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WORD COUNT	7840	TIME SUBMITTED	22-JUN-2022 07:54PM
		PAPER ID	87564794

Sirolimus increases the anti-cancer effect of Huai Er by regulating HIF-1α-mediated glycolysis in HCC

Zhou L *et al*. SRL plus Huai Er regulated HIF-1α in HCC

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Abstract

BACKGROUND

Glycolysis caused by hypoxia-induced abnormal activation of hypoxia inducible factor-1α (HIF-1α) in the immune microenvironment promotes the progression of hepatocellular carcinoma (HCC), leading to enhanced drug resistance in cancer cells. Therefore, altering the immunosuppressive microenvironment by improving the hypoxic state is a new goal in improving cancer treatment.

AIM

To analyse the role of HIF-1α, which is closely related to tumour proliferation, invasion, metastasis, and angiogenesis in the proliferation and invasion of liver cancer. In addition, to explore the HIF-1α pathway-mediated anti-cancer mechanism of sirolimus (SRL) combined with Huai Er.

METHODS

Previous studies on HCC tissues identified the importance of HIF-1α, glucose transporter 1 (GLUT1), and lactate dehydrogenase A (LDHA) expression. In this study, HepG2 and Huh7 cell lines were treated, under hypoxic and normoxic conditions, with a combination of SRL and Huai Er. The effects on proliferation, invasion, cell cycle, and apoptosis were analysed. Proteomics and genomics techniques were used to analyze the HIF-1α-related signalling pathway during SRL combined with Huai Er treatment and its inhibition of the proliferation of HCC cells.

RESULTS

High levels of HIF-1α, LDHA, and GLUT-1 were found in poorly differentiated HCC, with lower patient survival rates. Hypoxia promoted the proliferation of HepG2 and Huh7 cells and weakened the apoptosis and cell cycle blocking effects of the SRL/Huai Er treatment. This was achieved by activation of HIF-1α and glycolysis in HCC, leading to the upregulation of LDHA, GLUT-1, Akt/mammalian target of rapamycin (mTOR), vascular endothelial growth factor (VEGF), and Forkhead Box P3 and downregulation of phosphatase and tensin homolog deleted on chromosome ten (PTEN) and p27. The hypoxia-induced activation of HIF-1α showed the greatest attenuation in the SRL/Huai Er (S50 + H8) group compared to the drug treatments alone ($P < 0.001$). The S50 + H8 treatment significantly downregulated the expression of mTOR and HIF-1α, and significantly reduced the expression of VEGF mRNA. Meanwhile, the combined blocking of mTOR and HIF-1α enhanced the downregulation of Akt/mTOR, HIF-1α, LDHA, and GLUT-1 mRNA and resulted in the downregulation of PTEN, p27, and VEGF mRNA ($P < 0.001$).

CONCLUSION

SRL increases the anti-cancer effect of Huai Er, which reduces the promotion of hypoxia-induced HIF-1α on the Warburg effect by inhibition of the PI3K/Akt/mTOR-HIF-1α and HIF-1α-PTEN signalling pathways in HCC.

Key Words: Hepatocellular carcinoma; Sirolimus; Huai Er; Warburg Effect; HIF-1 alpha

Core Tip: Hypoxia-mediated glycolysis is associated with poorly differentiated hepatocellular carcinoma (HCC) and poor prognosis. Hypoxia inducible factor-1α (HIF-1α), induced by hypoxia, promotes the growth of HepG2 and Huh7 cells and weakens the anti-cancer effect of sirolimus (SRL) and Huai Er. SRL increased the anti-cancer effect of Huai Er, which reduced the promotion of hypoxia-induced HIF-1α on the Warburg effect by inhibiting the PI3K/Akt/mammalian target of rapamycin-HIF-1α

and HIF-1α-phosphatase and tensin homolog deleted on chromosome ten signalling pathways in HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide. Its incidence and mortality are increasing annually, particularly in the younger age groups[1]. More than half of the cases are in China, with more than 90% being related to hepatitis B[1]. Therefore, cancer death is the primary cause of death in China, with liver cancer ranking second with 390000 cases[1,2]. Immunosuppressive cells of Forkhead Box P3$^+$ (Foxp3$^+$) Tregs, neutrophils, MDSC, and others infiltrate the tumour[3,4]. Hypoxia-induced hypoxia inducible factor-1α (HIF-1α)-mediated glycolysis, infiltration of extracellular matrix, and accumulation of lactic acid in the anoxic microenvironment promote abnormal metabolism of cancer cells. This, combined with abnormal tumour immunity leads to downregulation of immune surveillance, increased immune escape, and promotion of proliferation, invasion, and metastasis[4].

Hanahan proposed "abnormal energy metabolism" as the seventh feature of cancer[5], in which the metabolism of tumour cells, mainly represented by abnormal glucose metabolism (Warburg effect) and abnormal lipid metabolism, is closely related to tumour occurrence and metastasis. Recent studies have suggested that abnormal glucose metabolism in cancer is not only related to metabolic enzymes, metabolic pathways, and other related signal transduction pathways, but also to the immune status and local microenvironment of tumour tissues[6-8].

The anoxic environment induces the activation and upregulation of HIF-1α expression which promotes glycolysis, increases glucose transporter 1 (GLUT1) expression, and accelerates tumour cell metabolism[9]. It has been confirmed that HIF-1α can upregulate the activity of glycolytic enzymes by 90%[10] and produce a large amount of lactic acid through metabolism. Lactic acid is transported out of the cells and forms a high lactic acid environment in cancer tissue, leading to metabolic competition with T cells. This further inhibits the function, proliferation, and activation of infiltrated

lymphocytes; destroys the killing effect and anti-tumour function; and promotes tumour immune escape[9-12]. However, cancer cells can efficiently use circulating lactic acid to produce glucose by anaerobic glycolysis, and a large number of electrons produced by metabolism adhere to lactic acid to compensate for the voltage instability inside and outside the cell caused by the high-speed electron movement of cancer cells, while ensuring the steady-state demand for energy and microenvironment for rapid growth and proliferation[10-12].

HIF-1α induction by Akt/mammalian target of rapamycin (mTOR)-mediated growth factors only involves the translation of mRNA under normoxia[13]; however, the regulatory mechanism of PI3K/Akt/mTOR on HIF-1α and the activation mechanism of PI3K are not clear under hypoxia. There may be alternative regulated signalling pathways because our results showed that the PI3K/Akt inhibitor, LY294002, cannot completely inhibit the expression of HIF-1α. Inhibiting the expression of HIF-lα and blocking the transmission of oxygen deficiency signals has become a new target for tumour therapy. Huai Er, also known as Trametes robiniophila Murr, is a traditional Chinese medicine. Various studies have demonstrated that Huai Er inhibits cancer progression and improves patient prognosis[14,15]. The purpose of this study was to explore the regulatory mechanism of sirolimus (SRL) and Huai Er on HepG2 cell proliferation caused by abnormal activation of the HIF-1α pathway induced by hypoxia and to provide a theoretical basis for new clinical treatments of liver cancer.

MATERIALS AND METHODS

Regent and ethics approval

The hepatoma cell lines, HepG2 and Huh 7, were purchased from the Institute of Basic Medicine, Chinese Academy of Medical Sciences. Liver tissue was obtained from the pathological laboratory with written informed consent obtained in accordance with the Declaration of Helsinki of the World Medical Association. The study complied with the Institutional Guidelines for the Care and Use of Laboratory Animals and was approved by the Ethics Committee of Beijing Chaoyang Hospital (No. 2021-1-19-3) and PLA

General Hospital (S2108-013-01). The experimental flowchart is shown in Supplementary Figure 1.

Cell proliferation experiment

HepG2 and Huh 7 cells were cultured at a density of 1×10^3/well with 100 μL complete medium (10%FBS + RMPI-1640, Gibco, United States) overnight for 24 h, then treated with SRL, Huai Er, Ly294002 (Ly), and KC7F2 (KC7) for 24-48 h, discarded and a mixture of 50 μL 1 × PBS and 20 μL MTS (Promega, United States) was added and co-cultured for 2-4 h. The OD value at 490 nm was used to calculate the IC_{50} values of the drugs and blockers.

Cell scratch assay

HepG2 and Huh7 cells were cultured at a density of 1×10^6/well for 24 h before scratching with a "cross" and culturing with 2 mL of treatment solution. Imaging identified the clear position of scratches, and images were acquired at 0 h, 24 h, and 48 h, the position of the scratch was marked and the scratch images were analysed using Image J[16]. The treatment groups were as follows: Huai Er (H) (Gaitianli Co.), SRL (S) (Sigma, United States), Ly294002 (Ly) (TargetMol, United States), KC7F2 (KC7) (TargetMol, United States), H + S, H + Ly, H + KC7, S + Ly, S + KC7, Ly + KC7, and S + H + Ly + KC7.

Plane cloning experiment

HepG2 and Huh7 cells at a density of 500/well were cultured for 24 h, the culture medium was then changed before the addition of SRL, Huai Er, hypoxia (simulated by 200 μM $CoCl_2$, Sigma, United States), and SRL + Huai Er, and the cells cultured for 10 d. After washing with 1 × PBS, the cells were fixed with 1% paraformaldehyde, stained with crystal violet, and photographed to observe the size of cancer colonies.

Cell cycle and apoptosis experiment

Preparation of single-cell suspension: A single-cell suspension was prepared using 0.25 % non-EDTA trypsin (Gibco, United States) to digest the cultured cells treated with Huai Er, SRL, and Huai Er + SRL.

Apoptosis detection: The prepared single-cell suspension was co-incubated with 5 μL of fluorescein isothiocyanate labelled Annexin V antibody and 2.5 μL of phycoerythrin-labelled PI antibody (BD, United States) for 15 min in a dark room at room temperature, washed with 1 ml precooled 1× Binding Buffer at 1200 rpm/min for 5 min, and the supernatant was discarded. The apoptosis rates of HepG2 and Huh 7 cells were determined by flow cytometry.

Cell cycle detection: The prepared single-cell suspension was fixed with 1 mL 70% precooled ethanol at 4 °C for 12 h, the supernatant discarded after centrifugation at 1000 g for 5 min, washed with 1 ml precooled 1 × PBS, adding 500 μL PI/RNase staining buffer (BD Biosciences, United States) for 10^6-10^7 cells, re-suspending cell to flow detection.

Immunohistochemistry

Paraffin-embedded tissues were sliced into thin tissue sections of 5 μm and heated at 60 °C for 30 min. The sections then underwent a dewaxing step, 3% hydrogen peroxide inactivation, antigen retrieval in a microwave at 100 °C for 20 min, and closed antibody incubation for 30 min. Next, the cells were incubated with primary antibodies against HIF-1α, lactate dehydrogenase A (LDHA), and GLUT-1 (1:100) (Proteintech, United States) overnight. The cells were then incubated with the secondary antibody from PV-9000 kits (OriGene Technologies, United States) for 60 min, before staining with the DAB kit (Vector, United States) under a Nikon microscope system.

Protein isolation and Western blotting

Cultured cancer cells were lysed by trypsinisation at 4 °C and the total protein was extracted. Protein purity was determined using a bicinchoninic acid assay, according to the manufacturer's instructions and western blotting was performed.

Reverse transcription-polymerase chain reaction gene detection

Total RNA was extracted using the TRIzol method (Life Technologies, Carlsbad, CA, United States), and the OD value was determined using a spectrophotometer. Primers were designed using the NCBI online primer design software Primer-BLAST and synthesised by Beijing Jingke Xinye Biotechnology Co., Ltd (China). The names of the primers and the corresponding sequence information are listed in Table S1. Then, 24 μL of the reaction system was prepared for cDNA synthesis and stored at -20 °C for later use. After that, a 20 μL reaction system was prepared, and then instantaneous centrifugation was performed in the fluorescence quantitative polymerase chain reaction (PCR) instrument to carry out the reverse transcription (RT)-PCR reaction according to the manufacturer's protocol (Life Technologies, United States).

Statistical analysis

Images from an optical microscope were captured and stored, and the results of flow cytometry were analysed using FlowJo10.0 software. The data were analysed using SPSS 22.0, each index was expressed as mean ± SD, and the difference between the two groups was tested using the *t*-test. Univariate and multivariate analyses of variance (ANOVA) were used to compare the difference between the two groups, and the *t*-test was used for data comparison. Statistical significance was set at $P < 0.05$. Single-factor analysis of variance was used to determine statistical differences among different experimental groups. Differences between groups were considered significant at $P < 0.05$. When $P < 0.01$, there was a significant difference between the groups.

RESULTS

High level of HIF-1a and the Warburg effect indicated a poor survival benefit

We identified high expression levels of HIF-1α in patients with poorly differentiated HCC, with expression being higher in the cancer tissue than in para-carcinoma tissue (Figure 1 A, B, and G) ($P < 0.001$). Higher levels of GLUT-1 ($P < 0.05$) and LDHA ($P < 0.001$) were identified in cancer tissues compared to para-carcinoma tissues, indicating an enhanced glycolytic effect (Warburg effect); these higher levels of expression were also observed in patients with poorly differentiated HCC compared to those with moderately differentiated HCC (Figure 1C-F, H, and I) ($P < 0.001$). The high levels of HIF-1α, GLUT-1, and LDHA were all associated with poorer survival outcomes ($P < 0.01$). Based on these results and our previous research on the effect of Huai Er and SRL on HCC in a rat model[15], we explored the effect of Huai Er combined with SRL on the glucose metabolism pathway in HepG2 and Huh7 cell lines, to determine the synergistic mechanism of tumour growth inhibition.

Optimal inhibitory concentration of SRL, Huai Er, Ly294002, and KC7F2 on HepG2 cells

The suppression of proliferation of HepG2 and Huh7 cells increased gradually on the administration of SRL at concentrations ranging from 0.1 nM to 1000 nM. A gradual increase was also observed on the administration of Huai Er at concentrations ranging from 4 mg/mL to 8 mg/mL. The optimal inhibitory concentration was identified as 50 nM for SRL (S50) and 8 mg/mL for Huai Er (H8), using IC_{50} curve calculation. An optimal inhibitory concentration of 25 μM was calculated for Ly294002 (a specific inhibitor of PI3K) (Ly25) and 20 μM for KC7F2 (a selective inhibitor of HIF-1α) (KC7). These optimal concentrations were used in the following study with S50, H8, Ly25, and KC7 groups.

Huai Er displayed a greater inhibition than SRL on the invasion of HepG2 and Huh7 cells

The scratch test results showed that H8 treatment significantly inhibited the invasion of HepG2 and Huh 7 cells compared to S50 treated cells and the control group (Figure 2A

and B). The addition of S50 enhanced the inhibitory effect of the H8 treatment, showing a greater inhibition of invasion compared to the single therapy treatments (Figure 2C and E).

Huai Er plus SRL treatment showed a greater inhibitory effect on invasion than other combined applications

Compared with the control group, the combination of any two drugs, Huai Er, SRL, Ly25, or KC7, significantly inhibited the invasion of cancer cells ($P < 0.001$) (Figure 2A, B, D, and F). Huai Er application combined with S50, Ly25, or KC7 revealed a stronger inhibitory effect on the invasion of HepG2 and Huh 7 cells than other combined applications, with the strongest effect being in the H8 + S50 treatment group ($P < 0.001$) (Figure 2A, B, D, and F).

Hypoxia promotes the proliferation of HepG2 and Huh7 cells

High proliferation of HepG2 and Huh7 cells was observed when maintained in a hypoxic environment at an appropriate concentration of $CoCl_2$, compared to when maintained under normoxic conditions (Figure 2G and H). The inhibitory effect of H8 + S50 on the proliferation of these cells was weakened after culturing under hypoxia (Figure 2G and H).

Promotion of cell apoptosis in both HepG2 and Huh7 cells after SRL and Huai Er treatment

SRL and Huai Er promote the apoptosis of HepG2 and Huh7 cells under normoxia: The apoptosis rate of HepG2 (Figure 3A-H) and Huh7 (Figure 3I-P) cells increased gradually 24 h to 48 h after treatment with S50 and H8 ($P < 0.001$) (Figure 3A, B, G and Figure 3I, J, and O). The combination of S50 + H8 caused the highest promotion of apoptosis in this time period, compared to the single-drug treatments ($P < 0.001$) (Figure 3E, G; Figure 3M, O). These results indicate that Huai Er may enhance the pro-apoptotic effect of S50 on HCC cells.

Hypoxia reduces the apoptosis effect of SRL and Huai Er on HepG2 and Huh7 cells: Despite the longer treatment time under hypoxia, the increased apoptosis rate of HepG2 and Huh7 cells (Figure 3C, D, and H; Figure 3 K-L and P), observed on treatment under normoxia, was significantly reduced ($P < 0.05$, Figure 3). There was also a decrease in apoptosis levels after S50 + H8 treatment under hypoxia (Figure 3E-F, H and Fig 3 M, N, P) in both HepG2 and Huh7 cells.

Block cell cycle processes of HepG2 and Huh7 cells with SRL and Huai Er treatment

Hypoxia weakened the blocking effect of SRL and Huai Er on the cell cycle： Treatment with S50 and H8 showed a significant blocking effect, in the form of mitotic stagnation in the G2 phase. An increased proportion of S and G2 phase HepG2 cells were observed (Figure 4A-H), while Huh 7 cells showed an increased proportion in the G2 phase (Figure 4I-P). Both cell types had a decreased proportion in the G1 phase ($P < 0.01$) (Figure 4A-D; Fig 4I-L). The blocking effect occurred gradually over time (Figure 4A-D; Figure 4I-L) ($P < 0.01$). When cultured under hypoxia, the blocking effect of SRL and Huai Er on HepG2 and Huh7 cells was weakened, and there was no significant difference in the S50 treatment group, compared with the hypoxia group after treatment at either time point, ($P > 0.05$) (Figure 4E, F, and H; Figure 4M, N, and P); however, the difference in H8 remained statistically significant ($P < 0.001$).

SRL application with Huai Er has a synergistic effect on blocking the cell mitotic cycle: S50 + H8 treatment significantly inhibited the cell division of HepG2 cells in the S phase under normoxia, while the ratio of G1 phase to G2 phase decreased both at 24 h and 48 h (Supplementary Figure 2A-C) ($P < 0.05$) (Fig S2 C). This ratio was significantly higher than that observed for the single drug treatments ($P < 0.01$) (Supplementary Figure 2E and F), suggesting that SRL and Huai Er may have synergistic inhibitory effects. Hypoxia attenuated the synergistic inhibitory effect of S50 + H8 on the cell cycle of HepG2 (Supplementary Figure 2A-D), decreasing the proportion of S-phase cells ($P <$

0.01). The ratio of after S50 + H8 treatment was significantly higher than that of the S50 group ($P < 0.01$), but not significantly different from that in the H8 group (Supplementary Figure 2G and H), at both timepoints.

Thus, we believe that the combined SRL and Huai Er treatment can significantly arrest the cell cycle of HepG2 and Huh7 cells; therefore, protein and gene-level analyses were mainly focused on HepG2 cells.

SRL combined with Huai Er significantly decreased the expression of mTOR and HIF-1a

To further explore the molecular mechanism of SRL combined with Huai Er, western blotting was used to analyse the expression levels of the key proteins of mTOR, in the PI3K signalling pathway, and HIF-1α, in hypoxia-induced factor-mediated glycolysis. Compared with the control and the monotherapy treatments, S50 + H8 significantly reduced the expression level of mTOR and the expression of HIF-1α (Supplementary Figure 3A-C).

Hypoxia-induced HIF-1a promotes HepG2 cell proliferation by enhancing the Warburg Effect

Hypoxia can significantly upregulate the expression of HIF-1α mRNA, while increasing the expression of LDHA and GLUT-1 mRNA in anaerobic glycolysis, leading to the promotion of vascular endothelial growth factor (VEGF) mRNA expression (Supplementary Figure 3D and E). Hypoxia also downregulates the mRNA expression of FoxP3, p27, and Phosphatase and tensin homolog deleted on chromosome ten phosphatase and tensin homolog deleted on chromosome ten (PTEN). Although there was an increase in the expression of Akt and mTOR, no significant difference was observed (Supplementary Figure 3D and E). These results suggest that hypoxia-induced HIF-1α upregulation can mediate downstream activation, leading to an enhancement of HepG2 cell proliferation by upregulating the expression of LDHA and GLUT-1.

Effect of SRL and Huai Er on the expression of target gene mRNA under normoxia

SRL promoted the expression of Akt mRNA, p27 mRNA, and PTEN mRNA, and decreased the expression of FoxP3 mRNA and VEGF mRNA: Compared to the control group, the expression of mTOR mRNA showed no significant expression changes. However, SRL significantly upregulated the expression of Akt mRNA, p27 mRNA, and PTEN mRNA; this upregulation changed significantly over time (Figure 5A). In addition, SRL treatment significantly downregulated the expression of FoxP3 and VEGF mRNA over the same timeperiod (Figure 5A).

Effect of Huai Er on the expression of Akt mRNA, mTOR mRNA, FoxP3 mRNA, and VEGF mRNA: Compared with the control group, Huai Er treatment significantly downregulated the expression of Akt mRNA, mTOR mRNA, FoxP3 mRNA, and VEGF mRNA; this downregulation was significantly increased over time (Figure 5B). The level of p27 mRNA ($P_{24\,h}$ = 0.0066, $P_{48\,h}$ = 0.0038) and PTEN mRNA ($P_{24\,h}$ = 0.003, $P_{48\,h}$ = 0.0007) were increased at both time points. Akt and mTOR expression showed the most significant downregulation after treatment, leading to a reduction in FoxP3 and VEGF mRNA levels from 24 h to 48 h.

SRL and Huai Er downregulate the expression of HIF-1α induced by hypoxia

SRL treatment blocked the downstream amplification effect caused by the expression of mTOR mRNA under hypoxia, which significantly downregulated the enhancement effect of hypoxia on HIF-1α mRNA expression ($P < 0.01$) (Figure 5C). The treatment also decreased the expression levels of VEGF, LDHA, and GLUT-1 mRNA ($P < 0.01$), and simultaneously upregulated the expression of p27, PTEN, and FoxP3 mRNA ($P < 0.001$) (Figure 5C). Compared with the hypoxia group, the Huai Er treatment attenuated the hypoxia-induced enhanced expression of HIF-1α mRNA and downregulated the expression of Akt, mTOR, VEGF, and FoxP3 mRNA ($P < 0.01$) (Figure 5C). It also significantly decreased the expression levels of LDHA and GLUT-1 mRNA ($P < 0.001$) and upregulated the expression of p27 and PTEN mRNA. ($P < 0.001$) (Figure 5C)

LY294002 treatment downregulates the hypoxia-induced HIF-1a expression and weakens its mediated glycolytic effect

LY294002 specifically inhibits PI3K, leading to a downstream blocking effect. Ly25 treatment significantly downregulated the expression of Akt and mTOR mRNA and caused a reduction in VEGF and FoxP3 mRNA expression. Downregulation of HIF-1 α, LDHA, and GLUT-1, and upregulation of p27 and PTEN (Figure 6A), was also observed. When compared with the control group, only the expression of Akt mRNA was significantly downregulated at 24 h ($P < 0.05$) (Figure 6); significant differences among the other groups only became apparent after 48 h ($P < 0.05$) (Figure 6A). Except for FoxP3, there were significant differences between 48 h and 24 h in the other groups ($P < 0.05$) (Figure 6A).

KC7F2 blocked HIF-1a, resulting in a decreased expression of LDHA and GLUT-1 mRNA

Under normoxia, HIF-1α in tissues was quickly degraded by the intracellular, oxygen-dependent, ubiquitin protease degradation pathway, and its stable expression was weak. Therefore, we used the KC7F2-specific blockade of HIF-1α as a reference. On treatment with KC7F2, expression of Akt and mTOR mRNA was increased, the expression of PTEN and p27 mRNA was upregulated, while the expression of VEGF, HIF-1α, LDHA, and GLUT-1 mRNA were downregulated (Figure 6B).

Ly25 and KC7 treatment weaken the promotion of anaerobic glycolysis, mediated by hypoxia-induced HIF-1a accumulation

The expression of Akt, mTOR, VEGF, HIF-1α, LDHA, and GLUT-1 mRNA was decreased after treatment with Ly25 ($P < 0.05$) (Figure 7A), while the expression levels of p27 and PTEN mRNA were increased, and the expression of FoxP3 was upregulated ($P < 0.05$) (Figure 7A). KC7F2 specifically inhibited the expression of HIF-1α, which downregulated the hypoxia-induced accumulation and activation of HIF-1α mRNA

expression ($P < 0.05$). There was also a significant downregulation of LDHA, GLUT-1, and VEGF mRNA expression ($P < 0.05$) (Figure 7A), while upregulation of p27 and PTEN mRNA expression was observed. However, blocking with KC7 did not inhibit the Akt/mTOR signalling pathway ($P < 0.05$) (Figure 7A), indicating that other regulatory pathways may exist.

Combined treatment of SRL with Huai Er significantly downregulated the expression of Akt/mTOR

The S50 + H8 combined treatment significantly downregulated the levels of Akt and mTOR mRNA to a greater extent than the downregulation observed with the H8 treatment alone. In contrast, individual S50 treatment showed an upregulation (Figure 8A). The downregulation of Akt and mTOR mRNA resulted in a significant downregulation of VEGF ($P < 0.01$) and FoxP3 mRNA ($P < 0.05$) (Figure 8A), and an increase in the upregulation of p27 ($P < 0.01$) and PTEN mRNA ($P < 0.05$) (Figure 8A).

SRL combined with Ly25 enhanced the inhibition of anaerobic glycolysis

Compared with S50 and Ly25 individual treatments, the S50 + Ly25 combination significantly reduced the expression of VEGF and FoxP3 mRNA ($P < 0.05$) (Figure 7B). It significantly upregulated the expression of PTEN mRNA and p27 mRNA and there were significant differences in p27 mRNA between the two groups ($P < 0.05$). PTEN showed the only significant difference in expression between the S50 and S50 + Ly25 groups ($P < 0.05$) (Figure 7B). Downregulation of Akt, LDHA and GLUT-1 was observed in the S50 + Ly25 group and was more significant than in the hypoxia group. The difference between groups was significant ($P < 0.01$) (Figure 7B).

SRL combined with KC7F2 enhances the inhibition of the Akt/mTOR signalling pathway

Compared with the S50 and KC7F2 groups, the combined treatment of S50 + KC7F2 significantly downregulated the expression of VEGF mRNA ($P < 0.01$) (Figure 8B) and

upregulated the expression of p27 ($P < 0.05$) and PTEN mRNA ($P < 0.01$) (Figure 8B). S50 + KC7F2 treatment also significantly downregulated the levels of Akt and mTOR mRNA ($P < 0.01$) (Figure 8B), and neutralised the downregulation of FoxP3 mRNA by S50 and upregulation of FoxP3 mRNA by KC7F2 (Figure 8B).

DISCUSSION

A large number of infiltrated lymphocytes, fibroblasts, macrophages, neutrophils, MDSC, and other immune cells result in local immunosuppression and a hypoxic environment which activates HIF-1α[4]. The highly acidic microenvironment caused by the abnormal metabolism of cancer cells and local microvascular infiltration plays an important role in cancer recurrence, proliferation, and invasion[4]. In this study, we indirectly confirmed that high expression of HIF-1α, LHDA, and GLUT-1 in poorly differentiated HCC indicates active glycolysis.

The anoxic and hyperacidic environment of local cancer tissue leads to the inhibition of immune cell function and downregulation of immune cell activity. Cancer cells can constantly adapt to this microenvironment, absorb circulating lactic acid for energy supply, and thus compete with infiltrating immune cell metabolism. This results in a lack of nutrition for T cells in local tissues, low anti-tumour effect, and resultant immune escape of tumour cells[7,12].

It has been confirmed that highly infiltrated regulatory T cells and neutrophils in the local microenvironment are negatively correlated with tumour pathological differentiation and prognosis. In addition, the increase in FoxP3 $^{+}$ Tregs and NLR in peripheral blood before surgery is related to poor prognosis after surgery[17,18]. We confirmed the relationship between FoxP3 $^{+}$ Tregs and recurrence of liver cancer and its inhibitory effect on T cells[15,17]. We found that SRL combined with Huai Er and Thymus Faxin could reduce the level of FoxP3 $^{+}$ Tregs in peripheral blood, delaying the time before tumour recurrence after liver transplantation[19]. However, the specific mechanism of action of FoxP3^{+} Tregs remains unclear. The present study aimed to

explore whether SRL application in combination with Huai Er can achieve antitumour growth by affecting the glycolytic pathway.

Mechanism analysis of proliferation inhibition by SRL combined with Huai Er

Due to the immunosuppressive microenvironment of hypoxia and lactic acid accumulation, most of the $CD8^+$ T lymphocytes infiltrating HCC were in a non-functional state and had a low immune response[10,20]. The cytokines TGF-β, chemokine CXCR4, and CXCR7 produced during tumour proliferation tend to recruit circulating neutrophils and regulatory T cells to the local microenvironment; tumour-related neutrophils can recruit regulatory T cells in cancer tissues through the role of chemokines[3,21]. This interaction between neutrophils and regulatory T cells may enhance the inhibitory properties of the tumour microenvironment. Our previous studies confirmed that $FoxP3^+$ Tregs and $PD\text{-}L1^+$ NEUT are highly expressed in HCC patients, with or without recurrence[17]. The levels of IL-10 and TGF-β in peripheral blood are significantly higher in patients with cancer than in normal individuals, which is consistent with published results[15,17]. $FoxP3^+$ Tregs inhibit $CD8^+$ T cells to promote tumour growth by secreting IL-10 and TGF-β, which may be a target for the anticancer mechanism of Huai Er. In this study, the cell proliferation assay confirmed that SRL and Huai Er inhibited the growth and proliferation of HepG2 and Huh 7 cell lines in a time- and dose-dependent manner. Treatment with SRL at 50 nM and Huai Er at 8 mg/mL showed the greatest inhibition.

The inhibitory effect of SRL combined with Huai Er was significantly higher than that of a single drug and was significantly better than that of SRL or Huai Er combined with LY294002 and KC7F2 when observing cell invasion by cell scratch assay. This suggested a synergistic effect on inhibiting the invasion of HepG2 and Huh 7 cells. Furthermore, SRL combined with Huai Er displayed high inhibition of the proliferation of cancer colonies. These results indicate that Huai Er and SRL inhibit the proliferation and invasion of HepG2 and Huh 7 cells in a concentration- and time-dependent manner.

We confirmed that the apoptosis-promoting effects of SRL and HuaiEr on HepG2 and Huh 7 cells affected mainly early apoptosis, with the combined treatment having a greater effect. Furthermore, SRL and Huai Er significantly increased the proportion of cells in the S + G2 or G2 phase, this proportion was higher in the combined treatment compared to monotherapy.

We believe that the inhibitory effect of SRL combined with Huai Er on HepG2 and Huh 7 cells proliferation mainly affects cell cycle stagnation in the G2 phase and promotes apoptosis.

Effects of hypoxia and HIF-1a on the Anti-tumour effect of Huai Er and SRL

Hypoxia is a common pathophysiological change during the development of most solid tumours[22]. The expression of HIF-1α is first activated by hypoxia in tissue cells, which induces $CD4^+$ T cells to differentiate into $FoxP3^+$ Treg cells[23] and plays a role in the regulation of FoxP3 expression[24,25]. Hypoxia can induce the expression of PD-L1 (CD274) on the surface of immune cells (macrophages, neutrophils, dendritic cells, MDSC, *etc.*) and cancer cells[26], which competitively binds to PD-1 receptors on the surface of T cells resulting in impaired T cell activation function[27,28]. In this study, hypoxia promoted the formation and growth of cancer colonies, resulting in a significant increase in the number of cancer colonies in the S50 + H8 group. In addition, hypoxia increased the survival rate of HepG2 and Huh 7 cells, whereas Huai Er and SRL promoted their apoptosis.

Although the apoptotic effect increased gradually over time, the maximum apoptotic effect under normoxic conditions was significantly lower than that under hypoxic conditions in both the single drug and S50 + H8 groups. Although hypoxia could significantly downregulate the blocking effect of SRL on the S + G2 phase of HepG2 cells and G2 phase of Huh7 cells, it had less effect on the cell cycle stagnation caused by H8 treatment. Under hypoxic conditions, the S50 + H8 group still had an increased proportion of cells in the S + G2 phase although this proportion was lower than that observed in normoxia. This may be because Huai Er is involved in multiple signalling

pathways rather than acting within a single cell signalling pathway which is blocked during hypoxia.

Under normoxic conditions, SRL and Huai Er can act synergistically to inhibit tumour proliferation, but this effect is weakened under hypoxic conditions, possibly due to downstream effects activated by HIF-1α. This indicates that removing the hypoxic environment of tumours may be the key to increasing the efficacy of anti-cancer drugs.

Analysis of HIF-1a regulated tumour growth induced by the hypoxic environment

When cultured under hypoxia, the promotion of HepG2 and Huh 7 cell proliferation and the decrease in apoptosis and cell cycle effect of S50 + H8 treatment indicated that hypoxia plays an important role in HCC. Under hypoxic conditions, HIF-1α and mTOR in HCC cell lines were shown to be upregulated; in addition, LDHA, GLUT-1, and VEGF mRNA were upregulated and PTEN and p27mRNA expression was inhibited. The expression levels of Akt and mTOR mRNA in the PI3K-Akt-mTOR pathway were upregulated in the hypoxic environment, although this was not statistically significant. The relative decrease in Akt and mTOR mRNA expression over time may be related to the activation of the HIF-1α-PTEN-Akt pathway by hypoxia, thereby increasing the glucose metabolism mediated by HIF-1α.

The molecular mechanism of HIF-1a, regulated by Huai Er and SRL

The decrease in HIF-1α and mTOR levels in the S50, H8, and S50 + H8 treatment groups may indicate a potential target pathway for S50 + H8 application in the treatment of HCC. With the intervention of SRL, the expression of VEGF and FoxP3 mRNA was downregulated, while the expression of Akt, PTEN and p27 mRNA was upregulated. With the intervention of Huai Er, the expression of Akt, mTOR, VEGF and FoxP3 mRNA was downregulated, while the expression of PTEN and p27 mRNA was upregulated. The combined effects of downregulation of VEGF, Akt, and mTOR mRNA expression and upregulation of p27 and PTEN were found in the S50 + H8 group which

was more significant than that observed in the individual treatment groups. The downregulation of FoxP3 mRNA was lower than that in the H8 treatment group; this may be associated with the immunomodulatory function of SRL.

Under hypoxic conditions, the accumulation of HIF-1 α mRNA after treatment with SRL and Huai Er was downregulated. In addition, the expression of Akt, mTOR, VEGF, LDHA, and GLUT-1 mRNA were downregulated, and PTEN and P27 mRNA were upregulated. However, the effect of Huai Er combined with SRL was enhanced compared with that of monotherapy, which further confirmed that SRL combined with Huai Er exerted anti-tumour effects through the PI3K-Akt-mTOR-HIF-1α pathway. Previous studies have reported that hypoxia-induced cumulative activation of HIF-1 α can downregulate the expression of PTEN and P27 mRNA, enhance the glycolytic function of tumour cells, and promote the growth and proliferation of tumour cells by upregulating LDHA and GLUT-1 mRNA[29]. There have been debates in the literature regarding the mechanism by which[30], hypoxia decreases the expression of FoxP3, with some saying this is not HIF-1 dependent[31]. In our study, the expression changes of FoxP3 were not consistent with HIF-1 α under hypoxic conditions; this requires further research on co-culture with T cells. The final effect of a significant reduction in VEGF mRNA expression was observed, therefore, we believe that there exists a co-effect of the HIF-1α-PTEN-Akt /mTOR pathway.

Most anticancer drugs decrease the downstream activation effect by reducing the activity of the PI3K-Akt-mTOR pathway[32,33]. PTEN negatively regulates PI3K/Akt signalling and is a well-known tumour suppressor[34]. Studies have demonstrated a strong correlation between alterations in the PTEN/PI3K/Akt cascades and the carcinogenesis of human tumours, including HCC, therefore making it a promising therapeutic target for HCC[34-36]. It has been proved that an increase in PTEN can inhibit the expression of PI3K/pAkt[37]. The upregulation of PTEN can indirectly increase p27 by secreting signal factors[38,39], while the increased PTEN acts on the PI3K-Akt-mTOR pathway and downregulates the expression of pAkt[40-42], further weakening the downstream activation effect. We believe that in this study, the intervention of SRL and

Huai Er played a role in Akt by downregulating the activity of the Akt/mTOR pathway and increasing the expression of PTEN. SRL can increase the sensitivity of HepG2 cells to Huai Er and exert an anticancer mechanism through the PI3K-Akt-mTOR-PTEN and PTEN-Akt/mTOR-FoxP3 regulation pathways.

To further investigate the regulatory effect of the above-mentioned effector drugs on HIF-1α, LY294002 and KC7F2 blockers were selected to interfere with the expression of Akt and HIF-1α in the cell pathway. LY294002 inhibits downstream activation and the results showed that LY294002 could significantly reduce the hypoxia-induced upregulation of HIF-1α mRNA and attenuate the hypoxia-mediated upregulation of LDHA, GLUT-1, and VEGF mRNA expression. We observed that the expression of PTEN and p27 were significantly upregulated, while Akt and mTOR mRNA were downregulated, further confirming the existence of the HIF-1α-PTEN-Akt/mTOR intervention pathway. In addition, the expression of FoxP3 was significantly upregulated, confirming that the regulation of FoxP3 by hypoxia did not completely depend on changes in HIF-1α. We also blocked HIF-1α, using KC7F2 inhibitor, and found that the expression of HIF-1α, LDHA, GLUT-1, and VEGF were significantly downregulated, especially LHDA. The enhancement effect on PTEN and FoxP3 was higher than that with LY294002 treatment, which further confirmed that the effect of hypoxia on FoxP3 was independent of the change in HIF-1α and the existence of the HIF-1α-PTEN effect. The combined treatment of SRL + Ly and SRL + KC7 showed that the regulation of PTEN, p27, and the expression of VEGF and FoxP3 mRNA was consistent with the trend seen during single drug intervention. Based on the above discussion, we further summarized the functional pathways of this study (Supplementary Figure 4).

Thus, we believe that hypoxia can induce the accumulation of HIF-1α and enhance its mediated downstream promoting effect, thereby promoting the growth of tumour cells. SRL can enhance the hypoxia-induced HIF-1α downregulation by Huai Er and attenuate the enhanced effect of glucose metabolism, mediated by HIF-1α, thus exerting

an antitumour effect through the PI3K-Akt-mTOR-HIF-1α and HIF-1α-PTEN-Akt/mTOR pathways.

The overexpression of HIF-1α, the role of FoxP3 in regulating transcription, and the combined intervention of both on the expression of post-transcriptional proteins require further study. In addition, the co-culture of cells affected by T-cell killing function to determine changes in gene and protein levels will be the next research direction of this study.

CONCLUSION

SRL increased the anti-cancer effect of Huai Er, which reduced the promotion of hypoxia-induced HIF-1α on the Warburg effect by inhibiting the PI3K/Akt/mTOR-HIF-1α and HIF-1α-PTEN signalling pathways in HCC.

ARTICLE HIGHLIGHTS

Research background

Hypoxic and high lactate environment further aggravates the aerobic glycolytic effect of cancer and promotes the proliferation and metastasis of liver cancer. Hypoxia-inducible factor 1α plays an important role in the Warburg effect.

Research motivation

We found in clinical practice that the combination of sirolimus (SRL) and Huaier granules can prolong the survival time of liver transplant patients and delay tumor recurrence. The mechanism of combination therapy is unclear.

Research objectives

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. The activation of hypoxia-inducible factor caused by hypoxia and lactic acid accumulation leads to enhanced glucose metabolism in cancer cells, and a large amount of lactic acid is produced to further aggravate the hypoxic microenvironment. We aimed to clarify

the regulatory mechanism of SRL combined with Huaier granule intervention on Warburg effect.

Research methods

In order to solve the scientific problems raised in this study, immunohistochemistry, cell culture, cell scratching and cloning, and flow cytometry were used to analyze the changes of cell levels. Regulatory mechanisms of hypoxia-induced Warburg effect.

Research results

Hypoxia-mediated glycolysis is associated with poorly differentiation HCC and lower prognosis. Hypoxic-induced HIF-1α promotes the growth of HepG2 and Huh7 cell, which weakened with the treatment of SRL and Huai Er. SRL increased the anti-cancer effect of Huai Er which reduced the promotion of hypoxia-induced HIF-1α on the Warburg effect by inhibiting the PI3K/Akt/mammalian target of rapamycin (mTOR)-HIF-1α and HIF-1α-phosphatase and tensin homolog deleted on chromosome ten (PTEN) signaling pathways in HCC.

Research conclusions

SRL increased the anti-cancer effect of Huai Er which reduced the promotion of hypoxia-induced HIF-1α on the Warburg effect by inhibiting the PI3K/Akt/mTOR-HIF-1α and HIF-1α-PTEN signaling pathways in HCC.

Research perspectives

This study confirmed that SRL combined with Huaier granules can down-regulate the Warburg effect mediated by hypoxia-inducible factor 1α, laying a foundation for the combined treatment of HCC with traditional Chinese and western medicine.

Figure Legends

Figure 1 Expression of hypoxia inducible factor-1α, lactate dehydrogenase A and glucose transporter 1 in hepatocellular carcinoma. A: Expression of hypoxia inducible factor-1 (HIF-1α) in poorly hepatocellular carcinoma (HCC), (Magnification: 200 ×, scale bar 10 μm); B: Expression of HIF-1α in high-moderately HCC, (Magnification: 200 ×, scale bar 10 μm); C: Expression of lactate dehydrogenase A (LDHA) in poorly HCC, (Magnification: 200 ×, scale bar 10 μm); D: Expression of LDHA in high-moderately HCC, (Magnification: 200 ×, scale bar 10 μm); E: Expression of glucose transporter 1 (GLUT1) in high-moderately HCC, (Magnification: 200 ×, scale bar 10 μm); F: Expression of GLUT1 in high-moderately HCC, (Magnification: 200 ×, scale bar 10 μm); G: The difference of HIF-1α in different groups nalyzed by *t* tset; H: The difference of LDHA in different groups analyzed by *t* test; I: The difference of GLUT1 in different groups analyzed by *t* test. $^{a}P < 0.001$ *vs* poorly differentiated HCC (poorly-HCC); $^{b}P < 0.001$ *vs* cancer; $^{c}P < 0.05$ *vs* cancer.

Figure 2 Inhibition on HepG2 and Huh 7 cell invasion and proliferation of different drug with scratch test and plane cloning experiment. A: Image of cell scratch of HepG2 under different drug intervention; B: Image of cell scratch of Huh7 under different drug intervention; C: Effect of single drug treatment on HepG2 invasion ability; D: Effect of combination application treatment on HepG2 invasion ability; E: Effect of single drug treatment on Huh 7 invasion ability; F: Effect of combination application treatment on Huh 7 invasion ability; G: Clonal size of HepG2 cells with different drugs; H: Clonal size of Huh 7 cells with different drugs. $^{a}P < 0.05$ *vs* control; $^{b}P < 0.01$ *vs* control; $^{c}P < 0.001$ *vs* control. H8: 8 mg/mL for Huai Er; S50: 50 nM for Sirolimus; Ly25: Ly294002; KC7: 20 μM for KC7F2.

Figure 3 Effects of Sirolimus and Huai Er on apoptosis of HepG2 and Huh 7 with normal oxygen and hypoxia. A: Scatter diagram of HepG2 cell apoptosis intervened by Sirolimus (SRL); B: Scatter diagram of HepG2 cell apoptosis intervened by Huai Er; C: Scatter diagram of HepG2 cell apoptosis in SRL under hypoxia; D: Scatter diagram of HepG2 cell apoptosis in Huai Er under hypoxia; E: Scatter diagram of HepG2 cell apoptosis in S50+H8 under normal oxygen; F: Scatter diagram of HepG2 cell apoptosis in S50+H8 under hypoxia; G: Apoptosis rate analyzed by two-way analyses of variance (ANOVA) at 24 h and 48 h under normal oxygen; H: Apoptosis rate analyzed by two-way ANOVA at 24 h and 48 h under hypoxia; I: Scatter diagram of Huh 7 cell apoptosis intervened by SRL; J: Scatter diagram of Huh 7 cell apoptosis intervened by Huai Er; K: Scatter diagram of Huh 7 cell apoptosis in SRL under hypoxia; L: Scatter diagram of Huh 7 cell apoptosis in Huai Er under hypoxia; M: Scatter diagram of Huh 7 cell apoptosis in S50 + H8 under normal oxygen; N: Scatter diagram of Huh 7 cell apoptosis in S50 + H8 under hypoxia; O: Apoptosis rate analyzed by two-way ANOVA at 24 h and 48 h under normal oxygen; P: Apoptosis rate analyzed by two-way ANOVA at 24 h and 48 h under hypoxia. $^{a}P < 0.01$ *vs* H8 + S50/hypoxia; $^{b}P < 0.001$*vs* H8 + S50/hypoxia; $^{c}P < 0.05$ *vs* H8 + S50/hypoxia. H8: 8 mg/mL for Huai Er; S50: 50 nM for SRL.

Figure 4 Effects of Sirolimus and Huai Er on cell cycle of HepG2 and Huh 7 under normal oxygen and hypoxia. A-C: Peak graph of HepG2 apoptosis in control (A), Sirolimus (SRL) (B) and Huai Er (C) under normal oxygen; D: HepG2 cell cycle difference with two-way analyses of variance (ANOVA) at 24 h and 48 h under normal oxygen; E-G: Peak graph of HepG2 apoptosis in control (E), SRL (F) and Huai Er (G) under hypoxia; H: HepG2 cell cycle difference with two-way ANOVA at 24 h and 48 h under hypoxia; I-K: Peak graph of Huh 7 apoptosis in control (I), SRL (J) and Huai Er (K) under normal oxygen; L: Huh 7 cell cycle difference with two-way ANOVA at 24 h and 48 h under normal oxygen; M-O: Peak graph of Huh 7 apoptosis in control (M), SRL (N) and Huai Er (O) under hypoxia; P: Huh 7 cell cycle difference with two-way ANOVA at 24 h and 48 h under hypoxia. $^{a}P < 0.01$ *vs* control; $^{b}P < 0.001$ *vs* control; $^{c}P <$

0.01 *vs* hypoxia; [d]*P* < 0.001 *vs* hypoxia; [e]*P* < 0.05 *vs* control; [f]*P* < 0.05 *vs* hypoxia. H8: 8 mg/mL for Huai Er; S50: 50 nM for SRL.

Figure 5 Effects of Sirolimus & Huai Er on the expression of target genes under normal oxygen and hypoxia. A: The mRNA level of Akt, p27, PTEN, FOXP3 and VEGF treated with Sirolimus (SRL) under normal oxygen at 24 h and 48 h with two-way analyses of variance (ANOVA) analysis; B: The mRNA level of Akt, mammalian target of rapamycin (mTOR), forkhead box P3 (FOXP3) and vascular endothlial growth factor (VEGF) treated with Huai Er under normal oxygen at 24 h and 48 h with two-way ANOVA analysis; C: The mRNA level of Akt, mTOR, VEGF, hypoxia inducible factor-1α, lactate dehydrogenase A, glucose transporter 1, FOXP3, p27 and phosphatase and tensin homolog deleted on chromosome ten treated with SRL and Huai Er under hypoxia at 24 h and 48 h with two-way ANOVA analysis. [a]*P* < 0.05 *vs* control; [b]*P* < 0.05 *vs* control; c, down-regulated [c]*P* < 0.01 *vs* hypoxia; d, up-regulated [d]*P* < 0.001 *vs* hypoxia. PTEN: Phosphatase and tensin homolog deleted on chromosome ten; FoxP3: Forkhead Box P3; VEGF: Vascular endothlial growth factor; mTOR: Mammalian target of rapamycin; HIF-1α: Hypoxia inducible factor-1α; LDHA: Lactate dehydrogenase A; GLUT-1: Glucose transporter 1.

Figure 6 Intervention of LY49002 and KC7F2 on mRNA expression of target gene. A: Effect of LY294002 intervention on gene expression under normoxic condition with two-way analyses of variance (ANOVA) analysis; B: Effect of KC7F2 intervention on gene expression under normoxic condition two-way ANOVA analysis. [a]*P* < 0.05 *vs* control; [b]*P* < 0.05 *vs* 24 h; [c]*P* < 0.05 *vs* control; [d]*P* < 0.01 *vs* control. mTOR: Mammalian target of rapamycin; VEGF: Vascular endothlial growth factor; FoxP3: Forkhead Box P3; HIF-1α: Hypoxia inducible factor-1α; LDHA: Lactate dehydrogenase A; GLUT-1: Glucose transporter 1; PTEN: Phosphatase and tensin homolog deleted on chromosome ten.

Figure 7 Effect of single or combined drug intervention on target gene mRNA expression with one-way analyses of variance. A: Effects of LY294002 and KC7F2 on different gene expression under hypoxia; B: Effect of S50 combined with Ly25 on the expression of different genes. a, down-regulated, $^{a}P < 0.05$ *vs* hypoxia; b, up-regulated, $^{b}P < 0.05$ *vs* hypoxia; $^{c}P < 0.05$ *vs* S50 + Ly25; $^{d}P < 0.01$ *vs* S50 + Ly25. mTOR: Mammalian target of rapamycin; VEGF: Vascular endothlial growth factor; FoxP3: Forkhead Box P3; HIF-1α: Hypoxia inducible factor-1α; LDHA: Lactate dehydrogenase A; GLUT-1: Glucose transporter 1; PTEN: Phosphatase and tensin homolog deleted on chromosome ten.

Figure 8 Effect of S50 combined H8 or KC7 intervention on target gene mRNA expression with one-way ANOVA analysis. A: The regulatory effect of S50 + H8 on the gene expression under hypoxia condition; B: Effect of S50 + KC7 on the expression of different genes. $^{a}P < 0.05$ *vs* S50 + H8; $^{b}P < 0.01$ *vs* S50 + H8; $^{c}P < 0.05$ *vs* S50 + KC7; $^{d}P < 0.01$ *vs* S50 + KC7. mTOR: Mammalian target of rapamycin; VEGF: Vascular endothlial growth factor; FoxP3: Forkhead Box P3; PTEN: Phosphatase and tensin homolog deleted on chromosome ten.

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