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Inhibitory Effect of Extract of Fungi of *Huaier* on Hepatocellular Carcinoma Cells

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Summary: This study investigated the inhibitory effect of the extract of fungi of *Huaier* (EFH) on the growth of hepatocellular carcinoma (HCC) cells. Hep-G2 cells, a human HCC cell line, were cultured in DMEM containing 10% fetal bovine serum and treated with EFH of different concentrations (1, 2, 4, 8 mg/mL) for 24, 48 and 72 h respectively. The apoptosis rate of the cells was flow cytometrically measured. Thirty-six tumor-bearing New Zealand rabbits were randomly divided into 3 groups: group A (control group), in which the rabbits were infused with 0.2 mL/kg normal saline via the hepatic artery; group B (transhepatic artery chemoembolization [TACE] group), in which the rabbits were given lipiodol at 0.2 mL/kg plus MMC at 0.5 mg/kg via the hepatic artery; group C (TACE + EFH group), in which EFH (500 mg/kg) were orally administered after TACE. Two weeks after TACE, the rabbits were sacrificed and the implanted tumors were sampled. The tumor volume and the necrosis rate were determined. The tumor tissues were immunohistochemically detected for the expressions of factor Ⅷ, VEGF, P53, Bax and Bcl-2. The microvessel density (MVD) was calculated by counting the factor Ⅷ-positive endothelial cells. Our results showed that after treatment with EFH, the apoptosis rate of Hep-G2 cells was enhanced in a concentration- and time-dependent manner. Two weeks after the treatment, the average tumor volume, the necrosis rate and the growth rate of the implanted tumor in group C were significantly different from those in groups A and B ($P<0.05$). MVD and VEGF expressions were significantly decreased in the group C when compared with those in groups B ($P<0.05$ for all). The Bax expression was weakest in group A and strongest in group C. The expressions of P53 and Bcl-2 were minimal in group C and maximal in group A. There were significant differences in the expressions of P53, Bax and Bcl-2 among the 3 groups ($P<0.05$ for all) and there was significant difference between group B and group C ($P<0.05$). It was concluded that EFH could suppress not only the growth of HCC cells but also tumor angiogenesis and it can induce the apoptosis of HCC cells. EFH serves as an alternative for the treatment of HCC.
Key words: extract of fungi of *Huaier*; hepatocarcinoma; chemoembolization; rabbit VX2 tumor; apoptosis

Hepatocellular carcinoma (HCC), a common malignancy, occurs quietly with few early symptoms and can not be confirmed until the advanced stage. Up to now, the treatment efficacy is not satisfactory. Transcatheter arterial chemoembolization (TACE) has recently become one of the main therapeutic choices for the unresectable HCC. The long-term effect of TACE is uncertain due to the growth and metastasis of the remnant tumor cells in the lesioned tissues[1–3]. Recently, a Chinese traditional medicinal herb, *Huaier Granule*, of which the active ingredient was extract of fungi of *Huaier*, namely EFH, is reported to be a promising TACE-adjuvant agent for HCC treatment, and to be able to significantly improve the life quality of the HCC patients, but the exact mechanism is still elusive. In this study, we investigated the anti-cancer mechanisms of EFH by using cell culture and rabbit model of VX2 hepatocarcinoma after TACE.

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1 MATERIALS AND METHODS

1.1 Experimental Materials

Human liver cancer cell line Hep-G2 was provided by the Cell Storage Center of General Surgery Laboratory of Union Hospital, Wuhan, China. Hep-G2 apoptosis detection kit was from Jingmei Co., China. Kit for flow cytometry was purchased from BD Co., China. EFH was product of Jiangsu Qidong Gaitianli Pharmaceutical Co. Ltd., China (purity: 98%). Thirty-six white New Zealand rabbits were provided by the Center of Experimental Animals, Tongji Medical College, HUST, China. Rabbit VX2 tumor cells were from the Department of Liver and Gallbladder Surgery, Tongji Hospital, Wuhan, China. The monoclonal antibodies against VEGF, factor Ⅷ, P53, Bax and Bcl-2, used in this study, were purchased from Wuhan Boster Co., China (dilution: 1:100). Supervision™ two-step (universal type) immunohistochemical detection kit was obtained from Beijing Zhongshan Biotechnology Co. Ltd., China.

1.2 Flow Cytometrical Detection of the Apoptosis Rate of Hep-G2 Cells

Hep-G2 cells were cultured in DMEM containing 10% fetal bovine serum and seeded into 6-well plates at exponential phase with 3 mL DMEM (1×10^5 cells) in each well. After incubation for 24 h, the cells were treated with different concentrations of EFH (500 μL) which was diluted into 1, 2, 4 and 8 mg/mL with DMEM containing 10% fetal bovine serum in advance and then cultured for 24, 48 and 72 h. The 500 μL DMEM containing 10% fetal bovine serum in the absence of EFH served as blank control. The apoptosis rate of Hep-G2 cells was detected by flow cytometry by following the manufacturer's directions.

1.3 Grouping of the Animal Models and Treatment Program

White New Zealand rabbit VX2 liver cancer models (n=36) were established as previously described[4, 5], confirmed by MRI two weeks after the operation, and then they were randomly divided into three groups with 12 rabbits in each group: group A, in which the rabbits were infused with normal saline at the dosage of 0.2 mL/kg body weight via the hepatic artery; group B (TACE group), in which the rabbits were infused with super-liquid lipiodol (0.2 mL/kg) plus MMC emulsion (0.5 mg/kg) via the hepatic artery; group C (TACE + EFH group), in which EFH (500 $mg\cdot kg^{-1}\cdot day^{-1}$) was orally given after TACE. All the animals were sacrificed 2 weeks after the operation, and the tumor specimens were removed and fixed with 10% formalin.

1.4 Evaluation of Tumor Growth

The tumor was longitudinally cut for the evaluation of tumor necrosis. The tumor volume (TV) was calculated according to the following formula: $TV=a\times b^2/2$, where "a" and "'b" represented the maximum and minimum diameters of the tumor, respectively. The tumor necrosis rate was calculated as the necrosis area divided by the entire area of the tumor. The tumor growth rate was evaluated by the ratio of TV before and after the therapy.

1.5 Immunohistochemistry

The tumors and the adjacent tissues were sampled, paraffin-embedded and then sliced into sections of 4 μm thick. The samples were immunohistochemistrically detected for the expressions of VEGF, factor Ⅷ, P53, Bax and Bcl-2 by employing Supervision™ two-step method and stained with DAB/H_2O_2. VEGF-positive cells had brown-yellow granules in the cytoplasm. The VEGF expression was semi-quantificated with reference to Park's method[6]. Three views with the strongest VEGF expression were selected under microscope (×40), and then the VEGF-positive cells were counted under high-power microscope (×400). The positive cell rate ⩾ 5% was regarded as positive (+). The endothelial cytoplasm of factor Ⅷ-positive cells was also stained brown-yellow. Microvessel density (MVD) was counted as described by Weidner *et al*[7]. The five regions with highest MVD, i.e., vascular "hot spot" areas, were observed under low-power microscope. And then the microvessels were counted in five views under high-power microscope (×400). The values, which reflected MVD, were averaged. Bcl-2 and Bax proteins presented as brown-yellow granules in the cytoplasm of tumor cells. P53 protein was located in the nuclei of tumor cells and P53-positive cells presented as brown-yellow massive particles in the nuclei. Five views of each section were observed and the positive cells were counted. The results were considered to be positive (+) when the percentage of positive cells was more than 10%.

1.6 Statistical Analysis

The SPSS 12.0 software package was used for statistical analysis. Multi-group comparison of the apoptosis rate of Hep-G2 cells, the average volume, the necrosis rate, the growth rate and the MVD of the implanted tumor after TACE was made by using analysis of variance, and two-group comparison was made by using LSD-test. χ^2 test was employed to assess the differences of the expressions of VEGF, P53, Bax and Bcl-2 between groups. A $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Effect of EFH on the Apoptosis of Hep-G2 Cells

EFH significantly induced the apoptosis of Hep-G2 cells. Significant differences were found in the apoptosis rate between cells treated with different concentrations of EFH and non-treated cells ($P<0.05$ for all). The apoptosis rate of Hep-G2 cells was enhanced with the increasing concentration of EFH over time (table 1).

2.2 Effect of EFH on the Tumor Growth

Two weeks after the treatment, the TV and the growth rate were significantly decreased and the necrosis rate of the tumors increased in group C as compared with those in groups A and B ($P<0.05$) (table 2).

2.3 Immunohistochemical Detection

The group C had the lowest expressions of VEGF, P53 and Bcl-2 and the strongest expression of Bax when compared with groups B, with the differences being significant ($P<0.05$ for all) (table 3, fig. 1A–1J). MVD was significantly decreased in group C in comparison to that in groups B ($P<0.05$).

Table 1 Apoptosis rate of Hep-G2 cells (%, $\bar{x}\pm s$)

Groups	Concentration (mg/mL)	Apoptosis rate		
		24 h	48 h	72 h
Control	0	4.186±0.655	9.353±0.596	13.306±1.233
EFH	1	6.456±0.640*	12.653±0.701*	18.530±1.384*
	2	8.750±0.575*	21.546±1.277*	25.176±1.136*
	4	10.440±0.730*	28.386±1.316*	33.410±1.223*
	8	13.703±0.755*	34.456±1.423*	37.443±1.370*

*$P<0.05$ as compared with control group

Table 2 Tumor volume, tumor growth rate and tumor necrotic rate in different groups ($\bar{x}\pm s$)

Groups	Tumor volume (cm^3)		Tumor growth rate (%)	Tumor necrotic rate (%)
	1 day before TACE	14 day after TACE		
A	0.888±0.129	4.906±0.563	556.02±41.64	35.11±7.98
B	0.875±0.136	1.586±0.572	177.47±43.53	56.67±14.61
C	0.863±0.134	1.028±0.524*	114.97±40.59*	68.50±13.07*

*P<0.05 as compared with groups A and B

Table 3 The expression of VEGF, P53, Bax and Bcl-2 and the MVD values

Groups	MVD ($\bar{x}\pm s$)	VEGF		P53		Bax		Bcl-2	
		+	Positive rate (%)	+	Positive rate (%)	+	Positive rate (%)	+	Positive rate (%)
A	54.68±21.36	6	50.00	11	91.67	2	16.67	10	83.33
B	75.29±23.27	11	91.67	8	66.67	5	41.67	8	66.67
C	45.95±23.80*	5	41.67*	3	25.00*	10	83.33*	2	16.67*

*P<0.05 as compared with group B

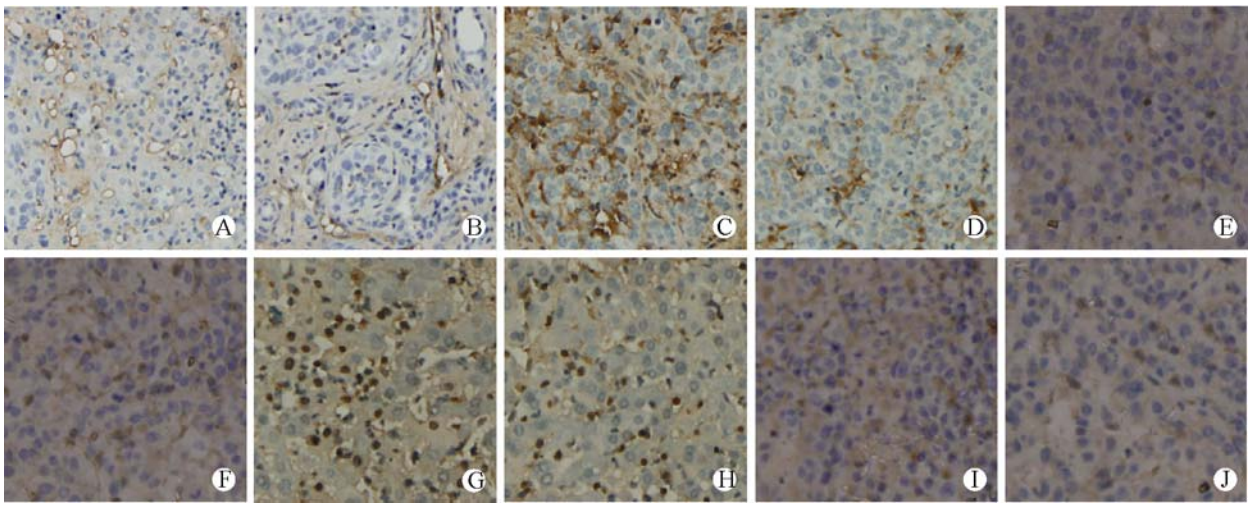

Fig. 1 Expression of factor Ⅷ, VEGF, Bax, P53, Bcl-2 in group B (A, C, E, G, I) and group C (B, D, F, H, J) in tumor tissues (×400)

3 DISCUSSION

Huaier, an important medicinal fungus, was extensively used for the treatment of cancer and inflammation in the practice of Chinese traditional medicine (CTM). EFH is extracted from *Huaier* and its main ingredient is polysaccharide protein which has been proved to be the main active ingredient of *Huaier* in the respect of anti-cancer effects and immunity-enhancing actions. As an anti-cancer drug, EFH has been used for the treatment of liver cancer with satisfactory results. Mounting evidence suggest that the anti-tumor mechanism of EFH may be associated with inhibition of the proliferation of endothelial cells, interference with tumor angiogenesis[8], induction of tumor cell apoptosis and suppression of tumor cell proliferation[9].

The tumorigenesis is mainly ascribed to the abnormal cell proliferation and dysfunction of cell death. Tumor cells thrive and local mass develops. Apoptosis is known as an active programmed cell death induced by some *in vitro* and *in vivo* factors, and plays a vital role in tumor development. It has been demonstrated that many drugs can treat cancer by inhibiting the tumor growth and inducing the apoptosis of the tumor cells[10–12]. In this study, we found that after treatment of TACE and EFH, the necrosis rate was increased and the TV and the growth rate were decreased significantly, suggesting that EFH can enhance the effect of intervention therapy by suppressing the tumor growth and stimulating the tumor necrosis.

There are many apoptosis-related genes under the physiopathological circumstances. Among them, P53, Bax and Bcl-2 are important genes intimately related to the apoptosis of HCC cells. P53 gene is categorized as wild-type and mutant-type. The wild-type P53, a tumor suppressor gene, can efficiently inhibit the proliferation of tumor cells and induce tumor cell apoptosis. The mutant-type P53 gene can inactivate the function of the former, resulting in cell differentiation and abnormal cell proliferation, and consequently the development of many malignant tumors. More than 50% of the human tumors have P53 gene mutation. Bax is a pro-apoptosis member of Bcl-2 family. Bax combines with Bcl-2 to form a dipolymer to inactivate Bcl-2 gene, and its over-expression promotes apoptosis[13, 14]. The over-expression of Bcl-2 gene can not only promote the development of tumor but also adversely affect the efficacy of tumor therapies[15, 16]. Our study showed that the expressions of P53 and Bcl-2

were significantly decreased and Bax expression was increased in group C as compared with those in groups A and B, suggesting that the EFH can induce tumor cell apoptosis by reducing the P53 and Bcl-2 expressions and enhancing the Bax expression.

Tumor angiogenesis, which plays an important part in the development of tumor, is regulated by angiogenic factors, which were generated by tumor cells *per se* and inflammatory cells[17]. Among numerous pro-angiogenic factors, VEGF is intensively studied, which has been found to be the most important and directly-acting factor that can induce tumor angiogenesis and play a critical role in the development of HCC[18, 19]. Factor Ⅷ-related antigen is a serum glucoprotein, which is expressed in endothelial cells of blood vessels, platelet and lymphatic endothelia cells and is usually used for the diagnosis of angiogenic tumors[20, 21]. In this study, factor Ⅷ-related antigen was used as an index for the evaluation of MVD. We found in the current study that, in the group C (TACE + EFH group), VEGF and MVD expressions were significantly decreased when compared with those in groups B, suggesting that EFH can treat HCC by inhibiting the VEGF expression and tumor angiogenesis.

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