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Activator of thyroid and retinoid receptor increases sorafenib resistance in hepatocellular carcinoma by facilitating the Warburg effect

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Abstract

Sorafenib resistance is a major challenge in the therapy for advanced hepatocellular carcinoma (HCC). However, the underlying molecular mechanisms of HCC resistance to sorafenib remain unclear. Activator of thyroid and retinoid receptor (ACTR, also known as SRC-3), overexpressed in HCC patients, plays an important oncogenic role in HCC; however, the link between ACTR and sorafenib resistance in HCC is unknown. Our study demonstrated that ACTR was one of the most upregulated genes in sorafenib-resistant HCC xenografts. ACTR increases sorafenib resistance through regulation of the Warburg effect. ACTR promotes glycolysis through upregulation of glucose uptake, ATP and lactate production, and reduction of the extracellular acidification and the oxygen consumption rates. Glycolysis regulated by ACTR is vital for the susceptibility of HCC to sorafenib in vitro and in vivo. Mechanistically, ACTR knockout or knockdown decreases the expression of glycolytic enzymes. In HCC patients, ACTR expression is positively correlated with glycolytic gene expression and is associated with poorer outcome. Furthermore, ACTR interacts with the central regulator of the Warburg effect, c-Myc, and promotes its recruitment to glycolytic gene promoters. Our findings provide new clues regarding the role of ACTR as a prospective sensitizing target for sorafenib therapy in HCC.

KEYWORDS
activator of thyroid and retinoid receptor, aerobic glycolysis, hepatocarcinoma, sorafenib resistance, treatment

L. Ma, W. Liu, A. Xu, Q. Ji and Y. Ma contributed equally to this work.

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common types of adult human malignancies worldwide, especially in South-East Asia.[1] Most HCC patients face a poor prognosis, with a >60% recurrence rate within 5 years after resection.[2] Many molecular targeted drugs hold promise for prolonging the overall survival of patients with advanced liver cancer, of which sorafenib is the most widely used. However, its effect is often hampered by the occurrence of sorafenib resistance.[3,4] Hence, further understanding the potential molecular mechanisms of sorafenib resistance and seeking an effective systemic therapy for patients after failure of sorafenib treatment are of great importance.

The Warburg effect (aerobic glycolysis), a common phenomenon in tumors, is associated with abnormal metabolism with high glycolysis rates despite abundant oxygen. Currently, this property is accepted as a hallmark of cancer that can facilitate tumor initiation and drug resistance. The Warburg effect is characterized by enhanced glucose uptake and lactate production,[5-7] which has been used in clinical diagnosis through ^{18}F-deoxyglucose positron emission tomography to image tumors with an enriched glucose uptake.[8] As a metabolically heterogeneous cancer, HCC can respond to unfavorable and stressful environmental conditions through reprogramming their metabolism in multiple ways, and this metabolic plasticity can allow them to thrive in a wide range of glucose concentrations.[9,10] This metabolic switch meets the proliferation demand of cancer cells, which is related to drug resistance. Thus, understanding the control of this process is critical for identification of potential targets for effective cancer therapy.

Activator of thyroid and retinoid receptor (ACTR, also known as SRC-3) is a member of the p160 coactivator family, which regulates target gene expression by interacting with nuclear hormone receptors and other transcription factors.[11-14] ACTR protein has been demonstrated to be overexpressed in 68% of human HCC specimens and promotes HCC cell proliferation both in vitro and in vivo.[15-17] However, the link between ACTR and sorafenib resistance in HCC is unknown. Given that ACTR promotes HCC proliferation by controlling the Warburg effect, we aim to demonstrate whether ACTR influences sorafenib resistance by controlling the Warburg effect in HCC patients.

In this study, we examined ACTR's crucial role in sorafenib resistance through regulation of the Warburg effect. We found that ACTR knockout (KO) or knockdown (KD) decreased sorafenib resistance in hepatoma cells both in vitro and in vivo. Mechanistically, the decreased sorafenib resistance regulated by ACTR KO or KD was accompanied by decreased glycolysis. We confirmed that aerobic glycolysis is involved in the regulation of HCC sorafenib resistance both in vitro and in vivo. Our results reveal a new mechanism for ACTR, as a potential molecular target of sorafenib in the treatment of HCC, and provide new ideas for the effective treatment of HCC.

2 | MATERIALS AND METHODS

2.1 | Data collection

The GSM1898060-1898062 and GSM1898056-1898058 datasets were downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database. The GSM1898060-1898062 datasets contained the gene expression profiles of three sorafenib-sensitive HCC xenografts in mice. GSM1898056-1898058 datasets contained the gene expression profiles of three sorafenib-resistant HCC xenografts in mice.[18]

2.2 | Activator of thyroid and retinoid receptor knockout hepatocellular carcinoma cells

CRISPR/Cas9 was used for ACTR KO HepG2 cell generation (Genloci Biotechnologies). The CRISPR cell lines were clonal. We applied the CRISPR design web tool to CRISPR sgRNA design (http://crispr.mit.edu) and then cloned ACTR sgRNA into pGK1.1/CRISPR/Cas9 vector (Genloci Biotechnologies) according to manufacturer's instructions. Cells were transfected with sgRNA vectors and amplified, and we screened for mutant genes of nuclease target sites. Then DNA sequencing and immunoblotting were performed. Rescue experiments were performed to avoid the off-target effects.

2.3 | siRNA, plasmids and antibodies

Human ACTR gene of 4275 bp in length was obtained from a mammary gland cDNA library through PCR with the following primers: 5′-GGGGTACCATGAGTGGATTAGGAGAAAACTTG-3′(forward) and 5′-CCGCTCGAGTCAGCAGTATTTCTGATCAGGACC-3′ (reverse). Then the products and pcDNA3.0 vector were digested by enzymes *Kpn*I and *Xho*I. Next, enzyme connection and transformation were accomplished. The recombinant plasmid pcDNA3.0-Flag-ACTR was extracted and identified by enzyme digestion and sequencing. The cDNA target sequence of siRNA for ACTR was GGTGAATCGAGACGGAAAC (GenePharma). Anti–ACTR, anti–GPI, anti–PFKL, anti–ENO1 and anti–β-actin antibodies (Santa Cruz Biotechnology), anti–ALDOA, anti–GLUT1, anti–PGAM1, anti–GAPDH, anti–c-Myc and anti–PGK1 antibodies (Proteintech), and anti–pyruvate kinase M2 (PKM2) and anti–HK2 antibodies (Cell Signaling Technology) were incubated at appropriate concentrations.

2.4 | Analysis of cell survival and apoptosis

Cell lines were routinely cultured in a 95% humidified and 5% CO_2 atmosphere at 37°C. High glucose DMEM (25 mmol/L, Gibco) with 10% FBS was used for cell culture. Following the manufacturer's protocols, Cell Counting Kit-8 (Dojindo) was used to estimate cell survival. Cells were seeded at 3000 cells per 96 well and then treated with sorafenib (Bayer Pharma AG) at concentrations of 0, 2, 4, 6, 8 and 10 μmol/L. At the same time, the sensitivity of low glucose DMEM (5.5 mM, Gibco) to sorafenib was detected. Then cells of colony formation assays were allowed to grow for 10-14 days in 35-mm plates before staining with crystal violet (Sigma) for cell counting. Diameters of colonies more than 1.0 mm were counted. Flow cytometry analysis was used to estimate cell apoptosis. Cells were labeled with Annexin V and propidium iodide, following the manufacturer's instructions (BD Biosciences). Samples were collected and analyzed using a FACSCalibur Flow Cytometer for a minimum of 10 000 events for each one (Becton Dickinson, BD Biosciences).

2.5 | Glucose uptake, ATP and lactate assays

Glucose Uptake Colorimetric Assay (Cat# K676, BioVision), ATP Colorimetric Assay (Cat# K354, BioVision) and Lactate Assay II (Cat# K627, Bio, BioVision) were used to measure the glucose uptake, ATP and lactate production. Glucose uptake colorimetric analysis was carried out in 96-well plates with a density of 1×10^4 cells per well. 2-Deoxy-d-glucose (2-DG) (2.5 mmol/L) and sorafenib (6 μmol/L) were added to the plate for 6 hours. Pre–culture of the cells with 100 μL Krebs-Ringer-Phosphate-HEPES buffer containing 2% BSA for 40 minutes resulted in glucose deficiency. The supernatant was obtained by centrifugation to remove the insoluble fraction and ready for assay.

2.6 | Extracellular acidification rate and oxygen consumption rate assays

The cellular oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) were measured by Seahorse XFe 96 Extracellular Flux Analyzer (Seahorse Bioscience) with the kit of Seahorse XF Glycolysis Stress Test (Cat# 103020, Agilent Technologies) and the Seahorse XF Cell Mito Stress Test (Cat# 103015, Agilent Technologies), respectively. For ECAR, glucose, oligomycin and 2-DG were given sequentially after baseline measurements. For OCR, oligomycin (p-trifluoromethoxy carbonyl cyanide phenylhydrazone), mitochondrial complex I inhibitor rotenone and mitochondrial complex III inhibitor antimycin A (Rote/AA) were sequentially given into each well at the reference time points. Sorafenib (6 μmol/L) was added to the indicated groups for 6 hours. The Seahorse XF96 Wave software was used for the evaluation of data. The ECAR was presented in mpH/min and the OCR in pmoles/min.

2.7 | Quantitative RT-PCR

The mRNA expression levels were measured using SYBR Premix Ex Taq Master Mix (2×) (Takara). The primer sequences used for quantitative RT-PCR analysis are presented in Table S1. Relative expression was computed by the comparative *Ct* method.

2.8 | ChIP and re–immunoprecipitation

ChIP assay was conducted using the Magna ChIP G Assay Kit (Cat# 17-409, Merck, Millipore). The precipitated chromatin complexes of cells were collected according to the manual of the kit and then analyzed by RT-PCR with primers (Table S1). For re–immunoprecipitation (re–ChIP), complexes were eluted from the primary immunoprecipitation by incubation with 10 mmol/L DTT at 37°C for 30 minutes and diluted 1:50 in re–ChIP buffer (150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl, pH 8.1) followed by re–immunoprecipitation with the second antibodies. Samples were analyzed by RT-PCR with the primers listed in Table S1.

2.9 | Tumor growth in vivo

Different HepG2 types were subcutaneously injected into the hind limbs of 6-week-old male nude mice (n = 7). An aliquot of 2-DG (500 mg/kg) was injected intravenously via the lateral tail vein of nude mice at indicated times. Sorafenib was given by oral administration. Then mice were killed at indicated times. Each tumor was excised, measured and fixed in formalin. We calculated the tumor volume using the following formula: volume = (longest diameter × shortest diameter^2)/2.

2.10 | Immunohistochemistry

The immunohistochemistry (IHC) procedure was performed as described previously.[19] The antigens were retrieved using the high-pressure method and incubated with rabbit anti–ACTR antibody (Santa Cruz Biotechnology), rabbit anti–lactate dehydrogenase A (LDHA) antibody (Proteintech) or rabbit anti–PKM2 antibody (Cell Signaling Technology). The binding primary antibodies were determined by adding biotin goat anti–rabbit secondary antibody and streptavidin HRP (Zymed Laboratories). In the negative control group, primary antibodies were replaced by PBS or normal rabbit IgG (Santa Cruz Biotechnology). All IHC staining was evaluated by two experienced pathologists blinded to the source of each specimen. The LDHA score was calculated by multiplying the percentage of stained cells (0%-100%) with the intensity of the staining (low: 1+; medium: 2+; strong: 3+), with the score between 0 and 3. The optimal cut-off values of the IHC scores were determined by receiver operating characteristic

(ROC) curve analysis.[20] In the correlation analysis, we defined a score <0.25 as low ACTR, ≤0.25 to ≤0.75 as medium ACTR, and a score >0.75 as high ACTR. A score <0.5 was considered low LDHA or PKM2, with ≤0.5 to ≤1.0 being medium and >1.0 high.

2.11 | Co–immunoprecipitation

To detect the interaction of endogenous protein ACTR with c-Myc, 0.5 mL lysis buffer (50 mmol/L Tris at pH 8.0, 0.5% NP-40, 500 mmol/L NaCl, 1 mmol/L DTT and protease inhibitor tablets from Roche Applied Science) was used for cell lysis and anti–ACTR or control serum (Santa Cruz Biotechnology) for immunoprecipitation. The immunoprecipitates were separated by SDS-PAGE after extensive washing with the lysis buffer, and then analyzed by western blot.

2.12 | Statistical analysis

All in vitro experiments were repeated three times in triplicate. The cell proliferation, glucose uptake, ATP, lactate, OCR and ECAR measurements were analyzed by the two-tailed Student's *t* test, and the difference was statistically significant. The difference in the expression of ACTR and LDHA was assessed by Spearman correlation. The statistical calculations were performed using the SPSS 21.0 statistical software package. In all analyses, $^{*/\#}P < 0.01$ and $^{**/\#\#}P < 0.05$ were considered statistically significant.

3 | RESULTS

3.1 | Gene Expression Omnibus dataset analysis in sorafenib-sensitive and sorafenib-resistant hepatocellular carcinoma xenografts

We used microarray data of sorafenib-resistant xenografts and sorafenib-sensitive xenografts in mice based on datasets from the GEO database to investigate the critical genes that are potentially associated with sorafenib resistance in HCC (Figure 1A). The transcripts of 739 genes were differentially expressed between sorafenib-resistant and sorafenib-sensitive xenografts. Among them, ACTR was one of the most differentially expressed genes detected, with more than eightfold higher expression in sorafenib-resistant xenografts when compared with sorafenib-sensitive xenografts (Figure 1B). Therefore, we hypothesized that ACTR might play a role in regulating sorafenib resistance of HCC patients.

3.2 | Activator of thyroid and retinoid receptor enhances sorafenib resistance by affecting aerobic glycolysis in vitro

Because aerobic glycolysis is of great importance in drug resistance and ACTR functions as an oncogene, we examined whether aerobic glycolysis plays a role in ACTR-mediated enhancement of sorafenib resistance

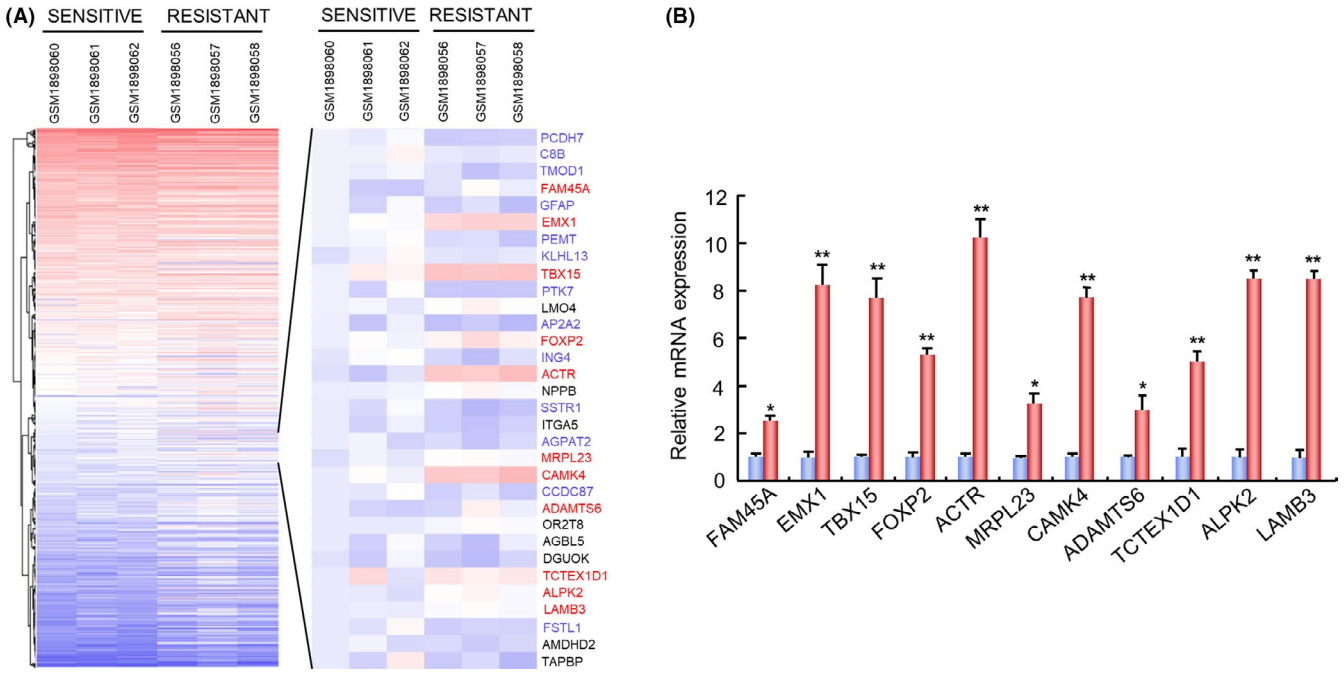


FIGURE 1 Differential expression of genes in sorafenib sensitive and resistant hepatocellular carcinoma (HCC) xenografts. A, A heat map showing the microarray profiles and supervised hierarchical clustering analysis for sorafenib sensitive and resistant HCC xenografts. Significantly differentially expressed genes matching the threshold (difference ≥2-fold and signal value >500) and statistical analysis standard adjust *P*-value < 0.05 were selected. Each column represents xenografts and each row shows the relative expression level for individual genes. The magenta and cyan colors indicate high or low expression, respectively. B, Average values of 11 upregulated genes in the sorafenib-resistant HCC xenografts compared with sorafenib-sensitive HCC xenografts. The *P*-values were generated using a two-tailed Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$ vs matched sorafenib-sensitive HCC xenografts

in hepatoma cells. As expected, ACTR KO or KD decreased the resistance of sorafenib in hepatoma cells. Indeed, the glycolytic inhibitor 2-DG reduced the sorafenib resistance of hepatoma cells. Importantly, the reduction of sorafenib resistance led by ACTR KO or KD was almost abolished with 2-DG treatment (Figure 2A,B & Figure S1A). Next, we examined the effect of ACTR on sorafenib resistance with low glucose. Our results showed that low glucose culture conditions further enhanced sorafenib sensitivity mediated by ACTR KO or KD, suggesting that the ACTR regulation of sorafenib resistance is glucose-dependent (Figure 2C,D). The sorafenib response in the ACTR KO cells and KD cells could be reversed by ACTR re–expression through transfection with the ACTR expression vector (Figure 2A-D & Figure S1). Colony formation assays revealed similar trends to those observed in the cell viability assay (Figure 2E,F). Annexin-V assay demonstrated that either ACTR KO or treatment with 2-DG could increase the apoptosis rate of hepatoma cells treated with sorafenib, and, moreover, the enhancement of apoptosis that led by ACTR KO was almost abolished when treated with 2-DG (Figure 2E). These data collectively suggest that aerobic glycolysis is involved in the regulation of sorafenib resistance by ACTR.

3.3 | Activator of thyroid and retinoid receptor attenuates the inhibitory effect of sorafenib on glycolysis

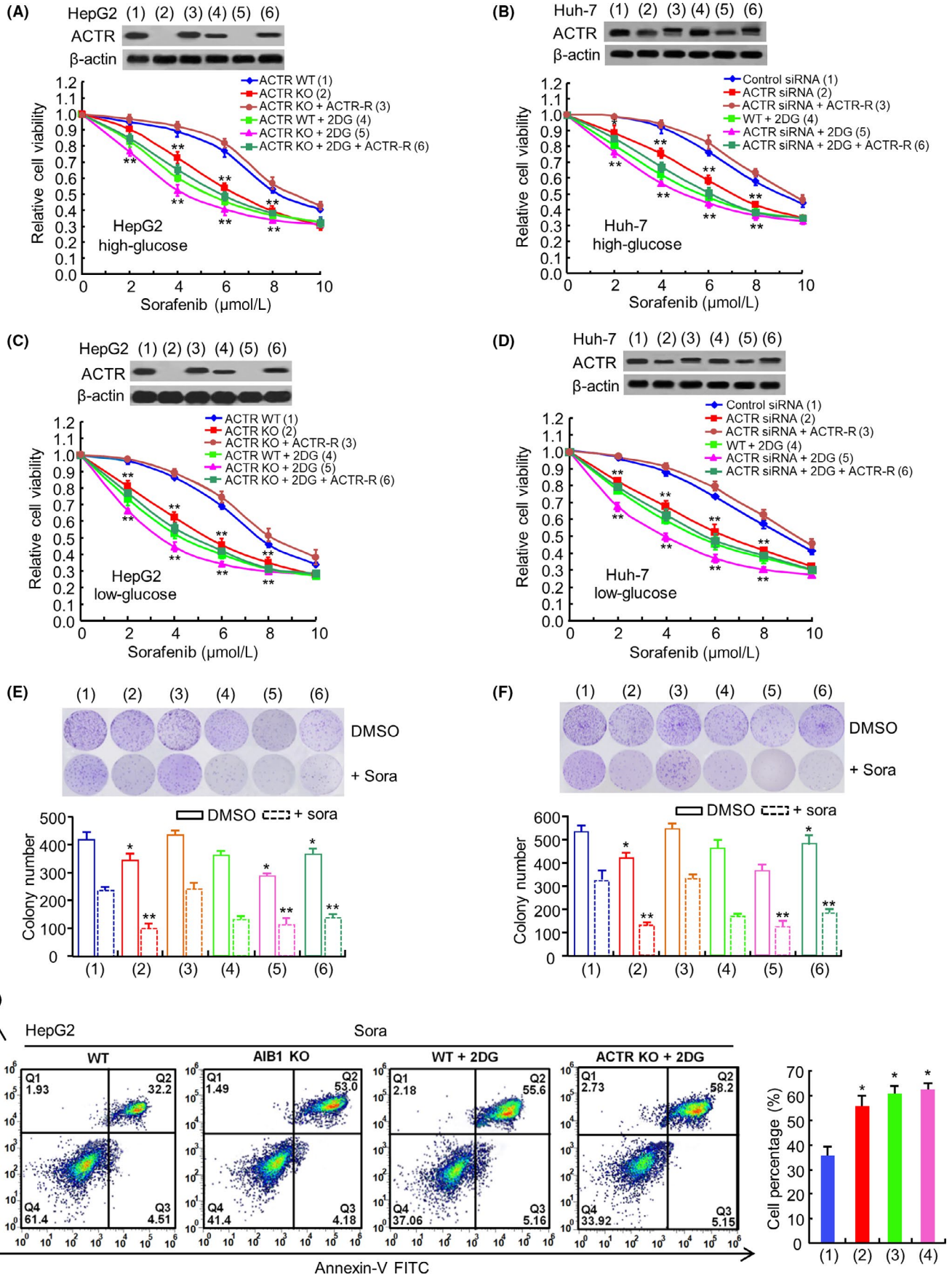
In glycolysis, each molecule of glucose can produce a net of two ATP. Cancer cells consume glucose avidly and generate lactate from pyruvate, even in the presence of ample oxygen. We investigated whether ACTR influences glycolysis in liver cancer cells and relationship between sorafenib and ACTR in glycolysis.

First, we tested the effect of ACTR on the regulation of glucose uptake, lactate production and ATP generation. As expected, ACTR KO and KD reduced glucose uptake, lactate production and the ATP level in HepG2 and Huh-7 hepatoma cells. These effects were reversed by ACTR re–expression in ACTR KO or KD cells transiently transfected with the ACTR expression vector. With sorafenib treatment, glucose uptake, lactate production and ATP level decreased more significantly compared with the untreated group, especially in ACTR KO and KD cells (Figure 3A,B). We also found that ACTR KO or KD could reduce the ECAR, a representative of total glycolytic flux, and increase the OCR, which indicated mitochondrial respiration. More importantly, the ECAR in the ACTR KO (or KD) cells together with sorafenib group decreased more significantly than in the ACTR KO (or KD) group or in the sorafenib group alone, while the OCR in the ACTR KO (or KD) cells together with sorafenib group increased more significantly than in the ACTR KO (or KD) group or in the sorafenib group alone. Again, ACTR re–expression in the ACTR KO or KD cells transiently transfected with ACTR expression vector almost rescued these effects in HepG2 and Huh-7 cells (Figure 3C-F). Taken together, these data collectively suggest that ACTR enhances glycolysis in hepatoma cells and promotes transformation of cell glucose metabolism from oxidative phosphorylation to glycolysis. Moreover, ACTR attenuates the inhibitory effect of sorafenib on HCC glycolysis, which may explain for the effect of ACTR on sorafenib resistance.

3.4 | Activator of thyroid and retinoid receptor impacts the effect of sorafenib by regulating the expression of glycolytic enzymes

To investigate the mechanisms of how ACTR regulates glycolysis and influences sorafenib function, we tested the effect of ACTR on glycolytic gene expression under sorafenib. Real-time RT-PCR and western blot confirmed that ACTR could alter the expression of five glycolysis-related genes: GLUT1 (glucose transporter 1 or SLC2A1), PFKL (6-phosphofructokinase, liver type), ENO1 (enolase 1), PKM2 and LDHA. The effect of ACTR KO on glycolytic gene expression could be rescued by ACTR re–expression in ACTR KO HepG2 hepatoma cells transiently transfected with the ACTR expression vector. Sorafenib decreased the expression of five glycolytic enzymes, including GLUT1, GPI, PFKL, PGAM1 and PKM2, in HepG2 cells. Furthermore, the effect of ACTR KO or KD on the glycolytic expression was more severe with sorafenib treatment. Similarly, this effect could be rescued by ACTR re–expression (Figure 4A,B). ACTR KD in Huh-7 cells also showed a similar tendency of glycolytic enzymes when treated with sorafenib (Figure 4C,D). Next, we tested how ACTR regulated glycolytic gene transcription. ChIP demonstrated that ACTR was recruited to the promoters of glycolytic genes GLUT1, PFKL, ENO1, PKM2 and LDHA but not to the regions upstream of their promoters, suggesting that ACTR promotes glycolytic gene transcription by binding to the indicated glycolytic gene promoters. Moreover, the recruitment of glycolytic genes GLUT1,

FIGURE 2 Activator of thyroid and retinoid receptor (ACTR) enhances sorafenib resistance by affecting aerobic glycolysis in vitro. A and B, The relative viability curves of ACTR WT or KO HepG2 cells or ACTR KO HepG2 cells transiently transfected with ACTR, as well as Huh-7 cells in DMEM with high glucose (25 mmol/L), transfected with ACTR siRNA or ACTR siRNA plus ACTR expression vector or non-specific control for siRNA (Control siRNA); they were treated with Deoxy-d-glucose (2-DG) (2.5 mmol/L) and increasing concentrations of sorafenib as indicated above. After 72 h, cell viability assays were performed using the CCK-8. The group without treatment of sorafenib had 100% viable cells and was used as an internal control for comparison. The representative immunoblot with ACTR indicates ACTR expression levels. C and D, The relative viability curves of HepG2 cells (C) or Huh-7 cells (D) transfected and treated as in (A) or (B) and cultured in DMEM with low glucose (5.5 mmol/L). E and F, Colony formation assays of HepG2 and Huh-7 cells treated as in (A) and (B) with sorafenib (6 μmol/L) or not. G, Representative flow cytometry analysis of Annexin V (1:1000) and propidium iodide (1:1000) staining was carried out in HepG2 ACTR WT cells, KO cells, WT cells and KO cells treated with 2-DG (2.5 mmol/L) and sorafenib (6 μmol/L) for 6 h. Data shown are mean ± SD of triplicate measurements that have been repeated three times with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$ vs corresponding ACTR WT group


(A)
HepG2 (1) (2) (3) (4) (5) (6)
ACTR
β-actin
ACTR WT (1)
ACTR KO (2)
ACTR KO + ACTR-R (3)
ACTR WT + 2DG (4)
ACTR KO + 2DG (5)
ACTR KO + 2DG + ACTR-R (6)
Relative cell viability
HepG2
high-glucose
Sorafenib (μmol/L)
(B)
Huh-7 (1) (2) (3) (4) (5) (6)
ACTR
β-actin
Control siRNA (1)
ACTR siRNA (2)
ACTR siRNA + ACTR-R (3)
WT + 2DG (4)
ACTR siRNA + 2DG (5)
ACTR siRNA + 2DG + ACTR-R (6)
Relative cell viability
Huh-7
high-glucose
Sorafenib (μmol/L)
(C)
HepG2 (1) (2) (3) (4) (5) (6)
ACTR
β-actin
ACTR WT (1)
ACTR KO (2)
ACTR KO + ACTR-R (3)
ACTR WT + 2DG (4)
ACTR KO + 2DG (5)
ACTR KO + 2DG + ACTR-R (6)
Relative cell viability
HepG2
low-glucose
Sorafenib (μmol/L)
(D)
Huh-7 (1) (2) (3) (4) (5) (6)
ACTR
β-actin
Control siRNA (1)
ACTR siRNA (2)
ACTR siRNA + ACTR-R (3)
WT + 2DG (4)
ACTR siRNA + 2DG (5)
ACTR siRNA + 2DG + ACTR-R (6)
Relative cell viability
Huh-7
low-glucose
Sorafenib (μmol/L)
(E)
(1) (2) (3) (4) (5) (6)
DMSO
+ Sora
DMSO + sora
Colony number
(F)
(1) (2) (3) (4) (5) (6)
DMSO
+ Sora
DMSO + sora
Colony number
(G)
HepG2
Sora
WT
AIB1 KO
WT + 2DG
ACTR KO + 2DG
PI
Annexin-V FITC
Q1 1.93
Q2 32.2
Q4 61.4
Q3 4.51
Q1 1.49
Q2 53.0
Q4 41.4
Q3 4.18
Q1 2.18
Q2 55.6
Q4 37.06
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Q1 2.73
Q2 58.2
Q4 33.92
Q3 5.15
Cell percentage (%)
(1) (2) (3) (4)

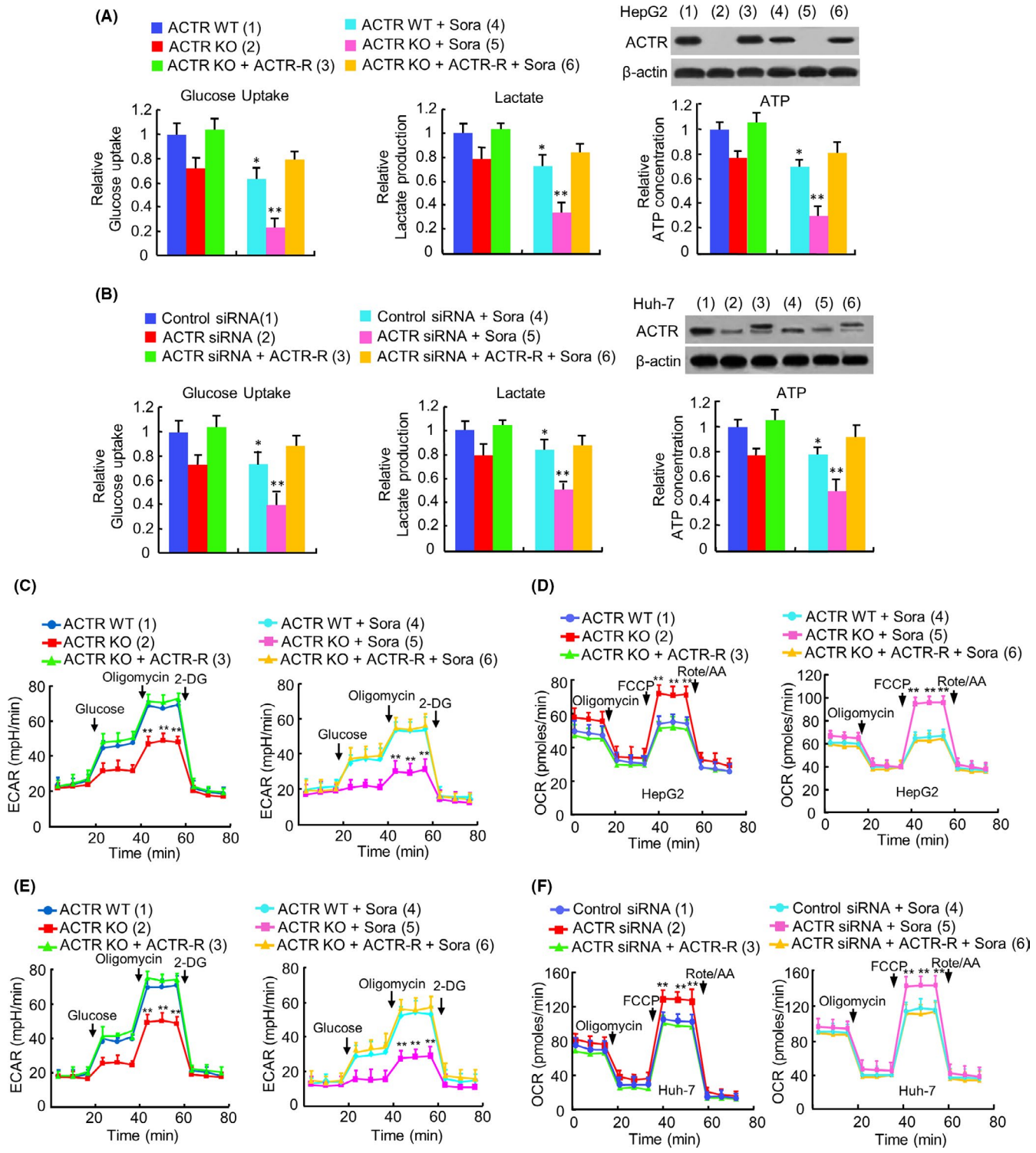


FIGURE 3 Activator of thyroid and retinoid receptor (ACTR) attenuates the inhibitory effect of sorafenib on glycolysis. A, Glucose uptake, lactate production and ATP level were determined in ACTR WT or KO HepG2 cells or ACTR KO HepG2 cells transiently transfected with ACTR, and they were treated with sorafenib (6 μmol/L) for 6 h or not treated. $^{**}P < 0.01$ vs corresponding WT cells. B, Huh-7 cells were transfected with ACTR siRNA or ACTR siRNA plus ACTR expression vector, and they were treated with sorafenib as above. Glucose uptake, lactate production and ATP level were determined. A representative immunoblot reveals the expression of ACTR. $^{*}P < 0.05$ vs corresponding control siRNA. C and D, The extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) were analyzed in ACTR WT or KO HepG2 cells or ACTR KO HepG2 cells transiently transfected with ACTR, treated with sorafenib or not treated, as above. $^{**}P < 0.01$ vs corresponding WT cells. E and F, ECAR and OCR assays of Huh-7 cells were transfected as in (C) and (D). $^{**}P < 0.01$ vs corresponding control siRNA. All values shown are mean ± SD of quintuplicate measurements and were repeated three times with similar results

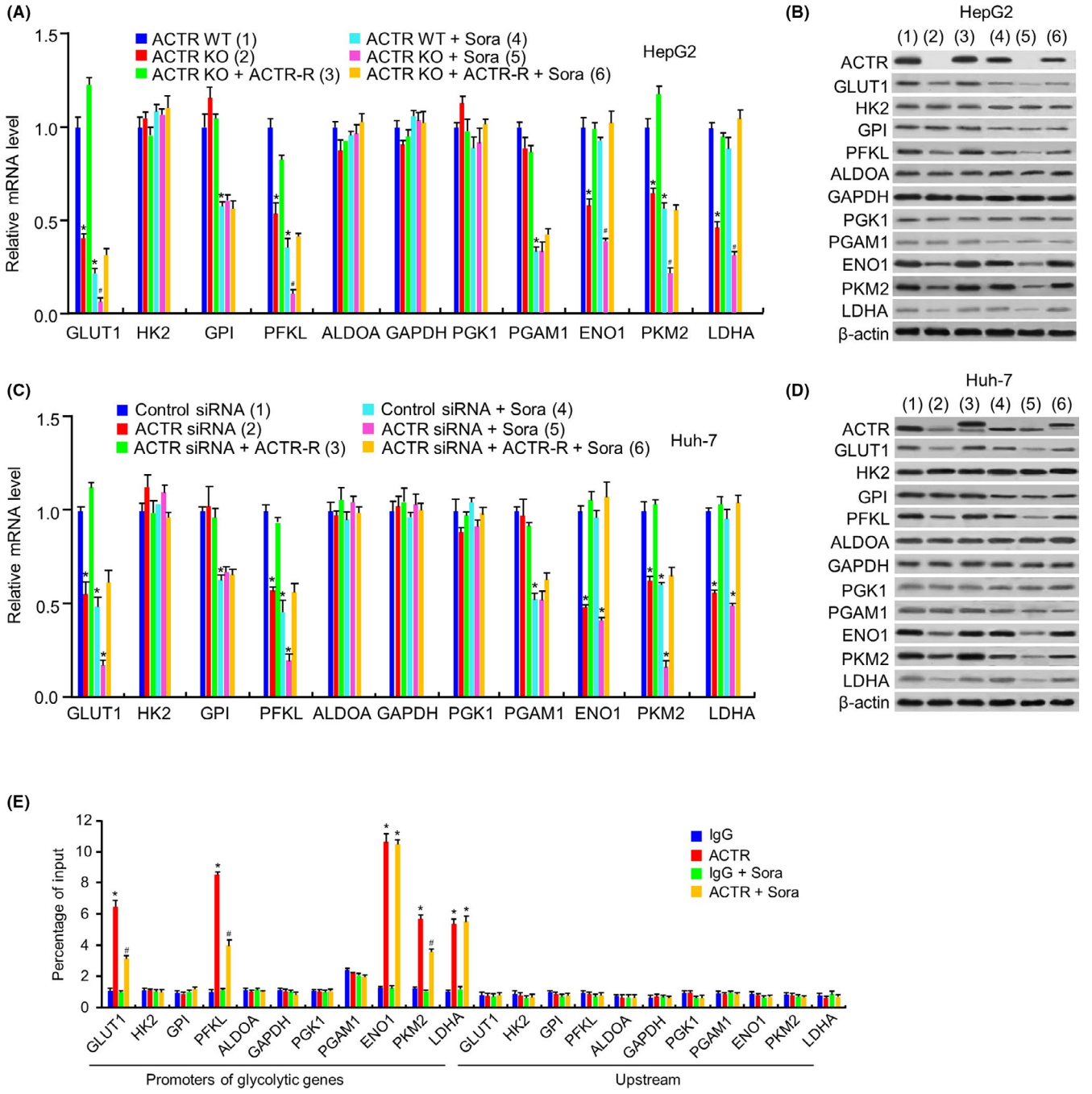


FIGURE 4 Glycolysis gene expression is regulated by activator of thyroid and retinoid receptor (ACTR) and related to sorafenib treatment. A and B, Quantitative RT-PCR and immunoblotting analyses of glycolytic gene expression in ACTR WT or KO HepG2 cells, or ACTR KO HepG2 cells transiently transfected with ACTR; they were treated with sorafenib (6 μmol/L) for 6 h or not treated. $^{**}P < 0.01$ vs corresponding WT cells. C and D, Quantitative RT-PCR and immunoblotting analyses of glycolytic gene expression in Huh-7 cells transfected with ACTR siRNA or ACTR siRNA plus ACTR expression vector, and treated with sorafenib as above. $^{*}P < 0.05$, $^{**}P < 0.01$ vs corresponding control siRNA cells. E, ChIP analysis of ACTR occupancy on the indicated glycolytic gene promoters in HepG2 cells treated with sorafenib (6 μmol/L) or not treated. $^{*}P < 0.05$ vs transfected with empty vector group. $^{\#}P < 0.05$ vs transfected with ACTR group. All data shown are mean ± SD of triplicate measurements that have been repeated three times with similar results

PFKL, PKM2 was decreased under sorafenib treatment, indicating that sorafenib inhibits the recruitment of glycolysis genes by ACTR (Figure 4E). Taken together, these data collectively suggest that ACTR affects sorafenib resistance by regulating the expression of glycolytic enzymes.

To examine the link between ACTR and the glycolytic key regulator c-Myc, we first confirmed that ACTR interacted with c-Myc in hepatoma cells by co–immunoprecipitation (Figure S2A). We then tested the effect of ACTR on c-Myc gene expression. Real-time RT-PCR and western blot data showed that ACTR KO decreased

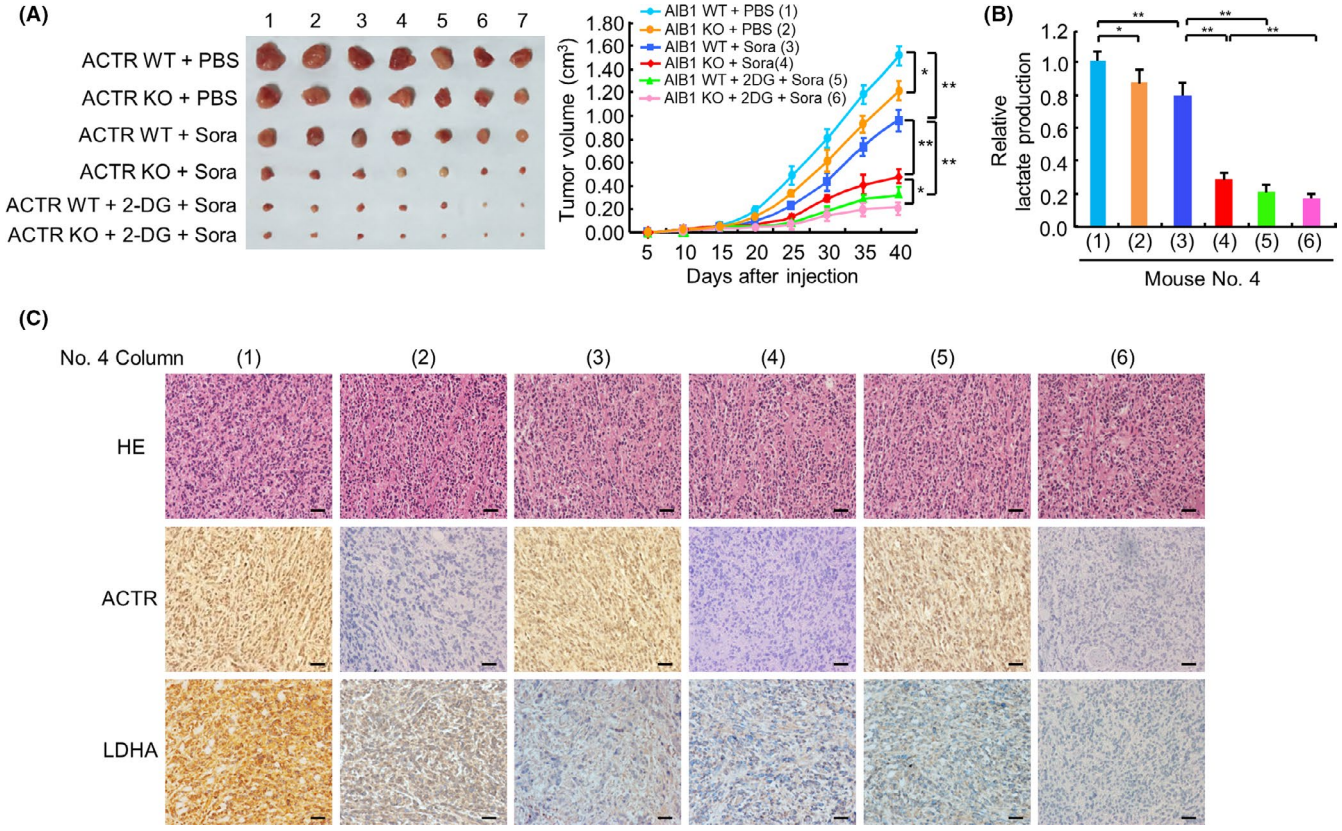


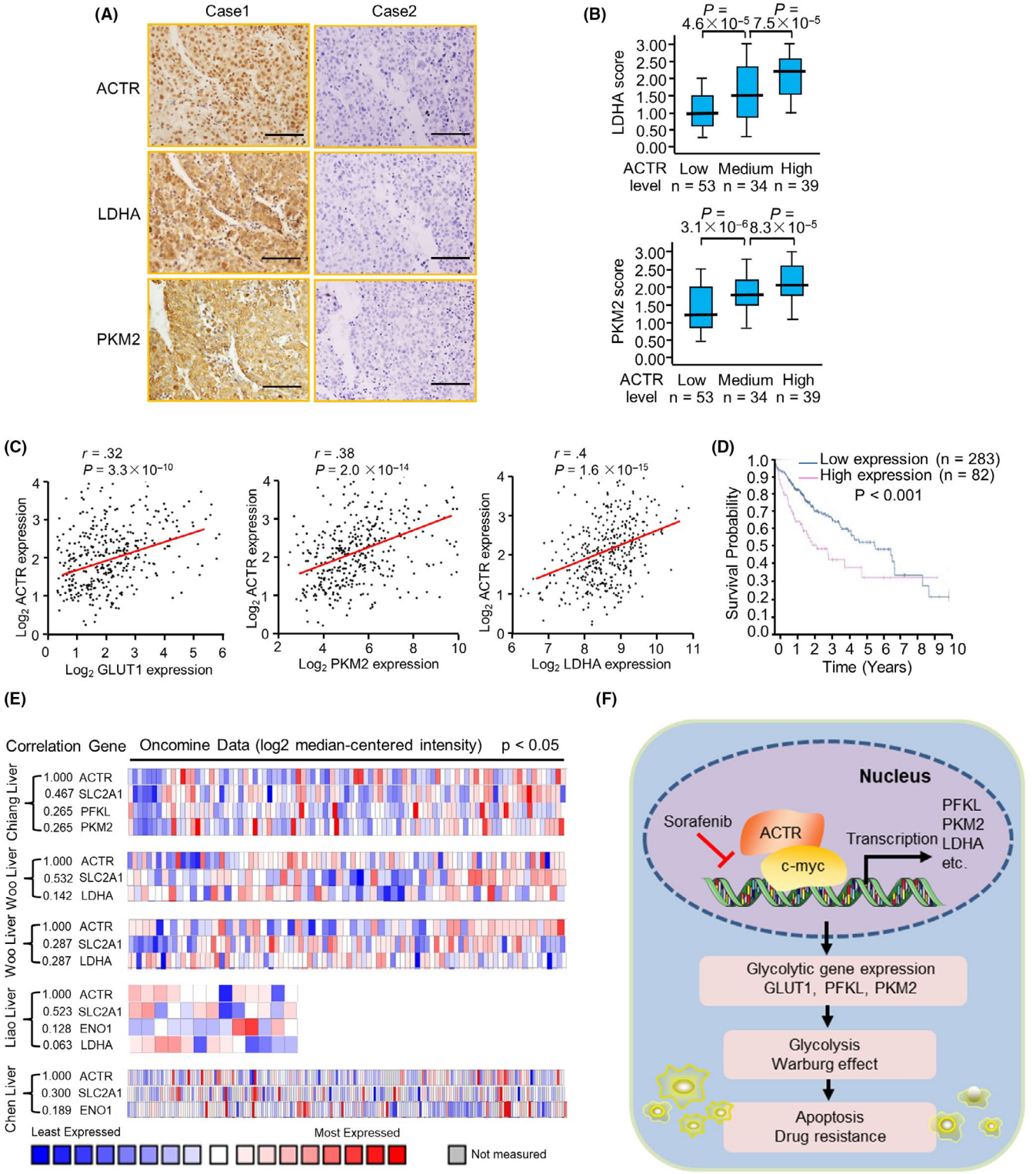
FIGURE 5 Activator of thyroid and retinoid receptor (ACTR) regulates glycolysis, promotes tumor growth and inhibits sorafenib sensitivity in vivo. A, ACTR WT or KO HepG2 cells were injected into nude mice. Nude mice were treated with sorafenib (100 mg/kg body weight), together with Deoxy-d-glucose (2-DG) or not treated, or equal volume of PBS for 7 days after injection of hepatocellular carcinoma (HCC) cells 30 days. After injection of HCC cells 40 days, mice were killed to harvest tumors. The tumors were measured and the growth curve was plotted. $^{**}P < 0.01$ at day 40 (mean ± SD, n = 7). B, Lactate production of representative tumor tissues from (A). Data shown are mean ± SD of quintuplicate measurements and have been repeated three times with similar results. $^{**}P < 0.01$ vs ACTR WT HepG2 cells. C, Immunohistochemical staining of the expression of glycolytic genes in a representative excised tumor from (A). Scale bar: 50 μm

the expression of c-Myc. These effects were reversed by ACTR re–expression in the ACTR KO cells transiently transfected with the ACTR expression vector (Figure S2B,C). Moreover, ChIP assay revealed that ACTR facilitated c-Myc recruitment to the promoters of glycolytic genes, including GLUT1, PFKL, PKM2, ENO1 and LDHA (Figure S2D). Re–ChIP experiments further showed that c-Myc was associated with ACTR on the corresponding binding sites, suggesting the co–occupancy of ACTR and c-Myc (Figure S2E).

3.5 | Activator of thyroid and retinoid receptor regulates glycolysis and modulates hepatocellular carcinoma sorafenib resistance in vivo

Based on the fact that ACTR regulates sorafenib resistance in HCC cells in vitro, we investigated the phenotype of cells with ACTR depletion in vivo. ACTR KO cells or WT cells were subcutaneously injected into the backs of BALB/c nude mice. After the mice developed palpable tumors, they were randomly assigned into sorafenib or PBS or sorafenib

FIGURE 6 Correlation between activator of thyroid and retinoid receptor (ACTR) and glycolytic gene expression in hepatocellular carcinoma (HCC) patients. A, Representative immunohistochemistry (IHC) of 126 HCC patients' ACTR, lactate dehydrogenase A (LDHA) and pyruvate kinase M2 (PKM2) were assessed by IHC. Case 1 represents high ACTR expression and Case 2 low ACTR expression. Scale bar: 100 μm. B, The correlation of ACTR with LDHA and PKM2 in liver cancer patients from (A) was analyzed using the one-way ANOVA test with Games-Howell's correction. The *P*-value was generated using Spearman's rank correlation test. C, Analysis of correlation of ACTR expression with LDHA and PKM2 mRNA using the data from The Genome Cancer Atlas (TCGA) (https://cancergenome.nih.gov/) and Spearman's rank correlation test. We used the following filters to analyze the correlation. Disease type: liver hepatocellular carcinoma. Data category: transcriptome profiling. Data type: gene expression quantification. Experimental strategy: RNA-Seq. Workflow type: HTSeq–FPKM. Access level: open. D, Kaplan-Meier estimates of overall survival of HCC patients based on TCGA database as described in (C). Marks on graph lines represent censored samples. E, Analysis of correlation of ACTR mRNA expression with mRNA expression of glycolytic genes using the data from Oncomine. For Oncomine, we used the following filters to determine the correlation. Gene: NCOA3. Analysis type: co–expression analysis. Data type: mRNA. Color changes according to weaker (blue) or higher (red) expression, passing by white, with fluctuating color intensity. F, A proposed model underlying the role of the ACTR in regulating HCC glycolysis and sorafenib resistance



combined with 2-DG treatment groups. There was no significant difference in initial tumor volumes among groups. Like sorafenib, ACTR KO or 2-DG induced inhibition of tumor growth (Figure 5A). The 2-DG enhanced the effect of sorafenib on HCC tumor. More importantly, the effect of ACTR on sorafenib resistance was almost abolished under 2-DG treatment, accompanied by the altered expression of LDHA and lactate production (Figure 5B,C). These findings suggest that ACTR enhances sorafenib resistance of HCC cells by regulating glycolysis in vivo.

3.6 | Correlation between activator of thyroid and retinoid receptor and glycolytic gene expressions in hepatocellular carcinoma patients

We assessed the expression of two representative glycolytic genes by IHC and ACTR expression in 126 human liver cancer samples. In agreement with ACTR promotion of LDHA and PKM2 in cultured cells, the expression of ACTR was positively correlated with LDHA

and PKM2 expression (Figure 6A,B). The correlation between ACTR and glycolytic genes GLUT1, PKM2 and LDHA was further confirmed by external datasets from The Cancer Genome Atlas (TCGA) (Figure 6C). Higher ACTR expression predicted poorer outcome in a cohort of 365 HCC patients collected from TCGA, whose OS data were available ($P < 0.001$) (Figure 6D). The specificities of the ACTR, LDHA and PKM2 antibodies were validated through IHC of liver cancer tissues or immunoblot of cell lysates (Figure S3). Moreover, from Oncomine data, we observed a positive correlation of ACTR expression with multiple glycolytic genes (Figure 6E).

4 | DISCUSSION

Sorafenib is currently the main drug in the treatment of patients with advanced HCC and resistance occurs frequently.[21-24] Our study established ACTR as a glycolytic inducer in regulating sorafenib resistance in vitro and in vivo. First, ACTR can result in sorafenib resistance, and glycolytic inhibitor 2-DG weakens the effect of ACTR and reduces sorafenib resistance in HCC. ACTR KO or KD dampens glycolysis by decreasing glucose uptake, lactate production, ATP generation and ECAR, and promotes mitochondrial respiration by increasing OCR in cultured liver cancer cells. Meanwhile, sorafenib has the same function and it enhances the inhibitory function of ECAR and the promotive function of OCR in ACTR KO or KD cells. Nude mice treated with ACTR-KO or 2-DG had reduced resistance to sorafenib, and the production of lactic acid decreased more significantly. Mechanistically, ACTR enhances glycolysis through binding to the promoters of five glycolytic genes, including GLUT1, PFKL, ENO1, PKM2 and LDHA; three of them (GLUT1, PFKL, PKM2) are correlated to sorafenib resistance. Furthermore, ACTR interacts with the central regulator of the Warburg effect, c-Myc, and promotes its recruitment to glycolytic gene promoters. Clinically, ACTR expression positively correlates with glycolytic genes in liver cancer tissues, and public databases further validate the correlation. These results suggest that the impact of ACTR on the Warburg effect is crucial for regulating susceptibility of HCC to sorafenib.

Activator of thyroid and retinoid receptor (known as SRC-3), located in a frequently amplified region, 20q12, is overexpressed in multiple cancers, such as breast, liver, gastric and prostate cancer.[14,25-27] However, the detailed mechanisms and influence of ACTR on carcinoma treatment still require investigation. Mo et al report that KD of ACTR decreased the mRNA levels of Cyclin A2, Cyclin B1, Cyclin E2 and Hes1, inhibited cell cycle progression at G1 phase, and decreased colorectal cancer cell invasiveness, suggesting that ACTR was a potential molecular target for colorectal cancer treatment.[28] Liu et al[16] report that HBx could stabilize the ACTR protein and cooperate with it to promote human HCC cell proliferation and invasiveness, and Li et al[29] demonstrate that downregulation of ACTR contributes to the anti–tumor effect of sorafenib. Multiple lines of evidence from the latest reports reveal that ACTR is related to intracellular glucose metabolism. The Warburg pathway enzyme PFKFB4 activated transcriptional coactivator ACTR by phosphorylating serine 857, thereby stimulating its transcriptional activity and promoting breast cancer aggressiveness.[30] Transcription factor SIX1 directly enhanced the expression of some glycolytic genes, promoting the aerobic glycolysis and tumor growth through ACTR histone acetyltransferases.[31] Our study provides a molecular explanation linking the ACTR's tumor-inductive role with its enhanced glycolysis in regulating sorafenib resistance.

The c-Myc proto-oncogene commonly amplified in human tumors is involved in the control of cell proliferation, differentiation and apoptosis. It performs as a transcription factor that regulates these processes by inducing expression of multiple required genes and has been demonstrated to participate in growth factor signaling in glucose metabolism. Similar to other oncogenic transcription factors, targets of c-Myc include glycolytic enzymes and LDHA.[32] Investigations of transgenic animals overexpressing MYC in the liver demonstrated induction of glycolysis by MYC in *vivo*. These animals had increased glycolytic enzyme activity and lactic acid generation in the liver.[33] In the present study, we provide evidence of a correlation between ACTR and c-Myc. ACTR facilitates c-Myc recruitment to the glycolytic gene promoters.

Metabolic reprogramming contributes to tumor growth and introduces metabolic liabilities that could be exploited to treat cancer.[34,35] Oncogenic Myc and HIF synergistically enhance the metabolic needs of cancer cells by increasing glucose uptake and converting to lactate. It has been demonstrated that this cooperation accelerates glycolytic metabolism and angiogenesis by inducing key proteins, HK2, PDK1 and vascular endothelial growth factor.[36] In addition, epigenetic regulation of metabolism affects tumorigenesis and drug resistance; this represents a new treatment strategy for utilizing metabolic changes, some of which are being evaluated in preclinical models or clinical trials.[37-39] ACTR is overexpressed in many cancers, and is correlated with poor survival and unsatisfactory therapeutic effects for patients. Because ACTR KO or KD enhances sorafenib sensitivity through suppressing glycolysis and downregulating the expression of key glycolytic enzymes, it is a promising target in HCC treatment.

In conclusion, our study demonstrates that ACTR enhances glycolysis through upregulation of glycolytic enzyme expression that is related with sorafenib resistance, inhibiting the susceptibility of tumor cells to sorafenib. ACTR abundance is positively correlated with glycolytic gene expression in HCC patients. These findings outline the importance of ACTR in the Warburg effect, HCC tumorigenesis and resistance to tumor therapy. Therefore, inhibiting ACTR in patients with overexpression of ACTR is a promising treatment for sorafenib resistance.

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DISCLOSURE

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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