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Original Article

STOML2 inhibits sorafenib-induced ferroptosis in hepatocellular carcinoma via p-AKT signaling pathway

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Abstract: Tyrosine kinase inhibitor resistance is a key factor affecting the prognosis of patients with advanced hepatocellular carcinoma (HCC). Previously, our group demonstrated that HCC patients with high stomatin-like protein 2 (STOML2) expression were poorly sensitive to systemic therapy. Whether STOML2 is involved in sorafenib resistance is unclear. Recent mechanistic studies have demonstrated that selective activation of ferroptosis pathways is expected to restore sorafenib sensitivity. The aim of the present study was to investigate the STOML2-ferroptosis axis and its contribution to sorafenib resistance. In this study, STOML2 expression was detected in tissue microarrays from patients with primary HCC and in human cell lines. Functional proliferative clone formation assay was used to study the biological function of STOML2. Ferroptosis was detected by flow cytometry, cellular lipid peroxidation and the malondialdehyde (MDA) test. Western blotting and qPCR assays were used to verify the STOML2-AKT-solute carrier family 7 membrane 11 (SLC7A11) axis and to explore the possible mechanism of the combination of LY294002 (an AKT inhibitor) in patients with advanced HCC. The results indicated that patients with poor efficacy demonstrated higher expression of STOML2 compared with that in samples derived from patients with good efficacy. Knockdown of STOML2 expression inhibited colony formation and IC_{50} in HCC cell lines treated with sorafenib. High STOML2 expression was negatively correlated with ferroptosis as shown by Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis. STOML2 inhibited ferroptosis by activating the AKT-SLC7A11 axis, which promoted increased intracellular antioxidant capacity. The AKT inhibitor LY294002 exhibited synergistic antitumor effects with sorafenib in HCC. In conclusion, the present study demonstrated that STOML2 could enhance the AKT-SLC7A11-mediated antioxidant capacity in HCC, inhibit ferroptosis and reduce the sensitivity of HCC to sorafenib, providing a theoretical basis for the combination of LY294002 and sorafenib.

Keywords: Stomatin-like protein 2 (STOML2), hepatocellular carcinoma (HCC), ferroptosis, AKT, sorafenib, drug resistance

Introduction

Hepatocellular carcinoma (HCC) ranks among the most common malignancies worldwide and represents the fourth leading cause of cancer-related mortality [1]. A pivotal development in systemic treatment for HCC is the approval of sorafenib by the Food and Drug Administration. This multi-target kinase inhibitor serves as a primary treatment for advanced HCC by inducing ferroptosis, suppressing angiogenesis, and inhibiting tumor proliferation [2-4]. However, the efficacy of sorafenib in treating patients with advanced HCC is still limited [5, 6]. Therefore, there is an imperative need to identify potential mechanisms related to sorafenib resistance.

Ferroptosis, an emerging form of programmed cell death, is uniquely characterized by the iron-dependent accumulation of lipid peroxidation products [7-9]. Mounting evidence has highlighted that ferroptosis can be inhibited by overexpressing the solute carrier family 7 membrane 11 (SLC7A11), also commonly known as System x_c^- [10, 11]. This transporter is integral to cystine import, which is essential for glutathione biosynthesis and the body's antioxidant defense mechanisms [12-14]. Sorafenib limits cystine uptake through the inhibition of system

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x_c-, leading to depletion of glutathione, restriction of antioxidant capacity, and ultimately triggering ferroptosis [10, 15-18]. These insights suggest that SLC7A11 plays a crucial role in mediating resistance to sorafenib via ferroptosis.

Stomatin-like protein 2 (STOML2), also referred as SLP-2 or mitochondrial SLP-2 is an approximately 1.5-kilobase mRNA located on chromosome 9p13 [19, 20]. STOML2 is predominantly localized in the inner mitochondrial membrane and functions as a key regulator of mitochondrial biogenesis and activity [21, 22]. Over the past two decades, the oncogenic potential of STOML2 has been increasingly recognized. Extensive research has revealed its involvement in promoting the progression of various cancer types, including HCC, pancreatic cancer, head and neck squamous cell carcinoma and colorectal cancer [23-27]. In our previous study, it was discovered that STOML2 enhanced the metastatic capabilities of HCC. This was achieved by facilitating PTEN-induced kinase 1 (PINK1)-mediated mitophagy and influencing the response to lenvatinib [26]. However, the exact interplay between STOML2 and the efficacy of sorafenib in the treatment of HCC remains uncertain.

This study systematically investigated the regulatory role of STOML2 in sorafenib-induced ferroptosis in HCC cells. The present research demonstrated that sorafenib enhanced STOML2 expression. A novel mechanistic pathway was identified whereby STOML2 facilitated the phosphorylation of AKT, which in turn upregulated SLC7A11 expression and reversed sorafenib-induced ferroptosis, leading to sorafenib resistance. The findings provide information for a new mechanistic pathway involving STOML2 and SLC7A11 in the context of sorafenib-induced ferroptosis. This offers insights into potential targets for enhancing the efficacy of sorafenib.

Materials and methods

Clinical sample analysis

In the present study, clinical samples of HCC tumor tissues were collected from five pairs of patients with HCC treated with combined targeted therapy and chemotherapy at the Infectious Disease Department of Huashan Hospital. The patients were categorized into two groups based on their treatment outcomes. The inclusion criteria for the study were the following: Age >18 years and the availability of paraffin-embedded tissue samples. The research was conducted in accordance with ethical standards and was approved by the Ethics Committee of Huashan Hospital.

Cell culture

The human HCC cell lines Huh7, LM3 and Hep3B were obtained from the Shanghai Institute of Cell Research, Chinese Academy of Sciences. The cell lines were cultured in DMEM (HyClone; Cytiva) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), at 37°C containing 5% CO_2.

Colony formation

Huh7, LM3 and Hep3B cells were seeded in 6-well plates at a density of 1,000 cells/well. Sorafenib (Selleck Chemicals) was added following 3 days of cell incubation and the preparation was replaced following 72 h. Following 2 weeks of cell incubation, visible colonies were washed and stained with crystal violet (1% methanol) for 10 min. The images were obtained using a digital microscope.

Antibodies and reagents

The antibodies used for the detection of STOML2 (Cell Signaling Technology, Inc.), p-AKT (Cell Signaling Technology, Inc.), AKT (Cell Signaling Technology, Inc.), SLC7A11 (Cell Signaling Technology, Inc.) and β-actin (Cell Signaling Technology, Inc.) were obtained from Proteintech Group, Inc. The antibodies used in immunohistochemical analysis against STOML2 (Proteintech Group, Inc.), p-AKT (Proteintech Group, Inc.) and SLC7A11 (Proteintech Group, Inc.) were obtained from Proteintech Group, Inc. The detailed use of antibodies is presented in Table S1. Sorafenib (Selleck Chemicals, cat. no. S7397), erastin (Selleck Chemicals, cat. no. S7242), SC79 (Selleck Chemicals, cat. no. S7863) and LY294002 (Selleck Chemicals, cat. no. S1105) were purchased from Selleck Chemicals.

Western blot analysis

Total protein samples from cells were extracted using RIPA (Novalzone) with protease and phosphatase inhibitor cocktails. The protein concentration was determined using the bicinchoninic acid (BCA) quantification method with the BCA assay kit (Thermo Fisher Scientific, Inc.). An equivalent amount of protein samples (20 μg of total protein per lane) was separated by 10% SDS-polyacrylamide gel electrophoresis and subsequently transferred on polyvinylidene fluoride membranes (Merck KGaA). Following a 2-h blocking step with 5% FBS in TBST at room temperature, the membranes were exposed to primary antibodies overnight at 4°C. Subsequently, the membranes were incubated with secondary antibodies for 1 h at room temperature. The protein bands were visualized using enhanced chemiluminescence substrate kits (Tanon Science and Technology Co., Ltd.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol® reagent (Absin) and the cell samples were pre-washed with 1 × PBS prior to use. cDNA was performed using 5 × PrimeScript Buffer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reverse transcription reaction was conducted as follows: 25°C for 5 min, 55°C for 15 min and 85°C for 5 min. Gene expression analysis was carried out on a 7500 Real-Time PCR System using the SYBR Green RT-qPCR system (Takara Bio, Inc.). qPCR was performed as follows: 95°C for 5 min, 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 20 sec. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method and GAPDH was used as the internal control for normalization. The primer sequences employed for RT-qPCR are provided in Table S2.

Intracellular reactive oxygen species (ROS) detection

The levels of intracellular ROS were assessed using fluorescence-based measurements. Briefly, cultured HCC cells in 6-well plates were incubated with the cellular ROS indicator DHE (MCE) in serum-free medium at 37°C for 30 min. Following washing with PBS three times, the cells were suspended in 500 μl PBS and subsequently analyzed using flow cytometry (Beckman CytoFlex S; Beckman Coulter, Inc.).

Malondialdehyde (MDA) detection

Lipid peroxidation was analyzed by quantification of MDA concentration using a lipid peroxidation MDA assay kit (Abbkine Scientific Co., Ltd.) as per the manufacturer's guidelines. The values of MDA were detected using a fluorescence microplate reader (Thermo Fisher Scientific, Inc.) at 532 and 600 nm.

Half-maximal inhibitory concentration (IC_{50}) assay

For IC_{50} assay analysis, the cells were initially plated in 96-well plates at a density of 1×10^4 cells per well and treated with different concentration of sorafenib. The cells were incubated with 5% CCK-8 solution (Meilunbio) diluted in DMEM for 1 h at 37°C. The absorbance at a wavelength of 450 nm was measured to determine the cell proliferation in each well.

Bioinformatic database analysis

For bioinformatic analysis, the gene expression data were annotated with official gene symbols and subsequently normalized (log2). Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene set enrichment analysis (GSEA) were utilized to explore the biological functions and downstream regulator analysis of STOML2 based on the data from The Cancer Genome Atlas (TCGA; ID: TCGA-LIHC; http://portal.gdc.cancer.gov/projects/TCGA-LIHC).

Immunohistochemical (IHC) staining

Formalin-fixed and paraffin-embedded tumor tissues were used for IHC. Tumor tissues from patients were collected to detect the levels of STOML2, SLC7A11 and p-AKT expression. Briefly, following deparaffinization, rehydration and antigen retrieval, IHC staining was performed. The antibodies used are listed in Table S1. Antigens were repaired (20 × Tris-EDTA antigen repair solution; Wuhan Servicebio Technology Co., Ltd.), endogenous peroxidase activity was quenched for 25 min away from light (3% hydrogen peroxide solution) and 3% BSA was used for blocking at room temperature for 30 min. Subsequently, primary antibod-

ies were applied to slides, incubated at 4°C overnight and followed with secondary antibody incubation at 37°C for 30 min. Staining was carried out with 3,3'-diaminobenzidine (DAB) chromogen (Wuhan Servicebio Technology Co., Ltd.) and counter-staining was performed with hematoxylin. Finally, the prepared slides were digitized using a Pannoramic MIDI scanner (3DHISTECH, Ltd.), ensuring high-resolution imaging of the histological details.

Statistical analysis

Statistical analysis was carried out using SPSS 22.0 (SPSS Inc.) and GraphPad Prism 9.0 (GraphPad Software Inc.). The data are presented as mean ± standard deviation (SD) and compared using either a student's t-test or one-way ANOVA. $P<0.05$ was used to indicate a statistically significant difference ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$).

Results

Upregulation of STOML2 expression inhibits systemic therapeutic sensitivity in HCC

To further investigate the role of STOML2 in patients with advanced HCC, 10 samples from primary HCC were collected and divided into two groups according to the therapeutic effect (Table S3). IHC staining revealed significantly elevated STOML2 expression in patients exhibiting poor therapeutic outcomes (**Figure 1A**). Furthermore, the expression levels of STOML2 were investigated between susceptible and resistant HCC cell lines using the GEO database (GSE182593). The results indicated that STOML2 mRNA expression levels were considerably higher in resistant cell lines than those noted in normal cell lines (**Figure 1B**). To gain insight on the ability of sorafenib to induce STOML2 expression, Huh7 and LM3 (human HCC cell lines) cells were treated with sorafenib for 24 and 48 h. The mRNA and protein levels of STOML2 in HCC cells increased over time following sorafenib treatment (**Figure 1C**). Moreover, the expression levels of STOML2 were investigated at different sorafenib concentrations. RT-qPCR and western blot analyses illustrated that STOML2 expression was upregulated with increasing sorafenib treatment concentration (**Figure 1D**). Collectively, the data indicated that upregulation of STOML2 expression occurred in HCC with poor therapeutic sensitivity and may mediate acquired resistance to sorafenib.

STOML2 mediates acquired resistance to sorafenib in human HCC cell lines

To further elucidate the relationship between STOML2 expression and the therapeutic effect of sorafenib, a subsequent series of functional assays were performed. Based on STOML2 expression levels in HCC cell lines (Figure S1), Huh7 and LM3 cells were used to knockdown STOML2 expression and enhance its expression in Hep3B cells using lentiviral-based short hairpin (sh) RNA construction (Table S2). The efficiency of knocking down STOML2 expression and overexpressing it was verified by RT-qPCR and western blot analyses (**Figure 2A-C**). Subsequently, cell viability following STOML2 overexpression (OE) and knockdown of STOML2 expression was analyzed following treatment with sorafenib at different concentrations for 48 h, as indicated by the cell growth curve. As expected, the IC_{50} value of sh-STOML2 cells was decreased compared with that of the control groups, while the IC_{50} value of STOML2-OE cells was enhanced (**Figure 2D-F**). Colony formation assays further demonstrated that the downregulation of STOML2 expression exhibited a suppressive effect on the prolonged proliferation of Huh7 cells with sorafenib treatment (**Figure 2H**). Consistently, STOML2 OE markedly promoted cell proliferation in Hep3B cells (**Figure 2G**). Therefore, these results indicated that the upregulation of STOML2 expression was a crucial factor affecting the therapeutic efficacy of sorafenib.

STOML2 negatively regulates ferroptosis and inhibits sensitivity to sorafenib in human HCC cells

The underlying mechanisms by which STOML2 affects sorafenib sensitivity was investigated by GO and KEGG pathway enrichment analyses utilizing the GSEA database (Figure S2). As depicted in Figure S2A, STOML2 expression was negatively associated with fatty acid metabolism, lipid catabolic process and the iron ion import pathway, indicating a potential involvement of STOML2 in cellular ferroptosis. Previous studies have demonstrated the association between sorafenib resistance and ferroptosis activity [17, 28-31]. Furthermore, the relationship between STOML2 and vital pro-

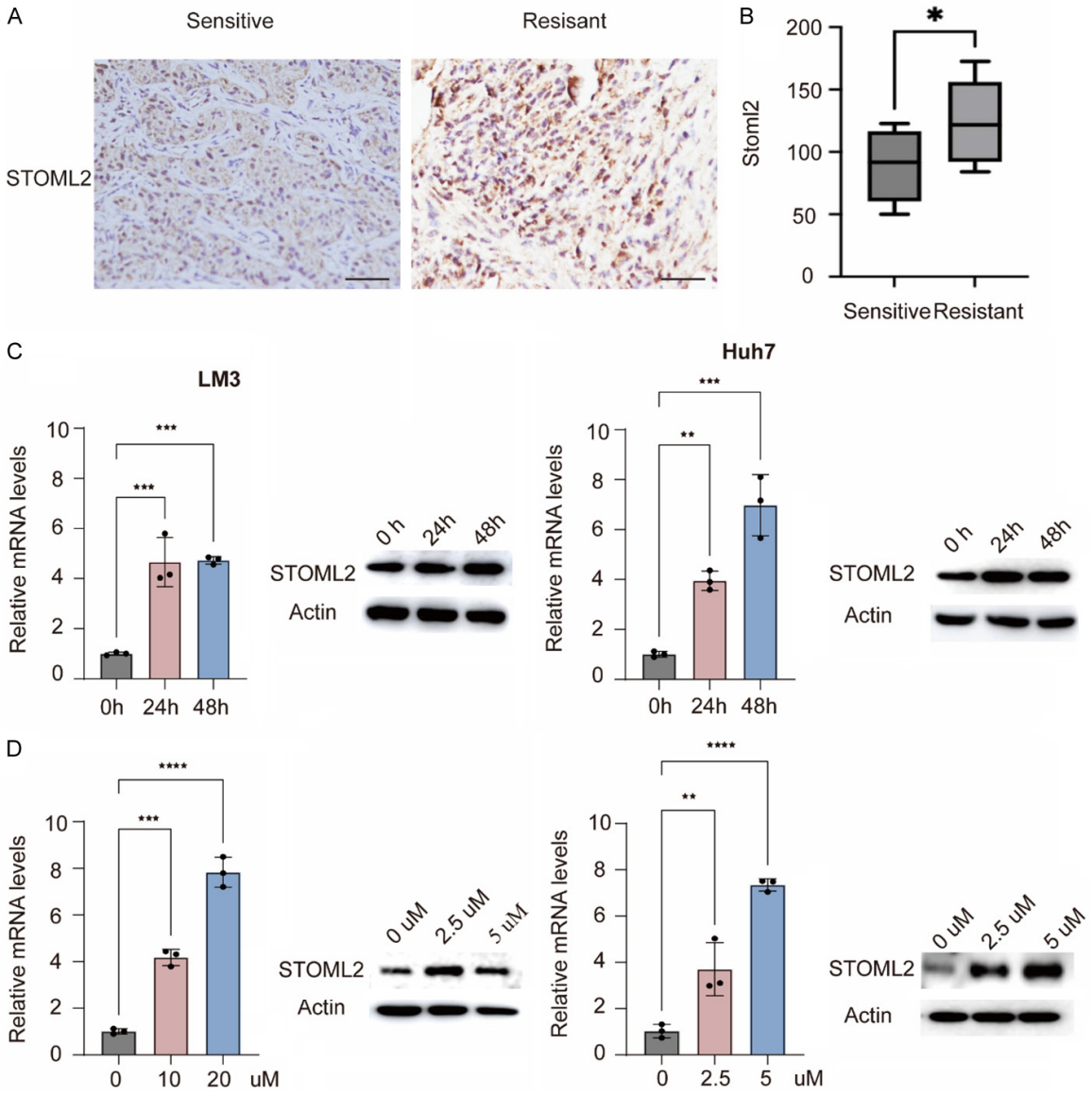

Figure 1. Upregulation of STOML2 expression inhibits systemic therapeutic sensitivity in HCC. A. The mRNA expression levels of STOML2 in 10 primary HCC samples were detected by IHC. Scale bars, 50 μm. B. Relative mRNA levels of STOML2 in sorafenib-resistant cells and normal cells from the HCC dataset (GSE182593) and as shown by box plots. The values are expressed as mean ± SD, $P<0.05$, two-tailed t-test. C. Human HCC cell lines (Huh7 and LM3 cells) were treated with sorafenib (2.5 and 10 μM) for 24 and 48 h. The mRNA expression levels of STOML2 were examined by RT-qPCR (n=3, $P<0.05$ vs. the untreated group); western blot analysis was used to detect the expression levels of STOML2. D. The mRNA and protein expression levels of STOML2 were examined in Huh7 (2.5 and 5 μM) and LM3 (10 and 20 μM) cells treated with different concentration gradients of sorafenib for 24 h. STOML2, stomatin-like protein 2; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; GSE, Gene set enrichment; SD, standard deviation; RT-qPCR, reverse transcription-quantitative PCR.

teins integral to ferroptosis was investigated. Notably, STOML2 was positively associated with glutathione peroxidase 4, atlastin-1, solute carrier family 7 member 11 (SLC7A11) and solute carrier family 3 member 2 (SLC3A2) (**Figure 3A**). The present study further elucidated whether STOML2 played a role in ferroptosis and contributed to sorafenib resistance in HCC. The expression levels of STOML2 were manipulated and their impact was evaluated on ferroptosis (**Figure 3B**). The results indicated that cell death in HCC cell lines induced by sorafenib or erastin could be counteracted by ferrostatin-1 (Fer-1, a ferroptosis inhibitor). In contrast to

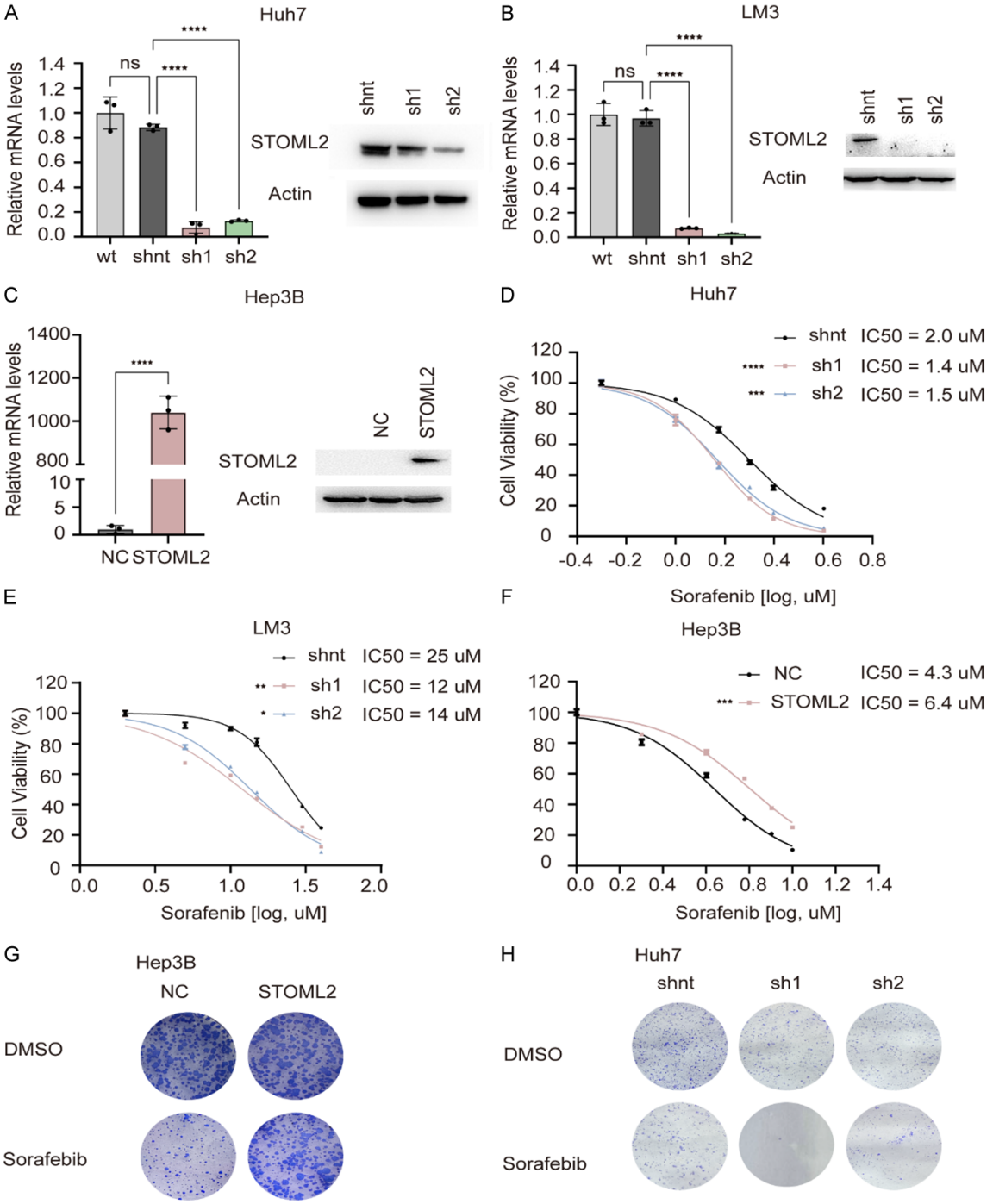

Figure 2. STOML2 mediates acquired resistance to sorafenib in human HCC cell lines. A-C. RT-qPCR and western blot analyses were used to detect the expression levels of STOML2 in HCC cells following its knockdown and overexpression. D-F. CCK-8 assay of sensitivity in knocked down and overexpressed (STOML2) human HCC cell lines and determination of the corresponding IC_{50} value. G, H. The effect of STOML2 overexpression and knockdown of its expression on the sensitivity to sorafenib in HCC cells was evaluated by the clonogenic cell survival assay (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). STOML2, stomatin-like protein 2; HCC, hepatocellular carcinoma; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8; IC_{50}, half-maximal inhibitory concentration.

these observations, cell death was not significantly altered by Z-VAD-FMK (Z-VAD, an apoptosis inhibitor) or necrostatin-1 (necroptosis inhibitor). This implied that sorafenib-induced or erastin-induced cell death in HCC cell lines was primarily attributed to ferroptosis, rather

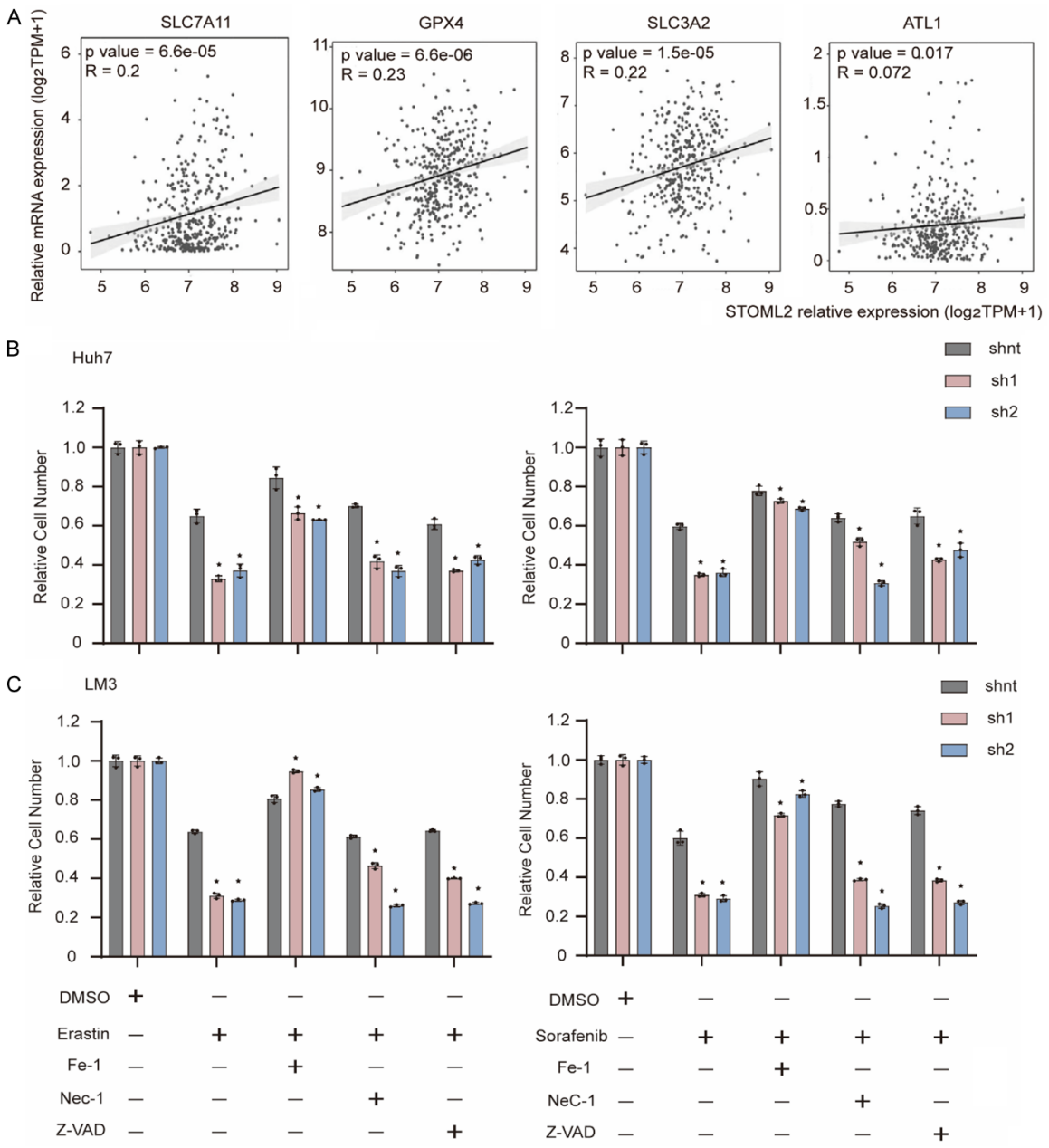

Figure 3. Knockdown of STOML2 expression promotes sorafenib-induced ferroptosis. (A) STOML2 expression was positively correlated with key inhibitory proteins of ferroptosis in the TCGA database. (B, C) Huh7 (B) and LM3 (C) cells were treated with sorafenib (2 and 10 μM) with or without a ferroptosis inhibitor (Fe-1, 1 μM), an apoptosis inhibitor (Z-VAD, 10 μM) and a necrosis inhibitor (Nec-1, 5 μM) for 24 h. STOML2, stomatin-like protein 2; TCGA, The Cancer Genome Atlas; Nec-1, necrostatin-1.

than apoptosis and necroptosis, aligning with earlier research studies. Moreover, STOML2 knockdown cells exhibited higher sensitivity to sorafenib-induced ferroptosis compared with that of the control cells (**Figure 3B** and **3C**). In addition, the levels of total cellular ROS and lipid peroxidation (MDA) were evaluated, which are known to be major contributors to ferroptosis. The results demonstrated that downregulation of STOML2 expression notably increased ROS levels (**Figure 4A**) and MDA production (**Figure 4B**) induced by sorafenib and erastin in HCC cells. Collectively, these results indicated that STOML2 was a negative regulator of ferroptosis and contributed to sorafenib resistance.

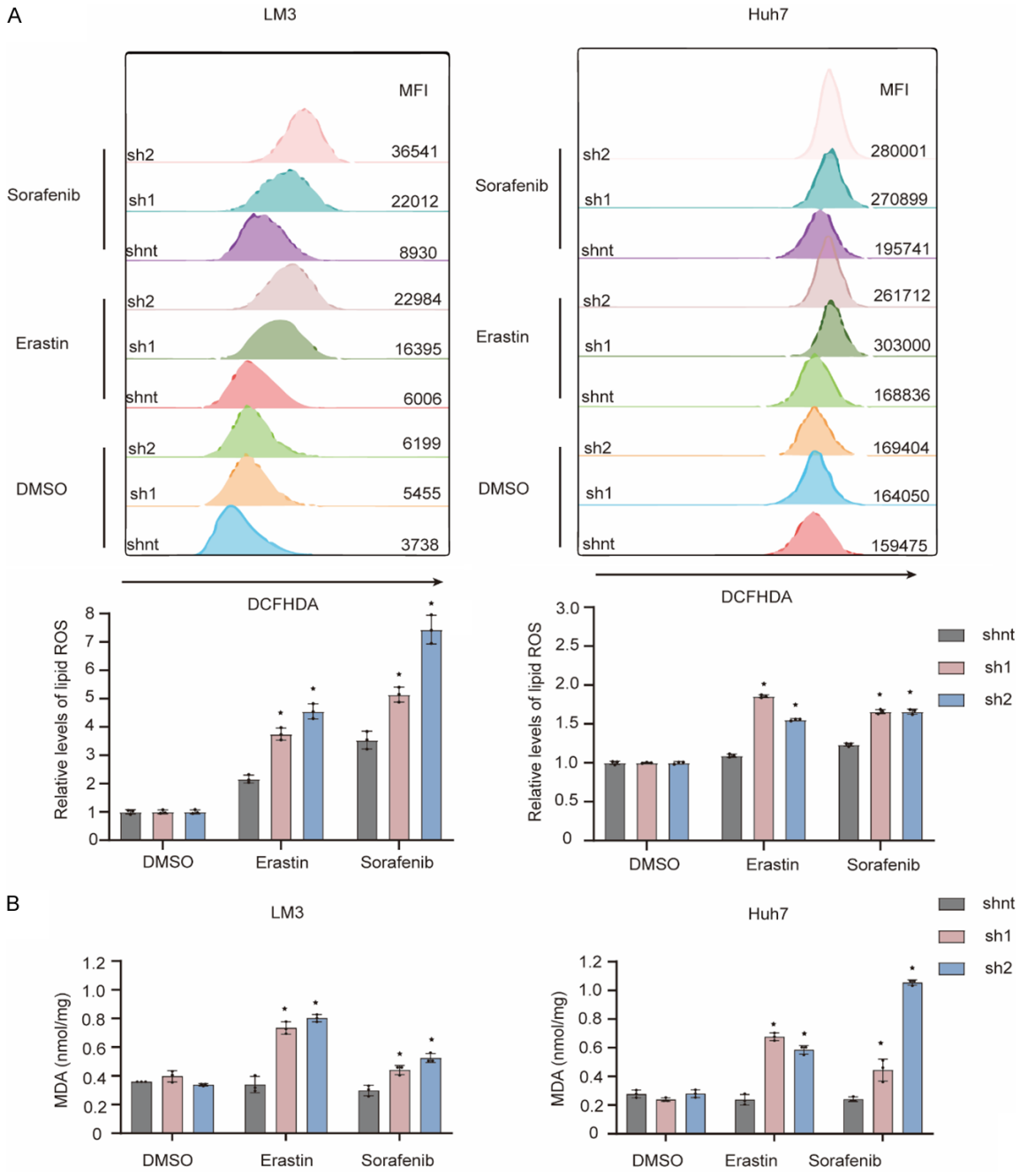

Figure 4. Knockdown of STOML2 expression reduces the antioxidant capacity in HCC. A. Lipid ROS levels were detected by flow cytometry and knockdown of STOML2 expression increased ROS levels in HCC cells compared with those noted in the control cells, P<0.05. B. MDA concentration levels of Huh7 and LM3 cell lines exposed to sorafenib (2 and 10 μM) and erastin (10 and 15 μM) for 24 h, P<0.05. STOML2, stomatin-like protein 2; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; MDA, malondialdehyde.

STOML2 inhibits tumor cell ferroptosis by regulating SLC7A11 levels

Sorafenib is known to increase intracellular lipid peroxidation levels by inhibiting SLC7A11, a specific light-chain subunit of the cystine/glutamate antiporter [16, 17]. Subsequently, the potential interaction between STOML2 and SLC7A11 was explored in modulating ferroptosis. Bioinformatic analysis identified a significant correlation between the expression levels of STOML2 and SLC7A11 (**Figures 3A** and S2B).

To validate the underlying mechanism, the mRNA levels of key molecules in ferroptosis were examined. The results revealed that knockdown of STOML2 expression decreased the expression levels of key inhibitory molecules of ferroptosis, with the most pronounced declining in SLC7A11 (**Figure 5A** and **5B**). Knockdown of STOML2 expression also caused a decrease in SLC7A11 protein levels and conversely, OE of STOML2 (OE-STOML2) was shown to upregulate SLC7A11 expression (**Figure 5C**). It is important to note that the IHC analysis provided further support for these observations, revealing that samples with high expression of STOML2 also exhibited an increase in SLC7A11 expression (**Figure 5D**). Collectively, these findings suggested that STOML2 may inhibit ferroptosis in HCC cells by upregulating SLC7A11 expression.

STOML2 upregulates SLC7A11 levels via activating AKT phosphorylation

Previous studies have established that AKT pathway activation confers protection against sorafenib-induced ferroptosis in HCC cells [17, 32, 33]. To confirm whether STOML2 modulates sorafenib-induced ferroptosis via the AKT signaling pathway, the levels of p-AKT were assessed in HCC cell lines. STOML2 knockdown cell lines were examined and the data indicated that p-AKT expression was decreased, while application of SC79 (an agonist of AKT) in shSTOML2 cells could compensate the SLC7A11 downregulation caused by STOML2 knockdown (**Figure 6A** and **6B**). p-AKT activation reduced cell death and downregulated ROS levels subsequent to knockdown of STOML2 expression (**Figure 6A** and **6B**). In addition, blockade of the AKT pathway with LY294002 (an inhibitor of AKT) counteracted the effect of STOML2 OE in Hep3B cell lines (**Figure 7A**), promoting cell death (**Figure 7B**) and elevating ROS levels (**Figure 7C** and **7D**). The aforementioned data suggested that STOML2 OE alleviated sorafenib-induced ferroptosis via activating the AKT pathway.

Discussion

Although there has been a notable decline in both the incidence and mortality rates of HCC in recent years, the effectiveness of current treatment modalities remains suboptimal. Sorafenib, a commonly used targeted therapy, frequently encounters varying degrees of drug resistance, which substantially limits its clinical effectiveness [34-36]. In HCC, sorafenib primarily inhibits tumor cell proliferation and delays tumor progression via suppressing the System x_c^- and inducing ferroptosis [37, 38]. Therefore, the aim of the present study was to explore the mechanism of sorafenib resistance involved in the ferroptosis pathway.

In the present study, the tissue samples were obtained from five pairs of patients with HCC. IHC staining revealed an increase in STOML2 expression in the group with poor therapeutic response. *In vitro* experiments demonstrated that OE of STOML2 inhibited cell death induced by sorafenib and decreased sensitivity to sorafenib, accompanied by reduced iron levels, lipid peroxidation and subsequent ferroptosis. Therefore, blocking the AKT/SLC7A11 pathway and enhancing sorafenib's anticancer activity via the induction of ferroptosis presents a promising therapeutic strategy for the treatment of HCC.

The biological function of STOML2 is based on the regulation of mitochondrial membrane stability and activity [21]. The previous research study has highlighted an upregulation of STOML2 expression in HCC at both tissue and molecular levels and its association with various clinical parameters [26]. Previous work also indicated that STOML2 enhanced mitophagy by interacting and stabilizing PINK1. This interaction contributes to the metastasis of HCC and influences the way by which HCC responds to lenvatinib treatment. Hu *et al* (29) demonstrated that STOML2 activated the MEK/ERK pathway while inhibiting the mitochondrial apoptotic pathway in HeLa cervical cancer cells. Other recent studies have suggested that STOML2 can suppress PINK1-induced mitophagy by directly stabilizing PARL, thereby reducing chemoresistance in pancreatic cancer [24]. Zhu *et al* (30) revealed that silencing STOML2 expression reduced the migratory and invasive capabilities in liver cancer by inhibiting the NF-κB pathway. A recent related study also revealed that STOML2 expression was markedly upregulated in colorectal cancer and contributed to the proliferation of cancer cells and tumor growth by engaging with PHB to activate the MAPK signaling pathway [23]. The present study indicated that

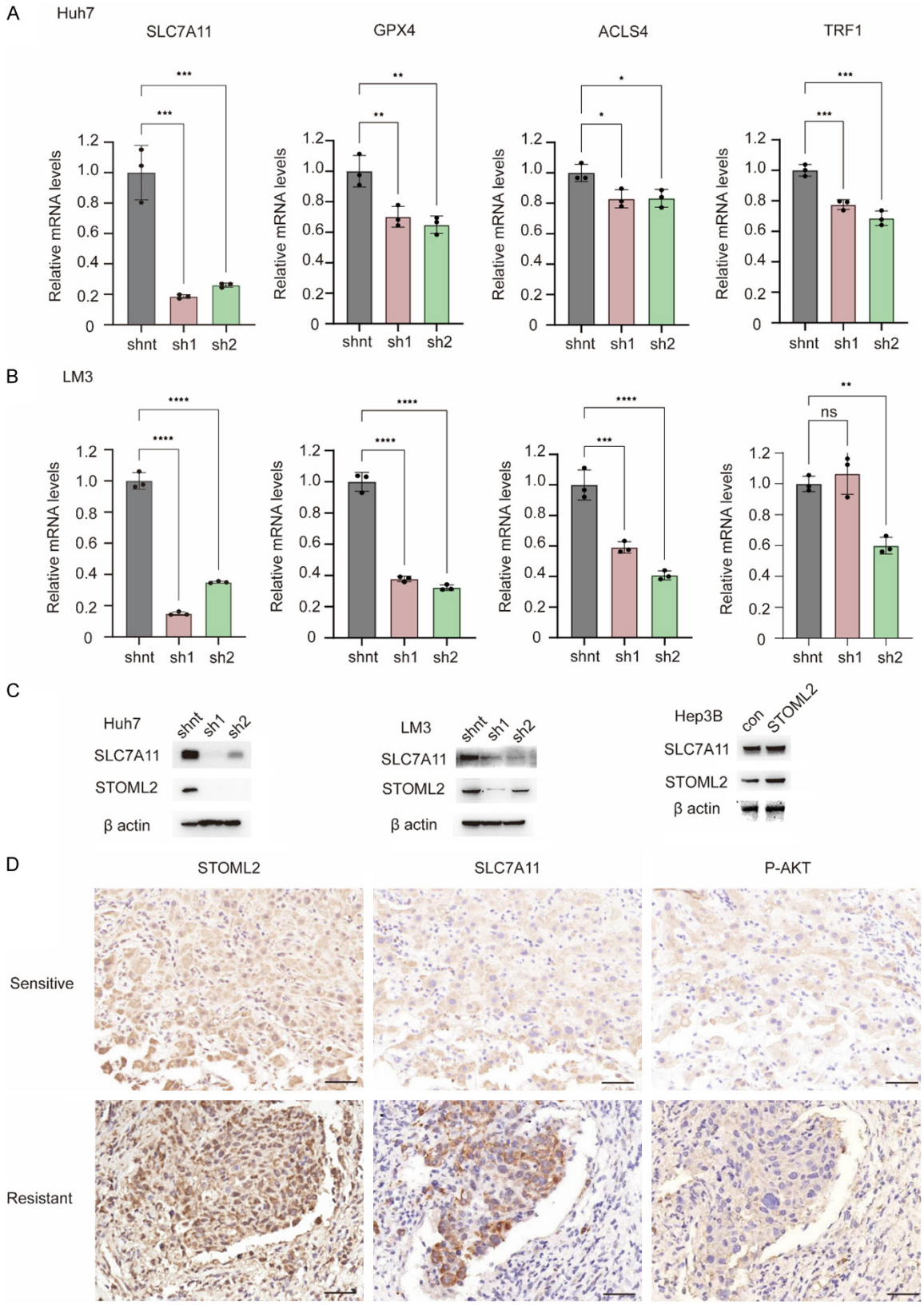

Figure 5. STOML2 inhibits HCC cell ferroptosis by regulating SLC7A11 levels. (A and B) mRNA expression of key inhibitory molecules of ferroptosis in Huh7 cells (A) and LM3 cell (B) lines. (C) Human HCC cell lines (HuH7, LM3 and Hep3B cells) were treated with sorafenib (2.5, 10 and 5 μM) for 24 h and western blot analysis was used to

evaluate the expression levels of the SLC7A11 protein. (D) Immunohistochemical staining (expression of SLC7A11 and p-AKT) was used to assess the expression levels of STOML2 in the tumor tissue. Representative figures were shown. Scale bars, 50 μm. STOML2, stomatin-like protein 2; HCC, hepatocellular carcinoma; SLC7A11, solute carrier family 7 membrane 11; p-, phospho.

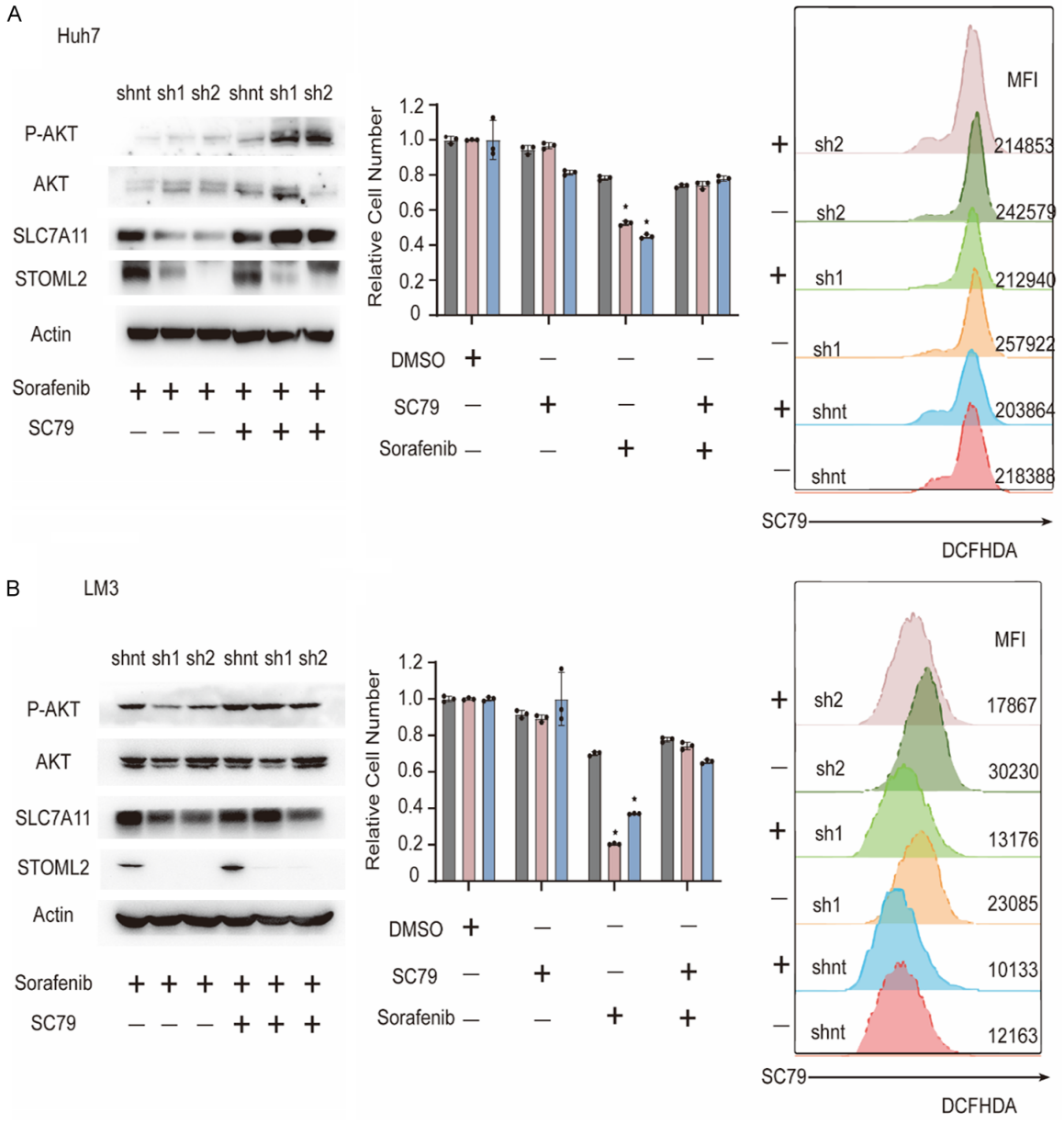

Figure 6. Knockdown of STOML2 reduces AKT phosphorylation-related SLC7A11 activation. A, B. Human HCC cell lines (Huh7 and LM3 cells) were treated with sorafenib (2.5 and 10 μM) and SC79 (10 μM) for 24 h. The protein expression levels of SLC7A11 were examined by western blot analysis. Cell proliferation was examined by the CCK-8 assay and the cellular ROS levels were examined by flow cytometry, $P<0.05$. STOML2, stomatin-like protein 2; SLC7A11, solute carrier family 7 membrane 11; HCC, hepatocellular carcinoma; CCK-8, Cell Counting Kit-8; ROS, reactive oxygen species.

STOML2 was upregulated in sorafenib-treated HCC cells. In addition, knocking down STOML2 expression increases the sensitivity of HCC cells to sorafenib, suggesting that STOML2 acts as a positive regulator in the resistance to sorafenib.

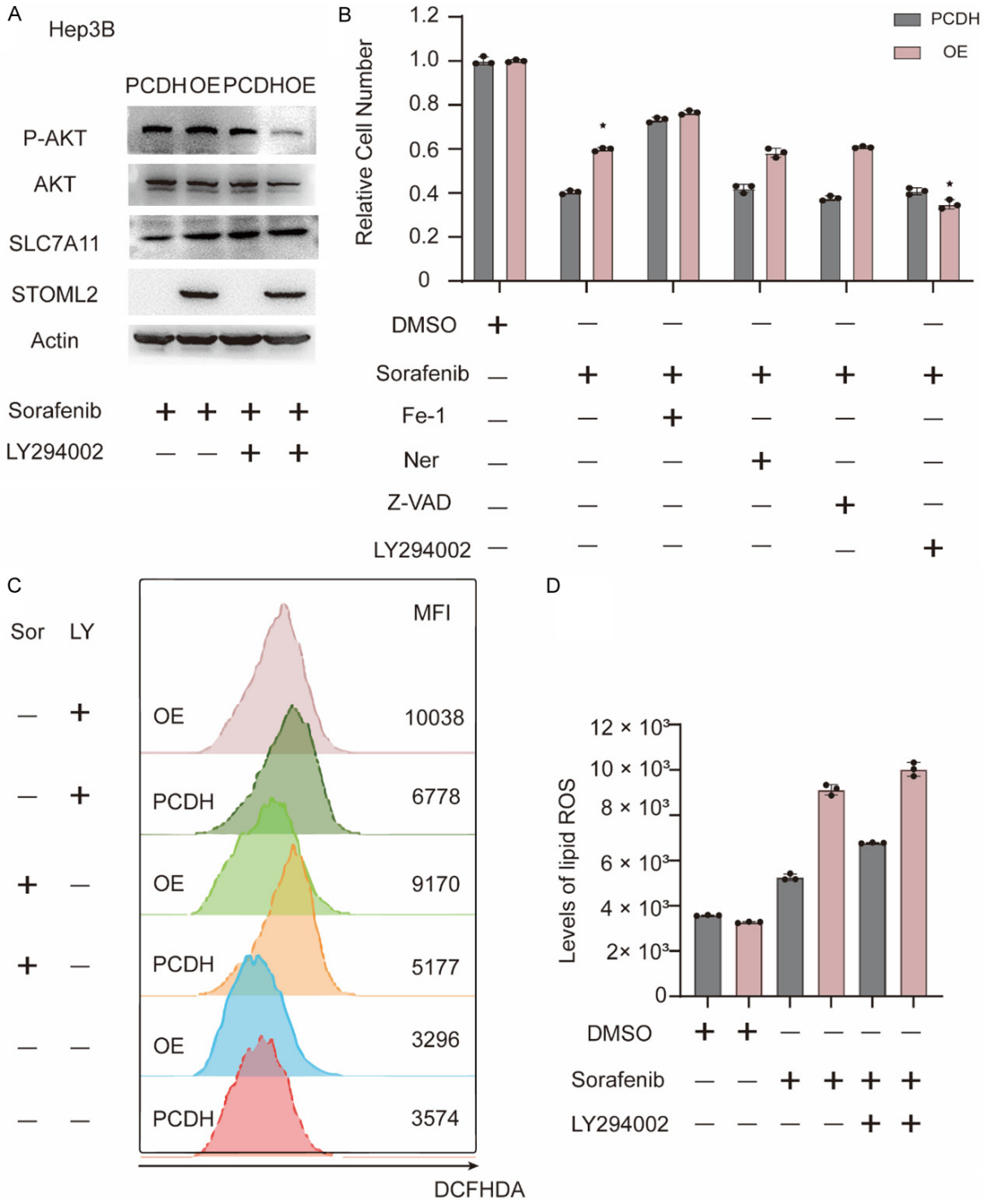

Figure 7. STOML2 upregulates SLC7A11 levels via activating AKT phosphorylation. (A) Hep3B cells were treated with sorafenib (5 μM) and LY294002 (10 μM) for 24 h. The protein expression levels of SLC7A11 were examined by western blot analysis; the proliferation rate was examined by the CCK-8 assay (B) and the cellular ROS levels (C, D) were examined by flow cytometry. P<0.05. STOML2, stomatin-like protein 2; SLC7A11, solute carrier family 7 membrane 11; CCK-8, Cell Counting Kit-8; ROS, reactive oxygen species.

Ferroptosis is a form of regulated cell death, distinct from other forms such as apoptosis, necrosis and autophagy. It is characterized by the iron-dependent accumulation of lipid peroxides to lethal levels [39, 40]. Recent studies have highlighted that targeting ferroptosis-

related signaling pathways, particularly via the modulation of SLC7A11, a key component of the system x_c-, may offer novel avenues to overcome treatment resistance in cancer. SLC7A11 plays a critical role in the uptake of cystine for GSH synthesis and in inhibiting lipid peroxide accumulation, thus regulating ferroptosis. Overexpressed SLC7A11 is closely associated with the effectiveness of various chemotherapeutic agents, targeted therapies and immunotherapy [28, 40]. Sorafenib, a known ferroptosis activator that targets SLC7A11, suggests a probable link between sorafenib resistance in HCC and SLC7A11 levels.

In the present study, it was discovered that STOML2 deficiency heightened the sensitivity to sorafenib treatment via downregulating SLC7A11 expression. This is characterized by decreased cell proliferation and enhanced production of lipid peroxidation, along with an increase in ROS levels. Conversely, OE of STOML2 decreased the sensitivity of HCC cells to sorafenib by inhibiting ferroptosis *in vitro*. Previous studies have demonstrated that the SLC7A11 can be activated and regulated by the PI3K/AKT pathway. It was hypothesized that STOML2 could promote resistance to ferroptosis by enhancing the expression of SLC7A11 via the phosphorylation of AKT. The results indicated that knockdown of STOML2 expression led to a decrease in both phospho (p-) AKT and SLC7A11 expression. Pharmacological activation of p-AKT with an AKT activator was able to restore SLC7A11 expression. Further experimentation revealed that administering an AKT agonist in STOML2 deficient cell lines promoted cell proliferation, increased the levels of ROS and elevated lipid peroxidation.

In summary, our findings establish that STOML2 enhances AKT phosphorylation, which subsequently upregulates SLC7A11 expression to suppress sorafenib-induced ferroptosis, ultimately reducing HCC sensitivity to sorafenib treatment. The present study demonstrates the feasibility of targeting STOML2/AKT as an effective therapeutic strategy for overcoming sorafenib resistance in HCC patients.

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Patients provided informed consent for the publication of any associated data and accompanying images.

Disclosure of conflict of interest

None.

Abbreviations

HCC, hepatocellular carcinoma; TKI, multi-target kinase inhibitor; SLC7A11, solute carrier family 7 membrane 11; mRNA, messenger RNA; qRT-PCR, quantitative real-time PCR; STOML2, stomatin-like protein 2; shNC, negative control shRNA; shS#1, short hairpin STOML2 #1; shS#2, short hairpin STOML2 #2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E staining, Hematoxylin-eosin staining; DHE, dihydroethidium.

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Table S1. Summary of primary antibodies used in Western Blot and Immunochemistry, immunofluorescence, Co-immunoprecipitation and Chromatin

Antibody	Concentration		Cat. No.	Company
	for WB	for IHC		
STOML2	/	1:200	10348-1-AP	Proteintech
STOML2	1:1000	/	#73956	Cell Signaling Technology
SLC7A11	/	1:100	26864-1-AP	Proteintech
SLC7A11	1:1000	/	#98051	Cell Signaling Technology
Phospho-AKT (Ser473)	/	1:400	66444-1-Ig	Proteintech
AKT (pan)	1:1000	/	#4691	Cell Signaling Technology
Phospho-Akt (Ser473)	1:1000	/	#9271	Cell Signaling Technology
β-actin	1:1000	/	#4967	Cell Signaling Technology
HRP Goat Anti-Rabbit IgG (H+L)	1:3000		AS014	Abclonal
HRP Goat Anti-Mouse IgG (H+L)	1:2000		AS003	Abclonal

Abbreviation: WB, western blot; IHC, immunohistochemistry.

Table S2. Primers and target sequences in this study

Identifier	Forward (5'-3')	Reverse (5'-3')
For qPCR		
STOML2	CCCGCTGCAAGTATGATGG	GTTCCTGAGACGCTGTTC
GAPDH	TGCGAGTACTCAACACCAACA	GCATATCTTCGGCCCACA
For STOML2-Knockdown		
STOML2 sh#1	CCGGGATGCAAGTCTTGATGAGGAACTC-GAGTTCCTCATCAAGACTTGCATCTTTTTTG	AATTCAAAAAGATGCAAGTCTTGATGAG-GAACTCGAGTTCCTCATCAAGACTTGCATC
STOML2 sh#2	CCGGCCGTTATGAGATCAAGGATATCTCGAGA-TATCCTTGATCTCATAACGGTTTTTTG	AATTCAAAAACCGTTATGAGATCAAGGATATCTC-GAGATATCCTTGATCTCATAACGG

Abbreviation: qPCR, quantitative real-time polymerase chain reaction.

Table S3. Relationship between low and high STOML2 and Clinicopathologic features

Number	Sex	Age	Extrahepatic metastasis	Classification of PVTT	Treatment
#1	F	52	N	VP0	Che + Sora
#2	F	53	N	VP0	Che + Sora
#3	M	59	Y	VP0	Sora
#4	M	62	N	VP0	Che + Sora
#5	M	60	N	VP3	Che + Sora
#6	M	38	N	VP4	Che + Sora
#7	M	67	N	VP2	Che + Sora
#8	M	59	Y	VP0	Che + Sora
#9	F	48	N	VP0	Che + Sora
#10	M	39	N	VP4	Che + Sora

Abbreviations: F: female; M: male; Y: yes; N: no; Che: Chemotherapy; TKI: target kinase inhibitor; Immu: immunotherapy.

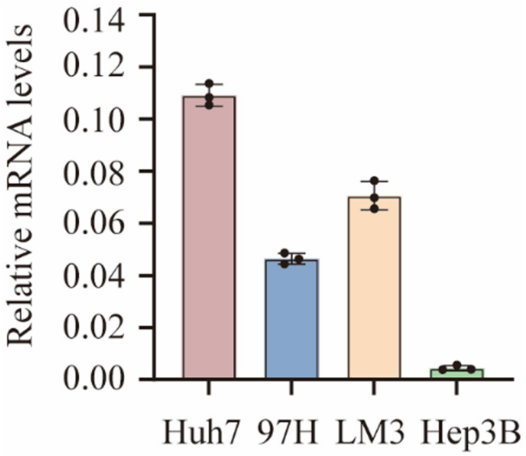

Figure S1. Basic mRNA expression levels of STOML2 in human HCC cell lines. STOML2, stomatin-like protein 2; HCC, hepatocellular carcinoma.

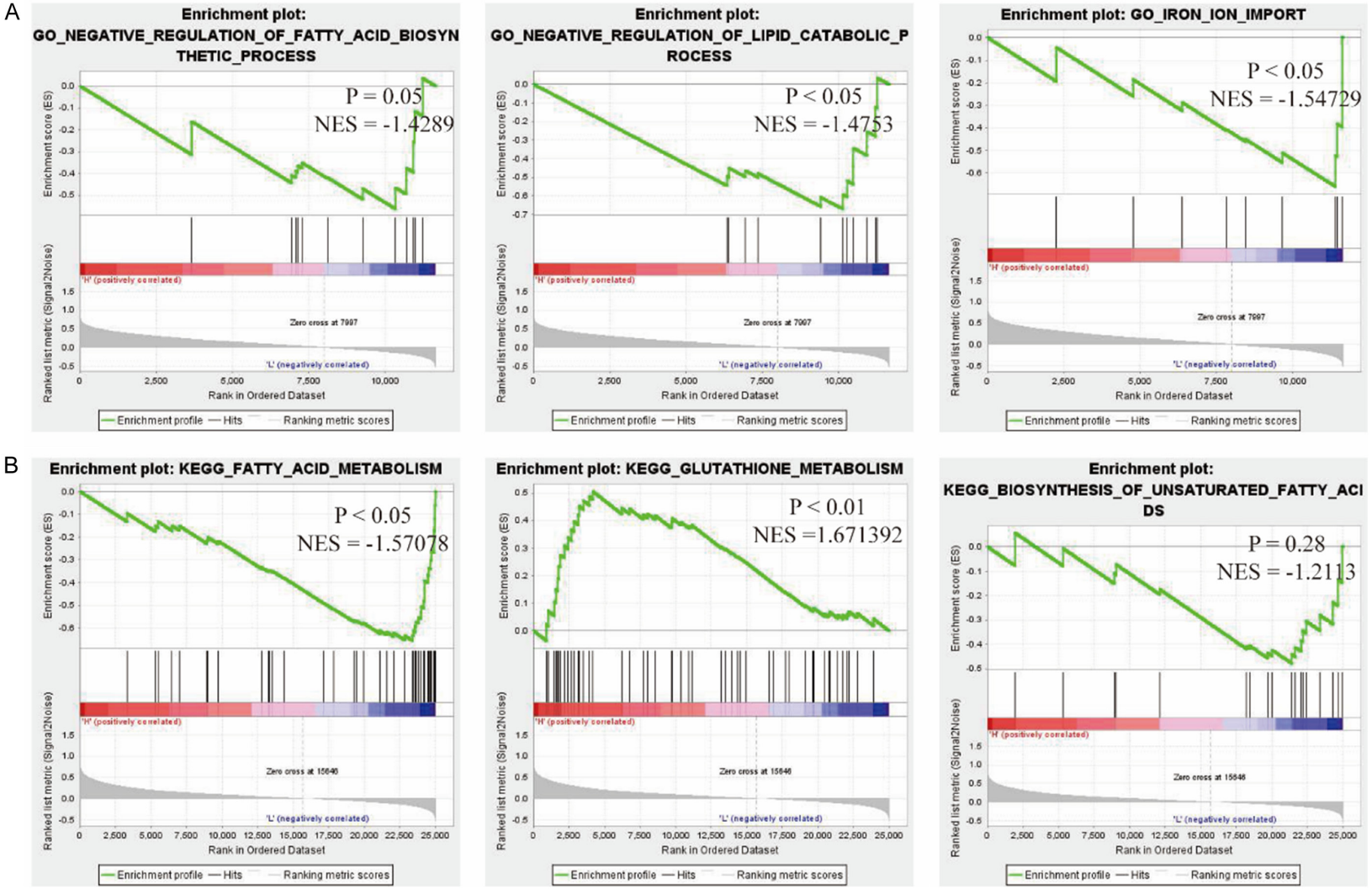

Figure S2. GSEA database. NES, normalized enrichment score. (A) GO analyses of the correlation between STOML2 expression and fatty acid metabolism (P=0.05), lipid catabolic process (P<0.05) and iron ion import pathway (P<0.05). (B) The data shown are derived from KEGG pathway enrichment analyses for the correlation between the expression of STOML2 and fatty acid metabolism (P<0.05), glutathione metabolism (P<0.01) and biosynthesis of unsaturated fatty acids (P=0.28). GSEA, Gene Set Enrichment Analyses; NES, normalized enrichment score; GO, Gene Ontology; STOML2, stomatin-like protein 2.