Inhibition of eukaryotic elongation factor-2 ~~Confers~~confers to tumor suppression by a herbal formulation Huanglian-Jiedu decoction in ~~Human~~human hepatocellular carcinoma

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Abstract

Ethnopharmacological Relevance

An oriental medicinal formulation, Huanglian Jiedu Decoction (HLJDD), has been well documented in few Traditional Chinese Medicine Classics 1300 years ago for treatment of heat and dampness-related diseases. Its effect is well accepted in Asian community, including China, Japan and Korea. Recent studies have postulated HLJDD as a regimen for cancer treatment, especially liver cancer, but the underlying mechanism is unknown. The aim of this study was ~~te~~to examine the suppressive effect of HLJDD on the growth of hepatocellular carcinoma (HCC) and its possible underlying mechanism.

Methods

Chemical composition of HLJDD was analyzed by high performance liquid chromatography. The tumor suppressive effect of HLJDD was determined on both HCC cells and xenograft model. Nascent protein synthesis was detected with Click-IT protein labeling technology; protein expression was determined by immunoblotting and imunnohistochemical analysis.

Results

Quality analysis revealed that HLJDD of different batches is consistent in both chemical composition and bioactivities. HLJDD inhibited HCC cell proliferation at its non-toxic doses, and suppressed growth and angiogenesis in xenografted murine model. HLJDD suppressed the synthesis of nascent protein via inactivation of eEF2 without deregulating the translation initiation factors. The major components in HLJDD, geniposide, berberine and baicalin, additively act on eEF2, and contributed to the responsible activity. HLJDD-activated eEF2 kinase (eEF2K) led to eEF2 inactivation, and activation of AMPK signaling may be responsible for the eEF2K induction. Blocked AMPK activity in HLJDD-treated HCC cells attenuated eEF2K activation as well as the inhibitory effect of the formula. In nutrient deprived HCC cells with inactivated eEF2, the inhibitory effect of HLJDD in tumor cell expansion was interfered.

Conclusion

Our results indicate that HLJDD has potential in blocking HCC progression with involvement of eEF2 inhibition.

Keywords: Nascent ~~Protein Synthesis~~protein synthesis; Hepatocellular ~~Carcinoma~~carcinoma; AMPK; eEF2; Huanglian Jiedu ~~Decoction~~decoction

1 Introduction

Hepatocellular carcinoma (HCC) is one of the world leading causes of death and the fifth ranked common human malignant tumor (Ng et al., 2010). HCC is more threatening in south-eastern Asia, with 33 out of 100,000 deaths per year was reported in male inhabitants. In Hong Kong, HCC is the second major cause of cancer deaths (Yip et al., 2009). For HCC patients, orthotropic liver transplantation is the best option amongst variety therapeutic strategies. However, only a small proportion of HCC patients is suitable for liver surgery (Liu et al., 2011). Different treatments have been developed in fighting against the unresectable HCC; nevertheless, the outcome remains poor due to the rapid growth of HCC cells.

Herbal medicine has been shown to be effective in treating HCC in clinical practice (Feng et al., 2008, 2011; Lin et al., 2005; Wang et al., 2013). However, the evidence-based research of herbal medicine remains challenging due to the concerns on the reproducibility of its pharmacological actions. Recent study on PHY906, a herbal product derived from ancient formula Huangqin Decoction has postulated its beneficial effect in reducing chemotherapy-induced gastrointestinal toxicity (Lam et al., 2010). Furthermore, another study on the Realgar-Indigo naturalis, suggested the synergy of the compounds rendering pharmacological actions of the whole formula in treatment of promyelocytic leukemia (Wang et al., 2008). These impacted and pioneered studies indicate that reproducibility of the pharmacological effects of herbal medicine could be achieved with stringent quality control of bioactive components. The concept of cancer appeared in modern medical theories; however, it is not very difficult to find some description of cancer-like symptoms in ancient medical records of China. It was recorded in an ancient Chinese medical monograph “Zhong-Zang-Jing” that “Yong, Yang, Chuang and Zhong” (which described the cancer-like symptoms) are caused by retention of various pathogens including heat and dampness. Huanglian Jiedu decoction (HLJDD, Oren-gedoku-to in Japanese and Hwangryun-Hae-Dok-tang in Korean) is an ancient herbal formula and its therapeutic effects have been well-documented in many Traditional Chinese Medicine Classics such as Medical Secretes of an Official (wai tai mi yao in Chinese) and Prescriptions for Emerent Reference (zhou hou fang in Chinese) (Ge. et al., 1996; Wang, 1955). It was used to treat internal heat-related mania, delirium, insomnia, irritability, dry mouth and throat, heat-induced blood omitting, skin spots, and sore furuncle (Chuang and Yang in Chinese) according to Medical Secretes of an Official. The formula was also used to treat heat pathogen-induced pyrostagnant rhinorrhagia, jaundice, and carbuncle (Yong in Chinese) as summarized by Prescriptions for Emerent Reference. It is composed of four herbal species: the rhizome of *Coptis chinensis* Franch. (Huang lian; Rhizoma Coptidis), the bark of *Phellodendron amurense* Rupr. (Huang bo; Amur cork tree), the fruit of *Gardenia jasminoides* J. Ellis (Zhi zi; Fructus Gardenia or cape jasmine), and the root of *Scutellaria baicalensis* Georgi (Huang qin; Chinese Skullcap). Modern studies have revealed the various pharmacological effects of HLJDD such as anti-ulcer, anti-trypanosomal, anti-inflammation and anti-oxidation (Mizukawa et al., 1993; Stefek and Benes, 1994; Takase et al., 1989; Yabu et al., 1998). It is also found to reduce neutrophil infiltration (Hwang et al., 2002) and suppress pre-adipocyte differentiation (Ikarashi et al., 2012). These actions rendered possibility of the decoction in treating various diseases including acute gastric mucosal lesions, liver injury, colitis, hypercholesterolemia, transient cerebral ischemia, allergic dermatitis and Alzheimer′s disease (Gao et al., 2005; Ohta et al., 1999; Ohta et al., 1997; Sekiya et al., 2005; Xu et al., 2000; Yu et al., 2010; Zhou and Mineshita, 1999). Our clinical observation and review studies also showed that HJLDD has liver protection and anticancer effects and therefore possesses high potential in treatment of liver diseases and cancer (Feng et al., 2008; Tang et al., 2009). A recent study showed that HLJDD inhibited human liver cancer in vitro (Hsu et al., 2008), however, the underlying mechanism regarding its anti-tumor effect has not been fully understood.

In this study, we reported eukaryotic elongation factor-2 (eEF2) as a new target of HLJDD in inhibiting HCC progression. We found that HLJDD inhibited proliferation of HCC cells in vitro, and suppressed the tumor growth in vivo. This inhibitory effect of HLJDD may be due to the suppression of nascent protein synthesis, which supports proliferation of the cancer cells (Shin et al., 2014). HLJDD controlled nascent protein synthesis by suppressing eEF2 activity, the protein essential for nascent peptide elongation (Kaul et al., 2011). The effect may be contributed by the activation of eEF2 kinase (eEF2K), and the regulation of AMPK/mTOR activity by HLJDD. In addition, we found that the combinatory effect of major active compounds of HLJDD inhibited eEF2 activity. Berberine is the major active component in inhibiting eEF2 while other compounds such as baicalin, geniposide and palmatine have additive actions. Our study reveals a novel mechanism involved in anti-cancer effect of HLJDD and may indicate the translational potential of the decoction as a complementary agent in HCC treatment.

2 Material and ~~Methods~~methods

2.1 Herbal preparation, chemicals and reagents

Coptis chinensis Franch. (voucher No. 120914), *Phellodendron amurense* Rupr. (voucher No. 120828), *Gardenia jasminoides* J. Ellis (voucher No. 120925) and *Scutellaria baicalensis* Georgi (voucher No. 120730) were collected and authenticated by Dr. Feng Yibin according to Chinese Pharmacopeia 2010 edition. The herbs were vouched and stored in specimen room of School of Chinese Medicine, The University of Hong Kong. To prepare the herbal extract, 900 g rhizomes of *C. chinensis* Franch., 900 g fruits of *G. jasminoides* J. Ellis, 600 g bark of *P. amurense* Rupr. and 600 g root of *S. baicalensis* Georgi were mixed together and boiled in 30 L of distilled water for 2 h and then filtered. The extraction was repeated twice and the filtrates were collected and evaporated to dryness. The extract powder was kept in –20 °C. Three batches of HLJDD were produced. Berberine chloride, palmatine chloride, baicalin and geniposide were purchased from Sigma-Aldrich (USA).

2.2 Antibodies

Rabbit monoclonal to GAPDH (14C10, #2118), rabbit monoclonal to phosphor-AMPKα (Thr172, 40H9, #2535), rabbit polyclonal to AMPKα, #2532), rabbit polyclonal to phosphor-eEF2K (Ser366, #3691), rabbit polyclonal to eEF2K (#3692), rabbit polyclonal to phosphor-eEF2 (Thr56, #2331), rabbit polyclonal to eEF2 (#2332), rabbit monoclonal to eIF4A (C32B4, #2013), rabbit polyclonal to eIF4H (#2444), rabbit polyclonal to phosphor-eIF4E (Ser209, #9741), rabbit monoclonal to eIF4E (C46H6, #2067), rabbit polyclonal to phosphor-eIF4G (Ser1108, #2441) and rabbit monoclonal to eIF4G (C45A4, #2469) were purchased from Cell Signaling Technology Inc. (USA). Rabbit polyclonal to mTOR (ab2732), rabbit polyclonal to mTOR (phosphor S2448, ab51044),

rabbit polyclonal to Ki67 (ab15580) and rabbit polyclonal to CD31 (ab28364) were purchased from abcam (UK).

2.3 Chemical analysis

The chemical profile of HLJDD was obtained with high performance liquid chromatography (HPLC). Briefly, standards and samples solutions were injected into the HPLC system (Waters, USA) and separated on C18 ODS HPLC column ~~(250×4.0mm,~~(250×0.0250x4.0 ~~5μm,~~mm, 5 μm, ACE, Scotland) with gradient elution. 50 mM KH_2PO_4 buffer (A) and acetonitrile (B) were used as mobile phase (0 min: A:B=80:20; 7 min: A:B=75:25; 50 min: A:B=75:25; 63 min: A:B=52:48; 65 min: A:B=20:80; 75 min: A:B=20:80). The flow rate was 1.0 mL/min and the displayed wavelength was ~~265nm.~~265 nm. Three batches of HLJDD were analyzed and the common chemical profile of the decoction was generated by recognizing relevant peaks on the chromatogram. Analysis of any heavy metals and organic pesticides contamination were conducted with methods described in our previous study (Tan et al., 2013).

2.4 Cells and cell culture

Human hepatocellular carcinoma cell line HepG2 was purchased from American Type Culture Collection (ATCC, USA). Human hepatocellular carcinoma cell line MHCC97L was kindly gifted by Dr. Man Kwan (Department of Surgery, The University of Hong Kong). Cells were maintained in DMEM medium with high glucose and incubated in a humidified atmosphere containing 5% CO_2 at ~~37°C,~~37 °C.

2.5 Cell viability assay

MTT assay was introduced to detect the cytotoxicity of HLJDD. In brief, cells were cultured in 96-well plate and treatment was made. All experiments were conducted parallel with controls (0.1% DMSO). 10 μl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml, Sigma, USA) were added to each well at the end of treatment and incubated at ~~37°C~~37 °C for 4 h. The medium was removed and 100 μl of DMSO was added to each well. The absorbance of formazan formed was measured at ~~595nm~~595 nm by Multiskan MS microplate reader (Labsystems, Finland). The percentage of viability was calculated as the following equation: (viable cells)%=(OD of drug-treated sample/OD of vehicle-treated sample)×100.

2.6 Clonogenic assay

10,000 cells were seeded into 6-well plate and treated with HLJDD for 12 days. At the end of treatment, medium was removed and the cells were fixed with 4% paraformaldehyde in PBS and stained with 2% of crystal violet.

2.7 Nascent protein synthesis assay

Detection of nascent protein synthesis was conducted using the Click-IT Chemistry Technology (Life Technologies, USA). HCC cells were incubated with methionine-free DMEM medium containing HLJDD. The cells were washed with PBS and incubated with methionine-free DMEM containing L-azidohomoalanine (AHA, 50 μM) at ~~37°C,~~37 °C, 5%CO_2. Cells were harvested and protein was collected. A total of 200 μg azide-labeled protein was used for reaction using the Click-IT protein reaction buffer kit (Life Technologies, USA). Briefly, the protein was labeled with biotin in reaction buffer containing $CuSO_4$ for 20 min at room temperature, and then protein was precipitated with methanol/chloroform and washed with methanol. The air-dried protein was dissolved in RIPA buffer for further analysis.

2.8 Immunoblotting

Cells were lysed with Radioimmunoprecipitation assay (RIPA) buffer with complex cocktailed proteinase inhibitor (Roche, USA) and phosphatase inhibitor (1 mM Na_3VO_4 and 1 mM NaF) on ice for 30 min followed by centrifugation at 14,000 rpm at ~~4°C~~4 °C for 15 min. Supernatant was transferred and protein concentration was determined by BSA assay (Bio-rad, USA). Equal yield of protein was separated on SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF, Biorad). The membrane was then blocked in buffer containing 5% BSA, Tris (10 mmol/L, ~~pH7.4),~~ pH 7.4), NaCl (150 mmol/L) and Tween 20 (1%) at room temperature for one hour with gentle shaking. The membrane was then incubated with primary antibodies at ~~4°C~~4 °C overnight followed by incubation with appropriate secondary antibody (Abcam, UK) at room temperature for 1 h. The immunoreactivites were detected using ECL advanced kit (GE Healthcare, UK) and visualized by chemiluminescence imaging system (Bio-Rad, USA).

2.9 Xenograft model

The tumor xenograft model was used in this study. Briefly, 6-week-old female BALB/c nu/nu athymic nude mice received injection of 1×10^7 MHCC97L cells suspended in 0.2 ml DMEM medium subcutaneously at its left side of waist. One week after injection, mice were received HLJDD (25, 50, and 100 mg/kg) or PBS every two days via gavage for 3 weeks. At the end of experiment, mice were sacrificed by injecting overdose of pentobarbital (200 mg/kg). Tumor volume and body weight were measured with the tumor volume=1/2 ~~(length ×width~~(length×width^2). All animals received human care and study protocols complied with guidelines of the Laboratory Animal Centre of The University of Hong Kong. Moreover, animals were processed according to the suggested international ethical guidelines for the care of laboratory animals throughout the experiments.

2.10 Immunohistochemistry

Immunohistochemistry was performed on paraffin-based section of the tumor. In brief, the slide was deparaffinized in xylene for 2 times, and then the slide was transferred to 100%, 95%, 70% and 50% alcohols respectively for 2 changes. The endogenous peroxidase activity was blocked by incubating the sample with 3% H_2O_2 at room temperature for 10 min. Then the slide was rinsed with PBS, and antigen retrieval was performed to unmask the antigenic epitope by incubating the slides with citrate buffer (10 mM, ~~pH6.0)~~ pH 6.0) at ~~100°C~~100 °C for 10 min. The slide was cooled down and rinsed with PBS for 2 changes. The slide was blocked with 10% normal goat serum (Invitrogen, USA) in TBS-Tween 20 buffer at room temperature for 1 h to avoid the non-specific binding of the antibody, and then drained. After that 100 µl diluted primary antibody was dropped on the top of the sample and the slide was incubated in a humidified chamber at ~~4°C~~4 °C overnight. After washed with PBS for 2 times, the sample was applied with diluted secondary antibody and incubated in a humidified chamber at room temperature for 1 h followed by wash. Then the slide was stained with DAB substrate kit for peroxidase (vector laboratories, USA) under ~~manufacturer's~~manufacturer's instruction. Counter stain was conducted by immersing the slide in Hematoxylin for 1 min. The slide was then dehydrated and visualized under microscope (Leica Microsystems Digital Imaging, Germany) with magnification of 10×10 to determine the level of liver damage. Images were captured with Leica DFC 280 CCD camera.

2.11 Statistical analysis

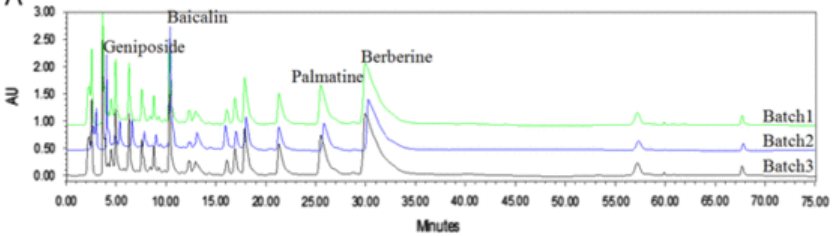
All the studies were conducted in triplicate except particular notice. Data was expressed as mean±SD. Statistical analysis was performed with Student *T*-Test.

3 Results

3.1 Critical quality control on HLJDD

Stringent quality control was conducted to ensure consistent chemical composition and bioactivity of different batches of decoctions. With HPLC-UV analysis, we generated the common chemical profile among 3 batches of HLJDD samples (Fig. 1A). By comparing the major peaks of HLJDD with the standard compounds, we found that HLJDD comprises four distinct constituents including berberine, palmatine, baicalin and geniposide. Berberine and palmatine are majorly contributed by *C. chinensis* Franch. and *P. amurense* Rupr. whereas baicalin and geniposide are derived from *S. baicalensis* Georgi and *G. jasminoides* J. Ellis respectively. Quantification of the four compounds is shown in Fig. 1B. The content ratio of the four compounds in HLJDD is approximately 3 (geniposide, GEN): 3 (berberine, BBR): 1 (baicalin, BAI): 1 (palmatine, PAL). There are no harmful heavy metal and organic pesticides detected in all three batches (data not shown).

A



B

Component	Retention time (min)	Content (mg/g in HLJDD)
Geniposide	5.04	23.5
Baicalin	9.88	7.5
Palmatine	24.07	8
Berberine	29.45	23.9

Fig. 1 Quality analysis, ~~A~~A shows the chemical analysis of HLJDD by HPLC. The results indicate that the major active components in HLJDD are geniposide, baicalin, berberine and palmatine; ~~B~~B shows quantification of major compounds. The ratio of content in HLJDD is about 3:1:3:1.

3.2 HLJDD suppresses neoangiogenesis and xenografted growth of HCC

To further determine the anti-cancer effect of HLJDD on HCC, we developed a HCC xenograft murine model. The HCC cells were subcutaneously injected into the right flank of mice and the mice were treated with different doses of HLJDD via oral administration. It was found that mice with treatment of HLJDD had no significant body weight loss, which may reveal that HLJDD has no potent toxicity to the mice (Fig. 2A). The tumor size was measured three times per week, and HLJDD was shown to suppress the xenografted growth of HCC in dose-dependent manner (Fig. 2B). The tumor was then dissected out at the end of experiment, and significant reduction of tumor size was observed, which may indicate that HLJDD inhibited in vivo tumor growth dose-dependently (Fig. 2C). Immunohistochemical analysis showed that the expression of tumor proliferation marker, Ki67, was potently inhibited by HLJDD. Furthermore, the neoangiogenesis of HCC may be suppressed by HLJDD intervention, as evidenced by the observation of potent reduced expression of endothelial cell marker, CD31in the tumors from HLJDD-treated mice (Fig. 2D). Our results indicate that HLJDD may be able to inhibit the growth and neoangiogenesis of xenografted HCC in vivo.

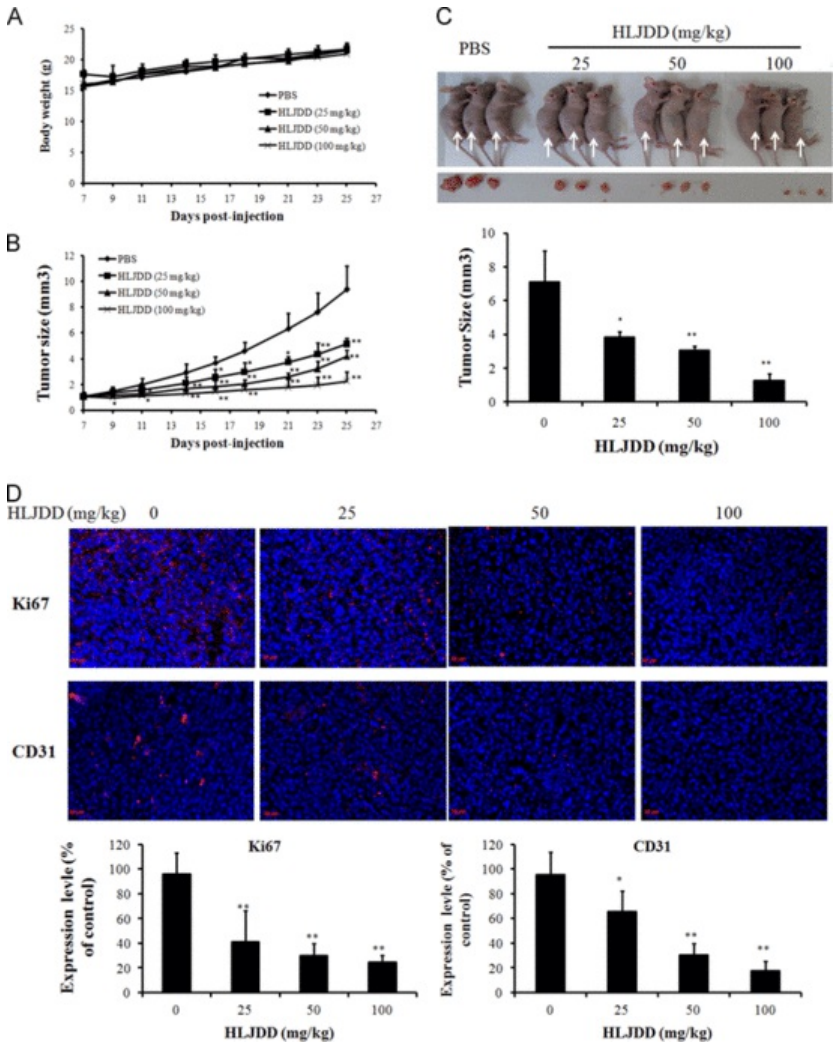


Fig. 2 HLJDD suppresses xenografted tumor growth in vivo. ~~A~~A shows the body weight of mice has no significant differences among groups. Mice were subcutaneously injected with 1×10^7 MHCC97L cells at the left side of waist. One week after injection, mice were received either PBS or HLJDD (25, 50, 100 mg/kg, three times per week for 3 weeks) via gavage. Body weight was measured once per two days. The result indicates that HLJDD exhibited no potent toxicity to the mice. ~~B~~B shows the tumor growth was reduced dose-dependently in mice with HLJDD treatment. The tumor sizes were measured once per two days. The result indicates that HLJDD may suppress tumor growth in vivo in dose manner. **C** shows that end-point tumor size was reduced by HLJDD. At the end of treatment, mice were sacrificed and tumors were dissected out. ~~D~~D shows that HLJDD reduced the tumor biomarker in xenografted model. The tumor cell proliferation marker Ki67 and the blood endothelial cell marker CD31 (as indicator of tumor angiogenesis) were determined by immunofluorescence. HLJDD treatment dose-dependently reduced the expression of both biomarkers, which confirms the suppressive effect of HLJDD on in vivo tumor growth. *p<0.05, **p<0.01 when compared to control.

3.3 Nascent protein synthesis of HCC was blocked by HLJDD

Previous study has shown that HLJDD has potent anti-cancer effect via induction of cell cycle arrest and apoptosis in HCC cells at its cytotoxic doses (Hsu et al., 2008). It is consistent with our study that HLJDD has potent inhibition on cell viability of HCC cell lines HepG2 and MHCC97L (Fig. 3A). The IC_{50} of HLJDD in HepG2 cells for 48 h treatment was approximately 150 μg/mL; while in MHCC97L cells, which exhibited highly resistant to the toxicity induced by HLJDD, the IC_{50} was around 500 μg/mL. Interestingly, it was observed in clonogenic assay that HLJDD suppressed the growth of HCC cells at its non-toxic doses. The 50 μg/mL and 100 μg/mL of HLJDD could effectively suppress the proliferation of HCC cells according to our study (Fig. 3B). To further

elaborate this pharmacological action, we then examined whether HLJDD could block the synthesis of nascent protein in both HepG2 and MHCC97L cells. By monitoring the incorporation of AHA, the rate of protein synthesis was potently reduced in HLJDD-treated HCC cells (Fig. 3C). This may indicate that HLJDD could globally suppress the protein expression of HCC cells. To further elucidate the mechanism underlying the translation inhibition of protein synthesis by HLJDD, the involvement of possible regulators in translational control was determined. It was found that HLJDD has no significant effect on the expression and activity of protein translation initiation regulators, including ~~eIF4~~eIF4A, eIF4E and eIF4G (Fig. 3D).

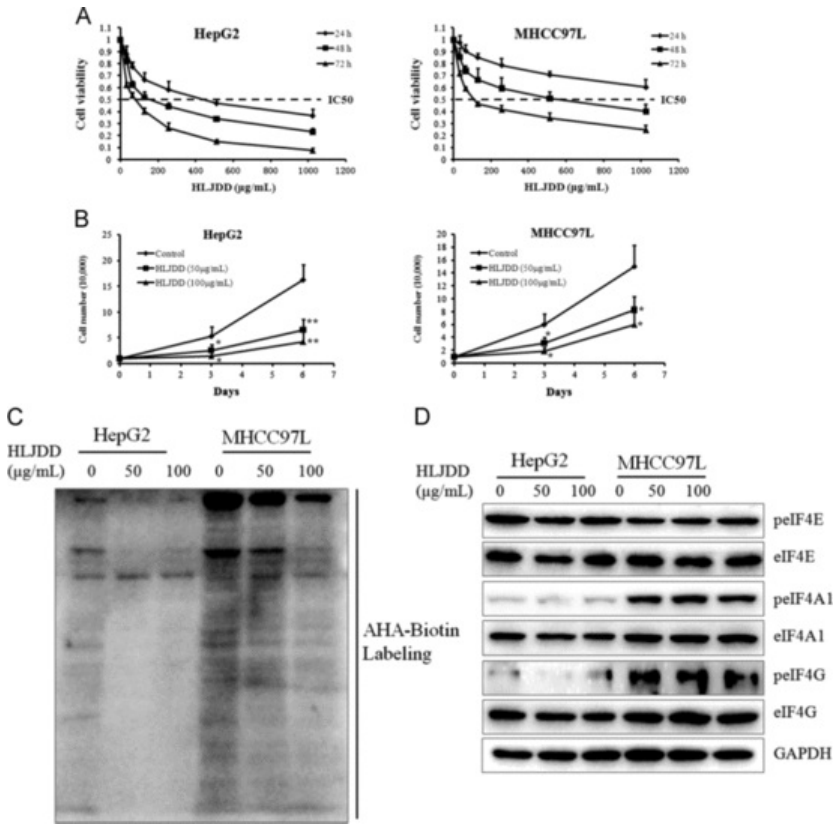


Fig. 3 Non-toxic dose of HLJDD inhibits nascent protein synthesis. ~~A~~A shows the cytotoxicity of HLJDD on HCC cell lines HepG2 and MHCC97L. Cells were treated with HLJDD and the cell viability at 24, 48 and 72 h was determined by MTT assay. The results show that high-dose of HLJDD exhibited potent toxicity to HCC cells; ~~B~~B shows non-toxic doses of HLJDD inhibits HCC cells proliferation in vitro. The proliferation of HCC cells were determined by growth curve. 10,000 cells were seeded into the plate and treated with 50 or 100 μg/mL of HLJDD for 3 and 6 days. The cell number was counted. It was observed that non-toxic doses of HLJDD could significant inhibit cell growth and proliferation. **C** shows that HLJDD suppresses the synthesis of nascent proteins in HCC cells. Cells were pre-treated with HLJDD and then cells were incubated with methionine free medium containing AHA to label the newly synthesized proteins. The protein was then collected and biotinylated labeled. The nascent protein was then detected by immunoblotting with streptavidin-conjugated antibody. ~~D~~D shows that HLJDD has no effect on the expression and activity of translation initiation regulators.

3.4 Components in HLJDD inhibit eEF2 activity in HCC cells

Instead, HLJDD was able to block the activity of translation elongation regulator eEF2 by inducing its phosphorylation at Th56 site (Fig. 4A). Inducing Th56 phosphorylation of eEF2 blocks its activity in mediating the elongation of newly synthesized peptides, which consequently suppresses the nascent protein synthesis (Kaul et al., 2011). Three batches of HLJDD consistently suppressed eEF2, further confirms the biological activity of HLJDD across different batches could be consistently achieved (Fig. 4B). Interestingly, the four major components were found to induce the Th56 phosphorylation of eEF2 in both HepG2 and MHCC97L cells, which may indicate that all the four compounds could inactivate eEF2 in HCC independently (Fig. 4C). However, induction of eEF2 phosphorylation in berberine and baicalin-treated HCC cells was much more remarkable than in geniposide and palmatine-treated cells. To further examine whether the four compounds could have combinatory effect on eEF2 phosphorylation, we treated the HCC cells with mixed compounds. It was observed that the effect of four compounds on eEF2 phosphorylation was additive and combination treatment exhibited stronger action on eEF2 inactivation than any of the single compound (Fig. 4D). This combinatory effect was further evidenced by the observation that combination exhibited more potent inhibition on nascent protein synthesis in HCC cells than any of the single treatment. Our results indicate that active compounds in HLJDD may complementarily inhibit

nascent protein synthesis in HCC cells via targeting on eEF2.

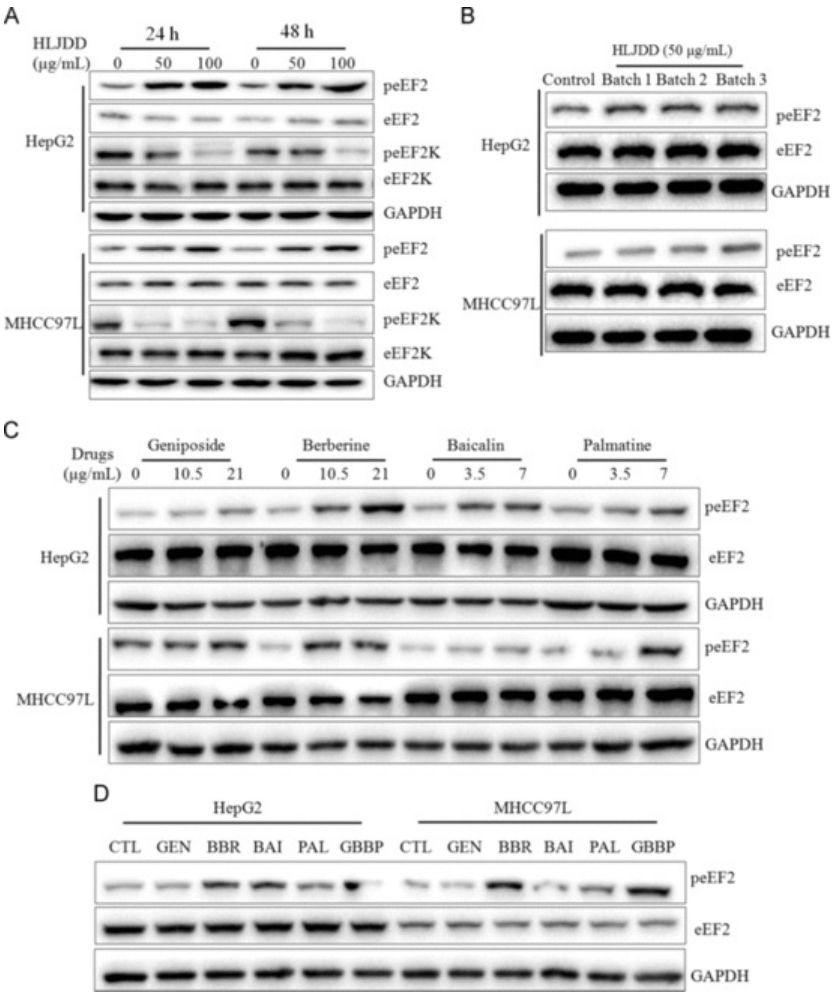


Fig. 4 Components in HLJDD inhibits eEF2. ~~A~~A shows that HLJDD could induce the phosphorylation of eEF2 and suppress the eEF2K phosphorylation in dose- and time-dependent manners. Cells were treated with HLJDD for 24 and 48 h and then expression of phosphorylated eEF2 and eEF2K was detected by immunoblotting. ~~B~~B shows three batches of HLJDD could consistently inhibit eEF2; **C** shows that the four compounds could up-regulate the phosphorylation of eEF2 respectively. HepG2 and MHCC97L cells were treated with geniposide, baicalin, berberine and palmatine for 24 h then collected. eEF2 phosphorylation was analyzed by immunoblotting. All the four compounds could induce phosphorylated eEF2 expression at different doses. ~~D~~D shows that combination of the four compounds additively increases eEF2 phosphorylation.

3.5 eEF2K/AMPK inhibition by HLJDD mediates eEF2 inactivation in HCC cells

The activity of eEF2 majorly regulated by eEF2 Kinase (eEF2K), which responsible for eEF2 phosphorylation at Th56 site (Hizli et al., 2013). We therefore examined if HLJDD could up-regulate the activity of eEF2K. Consistently, we found that HLJDD potently reduced the phosphorylation of eEF2K at its Ser366 site (Fig. 4A), which in consequence initiated eEF2K activity by suppressing ~~eEF2(~~eEF2 (Sataranatarajan et al., 2007). To further determine if eEF2K is responsible for the phosphorylation of eEF2 by HLJDD, we used A-484954 to block the activation of eEF2K. Presence of A-484954 recapitulated the up-regulation of phosphorylated eEF2 (Fig. 5A), indicating that HLJDD-induced eEF2 inactivation may be controlled by eEF2K-related mechanism. These results also reveal the possible involvement of eEF2K/eEF2 signaling in the inhibitory effect of HLJDD on nascent protein synthesis in HCC cells. Furthermore, we observed that HLJDD activated the AMP kinase in HCC cells (Fig. 5B). Activation of AMPK was reported to suppress the mTOR activity, followed by blockade of mTOR-mediated eEF2K phosphorylation (Sataranatarajan et al., 2007). Blockade of AMPK by treating the cells with compound C restored the mTOR activity and eEF2K phosphorylation (Fig. 5B). Our results reveal that the AMPK/mTOR/eEF2K/eEF2 may be involved in the HLJDD-mediated regulation on nascent protein synthesis in HCC cells. Presence of compound C mitigated the inhibitory effect of HLJDD on HCC expansion, indicating that AMPK

activation may confer to the anti-tumor action of this formula (Fig. 5C). Interestingly, HLJDD has minimal effect on HCC cell under nutrient deprivation condition, in which eEF2 is inactive (Fig. 5D). These observations further demonstrated that the AMPK/eEF2K/eEF2 axis may contribute to the tumor inhibition by HLJDD in human HCC.

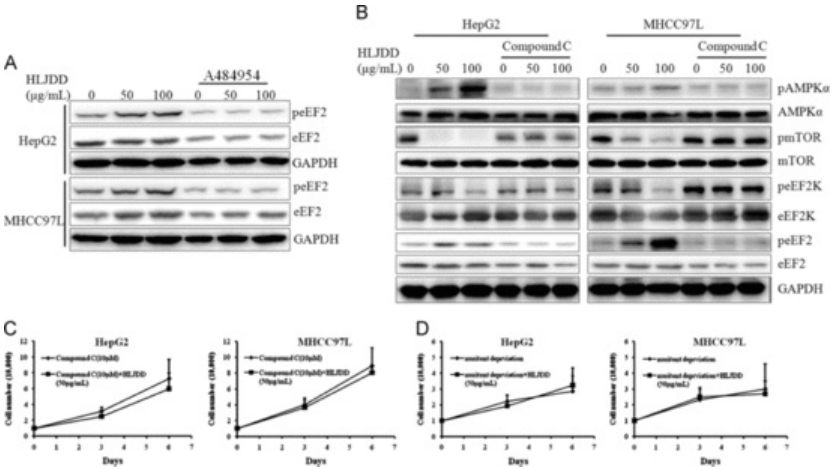


Fig. 5 ~~eEF2K/AMPK inhibition by HLJDD contributes to eEF2 suppression. A~~eEF2K/AMPK inhibition by HLJDD contributes to eEF2 suppression. A shows that the inactivation of eEF2 by HLJDD was associated with eEF2K. Cells were treated with HLJDD for 24 h in the presence or absence of A484594, the eEF2K inhibitor, and then protein was analyzed by immunoblotting. The results show blockade of eEF2K activity by A484594 attenuate HLJDD-induced eEF2 inactivation. ~~B~~B shows HepG2 and MHCC97L cells were treated with HLJDD in the presence or absence of Compound C for 24 h. It was observed that HLJDD could up-regulate the activity of AMPK and suppress the phosphorylation of mTOR. Presence of Compound C, the AMPK inhibitor, restored the phosphorylation of mTOR and eEF2K. Consequently, the phosphorylation of eEF2 by HLJDD was attenuated. ~~C~~C shows that inhibition of AMPK abolishes the tumor suppressive effect of HLJDD. Cells were treated with 50 μg/mL of HLJDD in the presence or absence of AMPK inhibitor compound C. Cell number was counted at 3 and 6 days. Presence of compound C attenuates inhibitory effect of ~~HLJDD.~~HLJDD. ~~D~~D shows that HLJDD could not further inhibit tumor cell growth under nutrient deprivation. Cells were conditioned at nutrient deprivation condition (amino acid-, glucose-free medium with 1% FBS) for 3 and 6 days in the presence or absence of 50 μg/mL HLJDD. It was shown that HLJDD has no further inhibition on HCC under nutrient deprivation condition.

4 Discussion

Huanglian Jiedu Decoction is an ancient Chinese Medicinal formulation that has been recorded for approximately 1300 years in treating human diseases related to inner heat and toxics. According to Medical Secretes of an Official (wai tai mi yao in Chinese) and Prescriptions for Emerent Reference (zhou hou fang in Chinese), HLJDD was mainly used to remove heat and scavenge toxins from human body, which represents the symptoms of fever and diarrhea (Feng et al., 2009). Its treatment on jaundice, inflammation and hypertension is well accepted in the early days in China, and it is now also approved by Ministry of Health, Labour and Welfare of Japan and Korean Food and Drug Administration, for palliative cares and atopic dermatitis treatment (Kim et al., 2011; Okumi and Koyama, 2014). Our clinical practice with HLJDD also indicates that this formula may be effective in treating patients with hepatocellular carcinoma. Patients receiving HLJDD were showed to have reduced tumor growth and metastasis, and improved quality of life (Feng et al., 2008). Although the clinical observation looks promising, it is not clear how HLJDD could restrict the tumor growth and invasion. A previous study has reported that the anti-tumor effect of HLJDD on liver cancer and the toxic dose of decoction may be able to induce cell cycle arrest and apoptosis of liver cancer cells (Hsu et al., 2008). However, the use of high dose Oriental Medicine decoction is always challenging and therefore the possible target of HLJDD in HCC is worth to be further investigated. In this study, we found that non-toxic doses of HLJDD could suppress the proliferation, angiogenesis and invasiveness of HCC cells both in vitro and in vivo. Non-toxic doses of HLJDD targets on eEF2, which controls translation elongation step during protein synthesis. Phosphorylation at Th56 site by eEF2K in HLJDD-treated HCC cells leads to the loss of eEF2 activity, and therefore blocks the elongation of nascent peptide. As shown in our study, HLJDD could activate AMPK signaling, and thereby suppress mTOR pathway. Inhibition of mTOR reduces the phosphorylation of eEF2K, which in consequence activates eEF2K, the negative regulator of eEF2. Our results reveal eEF2 as a novel target of HLJDD in treating HCC, and its effect in inhibiting nascent protein synthesis may be a new mechanism underlying the anti-HCC effect of HLJDD. The regulatory scheme underlying the anti-HCC action of HLJDD is shown in Fig. 6.

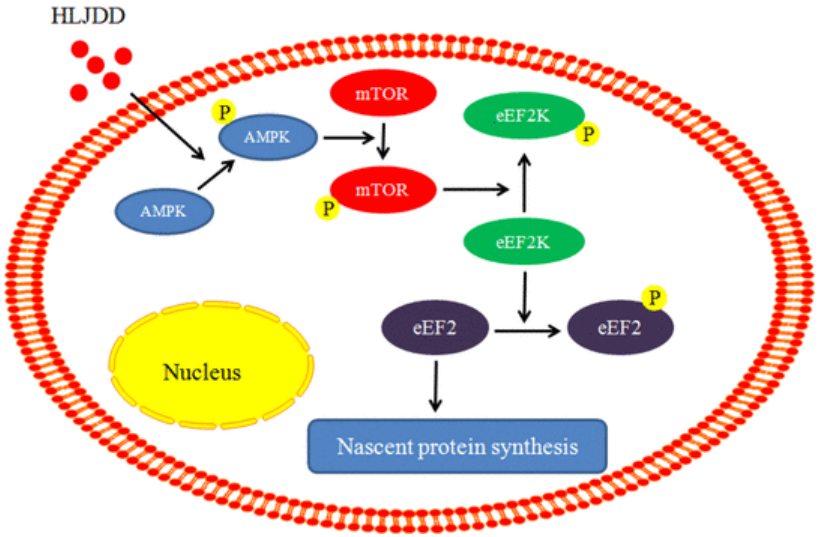


Fig. 6 Regulatory scheme of HLJDD on eEF2 activity in HCC.

The rapid growth of cancer cells is associated with an overall increase of protein synthesis(Grzmil and Hemmings, 2012). The mRNA translation is a process where cellular protein is synthesized in the cytoplasm using mRNA as the template. The process is well-controlled in eukaryotic cells, involving a multitude of signaling transduction pathways. In normal eukaryotic cells, these signaling transduction pathways are converted into several components of translational machinery, however, it was found that these components are often deregulated in human cancerous cells, renders the translated proteins become ~~oncogenic(~~oncogenic (Blagden and Willis, 2011). In particular, some anti-apoptotic proteins that confer to cancer progression and resistance to cancer treatment are selectively hyper-synthesized in tumor ~~cells(~~cells (White-Gilbertson et al., 2009). In this case, the dysregulation of translation control in cancer cells has a dominant role in enhancing cancer cell protein level as well as tumor progression. A recent study has shown that in human glioma the genes expression regulated via translation was 10-fold higher than those regulated through transcription when cells are exposed to ~~radiation(~~radiation (Lu et al., 2006). As a result, the differences in mRNA translational control between normal cells and cancerous cells make it a possible therapeutic opportunity against cancer. Many recent studies have revealed the important role of eEF2 in regulating cancer progression. It was observed that overexpression of eEF2 could promote the cellular G2/M progression and enhance tumor cell growth in vitro and in vivo in human gastrointestinal cancer cells (Nakamura et al., 2009). Overexpression of eEF2 was also observed in human ovarian tumors (Alaiya et al., 1997). As the expression level of eEF2 in eukaryotic cells are more tightly regulated than most proteins, which makes the overexpression of eEF2 become challenging. The observation that cancer cells could achieve greater level of eEF2 may be impressive and indicative to the pursuit of therapeutic opportunity against human cancer. In our study, we found that both HLJDD itself and its active components targeted on eEF2 and treatment of HLJDD results in eEF2 phosphorylation. Induction of Th56 phosphorylation of eEF2 disrupts the translation elongation and therefore blocks the global synthesis of cellular proteins (Schwer et al., 2013), leads to growth inhibition of tumor. By monitoring nascent protein synthesis, it showed that the blockade of eEF2 activity by HLJDD resulted in reduced production of protein. These results reveal that eEF2 could be the potential therapeutic target of HLJDD in treating HCC and inhibition of protein synthesis may be its underlying mechanism. Recent investigations have revealed that some commonly used anti-cancer drugs including ~~doxorubicin(~~doxorubicin (White et al., 2007) and taxol (Pineiro et al., 2007) inhibited eEF2, and this may be associated with the role of chemotherapeutic drugs in induction of apoptosis and suppression of tumor growth in human cancer cells. Our study may include HLJDD as a candidate in the list of complementary anti-HCC agents which target on eEF2.

We noticed that the major components in HLJDD decoction, including geniposide, berberine, baicalin and palmatine, could target on eEF2 spontaneously. The anti-tumor effect of each compound has been reported in previous studies (Lv et al., 2012; Wang et al., 2010; Zhang et al., 2012) and these compounds have been shown to target on various oncogenes in cancer cells. The diversified anti-cancer mechanisms increase the possibility of these compounds affecting different signaling pathways involved in cancer progression. Since HLJDD could potently inhibit the translation elongation and subsequent nascent protein synthesis, the very initial stage in the expression of oncogenic proteins; it would be very interesting to investigate whether the major active components in HLJDD have the same target on eEF2. Our results showed the additive action of combination treatment on eEF2 activity and nascent protein synthesis, which may indicate that the active components in HLJDD make a perfect harmony by targeting one particular protein to limit the HCC progression. The effect of geniposide, berberine, baicalin and palmatine in suppressing eEF2 activity has never been reported and it is not yet conclusive in our current study that they share the same mechanism in inhibiting eEF2. However, in our study, we found that AMPK activation may be responsible for the inactivation of eEF2 in HLJDD-treated HCC cells. Berberine and baicalin have been reported as AMPK activators in some previous ~~studies(~~studies (Lv et al., 2012; Ma et al., 2012), suggesting that these two compounds in HLJDD may majorly trigger the AMPK activation in HCC cells. Geniposide and palmatine, which though have never shown to activate AMPK, could slightly induce eEF2 phosphorylation. This may reveal that AMPK activation-independent mechanism may involve in the HLJDD-induced eEF2 phosphorylation. The exact

mechanism underlying the combinatory effect of active compounds in HLJDD needs further investigation.

In conclusion, we reported a novel mechanism underlying the global inhibitory effect of Huanglian Jiedu Decoction on HCC. Strict quality control showed that the consistency of HLJDD in chemical composition and bioactivity. HLJDD suppressed the cancer cell proliferation in vitro at its non-toxic doses, and inhibited tumor growth in vivo. HLJDD was also found to inhibit the nascent protein synthesis in HCC cells at its non-toxic doses. The inhibition on nascent protein was due to the reduced activity of translation elongation regulator eEF2, and HLJDD was shown to induce the Th56 phosphorylated-inactivation of eEF2 in HCC cells. Active components in HLJDD exhibited combinatory effect in suppressing eEF2 activity. While berberine was the major active compound in the decoction, baicalin, palmatine and geniposide exerted additive effects to berberine. Combination treatment exhibits more inhibition on eEF2 activity than any of the single treatment. HLJDD could trigger the activation of eEF2K by dephosphorylating the molecules at Ser366 site. Presence of Compound C, an AMPK inhibitor, attenuated the inhibitory effect of HLJDD on eEF2 activity and tumor cell proliferation, while HLJDD had minimal inhibition on HCC cells with inactive eEF2.Our results reveals that eEF2 may be the core target of HLJDD in inhibiting HCC progression and shed lights on the potential of HLJDD as a new complementary agent in treating hepatocellular carcinoma.

Conflict of ~~Interest~~interest

The authors declare no conflict of interest for this study.

~~Acknowledgement~~Acknowledgment

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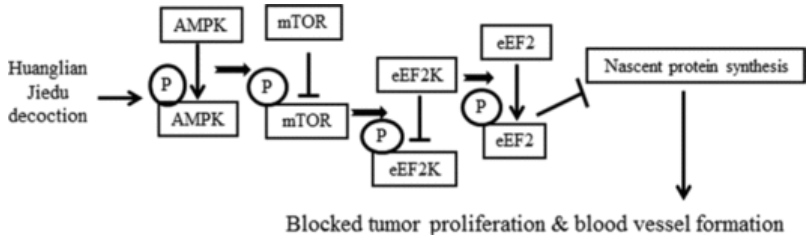

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