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Original Article

Polydatin regulates Wnt/β-catenin signaling to inhibit proliferation and migration of hepatocellular carcinoma cells

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Abstract: Objective: To investigate the effect of Polydatin on proliferation and migration of hepatocellular carcinoma cells and the related mechanism. Methods: Hepatocellular carcinoma (SMMC7721 and HepG2) were cultured in vitro, and these cells were treated with different concentrations of Polydatin. The inhibiting effect of Polydatin on proliferation of hepatocellular carcinoma cells was observed by methyl thiazolyl tetrazolium (MTT) method. The half maximal inhibitory concentration (IC_{50}) of Polydatin on cell proliferation was calculated. The migration and invasion of cells were studied by transwell experiment. Western blot method was adopted to analyze the effects of Polydatin on the expression of β-catenin, downstream target gene Cyclin D1 and c-Myc. To evaluate the mechanism, we treated SMMC7721 and HepG2 cells with Polydatin and Polydatin plus β-catenin activator SB216763, and observed the proliferation of hepatocellular carcinoma cells by methylthiazole tetrazolium (MTT) assay. Results: Polydatin inhibits proliferation of SMMC7721 and HepG2 cells. There was a statistically significant difference in IC_{50} between the two cells, and the inhibitory effect of polydatin on HepG2 proliferation was significant. Polydatin inhibits the expression and transcription of β-catenin, Cyclin D1 and c-Myc. SB-216763 accelerated proliferation and inhibited the apoptosis of Polydatin-treated SMMC7721 and HepG2 cells. Western blot analysis showed that SB-216763 up-regulated β-catenin, Cyclin D1 and c-Myc in Polydatin-treated SMMC7721 and HepG2 cells. Conclusions: Polydatin can inhibit the proliferation, invasion and migration of hepatocellular carcinoma cells. The anti-hepatoma mechanism may be related to Wnt/β-catenin signaling inhibition.

Keywords: Hepatocellular carcinoma cells, polydatin, Wnt/β-catenin, cell proliferation, cell migration, cyclin D1, c-Myc protein

Introduction

Polydatin (PDT) is a monomer extracted from the rhizome of polygonum cuspidatum (Herbaceous plant, perennial, polygonaceae). It is one of the most important components of polygonum cuspidatum and also known as polydatin [1]. Polydatin (PDT) is widely found in plants, especially in vitis plants. Its effect includes promoting the development of myocardial cells, improving the microcirculation in vivo, enhancing the resistance to oxidation, and protecting the liver, etc. [2, 3]. The latest studies have also demonstrated that Polydatin has an obvious preventive effect on liver cancer in rats with the specific molecular biology mechanism needing to be studied [4].

Hepatocellular carcinoma (HCC) mortality ranks third among all tumor-related deaths. HCC is mainly caused by chronic infection, alcoholic hepatitis and other cirrhosis [5]. Related studies have shown that Wnt protein may play an important role in the differentiation, proliferation and migration of liver cancer cells [6]. B-catenin is overexpressed in HCC, which activates downstream cellular signaling pathways and regulates biological processes such as cell proliferation, apoptosis, and invasion, possibly via Wnt protein and low density lipoprotein receptor-associated protein 5/6 interaction and inhibition of β-catenin phosphorylation and degradation [7]. Koneru [8] analyzed the effects of pirfenidone on cell proliferation and apopto-

sis and expression of Wnt/β-catenin signaling pathway in HepG2 cells. Zhang [9] found that β-catenin activator SB-216763 accelerates proliferation and inhibits apoptosis of HepG2 cells. Zhao [10] found that carotenoids can inhibit the proliferation and invasion of hepatoma cells through the Wnt/β-catenin signaling pathway. In addition, β-catenin activator SB-216763 up-regulates β-catenin expression, but its cytotoxicity is limited, limiting further clinical applications [11]. Therefore, there is an urgent need to develop new targeted drugs for the treatment of hepatocellular carcinoma.

Polydatin has low cytotoxicity and has a tumor suppressing effect. However, its effect on the Wnt/β-catenin signaling pathway has not been reported. This study investigated the effect of Polydatin on proliferation and migration of hepatocellular carcinoma cells and the related Wnt/β-catenin signaling mechanism.

Materials and methods

Reagents

Polydatin was purchased from Melon Biotechnology Co., Ltd.; MTT was purchased from Sinopharm.

Cell culture

SMMC7721 and HepG2 cell lines were provided by ATCC Company. They were cultured with 10% serum-contained DMEM under the condition of 5% CO_2 and 37°C. The liquid was replaced every 2 days. 0.25% pancreatin solution (containing 0.02% EDTA) was added when cells grew to 70%~90% confluency. The cells were subcultured every 2~3 days. The cells used in the experiments were in logarithmic phase. The SMMC7721 and HepG2 cell lines were divided into 6 groups (A/B/C/D/E/F), and 4 groups (A/B/C/D) were treated with different concentrations of Polydatin (0, 25, 50, 100 μg/mL). (E) group was treated with 100 μg /mL Polydatin, and the other group (F) was treated with Wnt signaling pathway inhibition (SB-216763, 9 nM).

Detection of cell proliferation by MTT

Two lines of cells in the log phase after treatment were collected. The concentration of the cell suspension was adjusted to 5×10^4/mL, and after 48 hours of incubation, 20 μL of LTTT solution (5 mg/mL, i.e., 0.5% MTT) was added to each well. The culture solution was aspirated from the well immediately after 4 hours of culture. 150 μL of dimethyl sulfoxide was added. The resulting material was shaken at a low speed for 10 minutes at 37°C. The OD value at 490 nm was measured and recorded by a microplate reader. This study has been approved by the Ethics Committee of Affiliated Hospital of North Sichuan Medical College.

Cell viability of control = A drug group - A zero setting well)/(A control well - A zero setting well) × 100%

Detection of cell migration and invasion by transwell experiment

The cells were digested with trypsin and resuspended in serum-free medium. The cell density was adjusted to 2×10^8/L. 100 μL of cell suspension was added to the upper layer of the transwell chamber. 500 μL of 10% serum contained medium was added to the lower layer. The chamber was taken out after culture was continued for 1 day. After washing with PBS, the unmigrated cells were slowly wiped off with wet swab. The cells were fixed with 4% paraformaldehyde for 20 min. After washing with PBS 3 times, 0.1% crystal violet was added for staining for 10 min. After washing with PBS 3 times, five fields of view were randomly selected. The number of migrated cells was counted under optical microscope.

Analysis and determination of effect of polydatin on expression level of β-catenin, D1 (cyclin D1) and c-Myc by western blot

The (A/B/C/D) group SMMC7721 and HepG2 cells were seeded in 6-well plates and treated with (0, 25, 50, 100 μg/mL) of polydatin at 37°C, in a 5% CO_2 incubator for 24 hours. Total protein was obtained using a lysis buffer containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Total protein concentration was measured using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The protein sample is transferred to the membrane after gel electrophoresis. Add % skim milk powder. The sample was sealed at 4°C for 2 hours. Mouse anti-human β-catenin, cyclin D1, c-Myc and GADPH monoclonal antibodies (1:1000) were

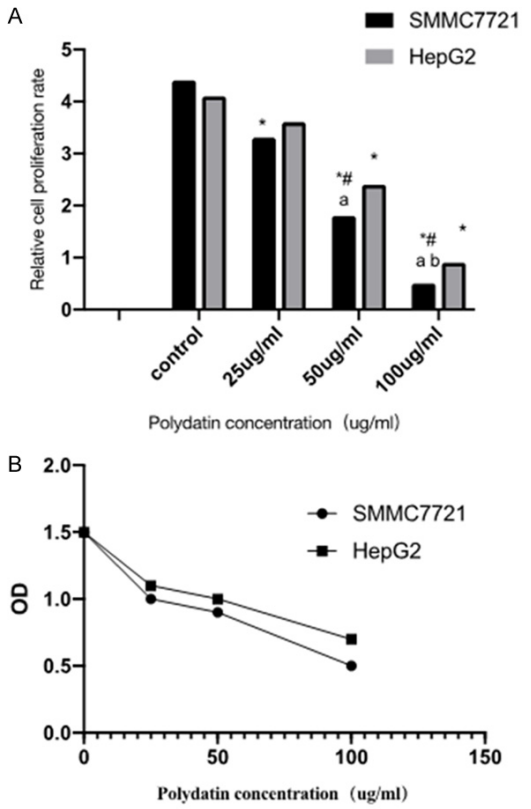

Figure 1. A. Polydatin reduces the proliferation of SMMC-7721 and HepG2 cells. MTT cell proliferation assay was performed to determine the effect of polydatin on SMMC-7721 and HepG2 cells at different concentrations. With the increase of the concentration of polydatin, the inhibitory effect on the proliferation of SMMC-7721 and HepG2 cells was more obvious, and the inhibitory effect of polydatin on SMMC-7721 was stronger than that of HepG2 cells. B. The half maximal inhibitory concentration (IC_{50}) of Polydatin on cell proliferation was calculated. The migration and invasion of cells were studied by transwell experiment. *indicates a comparison with control group P < 0.05, the difference is statistically significant; [a]indicates a comparison with 25 ug/ml group P < 0.05, the difference is statistically significant; [b]indicates a comparison with 50 ug/ml group P < 0.05, the difference is statistically significant; #indicates that SMMC-7721 is compared with HepG2 P < 0.05, the difference is statistically significant.

added, respectively. After washing, incubation was carried out and a second antibody labeled with HRP (1:2000) was added. Incubation was carried out for 2 hours at room temperature. After washing, ECL chemiluminescent reagents are used for color development. Bio-Rad Gel Imaging system is used for array calculations and statistical analysis of each set of bands. Relative expression of protein = expression of target protein/expression of GADPH protein. This experiment was repeated 3 times.

Group E SMMC7721 and HepG2 cells were treated with 100 μg/mL of polydatin, group F was treated with SB-216763, and treated at 37°C in a 5% CO_2 incubator for 24 hours for WB experiments. Verify the inhibition of SB-216763.

Statistical analysis

Statistical analysis of the data was performed using SPSS 22.0. All experimental result data are expressed as mean ± standard deviation (x ± sd). Statistical significance was determined using one-way ANOVA, and the LSD-t test was compared between groups (test level α=0.05).

Results

Polydatin inhibited the proliferation of SMMC7721 and HepG2 cell lines which can be reversed by SB-216763

In order to further study the role of Polydatin in the development and progression of hepatoma, the human SMMC7721 and HepG2 cell lines were used as the study objects. The cell proliferation was detected by MTT method. As shown in **Figure 1A**, the results showed that Polydatin could inhibit the proliferation of hepatocellular carcinoma cells compared with the blank control group, especially in high dose group (P < 0.05), There was a statistically significant difference in IC_{50} between the two cell lines, and the inhibitory effect of polydatin on HepG2 proliferation was significant, shown as in **Figure 1B**. SB-216763 inhibited the reduction of proliferation of SMMC-7721 and HepG2 cells mediated by polydatin. MTT cell proliferation assay for SB-216763 inhibition of Polydatin mediated SMMC-7721 and HepG2 Cells. In addition, SMMC-7721 cells were more sensitive to SB-21676 than HepG2 cells (**Figure 2**).

Polydatin inhibited the migration of SMMC7721 and HepG2 cell lines which can be reversed by SB-216763

The effect of Polydatin on migration of SMMC-7721 and HepG2 cells was detected by transwell method. The results showed that Polydatin could significantly inhibit the migration of SMMC7721 and HepG2 cells compared with the control group (Group A) (P < 0.05). Namely,

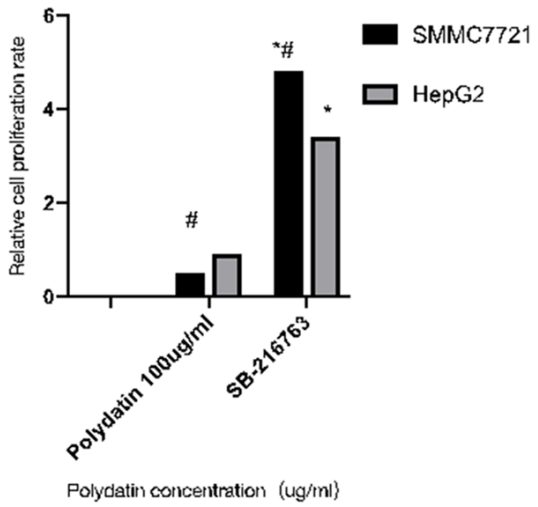

Figure 2. SB-216763 inhibited the reduction of proliferation of SMMC-7721 and HepG2 cells mediated by polydatin. MTT cell proliferation assay for SB-216763 inhibition of Polydatin mediated SMMC-7721 and HepG2 Cells. SB-216763 was more sensitive to scutellarin-mediated SMMC-7721 than HepG2 cells. *indicates a comparison with control group $P < 0.05$, the difference is statistically significant; #indicates that SMMC-7721 is compared with HepG2 $P < 0.05$, the difference is statistically significant.

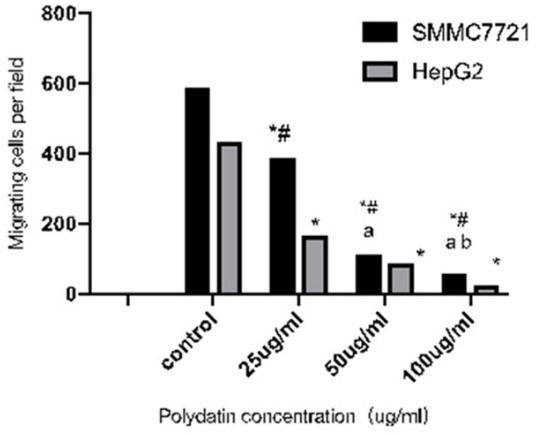

Figure 3. Polydatin reduces migration of SMMC-7721 and HepG2 cells. Transwell assays were performed to assess migration of SMMC-7721 and HepG2 cells treated with 0, 25, 50 and 100 μg/mL polydatin for 48 hours. The study group was significantly higher than the control group. The inhibitory effect of polydatin on HepG2 is stronger than that of SMMC-7721 cells. *indicates a comparison with control group $P < 0.05$, the difference is statistically significant; [a]indicates a comparison with 25 ug/ml group $P < 0.05$, the difference is statistically significant; [b]indicates a comparison with 50 ug/ml group $P < 0.05$, the difference is statistically significant; #indicates that SMMC-7721 is compared with HepG2 $P < 0.05$, the difference is statistically significant.

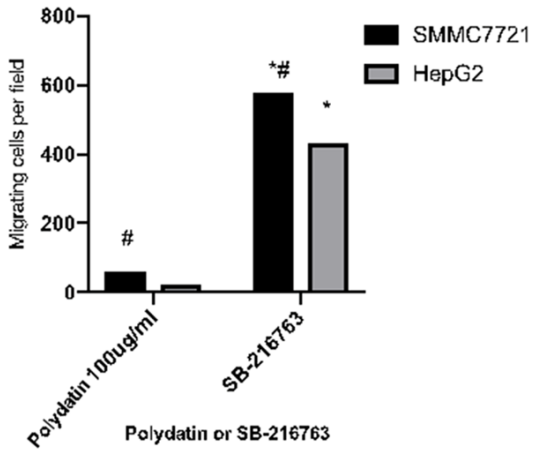

Figure 4. SB-216763 inhibits the reduction of polydatin-mediated migration of SMMC-7721 and HepG2 cells. Transwell assayed that SB-216763 inhibited polydatin, resulting in increased migration of SMMC-7721 and HepG2 cells. In addition, SB-216763 is more sensitive to SMMC-7721 than HepG2 cells. *indicates a comparison with control group $P < 0.05$, the difference is statistically significant; #indicates that SMMC-7721 is compared with HepG2 $P < 0.05$, the difference is statistically significant.

Polydatin had significant inhibiting effect on migration of hepatocellular carcinoma cells (**Figure 3**). SB-216763 inhibits the reduction of migration of SMMC-7721 and HepG2 cells induced by polydatin. SB-216763 inhibited polydatin-induced cell migration. The results showed an increase in migration of SMMC-7721 and HepG2 cells. In addition, SMMC-7721 cells were more sensitive to SB-21676 than HepG2 cells (**Figure 4**).

Polydatin inhibited the invasion of SMMC7721 and HepG2 cell lines which can be reversed by SB-216763

The effect of polydatin on the invasion of SMMC7721 and HepG2 cells was detected by transwell method. The results showed that compared with the control group (group A), polydatin significantly inhibited the invasion of SMMC7721 and HepG2 cells ($P < 0.05$). That is, polydatin has a significant inhibitory effect on hepatocellular carcinoma cell invasion (**Figure 5**). SB-216763 inhibits polydatin-mediated reduction of SMMC-7721 and HepG2 cell invasion. B-216763 inhibited polydatin-induced cell invasion. The results showed an increase in migration of SMMC-7721 and HepG2 cells. In addition, SMMC-7721 cells were more sensitive to SB-21676 than HepG2 cells (**Figure 6**).

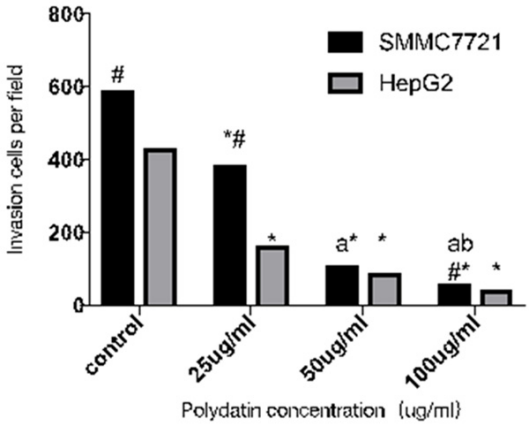

Figure 5. Polydatin reduces Invasion of SMMC-7721 and HepG2 cells. Transwell assays were performed to assess Invasion of SMMC-7721 and HepG2 cells treated with 0, 25, 50 and 100 μg/mL polydatin for 48 hours. The study group was significantly higher than the Control group. The inhibitory effect of polydatin on HepG2 is stronger than that of SMMC-7721 cells. *indicates a comparison between groups P < 0.05, the difference is statistically significant; [a]indicates a comparison with 25 ug/ml group P < 0.05, the difference is statistically significan; [b]indicates a comparison with 50 ug/ml group P < 0.05, the difference is statistically significant; #indicates that SMMC-7721 is compared with HepG2 P < 0.05, the difference is statistically significant.

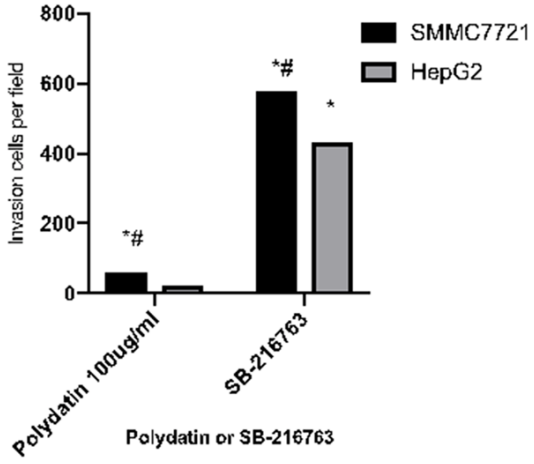

Figure 6. SB-216763 inhibits polydatin-mediated reduction of SMMC-7721 and HepG2 cell invasion. Transwell assayed that SB-216763 inhibited polydatin, resulting in increased invasion of SMMC-7721 and HepG2 cells. In addition, SB-216763 is more sensitive to SMMC-7721 than to HepG2 cells. *indicates a comparison with control group P < 0.05, the difference is statistically significant; #indicates that SMMC-7721 is compared with HepG2 P < 0.05, the difference is statistically significant.

Polydatin reduces the expression levels of β-catenin factor, Cyclin D1 and c-Myc in SMMC7721 and HepG2 cells which can be reversed by SB-216763

After the intervention of Polydatin, polydatin can inhibit the expression of β-catenin and downstream target genes Cyclin D1 and c-Myc (**Figures 7**, **9A**, **9B**). After SB-216763 intervention, the inhibitory effect of polydatin on the expression of β-catenin and downstream target genes Cyclin D1 and c-Myc was attenuated. In addition, SB-216763 is more sensitive to SMMC-7721 than to HepG2 cells. The study group was significantly higher than the control group (**Figures 8**, **9C**, **9D**).

Discussion

Primary hepatocellular carcinoma (HCC) is a high-grade malignant tumor, and its mortality rate ranks third among malignant tumors of the digestive system in China. In recent years, the rapid development of molecular biology has made tumor gene therapy a hot topic. Finding a key specific molecular target involved in the majority of liver cancers is an urgent problem to be solved in HCC gene therapy at this stage.

The anti-tumor effect of polydatin has been widely concerned by scholars. Cremon, Indraccolo et al [12, 13] believe that Polydatin can inhibit tumor growth and invasion, but the effect on hepatocellular carcinoma has not been examined.

The results of this study confirmed that Polydatin can significantly inhibit the proliferation of liver cancer cells in a dose- and time-dependent manner, and Polydatin can also promote the apoptosis of liver cancer cells, similar to the above results. From the above research results, Polydatin has anti-hepatocellular carcinoma effect and can be used as a potential therapeutic drug for hepatocellular carcinoma.

The classical Wnt/β-catenin signaling pathway has become a hot research topic in the biological filed of tumor molecules in recent years [14]. It has been demonstrated that the abnormal activation of Wnt/β-catenin signaling pathway is related to multiple tumors, such as cervical cancer, bladder cancer, and colorectal cancer, etc. [15-17]. As the most important transduction factor in Wnt signaling pathway, β-ca-

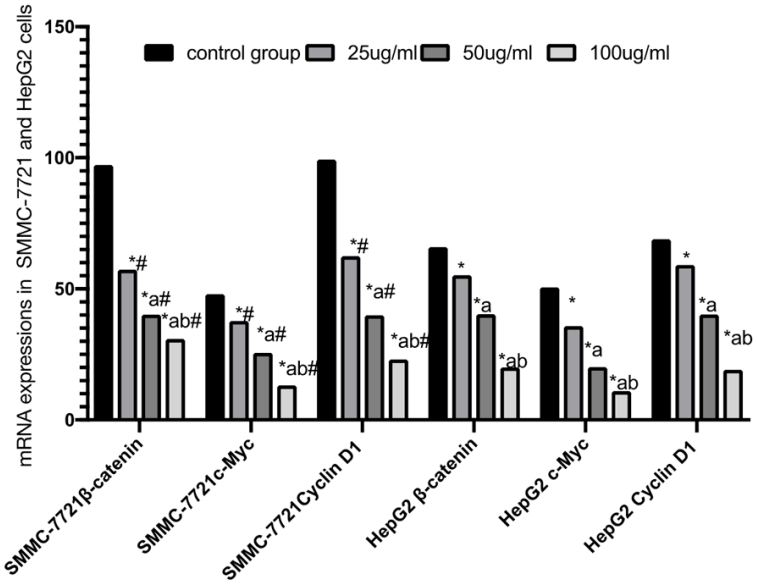

Figure 7. After the intervention of Polydatin, polydatin can inhibit the expression of β-catenin and downstream target genes Cyclin D1 and c-Myc. The study group was significantly higher than the control group. *indicates a comparison with control group $P < 0.05$, the difference is statistically significant; [a]indicates a comparison with 25 ug/ml group $P < 0.05$, the difference is statistically significan; [b]indicates a comparison with 50 ug/ml group $P < 0.05$, the difference is statistically significant; #indicates that SMMC-7721 is compared with HepG2 $P < 0.05$, the difference is statistically significant.

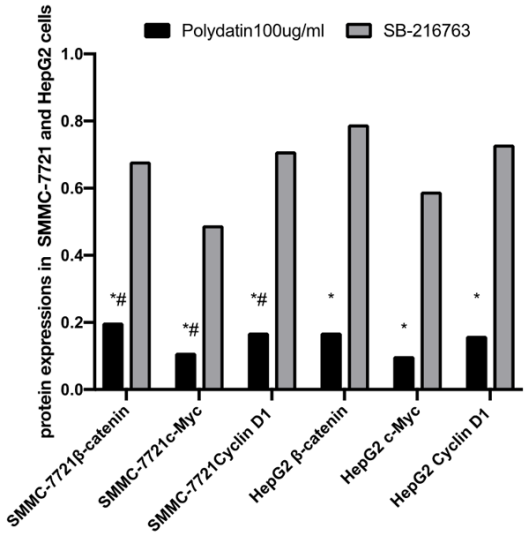

Figure 8. After SB-216763 intervention, the inhibitory effect of polydatin on the expression of β-catenin and downstream target genes Cyclin D1 and c-Myc was attenuated. In addition, SB-216763 is more sensitive to SMMC-7721 than to HepG2 cells. The study group was significantly higher than the control group. *indicates a comparison with SB-216763 group $P < 0.05$, the difference is statistically significant; #indicates that SMMC-7721 is compared with HepG2 $P < 0.05$, the difference is statistically significant.

tenin participates in intercellular adhesion and regulates the growth, differentiation, apoptosis and other key processes of cells. The appearance of intranuclear β-catenin factor indicates that the Wnt/β-catenin signaling pathway is activated [18, 19]. Study has demonstrated that a β-catenin/TCF complex with transcription function can be formed after β-catenin factor enters the nucleus and binds with the intranuclear transcription factor TCF. The complex regulates the transcription and expression of Cyclin D1 and c-Myc, and induces the malignant transformation of cells. Thus, the final effect of Wnt signaling is completed [20-22]. Other studies have shown that the abnormal activation of Wnt/β-catenin signaling pathway is closely related to hepatoma, and also determines the degree of metastasis after deterioration, the survival period and prognosis of patients with hepatoma. The abnormal transposition of β-catenin and the overtranscription of its downstream target genes are considered to be one of the key events in occurrence of hepatocellular carcinoma [23, 24]. Inhibiting the activation of Wnt/β-catenin signaling pathway may inhibit the self-renewal of liver cancer stem cells, and alleviate the malignant phenotype of tumor [25]. The study results further demonstrated that the expression and transcription level of β-catenin factor, Cyclin D1 and c-Myc increased significantly in hepatocellular carcinoma cells. After the intervention of Polydatin, the expression of β-catenin, Cyclin D1 and c-Myc was inhibited. To assess its mechanism, we treated SMMC7721 and HepG2 cells with Polydatin and resveratrol plus β-catenin activator SB-216763. The results showed that SB-216763 accelerated proliferation and inhibited apoptosis of resveratrol-treated SMMC7721 and HepG2 cells. Western blot analysis showed that SB-216763 up-regulated β-catenin in resveratrol-treated SMMC7721 and HepG2 cells. Expression. Ivanova [26] inhibited the prolifera-

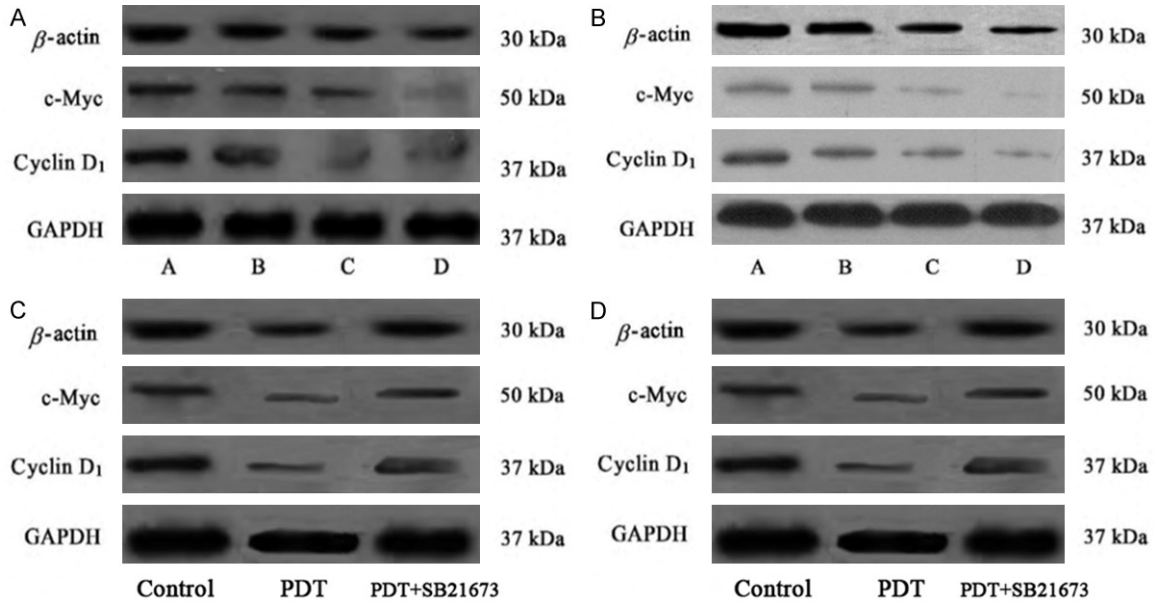

Figure 9. (A) Effect of Polydatin on protein of β-catenin and downstream target genes in SMMC-7721 cells. (B) Effect of Polydatin on protein expressions of β-catenin and downstream target genes in HepG2 cells. A. control; B. 25 ug/ml; C. 50 ug/ml; D. 100 ug/ml. (C) Effect of Resveratro+.SB-216763l on protein expressions of β-catenin and downstream target genes in SMMC-7721 cells. (D) Effect of Resveratro+.SB-216763l on protein expressions of β-catenin and downstream target genes in HepG2 cells.

tion of lung cancer cells by the Wnt/β-catenin pathway, and found that polydatin can increase the expression of GSK-3β. Concentration-dependent and Wnt5α expression were significantly reduced. Fako V [27] used the Wnt signaling pathway inhibitor SB-216763 to study the effects of lung cancer mediated by Wnt/β-catenin signaling. The results indicated that polydatin inhibits the migration and invasion of lung cancer cells through Wnt/β-catenin signaling pathway. Waisberg [28] and Brafman [29] found that Wnt/β-catenin signaling pathway promotes apoptosis of gastric cancer cells.

In addition, Giakoustidis [30] reported that Polydatin inhibits breast cancer metastasis via the Wnt pathway. The results of our study indicate that polydatin can inhibit the proliferation, migration and invasion of liver cancer cells through the Wnt pathway, providing a new idea for the treatment of hepatocellular carcinoma.

In this experiment, the pathway was studied only at the cellular and protein levels, and the results have certain limitations. Further animal experiments and molecular level related studies are needed for verification.

In summary, Polydatin can inhibit the proliferation, invasion and migration of hepatocellular carcinoma cells and promote their apoptosis. The anti-hepatoma mechanism may be related to its following effects: Polydatin can inhibit the expression of β-catenin factor in the signaling pathway of Wnt/β-catenin, down-regulate the transcription activity of β-catenin/TCF and inhibit the expression and transcription level of β-catenin, Cyclin D1 and c-Myc.

Disclosure of conflict of interest

None.

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