Ingredients, Anti-Liver Cancer Effects and the Possible Mechanism of DWYG Formula Based on Network Prediction

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Background: Hepatitis virus infection plays a critical role in liver cancer initiation and development; so the purpose of this study was to investigate the anti-liver cancer effects of DiWuYangGan (DWYG) which was effective for hepatitis.

Methods: Network predictions were performed. Next, several tests, including HPLC, Caco-2 absorption models, MMT, protein chip, Western blotting and H₂₂-tumor-bearing mouse, were carried out to investigate the effects and possible mechanism of DWYG.

Results: Network results showed DWYG might be involved in some processes such as STAT cascade. Some target genes may correspondingly participate in these procedures, such as IL-6, CASP3, AKT1, PPAR, and TP53. Diseases associated with DWYG formula may be liver cancer and hepatitis. Potential active compounds might be CUR and ISO. Chemical analysis results showed that ingredients in the formula, including DEO, SCHB, SOLA, SOLB, SCHA, LIQ, ISO, POT, and CHL, could be determined, indicating that DWYG samples for the following experiments were controllable and consistent. Caco-2 absorption of ingredients in DWYG, including DEO, SCHB, SOLA, SOLB, and LIQ, worked very well. In vitro experiment results showed that DWYG could inhibit the growth of cell lines and its effective ingredients might be SCHB, SOLB, SINA, SINB, SOLB, CUR, DEM, BIS, and GER. Further protein results showed that DWYG could upregulate the expressions of some proteins, including ERK1/2, AKT Ser473, BAD Ser112, PRAS40, Thr246, P38, Gsk-3 β , and Ser9. In vivo experiment results showed that DWYG could shrink tumor size, recover ALT and AST, and decrease IL-6 levels. Their possible mechanism might be through the JAK/STAT3 pathway.

Conclusion: Besides the known pharmacological function of anti-hepatitis, DWYG extract expressed anti-liver cancer effects and the results were consistent partly with network predictions.

Keywords: DWYG, liver cancer, JAK/STAT3 pathway, network prediction

Background

Liver cancer represents the fifth most common malignancy and the third leading cause of cancer-related death worldwide.¹ Although several compounds, such as 5-fluorouracil (5-Fu) and cisplatin, have been approved for hepatoma carcinoma (HCC) treatment, their effects are restricted by cell resistance and a series of toxicities. For patients with advanced stages of the disease, it is very important to find new drugs with good effect and low toxicity.

Diwuyanggan (DWYG), a new Traditional Chinese medicine (TCM), is authorized by the Hubei Food and Drug Administration (Grant No. Z20113160). This formula

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consists of *Schisandra chinensis* (Turcz.) Baill., *Artemisia capillaris* Thunb., *Curcuma longa* L., *Glycyrrhiza uralensis* Fisch., and *Rehmannia glutinosa* Libosch. Among them, *Schisandra chinensis* (Turcz.) Baill. has pure compounds such as deoxyschizandrin (DEO), schisandrin B (SCHB), schisandrol A (SOLA), schisandrol B (SOLB), schisantherin A (SINA), schisantherin B (SINB);² *Artemisia capillaris* Thunb. contains, eg, scoparone (SCOP), chlorogenic acid (CHL) and caffeic acid (CAF);³ *Curcuma longa* L. has eg, curcumin (CUR), demethoxycurcumin (DEM), bisdemethoxycurcumin (BIS), germacrone (GER);⁴ *Glycyrrhiza uralensis* Fisch. contains liquiritin (LIQ), isoliquiritoside (ISO), Liquiritigenin (LIG), potenline (POT), et al;⁵ and *Rehmannia glutinosa* Libosch. has meyerhmannioside D (REH D), catalpol (CAT), acteoside (ACT), et al⁶ (Figure 1). According to TCM theory, the above five herbs are combined to promote liver regeneration by tonifying the kidney and repairing the liver by affecting stem cells and the microenvironment.⁷ The formula has been used in Hubei provincial hospital of

TCM for more than 30 years, showing good therapeutic effects on hepatitis.

Pharmacological results showed that DWYG extract could relieve the degree of fibrosis in CCl₄-induced fibrotic rats,⁸ increase 2-AAF/PH-rat survival rates, suppress rats' hepatic pre-carcinoma changes.⁹ It could also rectify liver regeneration disorders of rats with kidney deficiency.¹⁰ A clinical study demonstrated that DWYG extract had significant hepato-protective effects on hepatitis B patients by decreasing the levels of IL-17 (interleukin) and IL-10 in serum¹¹ and increasing the percentages of CD⁴⁺ and CD⁴⁺/CD⁸⁺ T-lymphocytes¹² when combined with entecavir (ENT). It could effectively decrease the degree of fibrosis¹¹ and treat hepatitis B virus-associated nephritis (HBV-GN) for hepatitis B patients.¹³

As we know, chronic liver inflammation, often infected by hepatitis virus, is very important for liver cancer initiation and development.⁷ Our previous study also found that DWYG could inhibit occurrence and development of liver

Figure 1 Chemical structure of compounds in DWYG.

cancer for Solt-farber rat models.¹⁴ However, the effects and mechanism of DWYG for treatment of liver cancer needs be further clarified, so the emphasis of this study was the effects of DWYG extract on liver cancer using SMMC-7721 cell lines and H₂₂-tumor-bearing mice as subjects.

Methods

Network Prediction

Each ingredient in the TCM formula may impact on multiple target genes while each target gene may have interactions with multiple compounds. Here network prediction was performed according to the following Equation (1).

$$p(x \geq k) = \sum_{m=k}^n C_n^m \left(\frac{g}{n}\right)^m \left(1 - \frac{g}{n}\right)^{n-m} \quad (1)$$

n: the total number of ingredients; g: the average number of ingredients with which each target gene has interaction; k: the number of ingredients with which the current target gene has interaction. When P is less than 0.05, the probability of event that the target has interaction with the current ingredient is rare.

Prediction of possible Gene/Locus for liver cancer was carried out by searching OMIM,¹⁵ therapeutic target database (TTD)¹⁶ and Gene Card Database using liver cancer as the key word. Prediction of possible target gene and chemical compounds for DWYG was performed by searching HIT,¹⁷ TCM Integrated Database (TCMID),¹⁸ STITCH¹⁹ and TCM Pharmacology Database (TCMSP)²⁰ using DWYG herbs as key words. The Relevance Threshold value of the Gene target was 400. The threshold value of the chemical compound was 0.1. The significant threshold value of the Gene target: 0.05.

Biological function and disease enrichment were analyzed using functional enrichment tool (DAVID 23) to calculate KEGG enrichment. Only KEGG pathways with P-values <0.05 were included (corrected using the Benjamin method).

Chemical Analysis

Study on Chemical Ingredients of DWYG Formula

The pure compounds including DEO, SCHB, SOLA, SOLB, SINA, SINB, SCOP, CHL, CAF, CUR, DEM, BIS, GER, LIQ, LIG, ISO, POT, REHD, CAT, and ACT were purchased from the Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, People's Republic of China). Purity of all compounds was above 99.5%. Accurately weighed compounds

were dissolved and diluted for the calibration curves. About 0.2 g powder in each batch of DWYG extract (Hubei Provincial Hospital of TCM) was accurately measured, extracted under ultrasonic for 30 minutes, centrifuged and filtered through a 0.22 µm membrane (Millipore Corporation, USA) for HPLC analysis.

HPLC analysis was performed using an Agilent Technologies 1290 Infinity HPLC system (Waldbronn, Germany) coupled with a proshell 120 SB-C₁₈ column (2.7 µm, 100 mm×2.1 mm). The mobile phase consisted of deionized water (prepared by Milli-Q water system, USA) and grade methanol (Merck, Germany) or water and acetonitrile (Merck, Germany) with 0.3 mL/min flow rate at 30°C. The conditions for different compounds were listed using isocratic elution ([Supplementary Table 1](#)). The fingerprint chromatograms of DWYG were also obtained according to the gradient elution conditions ([Supplementary Table 2](#)). The injection volume was 1 µL.

The same solution was injected into HPLC six times to assess the precision. Powders in the same batch of DWYG extract were processed into six samples using the same method to evaluate the repeatability. An aliquot was injected respectively at 0, 2, 4, 6, 8 and 24 hours to evaluate the stability of the sample. The known amounts of analytes were added into the DWYG sample and analyzed in triplicate to calculate the recovery rate with the equation: recovery (%) = 100× (found amount-original amount)/amount spiked).

Similarity analysis for HPLC fingerprint was performed using similarity evaluation system for chromatographic fingerprint of Traditional Chinese Medicine (2004 A), which was developed by Shenyang Pharmaceutical University (China). The software was employed to evaluate the similarities between different chromatograms by calculating the cosine values of the vectorial angle and the correlation coefficient (γ_{ir}) among the samples [Equations (2) and (3)], as well as to compute and generate the mean chromatogram as a representative standard fingerprint. Furthermore, Relative Retention Time (RRT) and Relative Peak Area (RPA) of each characteristic peak related to a reference peak were constructed for quantitative measurement of the chemical composition of samples.

$$\cos \theta = \frac{\sum_{i=1}^n x_{in} y_{in}}{\sqrt{\sum_{i=1}^n x_{in}^2} \sqrt{\sum_{i=1}^n y_{in}^2}} \quad (2)$$

$$\gamma_{ir} = \frac{\sum_{i=1}^n n(x_{in} - \bar{x})(y_{in} - \bar{y})}{\sqrt{\sum_{i=1}^n n(x_{in} - \bar{x})^2} \sqrt{\sum_{i=1}^n n(y_{in} - \bar{y})^2}} \quad (3)$$

In vitro Absorption Experiment

Caco-2 cell lines (ATCC, Manassas, VA, USA) were cultured for in-vitro absorption.²¹ Each compound was dissolved in Dimethyl sulfoxide (DMSO, Sangon biological company). After washing, the transport buffer containing compound was added to either the apical chamber (400 μ L) in apical (AP) to basolateral (BL) directional studies or the basolateral chamber (600 μ L) in BL to AP directional studies. An aliquot solution (100 μ L) was taken from the receiver chamber at different time intervals (10, 20, 30, 40, 60, 80 and 120 minutes) for the analysis. The same volume of fresh transport buffer was replenished to the receiver chamber after each sampling. At 120 minutes, samples in the donor chamber were collected. All samples were stored at -20°C until analysis.

Pharmacological Study

In vitro Effects of DWYG Extract on SMMC-7721 Cells

SMMC-7721 cells (Shanghai Institute of Cell Biology, People's Republic of China) were incubated with different drugs for MTT analysis.²² Antibody array and Hoechst test were also performed using SMMC-7721 cells according to kit instructions to study the effects of drugs on protein expressions and apoptosis.

In vivo Effects of DWYG Extract on H₂₂-Tumor-Bearing Mice

Male Kunming mice (20–22 g per mouse) were purchased from Shanghai Laboratory Animals Ltd (People's Republic of China). The animals were maintained at a constant temperature (21–25°C) and humidity (50–70%) on a 12 hour light/12 hour dark cycle with free access to food and water. Animals were cultured to adapt to the laboratory environment for at least 7 days prior to the experiments. All procedures were performed according to the guidelines of the National Animal Welfare Law of China. The Experimental Animal Ethical Committee of Shanghai University of TCM care guidelines were complied with to ensure that the mice received human care (Ethics Approval Number: SCXK2016-0003).

H₂₂-tumor-bearing mice model was established by injecting mouse H₂₂ cells (ATCC, USA) to mice.²³ After 7 days, inoculated mice with tumor sizes of 100–150 mm³ were selected and randomly divided into different groups (10–12 mice/group). Twelve mice without inoculation injected with normal saline were characterized as the normal group. Among the inoculated groups, one group was

injected with Normal saline (NS) as the model group (Mod) and the other groups were respectively injected intraperitoneally or orally administered with SOLB (ip, 100 mg/kg), DWYG extract (po, 720 mg/kg), DWYG extract (po, 400 mg/kg) and 5-Fu (ip, 200 mg/kg). All mice were treated once daily between 09:00 and 10:00 continuously for 8 days except for the 5-Fu group, in which the drug was administered twice a week. The appearances and behaviors were carefully observed. Any abnormal situation such as diarrhea, irritation and emaciation should be recorded. Tumor sizes of the animals were also measured with Vernier calipers. When the treatments finished, all animals were deprived of food but allowed free access to water for 16 hours. On the following morning, the mice were euthanized by inhaling ether. The eyes of the animals were picked out to collect blood for enzyme-linked-immunosorbent assay (ELISA) and liver function determination including Alanine transaminase (ALT) and Aspartate transaminase (AST). Then the mice were euthanized for collection of the tumors and liver. Tumor inhibition rate was obtained with mean tumor indices of the drug group divided by mean tumor indices of the model group. Then, each tissue of liver and tumor was divided into two parts. One part of the tissue was immediately frozen in liquid nitrogen for Western blotting [JAK2 (Janus-activated kinase), STAT3 (Signal transducer and activator of transcription), P-STATtyr705, PSTATser725, β -actin (Santa Cruz Biotechnology Inc., Dallas, TX, USA)] and ELISA test (IL-6). The other part of the tissue was immediately placed in 4% buffered paraformaldehyde and fixed overnight for Hematoxylin and Eosin (HE) observation.

Statistical Analysis

The quantitative results of chemical analysis were expressed as the mean $\pm$ SD. The quantitative results of cells and animals were expressed by mean $\pm$ standard errors. Statistical analyses were performed by ANOVA using SPSS 17.0 for Windows software (SPSS Inc., USA). P-values less than 0.05 were considered statistically significant.

Results

Results of Network Prediction

The results of the network prediction showed that about 98 genes were associated with liver cancer, including TNF, JAK, IL-6, P53, STAT1, STAT3, TLR, et al; about 125 genes might be involved in the therapeutic process for

DWYG formula application to liver cancer, including IL-6, CASP3, AKT1, PPAR, TP53, BCL, BAX, TLR4, and so on (Supplementary Table 3); and about 96 chemical compounds in the DWYG formula might be bioactive ingredients, including CUR, ISO and so on (Supplementary Table 4).

The significant molecular functions included protein-lipid complex binding, and lipoprotein particle binding

(Figure 2A); the significant biological processes included STAT cascade, and regulation of JAK-STAT cascade (Figure 2B); significant cellular components were the nuclear transcription factor complex, the transcription factor complex and so on; the obvious KEGG signaling pathways were apoptosis, and PI3K-Akt signaling pathway (Figure 2C); the obvious diseases were liver diseases, liver cancer, hepatitis B, etc (Figure 2D).

Figure 2 Analysis on DWYG influence on enrichment of molecular function (A), biological process (B), KEGG signaling pathway (C), and diseases (D).

To sum up, the network results showed that the DWYG formula had a relationship with liver cancer. The possible mechanism might be associated with apoptosis and inflammation.

Chemical Analysis Results

Among the obtained chromatograms of DEO, SCHB, SOLA, SOLB, SCHA, LIQ, ISO, POT and CHL (Figure 3), the peaks of the mark ingredients showed satisfactory baseline separation.

The standard curves of the mark ingredients exhibited a good linearity (correlation coefficient above 0.9998) (Supplementary Table 5). The Relative Standard

Deviations (RSDs) of the precision, stability and repeatability were found to not exceed 11% (Supplementary Table 6), recovery rates of mark ingredients were between 95% and 115% (Supplementary Table 7). These all demonstrated the proposed methods exhibited a reliable resolution and satisfactory linearity. Their precision, stability, repeatability and accuracy were also good. Therefore, these methods could be used for determination of samples.

The content results showed (Supplementary Table 8) that RSDs of different ingredient contents in 10 batches were below 15% except SCHA and ISO, demonstrating that although the mark ingredients contents were variable

Deoxyschizandrin DEO t_R 12.207 min

SchisandrinB SCHB t_R 19.680 min

Schisandrol A SOLA t_R 10.764 min

Schisandrol B SOLB t_R 19.630 min

Isoliquiritoside ISO t_R 10.274 min

1 DEO, 2 SCHB
 t_R 12.278 min, 19.871 min

1 SOLA, 2 SOLB
 t_R 10.760 min, 19.667 min

1 ISO t_R 10.307 min

Figure 3 Continued.

Figure 3 HPLC chromatograms for ingredients in DWYG under the isocratic elution.

in some degree, the stability of different batches was still under control and suitable for further experiment.

The results of fingerprint showed that the peaks were separated well under the wavelength of 254 nm and 13 common peaks were obtained, including LIQ (3th), LIG (4th), SOLA (6th), SOLB (7th), SINA (9th), DEO (12th) and SHB (15th) (Figure 4). Similarities of ten batches of DWYG samples were good with RSDs <6% (Supplementary Table 9). RSDs of RRT for different batches were good while RSD of RPA were not good (Supplementary Table 10).

Absorption results (Figure 5) showed Papps (AP→BL) of DEO, SCHB, SOLA, SOLB, and LIQ were above 10^{-6}

cm/s, indicating absorbable permeability of these compounds were good. Ratios of Papp (AP→BL) to Papp (BL→AP) were below or near 1, indicating that the absorption manner of these compounds was passive transportation, demonstrating no efflux pump functions.

Pharmacological Results

In vitro Effects of DWYG Extract on SMMC-7721 Cells

MTT results showed (Figure 6A): DEO, SCHB, SOLB, SINA, SINB, CUR, DEM, BIS, GER, and DWYG extract could inhibit the growth of SMMC-7721 cells in a dose-dependent manner, indicating these compounds might be

Figure 4 HPLC fingerprints for DWYG under the gradient elution (254 nm). 3 LIQ; 4 LIG; 6 SOLA; 7 SOLB; 9 SINA; 12 DEO; 15 SHB.

anti-liver cancer effective ingredients. In contrast, other ingredients such as REHA, REHD, CHL, CAT, CAF, LIQ, and ISO had no obvious effects.

The protein chip results showed (Figure 6B and C, [Supplementary Table 11](#)): DWYG extract and its compounds could up-regulate the expressions of some proteins significantly, including ERK1/2, AKT Ser473, BAD Ser112, PRAS40, Thr246, P38, Gsk-3 β , and so on, indicating DWYG extract and its compounds had different influences on expression of proteins.

Hoechst results (Figure 6D) showed that at the range between 50 $\mu\text{g/mL}$ and 300 $\mu\text{g/mL}$ DWYG extract had no obvious effects on apoptosis. However, when the dose was increased to between 500 $\mu\text{g/mL}$ and 1000 $\mu\text{g/mL}$, effect of DWYG extract was good. Among the pure compounds of DWYG extract, effects of DEO and SOLA were not good (data not shown), but SOLB was an active ingredient of DWYG extract.

In vivo Effects of DWYG Extract on H₂₂-Tumor-Bearing Mice

A mouse was injected with H₂₂ cell lines and ascites were produced (Figure 7A) which were injected into other mice to induce liver tumor (Figure 7B).

The Normal group (12/12), Model group (12/12), 5-Fu group (10/12), SOLB group (12/12), DWYG extract group (po, 720 mg/kg) (12/12), DWYG extract group (po, 400 mg/kg) (12/12) were analyzed. In the 5-Fu group, two

mice could not tolerate the treatment and died, so only 10 mice were analyzed.

Pictures of tumor tissue (Figure 7C) showed: tumor tissue of the model group was the largest, indicating the model was successful; 5-Fu group was the smallest, indicating the effect of 5-Fu was the best. Drugs were effective but not better than 5-Fu. Effect of DWYG extract (high dose) was better than DWYG extract (low dose); SOLB was effective but not better than 5-Fu, indicating SOLB might be an effective ingredient in DWYG extract.

HE staining pictures showed that normal tissues of the model and drug groups were similar (Figure 7D), indicating the drugs had no significant toxicity on normal tissue. For tumor tissue (Figure 7E), there were differences among the model group and drug groups. Compared to the model group, tumor tissues of the drug groups expressed some phenomena including chromatin condensation and nuclear shrinkage, suggesting that these drugs could suppress growth and development of tumor tissues. Effect of 5-Fu was good. DWYG groups were effective and appeared to have a dose-dependent manner. SOLB might be a possible ingredient of DWYG extract.

After inoculation, the weights of mice steadily increased during the therapeutic period except in the 5-Fu group (Figure 8A). Animals in the 5-Fu group had diarrhea and alopecia, their appetite was poor. Compared with the 5-Fu group, the lifestyle of the other drug groups

Figure 5 Absorption curves of ingredients in DWYG using Caco-2 experiments.

Figure 6 MTT results (A), protein chip (B, C) and hochechst (D) of DWYG formula and its ingredients for SMMC-7721 cell lines. -: ctrl * $P < 0.05$, ** $0.05 < P < 0.001$, *** $P < 0.001$.

was normal. These indicated that the adverse reactions to 5-Fu chemotherapy were serious.

Tumor size results (Figure 8B) showed: each group grew up significantly as the days went on; tumor size of the model group was the largest while the 5-Fu group was the smallest, indicating the effect of 5-Fu was the best. DWYG extract was also effective and SOLB might be an active ingredient of DWYG extract.

Tumor weight results (Figure 8C) showed: 5-Fu had the strongest effect with a tumor inhibition rate of 91.94%. The tumor weights in DWYG extract (Low dose), DWYG extract (High dose) and SOLB groups also decreased, with inhibition rates of 45.4%, 60.57%, and 34.98%, respectively. These results were similar to tumor volume. 5-Fu was the best. DWYG was effective in a dose-dependent manner and SOLB might be an effective ingredient of DWYG.

For liver function (Figure 8D and E), ALT and AST levels of the model group were higher than the normal

group, indicating liver function of animals in the model group were damaged. After therapy by drugs, liver functions of the animals were recovered in some degree. The effect of 5-Fu was the best. Both SOLB and DWYG extract at high and low dose were effective.

For serum ELISA results (Figure 8F), IL-6 concentrations of the model and drug groups were higher than the normal group, indicating inflammatory reaction boosted after tumor cells were seeded into axilla. DWYG extract could decrease IL-6 in a dose-dependent manner. However, 5-Fu was increasing not decreasing IL-6, indicating that 5-Fu enhanced inflammatory injury. The IL-6 value of SOLB was also less than 5-Fu but more than DWYG extract.

Tumor ELISA results (Figure 8G) showed the trend was similar. IL-6 concentrations of 5-Fu groups were higher than the model group. IL-6 of DWYG extract and SOLB group were less than in the model group, indicating

Figure 7 Tumor tissue (C) of H₂₂ bearing mice (A, B), HE staining results of normal liver (D) and tumor tissue (E).

DWYG extract could relieve inflammatory reaction and SOLB might be its effective ingredient.

The western results of tumor tissue (Figure 8H–K) revealed that compared with the model group, 5-Fu, DWYG extract and SOLB could inhibit protein expressions of JAK2, Tyr705-p-STAT3, and Ser725-p-STAT3. Among the drugs, the effects of 5-Fu were the best. DWYG extract suppressed expressions of the three proteins in a dose-dependent manner, indicating DWYG extract had influences on inflammatory pathways and SOLB could be its effective ingredient.

Discussion

There are many factors which impact on the development of cirrhosis and HCC. Among them, Hepatitis B and C viruses are the principle reasons. Our team has also discovered that liver micro-environment influences the construction of liver cancer,²⁴ the inhibition of cytokine production and the control of macrophage infiltration rate were important for HCC prevention and therapy.²⁵

TCM has attracted attention all over the world in recent years. Now more than two-thirds of novel anti-cancer

drugs are natural products.²⁶ DWYG, a TCM formula, which has been proved to have significant effects on chronic hepatitis B,¹¹ might play a protective role in the formation and development of liver cancer, so our study focused on the effects of DWYG extract on liver cancer.

As we know, any TCM formula including DWYG is a complicated system with multiple ingredients and targets, which is designed according to special syndromes or patterns instead of targeting disease, so it is difficult to systematically investigate herbal formulae mechanisms using routine methods. Our group has accumulated some experience about network prediction.^{27–29} Therefore, here we adopted a network pharmacology approach to predict possible bio-active compounds, targets and mechanisms of DWYG. Then experiments in vivo and in vitro were designed according to the above results. Combining network approach and experiments, the possible targets of DWYG for liver cancer could be more easily focused on.

First, our network predicted DWYG might be involved in many processes, such as STAT cascade and JAK-STAT cascade. Accordingly, some target genes may participate in

Figure 8 Weights (A), tumor volume (B), tumor inhibition (C), liver function (D, E), ELISA (F, G), Western blotting (H–K), results of H₂₂ bearing mice and pathway of JAK/STAT3 (L). DNA strands depicted are our own.

these procedures, such as IL-6, CASP3, and AKT1. And disease associated with DWYG formula may be, eg, liver cancer, or hepatitis B. Potential active compounds might be CUR and ISO. These predicted results should be confirmed by experiments.

Compounds moving across a Caco-2 cell monolayer have been correlated to human absorption.²¹ An apical (A) to basolateral (B) directional study was used to evaluate the intestinal absorption of drugs while a basolateral (B) to apical (A) directional study was used for the delineation of transport mechanisms such as the involvement of active transporters, so this study adopted Caco-2 cells to study intestinal transport characteristics of ingredients in DWYG extract. Our data showed that absorption of some

ingredients worked very well, suggesting they might be effective ingredients.

After that, the pharmacological functions of DWYG on liver cancer were investigated using in vitro and in vivo experiments. As for in vitro experiments, data of MTT showed effective ingredients might be SCHB, SOLB, and SINA amongst others. Hoechst staining results further confirmed pro-apoptosis effects of DWYG extract and its ingredients. However, some chemical structures such as SCHB and SOLB, which were abundant, well absorbed and confirmed effective by experiments, were not in the list predicted by the network, indicating that the network results had some kinds of limitations. Proteomic profiling, which has been widely used for clinical bio-marker

identification and new drug discovery,³⁰ enables parallel detection of multiple proteins with low sample volume. Our data of protein chip showed that DWYG extract could up-regulate the expressions of some proteins, including ERK1/2, AKT Ser473, and BAD Ser112, some of which named as AKT were predicted by the network.

As for *in vivo* experiments, the dosage of animals needed to be confirmed. As was known, the preparation of DWYG is in capsule form, 0.38 g/cap and dosage of DWYG for humans is five capsules twice to three times daily. Therefore, the total daily amount for DWYG is 3.85.7 g for humans. Transferring to animal dosage, mice dosage is 543 mg/kg to 814 mg/kg. In our mice experiment, the dosage was 400 mg/kg and 700 mg/kg, roughly equaling the required dosage. According to human instruction of 5-Fu, 15–30 mg/kg is intravenously administered and the total dosage for one period is no more than 10 g. Transferring to mouse dosage it is 150–300 mg/kg and total dosage for each period is below 2.86 g. In our experiment, 200 mg/kg 5-Fu was administered twice per week to the mice. The dosage per day was between the recommended dosage range and the total dosage was below the recommended total dosage. Therefore, the dosage of DWYG and 5-Fu for mouse was calculated referring to the human dose. Results of the animals may afford the basis for clinical experiment. The data of H₂₂-tumor-bearing mouse model showed DWYG could also shrink tumor size, reduce tumor weight and recover liver function index such as ALT and AST. HE also expressed that DWYG could inhibit tumor growth and SOLB might be a possible ingredient of DWYG.

In order to further examine possible mechanisms, ELISA and Western blotting were adopted. Continuous activation of JAK2/STAT3 pathway plays a central role in the development and progression of liver cancer. Cytokine-induced STAT3 activation was a transient event in normal cells while in cancer cells STAT3 was often found to be activated.³¹ In approximately 60% of human hepato-carcinoma specimens, phosphorylation of STAT3 was detected and STAT3-positive tumors were more aggressive with poor prognosis.³² Expression of elevated IL-6 in tumor tissue could activate phosphorylation of STAT via JAK (especially JAK2), which could respectively combine with Tyr705 outside nuclear and Ser 707 inside nuclear to come into the complex of STAT-p-Tyr705 and STAT-p-Ser 707 (Figure 7L), to promote DNA transcription. Our network also predicted that DWYG involved in anti-liver cancer through JAK-STAT pathway.

Therefore, we investigated this inflammatory pathway. Results showed that DWYG extract could reduce the expressions of inflammatory cytokine IL-6 and suppress the expressions of JAK2, Tyr705-p-STAT3, and Ser725-p-STAT3, suggesting that DWYG could inhibit inflammatory reaction and further reduce the risk of liver cancer via JAK/STAT3 pathway (Figure 8L).

The above cell and animal experiments suggested that DWYG could play a protective role in the formation and development of liver cancer. Of course, further clinical observations are required. Based on these results, we should consider the possibility of widening indications of DWYG – not only for anti-hepatitis but also anti-liver cancer.

Conclusions

In summary, the quality of DWYG formula was controllable and DEO, SCHB, SOLA, SOLB could be the effective ingredients. Besides its well-known pharmacological function of anti-hepatitis, DWYG extract also expressed anti-liver cancer effects, which could be associated with inhibiting cell proliferation and affecting inflammatory cytokines. Its mechanism might be via the JAK/STAT3 pathway. These data supplied the foundation for adding new clinical indications of DWYG. In addition, the current research also demonstrated only part of the results predicted by the network can be verified by experiments. Therefore, we should hold a prudent attitude on network prediction.

Abbreviations

ACT, Acteoside; ALT, Alanine Transaminase; ANOVA, One-way Analysis of Variance; AP, Apical chamber; AST, Aspartate transaminase; ATCC, American Type Culture Collection; BIS, Bisdemethoxycurcumin; BL, Basolateral chamber; CAF, Caffeic acid; CAT, Catalpol; CHL, Chlorogenic acid; CUR, Curcumin; DEM, Demethoxycurcumin; DEO, Deoxyschizandrin; DMSO, dimethyl sulfoxide; DWYG, DiWuYangGan capsule; ELISA, Enzyme-Linked Immunosorbent Assay; EMT, Epithelial-to-Mesenchymal Transition; ENT, Enticavir; FBS, Fetal Bovine Serum; GER, Germacrone; GGT, Glutamyl Transpeptidase; HBV, Hepatitis B Virus; HE, Hematoxylin and Eosin; HPLC, High Performance Liquid Chromatography; IFN, Interferon; IL, Interleukin; ISO, Isoliquiritoside; JAK, Janus-Activated Kinase; LIQ, Liquiritin; LIG, Liquiritigenin; MET, Mesenchymal-to-Epithelial Transition; MTT, Methyl Thiazolyl Tetrazolium; OD, Optical Density; PBS, Phosphate-Buffered Saline; POT,

Potenline; REH D, Mey Rehmannioside D; REHA, Rehmannioside A; RRT, Relative retention time; RPA, Relative peak area; RSD, Relative Standard of Deviation; DEO, Deoxyschizandrin; SCH B, SchisandrinB; SCOP, Scoparone; SINA, Schisantherin A; SINB, SchisantherinB; SOL A, Schisandrol A; SOLB, Schisandrol B; SPSS, Statistics Package for Social Science; STAT-3, Signal Transducer and Activator of Transcription 3; TBIL, Total Bilirubin; TCM, Traditional Chinese Medicine; TCMID, Traditional Chinese Medicine Integrated Database; TCMSP, Traditional Chinese Medicine System Pharmacology Database; TEER, Transpethelial Electric Resistance; TTD, Therapeutic Target Database.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author.

Ethics and Consent Statement

In our study, all animal procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai University of TCM.

Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work. Yao Li and Han-Min Li are co-first authors.

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Disclosure

The authors declare that they have no competing interests in this work.

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