Study on the mechanism of inhibiting proliferation of hepatocellular carcinoma HepG2 cells by Grifola polysaccharide

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Abstract: Objective: To observe the effects of Grifola frondosa polysaccharides (GFP) on proliferation, apoptosis, cell cycle, expression of cyclin and apoptotic proteins of hepatocellular carcinoma HepG2 cells of human, and to explore the mechanism of its inhibition on proliferation of HepG2 cells. Methods: The experiment was divided into PGF/control group. Different concentrations of PGF solution were used to interfere with human hepatocellular carcinoma cells HepG2 in vitro. MTT assay was used to detect the effects of different concentrations of PGF on the survival of HepG2 cells. The apoptosis rate and cell cycle distribution of HepG2 cells induced by PGF were detected by flow cytometry. The expressions of Bcl-2, Bax and Caspase3, Cyclin-A1 and Cyclin-B1 were detected by immunohistochemistry. Results: Compared with the control group, PGF could effectively decreased proliferation of human hepatoma HepG2 in a concentration-dependent manner. Cell cycle detection showed that the proportion of S phase in each group was 24.71%, 28.78%, 36.26 and 42.39%, respectively, indicating that cells were blocked in S phase. The immunocytochemical results showed that the expression of Cyclin A1 protein decreased significantly, and the expression of cyclin-B1 was not significantly different before and after treatment. Flow cytometry showed that the apoptosis rates in control group and PGF group were 0, 18.0%, 30.5% and 49.5%, respectively. The difference between PGF group and control group was significant. Immunocytochemical results showed that PGF could significantly inhibit the expression of mitochondrial apoptosis inhibitor Bcl-2, increase the expression of pro-apoptotic factor Bax in a concentration-dependent manner, and up-regulate the percentage of Bcl-2/Bax to induce apoptosis of hepatocellular carcinoma cells. Conclusion: PGF can inhibit the proliferation of human hepatoma HepG2 cells by inducing apoptosis, inhibiting the proliferation of cancer cells, blocking cell cycle, inhibiting the expression of Bcl-2, and upregulating the expression of Bax and Caspase3.

1. Introduction

Hepatocellular carcinoma (HCC) is the main cause of cancer-related death [1]. The annual incidence of new liver cancer in China accounts for more than half of the world, and the mortality rate ranks second among all malignant tumors [2]. At present, surgery, radiotherapy and chemotherapy are the most common treatments. However, the curative effect is not ideal, and the 5-year survival rate is almost zero. The side effects of chemotherapy drugs are great, and long-term drug use is easy to produce drug resistance. Chemotherapy for advanced liver cancer patients is still lacking of effective and low-toxicity drugs. Compared with modern medicine, traditional Chinese medicine has low toxicity, good curative effect and relatively low price. In recent years, the anti-tumor effect of fungal polysaccharides has attracted wide attention, and it has unique efficacy in preventing and treating the occurrence and metastasis of liver cancer, prolonging the survival period of liver cancer patients, and improving the quality of life of liver cancer patients [3]. Among them, Grifola frondosa, also known as Phyllophora, belongs to Basidiomycetes, Lamycetes, Nonplicata, Polyporaceae, and Dendrofloris. It is a fungal Chinese medicine used for both medicine and food, and belongs to the tonifying or strengthening medicine of Chinese medicine [4]. According to literature reports, grifola polysaccharide has the effect of inhibiting tumor proliferation, hypoglycemic, bacteriostatic, antiviral and immunomodulatory. Grifolia polysaccharide (GFP) is an immunoactive component of grifolia, consisting of beta -(1-6) glucan with beta -(1-3) side chain and beta -(1-3) glucan with beta -(1-6) side chain. Studies have shown that this specific structure makes it possess stronger biological regulatory activity [5].

This study investigated whether GFP could inhibit the proliferation of human hepatocellular carcinoma cells HepG2 by inducing apoptosis and blocking cell division through its effect on human hepatocellular carcinoma cells cultured in vitro.
2. MATERIALS AND METHODS

2.1. Materials

Human hepatoma HepG2 cells, provided by Translational Medicine Center, the Second Affiliated Hospital of Shanxi University of Chinese Medicine.

Drugs and reagents PGF (Zhejiang Square Pharmaceutical Company), purity 90%. Tetramethyl azazole Blue (MTT) (Sigma Corporation), RPMI1640 culture solution and trypsin (Hyclone Corporation); Fetal bovine serum (Hangzhou Sijiqing); Mouse anti-human Bel-2, Bax, Caspase-3 antibody; Cyclin-A1, Cyclin-B1 antibody and HRP labeled Rabbit anti-mouse IgG (Wuhan Bode Biological Company).

Main instruments Ultra-clean table (ESCO), inverted microscope (Otte BDS-200), carbon dioxide incubator (BindCD-150), ultra-low temperature refrigerator (Thermo 702), High speed low temperature centrifuge (Gene Company Limited), Enzyme labeling instrument (Bio-Tek ELX808IU), flow cytometer (Beckman Coulter), decolorization shaker (Kylin-Bell BETS-M5).

2.2. Methods

After pre-experimental screening, after 48 h of drug action, the best effect was achieved when the dose concentration of GFP was 200, 400, 600ug/ml, and the inhibition rate showed an obvious time-dose dependent concentration. The operation is as follows: accurately weigh 20mg of grifolia polysaccharide, dissolve in 25ml double steaming water at a concentration of 800ug/ml, that is, GFP stock solution, autoclaved, stored at 4℃, and set aside. Before the experiment, 0.1ml, 0.2ml and 0.3ml of raw solution were added into 4mlRPMI1640 culture solution respectively, and the final concentration was 20ug/ml, 40ug/ml and 60ug/mlGFP solution, namely low, medium and high doses [6].

HepG2 Cell Proliferation Inhibition test HepG2 cells were amplified at 37℃ and 5% CO2 saturated humidity, and HepG2 cells were taken at logarithmic growth stage and inoculated into 96-well culture plates with an adjusted density of 2×104 cells /mL and 100ul/ well. After the cells were attached to the wall, the supernatant was abandoned, blank control group was set, and only equal volume of 1640 complete culture solution was added to the blank group, and different concentrations of GFP was added to the GFP group at 37℃ for 48h. Different concentrations of GFP were added, and the same volume of 1640 culture solution was added to the blank control group for 48h, and 4% paraformaldehyde was fixed. The cells were attached to the wall, the supernatant was discarded, 1ml1640 was added to the blank group, and different concentrations of GFP was added to the GFP group at 37℃ for 48h. Different concentrations of GFP were added, and the same volume of 1640 culture solution was added to the blank control group for 48h, and 4% paraformaldehyde was fixed. The serum was sealed and incubated with mouse anti-human Bel-2, Bax and Caspase-3 antibodies (1:100) at 4℃ overnight, and the second antibody was cultured with rabbit anti-mouse IgG labeled HRP (1:200) at 37℃ for 1h, followed by DAB for 5min and PBS washing for 3 times. Restaining with hematoxylin for 7min was observed under an inverted microscope and preserved.

The expression detection procedures of Cyclin A1 and Cyclin B1 were the same as those in 1.2.5.

2.3. Statistical Analysis

The SPSS19.0 software package was used for statistical analysis. Analysis of variance was used to compare the data. Data with ±s indicated that P<0.05 was statistically significant.

3. Result

3.1. Effects of GFP on proliferation of HepG2 cells

Compared with the blank control group, low, medium, high and concentration of GFP can inhibit the growth of HepG2 cells, and with the increase of concentration, the inhibition rate of HepG2 cells gradually increases. At
60μg/ml, the inhibition rate reached 62.6%. The difference was statistically significant (P<0.01), as shown in Figure 1.

![Effect of Grifola polysaccharide on the inhibition rate of HepG2 cells](image)

Fig. 1. Effect of GFP on proliferation of HepG2 cells

### 3.2. Effects of GFP on apoptosis of HepG2 cells

Compared with the blank control group, different concentrations of GFP can induce apoptosis of HepG2 cells, and the apoptosis rate increases gradually with the increase of GFP concentration, suggesting that the effect is concentration-dependent. The difference was statistically significant (P<0.01). Immunohistochemical results showed that Bcl-2 expression was higher in blank group, while Bax and Caspase-3 expression was lower in GFP group, while Bcl-2 expression was gradually decreased and Bax and Caspase-3 expression was upregulated with the increase of drug concentration. The GFP MOD of 20μg/ml, 40μg/ml and 60μg/ml were significantly different compared with blank group (P<0.05). There were also significant differences in 40μg/ml and 60μg/ml GFP MOD compared with 20μg/ml group (P<0.05). See Figures 2A, B, C.

![Effect of GFP on apoptosis of HepG2 cells](image)

Fig. 2A. Effect of GFP on apoptosis of HepG2 cells

![Apoptosis rate (%) **P<0.01](image)

Fig. 2B. Apoptosis rate (%) **P<0.01

![Effect of GFP on the expression](image)

Fig. 2C. Effect of GFP on the expression

![Effect of GFP on apoptosis of HepG2 cells](image)

Fig. 2D. compared with blank group, *P< 0.05, of Bcl-2, Bax and Caspase-3 (x200) compared with 20μmol/Lcur, *P< 0.05

### 3. Discussion

In recent years, with the deepening of research on the antitumor effects of fungal polysaccharides, grifola polysaccharide has gradually become a research hotspot due to its significant anti-tumor effects. As the main active component of grifola, GFP is composed of β-(1-3) glucan with β-(1-3) side chain and β-(1-6) glucan with β-(1-6) side chain. Studies have shown that this specific structure makes it possess stronger biological regulatory activity [5]. Lv Dongxia et al observed the inhibitory effect of mafolia polysaccharide combined with cisplatin on the growth of transplanted tumor in H22 mice, and promoted the expression of Caspase-8 and Cytc, and then promoted the expression of Caspase-3 to induce the apoptosis of H22 tumor cells [8]. Professor Hiroaki Namba of Kobe Pharmaceutical University in Japan took 190 cancer patients aged 25 to 65 years as subjects, and gave them grifolia flower powder plus vitamin C preparation, plus the polysaccharide body liquid extracted from grifolia flower. As a result, 50% of liver cancer patients had significantly reduced tumors [9]. These results indicated that grifola polysaccharide could regulate the immune system of patients with liver cancer to inhibit tumor growth and play an anti-liver cancer role.

Qian Yu et al. ’s polysaccharide extracted from the fermentation broth of grey tree flower has obvious inhibitory effect on S180 sarcoma of mice [10]. Chen Pei et al extracted polysaccharide from grifola at low temperature of 4℃, and the treated HepG2 cells showed apoptotic morphology [11]. In this study, HepG2 cells were treated with GFP, and the results showed that different concentrations of GFP could inhibit the growth of HepG2 cells in a concentration-dependent manner.
with the inhibition rate reaching 66% at 60μg/ml. The results are the same as in the above study. Flow cytometry showed that HepG2 cells were blocked in S phase and could not carry out cell cycle. All cell life activities need to undergo a complete cycle of G1-S-G2-M, and tumor cells are no exception. When the DNA integrity of cells is damaged, the cells cannot successfully complete the cycle of G1-S-G2-M [12]. The continued growth of cancer cells depends on the normal turnover of their cycles.

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In this study, GFP was applied to human hepatocellular carcinoma cells HepG2 in vitro, and it was found that GFP could inhibit the proliferation of human hepatocellular carcinoma cells HepG2 by inducing apoptosis and blocking cell division. In order to provide a new idea for the study of antitumor mechanism of fungi.

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REFERENCE


