

Aberrant activation of the PI3K/AKT/HIF-1 α pathway promotes glycolysis and lenvatinib resistance in liver cancer

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Received February 13, 2025; Accepted July 8, 2025

DOI: 10.3892/mmr.2025.13666

Abstract. Lenvatinib, a multi-target tyrosine kinase inhibitor, has been approved as the first-line treatment for advanced liver cancer (LC). However, its efficacy is markedly hindered by the rapid emergence of drug resistance. The phosphatidylinositol 3 kinase/protein kinase B/hypoxia-inducible factor-1 α (PI3K/AKT/HIF-1 α) signaling axis represents a key oncogenic pathway that regulates diverse biological processes, including aerobic glycolysis, and is closely associated with tumor progression and therapeutic resistance. However, the specific contribution of the PI3K/AKT/HIF-1 α pathway and aerobic glycolysis to lenvatinib resistance in LC, as well as the potential mechanistic interplay between these processes, remains inadequately elucidated. In the present study, colony formation, flow cytometry and Transwell assays were performed to evaluate the proliferative, apoptotic and invasive capabilities of LC cells. Cell aerobic glycolysis was assessed by detecting glucose uptake, lactate production, intracellular ATP levels and the expression of key glucose metabolism-related genes. Compared with their parental counterparts, lenvatinib-resistant (LR) Huh7 and HepG2 cells exhibited an enhanced glycolytic phenotype, characterized by increased glucose uptake, elevated lactate production, higher intracellular ATP levels and upregulated expression of key glycolysis-related genes. Notably, aberrant activation of the PI3K/AKT/HIF-1 α signaling pathway was observed in LR LC cells. LY294002, a specific PI3K inhibitor, effectively inhibited the PI3K/AKT/HIF-1 α pathway and glycolytic activity in LR cells. Co-administration of LY294002 with lenvatinib markedly suppressed the PI3K/AKT/HIF-1 α pathway and attenuated the glycolytic activity of Huh7-LR/HepG2-LR cells. Moreover, this combination treatment inhibited proliferation and invasion while inducing apoptosis and G₀/G₁

phase cell cycle arrest in LR cells. This evidence indicated that inhibition of the PI3K/AKT/HIF-1 α signaling pathway effectively restored the sensitivity of LR cells to lenvatinib. The findings in the present study demonstrate that aberrant activation of the PI3K/AKT/HIF-1 α pathway is required to enhance glycolysis and confers resistance to lenvatinib in LC. The combination of LY294002 with lenvatinib offers a promising strategic approach for overcoming resistance and enhancing the clinical efficacy of lenvatinib in patients with LC.

Introduction

Liver cancer (LC) remains a significant affliction to public health. It is estimated that by 2040, there will be ~1.4 million newly diagnosed cases and 1.3 million mortalities from LC worldwide, representing a substantial increase of >55% in both incidence and mortality rates compared with 2020 (1). Currently, curative surgery remains the main strategy for achieving long-term survival in patients with early-stage LC (2). Nevertheless, due to the insidious nature of early symptoms and the rapid progression of LC, the majority of cases are diagnosed at an advanced stage, resulting in the loss of opportunity for curative surgery and ultimately leading to a poor prognosis.

With the evolving development of molecular targeted therapies and immunotherapy, systemic treatment has assumed an increasingly pivotal role in the overall management of LC (3). Lenvatinib, an oral tyrosine kinase inhibitor (TKI), exerts its antitumor effect by inhibiting vascular endothelial growth factor receptors 1-3, platelet-derived growth factor receptor- α , fibroblast growth factor receptors 1-4, and the proto-oncogenes KIT and RET (4). In 2018, lenvatinib was approved as the second first-line treatment for advanced LC following sorafenib. As the REFLECT study revealed, lenvatinib was comparable to sorafenib in terms of overall survival (OS) and displayed notable improvements in objective response rate and progression-free survival (5). Despite the substantial benefits of lenvatinib in advanced LC, its efficacy still falls short of expectations for long-term survival, with a median OS of <1 year (6). The poor outcomes are mainly attributed to the rapid emergence of drug resistance, with evidence indicating that >60% of patients with LC develop tolerance to lenvatinib within 1 year of treatment initiation (7). Resistance has emerged as a major obstacle in the treatment of advanced

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Key words: phosphatidylinositol 3 kinase/protein kinase B/hypoxia-inducible factor-1 α pathway, glycolysis, LY294002, lenvatinib resistance, liver cancer

LC, highlighting the urgent need to elucidate the underlying molecular mechanisms and develop novel therapeutic strategies for overcoming lenvatinib resistance.

LC develops resistance to lenvatinib through a complex process that involves stem cell enrichment, reshaping of the immune microenvironment, epithelial-mesenchymal transitions and aberrant activation of signaling pathways (8-10). Among these mechanisms, dysregulated signaling pathways play a crucial role in resistance development. The phosphatidylinositol 3 kinase/protein kinase B/hypoxia-inducible factor-1 α (PI3K/AKT/HIF-1 α) pathway is recognized as a key intracellular signaling axis, regulating a variety of biological processes, such as cell proliferation, invasion, metabolism and angiogenesis. Substantial evidence indicates that the aberrant activation of the PI3K/AKT/HIF-1 α signaling pathway is closely linked to tumor progression and metastasis (11,12). Notably, this pathway also plays a critical role in therapeutic resistance across multiple malignancies. A recent study showed that the PI3K/AKT/HIF-1 α signaling pathway was abnormally activated in drug-resistant colon cancer cells compared with sensitive cells, and its inhibition alleviated the resistance of colon cancer cells to 5-fluorouracil (13). Similarly, Tian *et al* (14) demonstrated that suppressing the PI3K/AKT/HIF-1 α signaling cascade restored the sensitivity of breast cancer cells to doxorubicin treatment. However, the role of the PI3K/AKT/HIF-1 α signaling pathway in lenvatinib resistance in LC remains to be elucidated.

The Warburg effect, also known as aerobic glycolysis, refers to the phenomenon in which tumor cells metabolize glucose through glycolysis even in the presence of oxygen (15), playing a pivotal role in tumor progression and drug resistance. Previous studies have demonstrated that sorafenib-resistant LC cells exhibit increased aerobic glycolysis, and inhibition of key glycolytic enzymes such as 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 or pyruvate kinase M2 (PKM2), can mitigate sorafenib resistance in LC (16,17). A recent study revealed that acylphosphatase 1 (ACYPI) enhances aerobic glycolysis through modulation of lactate dehydrogenase (LDHA) activity, thereby promoting proliferation and invasion of LC cells and conferring resistance to Lenvatinib (18). Based on the aforementioned findings, it is reasonable to infer that increased glycolysis is closely linked to resistance to TKIs in the treatment of LC.

Furthermore, mounting evidence suggests that the PI3K/AKT/HIF-1 α signaling pathway not only promotes tumor growth and metastasis but also plays a crucial role in regulating glycolysis. Abnormal activation of the PI3K/AKT signaling pathway can increase the transcription and translation of HIF-1 α in tumor cells under both normoxic and hypoxic conditions (19). This, in turn, leads to the upregulation of various rate-limiting enzymes and glucose transporters, thereby increasing glycolytic activity (20,21). Studies in ovarian cancer (22), breast cancer (23) and oral cancer (24) have provided compelling evidence that the aberrant activation of the PI3K/AKT/HIF-1 α signaling pathway promotes tumor progression and induces treatment resistance by enhanced glycolysis. However, whether the PI3K/AKT/HIF-1 α signaling pathway and glycolytic reprogramming contribute to lenvatinib resistance in LC, and their potential mechanistic interplay, remain to be elucidated.

The present investigation performed a series of systematic studies to elucidate the functional roles and potential mechanistic interplay between the PI3K/AKT/HIF-1 α signaling pathway and aerobic glycolysis in lenvatinib resistance in LC. The aim of the present study was to delve into the underlying mechanisms of lenvatinib resistance and explore effective therapeutic strategies that could be used to overcome it in LC.

Materials and methods

Reagents and antibodies. Lenvatinib (cat. no. HY-10981) and a PI3K inhibitor (LY294002; cat. no. HY-10108) were purchased from MedChemExpress.

Cell source and cell culture. The Huh7 parental (Huh7-P) and HepG2 parental (HepG2-P) LC cell lines were purchased from Zhejiang Meisen Cell Technology Co., Ltd., and their authenticity was verified through short tandem repeat profiling. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Beijing Solarbio Science & Technology Co., Ltd.) supplemented with 10% fetal bovine serum (FBS; Beijing Solarbio Science & Technology Co., Ltd.) in a 37°C incubator with a 5% CO₂ atmosphere. As previously reported, two lenvatinib-resistant (LR) LC cell lines (Huh7-LR and HepG2-LR) were established by gradually exposing parental Huh7 and HepG2 cells to increasing concentrations of lenvatinib (25). The Huh7-LR and HepG2-LR cells were continuously cultured with 5 or 15 μ M lenvatinib, respectively.

Clonogenicity assay. To assess the clonogenicity of the Huh7-LR and HepG2-LR after different drug treatments, 0.5x10³ cells were seeded per well in a six-well plate, and incubated at 37°C. Cells were treated with vehicle control (DMSO), lenvatinib monotherapy, LY294002 monotherapy (20 μ M) or combination therapy for 72 h. The lenvatinib concentration was 1 μ M for Huh7-LR cells and 10 μ M for HepG2-LR cells. To optimize drug efficacy based on pharmacokinetic profiles, lenvatinib was administered at 0 h for the entire 72-h period, while LY294002 was added at 24 h post-seeding and maintained for the subsequent 48 h. Following the completion of the treatment period, cells were cultured in a standard culture medium as aforementioned for the next 11 days. After fixation with 4% paraformaldehyde for 30 min at room temperature and staining with 0.1% crystal violet for 20 min at room temperature, the colonies consisting of >50 cells were photographed and analyzed using ImageJ v1.48 software (National Institutes of Health).

Flow cytometry analysis. For cell cycle analysis of Huh7-LR and HepG2-LR cells, the cell concentration was adjusted to 1x10⁶/ml. After washing the cells with ice-cold phosphate-buffered saline (PBS), 1 ml of DNA staining solution and 10 μ l of permeabilization solution were added, and the cells were incubated in the dark for 30 min at 4°C. A cell cycle staining kit (cat. no. CCS012; MultiSciences Biotech Co., Ltd.), which included the DNA staining solution and permeabilization solution, was employed for detection. The LR cells were arrested primarily at the G₀/G₁ phase of the cell cycle. Cell cycle analysis was performed using Attune™ NxT (Thermo Fisher Scientific, Inc.) and data were analyzed with ModFit

LT v4.1.7 (Verity Software House, Inc.). For apoptosis detection, cells were seeded at a density of 1×10^6 cells per well in a 6-well plate and incubated overnight at 37°C. Thereafter, the cells were exposed to different drug-containing media in a 37°C incubator. The treatment groups, drug concentrations and treatment durations for Huh7-LR and HepG2-LR cells were consistent with those utilized in the clonogenic assay. Subsequently, the cells were harvested and stained with Annexin V-FITC and propidium iodide. The apoptotic rate was measured by flow cytometry (FC500; Beckman Coulter, Inc.) and analyzed using CXP Analysis software (version 2.0; Beckman Coulter, Inc.).

Transwell assay. Matrigel (cat. no. 356234; BD Biosciences) was thawed and diluted 1:8 with serum-free culture medium at 4°C using pre-chilled consumables. Subsequently, 50 μ l of the diluted Matrigel was evenly applied to each Transwell insert and these were incubated at 37°C for 30 min to allow gel solidification. Huh7-LR and HepG2-LR cells (2×10^4) were trypsinized, resuspended in 200 μ l serum-free DMEM, and added to the upper chamber of the Transwell device, while 600 μ l of DMEM containing 10% FBS was added to the lower chamber. After a 72-h incubation, the chambers were removed, fixed by 4% paraformaldehyde at room temperature for 20 min, and stained with 1% crystal violet for 20 min at room temperature, while the cells in the upper chamber were wiped with a cotton swab. Images were acquired using an Olympus BX43 optical light microscope (Olympus Corporation), with three random fields of view captured per chamber.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from LC cells using the TRIzol reagent (Thermo Fisher Scientific, Inc.) and cDNA synthesis was performed using a reverse transcription kit (Proteinase Biotech Co., Ltd.) according to the manufacturer's protocol. Subsequently, RT-qPCR was conducted using the MagicSYBR Mixture with a CFX96 Touch™ real-time PCR detection system (Bio-Rad Laboratories Inc.) following instructions provided by the manufacturer. The thermocycling conditions were as follows: Initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec.

The primer sequences utilized in this study (synthesized by Sangon Biotech Co., Ltd.) were as follows: LDHA-forward (F), 5'-GATTCAGCCCCGATTCCGTTACC-3'; LDHA-reverse (R), 5'-AGAGACACCAGCAACATTCATTCC-3'; HK2-F, 5'-TGG AACTGGTGGA-AGGAGAAGAG-3'; HK2-R, 5'-TCTGTG CGGAAGTCATCTAGGC-3'; GLUT1-F, 5'-AGG-AAGAGA GTCGGCAGATGATG-3'; GLUT1-R, 5'-TGGAGTAATAGA AGACAGCGTT-GATG-3'; HIF-1 α -F, 5'-CGCAAGTCCTCA AAGCACAGTTAC-3'; HIF-1 α -R, 5'-ATTC-ATCAGTGGT GGCAGTGGTAG-3'; β -actin-F, 5'-TCG-TGCGTGACATTA AGGAG-AAGC-3'; β -actin-R, 5'-GGCGTACAGGTCTTT GCGGATG-3'.

The results were normalized to β -actin expression for mRNA measurement, and the relative expression of the target gene was calculated using the $2^{-\Delta\Delta C_q}$ comparative method (26).

Western blotting. Total protein was extracted by lysing liver cancer cells using radioimmunoprecipitation assay lysis

buffer (Beijing Solarbio Science & Technology Co., Ltd.). Equal amounts of protein samples ($\sim 25 \mu$ g/lane), quantified by bicinchoninic acid assays, were separated by SDS-PAGE on 8-12% polyacrylamide gels and subsequently transferred onto Immobilon®-P transfer membranes (cat. no. IPVH00010; Merck KGaA). After blocking with 5% non-fat milk at room temperature for 2 h, membranes were incubated at 4°C overnight with the following primary antibodies: Anti-GLUT1 (1:1,000; cat. no. A11727; ABclonal Biotech Co., Ltd.), anti-HK-2 (1:1,000; cat. no. A0994; ABclonal Biotech Co., Ltd.), anti-LDHA (1:1,000; cat. no. A21893; ABclonal Biotech Co., Ltd.), anti-PI3K (1:1,000; cat. no. A17433; ABclonal Biotech Co., Ltd.), anti-pPI3K (1:1,000; cat. no. AP0854; ABclonal Biotech Co., Ltd.), anti-AKT (1:1,000; cat. no. A22533; ABclonal Biotech Co., Ltd.), anti-pAKT (1:1,000; cat. no. AP0637; ABclonal Biotech Co., Ltd.) and anti-HIF-1 α (1:1,000; cat. no. bs0737R; BIOSS). Thereafter, the membranes were incubated with HRP-conjugated secondary antibodies (1:3,000; cat. nos. ZB2301 and ZB2305; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) at room temperature for 90 min, followed by incubation with an enhanced chemiluminescence reagent (cat. no. C05-07004; BIOSS). Finally, the bands were imaged using a Tanon 4800 automated imaging system (Tanon Science and Technology Co., Ltd.). The gray-scale intensity of protein bands was quantified using Tanon Gel Imaging System software (version 4.2; Tanon Science and Technology Co., Ltd.).

2-NBDG uptake assay. The glucose uptake ability of cells was measured by 2-NBDG (AbMole Bioscience Inc.). Briefly, cells were seeded into 6-well plates at a density of 1×10^6 per well and subjected to different treatments. The treatment groups, drug concentrations and exposure durations were identical to those employed in the clonogenic assay. Following treatment, 2-NBDG was added to the cells at a final concentration of 10 μ M, and the cells were incubated for 30 min at 37°C. The cells were subsequently washed twice with PBS buffer to eliminate any unabsorbed probes. The mean fluorescence intensity of samples was measured by flow cytometry (FC500; Beckman Coulter, Inc.), and the median was used for statistical analysis.

Extracellular lactate assay. The lactate contents of cell supernatant samples were determined using the Lactic Acid Content Assay Kit (cat. no. L256; Dojindo Laboratories, Inc.). After collecting the sample of cell culture supernatant, the extracellular lactate concentrations were determined using a microplate reader (Fluoroskan™ FL; Thermo Fisher Scientific, Inc.) at a wavelength of 450 nm, according to the instructions provided by the manufacturer.

Detection of cellular ATP levels. Intracellular ATP levels were determined using an ATP assay kit (cat. no. S0027; Beyotime Institute of Biotechnology). Cells were plated at a density of 2×10^5 per well in 6-well plates and treated according to the experimental design as described for the clonogenic assay. After lysis, the supernatants were obtained by centrifugation at 12,000 $\times$ g for 5 min at 4°C and mixed with ATP detection working solution included in the ATP assay kit. Fluorescence intensity was measured using a microplate reader (Varioskan

LUX; Thermo Fisher Scientific, Inc.) and ATP concentrations were calculated based on a standard curve. The results were normalized to protein content (BCA assay).

Statistical analysis. Statistical analyses were performed using SPSS Statistics version 26.0 (IBM Corp.) and the figures were generated using GraphPad Prism 8.0 (Dotmatics). All measurement data were obtained in triplicate and are shown as the mean $\pm$ SD. A two-tailed unpaired Student's t-test was used to compare variables between two groups. One-way ANOVA was performed for multi-group comparisons using Tukey's post hoc tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

LR cells exhibit an enhanced glycolytic phenotype. The present study aimed to investigate the mechanisms underlying lenvatinib resistance in LC. Thus, the previously established LR cell models, Huh7-LR and HepG2-LR, were used, which were generated through gradually exposing Huh7 and HepG2 cells to increasing concentrations of lenvatinib. Aberrant metabolism is a major hallmark of cancer. Aerobic glycolysis, an essential part of metabolic reprogramming, is closely associated with drug resistance in various tumors (27). Recent studies have reported that prolonged exposure to lenvatinib can enhance aerobic glycolysis, potentially driving lenvatinib resistance in LC (28,29). Therefore, the present study conducted a comparative analysis of glycolytic activity between parental cells and their LR derivatives. Glucose serves as the main energy source for tumor cells, and glucose uptake is a crucial indicator of glycolytic activity. The uptake of the fluorescence-labeled glucose analogue 2-NBDG was assessed, and a significantly increased 2-NBDG uptake in LR LC cells was observed (Fig. 1A and B). Lactate, a main metabolic byproduct of glycolysis, is of notable importance in assessing the glycolytic levels of tumor cells. Lactate assays revealed significantly higher lactate production in Huh7-LR and HepG2-LR cells compared with their parental counterparts (Fig. 1C and D). To further evaluate metabolic alterations, intracellular ATP levels were compared between the two groups. The results showed that, compared with the parental cells, the intracellular ATP levels were significantly elevated in LR LC cells (Fig. 1E and F). In addition, the expression of three key glycolysis-related genes was analyzed, namely GLUT1, LDHA and HK2. As shown in Fig. 1G and H, the mRNA expression levels of GLUT1, LDHA and HK2 were significantly upregulated in LR LC cells compared with their parental cells. Consistently, western blotting analysis further confirmed a significant increase in GLUT1, LDHA and HK2 protein expression in resistant cells (Fig. 1I and J). These findings collectively indicate that LR LC cells exhibit an enhanced glycolytic phenotype, suggesting an association between lenvatinib resistance and increased glycolysis.

PI3K/AKT/HIF-1 α pathway is aberrantly activated in LR cells. Increased aerobic glycolysis has been implicated in the acquisition of various malignant tumor phenotypes, including drug resistance. To further elucidate the mechanisms underlying lenvatinib resistance in LC, the regulation of glycolysis

was investigated with a focus on the PI3K/AKT/HIF-1 α signaling pathway, which serves as a key regulator of glycolytic activity and plays a crucial role in promoting tumor resistance (30). The expression levels of key components within the PI3K/AKT/HIF-1 α signaling pathway was compared between Huh7/HepG2 parental cells and their LR LC cells. Analysis revealed significantly elevated levels of phosphorylated PI3K/PI3K (pPI3K/PI3K), phosphorylated AKT/AKT (pAKT/AKT) and HIF-1 α in Huh7-LR/HepG2-LR cells compared with their respective parental cell lines (Fig. 2A and C). Additionally, HIF-1 α mRNA expression was assessed in Huh7/HepG2 parental and drug-resistant strains using RT-qPCR. The results demonstrated a significant upregulation of HIF-1 α mRNA expression in Huh7-LR and HepG2-LR cells compared with their parental counterparts (Fig. 2B and D). These findings indicate that the PI3K/AKT/HIF-1 α signaling pathway is aberrantly activated in LR LC cells. This dysregulation appears to be associated with acquired lenvatinib resistance in LC.

LY294002 significantly suppresses the PI3K/AKT/HIF-1 α pathway in LR cells. To further elucidate the role of the PI3K/AKT/HIF-1 α signaling pathway in increased glycolysis and lenvatinib resistance, LR cells were treated with 20 μ mol/l (31) LY294002, a broad-spectrum PI3K inhibitor. The expression levels of the key components within the PI3K/AKT/HIF-1 α signaling pathway were subsequently assessed using western blotting. LY294002 significantly reduced the phosphorylation levels of PI3K and AKT, as well as the protein levels of HIF-1 α in both Huh7-LR (Fig. 3A) and HepG2-LR cells compared with those in control cells and cells treated with lenvatinib (Fig. 3B). In addition, co-administration of LY294002 with lenvatinib further resulted in an even greater suppression of the PI3K/AKT/HIF-1 α pathway in LR cells compared with LY294002 treatment alone (Fig. 3A and B).

Dysregulated PI3K/AKT/HIF-1 α pathway contributes to enhanced glycolysis in LR cells. To decipher the relationship between the PI3K/AKT/HIF-1 α signaling pathway and glycolysis in LR LC cells, glycolytic activity following pharmacological inhibition of this pathway was investigated. The findings of the present study revealed that treatment with the PI3K inhibitor LY294002 significantly reduced glucose uptake (Fig. 4A and B), lactate production (Fig. 4C and D) and intracellular ATP levels (Fig. 4E and F), compared with both the control and lenvatinib monotherapy groups. In addition, the expression levels of glycolysis-related genes among different treatment groups were compared. In Huh7-LR cells, the mRNA expression levels of GLUT1, LDHA and HK2 were significantly decreased following LY294002 treatment compared with the control group (Fig. 4G). A similar trend was observed in HepG2-LR cells, although the reduction in GLUT1 expression compared with the control group did not reach statistical significance (Fig. 4H; $P = 0.053$). Notably, the combination of LY294002 and lenvatinib produced a more pronounced suppression of glycolytic activity than LY294002 alone, with both treatment groups showing a significant reduction compared with the control group (Fig. 4A-H). These results demonstrate that the PI3K/AKT/HIF-1 α signaling pathway plays a critical role in regulating glycolysis in LR

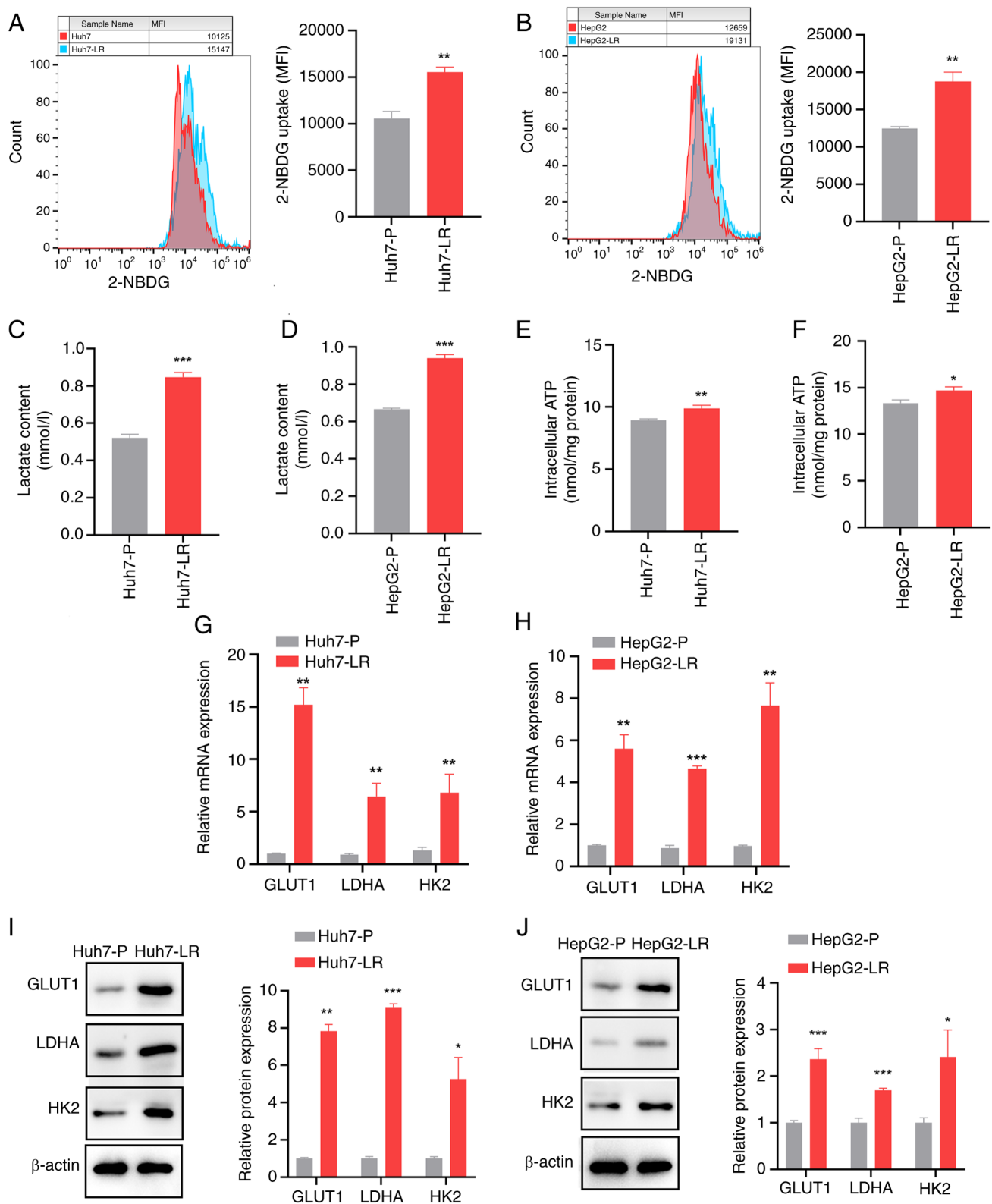


Figure 1. LR LC cells exhibit an enhanced glycolytic phenotype. Flow cytometry analysis of 2-NBDG uptake by (A) Huh7 and (B) HepG2 LR cells and their P cells. Lactate production in (C) Huh7 and (D) HepG2 LR cells and their P cells. Intracellular ATP levels assay in the P and LR (E) Huh7 and (F) HepG2 cells. Reverse transcription-quantitative PCR analysis of GLUT1, LDHA and HK2 in the P and LR (G) Huh7 and (H) HepG2 cells. Western blotting of GLUT1, LDHA and HK2 in the P and LR (I) Huh7 and (J) HepG2 cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with P cells. LC, liver cancer; GLUT1, glucose transporter 1; HK2, hexokinase 2; LDHA, lactate dehydrogenase; LR, lenvatinib-resistant; P, parental; MFI, mean fluorescence intensity.

cells. Furthermore, combined LY294002 and lenvatinib treatment attenuates glycolytic activity through synergistic suppression of the PI3K/AKT/HIF-1 α pathway.

LY294002 alleviates lenvatinib resistance by targeting the PI3K/AKT/HIF-1 α signaling pathway. To investigate the effect of inhibiting the PI3K/AKT/HIF-1 α pathway on

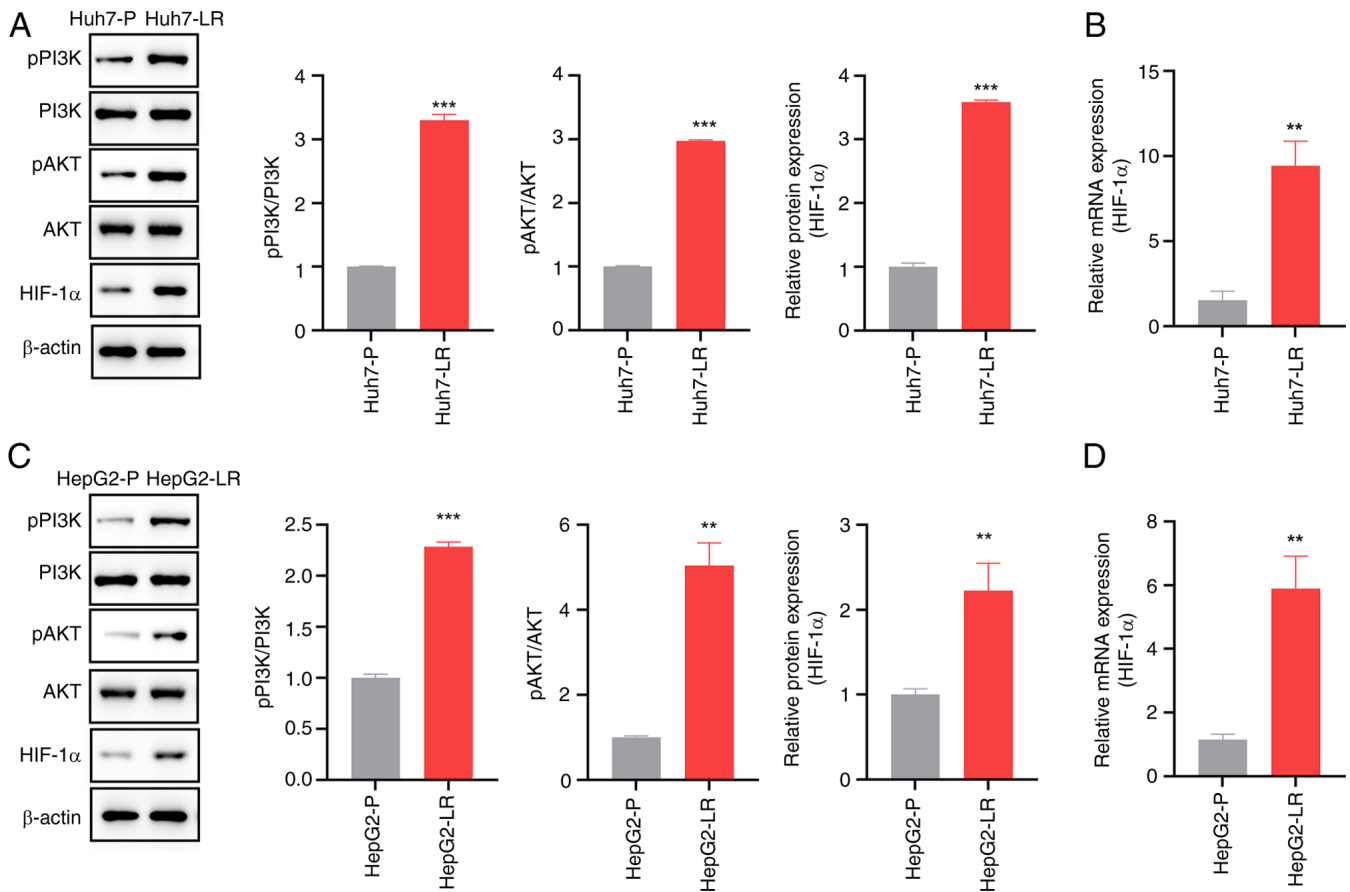


Figure 2. Aberrant activation of the PI3K/AKT/HIF-1 α signaling pathway in LR LC cells. Western blotting analysis of pPI3K, PI3K, pAKT, AKT and HIF-1 α in (A) Huh7 and (C) HepG2 LR LC cells and their P cells. Reverse transcription-quantitative PCR analysis of HIF-1 α expression in (B) Huh7 and (D) HepG2 LR LC cells and their P cells. ** $P < 0.01$ and *** $P < 0.001$ compared with their respective P cells. LC, liver cancer; LR, lenvatinib-resistant; P, parental; PI3K, phosphatidylinositol 3 kinase; pPI3K, phosphorylated phosphatidylinositol 3 kinase; AKT, protein kinase B; pAKT, phosphorylated protein kinase B; HIF-1 α , hypoxia-inducible factor-1 α .

lenvatinib resistance in LR cells, the clonogenic potential of Huh7-LR and HepG2-LR cells under different treatment conditions was evaluated. Lenvatinib monotherapy failed to inhibit colony formation in LR cells compared with the control. Notably, colony formation was significantly reduced following LY294002 treatment in both Huh7-LR and HepG2-LR cells compared with control or lenvatinib treatment alone. Additionally, the combination of LY294002 and lenvatinib led to a further reduction in colony formation compared with LY294002 monotherapy (Fig. 5A and B). Next, the invasive capacity of LC cells was assessed using Transwell invasion assays. LY294002 treatment significantly decreased the number of invading cells compared with control and lenvatinib treatment. Furthermore, the combined treatment with LY294002 and lenvatinib resulted in a significant reduction in tumor invasion in LR LC cells (Fig. 5C and D). These results demonstrated that inhibition of the PI3K/AKT/HIF-1 α signaling pathway significantly enhanced the anti-proliferative and anti-invasive effects of lenvatinib in LR cells.

Subsequently, apoptosis rates in LR cells were analyzed under various treatment conditions. While lenvatinib monotherapy did not alter apoptosis rates in resistant cell lines compared with control, LY294002 significantly induced apoptosis in both LR LC cell lines. As anticipated, the combination of LY294002 and lenvatinib enhanced apoptosis in LR cells

to a greater extent than LY294002 alone (Fig. 6A and B). Finally, the effect of LY294002 and lenvatinib on cell cycle distribution was examined by flow cytometry analysis. Results revealed that inhibition of the PI3K/AKT/HIF-1 α signaling pathway significantly induced G₀/G₁ phase cell cycle arrest in LR cells, with the combination of LY294002 and lenvatinib further enhancing this arrest in Huh7-LR and HepG2-LR cells compared with LY294002 treatment alone (Fig. 6C and D). These results indicate that inhibition of the PI3K/AKT/HIF-1 α signaling pathway significantly potentiates lenvatinib-induced G₀/G₁ phase cell cycle arrest and apoptosis. Collectively, these findings suggest that the PI3K/AKT/HIF-1 α pathway plays a pivotal role in mediating lenvatinib resistance in LC. Inhibition of this signaling pathway attenuates glycolytic activity in LR LC cells, thereby resensitizing them to lenvatinib treatment.

Discussion

Currently, systemic therapies have become the mainstay treatment for advanced-stage LC, with sorafenib, lenvatinib, and a combination of atezolizumab and bevacizumab approved as first-line treatments in LC. Compared with sorafenib, lenvatinib demonstrates a higher disease control rate, longer progression-free survival and superior health-related quality of life benefits in the treatment of LC (32). Recent evidence

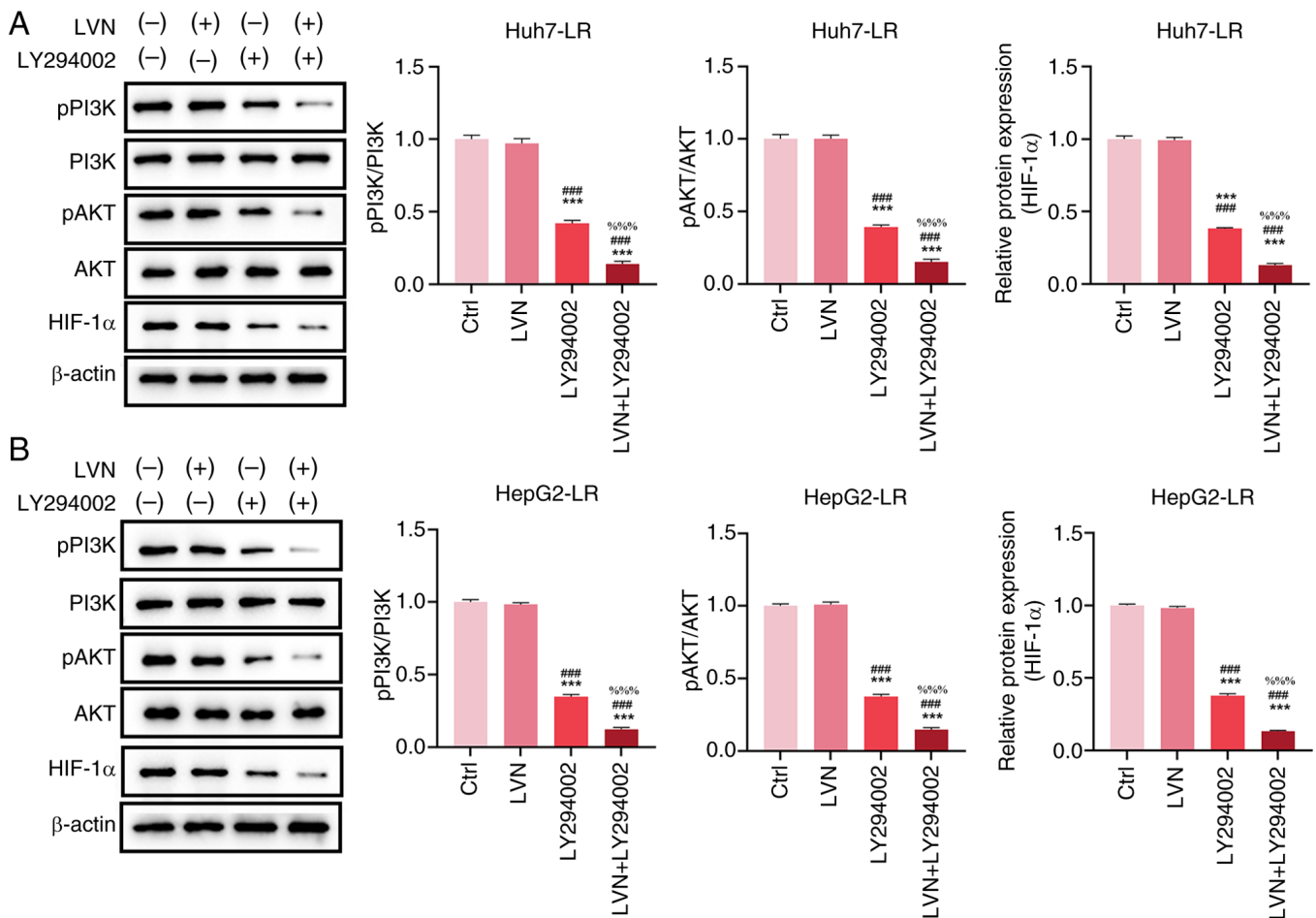


Figure 3. LY294002 suppresses the PI3K/AKT/HIF-1 α pathway in LR cells. Western blotting of pPI3K, PI3K, pAKT, AKT and HIF-1 α expression in (A) Huh7-LR and (B) HepG2-LR cells under various treatment conditions. ***P<0.001 vs. Ctrl; ###P<0.001 vs. lenvatinib; %%%P<0.001 vs. LY294002. LR, lenvatinib-resistant; Ctrl, control treatment; LVN, lenvatinib treatment; PI3K, phosphatidylinositol 3 kinase; pPI3K, phosphorylated phosphatidylinositol 3 kinase; AKT, protein kinase B; pAKT, phosphorylated protein kinase B; HIF-1 α , hypoxia-inducible factor-1 α .

also suggests that lenvatinib is more effective than the combination of atezolizumab and bevacizumab for patients with LC with non-viral etiology (33). Despite these clinical advantages, the overall response rate of lenvatinib is only ~24% (5). The emergence of resistance has become a major obstacle limiting the efficacy of lenvatinib. Elucidating the mechanisms underlying lenvatinib resistance and identifying strategies to overcome this therapeutic challenge are crucial for improving prognosis in patients with LC. The present study revealed that LR LC cells exhibited an enhanced glycolytic phenotype. The PI3K/AKT/HIF-1 α signaling pathway was aberrantly activated in LR LC cells compared with their parental counterparts. Based on the present study, targeting the PI3K/AKT/HIF-1 α pathway can reduce glycolytic levels and enhance the sensitivity of LR LC cells to lenvatinib.

The PI3K/AKT pathway is frequently hyperactivated in various malignancies and plays a pivotal role in multiple cellular processes that contribute to tumorigenesis and disease progression (34). HIF-1 α , a downstream target of PI3K/AKT, undergoes enhanced transcription and translation in response to activated PI3K/AKT signaling (30). The PI3K/AKT/HIF-1 α signaling pathway is an oncogenic pathway, and its aberrant activation is closely associated with tumorigenesis and progression. Ren *et al* (35) reported that activation of the

PI3K/AKT/HIF-1 α signaling pathway can enhance the invasion and metastasis of LC. Multiple investigations have indicated that targeting the PI3K/AKT/HIF-1 α signaling pathway effectively inhibits proliferation, metastasis and energy metabolism across diverse tumor types (36-38). Furthermore, increasing evidence suggests that aberrant activation of the PI3K/AKT/HIF-1 α pathway contributes to the development of therapeutic resistance. Collectively, the available data highlights the PI3K/AKT/HIF-1 α pathway as a promising therapeutic target for overcoming drug resistance (39,40).

The present study represents, to the best of our knowledge, the first comprehensive investigation of the role of the PI3K/AKT/HIF-1 α signaling pathway in lenvatinib resistance in LC. The data demonstrated that the PI3K/AKT/HIF-1 α signaling pathway was aberrantly activated in LR cells. In addition, pharmacological inhibition of this pathway suppressed cell proliferation and invasion, induced apoptosis and triggered G₀/G₁ phase arrest in LR cells. Notably, combining LY294002, a broad-spectrum PI3K inhibitor, with lenvatinib effectively overcame lenvatinib resistance by targeting the PI3K/AKT/HIF-1 α signaling axis. These findings are consistent with those previously reported by Yan *et al* (41), who demonstrated that ectopic overexpression of AKT in lenvatinib-sensitive Hep3B and

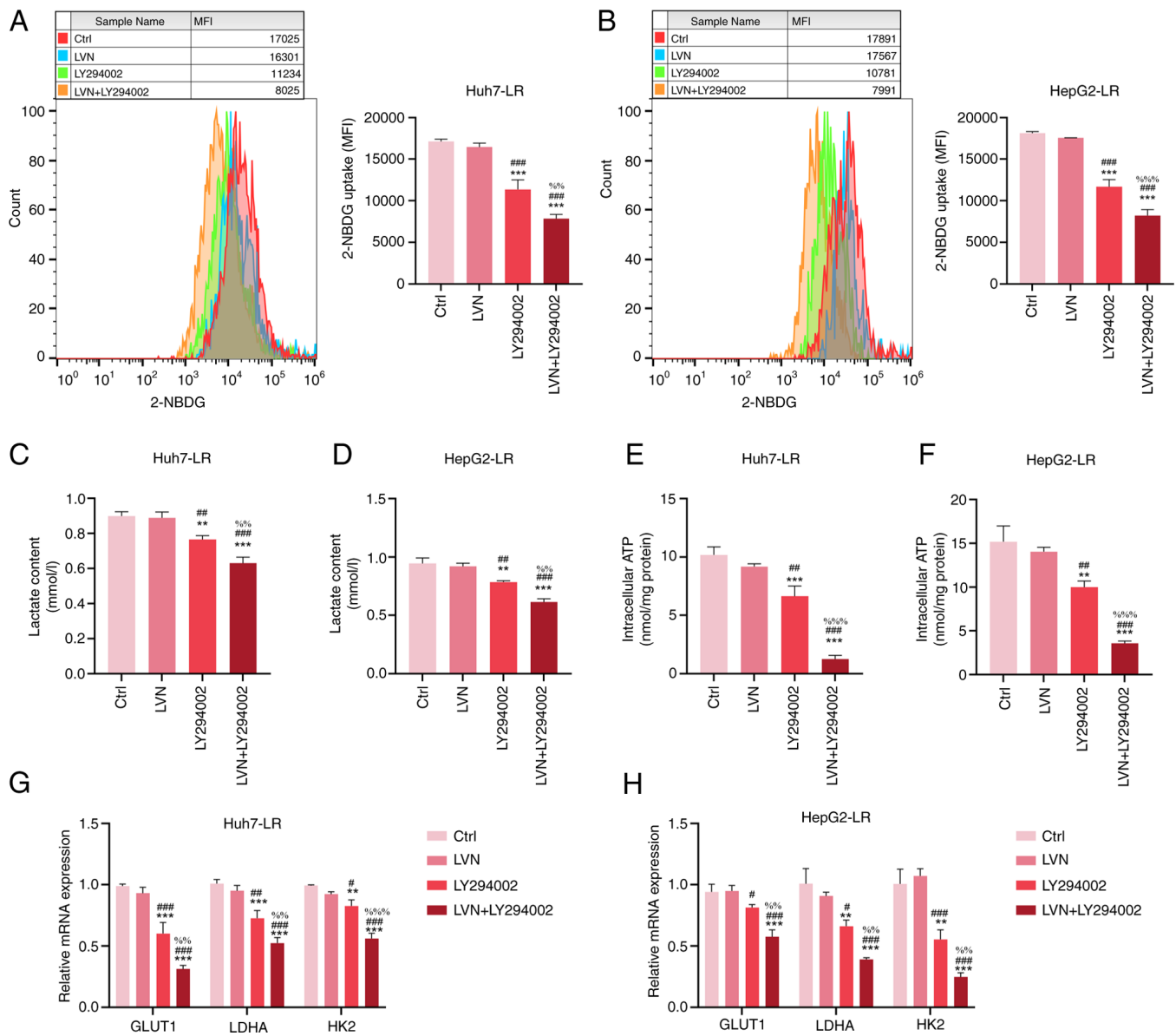


Figure 4. Dysregulated activation of the PI3K/AKT/HIF-1 α pathway contributes to increased glycolysis in LR cells. Flow cytometry analysis of 2-NBDG uptake by (A) Huh7 and (B) HepG2 LR cells following different treatments. Lactate analysis in the supernatant of (C) Huh7-LR and (D) HepG2-LR LC cells following different treatments. Intracellular ATP in (E) Huh7 and (F) HepG2 LR cells following different treatments. Reverse transcription-quantitative PCR analysis of GLUT1, LDHA and HK2 in (G) Huh7-LR and (H) HepG2-LR LC cells following different treatments. ** $P < 0.01$ and *** $P < 0.001$ vs. Ctrl; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs. lenvatinib; % $P < 0.01$ and %% $P < 0.001$ vs. LY294002. LR, lenvatinib-resistant; LC, liver cancer; Ctrl, control treatment; LVN, lenvatinib treatment; PI3K, phosphatidylinositol 3 kinase; pPI3K, phosphorylated phosphatidylinositol 3 kinase; AKT, protein kinase B; pAKT, phosphorylated protein kinase B; HIF-1 α , hypoxia-inducible factor-1 α ; GLUT1, glucose transporter 1; HK2, hexokinase 2; LDHA, lactate dehydrogenase; MFI, mean fluorescence intensity.

Huh7 cell lines conferred resistance to lenvatinib treatment in LC. Additionally, another study showed that MK-2206, an AKT inhibitor, resensitized LR LC cells to lenvatinib treatment (42). All of the evidence confirmed the crucial role of the aberrant activation of the AKT signaling pathway in lenvatinib resistance in LC. Specifically, the present study identified that HIF-1 α , a key downstream effector of the PI3K/AKT pathway, is also significantly upregulated in LR LC cells. The comprehensive PI3K/AKT/HIF-1 α axis was emphasized and elucidated its critical contribution to lenvatinib resistance in LC. While previous studies have suggested that the PI3K/AKT pathway regulates HIF-1 α expression under hypoxic conditions (43), Yeh *et al* (19)

indicated that this pathway upregulates HIF-1 α transcription and translation independent of oxygen levels. A recent investigation demonstrated that PI3K/AKT signaling regulates both HIF-1 α mRNA and protein expression in 5-fluorouracil-resistant colorectal cancer cells in response to reactive oxygen species (ROS) overload rather than oxygen deprivation (13). The present study also revealed that, under normoxic conditions, aberrant activation of the PI3K/AKT signaling pathway upregulated HIF-1 α , thereby contributing to lenvatinib resistance in LC. In addition, the accumulation of ROS has also been recognized as an important factor in inducing lenvatinib resistance in LC (44). However, whether ROS contribute to lenvatinib

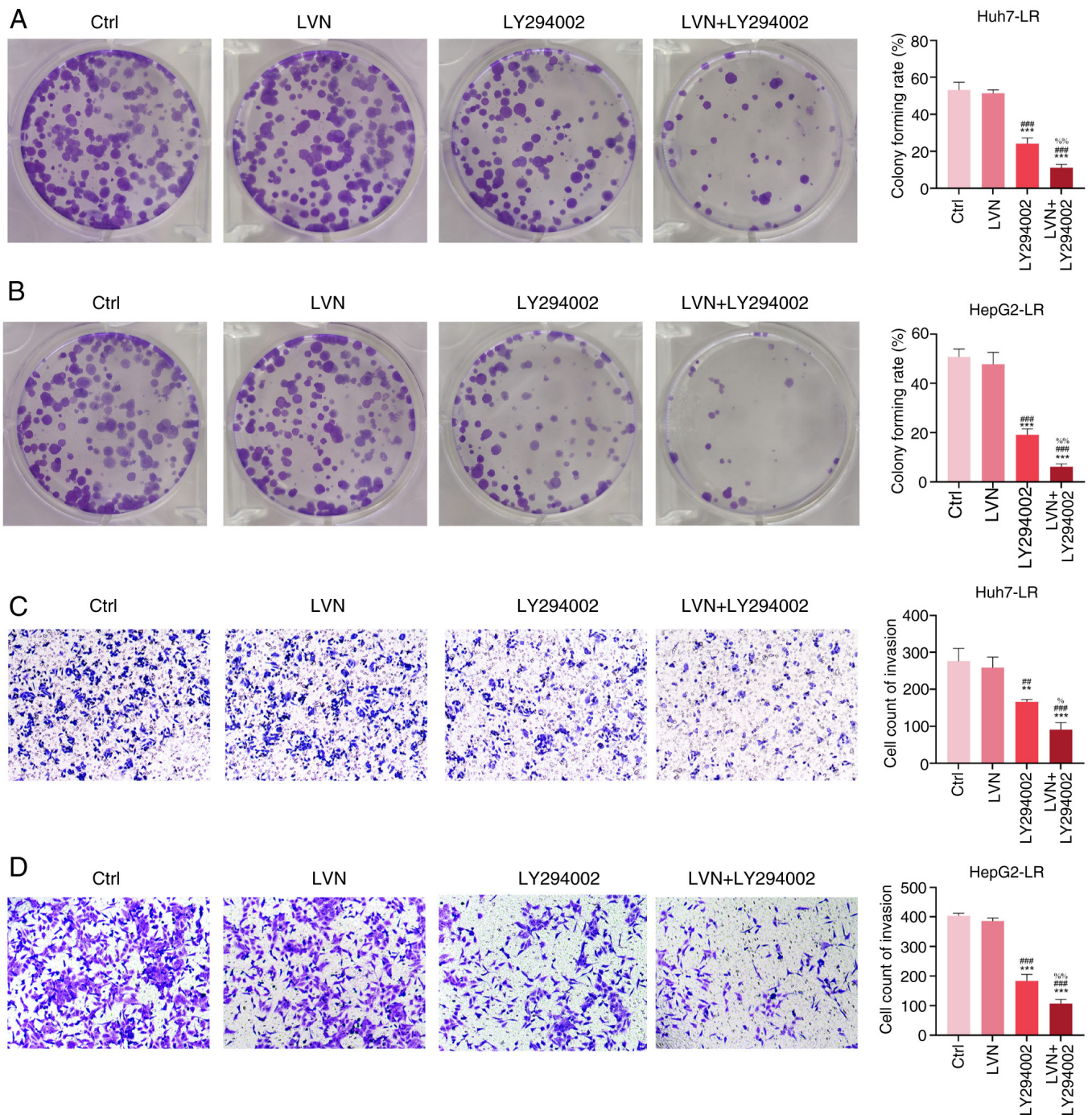


Figure 5. LY294002 alleviates LR by inhibiting colony formation and invasion in LR LC cell lines. Colony formation assays for (A) Huh7-LR and (B) HepG2-LR cells following different treatments. Invasion assays following different treatments in (C) Huh7-LR and (D) HepG2-LR LC cells. ** $P < 0.01$ and *** $P < 0.001$ vs. Ctrl; ## $P < 0.01$ and ### $P < 0.001$ vs. lenvatinib; % $P < 0.05$ and %% $P < 0.01$ vs. LY294002. LR, lenvatinib-resistant; LC, liver cancer; Ctrl, control treatment; LVN, lenvatinib treatment.

resistance through the activation of the PI3K/AKT/HIF-1 α signaling cascade requires further investigation.

Normal differentiated cells primarily rely on mitochondrial oxidative phosphorylation for energy supply, whereas most cancer cells preferentially utilize aerobic glycolysis (45). Growing evidence indicates that enhanced aerobic glycolysis facilitates tumor cell proliferation and contributes significantly to the development of therapeutic resistance across various malignancies. Compared with their parental cells, gemcitabine-resistant BxPC3 and PANC1 pancreatic cancer

cells exhibit a significantly enhanced glycolytic phenotype. Further studies have revealed that long non-coding RNA HIF1A-AS1 enhances glycolysis through regulation of the AKT/YB1/HIF-1 α pathway, thereby promoting gemcitabine resistance in pancreatic cancer (46). Li *et al* (16) demonstrated that sorafenib-resistant LC cells display increased glucose uptake, elevated lactate production and higher expression of key glycolytic enzymes relative to parental cells. Notably, inhibition of glycolysis effectively attenuates sorafenib resistance in LC. The present study found that, compared with

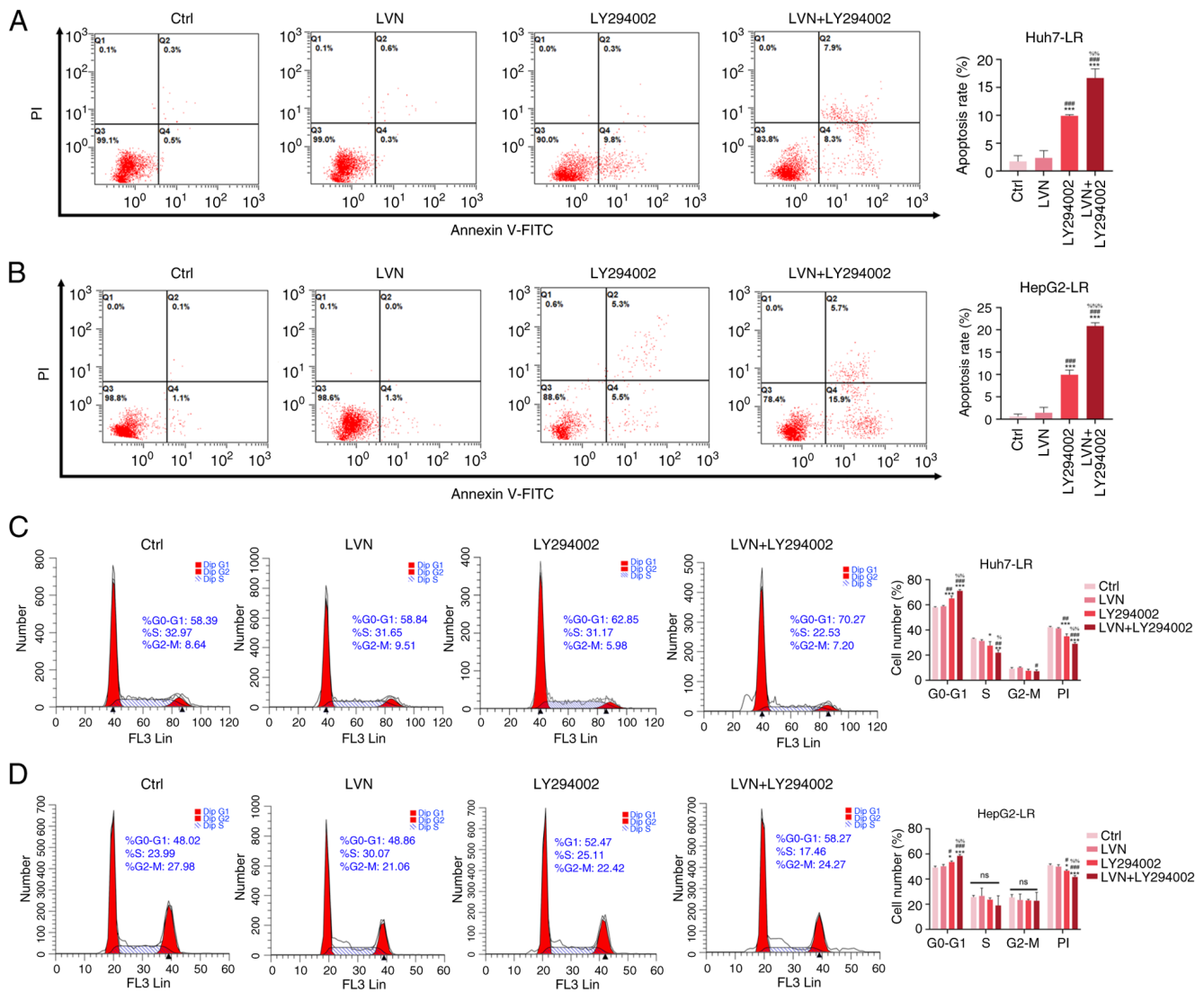


Figure 6. LY294002 alleviates LR by inducing apoptosis and G₀/G₁ phase arrest in LR LC cells following different treatments. Cell cycle distribution in (C) Huh7-LR and (D) HepG2-LR LC cells following different treatments. **P*<0.05, ***P*<0.01 and ****P*<0.001 vs. Ctrl; #*P*<0.05, ##*P*<0.01 and ###*P*<0.001 vs. Lenvatinib; %*P*<0.05, %%*P*<0.01 and %%%*P*<0.001 vs. LY294002. LR, lenvatinib-resistant; LC, liver cancer; Ctrl, control treatment; LVN, lenvatinib treatment; PI, propidium iodide; ns, not significant.

parental cells, LR LC cells exhibited significantly elevated glucose uptake, increased lactate production, higher intracellular ATP levels and upregulated expression of key glycolytic genes. These findings collectively demonstrated that LR LC cells manifest an enhanced glycolytic phenotype, and this metabolic reprogramming was intimately associated with lenvatinib resistance in LC. Consistent with observations in the present study, evidence from proteomics and metabolomics analyses have demonstrated that both sorafenib- and lenvatinib-resistant LC cells preferentially enhance glycolysis and glucose metabolism pathways compared with their sensitive counterparts (47).

Various factors drive the occurrence of aerobic glycolysis in tumor cells, with the PI3K/AKT/HIF-1 α pathway playing a pivotal role in regulating this metabolic process (48). In addition, an increasing number of studies have demonstrated that the PI3K/AKT/HIF-1 α signaling pathway promotes tumor resistance by enhancing aerobic glycolysis (49,50). Compared with their parental cells, cisplatin-resistant lung

cancer cells exhibit enhanced aerobic glycolysis. Inhibition of the PI3K/AKT/HIF-1 α signaling pathway attenuates glycolytic activity in cisplatin-resistant lung cancer cells, thereby enhancing their sensitivity to cisplatin treatment (49). Sun *et al* (50) found that Jiedu Sangen Decoction reverses 5-fluorouracil resistance in human colon cancer cells by suppressing glycolysis mediated by the PI3K/AKT/HIF-1 α signaling pathway. The present study revealed aberrant activation of the PI3K/AKT/HIF-1 α signaling pathway in LR LC cells, concomitant with enhanced glycolytic activity. Blockade of the PI3K/AKT/HIF-1 α signaling pathway significantly reduced glycolytic metabolism in resistant cells. Simultaneously, the ability of lenvatinib to inhibit proliferation and invasion, as well as to induce apoptosis in resistant cells, was markedly enhanced, thereby improving its efficacy against LR LC. Previous studies suggested that targeting the glycolytic pathway by inhibiting key enzymes such as HK2 (51), GLUT1 (52) and PKM2 (53) may serve as a potential broad-spectrum therapeutic strategy for overcoming drug

resistance. The present study demonstrated that inhibition of the PI3K/AKT/HIF-1 α signaling axis significantly attenuated glycolytic activity and subsequently reversed lenvatinib resistance. This therapeutic approach may confer superior efficacy compared to strategies targeting individual glycolytic enzymes, as the PI3K/AKT/HIF-1 α pathway functions as a critical upstream regulator governing the entire glycolytic cascade.

Lactylation, a recently identified post-translational protein modification, has been closely associated with the development of therapeutic resistance in various malignancies (54,55). The present study demonstrated that LR cells exhibited enhanced glycolysis and significantly elevated lactate levels compared with parental cells. The enhanced glycolysis metabolism may lead to substantial lactate accumulation, which subsequently serves as a substrate for histone lactylation. Of note, this metabolic alteration appears to be a characteristic molecular signature associated with acquired resistance to lenvatinib therapy in LC. Multiple studies have confirmed that LR LC cell lines exhibit increased lactate levels and demonstrate significantly elevated lactylation levels, which, as a direct promoter of gene transcription, confers resistance to lenvatinib therapy (56,57). A recent study identified a positive feedback regulation between glycolysis and histone lactylation that drives oncogenesis in pancreatic ductal adenocarcinoma (58). Moreover, another study revealed that histone lactylation can accelerate glycolysis by activating the PI3K/AKT/HIF-1 α signaling pathway in endometrial carcinoma (59). Whether there exists a potential positive feedback loop between histone lactylation, PI3K/AKT/HIF-1 α signaling pathway activation and enhanced glycolysis, as well as its contribution to lenvatinib resistance in LC, remains unclear. The next step in our research is to comprehensively characterize this proposed regulatory mechanism through *in vitro* and *in vivo* studies.

The critical role of the PI3K/AKT/HIF-1 α signaling pathway in lenvatinib resistance in LC has been elucidated in the present study, and the aforementioned findings may provide a novel therapeutic strategy to improve the prognosis of patients with LC. However, several potential limitations of the present study should be acknowledged. Firstly, the effects of combination treatment in two types of LC cells were examined, but this investigation did not extend to pre-clinical animal models of LC. Although such models could provide additional insights, our laboratory currently lacks the capability to perform *in vivo* imaging of small animals to assess glycolytic activity. Secondly, the upstream targets responsible for the activation of the PI3K/AKT/HIF-1 α signaling pathway in LR LC cells remain unidentified. In future studies, multi-omics and bioinformatics techniques will be used to further explore the upstream targets of this pathway and attempt to identify serum biomarkers associated with this resistance mechanism. These efforts aim to develop more effective strategies for overcoming lenvatinib resistance in LC and ultimately improving patient outcomes.

In conclusion, the present investigation demonstrated that aberrant activation of the PI3K/AKT/HIF-1 α signaling pathway represents a critical mechanism underlying lenvatinib resistance in LC. Targeted inhibition of this pathway effectively attenuated glycolytic metabolism and mitigated lenvatinib resistance in LC. These findings provide mechanistic insights

into lenvatinib resistance and may contribute to the development of novel and efficacious treatment strategies for patients with advanced LC.

Acknowledgements

Not applicable.

Funding

This research was funded by grants from the Hebei Natural Science Foundation (grant no. H2020206589), the Medical Science Research Project of Hebei (grant no. 20240474) and the 2024 Hebei Province Foreign Talent Introduction Program: A Multi-Omics Study on the Role of Tumor-Infiltrating Lymphocytes in Immunotherapy for Gastrointestinal Tumors.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JW and FY conceptualized the study. JS and LM designed the methodology and performed the data analysis. JW, MZ and GH conducted the experiments. JW drafted the manuscript. FY contributed to overall editing and supervision. All authors read and approved the final version of the manuscript. FY and JW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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