

The mechanism of Grifola polysaccharide inhibiting hepatocellular carcinoma cells

Jiayi Zhang¹, Jing Feng^{2*}, Yumin Huang³, Bing Xu²

¹Shaanxi University of Chinese Medicine, Xianyang City, Shaanxi Province, 712000, China

²School of Medical Technology, Shaanxi University of Chinese Medicine, Xianyang City, Shaanxi Province, 712000, China

³Ankang Vocational and technical College, Ankang City, Shaanxi Province, 725000, China

Abstract Objective: To observe the effects of Grifola frondosa polysaccharides (GFP) on proliferation, apoptosis, cell cycle, expression of cyclin and apoptotic proteins of human hepatocellular carcinoma HepG2 cells, and to explore the mechanism of its inhibition on proliferation of HepG2 cells. Methods: The experiment was divided into control group and PGF 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 inhibit the 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 cell cycle arrest and expression of related cyclin and apoptotic proteins.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading 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 is increasingly prominent in the treatment of cancer because of its 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, Nonplicate, 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 immunoreactive 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.

*Corresponding author: 44115692@qq.com

2. Materials and methods

2.1. Materials

Human hepatoma HepG2 cells, provided by Translational Medicine Center, the Second Affiliated Hospital of Shaanxi 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 Bcl-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°C, 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°C and 5% CO₂ saturated humidity, and HepG2 cells were taken at logarithmic growth stage and inoculated into 96-well cell 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. For GFP group, add low, medium and high doses of GFP solution 100ul/ well. After culture for 24h, 20ul 5mg/mlMTT solution was added to continue culture for 4h. The culture medium was abandoned, DMSO was added to 100 UL/well, and shook at 37°C for 10min. After the crystallization was fully dissolved, the optical density (OD) of each well was measured at 490nm wavelength by enzymograph. Cell survival rate (%)= experimental group OD average/control group OD average ×100%[7].

HepG2 cells were detected by flow cytometry and inoculated with 6-well plates. 1640 culture medium was added to the blank control group. Different concentrations of GFP5ml were added to the GFP group. After the cells of the above groups were treated for 48

hours, the cells of each group were digested and collected to make single-cell suspension, which was washed with cold PBS. The cells were suspended with 100ulBinding buffer and stained with Annexin V-FITC and pro-pidiumiodide (PI) in accordance with the Annexin V-FITC apoptosis kit instructions. The cells were incubated at 4°C without light for 15min and vibrated, and the cell concentration reached 1×10⁵/ml. The cell apoptosis rate was measured by Flow cytometry, and the software flow JoVer.10 was used to calculate the cell apoptosis rate. The experiment was repeated three times.

HepG2 cells were treated for 48h by the same flow cytometry method as 1.2.3 method. The cells were digested and collected by pancreatic enzyme, and the density was adjusted to 1x10⁵ cells /mL. The cells were fixed with 70% ice ethanol and stored at -20°C for later use. Meanwhile, the blank control group was treated with 1640 culture solution. Before staining, the fixative was washed off with PBS, the cells were suspended by PBS, and 1mg/mL RNaseA 20ul in 37°C water bath for 30min, then 50ug/mL PI 400ul in ice bath for 30min, and then detected by flow cytometry. Modfit software analyzes the cell cycle.

Expression of apoptotic proteins Bcl-2, Bax and Caspase-3 A sterile cover slide was placed at the bottom of a 6-well plate, the cell density was adjusted to 1×10⁵/ml, and a 6-well plate was added to 1ml per well, and cultured at 37°C. After the cells were attached to the wall, the superserum was discarded, 1ml1640 was added to the blank group, and different concentrations of GFPwas added to the GFP group at 37°C 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 Bcl-2, Bax and Caspase-3 antibodies (1:100) at 4°C overnight, and the second antibody was cultured with rabbit anti-mouse IgG labeled HRP (1:200) at 37°C 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

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 60ug/ml, the inhibition rate reached 62.6%. The

difference was statistically significant ($P < 0.01$), as shown in Figure 1.

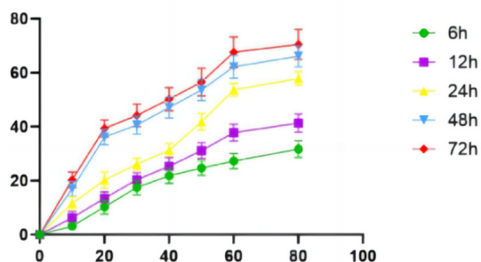


Fig. 1. Effect of GFP on proliferation of HepG2 cells

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 20 μ g/ml, 40 μ g/ml and 60 μ g/ml were significantly different compared with blank group ($P < 0.05$). There were also significant differences in 40 μ g/ml and 60 μ g/ml GFP MOD compared with 20 μ g/ml group ($P < 0.05$). See Figures 2A, B, C.

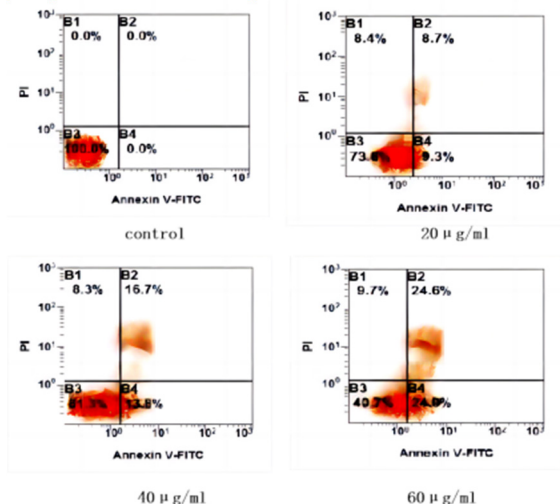


Fig. 2A. Effect of GFP on apoptosis of HepG2 cells

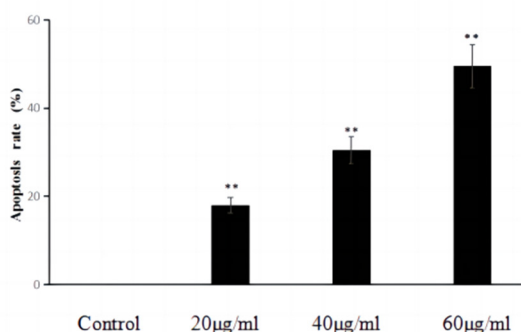


Fig. 2B. Apoptosis rate (%) $**P < 0.01$ vs control group

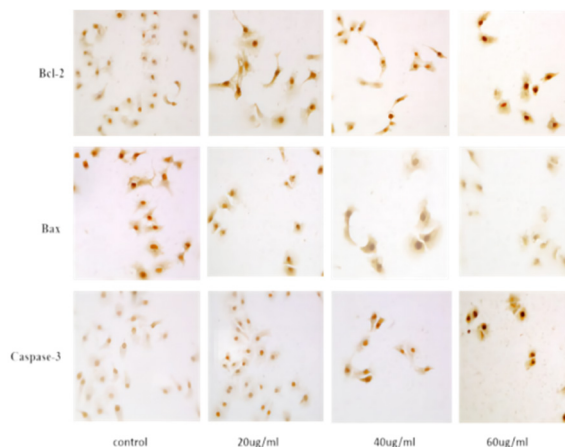


Fig. 2C. Effect of GFP on the expression

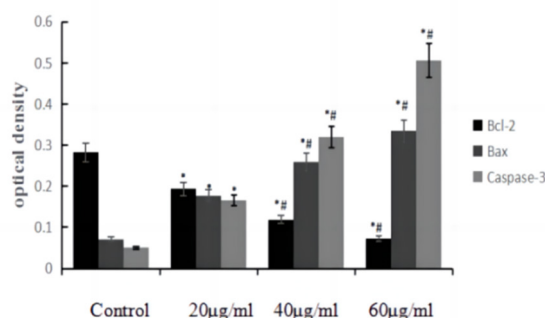


Fig. 2D. compared with blank group, $*P < 0.05$, of Bcl-2, Bax and Caspase-3 (x200) compared with 20 μ mol/Lcur, $*P < 0.05$

4. Discussion

In recent years, with the deepening of research on the antitumor effects of fungal polysaccharides, grifolia polysaccharide has gradually become a research hotspot due to its significant anti-tumor effects. As the main active component of grifolia, GFP is composed of β -(1-3) glucan with β -(1-3) side chain and β -(1-3) 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 grifolia 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 grifolia at low temperature of 4 $^{\circ}$ C, 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 60ug/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. In this process, Cyclins and CDK are the most critical [13]. Among them, Cyclin A1 and Cyclin B1 are important cyclins to ensure the cell cycle. Studies have found that the high expression of Cyclin A1 and Cyclin B1 will lead to abnormal cell proliferation, thus worsening the disease. Zhang Yuanyuan et al. [14] isolated and purified GFD-1, a polysaccharide component of *Maffolia*, and tested the apoptosis rate and cell cycle through in vitro proliferation inhibition experiment and flow cytometry technology. It was found that GFP could significantly inhibit the proliferation of human hepatoma cells HepG2. We treated HepG2 cells with GFP and found that the proportion of S phase increased from 24.11% to 32.68% at 20ug/ml, suggesting that GFP could block HepG2 cells in S phase. The expression of Cyclin A1 and Cyclin B1 was further detected by immunohistochemistry. It was found that GFP could inhibit the expression of Cyclin A1 and block HepG2 cells in S phase, thus inhibiting their proliferation, which was consistent with the results of Zhang's study. It is suggested that GFP can change the process of HepG2 cell cycle, and then block its further division and proliferation. Apoptosis is the programmed and active mode of cell death pathway, which is regulated by its own genes in normal physiological or pathological environment [15], among which Bcl-2 and Caspase are particularly important in cell apoptosis. As a key enzyme in the apoptosis cascade, caspase-3 is the core of the apoptosis cascade, and the hydrolytic activity of Caspase-3 can directly induce apoptosis [16]. Bcl-2 and Bax are the most important anti-apoptotic and pro-apoptotic genes in this family respectively, and they can jointly activate the pro-apoptotic factor caspase-3 and other factors in this family Bcl-2 are important anti-apoptotic genes, which can prevent the occurrence of apoptosis caused by various injuries. During the apoptosis process, they mainly regulate the increased expression of Bax protein. It can promote the occurrence of apoptosis [17]. Flow cytometry showed that GFP could induce apoptosis of HepG2 cells, and the apoptosis rate increased with increasing concentration. Further immunohistochemical detection of apoptotic proteins Bcl-2, Bax and Caspase-3 showed that GFP could significantly down-regulate the expression of Bcl-2 and up-regulate the expression of pro-apoptotic factor Bax in a dose-dependent manner. Meanwhile, GFP could up-regulate the percentage of Bcl-2/Bax, suggesting that GFP could induce apoptosis of hepatocellular carcinoma cells.

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.

Acknowledgment

1.This work was supported in part by key research project in shaanxi province department of education (key laboratory project) (2016JM8150).

2. This work was supported in part by Shaanxi Provincial College Student Innovation and Entrepreneurship Project 2023 (S202310716047).

Reference

1. Ferenci P, Fried M, Labrecque D, et al. Hepatocellular carcinoma (hcc): a global perspective. 2010; 44: 239-245.
2. Study on microfluidic digital isothermal amplification and its application in miREN detection of liver cancer [D]. University of Science and Technology of China, 2020.
3. Ye Lihong, Cheng Haibo, Zhang Yonghong, et al. Study on etiology of traditional Chinese medicine diseases of primary liver cancer [J]. *Shi Zhenguo Medicine*,2010,21(10) : 2627.
4. Dai Yucheng, Yang Zhuliang. List of medicinal fungi in China and revision of some names [J]. *Acta Mycologica Sinica*,2008, 6(12): 801-824.
5. Liu Jia, Bao Haiying, Tuligur. Research progress on chemical constituents and pharmacological activities of *Grifolia cinerea* [J]. *Journal of Fungal Research*,2018, (16) :150-157.
6. Cao L. Study on anti-oxidation, anti-tumor and immunomodulatory activities of four fungal polysaccharides [D]. Zhengzhou University,2010.
7. Liu Yueming, Zhu Jun, Zheng Sipeng, et al. Study on in vitro expansion of peripheral blood NK cells in healthy adults in Xinjiang [J]. *Journal of Xinjiang Medical University*,2013,35(6):763-767. (in Chinese)
8. LV Dongxia, Du Wei, Fan Xiaoyan et al. Inhibitory effect of grifola polysaccharide combined with cisplatin on H22 hepatocellular carcinoma transplanted mice [J]. *Chinese Journal of Gerontology*,2014,34(8):2165-2166.
9. Gan Changfei. Research progress of *Grifolia grifolia* and its pharmacological effects [J]. *Journal of Edible and Medicinal Fungi*,2014,22(5):264-267. (in Chinese)
10. Qian Yu, Qiao Hui, Zheng Tianjun, et al. Research progress of antitumor activity and mechanism of medicinal fungi [J]. *Chinese Journal of Mycology*, 2018,13(2):124-128. (in Chinese)

11. Chen Pei, Liu Huiping, Sun Naxin, et al. Isolation, purification and antitumor activity of polysaccharide from *Grifolia grifolia* [J]. *Modern Food Science and Technology*, 2018,34(6):107-114. (in Chinese)
12. SIEGEL R, DESANTIS C, VIRGO K, et al. Cancer treatment and survivorship statistics[J].*CA Cancer J Clin*. 2012 ,62(4):220-241
13. Yang P, Yin K, Zhong D, et al. Inhibition of osteosarcoma cell progression by MacroH2A via the downregulation of cyclin D and cyclin dependent kinase genes [J]. *Mol Med Rep*, 2015, 11(3): 1905-1910.
14. Zhang Yuanyuan, Meng Meng, Wang Mingfei, et al. Isolation, purification and anti-liver cancer effect of *Grifola* polysaccharide in vitro [J]. *Food Research and Development*, 2016,37(15):1-5. (in Chinese)
15. Qiu Qing-Yong, Peng Jin, Chen Li, et al. Effect of dogmoside on oxidative stress injury of H9c2 cardiomyocytes via Bcl-2 /Bax pathway [J]. *China Pharmaceutical Industry*, 2020,29(17):39-42.
16. Xu Bin, Yang Xiushu, Liu Xuan, et al. Expression of cysteine proteinase 3, Bax and Bcl-2 in intestinal epithelial cells in urine [J]. *Journal of Tissue Engineering*, 2019,25(20):3173-3177.
17. Yang Zhihong, Yang Xiaofang, Yi Wei, et al. Effect of moxibustion Shenque Point on the expression of bcl-2, bax, caspase-3 and p53 in brain tissue of aging mice [J]. *Chinese Journal of Gerontology*, 2019, 41 (2) 321-324.