Diwu Yanggan Modulates the Wnt/β-catenin Pathway and Inhibits Liver Carcinogenesis Signaling in 2-AAF/PH Model Rats*

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Summary: The activation of the Wnt/β-catenin signaling pathway in oval cells after liver injury is implicated in hepatocarcinogenesis. Diwu Yanggan capsule is a Chinese herbal medicine that has been used for treating liver disorder. The present study aimed to examine the mechanism by which Diwu Yanggan inhibits liver carcinogenesis, and the involvement of the Wnt/β-catenin signaling pathway. Diwu Yanggan capsule was administered to 2-acetaminofluorene/partial hepatectomy (2-AAF/PH) rats, a murine model of liver injury. The biomarkers of oval cells and key proteins in the Wnt/β-catenin signaling pathway were assessed on postoperative day 8, 10, 14, 17, 19 and 22. The results showed that treatment with Diwu Yanggan was associated with reduced expression of oval cell and stem cell biomarkers in the 2-AAF/PH animals. The expression pattern of key proteins in the Wnt/β-catenin pathway was altered in Diwu Yanggan-treated animals, indicating that the Diwu Yanggan treatment accelerated the activation of the Wnt/β-catenin pathway in the initial stage and contributed to its deactivation in the later stage. Histological findings indicated that hepatocyte proliferation was suppressed in Diwu Yanggan-treated animals, compared with untreated 2-AAF/PH animals. Taken together, Diwu Yanggan capsule may reduce the risk of hepatocarcinogenesis by modulating the Wnt/β-catenin signaling pathway.
Key words: Diwu Yanggan; 2-acetaminofluorene/partial hepatectomy (2-AAF/PH); liver regeneration; hepatocarcinogenesis; Wnt/β-catenin pathway

Hepatic carcinoma (HCC) is a leading cause of cancer mortality in China and in the United States, with a five-year survival rate for metastatic HCC being approximately 2.3%[1–3]. Current liver cancer treatment options offer limited survival benefits.

The Wnt/β-catenin signaling pathway is a complex and highly evolutionarily conserved pathway for cell homeostasis, and plays a key role in the liver repair process. The dysregulation of the pathway can contribute to the development of HCC[4, 5]. Upon binding of the Wnt protein with its receptors, β-catenin is released from the destructed complex, and the active β-catenin translocates into the nucleus where it activates the expression of genes that are known to promote the progression of the cell cycle and inhibit apoptosis, including cyclin D1, c-myc and survivin[6]. Furthermore, β-catenin has been found in HCC tumor cells[7], indicating an association between β-catenin and hepatocarcinogenesis.

Hepatic oval cells are a subpopulation of liver stem cells that arise in the periportal region of the liver and they participate in liver repair after injury. These cells can differentiate into hepatocytes or bile ductular cells, and are found after some types of hepatic injury when liver cell proliferation is inhibited[8, 9]. In addition, hepatic stem cells including oval cells have been demonstrated to be implicated in hepatocarcinogenesis, since the proliferation of oval cells was found to be associated with increased risk of HCC[10, 11].

In order to study the diverging point between the normal liver repair process and hepatocarcinogenesis, a 2-acetylaminofluorene/partial hepatectomy (2-AAF/

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PH) murine model was developed. This model has been commonly used in the study of oval cells, in which 2-AAF inhibits hepatocyte proliferation and partial hepatectomy triggers liver repair[12].

Diwu Yanggan (DWYG), a Chinese herbal medicine formula, is a new drug authorized by the Hubei Food and Drug Administration (Grant No. Z20113160). The mixture includes five Chinese medicinal herbal extracts, whose proportions (w/w) are as follows: *Rehmanniaglutinosa* (Gaertn.) DC., 20.0%; *Artemisia scoparia* Waldst. &Kitam., 33.3%; *Curcuma longa* L., 13.4%; *Schisandrachinensis* (Turcz.) Baill., 20.0%; and *Glycyrrhizauralensis* Fisch., 13.4%. Results from clinical trials showed that DWYG could reduce the risk of HCC in patients with hepatitis B infection[13–16]. In the present study, we aimed to examine the molecular effects of DWYG on the hepatocarcinogenesis by using the 2-AAF/PH model.

1 MATERIALS AND METHODS

1.1 Materials

Anti-albumin (ALB), anti-alpha fetoprotein (AFP), anti-cytokeratin-19 (CK19), anti-Thy1.1, anti-β-actin, anti-Wnt1, anti-Wnt3, anti-C-myc, anti-β-catenin, anti-glycogensynthase kinase-3β (GSK3β), anti-frizzled class receptor 2 (FZD2), anti-cyclinD1, anti-epithelial cell adhesion molecule (EpCAM) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). 2-Acetylaminofluorene was purchased from Sigma Aldrich (Saint Louis, USA). Polyvinylidene fluoride (PVDF), methanol, phosphate buffered saline (PBS) buffer, Tween 20, and the electro-chemi-luminescence (ECL) Western blotting kit were purchased from Guge Biotechnology (China). Electrophoretic apparatus and polychromatic fluorescence gel imaging system were purchased from Bio-Rad Laboratories (Hercules, USA).

The DWYG capsules[17] used in this study (batch number: 20120221) were provided by the Traditional Chinese Medicine Preparation Room of Hubei Provincial Hospital of Traditional Chinese Medicine. The DWYG capsules were obtained by decompression drying and granulating of the above-refined concentrates. DWYG capsules were suspended in distilled water at a final concentration of 36 mg/mL.

1.2 Establishment of Animal Models

Male Wistar rats (160–180 g) were provided by the Hubei Experiment Animal Research Center. The Solt-Farber model of carcinogenesis was developed by using previously published methods[18, 19]. Briefly, the rats were distributed into four groups: normal, sham, vehicle and DWYG. For the sham group, rats' abdomens were opened, and 1 mL whole blood was taken from the portal vein. No partial hepatectomy (PH) was performed. For vehicle and DWYG groups, the rats received PH. The three groups were then orally administered with 20 mg/kg 2-AAF once a day for one week. Meanwhile, animals in the sham and vehicle groups were orally administered with distilled water (10 mL/kg) until sacrificed, while those in the DWYG group were orally administrated with 10 mL/kg DWYG until sacrificed. Sixteen rats in each group were sacrificed on day 8, 10, 14, 17, 19 and 22 post-PH. At pre-determined interval, rats were sacrificed, and the livers collected and stored at –80°C for further analyses.

1.3 Histology Studies

Liver tissues obtained from the animals were sectioned and stained using the hematoxylin and eosin (H&E) standard protocol. Morphological changes were observed under a microscope. Images of five random areas were acquired from each slide and analyzed by Image Pro-Plus (version 6.0). The minimum area analyzed was 100 pixels. The number of nuclei, mean diameter of the nuclei, total surface area of the nuclei, and integrated absorbance (I*A*) of the nuclei were measured.

1.4 Western Blotting

The rats were euthanized, and the liver tissues (50 mg) were homogenized in 500 μL radio-immunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, China) containing phenylmethanesulfonyl fluoride (PMSF) (1 μmol/L) and phosphatase inhibitors (1 μmol/L). The homogenate underwent a freeze (–20°C)-thaw cycle, and was agitated using a rotator for 30 min. Then, the homogenate was centrifuged at 12000 g for 15 min. The supernatant was collected and denatured by boiling with 5× sample buffer. Proteins were separated by using electrophoresis, followed by transblotting onto a PVDF membrane (Wuhan Guge Biotechnology Co., Ltd., China). The membrane was incubated in 5% fat-free milk (in TBST), and the membrane with phosphorylated proteins was incubated with 5% bovine serum albumin (BSA) for an hour at room temperature. Then, the membranes were incubated with primary antibodies overnight at 4°C, followed by washing (15 min, three times) and incubation with appropriate secondary antibodies (1:3000) for an hour at room temperature. Next, the membranes were washed (15 min, three times) and the protein bands were visualized by using an ECL Western blotting kit (Wuhan Guge Biotechnology Co., Ltd., China). The primary antibodies used in the study were β-catenin (1:200), Wnt1 (1:500), c-myc (1:500), AFP (1:1 000), CK19 (1:1 000), Thy1.1 (1:1 000), ALB (1:1 000), β-actin (1:1 000), Wnt3 (1:1 000), FZD2 (1:1 000), GSK3β (1:1 000), EpCAM (1:1 000) and cyclinD1 (1:2 000) (Santa Cruz, USA). The images were acquired with Image Lab and analyzed by using Image J.

1.5 Statistical Analysis

Statistical analyses were performed using SPSS

19.0. All data were presented as mean±standard deviation (SD), unless otherwise noted. One-way ANOVA was used for between-group analyses. A *P*-value of <0.05 was considered statistically significant.

2 RESULTS

2.1 Effects of DWYG on Hepatic Pathology

In order to assess the possible protective effects of DWYG on hepatocytes, we examined the morphological characteristics of hepatocytes of the animals in different experimental groups (fig. 1). We found that there were significantly more nuclei in the hepatic tissues of the 2-AAF/PH animals (vehicle group) than in the sham-operated animals and the normal animals on post-surgery day 10, 14, 17, 19, and 22. For DWYG-treated animals, the nuclei were significantly increased when compared with those in the normal group on post-surgery day 10, 14, 17, 19, and 22.

The total surface area of the nuclei was significantly greater in the 2-AAF/PH animals than in the sham-operated ones on post-surgery day 10, 14, 17, and 19. On post-surgery day 14, 17, 19, and 22, the total surface area was significantly greater in 2-AAF/PH animals than in the normal animals (P<0.05). On post-surgery day 17, the total surface nuclei area in the DWYG-treated group was significantly greater than that in the normal group (P<0.05). In contrast, on post-surgery day 19, it was significantly lower in DWYG-treated group than in the 2-AAF/PH animals (vehicle group) (P<0.01).

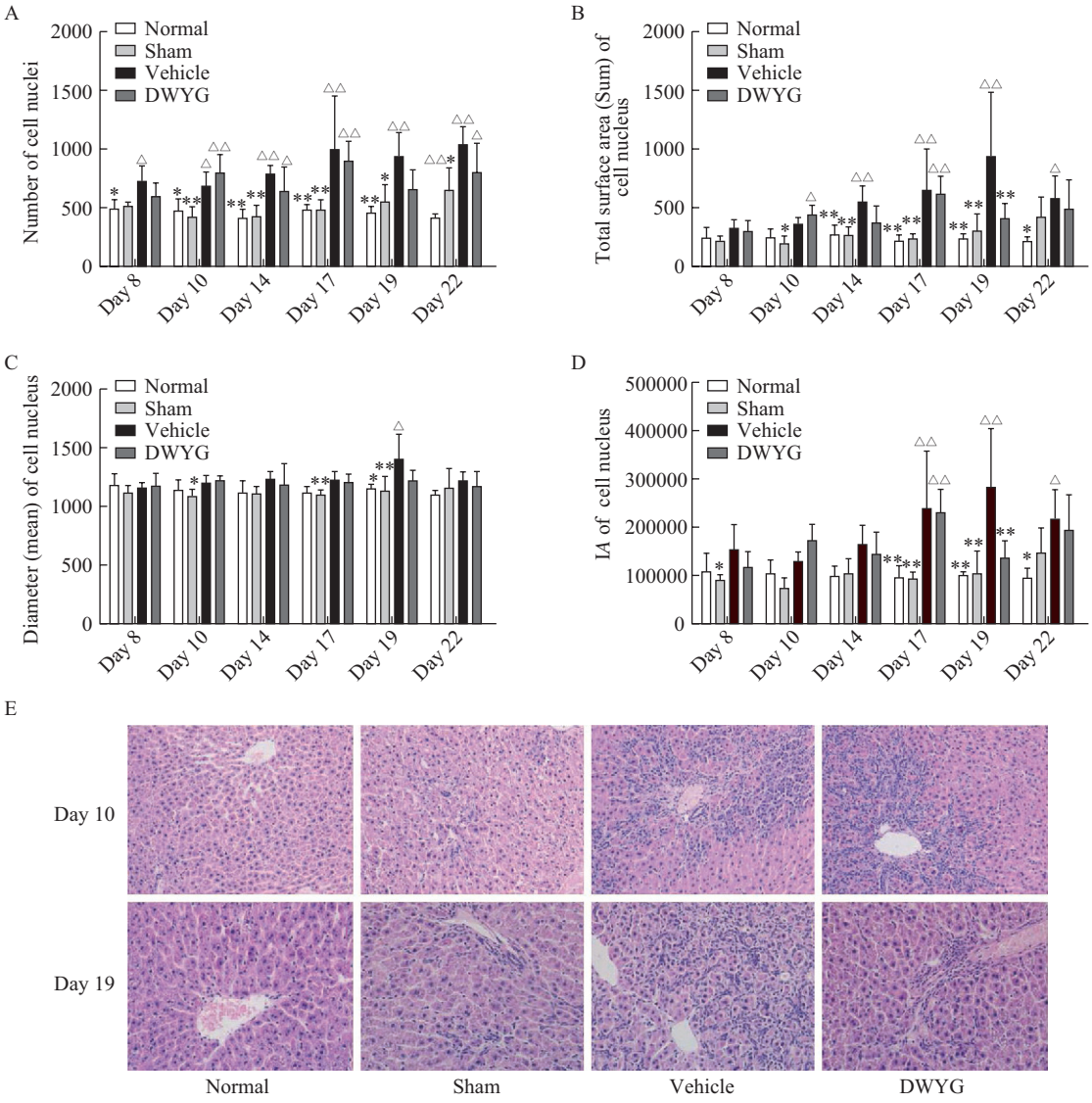

Fig. 1 Effects of DWYG treatment on the morphology of hepatic tissues

Normal Wistar rats (normal group), sham-operated rats (sham group) and 2-AAF/PH rats were orally administered with vehicle (distilled water, vehicle group) or DWYG (DWYG group, 10 mg/kg). A: the number of nuclei; B: total surface area of nucleus; C: mean diameters of the nucleus; D: I*A* of the nucleus; E: representative images of H&E staining. Original magnification (×200). $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01, compared with the normal group; $^{*}P$<0.05, $^{**}P$<0.01, compared with the 2-AAF/PH animals (vehicle group)

The mean diameter of the nuclei was significantly increased in the 2-AAF/PH animals (vehicle group) as compared with the sham-operated animals on post-surgery day 10, 17, and 19. Furthermore, it was significantly greater in the 2-AAF/PH animals (vehicle group) than in normal animals on post-surgery day 19.

The I*A* of the nuclei was significantly higher in 2-AAF/PH animals than in the sham-operated group on post-surgery day 8, 17, and 19. On post-surgery day 17, 19, and 22, the I*A* of the nuclei was significantly higher in the 2-AAF/PH animals than in the normal group. On post-surgery day 17, the I*A* was significantly increased in the DWYG-treated animals when compared with that in the normal animals, and on post-surgery day 19, it was significantly lower in the DWYG-treated animals than in the 2-AAF/PH animals.

2.2 Effects of DWYG on the Expression of Oval Cell Biomarkers

We first examined the expression of biomarkers for oval cells in the liver tissues, including AFP, CK-19, Thy1.1, and ALB with time (fig. 2). The expression of the biomarkers was assessed on days 8, 10, 14, 17, 19, and 22 post-surgery. The expression of AFP was significantly higher on day 17 in 2-AAF/PH animals that received only the vehicle (vehicle group) (n=4), than in either the normal animals (n=8) or animals in the sham surgery group (n=8) (P<0.01). This increase in AFP expression was significantly lower in the DWYG-treated 2-AAF/PH animals (P<0.05). Furthermore, AFP expression was significantly increased in DWYG-treated group as compared with that in the normal animals (P<0.05).

An increase in the expression of CK19 in the 2-AAF/PH animals was first observed on post-surgery day 14, and this increase was significant when compared with the normal animals on post-surgery day 22 (P<0.05). Treatment with DWYG appeared to promote the early increased expression of CK19,

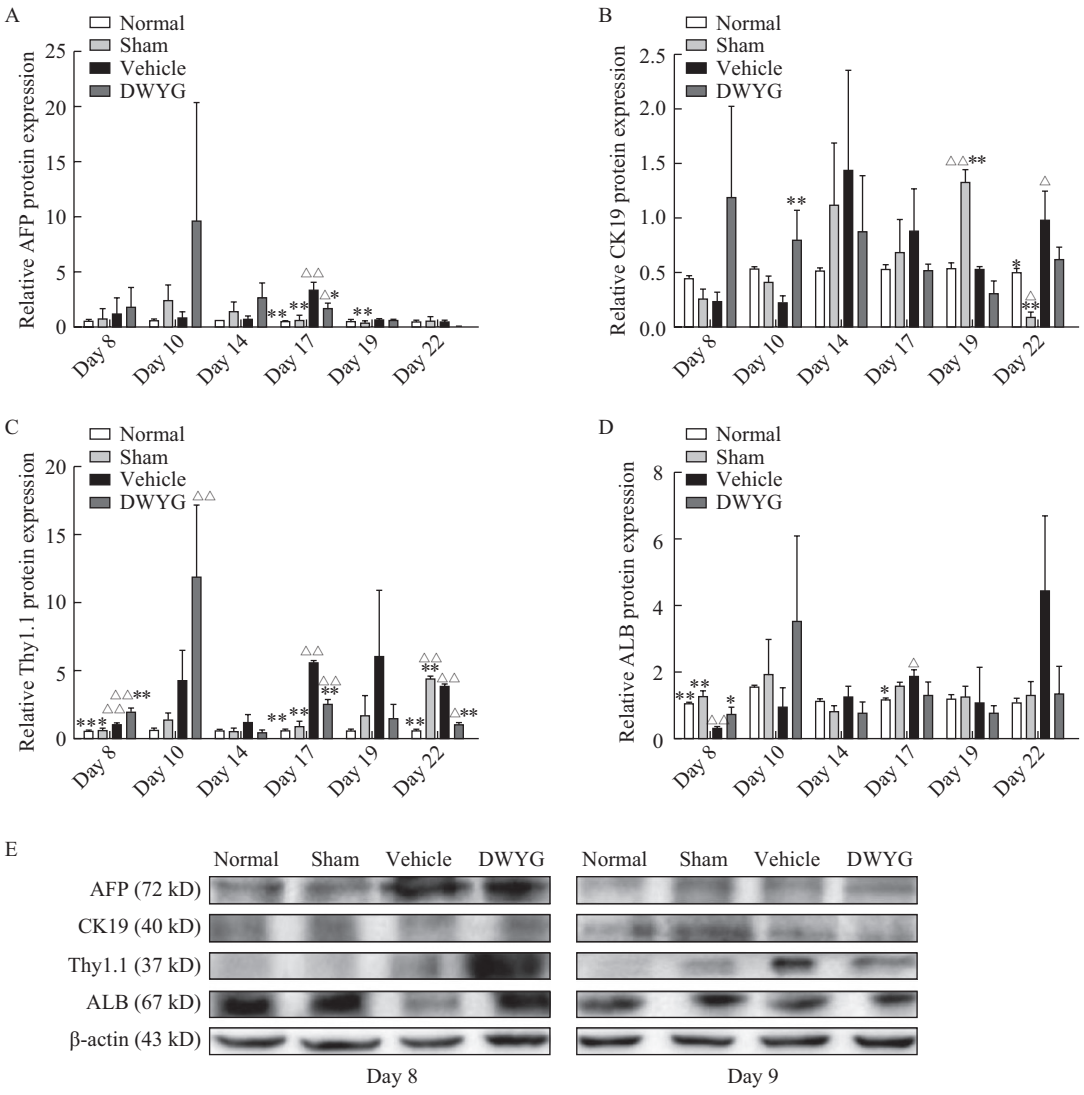

Fig. 2 Expression levels of the biomarkers of oval cells and liver stem cells

A: AFP protein level; B: CK19 protein level; C: Thy1.1 protein level; D: Alb protein level; E: representative blots of AFP, CK19, Ty1.1, and Alb. $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 compared with the normal group; $^{*}P$<0.05, $^{**}P$<0.01 compared with the 2-AAF/PH animals (vehicle group)

as evidenced by increased CK19 expression levels on post-surgery day 8, 10 in the DWYG-treated animals when compared with those in 2-AAF/PH animals (P<0.01).

The expression of Thy1.1 was significantly higher in the 2-AAF/PH animals than in the normal animals on post-surgery day 8, 17 and 22. Treatment with DWYG could transiently significantly increase the Thy1.1 expression on post-surgery day 10, followed by a decline. The expression of Thy1.1 was significantly increased in DWYG-treated animals on post-surgery day 8, but was significantly decreased on post-surgery day 17 and 22, when compared with that in the 2-AAF/PH animals. In contrast, ALB expression was significantly lower (P<0.05) on post-surgery day 8, but significantly higher on post-surgery day 17 (P<0.05) in the 2-AAF/PH animals (vehicle group) than in the normal animals, whereas ALB in the DWYG-treated animals was significantly higher than in the 2-AAF/PH animals on post-surgery day 8 (P<0.05).

2.3 Effects of DWYG on the Expression of Wnt/β-catenin Signaling Pathway Proteins

The effects of DWYG on the expression of key proteins in Wnt/β-catenin signaling pathway were examined (fig. 3). The levels of Wnt-1, Wnt-3, β-catenin, FZD2, GSK3β, c-myc, cyclin D1, and EpCAM were significantly higher in the 2-AAF/PH animals than in the normal group during the post-surgery day (P<0.05). Treatment with DWYG could significantly decrease the levels of these proteins except Wnt-1 in various post-surgery days (P<0.05), specifically Wnt-3 on days 10 and 19, β-catenin on day 17, FZD2 on days 14 and 17, GSK3β on days 14 and 22, c-myc on day 19, cyclin D1 on days 17 and 22, and EpCAM on day 14.

3 DISCUSSION

In the present study, we found that oval cell biomarker levels were significantly increased in the 2-AAF/PH animals relative to the normal animals from post-surgery day 17 to 22. This observation indicated that the oval cells continued to proliferate in 2-AAF/PH animals. In addition, CD45 and Thy1.1 levels were higher in the 2-AAF/PH animals than in normal animals from post-surgery day 8 to the end of study (day 22). CD45 and Thy1.1 are biomarkers for the hematopoietic stem cells that originate from the bone marrow. The elevated levels of these biomarkers indicated that the stem cells continued to proliferate at least up to the end of the study, which increased the risk of hepatocarinogenesis. The treatment of DWYG reduced the elevated levels of these biomarkers, suggesting that DWYG partially inhibited the proliferation of oval cells and hematopoietic stem cells in the latter phase of liver repair in the 2-AAF/PH animals. Furthermore, the expression of key proteins in the Wnt/β-catenin signaling pathway, including Wnt-1, Wnt-3, FZD2, and β-catenin, increased from 14 to 22 day post surgery, which possibly resulted in an increase in the downstream target gene expression products c-myc, cyclin D1, and EpCAM protein. The Wnt/β-catenin signaling pathway is involved in repair after liver injury. Therefore, the activation of the pathway in the 2-AAF/PH animals was an expected physiological response. However, the persistent activation of Wnt/β-catenin is demonstrated to be implicated in hepatacarcinogenesis[7]. Therefore, the timely deactivation of the signaling pathway could contribute to the prevention of hepatocarcinogenesis. We found that treatment with DWYG promoted Wnt/β-catenin signaling pathway activation, as evidenced by the higher expression of Wnt-1, β-catenin, and c-myc on post-surgery day 8, and higher level of FZD2 on post-surgery day 10 and cyclin D1 from post-surgery day 10 to 14 in DWYG-treated animals than in the 2-AAF/PH animals. Furthermore, the protein levels of Wnt-1, Wnt-3, and FZD2 from post-surgery day 14 to 22, and c-myc, cyclin D1, EpCAM from post-surgery day 17 to 22 were significantly lower in the DWYG-treated animals than in the 2-AAF/PH animals. In addition, the expression of GSK3β, a protein that targets β-catenin for degradation, was significantly higher in the DWYG-treated animals than in the 2-AAF/PH animals. Taken together, these results indicated that during liver repair in the 2-AAF/PH animals, DWYG accelerated the activation of Wnt/β-catenin signaling in the initial stage, and then contributed to the deactivation of the signaling pathway in the later phase. Since the persistent activation of Wnt/β-catenin signaling increases the risk of hepatocarcinogenesis, these effects of DWYG are potentially beneficial to liver repair and therefore reduce the risk of the pathogenesis of HCC.

The Wnt/β-catenin signaling pathway has been found to contribute to the carcinogenesis of HCC, since β-catenin has been found to accumulate in the nucleus of liver tumor cells[20]. Thus, this signaling pathway has become a therapeutic target for the treatment of HCC[21, 22]. We found in the current study that DWYG could modulate the Wnt/ β-catenin signaling pathway, implying a role of DWYG in the prevention or treatment of HCC.

DWYG is a traditional Chinese herbal medicine that contains five herbal medicinal extracts, and has a history of being used as a treatment for liver diseases, including hepatitis. However, the exact mechanism by which DWYG achieves the therapeutic effects is not understood. Furthermore, as with other traditional Chinese herbal medicines, it is not clear which compound(s) in DWYG confers the therapeutic benefits. Nevertheless, the molecular mechanism of DWYG as a composite has been investigated. DWYG has been reported to modulate the hepatic

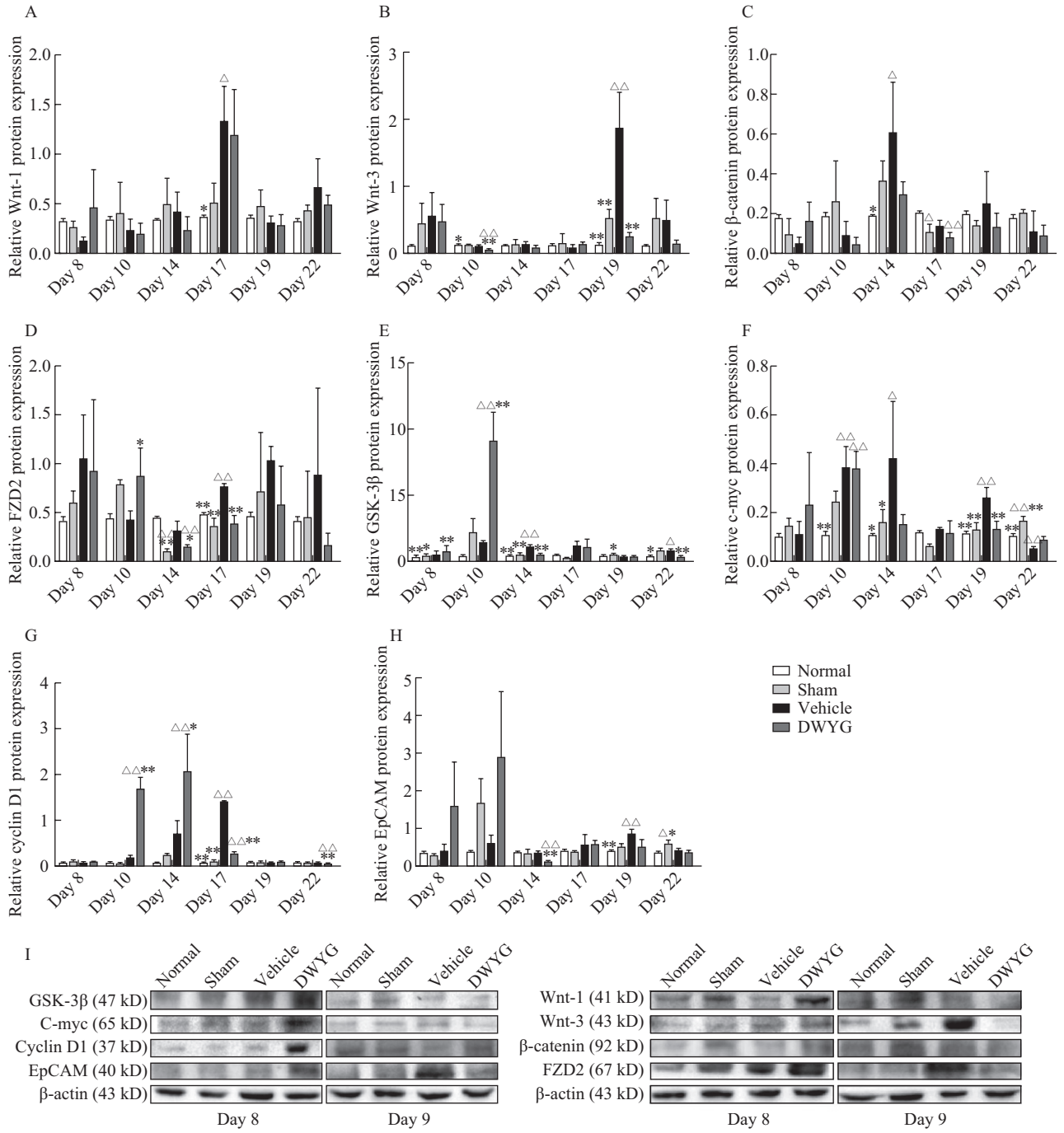

Fig. 3 Expression levels of key proteins in the Wnt/β-catenin signaling pathway
A: Wnt-1 protein levels; B: Wnt-3 protein levels; C: β-catenin protein levels; D: FZD2 protein levels; E: GSK-3β protein levels; F: c-myc protein levels; G: cyclineD1 protein levels; H: EpCAM protein levels; I: representative blots of individual proteins. $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ compared with the normal group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the 2-AAF/PH animals (vehicle group)

microenvironment and promote liver regeneration[17]. Since a hepatic microenironment could influence progenitor cell differentiation[23], and the cell matrix surrounding the cancerous cells changes the microenviroment to support cancer growth[24, 25], the regulation of microenvironements could contribute to the prevention of pathophysiological changes that are necessary for cancer growth. Therefore, it is possible that DWYG reduces the risk for liver cancer by modifying the hepatic microenvironments. In the present study, we found that the hepatic tissues from 2-AAF/PH animals contained more nuclei, had larger nuclei, and had higher density of nuclei, indicating ongoing cell proliferation, inflammatory cell infiltration, and an increase in nuclei density. These findings are consistent with our previous study that reported morphological changes in

the liver tissue that were typical of carcinogenesis[17]. The present findings that DWYG could reduce the risk of hepatocarcinogenesis via modulation of the Wnt/β-catenin signaling pathway add to our knowledge of the medicine. Future studies will direct toward identifying the effective ingredient of DWYG.

Overall, we showed that DWYG-treatment altered the expression pattern of key proteins in the Wnt/β-catenin signaling pathway, and possibly contributed to reducing the risk of hepatocarcinogenesis. DWYG possibly first accelerated the activation, and then contributed to the deactivation of the Wnt/β-catenin signaling pathway, thereby promoting liver injury repair and reducing the risk of pathological changes induced by persistent Wnt/β-catenin signaling, which could lead to carcinogenesis.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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