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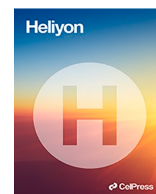

Research article

HuaChanSu suppresses the growth of hepatocellular carcinoma cells by interfering with pentose phosphate pathway through down-regulation of G6PD enzyme activity and expression

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ABSTRACT

HuaChanSu is active water extracts from the skin of *Bufo bufo gargarizans* Cantor. It has been already used to treat clinical cancers including HCC (Hepatocellular carcinoma, HCC), however, the molecular mechanisms under HuaChanSu's anti-cancer effects remain unclear. PPP (Pentose phosphate pathway, PPP), the major source of ribose and NADPH (Nicotinamide adenine dinucleotide phosphate, NADPH), is always over-activated and particularly critical for tumor cells growth. In this study, firstly, we illustrate that HuaChanSu restrains the growth of human hepatoma cells. More importantly, we demonstrate that the expression of G6PD (Glucose-6-phosphate dehydrogenase, G6PD), the first rate-limiting enzyme of the PPP, is restrained in human hepatoma cells after treatment with HuaChanSu. Additionally, our results show that G6PD enzyme activity and dimer formation are inhibited by HuaChanSu. Furthermore, we find that HuaChanSu could inhibit NADPH production and nucleotide level. In addition, we identify that expression of PLK1 (Polo-like kinase 1, PLK1) is also reduced in response to HuaChanSu, and knockdown of PLK1 restrains enzyme activity and dimer formation of G6PD, but has no effect on G6PD protein level. Subsequently, we demonstrate that inhibition of G6PD could restrain the proliferation of tumor cells and enhance the inhibitory effect of HuaChanSu on cell proliferation of human hepatoma cells. In conclusion, for the first time, our study reveals that HuaChanSu interferes with PPP via suppression of G6PD expression and enzyme activity to restrain growth of tumor cells, and these results provide a novel insight for the anti-hepatoma mechanisms of HuaChanSu and promote the innovation of the research model of TCM. Moreover, the development of drugs targeting abnormal tumor metabolism is currently a hot topic, our works provide theoretical support for further drug development from HuaChanSu, meanwhile, the revelation of the new molecular mechanism also provides a new perspective for the study of the pathogenesis of liver cancer.

Short abstract: HuaChanSu suppresses expression of G6PD, the first rate-limiting enzyme of the PPP, restrains G6PD enzyme activity and dimer formation via inhibition of PLK1, knockdown of G6PD could impair the growth of human hepatoma cells and increase the blocking effect of HuaChanSu on cell proliferation of cancer cells. In addition, HuaChanSu restrains NADPH production and nucleotide level, implying the suppression of PPP flux. Our study suggests that HuaChanSu interferes with PPP via G6PD inhibition to exert anti-hepatoma effects.

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1. Introduction

HCC is the sixth most common cancer worldwide, and its morbidity continues to rise [1]. For patients with HCC who meet the Milan criteria (HCC: single tumor diameter less than 5 cm or up to 3 nodules), the best treatment is liver transplant, but usually organ donors are scarce. Currently, surgery is still the first choice for HCC patients [2], followed closely by chemotherapy and radiation therapy [3]. However, most of HCC patients are diagnosed at advanced stage, and have missed the best time for surgery excision [4]. Drug treatment is still the primary medical therapy for patients with liver cancer.

TCM (Traditional Chinese medicine, TCM) has a long history and exerts an important role in cancer prevention and treatment, such as reversing precancerous lesions, impairing toxicity and enhancing efficacy of chemotherapy, inhibiting tumor recurrence and metastasis [5,6]. HuaChanSu is active water extracts from skin of *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider [7]. HuaChanSu capsules, HuaChanSu tablets, and Cinobufacini injection have been maturely used in clinic for the treatment of HCC and hepatitis B. Studies on the chemical composition and pharmacological activity of HuaChanSu begin in 1980s. HuaChanSu consists of two major categories of bioactive ingredients, which include steroidal cardiac glycosides (such as bufalin, resibufogenin, cinobufagin, cinobufotalin, bufotalin) and indole alkaloids (such as bufotenidine, bufotenine, cinobufotenine, and serotonin) [8]. Furthermore, for the past few years, the anti-tumor effects of HuaChanSu have been widely reported, and it has been used clinically to fight cancer including liver, lung, pancreatic, and colorectal cancers [9,10]. Nevertheless, the molecular mechanisms underlying its anti-tumor actions still need to be urgently elucidated.

Metabolic reprogramming is a pivotal hallmark of cancer cells. The metabolic flux in cancer cells is significantly reprogrammed to provide both excessive energy and intermediates which are essential for the rapid growth of cancer cells [11]. The Warburg effect is a unique metabolic phenomenon of cancer cells, even when oxygen is abundant, cancer cells are more inclined to catabolize high levels of glucose to lactate. The accumulated intermediates of glycolysis can branch into the PPP [12,13]. The PPP generates R-5-P (Ribose-5-phosphate, R-5-P) and NADPH for the biosynthesis of DNA/RNA and fatty acid, which are required for the rapidly growing tumors [14,15]. G6PD is the first rate-limiting enzyme of the PPP [16], which catalyzes the conversion of G6P (Glucose-6-phosphate, G6P) to 6-phosphogluconate and promotes the production of NADPH in the presence of NADP+. G6PD plays an important role in tumorigenesis and progression of many kinds of human cancers via increasing PPP flux [11,17]. Moreover, G6PD activity can be regulated by oncogenes such as mTORC1 [18] and PIKE-A/STAT3 [19], or anti-oncogene such as TAp73 [16] and P53 [20].

PLK1 belongs to serine/threonine protein kinase family, existing in eukaryotic cells [6], which has multiple functions during mitosis and is highly expressed in a broad range of human tumors, and its over-expression is associated with poor prognosis of cancer patients. Furthermore, increased PLK1 expression has been found to be correlated with chemotherapy resistance [21]. Many studies have shown that inhibition of PLK1 causes apoptosis in cancer cells by interfering with multiple stages of mitosis [22,23]. Meanwhile, studies have reported that PLK1 can directly phosphorylate G6PD and promote the formation of the active G6PD dimer, which ultimately activates PPP pathways to generate intermediates for the synthesis of ribonucleotide [24].

In this work, we confirm that HuaChanSu restrains the proliferation of human hepatoma cells. Moreover, we demonstrate that HuaChanSu suppresses G6PD expression and restrains G6PD enzyme activity and dimer formation via inhibition of PLK1, and knockdown of G6PD could impair the growth of human hepatoma cells and increase the blocking effect of HuaChanSu on cell proliferation of cancer cells. In addition, our results suggest that HuaChanSu restrains NADPH production and nucleotide level, implying the suppression of PPP flux. Our study provides a reliable theoretical foundation for the clinical application of HuaChanSu. It can also promote drug discovery from TCM.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's Modified Eagle's Medium (DMEM), 0.25 % trypsin-EDTA, fetal bovine serum (FBS), and penicillin-streptomycin mixture were obtained from Corning Life Sciences (Steuben County, New York, USA). Cell Counting Kit 8 was purchased from Beijing Bairuiji Biotechnology Co., Ltd. (Beijing, China). NADP+/NADPH detection kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). BCA protein assay kit was obtained from Solarbio (Beijing, China). G6PD enzyme activity detection kit was purchased from Beyotime Biotechnology (Shanghai, China). Antibodies against G6PD (ab210702) were purchased from Abcam (Cambridge, UK). Antibodies against PLK1 (Cat#208G4) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β-actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse (Cat#7076) and rabbit (Cat#7074) IgG-HRP antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Disuccinimidyl suberate (DSS) was purchased from Sangon Biotech (Shanghai, China). AMP (Adenosine monophosphate, AMP) was purchased from Aladdin (Shanghai, China). CMP (Cytidine monophosphate, CMP) was obtained from Yuanye biology (Shanghai, China). GMP (Guanosine monophosphate, GMP) was purchased from Macklin (Shanghai, China). HuaChanSu was obtained from Shanxi Eastantai Pharmaceutical Co., Ltd, the extraction method was shown in the patent (patent number: CN200510005277.4).

2.2. Cell culture

HepG2 and Huh-7 human hepatoma cell lines were acquired from American Type Culture Collection (Manassas, VA, USA). HepG2 and Huh-7 cells were cultured in DMEM medium supplemented with 10 % FBS in a humidified atmosphere with 5 % CO_2 at 37 °C.

2.3. Cell viability assay

Huh-7 cells (about 3.5×10^3 cells/well) were plated in 96-well cell culture plates. After 24 h, the cells were added with different doses of HuaChanSu for 24 h and 48 h, respectively. After, the cell viability was detected by CCK-8 assay kit. The absorbance of the reaction solution was read by a microplate reader (TECAN, SPARK, USA) at a wavelength of 450 nm.

2.4. Clone formation assay

Huh-7 cells were seeded in 6-well cell culture plates at a density of 500 cells/well, then incubated with different concentrations of HuaChanSu for 9 days. The colonies were fixed with methanol and stained with 0.1 % crystal violet at room temperature for 15 min. Then the dye, was washed with PBS and the stained colonies were photographed.

2.5. Metabolite extraction and UPLC-MS/MS analysis

2×10^6 HepG2 cells were collected and washed two times with cold PBS. Cell samples were treated with freeze-thaw cycle to fully release metabolites, followed by extraction with 1 mL of 80 % methanol. Next, the cell samples were completely dried under nitrogen gas. The powder containing metabolites was dissolved in 100 μL of 80 % methanol and injected into UPLC-MS/MS system for assay. The detail analysis conditions are as follows: mobile phase A was 2 mmol/L ammonium acetate solution, mobile phase B was methanol. Chromatographic separation was conducted on an Acquity UPLC® HSS T3 column (2.1 × 100 mm, 1.8 μm), the column temperature was 40 °C, flow rate was 0.2 mL/min, and the sample volume was 1 μL. Gradient program: 0–0.1 min, 0 % B; 0.1–4 min, 30 % B; 4–7 min, 60 % B. Detection was performed in multiple reaction monitoring (MRM) mode. Ion spray voltage was 5.5 KV, ion source temperature was 500 °C, curtain gas was 30 Psi, ion source gas1 and 2 was 50 Psi.

2.6. Measurement of intracellular NADPH

1×10^6 HepG2 or Huh-7 cells were plated in 6-well cell culture plates, then incubated with different doses of HuaChanSu. After 24 h, cells were lysed in 400 μL of extraction buffer, and centrifuged at 12,000×*g* for 10 min. The NADPH level was analyzed according to the instruction of test kit [25].The sample protein concentration was used to normalize the NADPH level.

2.7. G6PD enzyme activity assay

Briefly, 1×10^6 HepG2 or Huh-7 cells were plated in 6-well cell culture plates, then added with different doses of HuaChanSu. After 24 h, cells were extracted with 1 mL of G6PDH extraction buffer, G6PD enzyme activity was determined by using a G6PD enzyme activity assay kit [26]. The sample protein concentration was used to normalize the G6PD enzyme activity.

2.8. Western blotting analysis

HepG2 or Huh-7 cells were plated in 6-well cell culture plates and added with different doses of HuaChanSu for 24 h, then cells were harvested with cell lysis buffer for western blotting assay as previously described [27].

2.9. Protein cross-linking assay

HepG2 or Huh-7 cells were plated into 6-well cell culture plates and treated with HuaChanSu. After 24 h, the cells were washed twice with PBS, and subsequently incubated with 1 mM disuccinimidyl suberate (DSS) solution for 30 min. Next, the DSS solution was removed, tris buffer (10 mM) was added and the cells were incubated at room temperature for 15 min. Finally, the cells were washed with PBS, and harvested by cell lysis buffer for western blotting.

2.10. RNA extraction and quantitative RT-PCR

Total RNA was extracted using E.Z.N.A.® Total RNA Kit I (OMEGA). The concentration of RNA was determined using the NanoDrop 2000. Then using the Takara PrimeScript RT Reagent Kit (Otsu, Japan) to convert RNA into cDNA. The relative amount of mRNA was calculated using the relative quantification method. The primer pairs were as follows:

Human G6PD (Forward): 5′-TGAGCCAGATAGGCTGGAA-3';
Human G6PD (Reverse): 5′-TAACGCAGGCGATGTTGTC-3';
Human PLK1 (Forward): 5′-GGCAACCTTTTCCTGAATGA-3';
Human PLK1 (Reverse): 5′-AATGGACCACACATCCACCT-3';
Human β-actin forward: 5′-GGGACCTGACTGACTACCTC-3';
Human β-actin reverse: 5′-TCATACTCCTGCTTGCTGAT-3'.

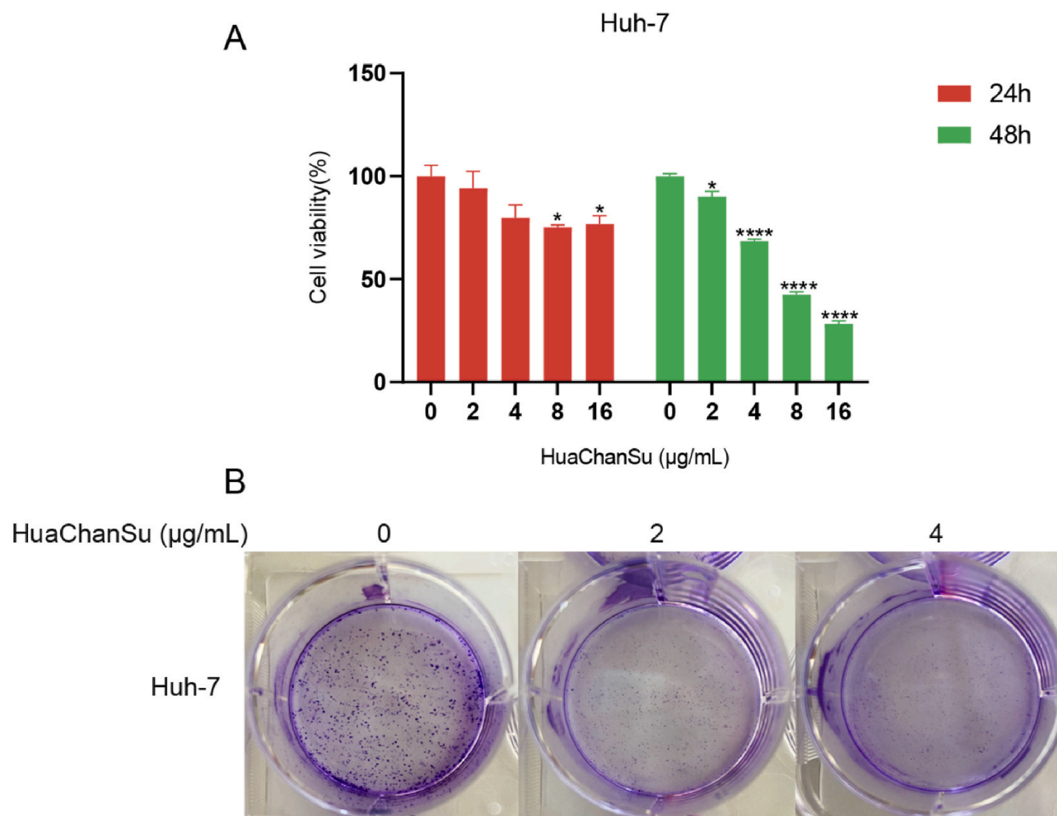

Fig. 1. HuaChanSu restrains the proliferation of human hepatoma cells. (A) Cell viability of Huh-7 cells treated with indicated concentrations of HuaChanSu (0–16 μg/mL) for 24 and 48 h. (B) Colony formation assay of Huh-7 cells treated with HuaChanSu (0, 2, 4 μg/mL). *$P < 0.05$, ****$P < 0.0001$ *vs* control group. The data are representative of three independent experiments, and each was performed at least in triplicate.

2.11. RNA interference

siRNAs targeting PLK1 or G6PD were synthesized from GenePharma (Shanghai, China). The cells were transfected with siRNAs by using Lipofectamine 2000 (Invitrogen, CA) as previously described [27].

The following siRNA sequences were used for cell transfection:

siG6PD: 5′-CAGAUGACGUCCGUGAUGA-3';

siPLK1: 5′-AUCACCCUCCUUAAAUAUUTT-3';

siNegative Control (siNC): 5′-UUCUCCGAACGUGUCACGUTT -3'.

2.12. Statistical analysis

Statistical analyses were performed with a two-tailed unpaired Student's *t*-test. The data was shown as mean ± SEM. All statistical tests were performed using the GraphPad Prism 8.0.2 software package. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. HuaChanSu inhibits cell viability of human hepatoma cells

Our previous study has shown that HuaChanSu can restrain the growth and colony formation ability of HepG2 cells [27]. In our present study, Huh-7 cells were treated with different concentrations of HuaChanSu for 24 and 48 h, the cell viability was investigated using the CCK-8 assay. As shown in Fig. 1A, HuaChanSu restrained the proliferation of Huh-7 cells. In addition, a similar result was discovered in the colony formation assay, after HuaChanSu treatment for 9 days, the Huh-7 cells exhibited weak proliferative potential (Fig. 1B). In conclusion, HuaChanSu exerts obvious anti-growth effect in human hepatoma cells.

3.2. HuaChanSu restrains G6PD level and enzyme activity in human hepatoma cells

G6PD is the first rate-limiting enzyme of PPP, and promotes the production of NADPH to fulfill macromolecular biosynthesis,

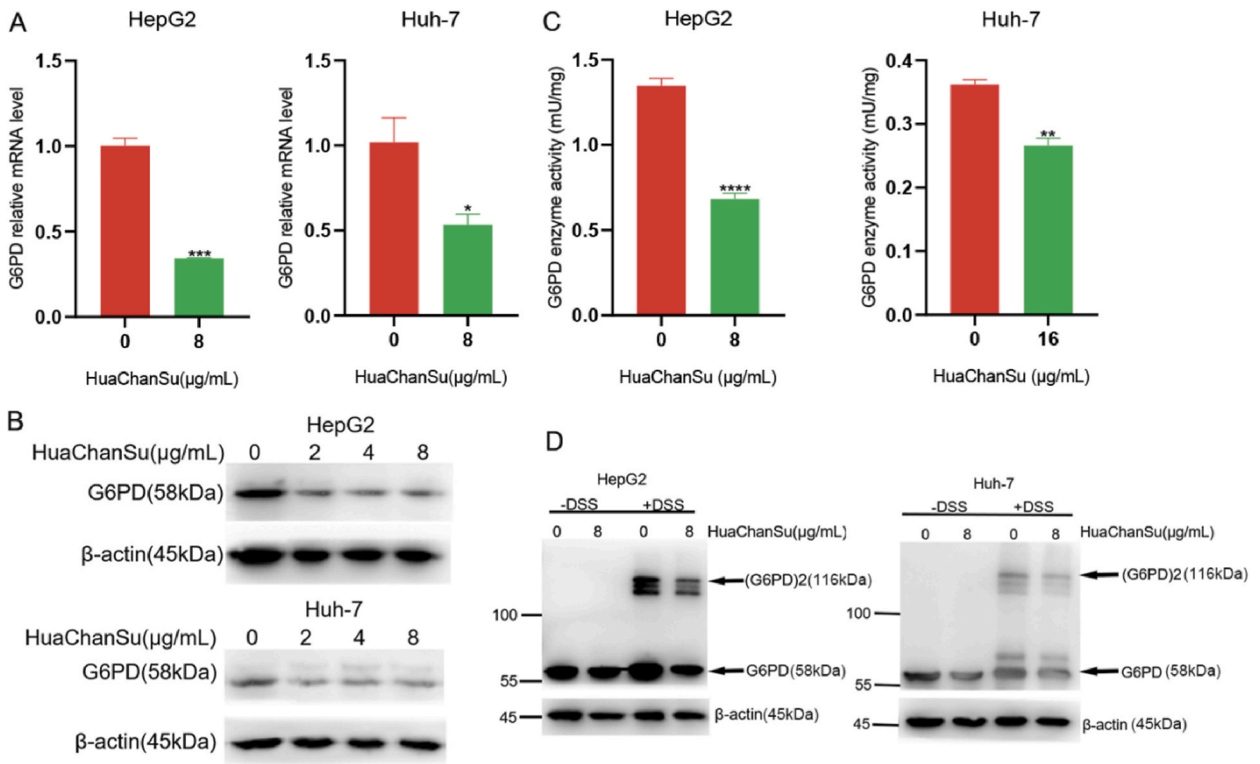

Fig. 2. HuaChanSu restrains G6PD level and enzyme activity in HepG2 and Huh-7 cells. (A) Quantitative RT-PCR assay of G6PD mRNA level in tumor cells after HuaChanSu (0, 8 μg/mL) treatment for 24 h. (B) Immunoblotting analysis of G6PD protein expression level in tumor cells treated with series concentrations of HuaChanSu (0, 2, 4, 8 μg/mL) for 24 h. (C) The G6PD enzyme activity of tumor cells treated with HuaChanSu for 24 h. (D) Tumor cells were treated with HuaChanSu at 0, 8 μg/mL for 24 h, then incubated with or without 1 mM DSS solution, followed by immunoblotting. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ *vs* control group. The data are representative of three independent experiments, and each was performed at least in triplicate.

meanwhile, increases the formation of R-5-P, which is an important precursor of nucleic acid synthesis [14,28]. In this study, firstly, we examined the mRNA level of G6PD in HepG2 and Huh-7 cells exposed to HuaChanSu, as shown in Fig. 2A, the quantitative RT-PCR data showed that HuaChanSu inhibited the mRNA level of G6PD. Meanwhile, western blotting showed that G6PD protein level was restrained in HepG2 and Huh-7 cells in response to HuaChanSu treatment (Fig. 2B). Furthermore, we also detected the activity of G6PD enzyme, as shown in Fig. 2C, HuaChanSu reduced G6PD enzyme activity in cancer cells. The G6PD protein exists in various oligomeric states, such as monomer, dimer, and tetramer to hexamer. In addition, the dimeric form of G6PD is catalytically active [14]. To detect whether HuaChanSu affects dimeric state of G6PD, we performed a protein cross-linking assay, the results showed that HuaChanSu could inhibit dimerization of G6PD (Fig. 2D).

3.3. HuaChanSu restrains NADPH and nucleotide level in human hepatoma cells

As a major source of cellular NADPH, the PPP provides the reducing capacity not only for supporting reductive biosynthetic reactions but also remaining cellular redox homeostasis [19,29,30]. Based on the inhibition of G6PD in tumor cells exposed to HuaChanSu treatment, firstly, we tested the effects of HuaChanSu on NADPH level. As shown in Fig. 3A, in HepG2 and Huh-7 cells, we discovered that HuaChanSu reduced NADPH level. PPP is an important pathway for nucleotide production [24]. Therefore, we examined the levels of several different nucleotides in liver cancer cells via UPLC-MS/MS analysis. As shown in Fig. 3B and C, the levels of AMP, GMP, and CMP were decreased in response to HuaChanSu.

3.4. HuaChanSu inhibits the G6PD enzyme activity via suppression of PLK1 in human hepatoma cells

Over-expression of PLK1 has been identified in various kinds of cancers such as liver cancer, prostate cancer, and lung cancer, and suppression of PLK1 could be considered as an effective strategy for cancer treatment [6]. Studies have confirmed that PLK1 affects the activity of G6PD [24]. In this study, firstly, we investigated whether HuaChanSu had any effects on the expression of PLK1 in HepG2 and Huh-7 cells. As shown in Fig. 4A, compared with the control group, there was a reduction of mRNA level of PLK1 in liver cancer cells exposed to HuaChanSu, suggesting that HuaChanSu treatment leads to transcriptional inactivation of the PLK1. Additionally, the

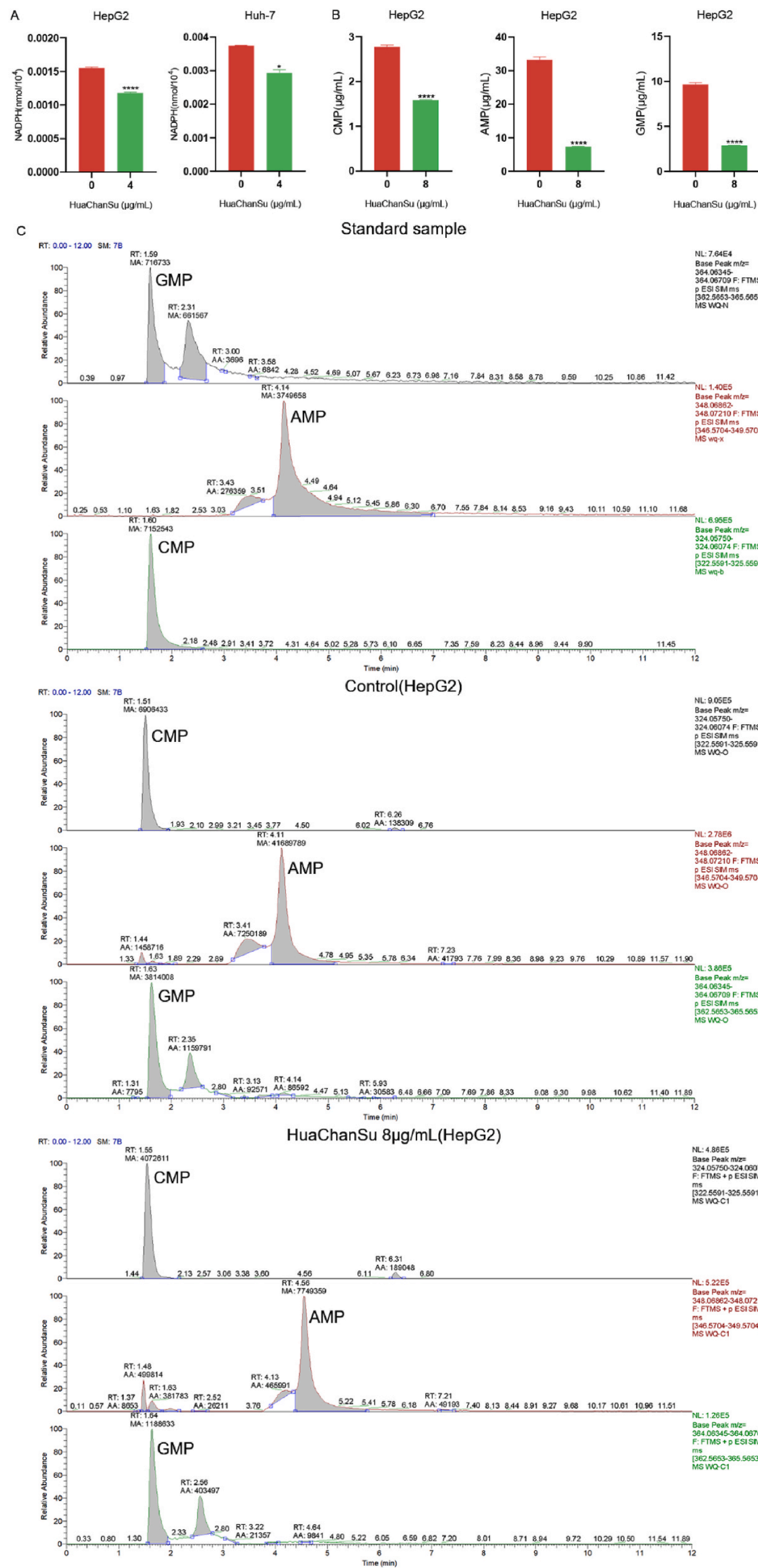

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Fig. 3. HuaChanSu restrains NADPH production and nucleotide level in HepG2 and Huh-7 cells. (A) The NADPH level in tumor cells treated with indicated doses of HuaChanSu (0, 4 μg/mL) for 24 h. (B) UPLC-MS/MS analysis results of intracellular nucleotides (AMP, CMP, and GMP) levels in tumor cells treated with HuaChanSu (0, 8 μg/mL) for 24 h. (C) MRM chromatograms of UPLC-MS/MS of standard sample and cell samples (HuaChanSu 0 and 8 μg/mL). MA: Peak area. *P < 0.05, ****P < 0.0001 *vs* control group. The data are representative of three independent experiments, and each was performed at least in triplicate.

western blotting results showed that PLK1 protein expression was down-regulated in HepG2 and Huh-7 cells exposed to HuaChanSu (Fig. 4B). Following that, we focused on how PLK1 affected G6PD. Tumor cells were transfected with siRNAs targeting PLK1 (siPLK1) or negative control (siNC), as shown in Fig. 4C, suppression of PLK1 had no obvious effects on G6PD protein level, but it's worth noting that G6PD enzyme activity and dimer formation were restrained by depletion of PLK1 in liver cancer cells (Fig. 4D and E). In conclusion, these results suggest that HuaChanSu could affect G6PD enzyme activity through down-regulation of PLK1.

3.5. HuaChanSu inhibits the proliferation of human hepatoma cells by restraining expression of G6PD

Subsequently, we detected the effects of G6PD on the proliferation of tumor cells. We first utilized siRNAs targeting G6PD to knockdown G6PD. As shown in Fig. 5A, knockdown of G6PD decreased the protein level of G6PD, and G6PD depletion caused the inhibition of growth of HepG2 and Huh-7 cells (Fig. 5B and C). Furthermore, suppression of G6PD increased the blocking effects of HuaChanSu on cell proliferation of liver cancer cells (Fig. 5D). The above results indicate that HuaChanSu induces tumor cells proliferation inhibition via down-regulation of G6PD.

4. Discussion

HCC is the primary liver cancer originating from hepatocytes, which approximately accounts for 85%–90 % of all primary liver cancers [31]. Published studies have demonstrated that only 10%–20 % of HCC patients can be treated with surgery, while most patients are appropriate candidates for chemotherapy [32]. Notably, chemotherapeutic drugs usually carry high risk of adverse effects. Furthermore, liver cancer has been confirmed to be highly resistant to traditional chemotherapy drugs, such as 5-fluorouracil and adriamycin. Hence, it is urgent to develop new effective drugs for liver cancer treatment. HuaChanSu is active water extracts from dry toad skin [33], which has been extensively used in clinical treatment of liver cancer, and could enhance the quality of life and prolong survival time of cancer patients [34,35]. Previous research has identified that bufadienolides are the primary anti-cancer active ingredients of HuaChanSu, such as cinobufagin, bufalin, and resibufogenin [33]. Research shows that HuaChanSu restrains

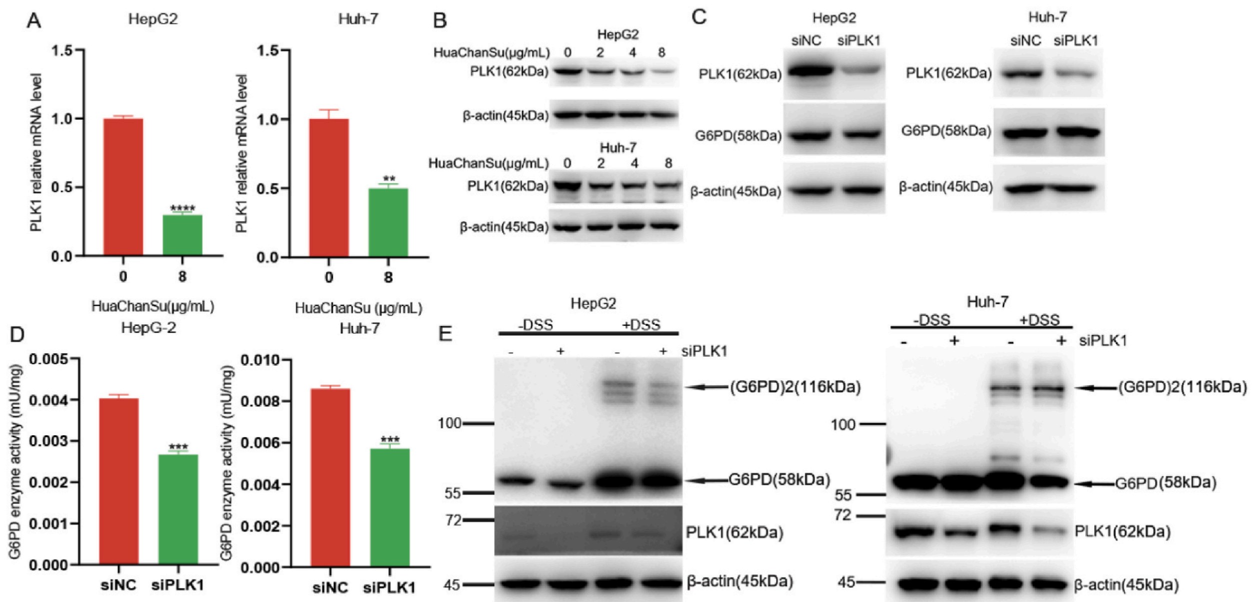

Fig. 4. HuaChanSu suppresses G6PD enzyme activity via down-regulation of PLK1 in HepG2 and Huh-7 cells. (A) Quantitative RT-PCR assay of PLK1 mRNA level in tumor cells treated with HuaChanSu (0, 8 μg/mL) for 24 h. (B) Immunoblotting analysis of PLK1 protein expression level in tumor cells treated with series concentrations of HuaChanSu (0, 2, 4, 8 μg/mL) for 24 h. Tumor cells were transfected with siRNAs targeting PLK1, (C) the PLK1 and G6PD protein expression levels were detected by immunoblotting, (D) the G6PD enzyme activity was examined. (E) Tumor cells transfected with siPLK1 or siNC were incubated with or without 1 mM DSS solution, followed by immunoblotting analyses. NC: Negative control. **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs* control group. The data are representative of three independent experiments, and each was performed at least in triplicate.

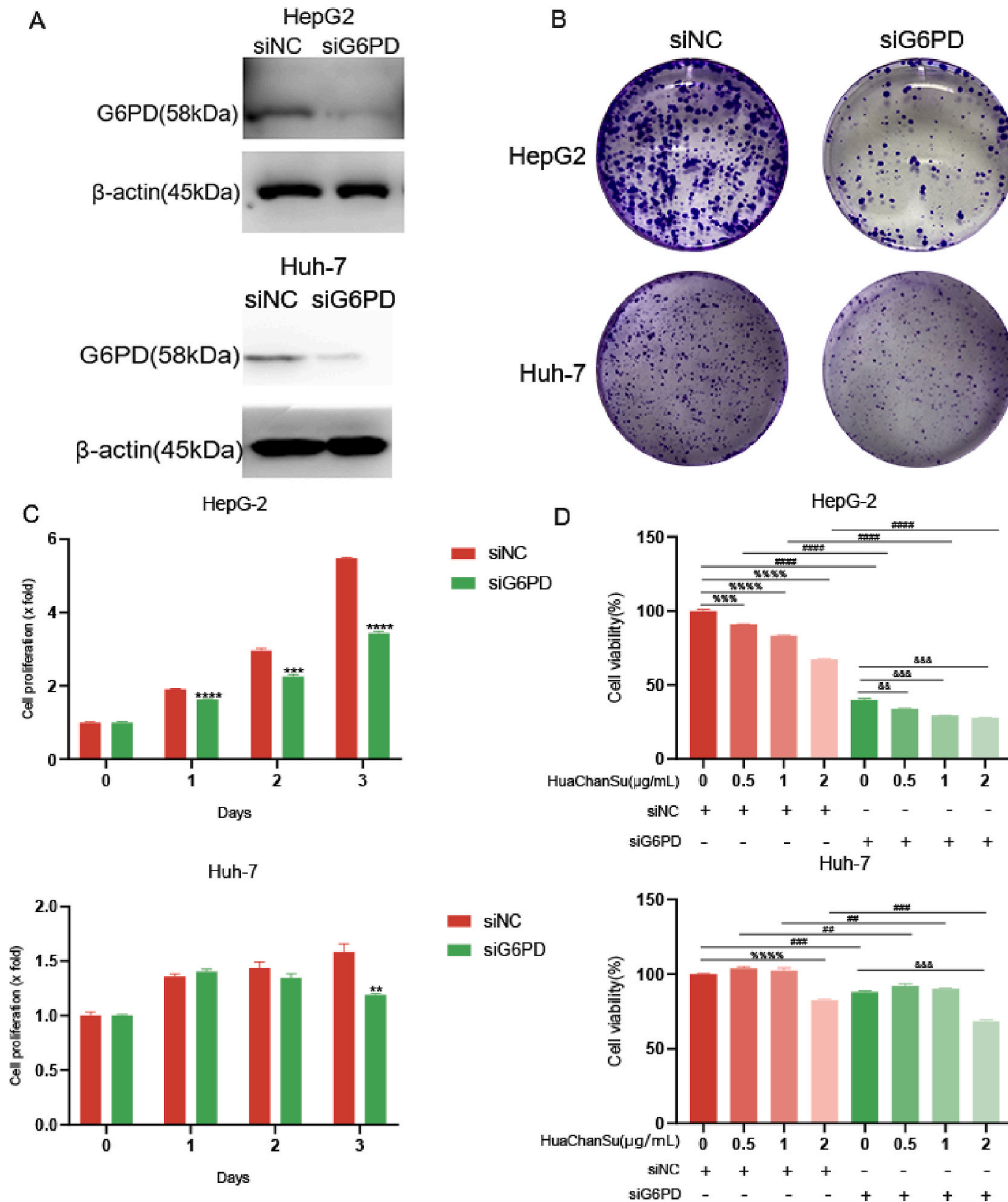

Fig. 5. HuaChanSu inhibits the proliferation of HepG2 and Huh-7 cells via down-regulation of G6PD expression. (A) Immunoblotting analysis of G6PD protein expression level in tumor cells transfected with siG6PD or siNC. (B) Cell proliferative potential was detected by colony formation assay in tumor cells transfected with siG6PD or siNC. (C) Cell proliferation rates were detected by CCK-8 assay in tumor cells transfected with siG6PD or siNC. (D) Cell viability of tumor cells transfected with siG6PD or siNC treated with or without HuaChanSu for 48 h. NC: Negative control. *: siNC (0, 1, 2, 3 Days) *vs* siG6PD (0, 1, 2, 3 Days), respectively; %: siNC control group *vs* siNC treated with HuaChanSu (0.5, 1, 2 μg/mL), respectively; #: siNC treated with HuaChanSu (0, 0.5, 1, 2 μg/mL) *vs* siG6PD treated with HuaChanSu (0, 0.5, 1, 2 μg/mL), respectively; &: siG6PD control group *vs* siG6PD treated with HuaChanSu (0.5, 1, 2 μg/mL), respectively. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; $^{\%\%\%}P < 0.001$, $^{\%\%\%\%}P < 0.0001$; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$; $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$.

tumorigenesis and development via multiple mechanisms. Ni T Y et al. have found that HuaChanSu restrains gastric cancer cell proliferation through regulation of Akt/mTOR signaling [35]. Published evidence has reported that HuaChanSu induces cell death via activating caspase-3 and inhibiting MAP kinase in non-Hodgkin's lymphoma [36]. Yang T et al. have found that HuaChanSu restrains human bladder cancer cell proliferation through regulation of the Fas/Fasl and TNF-alpha/TNFR1 pathway [37]. Our previous study has demonstrated that HuaChanSu down-regulates HK2 to impair the proliferation of HCC cells [27].

As a rate-limiting enzyme in PPP, G6PD plays a vital role, which exists in most normal tissues, such as liver, adrenal, and mammary

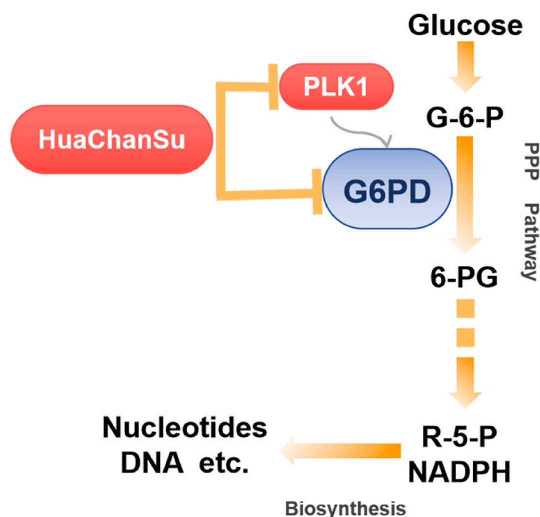

Fig. 6. Schematic diagram of HuaChanSu-mediated effects on human hepatoma cells by inhibition of G6PD.

adipose. Studies have reported that G6PD is highly expressed in several types of cancer, such as HCC, renal cancer, and oesophageal carcinoma, it is positively associated with worse prognosis [38,39]. Cancer cells activate G6PD to support cellular NADPH production and the synthesis of fatty acids and nucleic acids. Activation of pro-oncogenic pathways, for example TAp73, PI3K/Akt, and Src, has been confirmed to enhance G6PD activity [13,16,40]. Deletion of P53 could increase the PPP flux, suggesting that P53 may exert effect in the regulation of PPP. Further research has found that P53 directly inhibits the formation of G6PD dimer and restrains the enzyme activity of G6PD [20]. Glycosylation activates G6PD activity, which in turn promotes the PPP and tumor growth [11]. Furthermore, aberrant activation of G6PD is related to invasiveness, drug resistance, and unfavourable prognosis [14,38]. Targeting to G6PD is supposed to be an effective therapeutic strategy for anti-cancer treatment. In this study, firstly, we illustrate that HuaChanSu inhibits the expression, enzyme activity, and dimer formation of G6PD, secondly, we indicate that the NADPH production and nucleotide level are inhibited after treatment with HuaChanSu, indicating the suppression of PPP flux, thirdly, we confirm that knockdown of G6PD could inhibit the growth of HCC cells, and owing to the inhibition of G6PD, the blocking effects of HuaChanSu on the proliferation of tumor cells could be enhanced.

As a key regulator of the cell cycle, PLK1 has been confirmed to be a significant oncogene in cancer occurrence and progression. Studies have reported that PLK1 usually over-expresses in various kinds of human tumors, such as HCC, breast cancer, melanoma, and glioma, and it is positively related to poor patient survival. PLK1 deletion could reverse the drug resistance of cancer cells, and increase sensitivity to chemotherapy and radiotherapy [6,41]. At present, a series of PLK1 inhibitors have been tested in clinical. Volasertib, as the PLK1 inhibitor, has been proven to be effective against a variety of clinical tumors [42]. More importantly, published reports have identified that PLK1 inhibitor BI2536 can inhibit glycolysis and glutamine supplementation induced by metformin, which suggests that the inhibition of PLK1 could interfere with metabolic reprogramming [43]. In this study, we confirm that HuaChanSu suppresses PLK1 expression in HCC cells. Furthermore, we indicate that suppression of PLK1 could restrain the dimer formation of G6PD to restrain enzyme activity, but has no obvious effects on G6PD protein level.

In summary, our results show that HuaChanSu exerts anti-hepatoma activity via suppression of G6PD expression and enzyme activity to restrain PPP flux (Fig. 6), our study not only establishes a solid theoretical foundation for the clinical application of HuaChanSu, but also promotes the development of anti-cancer drugs from traditional chinese medicine.

5. Ethical standards

The manuscript does not contain clinical studies or patient data.

Additional information

No additional information is available for this paper.

6. Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Qi Wu: Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xue-li Ge:** Writing – original draft, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Zi-kai Geng:** Writing – original draft, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Hao Wu:** Methodology, Investigation, Formal analysis, Data curation. **Jing-yi Yang:** Investigation, Data curation, Conceptualization. **Shi-rong Cao:** Methodology, Investigation, Data curation, Conceptualization. **Ai-lin Yang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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