

**Jiedu Sangen decoction inhibits chemoresistance to 5-fluorouracil of colorectal cancer cells by suppressing glycolysis via PI3K/AKT/HIF-1α signaling pathway**

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[ABSTRACT] Drug resistance is a major obstacle in the development of effective colorectal cancer (CRC) therapy. Our study aimed to explore the reversal abilities of Jiedu Sangen decoction (JSD) on the 5-fluorouracil (5-FU) resistance and its underlying molecular mechanisms. Expression changes in HIF-1 of CRC tissues were firstly revealed by bioinformatics analysis. Afterwards, cell viabilities of JSD and 5-FU treatments on 5-FU resistant human colon cancer cells (HCT-8/5-FU) were determined. Expressions of phosphoinositide 3-kinase (PI3K), protein kinase B (AKT)/p-AKT, hypoxia-inducible factor 1 (HIF-1α), as well as glycolysis related proteins such as L-lactate dehydrogenase A (LDHA), Glucose transporter type 1 (Glut1), Hexokinase 2 (HKII), and cysteinyl aspartate specific proteinase (Caspase) family members in HCT-8/5-FU cells, HIF-1α silenced HCT-8/5-FU cells and tumor tissues were detected by western blotting. HIF-1α was found over expressed in CRC tissues according to public available datasets in Oncomine. Growth inhibition rates of HCT-8/5-FU cells were increased along with the increase of JSD concentrations. JSD caused down-regulated HIF-1α, PI3K, AKT/p-AKT, HKII and Glut1, as well as up-regulated Caspase3 and Caspase9 in HCT-8/5-FU cells and tumor tissues. In HIF-1α silenced HCT-8/5-FU cells, synergistic group showed significantly reduced expression levels of PI3K, AKT, p-AKT. Additionally, up-regulated expressions of Caspase6 and Caspase7 were observed. JSD combined with 5-FU also exhibited obvious inhibitory efficiency on tumor growth in vivo. JSD may reverse 5-FU resistance by suppressing glycolysis via PI3K/AKT/HIF-1α signaling pathway, thereby inhibiting glycolysis and induce apoptosis to enhance anti-tumor activity.

[KEY WORDS] Jiedu Sangen decoction; 5-Fluorouracil resistance; Glycolysis; Apoptosis; Colorectal cancer


**Introduction**

Colorectal cancer (CRC), one of the most common ma-

lignant tumors, is currently the predominant cause of cancer-

related deaths all over the world [1]. The mortality rate is high due to more than 50% of patients are diagnosed at or beyond stage III when distant metastasis has already occurred [2]. It is widely acknowledged that 5-fluorouracil (5-FU) is the first-

line chemotherapeutic agent for the treatment of CRC pa-

tients since it can induce cancer cell death by interfering DNA synthesis [3–4]. However, treatment efficacy of 5-FU is often influenced owing to the development of 5-FU-resistance, which results in the main chemotherapy failure for CRC treatment in routine clinical practice [5]. Although chemo-

therapeutic intervention coupled with surgery is initially ef-

fective for metastatic CRC, chemotherapy with 5-FU could result in poor long-term survival associated with high rates of relapse and drug resistance [6]. Thus, it is urgently needed to explain the molecular mechanisms involved in 5-FU-resistance of CRC and exploring new ways to inhibit 5-FU-resist-

ance.

In general, large-scale metabolic reprogramming (such as
metabolisms of glucose, lactate, pyruvate, hydroxybutyrate, acetate, glutamine, and fatty acids) of tumors driven by oncogene cooperation is quite complex since they contain multiple metabolic compartments and are linked by the transfer of these catabolites [9-10]. Increased utilization of glycolysis by tumor cells for their energy requirements under physiological oxygen conditions has been the basis for much speculation on the survival advantage of tumor cells, tumorigenesis, drug efficacy and the microenvironment of tumors [8-10]. The phosphoinositide 3-kinase (PI3K) pathway regulates multiple steps in glucose metabolism and also cytoskeletal functions (such as cell movement and attachment), and full activation of glycolysis by PI3K requires protein kinase B (AKT) activation [11]. LncRNA hypoxia-inducible factor 1 (HIF-1α) plays crucial roles in protection of dihydromyricetin during apoptosis and necrosis induced by ischemia-reperfusion treatment [15]. LncRNA hypoxia-inducible factor 1 alpha-antisense RNA 1 (HIF1A-AS1) could be up-regulated by tumor necrosis factor-α (TNF-α) and inhibition of HIF1A-AS1 markedly reduced mRNA level of cysteinyl aspartate specific proteinase 3 (Caspase 3) and significantly rescued cell apoptosis [16]. Additionally, it has been reported that increased 5-FU sensitivity, reduced MDR protein 1 (MDR1) expression levels and reduced cancer stem cells -like properties were associated with metastasis-associated colon cancer 1 (MACC1) knockdown, which may be associated with inhibition of the PI3K/AKT signaling pathway [14]. Therefore, inhibiting PI3K/AKT/HIF-1α-induced glycolysis may be a potential approach for the suppression of drug resistance.

Adopting appropriate glycolysis inhibitor in combination with chemotherapy drug might be a key for overcoming the drug resistance. Recently, traditional Chinese medicine combined with chemotherapeutic drugs (such as oxaliplatin, 5-FU and leucovorin) in the treatment of advanced colorectal cancer have received great interests since the combined therapies could significantly improve the life quality, prolong the survival time and reduce the side effects of chemotherapy [9].

Jiedu Sangen Decoction (JSD), consisting of Geum japonicum Thunmb, Polygonum Cuspidatum and Radix Actinidiae Chinensis, is an oral traditional Chinese medicine and has been shown obviously suppressive effects against migration and invasion of colon cancer cells [16-17]. In the present study, the expression of HIF-1α was found higher in CRC tissues than normal controls according to the bioinformatics analysis of published studies in Oncomine. Subsequently, effects of JSD and 5-FU on 5-FU sensitivity of CRC in vivo and in vitro were studied by using the 5-FU resistant human colon cancer cells (HCT-8/5-FU) and HCT-8/5-FU induced CRC mice model. The expression level changes in PI3K, AKT, p-AKT, HIF-1α, as well as glycolysis related proteins such as L-lactate dehydrogenase A (LDHA), Glucose transporter type 1 (Glut1) and Hexokinase 2 (HKII) and Caspase family members of different treatment groups were analyzed by western blotting to reveal the potential molecular mechanisms. There are several studies available in which animal models of herbal formula have been used to assess the efficacy against 5-FU-resistance on cell cycle, proliferation and apoptosis, but no animal model studies have been performed in which the efficacy of herbal formula against 5-FU-resistance by suppressing glycolysis has been conducted. Therefore, we explored the efficacy of Chinese herbal formula against 5-FU-resistance in a mouse model.

Materials and Methods

Preparations and and chemical composition analyses of JSD and JSAE

The medicinal materials of JSD, including Tengli Gen (Radix Actinidiae Argutae, Cat No. 20160721), Shuiyangmei Gen (Root of Adina rubella, Cat No. 20160810) and Huzhang Gen (Polygoni Cuspidati Radix, Cat No. 20160702) were purchased from the pharmacy of The First Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou, China). Three hundred grams each of the medicinal materials were mixed equally and soaked with 8 times volume of distilled water for 30 min, before being simmered for 1 h after initial short hard boil. The first batch of decoction was collected, and the herbs were decocted again with 3 times volume of distilled water for another 25 min. All decoctions were merged and filtered, concentrated to 1.2 g mL⁻¹ as JSD, and stored at 4 °C.

Two hundred grams each of Tengli Gen (Radix Actinidiae Argutae), Shuiyangmei Gen (Root of Adina rubella), and Huzhang Gen (Polygoni Cuspidati Radix) were mixed equally and pulverized to coarse powder, which was mixed and pulverized to coarse powder, was then mixed and pulverized to a coarse powder, and stored at 4 °C.

Moreover, chemical compositions of JSAE were determined by HPLC-UV and HPLC-MS (Figs. S1–S2, Table S1). It can be concluded that the main components in JSAE included 5-hydroxy-2-methylchromone-7-O-β-D-xylpyranosyl(1→6)-β-D-glucopyranoside (from Shuiyangmei Gen), polydatin (from Huzhang Gen), and 5-hydroxy-6-methoxy-coumarin-7-O-β-D-glucopyranoside (from Tengli Gen).

Bioinformatics analysis

We downloaded publicly available data sets related to colorectal cancer in Oncomine (https://www.oncomine.org/resource/main.html). These data sets were used to analyze the differences in HIF-1α expression among colorectal cancer patients with different histologic types, clinical stages, survival time and drug resistance. In addition, the mRNA and RNASeq expression profilings were obtained from The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/) by Bioconductor/TCGAbioblink function package for the colon adenocarcinoma reads.

Cell lines and cell culture

Human colon cancer cell line HCT-8 was obtained from Cell Bank of the Typical Culture Preservation Committee of the Chinese Academy of Sciences (Shanghai, China) after getting the approval of their Institutional Review Board (IRB).
from the State Administration of Traditional Chinese Medicine of Zhejiang Province, and approval number (2015ZZ013). The 5-FU induced resistant cell line HCT-8/5-FU was purchased from Hangzhou Haotian Biotech Co., Ltd. (Zhejiang, China). Both cell lines were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 100 U·mL⁻¹ penicillin, and 100 μg·mL⁻¹ streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. Besides, HCT-8/5-FU cells were cultured in the above medium supplemented with 15 μg·mL⁻¹ 5-FU. The morphological characteristics of HCT-8 and HCT-8/5-FU cells were monitored using high power microscope (Olympus, Japan).

**Cell viability assay**

Cell viability was determined using a Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Laboratories Co., Ltd., Kumamoto, Japan) following the manufacturer’s protocol. Cells in logarithmic growth phase were seeded in 96-well plates at a density of 7 × 10⁴ cells/well. After incubation for 24 h, HCT-8 cells were exposed to increasing concentrations of 5-FU (2, 4, 8, 16, 25, 50, 100, 200 μg·mL⁻¹) while HCT-8/5-FU cells were exposed to increasing concentrations of 5-FU (12.5, 25, 50, 100, 200, 400, 800, 1600 μg·mL⁻¹) for 48 h. Meanwhile, HCT-8/5-FU cells were also exposed to gradually increased concentrations of 5-FU (3, 4, 5, 6, 8, 10, 11, and 12 mg·mL⁻¹) for 48 h. Afterwards, cells were incubated with 9% CCK-8 solution for 1 h. The absorbance in each well was quantified at 450 nm using a microplate reader (Thermo, MA, USA). Inhibitory concentration of 50% of cells (IC₅₀) was calculated by SPSS version 22.0 software (SPSS, Inc., Chicago, IL, USA) with the linear regression method. In addition, the resistance index (RI) was determined according to the following equation: RI = IC₅₀ of drug resistant cells/IC₅₀ of parent cells. At least three experiments were performed, and each tested in triplicate. Based on the CCK-8 assay, the absorbance value was determined and the growth curve was also plotted. All experiments performed 6 wells and repeated 3 times.

**Synergistic effects of JSAE and 5-FU**

HCT-8/5-FU cells were exposed to the presence of 5-FU with increasing concentrations (12.5, 25, 50, 100, 200, 400, 800, and 1600 μg·mL⁻¹) along with or without JSD (Low-concentration group: 4 mg·mL⁻¹ JSAE, Medium-concentration group: 5 mg·mL⁻¹ JSAE, High-concentration group: 6 mg·mL⁻¹ JSAE, 5-FU control group: without JSAE) for 48 h. After the inhibition rate (IR) of HCT-8/5-FU cells were detected, the IC₅₀ was calculated according to the IR by SPSS. And the fold of drug resistance was calculated as the IC₅₀ with 5-FU alone divided by the IC₅₀ with 5-FU in combination with JSAE. All experiments performed 6 wells and repeated 3 times.

**Western blotting**

The total cellular proteins were extracted from cells and tumor samples using the radio immunoprecipitation assay (RIPA) lysis buffer (Applygen, Beijing, China) containing protease and protein phosphatase inhibitors. Protein concentrations of cell lysates were measured with spectrophotometer (Quawell, CA, USA). The aliquots of cellular protein were denatured for 7 min at 100 °C and restored at –80 °C after cooling. The denatured protein samples were loaded onto gradient sodium dodecyl sulfate (SDS) polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (BioWare, Shanghai, China). After the membranes were blocked in blocking solution (5% skim milk in TBST buffer, 50 mmol·L⁻¹ Tris, 500 mmol·L⁻¹ NaCl and 0.5% Tween 20 at pH 7.5) for 1.5 h to 2 h at 4 °C, the blots were incubated overnight at 4 °C with primary antibodies. The primary antibodies were against PI3K (1 : 1000; Cell Signaling Technology, MA, USA), AKT2 (1 : 1000; Abcam, MA, USA), phospho-AKT2 (p-AKT, 1 : 1000; Abcam, MA, USA), Caspase3 (1 : 1000; Abcam, MA, USA), Caspase6 (1 : 1000; Abcam, MA, USA), Caspase7 (1 : 1000; Cell Signaling Technology, MA, USA), Caspase9 (1 : 1000; Abcam, MA, USA), HIF-1α (1 : 2000; Proteintech, Wuhan, China), LDHA (1 : 2000; Proteintech, Wuhan, China), Glut1 (1 : 2000; Proteintech, Wuhan, China), and HKII (1 : 2000; Proteintech, Wuhan, China). Thereafter, the membranes were incubated with secondary antibodies at 4 °C for 2 h. Band intensities were quantified using the Quantity One (Quantity One, Inc., California, IL, USA).

**Cell transfection with small interfering RNA (siRNA)**

The siRNA oligonucleotides targeting HIF-1α and negative control siRNA-targeting luciferase (sh-NC) were obtained from Invitrogen. HCT-8/5-FU cells were transfected with siRNA of HIF-1α or scrambled negative control siRNA by using the Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. Transfected cells were harvested 48 h after transfection, and subjected to qRT-PCR or western blotting analysis to evaluate the efficiency of siRNA transfection. Stable clones were selected with 2 μg·mL⁻¹ puromycin (Sigma, MA, USA) treatment for 10 d.

**In vivo tumor xenograft model**

All animal experiments were approved by Zhejiang Chinese Medical University Laboratory Animal Research Center with the approval number of SYXK (Zhejiang) 2013-0184, and conducted according to the Animal Research Act, 1985 (New South Wales, Australia) and the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (2013). Tumor xenograft model was established in 5-week-old male BALB/c-𝑛u mice (Laboratory Animal Center of Zhejiang Chinese Medical University, Zhejiang, China) by right-forelimb subcutaneous injection of 100 μL cell suspension containing 1.5 × 10⁷ HCT-8/5-FU cells. All animals were kept in ventilated cages in compliance with institutional guidelines. After one week, subcutaneous tumor nodules with diameter of 2–4 mm developed in all mice. In order to evaluate cancer therapeutic efficacy in vivo, HCT-8/5-FU tumor-bearing nude mice were intraperitoneally (i.p.) injected with 5-FU solution, intragastrically (i.g.) given JSD, or combination of both. When the generated tumor reaches up to 100 mm³, mice were randomly assigned to 4 groups (15 mice per
group): blank control group (normal saline 0.1 mL/10 g i.p. and normal saline 0.1 mL/10 g i.g.), 5-FU group (5-FU 0.1 mL/10 g i.p. and normal saline 0.1 mL/10 g i.g.), JSD group (normal saline 0.1 mL/10 g i.p. and JSD 0.1 mL/10 g i.g.), synergistic group (5-FU 0.1 mL/10 g i.p. and JSD 0.1 mL/10 g i.g.).

Tumor volume \( (V_T) \) was calculated using the formula: \( V_T = L \times S^2 \), where \( L \) is the longest diameter (mm) and \( S \) is the shortest diameter (mm). After 10 days of treatment, mice were sacrificed (random 5 per group) and tumors were excised. The tumors were cut into small pieces, washed in normal saline, fixed in cell lysis buffer, and homogenized until the cellular structure was completely destroyed to extract total cellular protein for western blotting. Tumor volume and body weight were measured every 3 days. After experiments done, all mice were sacrificed by cervical dislocation with anesthesia.

**Statistical analysis**

All results were presented as mean ± standard deviation (SD) and analysis were conducted using the SPSS version 22.0 software (SPSS, Inc., Chicago, IL, USA). All \( P \) values were two-sided and \( P < 0.05 \) was considered as statistically significant difference.

**Results**

**Expressions of HIF-1α in colorectal tumor and normal colorectal tissues**

In Oncomine, expressions of HIF-1α in colorectal tumor and normal colorectal tissues were published in 13 studies containing 2061 tissue samples (Fig. 1A). Meta-analysis of these 13 studies showed that HIF-1α was highly expressed in colorectal tumor tissues compared with normal colorectal tissues \( (P = 0.002) \). Among different types of colorectal cancer, higher expressions of HIF-1α were observed in colon mucinous adenocarcinoma and rectal mucinous adenocarcinoma. The expression of HIF-1α was higher in the colorectal cancer patients with stage IV than the patients with stage I and stage II. Meanwhile, patients with higher expressions of HIF-1α had shorter survival time. These above results revealed that high expression of HIF-1α might be actually a predictor of development and prognosis in colorectal cancer.

**Morphological and cell viability changes between HCT-8 and HCT-8/5-FU cells**

HCT-8 cells grew by static adherence, and the cells were polygon or fusiform in shape, which were typical features of epithelial phenotype (Fig. 1B). In addition, HCT-8 cells were characterized by large nucleus, intact cell membrane and distinct cellular outline. By contrast, HCT-8/5-FU cells appeared as a disorganized and crowded monolayer, with adherent cells displaying a circle or polygon in appearance and containing more condensed smaller nuclei (Fig. 1B). Compared to HCT-8 cells, HCT-8/5-FU cells had enhanced cell density in confluent monolayers with more atypia, and these morphology changes were linked with increased chemoresistance of 5-FU. Cell growth results showed HCT-8 cells had characteristics of fast growth, short population doubling-time and speedy proliferation rate after entering the logarithmic phase (Fig. 1D).

**Inhibitory effects of 5-FU and JSAE on growth of HCT-8 and HCT-8/5-FU cells**

5-FU inhibited the growth of HCT-8 and HCT-8/5-FU cells in a concentration-dependent manner (Fig. 1C). Higher concentration of 5-FU solution is required for HCT-8/5-FU cells to inhibit cell proliferation than that required for HCT-8 cells. The IC\(_{50}\) of 5-FU against HCT-8 and HCT-8/5-FU cells were 21.050 ± 0.354 and 430.283 ± 162.689 μg·mL\(^{-1}\), respectively. Moreover, the RI was 20.441, suggesting drug resistance of cells met experimental requirements.

The growth inhibition rate was increased with higher concentration treatment of JSAE, which showed an ascending cytotoxic effect on HCT-8/5-FU cells (Fig. 1E, Table 1). JSAE enhanced the sensitivity of HCT-8/5-FU cells in a concentration-dependent manner. The growth inhibitory rate was significantly increased in high-concentration group \( (P < 0.05) \), whereas the differences of cell growth inhibitory rate between low and medium-concentration group were not significant \( (P > 0.05) \).

**Cell inhibition effects of JSAE combined with 5-FU on HCT-8/5-FU cells**

In the HCT-8/5-FU cells, IC\(_{50}\) of synergetic groups was obviously reduced from 430.283 to 359.990 μg·mL\(^{-1}\) in low-concentration group, to 321.450 μg·mL\(^{-1}\) in medium-concentration group, and to 98.000 μg·mL\(^{-1}\) in high-concentration group, when compared with 5-FU alone (Fig. 2). Beyond that, the IR of low-concentration group, medium-concentration group and high-concentration group calculated from IC\(_{50}\) was 1.195, 1.339 and 4.391, respectively.

Increased concentrations of 5-FU and JSAE caused higher inhibition ratios (Table 2). Combinations of 6 μg·mL\(^{-1}\) JSAE and 5-FU (> 50 μg·mL\(^{-1}\)) significantly led to larger degrees of cell inhibition than 5-FU control group \( (P < 0.05) \), whereas no significant difference was observed in the low-concentration group and medium-concentration group \( (P > 0.05) \).

**Combined treatment of JSAE and 5-FU suppressed glycolysis by activation of PI3K/AKT-HIF-1α pathway**

HCT-8/5-FU cells treated with JSAE or 5-FU alone had decreased expressions of LDHA and HKII \( (P < 0.05) \), and the expressions were further decreased by JSAE in combination with 5-FU (synergetic group) (Figs. 3A, 3B). In addition, we observed significant elevated expressions of Caspase3, Caspase7 and Caspase9 (28 kDa and 48 kDa) when compared to blank control group. HIF-1α expression was decreased when the cells were treated with JSAE alone or combined with 5-FU. Moreover, JSAE in combination with 5-FU effectively down-regulated the expression levels of PI3K, AKT, p-AKT, and Glut1.

In HIF-1α silenced HCT-8/5-FU cells, synergetic group showed significantly lower expression levels of PI3K, AKT and p-AKT than 5-FU group. HKII expression was lower in 5-FU.
Higher in the synergistic group than control group, while higher in synergistic group than 5-FU group. Additionally, up-regulated expressions of Caspase6 were observed in the synergistic group when compared with the 5-FU group (Figs. 3C, 3D).

**Combination therapy of JSD and 5-FU enhanced anti-tumor activity in vivo**

Both 5-FU and synergetic groups showed stronger inhibition effects on tumor growth than the JSD group and blank control group (Fig. 4A). We used HCT-8/5-FU cells for the 5-FU chemoresistant colorectal cancer mice models. A Kaplan-Meier survival curve (Fig. 4B) was generated to evaluate the

**Table 1** Growth inhibitory rate of HCT-8/5-FU cells treated with 3, 4, 5, 6, 8, 10, 11, and 12 mg·mL\(^{-1}\) JSAE (mean ± SD, All experiments performed 6 wells and repeated 3 times)

<table>
<thead>
<tr>
<th>JSAE concentration (mg·mL(^{-1}))</th>
<th>Growth inhibitory rate (IR, %)</th>
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<tbody>
<tr>
<td>3</td>
<td>5.71 ± 2.72</td>
</tr>
<tr>
<td>4</td>
<td>6.94 ± 2.04</td>
</tr>
<tr>
<td>5</td>
<td>11.91 ± 2.38</td>
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<tr>
<td>6</td>
<td>12.74 ± 1.71</td>
</tr>
<tr>
<td>8</td>
<td>21.51 ± 1.20</td>
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<tr>
<td>10</td>
<td>79.83 ± 0.25</td>
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<tr>
<td>11</td>
<td>98.54 ± 0.05</td>
</tr>
<tr>
<td>12</td>
<td>98.65 ± 0.06</td>
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</table>
overall survival of each group, which shows that JSD combined with 5-FU significantly prolonged mice survival compared with other groups. Intriguingly, body weight remained stable for JSD group during observation, while consistent weight loss was observed in 5-FU and synergetic groups (Fig. 4C). Meanwhile, JSD exhibited the highest inhibition of tumor volume (Fig. 4D) in relevant groups. Those \textit{in vivo} results suggested that JSD exhibited obvious tumor-cell inhibitory effects on tumor growth, without significant influence on body weight at the same time.

Caspase3, Caspase6, and Caspase9 were up-regulated in tumor samples of mice in synergistic group when compare to blank control group, and expressions of PI3K, AKT and HIF-1\(
\alpha
\) were decreased in tumor samples when the mice were

<table>
<thead>
<tr>
<th>5-FU concentration (μg·mL(^{-1}))</th>
<th>Growth inhibitory rate (IR, %)</th>
<th>5-FU Control Group</th>
<th>Low-concentration group (4 mg·mL(^{-1}) JSAE)</th>
<th>Medium-concentration group (5 mg·mL(^{-1}) JSAE)</th>
<th>High-concentration group (6 mg·mL(^{-1}) JSAE)</th>
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<tbody>
<tr>
<td>12.5</td>
<td>12.45 ± 4.51</td>
<td>20.58 ± 0.16(\uparrow)</td>
<td>17.10 ± 2.52</td>
<td>30.72 ± 1.27(\uparrow)</td>
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<tr>
<td>25</td>
<td>17.72 ± 6.85</td>
<td>30.67 ± 1.07(\uparrow)</td>
<td>31.90 ± 1.51(\uparrow)</td>
<td>30.60 ± 2.35(\uparrow)</td>
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<tr>
<td>50</td>
<td>28.39 ± 6.29</td>
<td>33.90 ± 0.63</td>
<td>38.16 ± 2.07</td>
<td>39.30 ± 3.04(\uparrow)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>35.74 ± 4.84</td>
<td>38.73 ± 1.73</td>
<td>42.21 ± 2.83</td>
<td>53.06 ± 2.91(\uparrow)</td>
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<tr>
<td>200</td>
<td>40.02 ± 6.93</td>
<td>39.43 ± 0.79</td>
<td>43.05 ± 2.76</td>
<td>56.01 ± 2.57(\uparrow)</td>
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</tr>
<tr>
<td>400</td>
<td>48.98 ± 4.40</td>
<td>49.33 ± 0.90</td>
<td>50.37 ± 1.59</td>
<td>63.24 ± 3.00(\uparrow)</td>
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<td>800</td>
<td>57.49 ± 5.74</td>
<td>61.84 ± 0.88</td>
<td>55.62 ± 0.77</td>
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<td>1600</td>
<td>65.00 ± 0.87</td>
<td>62.76 ± 0.72</td>
<td>66.50 ± 0.94</td>
<td>89.81 ± 0.55(\uparrow)</td>
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</table>

Notes: 5-FU Control Group: only treated with different concentration of 5-FU; Low-Concentration Group: 4 mg·mL\(^{-1}\) JSAE in combination with different concentration of 5-FU; Medium-Concentration Group: 5 mg·mL\(^{-1}\) JSAE in combination with different concentration of 5-FU; High-Concentration Group: 6 mg·mL\(^{-1}\) JSAE in combination with different concentration of 5-FU. \(\uparrow P < 0.05, \uparrow\uparrow P < 0.01\) vs the 5-FU control group.
Fig. 3  (A, B) Western blotting analysis of protein expressions of PI3K, AKT, p-AKT, HIF-1α, LDHA, Glut 1, HK II, Caspase3, Caspase7, and Caspase9 in HCT-8/5-FU cells of blank control group, 5-FU group, JSAE group and synergistic group. (C, D) Western blotting analysis of protein expressions of PI3K, AKT, p-AKT, HIF-1α, LDHA, HK II, Caspase3, Caspase6, Caspase7, and Caspase9 in HIF-1α silenced HCT-8/5-FU cells of blank control group, 5-FU group, JSAE group and synergistic group. Values were expressed as means ± SD (n = 3). *P < 0.05, **P < 0.01 vs blank control group; *P < 0.05, **P < 0.01 vs 5-FU group
treated with JSD, and this effect was further enhanced when combined with 5-FU. Moreover, JSD down-regulated the protein levels of HKII and Glut1, whereas these effects were further improved by combining JSD with 5-FU, the significant statistic difference was observed (P < 0.05). However, there was no significant difference in the protein levels of Caspase6.
between the synergetic group and 5-FU group ($P > 0.05$). In addition, no significant effect was observed on LDHA and Caspase7 protein levels in tumor samples of 5-FU group, JSD group, or synergetic group when compared with black control group (Figs. 4E and 4F).

**Discussion**

Despite 5-FU is widely used in the treatment of cancers, 5-FU drug resistance is one of the primary obstacles for chemotherapy failure in the patients with various solid tumors including colorectal cancer [18-19]. In our study, IC$_{50}$ of 5-FU against HCT-8/5-FU cells is obviously higher than that of HCT-8 cells and the RI of HCT-8/5-FU cells was 20.441, suggesting drug resistance of the used HCT-8/5-FU cells. Previously, we demonstrated that JSD inhibited the growth of colon cancer SW480 cells in a dose-dependent manner, and JSD repressed cancer cell migration and invasion [19]. In this present study, we found the growth inhibition rates of HCT-8/5-FU cells treated with JSD were increased along with the concentrations of JSD, suggesting JSD might be capable of suppressing 5-FU resistance to chemotherapy in colon cancer HCT-8/5-FU cells.

To investigate the underlying molecular mechanisms of 5-FU resistance, we used 5-FU-resistant colorectal cancer cell line (HCT-8/5-FU cells) and HCT-8/5-FU induced CRC mice model for *in vitro* and *in vivo* experiments. In recent years, HIF-1α has been recognized as an important cancer drug target on account of its elevated levels are strongly correlated with tumor metastasis, poor patient prognosis as well as tumor resistance therapy [21]. In the present study, the expression of HIF-1α was also found higher in CRC tissues than normal controls according to the bioinformatics analysis of publicly available data sets in Oncomicne.

It is reported that HIF-1 mediates metabolic conversion from oxidative phosphorylation to glycolytic pathway for hypoxic tumor cells through the induction of enzymes involved in the glycolysis pathway and overexpression of glucose transporters which increase glucose import into tumor cells [23]. Glycolysis is a major reason leading to drug resistance in solid tumors [16, 23]. It has been widely acknowledged that glycolysis is the main energy source for hypoxic cancer cells to produce enough energy, which promotes malignant tumor cells to adapt to the hypoxic environment [24]. Glycolytic enzymes, such as Glut1 and pyruvate dehydrogenase kinase-1 (PDK1), have been previously reported to be regulated by HIF-1α in response to hypoxia condition [25-26]. Furthermore, cells lost sensitivity to apoptosis under hypoxia condition which might be responsible for chemotherapy resistance, and HIF-1α is recognized as a major endogenous inhibitor to induce apoptosis through regulating Caspase family [27-29]. Western blotting analysis of HCT-8/5-FU cells showed significantly down-regulated HIF-1α expression under the treatment of JSD, as well as the obviously suppression of PI3K, AKT and p-AKT. Meanwhile, LDHA, Glut1 and HKII were significantly down-regulated in HCT-8/5-FU cells treated by JSD in combination with 5-FU. Similar protein expression changes were observed *in vivo*, while no significant effect was observed on LDHA protein levels. Moreover, JSD down-regulated the protein levels of HKII and Glut1, whereas these effects were further improved by combining JSD with 5-FU. Then, we speculated that JSD may overturn drug resistance of HCT-8/5-FU cells through suppression of glycolysis *via* signaling pathway involving PI3K/AKT/HIF-1α, as well as by activations of Caspase family (Caspase3, and Caspase9) to enhance anti-tumor activity.

Previous findings suggested that PI3K/Akt signaling pathway is involved in the resistance response to hypoxia-chemia through inactivation of apoptosis-associated proteins and regulate the HIF-1α which can further regulate the expression of downstream proteins involved in glucose metabolism and angiogenesis [10]. It has been reported wogonin could be a good candidate for the development of new multidrug resistance reversal agent by the suppression of HIF-1α expression via inhibiting PI3K/Akt signaling pathway [30]. Knockdown of kruppel-like factor 5 (KLFS) and astrocyte elevated gene-1 (AEG-1) could suppress hypoxia-induced chemoresistance via regulating PI3K/AKT/HIF-1α [32-33]. In our study, synergetic group showed significantly lower expression levels of PI3K, AKT and p-AKT than 5-FU group of HIF-1α silenced HCT-8/5-FU cells. HKII expression was lower in 5-FU group than control group, while higher in synergetic group than 5-FU group. Additionally, up-regulated expressions of Caspase6 and Caspase7 were observed in synergetic group.

**Conclusion**

Generally speaking, combination of JSD with 5-FU showed reversal potency of 5-FU resistance *in vitro* and anti-tumor efficiency *in vivo* by inhibiting signaling pathway involving PI3K/AKT/HIF-1α, thereby regulating glycolysis related proteins (HKII and Glut1) and Caspase family (Caspase3 and Caspase9) to suppress glycolysis and induce apoptosis. These molecules might be promising targets for development of effective therapy against chemoresistance in colorectal cancer.

**Abbreviations**

CRC, Colorectal cancer; JSD, *Jiedu Sangen* decoction; JSAE, *Jiedu Sangen* aqueous extract; 5-FU, 5-Fluouracil; PI3K, Phosphoinositide 3-kinase; AKT, Protein kinase B; HIF-1α: Hypoxia-inducible factor 1; HIF1A-AS1, Hypoxia-inducible factor 1 alpha-antisense RNA 1; TNF-α, Tumor necrosis factor-α; Caspase, Cysteiny1 aspartate specific proteinase; MDR1, MDR protein 1; MACC1, Metastasis associated colon cancer 1; HCT-8/5-FU, 5-FU resistant human colon cancer cells; LDHA, L-lactate dehydrogenase A; Glut1, Glucose transporter type 1; HKII, Hexokinase 2; IRB, Institutional review board; CCK-8, Cell counting Kit-8; IC$_{50}$, Inhibitory concentration of 50%; RI, Resistance index; RIPA, Radio immunoprecipitation assay; SDS, Sodium dodecyl sulfate; PVDF, Polyvinylidene fluoride; sh-NC, siRNA-targeting luciferase; i.p., intraperitoneally; i.g., intragastrically;
**References**


