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The Effect of Targeted Magnetic Nanopaticles on Hepatoma and the Expression of bcl-2/bax Protein*

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Summary: The effect of targeted magnetic nanoparticles on hepatoma and the underlying mechanism were examined. Nude mice transplanted with a human hepatoma cell line (HepG2 cells) were randomized into 5 groups, including: (1) group A, receiving normal saline, (2) group B, receiving 5-fluorouracil (5-Fu), (3) group C, receiving magnetic nanoparticles containing 5-Fu, (4) group D, consisting of treatment with magnetic nanoparticles containing 5-Fu and inside magnetic field and (5) group E, receiving pure magnetic nanoparticles and inside magnetic field. Morphological features of transplanted tumors in mice in each group were observed under transmission electron microscope (TEM). The expression of bcl-2/bax protein was immunohistochemically detected by SABC method. The results showed that a large number of apoptotic tumor cells were found in group B and group D under TEM. The expression of bcl-2 protein was significantly decreased and the expression of bax protein increased significantly in both group B and D as compared with those in group A, C and E (P<0.01 for all). The decrease in bcl-2 and the increase in bax were more in group D as compared with group B (P<0.01). It is concluded that the targeted magnetic nanoparticles containing 5-Fu can improve the chemotherapeutic effect of 5-Fu by decreasing bcl-2 expression, increasing bax expression and inducing apoptosis of the liver cancer cells.
Key words: liver neoplasms; cell line; bcl-2; bax; magnetic nanoparticles, 5-Fu

Magnetic nanoparticles containing chemotherapeutical agents provide a new treatment regimen for cancer. The targeted delivery of drugs can be controlled *in vivo* by using an external magnet to confine the magnetic carrier to the target site. Meanwhile, nanoparticles can move out of the endothelium of blood vessel, and then be endocytosed by the tumor cells. Using magnetic nanoparticles can improve the efficacy of some chemotherapeutics and alleviate their adverse effects on important organs[1]. In this study, magnetic nanoparticles containing 5-fluorouracil (5-Fu) were used for the treatment of transplanted liver cancer in nude mice and the effect of targeted magnetic nanoparticles containing 5-Fu on expression of bcl-2/bax protein in transplanted liver cancer cells in nude mice was studied.

1 MATERIALS AND METHODS

1.1 Chemicals and Reagents

Magnetic nanoparticles containing 5-Fu (10.1±1.2)% and pure magnetic nanoparticles were prepared by School of Pharmaceutics, Tongji Medical College, Huazhong University of Science and Technology (HUST), Wuhan, China. Magnetic metal stent for medicine was provided by Department of Material, Wuhan University of Technology, Wuhan, China. Culture media (RPMI-1640) were products of Gibco Co. Ltd., USA. Calf serum was purchased from Hangzhou Sijiqing Co. Ltd., China. Rabbit polyclonal antibody against human bcl-2 and bax protein and goat anti-rabbit polyclonal antibody were procured from Wuhan Boster Biological Technology Co. Ltd., Wuhan, China.

1.2 Cell line and Preparation

HepG2 cells were provided by the Laboratory of the Department of Surgery, Tongji Hospital, Wuhan, China and cultured in the RPMI 1640 media supplemented with 100 mL/L heat-inactivated calf serum, penicillin G (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 50 mL/L CO_2.

1.3 Animals

Forty male BALB/c nude mice, aged 3–5 weeks and weighing 17–20 g, were from the Experimental Animal Center, Tongji Medical College, HUST, Wuhan, China and were housed under specific pathogen-free conditions.

1.4 Tumor Induction in Nude Mice

HepG2 cells were grown in monolayer. The exponentially growing HepG2 cells in culture flasks were harvested, and adjusted to the concentration of 5×10^7/mL. Cell solution of 0.2 mL was injected subcutaneously into the right back of each nude mouse. Fourteen days later, when the tumor volume was about 130 mm^3, these mice were randomly divided into 5 groups in terms of different treatments (table 1). Nude mice in each group were administered the same volume of 0.2 mL of the treatment agents or normal saline by vena caudalis shot, respectively, once a day for 5 days.

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Table 1 The grouping and management of experimental animal

Groups	*n*	Treatments
A	8	Normal sodium
B	8	5-FU, 25 mg/kg
C	8	Magnetic nanoparticles containing 5-Fu, 250 mg/kg
D	8	Magnetic nanoparticles containing 5-Fu, 250mg/kg, and magnetic field with 300 gauss built inside tumor tissue
E	8	Magnetic nanoparticles without 5-Fu, 250mg/kg, and magnetic field with 300 gauss built inside tumor tissue

1.5 Tumor Assessment

The tumor was measured with a sliding caliper for maximal diameter (a) and minimal diameter (b) on the day before the treatment and on the 1st, 4th, 7th, 10th and 13th day after the treatment, and tumor volume was calculated by using the following formula: Tumor volume=a×b×b/2. All animals were killed and underwent complete examination of abdominal cavity on the 20th day after treatment. The tumor mass was then isolated.

1.6 Immunohistochemistry

Bcl-2 and bax proteins were detected by using SABC method. Tumor specimens were incubated with 3 mL/L hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity, washed with PBS and incubated in 100 mL/L normal goat serum for 20 min to reduce non-specific antibody binding. Thereafter, the specimens were incubated with a 1:100 dilution of rabbit polyclonal antibody against human bcl-2 or bax protein overnight at 4°C, washed 3 times with PBS, then incubated with biotinylated goat anti-rabbit polyclonal antibody at a dilution of 1:100 for 30 min, which was followed by washing 3 times. Afterwards, the slides were treated with streptavidinperoxidase reagent (dilution: 1/100) for 30 min and washed with PBS 3 times. In the end, the slides were incubated in phosphate-buffered saline containing diaminobenzidine and 10 mL/L hydrogen peroxide for 10 min, counterstained with hematine, and then mounted. For blank control, the rabbit antibody was replaced by PBS. Five high-power fields of each section were selected randomly and examined for staining intensity by using HM IAS-2000 image analysis system (Champion Co., China).

1.7 Statistical Analysis

Data were expressed as $\bar{x}\pm s$. Analysis of variance (ANOVA) was employed to compare the tumor volume among groups. The one-way ANOVA was used for the analysis of the expression of bcl-2 and bax in the 5 groups. All data were analyzed by utilizing SPSS 12.0 software package and a $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Comparison of Gross Tumor Volume

Fourteen days after subcutaneous injection, transplanted liver cancer masses with a volume of about 130 mm^3 were developed in each nude mice in all groups. As shown in table 2, the tumor shrank significantly in group B and group D as compared with that in group A, C and E ($P<0.01$) and the average tumor size was smaller in group B than in group D ($P<0.05$). No statistical differences in tumor volume were found among groups A, C and E ($P>0.05$).

Table 2 The comparison of gross tumor volume (mm^3, $\bar{x}\pm s$)

Group	*n*	Volume					
		Day 0	Day 1	Day 4	Day 7	Day 10	Day 13
A	8	131.9±4.3	261.0±29.7	362.0±52.9	513.1±67.3	643.9±83.4	776.1±116.2
B	8	134.4±4.7	228.7±26.7	294.8±31.6	364.9±34.5	416.9±64.4	465.0±91.1
C	8	133.7±8.3	253.0±27.8	341.5±34.6	473.4±53.2	600.2±72.4	764.7±100.6
D	8	135.8±6.8	213.0±18.1	261.5±22.0	315.5±27.6	337.2±32.6	367.8±44.0
E	8	136.9±7.2	250.3±31.0	343.1±46.4	500.6±66.2	644.3±75.5	774.4±93.4

2.2 TEM Observation

The tumor cells were irregularly-shaped with microvilli on the surface in group A, C and E under TEM. Excessive amount of endoplasmic reticula and mitochondria were found in the cytoplasm of these cells. The pathological karyokinesis and nuclear distortion were frequently seen and the nucleocytoplasmic ratio was increased. However, in groups B and D, especially in group D, few aberrant cells were found and the cell nuclei became round and small. The microvilli were markedly decreased. The intact cell membrane, nuclear condensation and apoptotic bodies were noted (fig. 1).

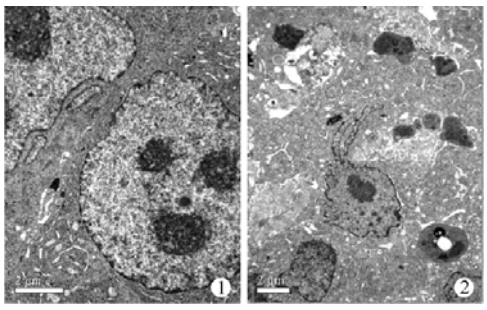

Fig. 1 TEM finding of tumor cells in group A (NS) and group D. 1: Group A; 2: Group D

2.3 Expression of bcl-2/bax Protein

The expression of bcl-2/bax protein was observed in tumor cells (fig. 2, 3). Table 3 showed that the expression of bcl-2 protein was markedly decreased whereas that of bax protein was significantly increased in group B and group D as compared with those in group A, C and E (P<0.01). The decrease in bcl-2 protein and the increase in bax protein were more in group D than in group B (P<0.01).

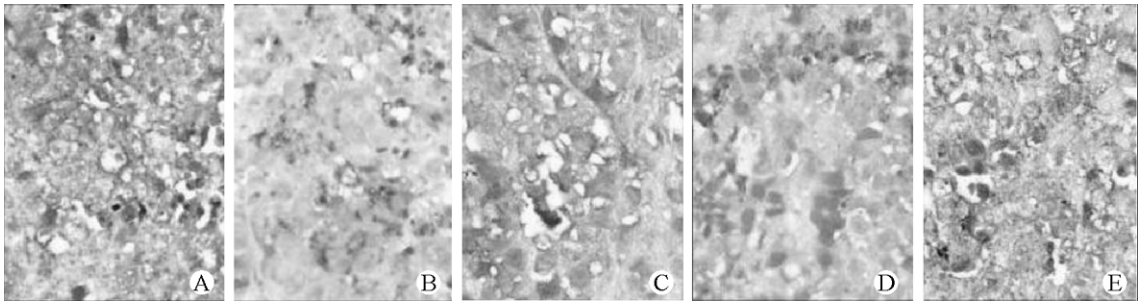

Fig. 2 Expression of bcl-2 protein (SABC×400)
A: Group A; B: group B; C: Group C; D: Gropu D; E: Group E

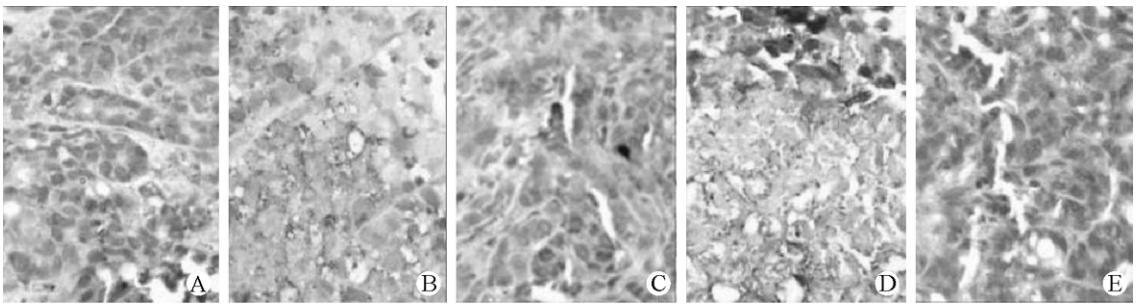

Fig. 3 Expression of bax protein (SABC×400)
A: Group A; B: group B; C: Group C; D: Gropu D; E: Group E

Table 3 Intensity of staining of bcl-2/bax protein ($\bar{x}\pm s$)

Groups	n	bcl-2	bax
A	8	0.1185±0.0111	0.1031±0.0119
B	8	0.0981±0.0121$^{\triangle}$	0.1375±0.0084$^{\triangle}$
C	8	0.1161±0.0094	0.1090±0.0141
D	8	0.0768±0.0119$^{\triangle\blacktriangledown}$	0.1587±0.0086$^{\triangle\blacktriangledown}$
E	8	0.1169±0.0105	0.1062±0.0154

$^{\triangle}P$<0.01 vs group A; $^{\blacktriangledown}P$<0.01 vs group B

3 DISCUSSION

Hepatocellular carcinoma (HCC) is a serious problem in developing countries, which makes up 81% of the total cases in the world. In China, the HCC patients account for 54% of the total number of the disease worldwide[2]. Even after curative resection of small HCC, the recurrent rate 5 years after operation remained as high as 40%–60%[3-5]. Furthermore, these tumors have been shown to be quite resistant to radiotherapy or chemotherapy[6]. The effective postoperative adjuvant chemotherapeutical agents have yet to be developed.

Nowadays the main chemotherapeutics for liver cancer were 5-FU, cis-diamminedichloroplatinum (CDDP), adriamycin (ADM), mitomycin (MMC) etc.[7-9]. The newly developed antineoplastic agents such as capecitabine[10] are expected to improve the therapeutic efficacy of liver cancer. However, the cost of the treatment is high and the therapeutic effect is still under investigation. The patients with liver cancer tended to be complicated with liver cirrhosis and chronic hepatitis. Moreover, all the chemotherapeutics have adverse effects, thus most patients have poor tolerance to chemotherapy[11]. Magnetic drug-coated nanoparticle provides a new alternative to the treatment of cancer. Under guidance of magnetic field, the distribution of magnetic nanoparticles containing drugs could improve the therapeutic efficacy of tumor treatment due to increased concentration of drugs in tumor tissue and dramatically minimize side effects [12,13].

Using magnetic nanoparticles containing 5-Fu under guidance of magnetic field built inside tumor tissue to treat transplanted liver cancer in nude mice can selectively target the tumor tissue with large dose. This study indicated that targeted magnetic nanoparticles containing 5-Fu under the guidance of the inside magnetic field could inhibit the growth of hepatocellular carcinoma and reduce tumor size, suggesting a strong anti-cancer effect.

Previous studies revealed that the anti-tumor effect of 5-FU, CDDP and MMC was positively related with apoptosis induction[14-16]. Apoptosis is a form of programmed cell death regulated by some gene or other factors[17]. Escape from apoptosis is associated with tu-

mor progression. Apoptosis-related genes fall into two categories: suppressor gene and trigger gene. Bcl-2, bcl-XL and mcl-1 are the main suppressor genes of apoptosis[18,19]. Bax, bcl-XS, p53 and c-myc are the main trigger gene of apoptosis[20,21]. Human bcl-2 gene is a proto-oncogene of tumor of hematopoietic system and is located on chromosome 18. Bcl-2 gene shows a depressant effect on apoptosis induced by many other factors[22,23]. Bax gene encodes 21 kD protein, and has 21% homology with bcl-2 gene. Overexpression of bax gene can inhibit the bcl-2 and promote apoptosis[24].

Our study showed that the targeted therapy of magnetic nanoparticles containing 5-Fu could decrease the expression of bcl-2 and increase the expression of bax, and there was a significant difference in the bcl-2/bax expression between group B (receiving 5-Fu) and group D (receiving magnetic nanoparticles containing 5-Fu in inside magnetic field) (P<0.01), indicating that the targeted therapy of magnetic nanoparticles containing 5-Fu can inhibit the growth of hepatocellular carcinoma and up-regulate apoptosis of the liver cancer cells by decreasing bcl-2expression and increasing bax expression. The efficacy of targeted therapy of magnetic nanoparticles containing 5-Fu is greater than 5-Fu only.

The targeted therapy of magnetic nanoparticles containing 5-Fu can selectively deliver 5-Fu to tumor tissue[1] and maintain a high concentration of the agent in tumor tissue for a long time[1,12]. Also, the concentrated nanoparticles can induce embolism in blood vessel. In addition, it causes ischemia and anoxyaemia of tumor tissue and increases the sensitivity of tumor to chemotherapy[13].

In conclusion, the targeted therapy of magnetic nanoparticle containing 5-Fu decreases the expression of bcl-2 gene, increases the expression of bax gene, thereby up-regulating apoptosis of the liver cancer cells and inhibiting the growth of hepatocellular carcinoma.

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