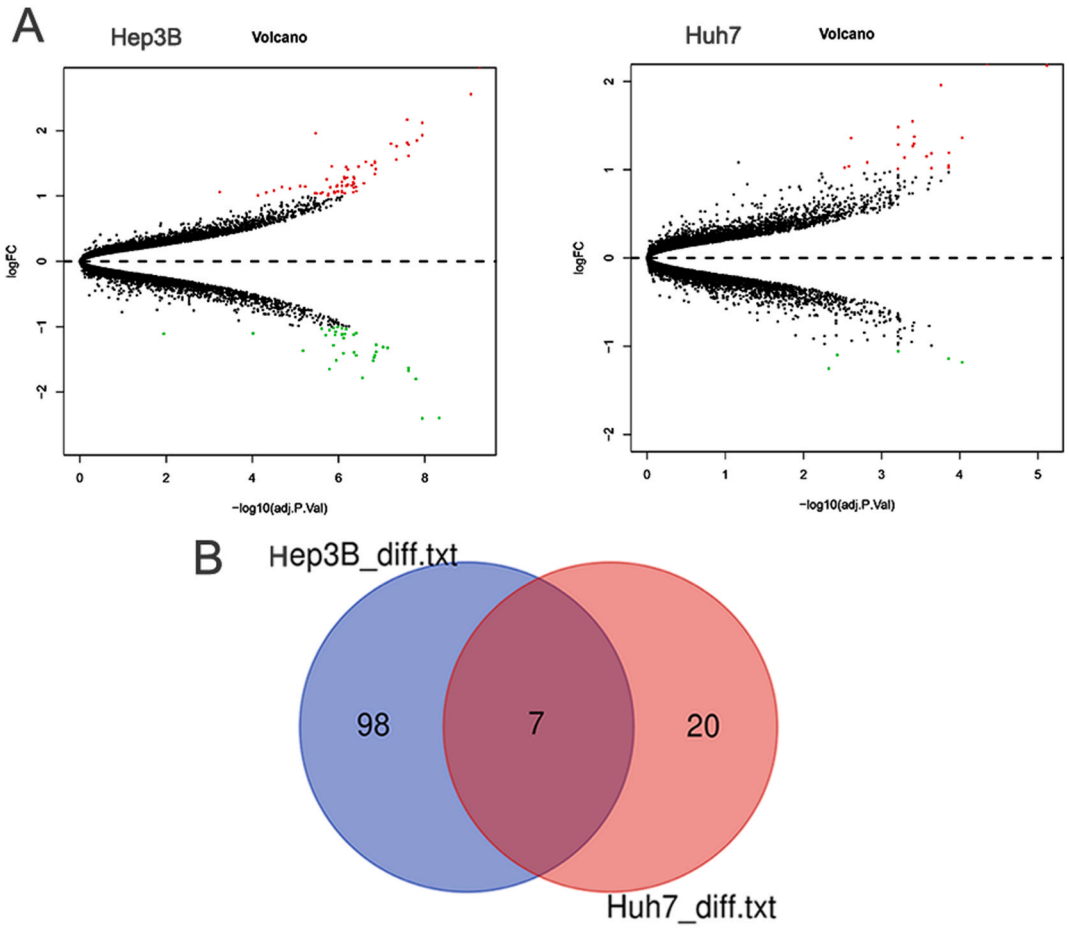

Fig. 2. DEGs in sorafenib-treated HCC cells obtained from the NCBI-GEO database. (A) Volcanoplot of the 105 and 27 identified DEGs in sorafenib-treated Hep3B and Huh7 cells, respectively. Red indicates DEGs with a |log2FC|>1.5. DEG, differentially expressed gene; FC, fold change. (B) The common DEGs between Hep3B and Huh7 cells were identified by Venn diagram analysis (http://bioinformatics.psb.ugent.be/webtools/Venn/). Table 1 showed the detailed genes in common DEGs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
The common DEGs of two gene expression profiles (adj. P val. <0.05, |logFC|>1.0).

Common DEGs Gene symbol
Common DEGs TRIB3; STC2; HOXD1; C2orf82; ADM2; RRM2; UNC93A
Upregulated DEGs **TRIB3; STC2**; C2orf82; ADM2; UNC93A
Downregulated DEGs HOXD1; RRM2
DEGs, differentially expressed genes

determined using GEPIA, a bioinformatic analysis website. Among the upregulated common DEGs, TRIB3, STC2, C2ORF82H, and ADM2 were highly expressed in HCC tissues compared to normal tissues. Interestingly, the levels of the downregulated DEGs HOXD1 and RRM2 were also elevated in HCC tissues compared to normal tissues (Fig. 4).

To further explore the roles of the upregulated common DEGs in HCC, gene-based survival analysis in KMplot, a web-based survival analysis tool, was conducted. The result showed that patients with high levels of TRIB3, STC2, HOXD1, and RRM2 in HCC were significantly associated with shorter overall survival (Fig. 5). In addition, cbioportal was applied for cancer genomics datasets analysis. The amplification and high mRNA levels of TRIB3 accounted for 5% of all HCC tissues. The amplification and high mRNA levels of STC2 accounted for 6% of all HCC tissues (Fig. 6A). Then, we assessed the TRIB3 and STC2 protein expression in human tumor tissues using the GTEx (Genotype-Tissue Expression) database, and TRIB3 and STC2 showed moderate protein expression levels in liver cancer tissues (Fig. 6B). Moreover, normal liver tissues display inferior protein expression levels of TRIB3 and STC2 relative to liver cancer tissues, which express these genes at medium protein levels (Fig. 6C).

Overall, these results indicated that TRIB3, STC2, HOXD1, and RRM2 might be potential oncogenes, while the increased

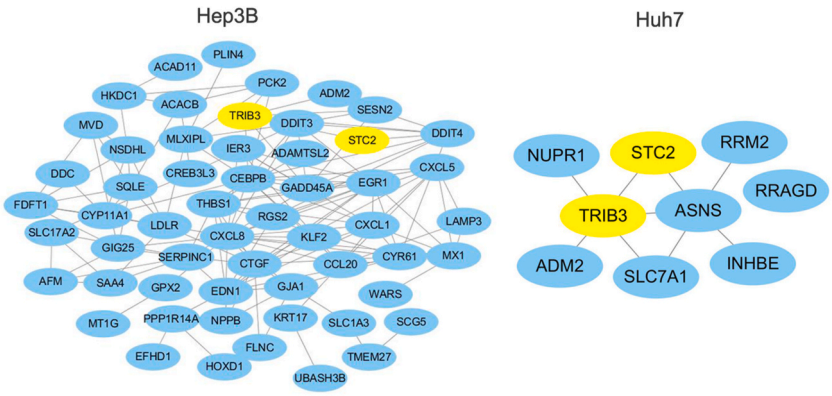

Fig. 3. Sorafenib treatment significantly upregulates the expression of TRIB3 and STC2 in HCC cells by identification DEGs. Using the STRING online database and Cytoscape, upregulated genes in the sorafenib groups were filtered into the DEG PPI network complex. Hub genes were marked in red, other linked genes were marked in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

expressions of TRIB3 and STC2 in response to sorafenib treatment might mediate sorafenib resistance in HCC.

3.4. Inhibition of TRIB3 or STC2 enhances the anti-cancer effect of sorafenib in HCC cells

To explore whether TRIB3 and STC2 mediated sorafenib resistance in HCC, the function of TRIB3 and STC2 were inhibited by small interfering RNAs (siRNAs). The siRNA knockdown efficiency was confirmed by western blotting (Fig. 7A). The CCK-8 and colony formation assays demonstrated that TRIB3 or STC2 knockdown significantly inhibited the cell viability of Hep3B and Huh7 cells (Fig. 7B and C), indicating that TRIB3 and STC2 might be essential for HCC growth. Finally, we combined sorafenib with TRIB3 or STC2 knockdown to investigate whether TRIB3 or STC2 inhibition could enhance the cytotoxicity of sorafenib against HCC cells. The results established an observable synergistic effect of this combination, culminating in a significant reduction of both cell proliferation (Fig. 8A and B) and colony formation (Fig. 8C and D) in the Hep3B and Huh7 cells.

4. Discussion

Sorafenib is an oral multi-kinase inhibitor that suppresses angiogenesis and tumor cell proliferation [10]. It was approved by the FDA as a unique target drug for advanced HCC in 2007 [11]. Sorafenib can significantly extend the median survival time of patients, but only by 3–5 months due to drug resistance. Therefore, it is necessary to elucidate the mechanisms underlying sorafenib resistance in HCC [12].

Due to the genetic heterogeneity of HCC, some patients exhibited primary resistance to sorafenib [13]. It has been reported that the aberrant activation of EGFR signaling can suppress the effect of sorafenib, and the combination of EGFR inhibitors can improve the anti-tumor abilities of sorafenib [14]. Another report also demonstrated that EGFR activation is a potential determinant of primary resistance of HCC cells to sorafenib via sustained activation of EGFR downstream signaling [15]. In addition, the aberrant activation of RAS/ERK signaling, JNK signaling, and VEGFR signaling have also been reported to mediate sorafenib primary resistance [16–18].

Studies have also revealed mechanisms underlying the acquired resistance to sorafenib in HCC [7]. In acquired drug-resistant HCC cell lines, many oncogenic signaling pathways have been found to be abnormally activated, such as the JAK/STAT pathway, which promotes the expression of Mcl-1 and cyclin D1, and lowers the expression of SHP-1 [19]. Sorafenib treatment can also activate autophagic flux in HCC cells, thus promoting tumor survival and suppressing drug efficacy [20]. In addition, several mechanisms, including hypoxia-induced signaling, crosstalk with PI3K/AKT pathway, epigenetic regulation, and tumor microenvironment might also be involved in the acquired resistance to sorafenib [21–23]. However, the relationship between sorafenib-acquired resistance and stress response remains unknown.

In this study, we analyzed the differentially expressed genes (DEGs) in sorafenib-treated hepatocellular carcinoma (HCC) cells obtained from the NCBI-GEO database and identified the critical genes that might be involved in the acquired resistance to sorafenib in HCC. The bioinformatic analysis revealed that the most significantly upregulated DEGs were TRIB3 and STC2, which are both stress proteins [24,25]. Stress proteins are a highly conserved family of proteins that are essential for cellular protection, protein homeostasis, and the preservation of cell viability under diverse stress conditions [8]. Studies have documented that several physiological conditions such as hypoxia, free amino acid insufficiency, and under-availability or oversupply of glucose can activate the TRIB3 promoter, which leads to an increase in TRIB3 levels [26–29]. The overexpression of TRIB3 is associated with metabolic dysfunctions and plays an important role in cancer development. Several tumors, such as colorectal cancer, lung cancer, and HCC, have been reported to exhibit higher TRIB3 mRNA levels [24]. In line with this, data from The Cancer Genome Atlas (TCGA) as described in our results also confirm that TRIB3 mRNA expression is higher in tumor tissues compared to normal tissues.

TRIB3 has been shown to enhance cell viability and survival during glucose deprivation by upregulating IGFBP2, a novel nutrient

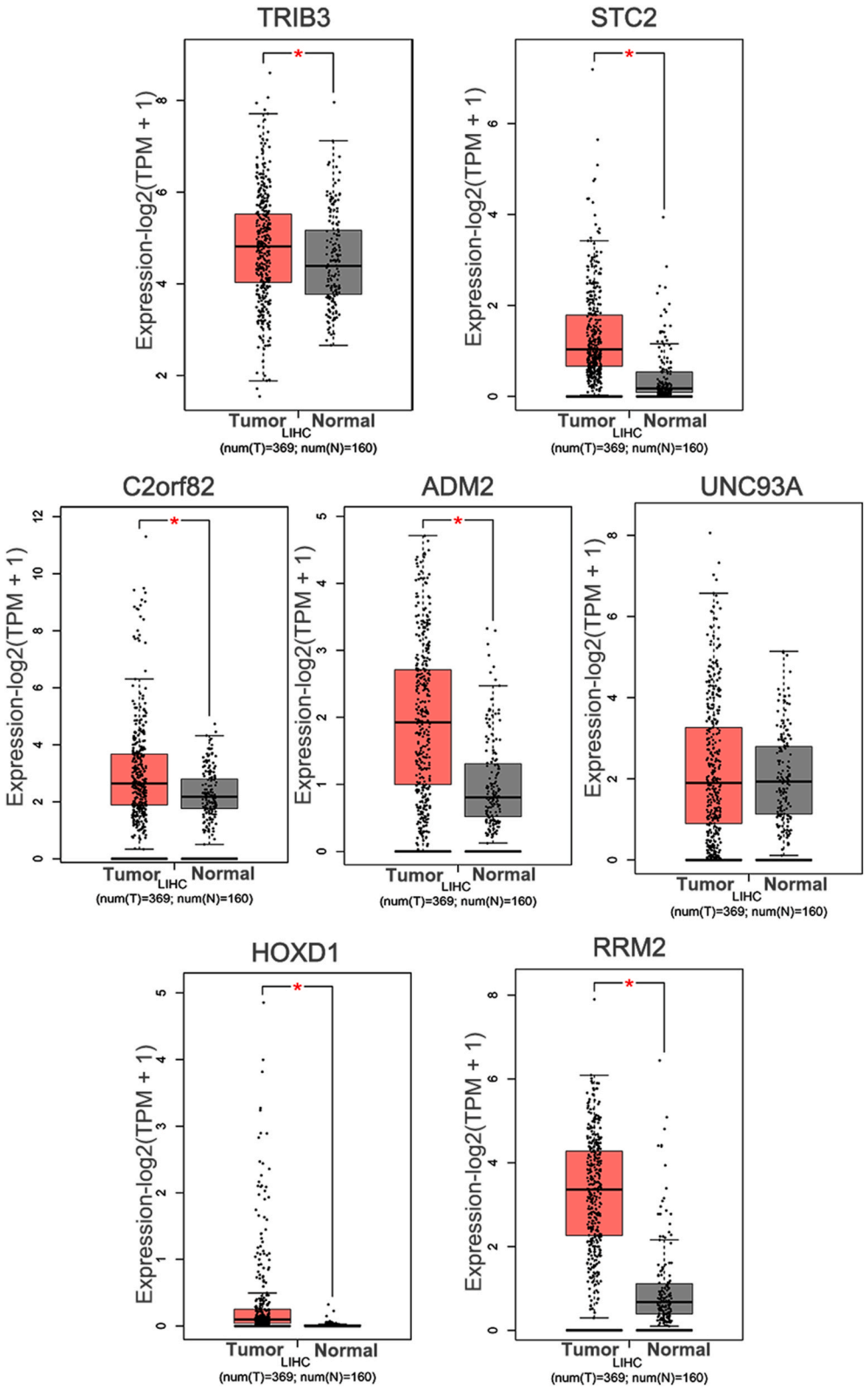

Fig. 4. The expression patterns of the common DEGs in HCC. Bioinformatic analysis in GEPIA (http://gepia.cancer-pku.cn/) showed the expression levels of the common DEGs in HCC. *$p < 0.05$.

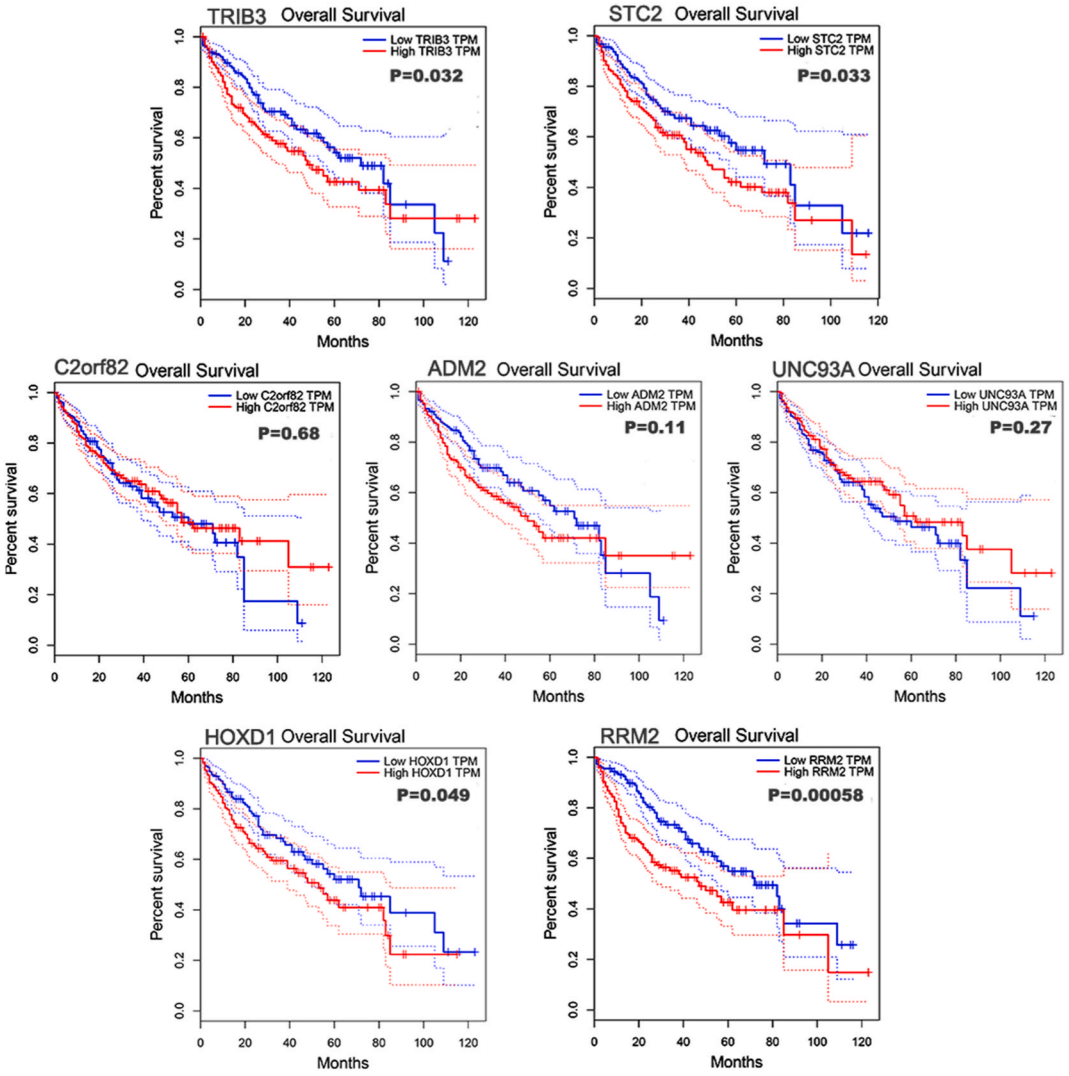

Fig. 5. Kaplan-Meier analysis of the common DEGs in HCC. Kaplan-Meier analysis indicated that high *TRIB3, STC2, HOXD1and RRM2* in HCC was significantly associated with shorter overall survival.

deficiency survival factor [30]. TRIB3 can also protect cells against the growth inhibitory and cytotoxic effects of ATF4 [31]. In cancers, numerous studies have demonstrated that TRIB3 can promote tumorigenesis and tumor progression through multiple mechanisms. For example, in colorectal cancer, TRIB3 increases cancer stem cell features and tumorigenesis by interacting with β-Catenin and TCF4 [32]. In breast cancer, TRIB3 has been reported to regulate Notch activation and its ligand JAG1 expression, which induces cell survival and tumor xenograft growth [33]. MAPK-ERK and TGFβ-SMAD4 signaling have been implicated in TRIB3-mediated Notch activation and the regulation of JAG1 expression. Additionally, TRIB3 can destabilize the tumor suppressor PPARα expression through ubiquitin-mediated proteasome degradation in acute myeloid leukemia [34].

STC2 is a highly conserved, secreted glycoprotein, which is expressed in a broad spectrum of tumor tissues [35]. Similar to TRIB3, STC2 can facilitate cells in dealing with stress conditions and prevent cells from apoptosis. Its levels are induced by various stress factors such as ER stress, hypoxia, and nutrient deprivation [25]. The best-described transcriptional factors that trigger STC2 expression levels are ATF4, a critical protein in mediating the integrated stress response. In addition, the STC2 promoter contains hypoxia response elements. Under hypoxic conditions, HIF-1 is translocated into the nucleus and induces the expression of STC2 [36]. Functionally, STC2 controls a variety of cellular processes such as cell survival and proliferation, tumor migration, and immune response. Many studies have shown that STC2 can activate the PI3K-AKT signaling, MAPK signaling, JUN signaling, and induce cyclin D expression and Rb phosphorylation [37,38]. It can also improve the production of matrix metalloproteinases (MMPs), drive epithelial-mesenchymal transition (EMT) through inducing *N*-cadherin and vimentin and suppressing *E*-cadherin, thus promoting tumor metastasis [39].

These studies indicated that TRIB3 and STC2 as universal tumor biomarkers and potential therapeutic targets. Consistent with

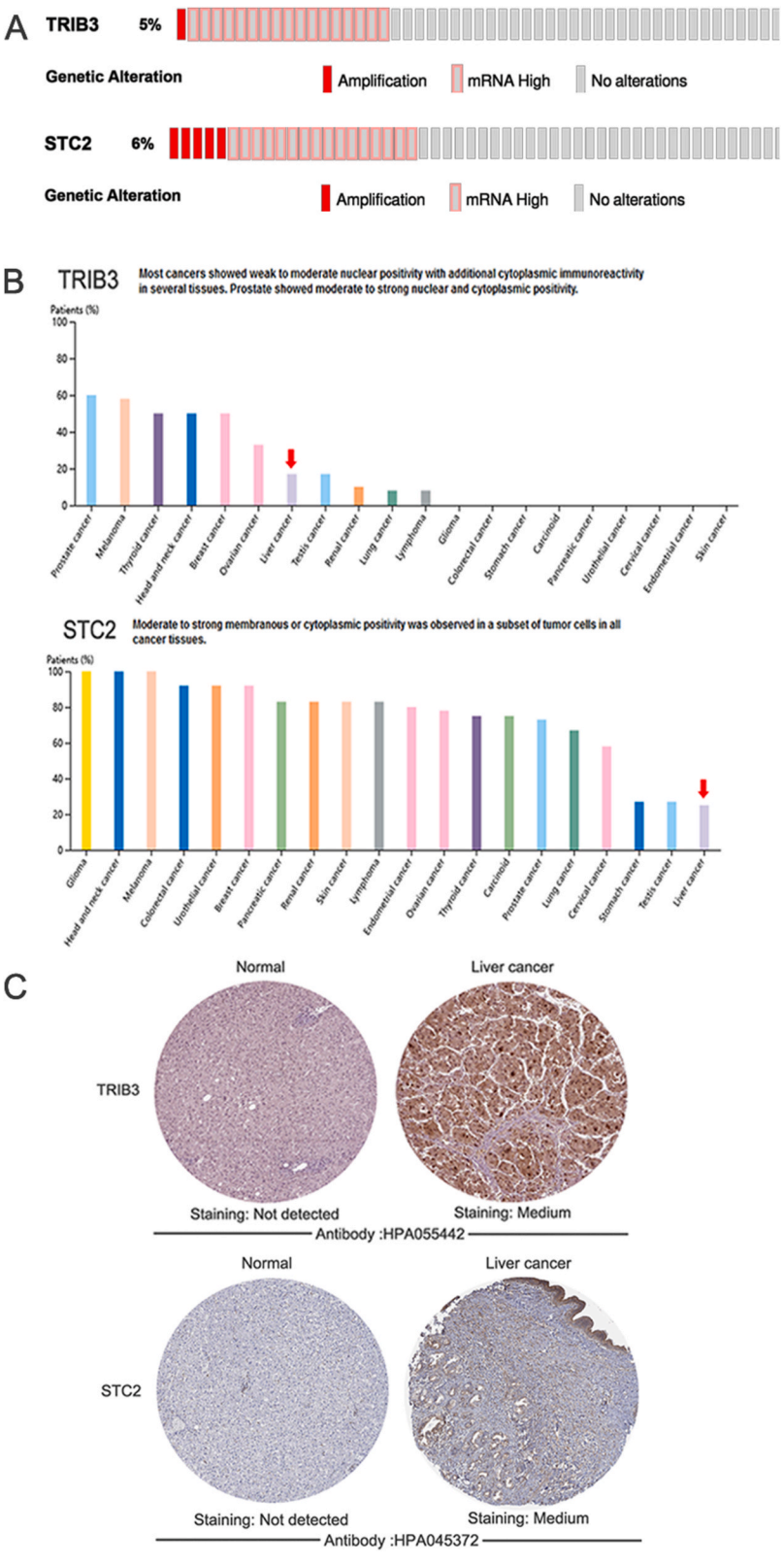

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Fig. 6. Expression analysis of TRIB3 and STC2 in HCC. (A) Gene alteration of TRIB3 and STC2 in HCC analyzed in Cbioportal (http://www.cbioportal.org/). (B) The protein expression profiles of TRIB3 and STC2 in human cancer tissues analyzed in the Human Protein Atlas project (https://www.proteinatlas.org/). (C) Representative immunohistochemistry images of TRIB3 and STC2 expression in normal liver tissues, liver cancer tissues analyzed in the Human Protein Atlas project.

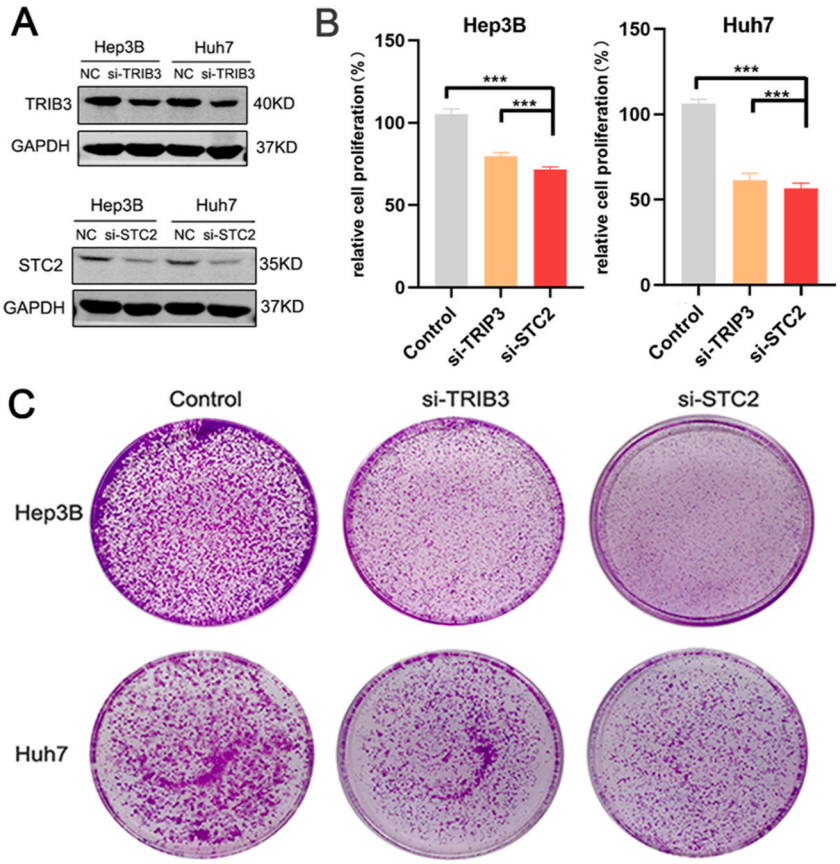

Fig. 7. TRIB3 and STC2 knockdown significantly inhibited the cell viability of Hep3B and Huh7 cells. (A) Protein levels of TRIB3 and STC2 in Hep3B and Huh7 cells infected with TRIB3 and STC2 siRNA. NC, Negative control. (B, C) Hep3B and Huh7 cells were transfected with TRIB3 or STC2 siRNA, respectively. The cell proliferation ability was detected by CCK-8 and colony formation assays. ***$p < 0.001$).

previous findings, TRIB3 and STC2 were found to be highly expressed in HCC tissues. Previous reports have also demonstrated that upregulation of the expression of TRIB3 and STC2 is a common phenomenon in drug-resistant tumor cells [25,40]. For example, in human HCC cells, TRIB3 was found to be critical in modulating apoptosis and chemotherapy resistance, and may regulate ER stress-induced cancer behaviors via modulation of the AKT signal pathway [41]. Similarly, ectopic expression of STC2 has been shown to compromise paclitaxel-induced apoptosis by upregulating *P*-glycoprotein and Bcl 2 in HCC cells [42]. These findings collectively suggest that TRIB3 and STC2 mediate chemoresistance in liver cancer cells, although their role in resistance to tyrosine kinase inhibitors (TKIs) like sorafenib in liver cancer cells remains unclear.

In our study, analysis of GEO database revealed upregulation of TRIB3 or STC2 expression in sorafenib-resistant HCC cells. Cellular proliferation and colony formation assays demonstrated that inhibiting TRIB3 or STC2 not only effectively suppressed HCC cell growth and colony formation but also enhanced the anti-tumor effect of sorafenib. Regarding the mechanisms underlying acquired resistance to TKIs, in non-small cell lung cancer, inhibition of TRIB3 can accelerate EGFR degradation through the TRIB3-PKCα-WWP1 regulatory axis, and STC2 knockdown can reverse TKI resistance through the Jun-Axl-Erk signaling axis by downregulating ERK kinase activation [38,43]. Therefore, the molecular mechanism underlying the inhibition of stress proteins TRIB3 or STC2 to enhance the inhibitory effect of sorafenib in liver cancer cells may be related to promoting EGFR degradation or downregulating MAPK pathway activation. In addition, previous study have also demonstrated that epithelial-mesenchymal transition (EMT) may also play a role in acquired resistance to sorafenib in HCC [44].

One limitation of our study is the lack of investigation into the mechanisms involving stress proteins TRIB3 or STC2 in the development of sorafenib resistance. Future investigations will be focused on illuminating the specific roles played by TRIB3 or STC2 in the EGFR pathway and EMT, as well as the specific molecular pathways leading to sorafenib resistance. These investigations will

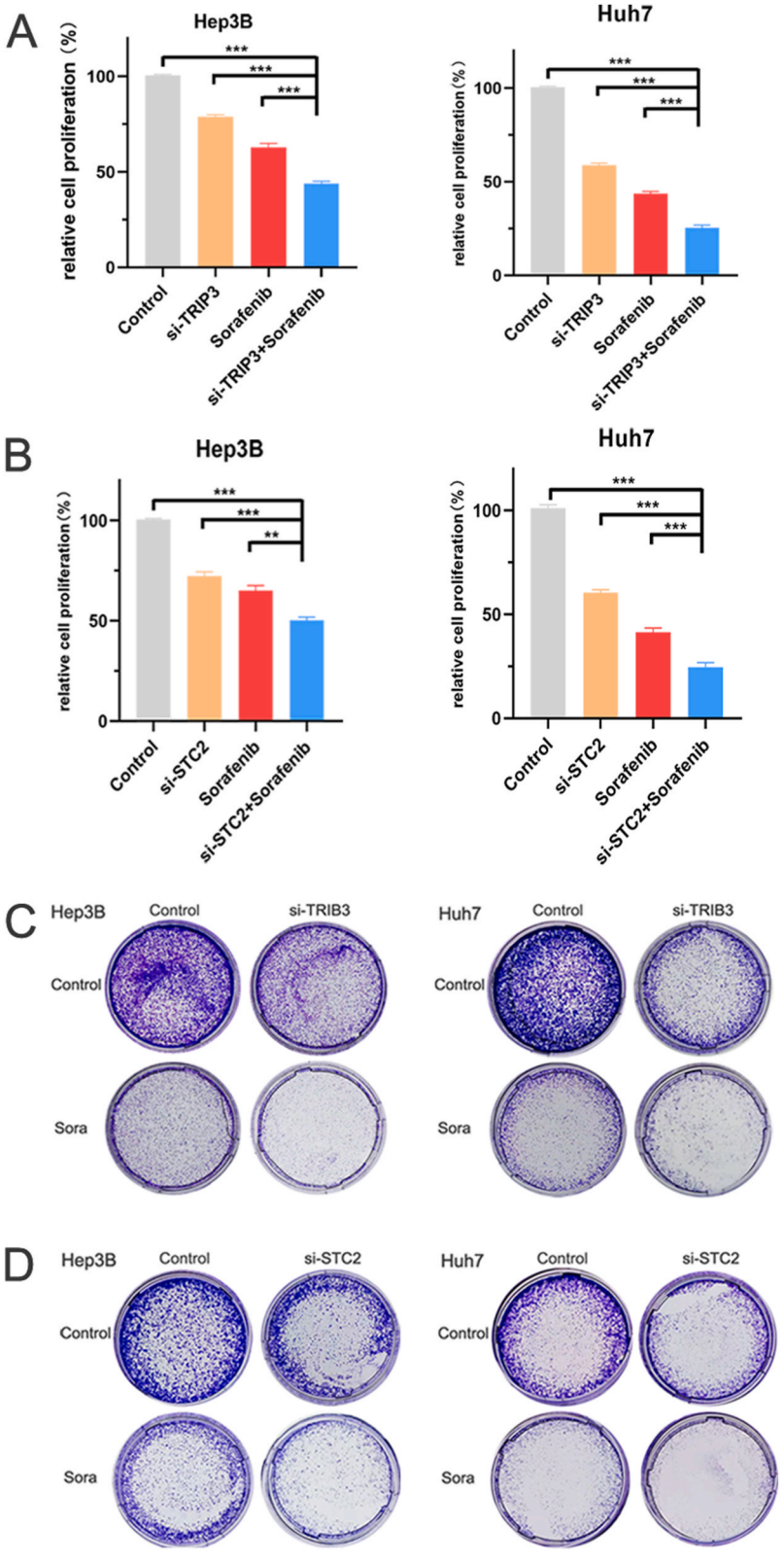

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Fig. 8. Targeting TRIB3 or STC2 synergizes with sorafenib in suppressing the viability of HCC cells in vitro. (A, B) Hep3B and Huh7 cells were transfected with TRIB3 or STC2 siRNA, respectively in the presence or absence of sorafenib for 3 d. Cell viability was detected by CCK-8 assay. ***$p < 0.001$). (C, D) Hep3B and Huh7 cells were transfected with TRIB3 or STC2 siRNA, respectively in the presence or absence of sorafenib for 3 d. Cell viability was detected by colony formation assays.

provide a better understanding of the underlying mechanisms of TRIB3 and STC2-mediated sorafenib resistance, ultimately aiding in the development of more effective treatment strategies.

In summary, these results suggest that the upregulation of TRIB3 or STC2 is associated with acquired resistance to sorafenib in HCC. The combination of TRIB3 or STC2 inhibition and sorafenib may be a promising therapeutic strategy for HCC.

Ethical approval

This study is based on open source database (TCGA and GEO), and approved by the Ethic Committee of the Xiangya Hospital of Central-South University.

Author contribution statement

Sheng Zhou: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.
Tian hong Wei: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Jin tang Liao: Performed the experiments; Analyzed and interpreted the data.

Data availability statement

Data associated with this study has been deposited at GEO database under the accession number GSE96796.

Funding

This work was supported by the Provincial Natural Science Foundation of Hunan (Grant No. 2022JJ40849) and the Youth Foundation of Xiangya Hospital of Central South University (2021Q20).

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.2023.e17295.

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