

GRB2 Promotes Sorafenib Resistance in Hepatocellular Carcinoma Cells Under Hypoxia by Activating the PI3K/AKT Signaling Pathway

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Purpose: Sorafenib resistance remains a major therapeutic challenge in advanced hepatocellular carcinoma (HCC). This study aimed to investigate the role of growth factor receptor-bound protein 2 (GRB2) in sorafenib resistance of HCC cells under hypoxic conditions and to elucidate the underlying molecular mechanisms involving the PI3K/AKT signaling pathway.

Methods: Bioinformatics analysis was performed using TCGA-LIHC, GEO datasets, and multiple databases to evaluate GRB2 expression, its correlation with hypoxia signatures, and prognostic significance in HCC. Western blot was used to detect GRB2 expression in HCC cell lines and normal hepatocytes. Huh7 cells were cultured under normoxic (21% O₂) or hypoxic (1% O₂) conditions, treated with sorafenib alone or combined with PI3K inhibitor LY294002. GRB2 knockdown was performed using lentiviral shRNA. Cell proliferation, migration, and apoptosis were evaluated by CCK-8 assay, wound healing assay, and flow cytometry, respectively.

Results: Bioinformatics analysis revealed that GRB2 was significantly upregulated in HCC tissues compared to normal tissues, positively correlated with hypoxia-related genes (HIF-1 α , VEGFA) and hypoxia scores, and associated with poor patient prognosis. Western blot confirmed that GRB2 was highly expressed in HCC cell lines compared to normal hepatocytes. Under hypoxic conditions, HIF-1 α expression increased in a time-dependent manner, validating the hypoxia model. Hypoxia attenuated sorafenib-induced inhibition of cell proliferation and migration while reducing apoptosis, as evidenced by increased IC₅₀ values (from 4.934 μ M to 8.676 μ M). Sorafenib treatment under hypoxia upregulated GRB2, PI3K, and p-AKT protein levels. PI3K inhibition by LY294002 or GRB2 knockdown restored sorafenib sensitivity, reduced PI3K and p-AKT expression, and promoted apoptosis in hypoxic HCC cells.

Conclusions: Our findings suggest that hypoxia reduces the sensitivity of HCC cells to sorafenib, and that GRB2 contributes to this process through activation of the PI3K/AKT pathway. Targeting GRB2 may represent a potential strategy to enhance sorafenib efficacy in HCC treatment under hypoxic conditions.

Keywords: hepatocellular carcinoma, hypoxia, GRB2, sorafenib, drug sensitivity, PI3K/AKT pathway

Introduction

HCC is the sixth most common cancer and the third leading cause of cancer-related death globally.^{1,2} Due to the difficulty in early diagnosis, most patients are diagnosed at advanced stages, leaving limited treatment options and making systemic therapy the only viable approach.³ Sorafenib, a multi-kinase inhibitor approved as the first-line systemic therapy for advanced HCC in 2007,⁴ targets multiple tyrosine kinases including RAF, c-Kit, FLT-3, RET, VEGFR, and PDGFR to suppress tumor cell proliferation and disrupt tumor angiogenesis.⁵ Clinical trials have demonstrated that sorafenib extends the overall survival of advanced HCC patients by approximately 2–3 months.⁶ However, sorafenib

resistance has become a major obstacle to its clinical application, with only one-third of advanced patients responding to treatment.⁷ Therefore, exploring the mechanisms of sorafenib resistance and formulating corresponding therapeutic strategies for HCC are of great significance.

Hypoxia is a hallmark feature of HCC and other solid tumors, resulting from aberrant tumor vasculature and uncontrolled cell proliferation.⁸ This microenvironmental characteristic directly promotes chemoresistance, angiogenesis, and tumor metastasis.⁹ Liang et al demonstrated that hypoxic liver cancer cells exhibit significantly higher resistance to multiple chemotherapeutic drugs, including sorafenib.¹⁰ HCC cells under hypoxic conditions show reduced apoptosis and enhanced migration compared to normoxic cells.¹¹ Notably, long-term sorafenib treatment exacerbates the hypoxic microenvironment of HCC by inhibiting tumor angiogenesis.¹² In HCC patients, overexpression of hypoxia-inducible factors HIF- α (including HIF-1 α and HIF-2 α) predicts a poor prognosis and is closely associated with HCC invasion and metastasis.¹³ Previous studies have confirmed that HIF- α promotes proliferation, inhibits apoptosis, and enhances invasion of HCC cells under hypoxic conditions,^{14,15} while the PI3K/AKT signaling pathway has also been identified as a critical mediator of sorafenib resistance.¹⁶

The PI3K/AKT signaling pathway is a critical regulatory pathway for hypoxic resistance in HCC. Under normoxic conditions, this pathway is strictly regulated, while hypoxia triggers its activation through HIF-1 α -dependent mechanisms.¹⁷ Activated PI3K generates phosphatidylinositol phosphates (PIPs), which recruit and phosphorylate AKT, thereby promoting cell survival, proliferation, and drug resistance.¹⁸ Recent studies have highlighted extensive crosstalk between PI3K/AKT and other signaling cascades, including mTOR and NF- κ B, which collectively amplify survival signaling under hypoxic stress.^{19,20} Emerging evidence also identifies novel upstream regulators and feedback mechanisms that fine-tune PI3K/AKT activation in the context of HCC drug resistance.²¹ Chen et al demonstrated that sorafenib resistance in HCC cells is associated with increased phosphorylation of PI3K/AKT, and inhibition of this pathway can restore drug sensitivity.²²

Growth factor receptor-bound protein 2 (GRB2) is a 25-kDa adapter protein containing SH2 and SH3 domains. Its SH2 domain binds to tyrosine phosphorylated sites of EGFR and VEGFR, while the SH3 domain interacts with proline-rich sequences.²³ In HCC, GRB2 has been identified as significantly overexpressed and associated with tumor progression and poor prognosis. Lv et al demonstrated that the deubiquitinase PSMD14 enhances HCC growth and metastasis by stabilizing GRB2, highlighting its oncogenic role in liver cancer.²⁴ Existing studies have shown that GRB2 plays a role in drug resistance in breast cancer, non-small cell lung cancer, and ovarian cancer.^{25–27} Mechanistically, GRB2 activates the PI3K/AKT pathway through an indirect but well-characterized mechanism. Upon receptor tyrosine kinase activation, GRB2 recruits the docking protein Gab1 (GRB2-associated binder 1) via its SH3 domain. Gab1 is subsequently tyrosine-phosphorylated, creating binding sites for the p85 regulatory subunit of PI3K, thereby activating PI3K and its downstream AKT signaling. Notably, PI3K-generated PIP₃ binds to Gab1's PH domain, further enhancing Gab1 membrane recruitment and sustaining PI3K activation in a positive feedback loop.^{28,29} This GRB2-dependent, Gab1-mediated PI3K/AKT activation mechanism provides a strong rationale for investigating the GRB2/PI3K/AKT axis in sorafenib resistance under hypoxia. Therefore, this study aims to investigate the regulatory role of GRB2 in sorafenib resistance in HCC cells via the PI3K/AKT signaling pathway under hypoxic conditions.

Materials and Methods

Bioinformatics Analysis

GRB2 pan-cancer expression data, paired transcriptome data of tumor tissues and adjacent normal tissues of hepatocellular carcinoma (HCC) from the TCGA-LIHC cohort (50 tumor–normal pairs) were obtained from the SPARKLE database (<https://grswsci.top/>), and the GSE76427 dataset (115 HCC tumor samples and 52 adjacent non-tumor samples) was used to verify the expression level of GRB2 in HCC. Using the TCGA-LIHC dataset from this database, the receiver operating characteristic (ROC) curve of GRB2 in HCC diagnosis, the correlations between GRB2 and HCC TNM stage as well as hypoxia-related key factors (HIF-1 α /VEGFA) were analyzed. Meanwhile, combined with the CancerSEA database and the GSVA algorithm, the correlation between GRB2 and 14 functional state gene sets was evaluated in the ICGC-LIRI cohort. The TCGA-LIHC dataset from the UALCAN database (<https://ualcan.path.uab.edu/>) was used to

analyze the correlation between GRB2 expression level and the histological grade of HCC. The overall survival curve and recurrence-free survival curve of patients with high/low GRB2 expression were constructed via the GEPIA 2 database (<http://gepia2.cancer-pku.cn/>), and survival analysis was performed using the Kaplan–Meier method with the Log rank test. Immunohistochemical images of normal liver tissues and liver tumor tissues were retrieved from The Human Protein Atlas (<https://www.proteinatlas.org/>) to compare the expression of GRB2 between the two types of tissues. The hypoxia score of TCGA-LIHC samples was calculated using the single-sample gene set enrichment analysis (ssGSEA) algorithm in R software (version 4.5.1). The GRB2 expression levels between the high and low hypoxia score groups were compared using the Wilcoxon rank-sum test, and visualization was performed using the ggplot2 package. In all analyses, $P < 0.05$ was considered statistically significant.

Cell Lines and Hypoxia Assay

Human HCC cell lines Huh7, Li-7, SNU-182, and SNU-387 were obtained from the Chinese Academy of Sciences Stem Cell Bank (Beijing, China). Normal human hepatocytes HL-7702 were purchased from Shanghai Mingjin Biological Technology Co., Ltd. The cells were cultured in DMEM high-glucose medium or RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and maintained at 37 °C in 5% CO₂. For hypoxic experiments, cells were cultured in a hypoxia chamber maintained at 1% O₂, 5% CO₂, and 94% N₂ at 37 °C. The oxygen concentration was monitored by a real-time display sensor to confirm that 1% O₂ was maintained.

Reagents and Antibodies

Sorafenib (BAY 43–9006) was purchased from Selleck Chemicals (Houston, USA). LY294002 (HY-10108), the inhibitor of PI3K was obtained from MedChem Express (Monmouth Junction, NJ, USA). Antibodies to GRB2, PI3K was obtained from Abcam Biological Technology (UK), HIF-1 α (D1S7W) Cell Signaling Technology (Boston, MA, USA). Phospho-Akt (66444-1-Ig), Akt (60203-2-Ig) was purchased from Proteintech (Wuhan, China) GAPDH was purchased from Biosharp (BL006B, Beijing, China). Annexin V-FITC Apoptosis Detection Kit was purchased from Beijing Solarbio Science & Technology Co.Ltd. PrimeScript™ RT Master Mix (RR820A, Takara, Japan).

Cell Viability Assay

2×10^3 cells per well were inoculated in 96-well plates and incubated overnight to allow attachment and growth. The cells were then exposed to increasing concentrations of sorafenib (0, 5, 10, 15, 20 μ M) for 24 hours under normoxic or hypoxic conditions. After exposure, 10 μ L of CCK-8 solution was added to each well and incubated at 37°C for 2 hours. The absorbance was measured at 450 nm using a microplate reader. Cell viability (%) = (OD_{treatment} – OD_{blank}) / (OD_{control} – OD_{blank}) $\times$ 100%. The half maximal inhibitory concentration (IC₅₀) of sorafenib was calculated using GraphPad Prism software (v8.0).

Western Blotting

The cells were rinsed with PBS and then lysed in RIPA Lysis Buffer (Beijing Solarbio Science & Technology Co., Ltd) supplemented with protease and phosphatase inhibitors. Subsequently, the protein concentrations were quantified using BCA protein assay kit (Beyotime, Jiangsu, China). Each sample's supernatant was portioned and combined with loading buffer, separated via SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The polyvinylidene fluoride membrane was blocked with a 5% skim milk solution for 1 hour, after which it was immersed in the primary antibody solution and incubated at 4°C overnight. Then, membranes were incubated with secondary antibody at room temperature for 1 hour. Reactive proteins were detected using enhanced chemiluminescence reagents BeyoECL Star (P0018AS) (Beyotime, Jiangsu, China). Protein expression was analyzed using Image J software (v1.48, NIH, Bethesda, MD) with GAPDH as a loading control.

Cell Apoptosis Analysis

Cell apoptosis was measured by the Annexin V-FITC apoptosis detection kit. Cells were seeded in 6-well plates at 2×10^5 cells/well and cultured overnight. After exposure to sorafenib (5 μ M), DMSO, or LY294002 (20 μ M) under normoxic or

hypoxic conditions for 24 hours, cells were harvested, washed twice with cold PBS, and resuspended in binding buffer. Then the cells were harvested and washed twice with cold PBS and resuspended in a binding buffer to a concentration of 5×10^6 cells/mL. 100 μ L of the cell suspension was stained with 5 μ L fluorescein isothiocyanate (FITC) annexin V and 5 μ L propidium iodide (PI) at room temperature for 5 minutes in the dark, followed by the addition of 400 μ L of PBS. Finally, the cells were analyzed using a flow cytometer.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The expression of GRB2 after lentiviral transduction was measured using quantitative real-time PCR (qRT-PCR). Total RNA was isolated using HiPure Total RNA Mini Kit (Magen, Guangzhou, China) following the manufacturer's protocol. Complementary DNA (cDNA) was generated using PrimeScript RT reagent Kit (Takara Shuzo, Shiga, Japan) according to the manufacturer's recommendation. Quantitative real-time PCR (qPCR) was carried out using the TB Green Premix ExTaq II (Takara Shuzo, Shiga, Japan) and a LightCycler[®] 480 System (Roche). The primers utilized for gene amplification were β -actin F-TGGCACCCAGCACAATGAA and R-CTAAGTCATAGTCCGCCTAGAAGCA; GRB2: F-GACGGCTTCATTCCCAAGAAC and R-TCGTGCCGCTGTTTGCTA. The mRNA levels of GRB2 were measured by the cycle threshold (Ct) values, and β -actin served as the internal control. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative expression levels.

Wound Healing Assay

Huh7 cells were seeded in 6-well plates at 5×10^5 cells/well and incubated overnight. When cell density reached ~90%, a 200 μ L pipette tip was used to create a wound line. Serum-free DMEM containing sorafenib, DMSO, or LY294002 was added and cells were cultured under normoxic or hypoxic conditions for 24 hours. Scratch morphology was observed and photographed at 0 and 24 hours. Cell migration distance was calculated using ImageJ software.

Lentivirus Transfection and Stable Cell Lines

Lentiviral particles carrying shRNA targeting GRB2 (shGRB2-1: CAGATATTCCTGCGGGACATA, shGRB2-2: CGGCTTCATTCCCAAGAACTA) and negative control (shGRB2-NC: TTCTCCGAACGTGTACGT) were constructed by GeneChem (Shanghai, China). Huh7 cells were stably infected at an MOI of 5. Stable cells were selected with 2 μ g/mL puromycin for one week, and transfection effects were evaluated using RT-qPCR and Western blot.

Statistical Analysis

Data were analyzed with GraphPad Prism 8.0 (USA) and SPSS software 22.0 (IBM, USA). Data are reported as means $\pm$ SD from at least three independent experiments. The Shapiro–Wilk test was used to assess normality, and Levene's test was used to verify homogeneity of variances. The unpaired two-tailed Student's *t*-test was used for comparisons between two groups (eg, normoxia vs. hypoxia). One-way ANOVA followed by Dunnett's post-hoc test was used for comparisons among three or more groups (eg, shGRB2-NC vs. shGRB2-1 vs. shGRB2-2). The IC₅₀ values between normoxic and hypoxic groups were compared using the extra sum-of-squares *F*-test. *P* < 0.05 was considered statistically significant.

Results

The High Expression of GRB2 in Hepatocellular Carcinoma

Through bioinformatics screening by using SPARKLE (<https://grswsci.top>), we observed that GRB2 mRNA was widely upregulated in various solid tumors (Figure 1A), with a significant increase also noted in HCC. We subsequently validated this finding in the TCGA-LIHC paired-sample cohort, where GRB2 expression was markedly higher in tumor tissues than in adjacent normal tissues (Figure 1B). Further external validation was performed in the GEO dataset GSE76427 (Figure 1C), and the result consistently showed that GRB2 was significantly highly expressed in HCC. In order to verify the above findings at the protein level, we searched the Human Protein Atlas (HPA) database and obtained immunohistochemical images of normal liver tissues and hepatocellular carcinoma tissues: As shown in Figure 1D, GRB2 was moderately-highly expressed in liver cancer tissues, while weakly or no staining in normal liver tissues, suggesting that its protein expression was significantly

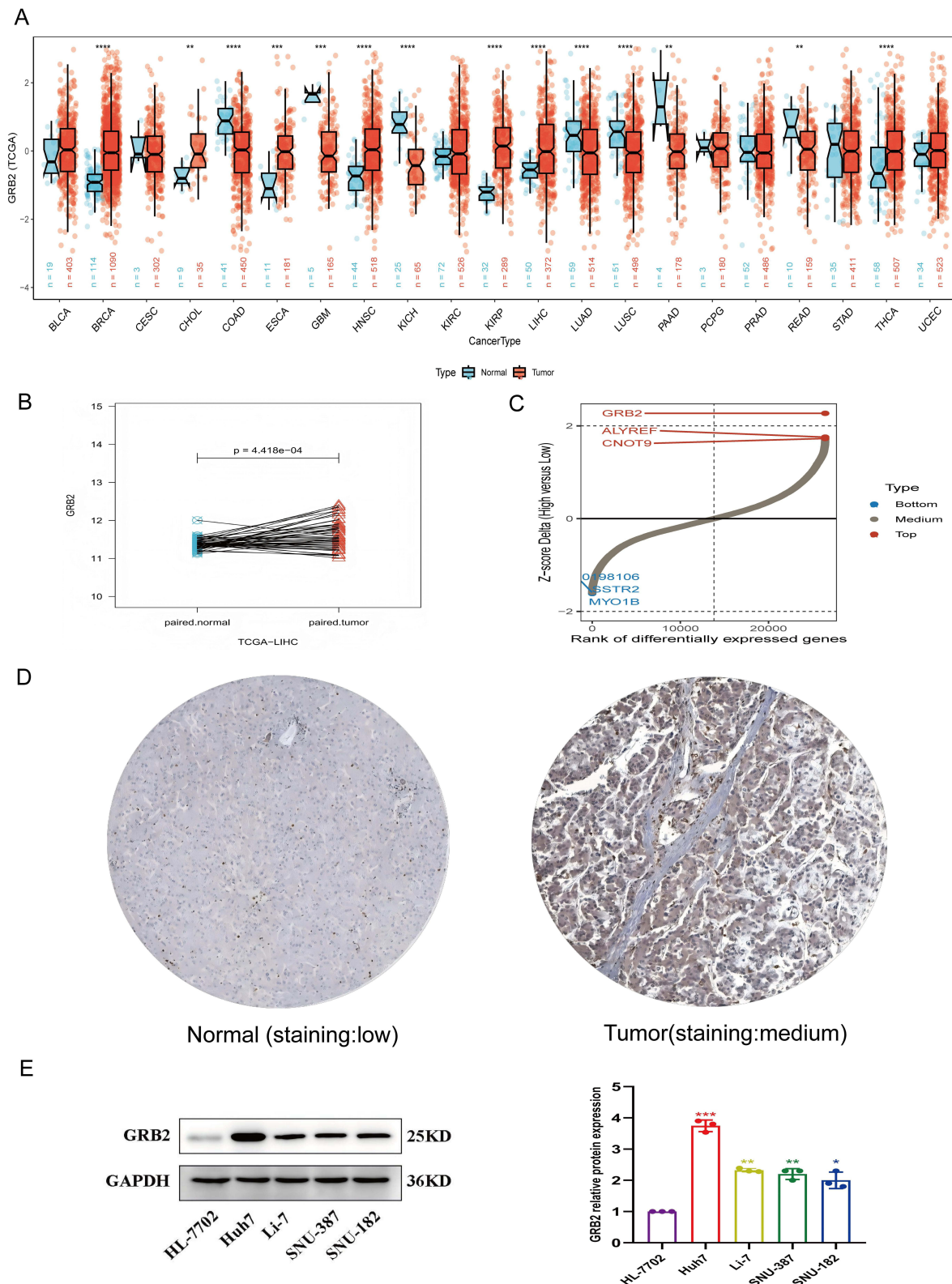


Figure 1 GRB2 is highly expressed in HCC. **(A)** The expression of GRB2 in pan-cancer; **(B)** Paired analysis of GRB2 transcript levels in HCC and normal tissues; **(C)** The ranking of GRB2 expression in GEO dataset GSE76427; **(D)** Representative images of GRB2 immunohistochemistry in The Human Protein Atlas database; **(E)** GRB2 expression in immortalized hepatocytes and four HCC cells. The data presented here are based on three independent experiments. “*” indicates $P < 0.05$, “**” $P < 0.01$, “***” $P < 0.001$, “****” $P < 0.0001$.

up-regulated. Finally, Western blot experiments further confirmed that the expression of GRB2 in various HCC cell lines was significantly higher than that in normal hepatocytes (Figure 1E). Additionally, analysis of publicly available RNA-seq data from the E-MTAB-7847 dataset confirmed that GRB2 expression in Huh7 cells was significantly higher than in THLE5B normal hepatocytes ($p = 0.012$, [Supplementary Figure 1](#)).

GRB2 Expression Is Associated with Poor Prognosis in HCC Patients

We further analyzed the relationship between GRB2 expression and clinicopathological features of HCC patients. Through analysis of the TCGA-LIHC dataset, the expression level of GRB2 increased significantly with histological grade (Figure 2A) and TNM stage (Figure 2B), suggesting a relationship with tumor progression. Kaplan-Meier survival analysis showed that overall survival and recurrence-free survival of patients with high GRB2 expression were significantly shorter than those in the low expression group (Figure 2C). In addition, ROC curve analysis demonstrated that GRB2 had a certain diagnostic accuracy for HCC (Figure 2D).

GRB2 Is Closely Related to Hypoxia Characteristics

HCC is characterized by aberrant vasculature, which can cause hypoxia in deeper tumor regions and contribute to therapeutic resistance.³⁰ Given the key role of tumor microenvironment hypoxia in HCC drug resistance, we further explored the correlation between GRB2 and hypoxia. Through TCGA data analysis, GRB2 expression was positively correlated with hypoxia key factors HIF-1 α and VEGFA (Figure 3A and B). Subsequently, the CancerSEA functional gene set was used to evaluate the correlation between GRB2 and 14 malignant phenotypes via the GSVA algorithm in the ICGC-LIRI cohort. The results showed that GRB2 expression was positively correlated with hypoxia, angiogenesis, and invasion (Figure 3C). To further verify this correlation, the hypoxia score of TCGA-LIHC samples was calculated based on the ssGSEA algorithm, and GRB2 expression in the high hypoxia score group was significantly higher than in the low hypoxia score group (Figure 3D). These multi-dimensional analyses suggest that GRB2 is closely associated with hypoxia signaling and may participate in hypoxia-related malignant progression of HCC.

Hypoxia Attenuates Sorafenib Efficacy in HCC Cells

First, we detected HIF-1 α protein expression in Huh7 cells under normoxic and hypoxic conditions. HIF-1 α was highly expressed in hypoxic Huh7 cells compared to the normoxic group, and hypoxia significantly increased HIF-1 α expression in a time-dependent manner (Figure 4A), validating the establishment of the cellular hypoxia model. To determine the optimal sorafenib concentration and treatment duration for subsequent experiments, a preliminary dose–time screening was performed using CCK-8 assay with sorafenib concentrations of 0, 5, 10, 20, and 40 μ M under both normoxic and hypoxic conditions for 24, 48, and 72 hours ([Supplementary Table S1](#)). Based on the preliminary results, the 24-hour treatment time and a concentration range of 0–20 μ M were selected for subsequent experiments, and 5 μ M sorafenib was used as the working concentration for functional assays. CCK-8 assay showed that sorafenib inhibited the viability of both normoxic and hypoxic cells in a concentration-dependent manner. The IC₅₀ of sorafenib on Huh7 cells was 4.934 μ M for normoxia and 8.676 μ M for hypoxia. Additionally, the viability of hypoxic Huh7 cells was significantly higher than the normoxic counterpart cells (Figure 4B). Flow cytometry demonstrated that the apoptosis rate induced by 5 μ M sorafenib was significantly decreased from approximately 17.04% under normoxia to 6.01% under hypoxia (Figure 4C). Wound healing assay demonstrated that hypoxia increased cell migration in Huh7 cells treated with 5 μ M sorafenib for 24 hours (Figure 4D). These data demonstrate that hypoxia attenuates the inhibitory effects of sorafenib on HCC cell proliferation, apoptosis, and migration, indicating reduced sensitivity to sorafenib under hypoxic conditions.

PI3K/AKT Pathway Mediates Sorafenib Resistance Under Hypoxia

The PI3K/AKT pathway is a critical signaling pathway involved in cell apoptosis and resistance to chemotherapy drugs. To further explore the potential mechanistic link between GRB2 and the PI3K/AKT pathway, we constructed a protein–protein interaction (PPI) network using the STRING database (v12.0). The results showed that GRB2 physically interacts with multiple key components of the PI3K/AKT pathway, including PIK3R1 (p85 α , score = 0.979) and PIK3CA (p110 α ,

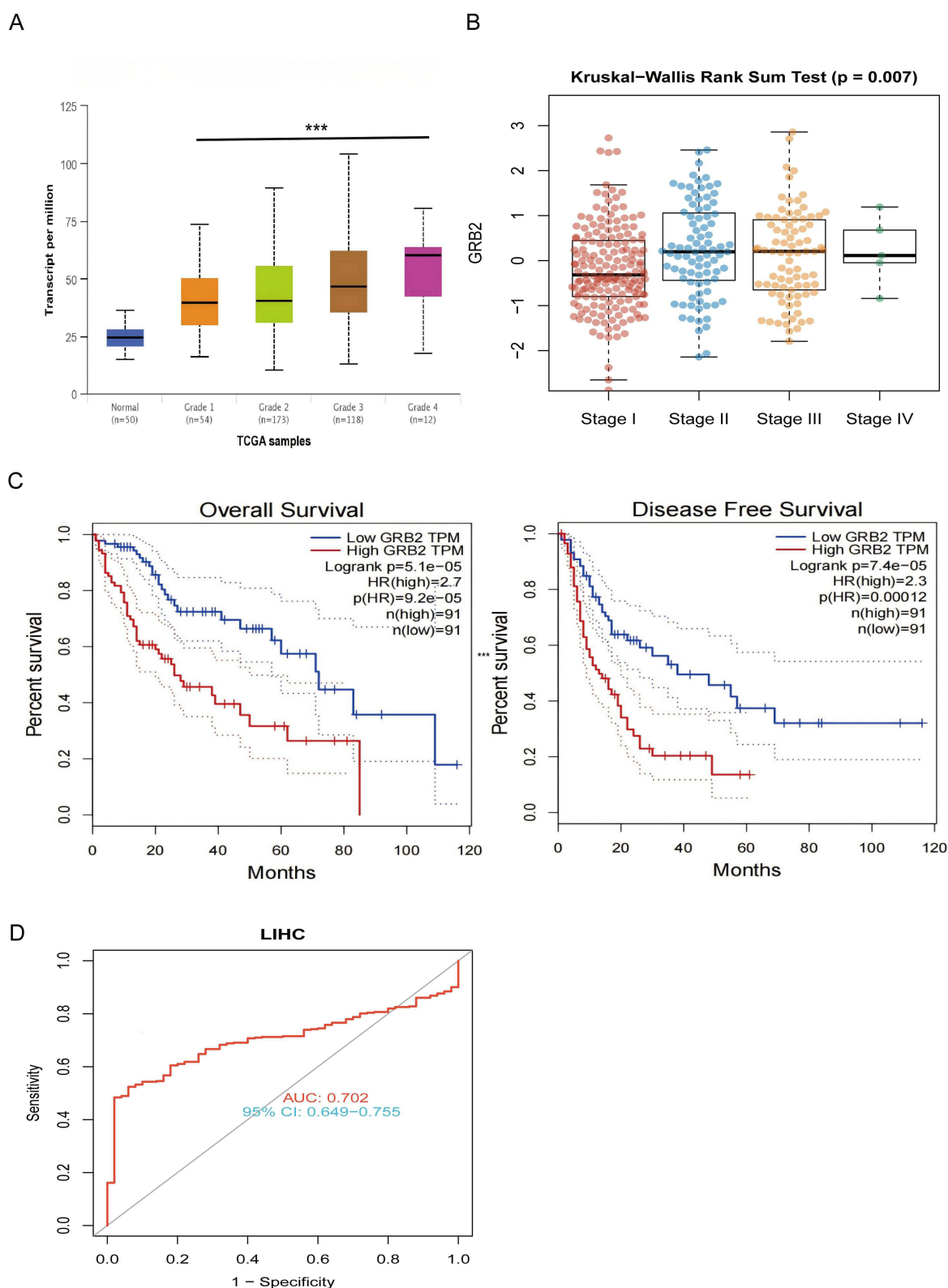


Figure 2 Correlation between GRB2 expression and poor prognosis of HCC patients. **(A)** GRB2 expression vs histological grade; **(B)** Association between GRB2 expression and TNM stage; **(C)** Overall and relapse-free survival curves of HCC patients; **(D)** The ROC curve of GRB2 in the diagnosis of HCC. “***” $P < 0.001$.

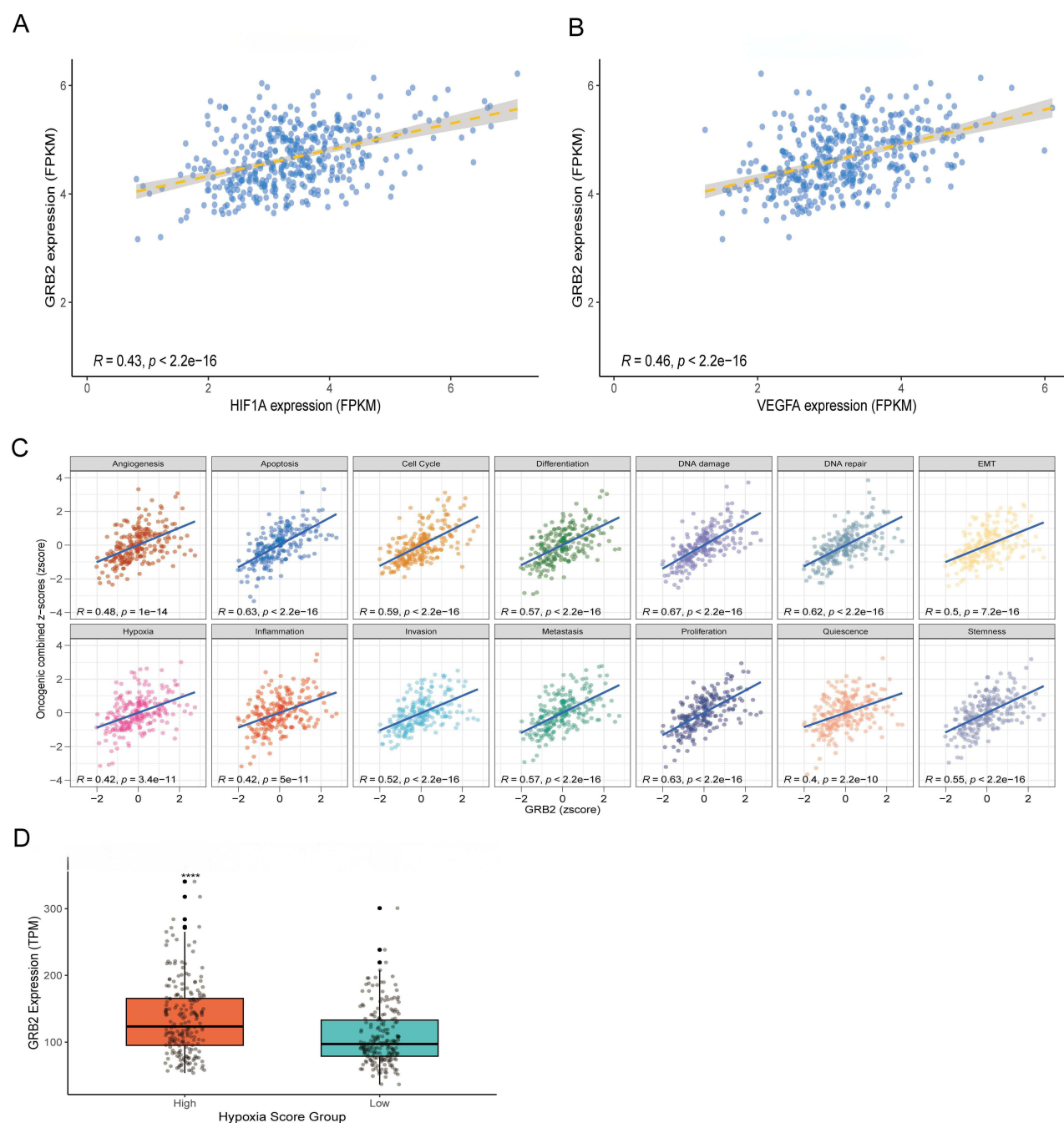


Figure 3 Correlation between GRB2 and hypoxia. **(A)** The correlation between GRB2 and hypoxia gene HIF-1 α expression.; **(B)** The correlation between GRB2 and hypoxia gene VEGFA expression.; **(C)** GSVA analysis of GRB2 in ICGC-LIRI; **(D)** Hypoxia score and GRB2 expression. **** $P < 0.0001$.

score = 0.702), as well as bridge adaptors GAB1 (score = 0.998) and GAB2 (score = 0.999), which are known to recruit and activate PI3K (Supplementary Figure 2A and 2B). These data provide bioinformatics evidence supporting a direct molecular connection between GRB2 and PI3K/AKT signaling.

To experimentally validate this association, we investigated the expression of GRB2, PI3K, AKT, and p-AKT in Huh7 cells after sorafenib treatment under hypoxia. Huh7 cells were treated with sorafenib (5 μ M) or DMSO under hypoxic conditions (1% O₂) for 24 hours. Western blotting demonstrated that PI3K, p-AKT, and GRB2 were dramatically upregulated in the sorafenib group compared to the DMSO control group (Figure 5A). We next investigated whether inhibition of the PI3K/AKT pathway could sensitize hypoxic HCC cells to sorafenib. Huh7 cells were treated with

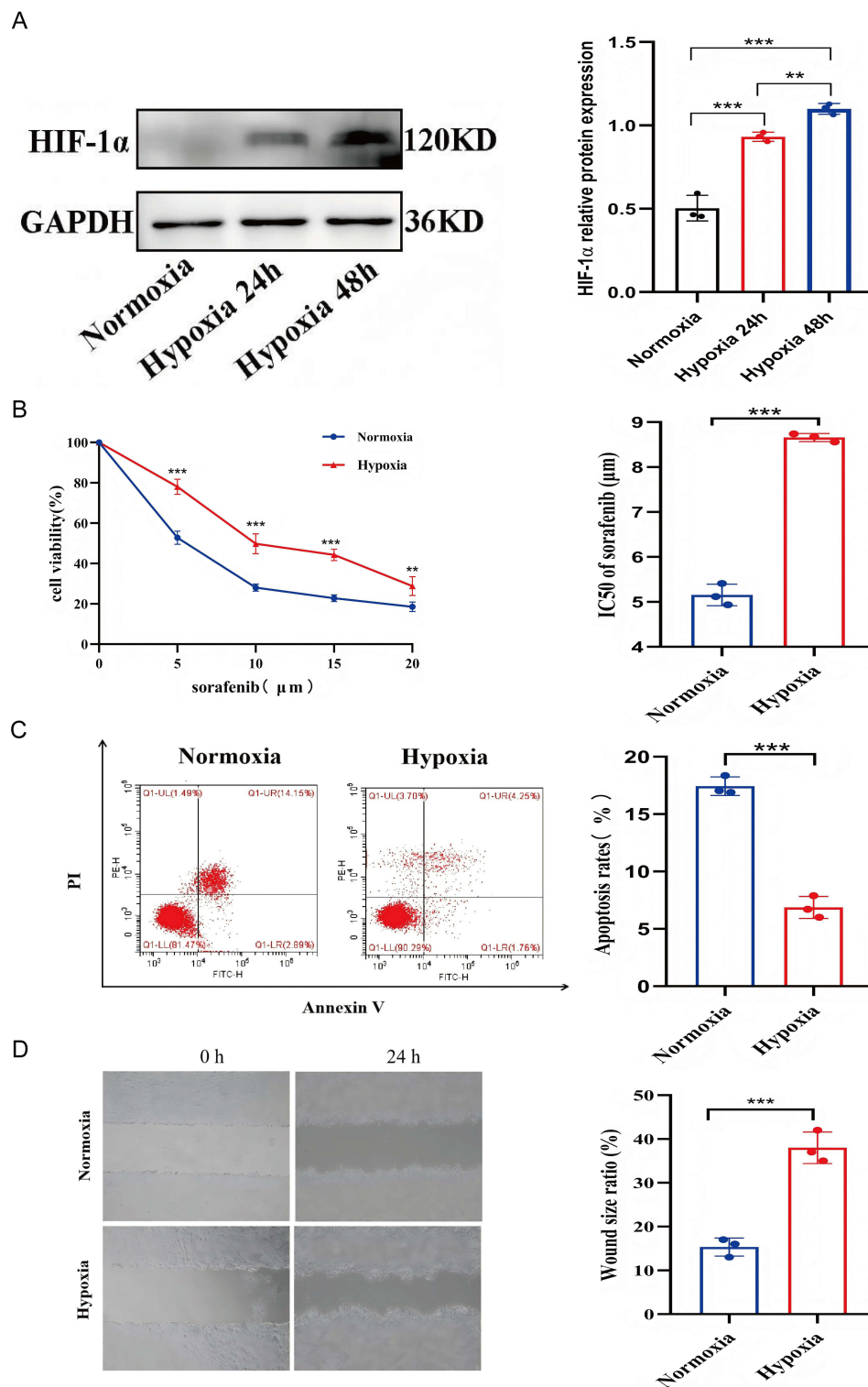


Figure 4 Hypoxia attenuates sorafenib efficacy in HCC cells. **(A)** HIF-1 α expression in Huh7 cells under normoxia and hypoxia at different time points by Western blotting; **(B)** CCK-8 cell viability assay under sorafenib treatment (0,5,10,15,20 μ M, 24 h). IC50 values compared by extra sum-of-squares *F*-test; **(C)** Apoptosis was measured by flow cytometry following treatment with sorafenib (5 μ M) for 24 hours under normoxic or hypoxic conditions; **(D)** Wound healing assay of Huh7 cells treated with sorafenib (5 μ M) under normoxic or hypoxic conditions for 24 h. The data presented here are based on three independent experiments. “***” *P* < 0.01, “****” *P* < 0.001.

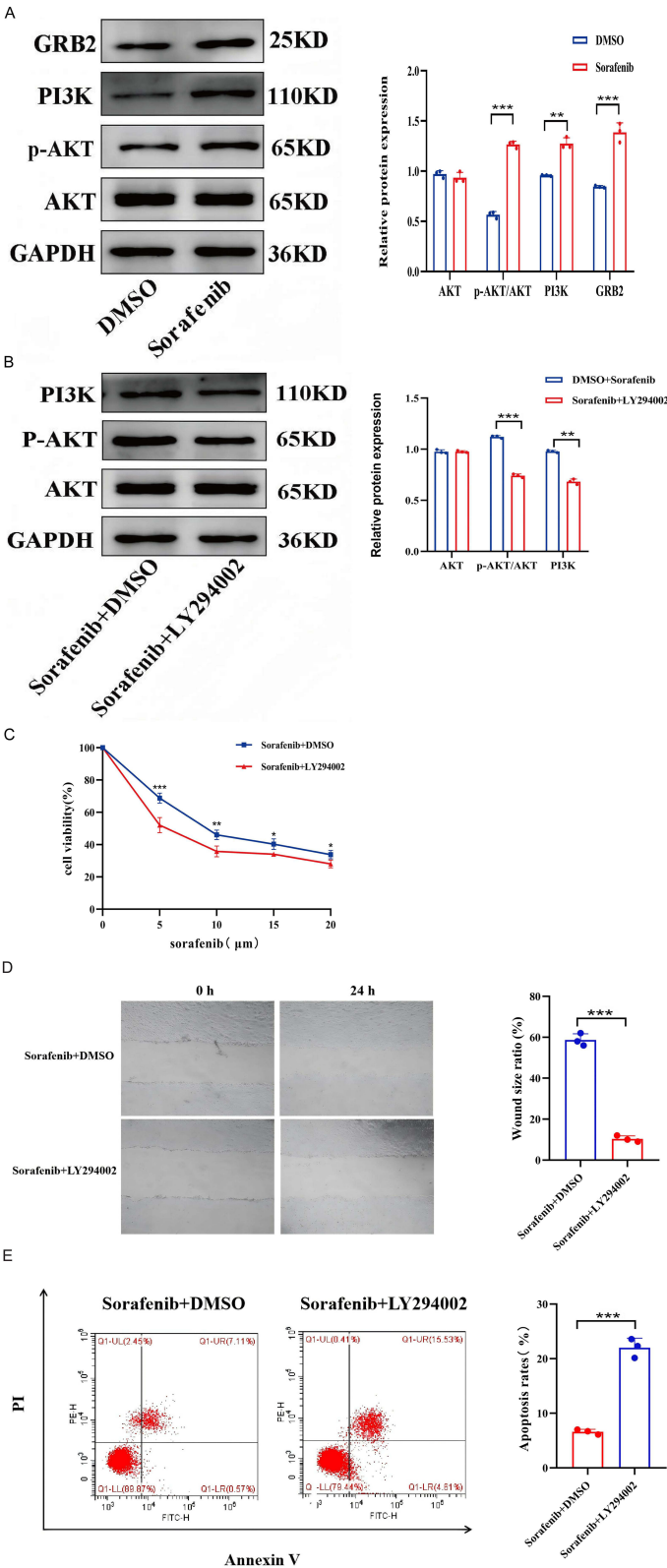


Figure 5 PI3K/AKT pathway contributes to sorafenib resistance under hypoxia. **(A)** Western blotting of GRB2, PI3K, AKT, p-AKT (sorafenib 5 μ M vs DMSO, hypoxia, 24 h); **(B)** Western blotting of PI3K, AKT, p-AKT (sorafenib 5 μ M $\pm$ LY294002 20 μ M, hypoxia, 24 h); **(C)** CCK-8 viability (sorafenib 0.5, 1.0, 1.5, 2.0 μ M $\pm$ LY294002 20 μ M, hypoxia, 24 h); **(D)** Wound healing (sorafenib 5 μ M $\pm$ LY294002, hypoxia, 24 h); **(E)** Flow cytometry (sorafenib 5 μ M $\pm$ LY294002, hypoxia, 24 h). The data presented here are based on three independent experiments. “*” indicates $P < 0.05$, “**” $P < 0.01$, “***” $P < 0.001$.

sorafenib (5 μ M) combined with the PI3K inhibitor LY294002 (20 μ M) or DMSO under hypoxic conditions for 24 hours. Western blotting showed that PI3K and p-AKT were significantly inhibited in the LY294002-treated group (Figure 5B). The CCK-8 assay indicated that hypoxic cells treated with sorafenib and LY294002 had significantly lower viability than those treated with sorafenib and DMSO (Figure 5C). Wound healing assay showed that the migration of cells treated with sorafenib and LY294002 was significantly reduced (Figure 5D). Furthermore, flow cytometry demonstrated that cells treated with sorafenib and LY294002 exhibited significantly increased apoptosis compared with the corresponding control group (Figure 5E). These data suggest that GRB2 and the PI3K/AKT pathway are associated with sorafenib resistance in HCC cells under hypoxia, and inhibition of the PI3K/AKT pathway can reduce sorafenib resistance.

Down-Regulation of GRB2 Enhances Sorafenib Sensitivity and Suppressed PI3K/AKT Pathway in HCC Cells Under Hypoxia

Our results showed that sorafenib significantly increased the expression of GRB2, p-AKT, and PI3K in hypoxic Huh7 cells. Since the results indicate a tight association between the PI3K/AKT pathway and sorafenib resistance, we further investigated whether the GRB2/PI3K/AKT pathway is involved in hypoxia-induced sorafenib resistance. Huh7 cells were stably transduced with lentiviral shGRB2-1, shGRB2-2, and shGRB2-NC, and selected with puromycin to generate stable cell lines. The knockdown efficiency of GRB2 was verified by Western blotting and RT-qPCR, confirming significant downregulation of GRB2 in shGRB2-1 and shGRB2-2 stable cell lines compared with shGRB2-NC ($P < 0.01$ and $P < 0.001$, respectively, Figure 6A). The cells were treated with different concentrations of sorafenib under hypoxic conditions for 24 hours, and the CCK-8 assay showed that Huh7 cells depleted of GRB2 had significantly lower viability than those transfected with shGRB2-NC (Figure 6B). Wound healing assay showed that Huh7 cells' migration was reduced by GRB2 knockdown while being treated with sorafenib under hypoxia (Figure 6C). Flow cytometry demonstrated that shGRB2-1 and shGRB2-2 groups exhibited significantly more apoptotic cells compared with shGRB2-NC (Figure 6D). Compared to shGRB2-NC, downregulation of GRB2 markedly reduced the expression of p-AKT and PI3K in hypoxic Huh7 cells treated with sorafenib for 24 hours (Figure 6E). These data suggest that GRB2 contributes to sorafenib resistance in HCC cells by activating the PI3K/AKT pathway under hypoxia, and its downregulation restores sorafenib sensitivity.

Discussion

The hypoxic microenvironment is a critical contributor to sorafenib resistance in hepatocellular carcinoma (HCC).³¹ As a core regulatory factor of the hypoxic response, HIF-1 α is closely involved in the development and therapeutic resistance of HCC by regulating downstream target genes related to cell proliferation, apoptosis, angiogenesis, and metabolic reprogramming.³² Our findings showed that hypoxia attenuated sorafenib-induced apoptosis and enhanced cell migration in Huh7 cells, consistent with previous reports that hypoxia promotes sorafenib resistance through FOXO3a-mediated autophagy¹⁰ and HSP90 α -mediated necroptosis inhibition.³³ Additionally, the PI3K/AKT pathway, whose activation can be facilitated by various receptor tyrosine kinase ligands,³⁴ has been widely implicated as a compensatory survival mechanism in sorafenib-treated HCC cells, and its inhibition can restore drug sensitivity.³⁵ In our study, we observed concomitant upregulation of GRB2 alongside PI3K/AKT activation under hypoxic conditions with sorafenib treatment, and further experiments demonstrated that inhibiting GRB2 expression suppressed PI3K/AKT pathway activity, thereby reducing sorafenib resistance.

GRB2 is a key adaptor protein that links receptor tyrosine kinases to downstream signaling cascades including PI3K/AKT and RAS/MAPK pathways.³⁶ Previous studies have demonstrated that GRB2 contributes to drug resistance in other cancer types: Chen et al showed that miR-27b-3p reverses multi-chemoresistance through the CBLB/GRB2 axis in breast cancer,²⁰ and Chen et al reported that lymecycline reverses acquired EGFR-TKI resistance by targeting GRB2 in non-small cell lung cancer.²¹ Our study extends these findings to the context of hypoxia-induced sorafenib resistance in HCC, specifically implicating the direct PI3K-activating function of GRB2 in the hypoxic microenvironment. STRING v12.0 PPI analysis revealed high-confidence interactions between GRB2 and key PI3K subunits (PIK3R1/p85 α and PIK3CA/p110 α) and adaptor proteins GAB1/2, while functional experiments confirmed that GRB2 knockdown led to concordant

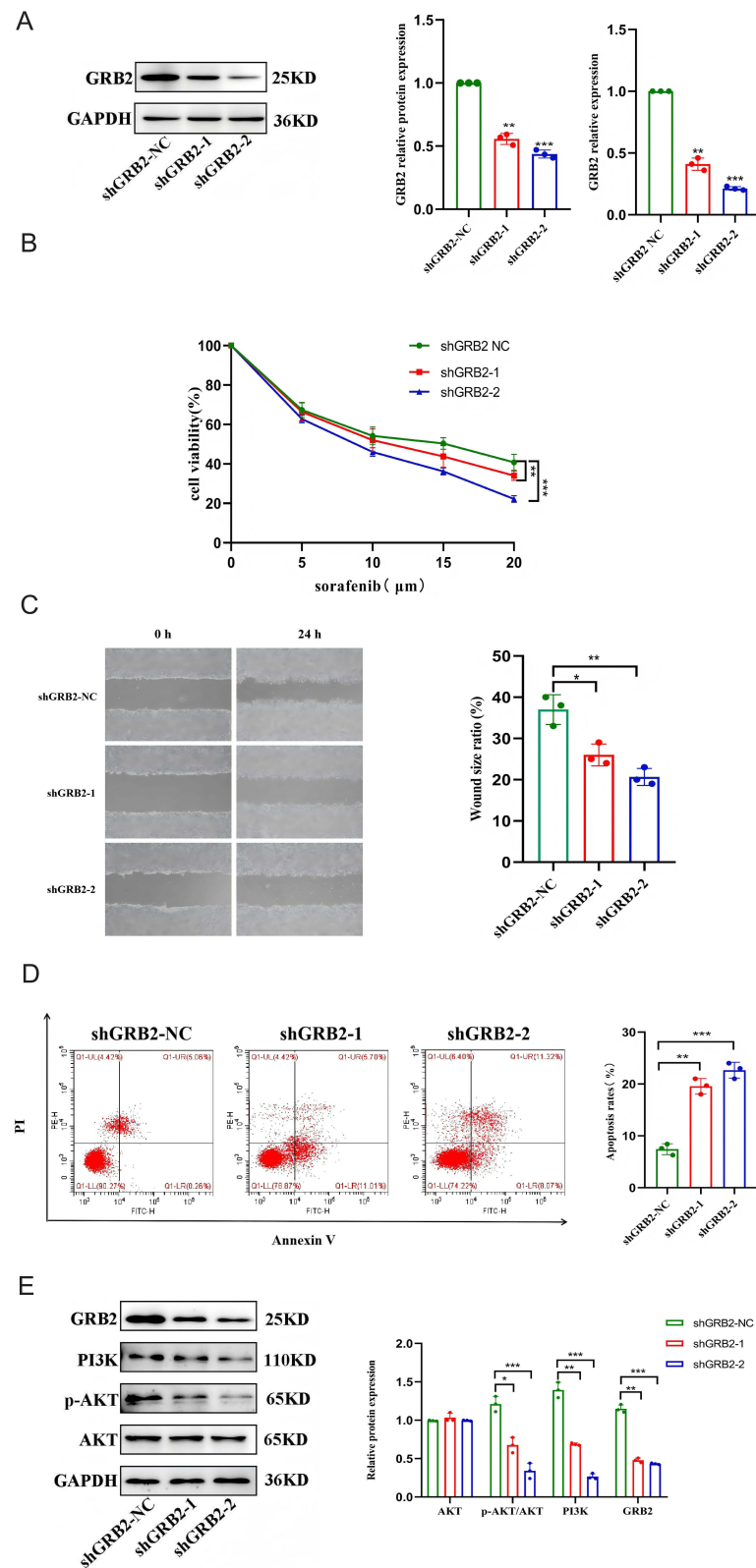


Figure 6 Down-regulation of GRB2 suppressed PI3K/AKT pathway in HCC cells. **(A)** GRB2 knockdown efficiency verified by Western blotting and RT-qPCR in stable cell lines; **(B)** CCK-8 viability (sorafenib 0.5,10,15,20 μM, hypoxia, 24 h) in shGRB2-NC, shGRB2-1, shGRB2-2; **(C)** Wound healing (sorafenib 5 μM, hypoxia, 24 h); **(D)** Flow cytometry (sorafenib 5 μM, hypoxia, 24 h); **(E)** Western blotting of GRB2, PI3K, AKT, p-AKT (sorafenib 5 μM, hypoxia, 24 h). The data presented here are based on three independent experiments. “*” indicates $P < 0.05$, “**” $P < 0.01$, “***” $P < 0.001$.

decreases in PI3K and p-AKT expression with restored sorafenib sensitivity. Notably, pharmacological PI3K inhibition with LY294002 produced highly similar outcomes, supporting that GRB2 and PI3K operate within the same signaling axis. Moreover, a recent study demonstrated that GRB2 interacts with BECN1 and regulates autophagy through modulation of VPS34 activity,³⁷ suggesting that GRB2 may contribute to drug resistance through additional mechanisms beyond PI3K/AKT signaling.

In the clinical context, sorafenib response is also affected by tumor heterogeneity, the immunosuppressive micro-environment, and epigenetic dysregulation.^{38,39} Modulation of the GRB2/PI3K/AKT pathway may need to be considered alongside these factors, and combination strategies targeting multiple resistance mechanisms may be more effective than single-pathway intervention.⁴⁰

This study has several limitations. First, mechanistic experiments were conducted primarily in Huh7 cells under short-term (24 h) hypoxia; validation in additional cell lines and long-term drug exposure models would strengthen the conclusions. The hypoxic model was also validated primarily by HIF-1 α expression; future studies should incorporate additional hypoxia markers for more comprehensive validation. Second, direct molecular interaction evidence (eg, co-immunoprecipitation) and in vivo validation are needed to further confirm the GRB2-PI3K regulatory relationship. Third, the HL-7702 cell line used as normal control has been reported as a potential HeLa derivative; future studies will employ authenticated hepatocyte lines such as THLE-3 or primary hepatocytes. The potential role of GRB2 as a therapeutic target requires further investigation, including in vivo studies and development of specific GRB2-targeted inhibitors.

Abbreviations

HCC, Hepatocellular carcinoma; GRB2, Growth factor receptor-bound protein 2; ROC, Receiver operating characteristic; ssGSEA, single-sample gene set enrichment analysis; FBS, Fetal bovine serum; IC50, Half maximal inhibitory concentration; CCK8, Cell Counting Kit-8; WB, Western Blot; IHC, Immunohistochemistry; qRT-PCR, quantitative real-time PCR.

Ethics Declaration

This study was exempt from Institutional Review Board approval in accordance with Article 32, Items 1 and 2 of the Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects (February 18, 2023, China), as it exclusively utilized publicly available data from public databases where specific individuals cannot be identified.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The author(s) report no conflicts of interest in this work.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2021;71(3):209–249. doi:10.3322/caac.21660
2. Vogel A, Meyer T, Sapisochin G, Salem R, Saborowski A. Hepatocellular carcinoma. *Lancet.* 2022;400(10360):1345–1362. doi:10.1016/S0140-6736(22)01200-4
3. Lu Y, Chan Y-T, Tan H-Y, et al. Epigenetic regulation of ferroptosis via ETS1/miR-23a-3p/ACSL4 axis mediates sorafenib resistance in human hepatocellular carcinoma. *J Exp Clin Cancer Res.* 2022;41(1):3. doi:10.1186/s13046-021-02208-x

4. Llovet JM, Kelley R, Villanueva A, et al. Hepatocellular carcinoma. *Nat Rev Dis Primers*. 2021;7(1):6. doi:10.1038/s41572-020-00240-3
5. Wang X, Hu R, Song Z, et al. Sorafenib combined with STAT3 knockdown triggers ER stress-induced HCC apoptosis and cGAS-STING-mediated anti-tumor immunity. *Cancer Lett*. 2022;547:215880. doi:10.1016/j.canlet.2022.215880
6. Xu J, Ji L, Liang Y, et al. CircRNA-SORE mediates sorafenib resistance in hepatocellular carcinoma by stabilizing YBX1. *Signal Transduct Target Ther*. 2020;5(1):298. doi:10.1038/s41392-020-00375-5
7. Lu H, Liu H, Yan R, et al. Carbonic anhydrase 2 facilitates sorafenib resistance by counteracting MCT4-mediated intracellular pH dysregulation in HCC. *Cell Reports*. 2024;43(12):114996. doi:10.1016/j.celrep.2024.114996
8. Ladd AD, Duarte S, Sahin I, Zarrinpar A. Mechanisms of drug resistance in HCC. *Hepatology*. 2024;79(4):926–940. doi:10.1097/HEP.0000000000000237
9. Mo Z, Liu D, Rong D, Zhang S. Hypoxic characteristic in the immunosuppressive microenvironment of hepatocellular carcinoma. *Front Immunol*. 2021;12:611058. doi:10.3389/fimmu.2021.611058
10. Liang C, Dong Z, Cai X, et al. Hypoxia induces sorafenib resistance mediated by autophagy via activating FOXO3a in hepatocellular carcinoma. *Cell Death Dis*. 2020;11(11):1017. doi:10.1038/s41419-020-03233-y
11. Prieto-Domínguez N, Méndez-Blanco C, Carbajo-Pescador S, et al. Melatonin enhances sorafenib actions in human hepatocarcinoma cells by inhibiting mTORC1/p70S6K/HIF-1 α and hypoxia-mediated mitophagy. *Oncotarget*. 2017;8(53):91402–91414. doi:10.18632/oncotarget.20592
12. Oura K, Morishita A, Tani J, Masaki T. Tumor immune microenvironment and immunosuppressive therapy in hepatocellular carcinoma: a review. *Int J Mol Sci*. 2021;22(11):5801. doi:10.3390/ijms22115801
13. Dong X-F, Liu T-Q, Zhi X-T, et al. COX-2/PGE2 axis regulates HIF2 α activity to promote HCC hypoxic response and reduce sorafenib sensitivity. *Clin Cancer Res*. 2018;24(13):3204–3216. doi:10.1158/1078-0432.CCR-17-2725
14. Ma Y, Xu R, Liu X, et al. LY3214996 relieves acquired resistance to sorafenib in hepatocellular carcinoma cells. *Int J Med Sci*. 2021;18(6):1456–1464. doi:10.7150/ijms.51256
15. Sun Y, Zhang H, Meng J, et al. S-palmitoylation of PCSK9 induces sorafenib resistance in liver cancer by activating the PI3K/AKT pathway. *Cell Rep*. 2022;40(7):111194. doi:10.1016/j.celrep.2022.111194
16. Dai Z, Wang X, Peng R, et al. Induction of IL-6R α by ATF3 enhances IL-6 mediated sorafenib and regorafenib resistance in hepatocellular carcinoma. *Cancer Lett*. 2022;524:161–171. doi:10.1016/j.canlet.2021.10.024
17. Pezzuto A, Carico E. Role of HIF-1 in Cancer Progression: novel Insights. A Review. *Curr Mol Med*. 2018;18(6):343–351. doi:10.2174/1566524018666181109121849
18. He Y, Sun MM, Zhang GG, et al. Targeting PI3K/Akt signal transduction for cancer therapy. *Signal Transduct Target Ther*. 2021;6(1):425. doi:10.1038/s41392-021-00828-5
19. Alzahrani AS. PI3K/Akt/mTOR inhibitors in cancer: at the bench and bedside. *Sem Cancer Biol*. 2019;59:125–132. doi:10.1016/j.semcancer.2019.07.009
20. Cheng Z, Ni Q, Qin L, Shi Y. MicroRNA-92b augments sorafenib resistance in hepatocellular carcinoma via targeting PTEN to activate PI3K/AKT/mTOR signaling. *Braz J Med Biol Res*. 2021;54(9):e10390. doi:10.1590/1414-431X2020e10390
21. Zhou X, Li T-M, Luo J-Z, et al. CYP2C8 suppress proliferation, migration, invasion and sorafenib resistance of hepatocellular carcinoma via PI3K/Akt/p27kip1 axis. *J Hepatocell Carcinoma*. 2021;8:1323–1338. doi:10.2147/JHC.S325554
22. Chen K-F, Chen H-L, Tai W-T, et al. Activation of PI3K/Akt signaling pathway mediates acquired resistance to sorafenib in HCC cells. *J Pharmacol Exp Ther*. 2011;337(1):155–161. doi:10.1124/jpet.110.175786
23. Gishizky ML, Cortez D, Pendergast AM. Mutant forms of growth factor-binding protein-2 reverse BCR-ABL-induced transformation. *Proc Natl Acad Sci USA*. 1995;92(24):10889–10893. doi:10.1073/pnas.92.24.10889
24. Lv J, Zhang S, Wu H, et al. Deubiquitinase PSMD14 enhances hepatocellular carcinoma growth and metastasis by stabilizing GRB2. *Cancer Letters*. 2020;469:22–34. doi:10.1016/j.canlet.2019.10.025
25. Chen D, Si W, Shen J, et al. miR-27b-3p inhibits proliferation and potentially reverses multi-chemoresistance by targeting CBLB/GRB2 in breast cancer cells. *Cell Death Dis*. 2018;9(2):188. doi:10.1038/s41419-017-0211-4
26. Chen Y, Wu J, Yan H, et al. Lymecycline reverses acquired EGFR-TKI resistance in non-small-cell lung cancer by targeting GRB2. *Pharmacol Res*. 2020;159:105007. doi:10.1016/j.phrs.2020.105007
27. Li B-Q, Liang Z-Y, Seery S, et al. WT1 associated protein promotes metastasis and chemo-resistance to gemcitabine by stabilizing Fak mRNA in pancreatic cancer. *Cancer Lett*. 2019;451:48–57. doi:10.1016/j.canlet.2019.02.043
28. Maus M, Medgyesi D, Kövesdi D, et al. Grb2 associated binder 2 couples B-cell receptor to cell survival. *Cell Signal*. 2009;21(2):220–227. doi:10.1016/j.cellsig.2008.10.002
29. Ding C-B, Yu W-N, Feng J-H, Luo J-M. Structure and function of Gab2 and its role in cancer (Review). *Mol Med Rep*. 2015;12(3):4007–4014. doi:10.3892/mmr.2015.3951
30. Xu J, Liang J, Meng Y-M, et al. Vascular CXCR4 expression promotes vessel sprouting and sensitivity to sorafenib treatment in HCC. *Clin Cancer Res*. 2017;23(15):4482–4492. doi:10.1158/1078-0432.CCR-16-2131
31. Fornari F, Giovannini C, Piscaglia F, Gramantieri L. Elucidating the molecular basis of sorafenib resistance in HCC: current findings and future directions. *J Hepatocell Carcinoma*. 2021;8:741–757. doi:10.2147/JHC.S285726
32. Chen ZX, Mu MY, Yang G, et al. Hypoxia-induced DTL promotes the proliferation, metastasis, and sorafenib resistance of hepatocellular carcinoma through ubiquitin-mediated degradation of SLTM and subsequent Notch pathway activation. *Cell Death Dis*. 2024;15(10):734. doi:10.1038/s41419-024-07089-4
33. Liao Y, Yang Y, Pan D, et al. HSP90 α mediates sorafenib resistance in human hepatocellular carcinoma by necroptosis inhibition under hypoxia. *Cancers*. 2021;13(2):243. doi:10.3390/cancers13020243
34. Glaviano A, Foo ASC, Lam HY, et al. PI3K/AKT/mTOR signaling transduction pathway and targeted therapies in cancer. *Mol Cancer*. 2023;22:138. doi:10.1186/s12943-023-01827-6
35. Scheiter A, Evert K, Reibenspies L, et al. RASSF1A Independence and early galectin-1 upregulation in PIK3CA-induced hepatocarcinogenesis: new therapeutic venues. *Mol Oncol*. 2022;16(5):1091–1118. doi:10.1002/1878-0261.13135
36. Wang D, Liu G, Meng Y, Chen H, Ye Z, Jing J. The configuration of GRB2 in protein interaction and signal transduction. *Biomolecules*. 2024;14(3):259. doi:10.3390/biom14030259

37. Montero-Vergara J, Plachetta K, Kinch L, et al. GRB2 is a BECN1 interacting protein that regulates autophagy. *Cell Death Dis.* 2024;15(1):14. doi:10.1038/s41419-023-06387-7
38. Yin H, Tan J, He J, Li M, Lin H. Multidimensional roles of EZH2 and its therapeutic potential in cancer therapy. *Life Conflux.* 2025;1(4):e252. doi:10.71321/q01vae55
39. Wang Y, Lu X, Tan J, et al. The cGAS-STING pathway: insights into regulatory mechanisms, disease dysregulation, and therapeutic development. *Life Conflux.* 2025;2(1):e300. doi:10.71321/dr57c347
40. Jiang Z, Dai C. Potential treatment strategies for hepatocellular carcinoma cell sensitization to sorafenib. *J Hepatocell Carcinoma.* 2023;10:257–266. doi:10.2147/JHC.S396231

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