



Effect of targeted magnetic nanoparticles containing 5-FU on expression of *bcl-2*, *bax* and *caspase 3* in nude mice with transplanted human liver cancer

Jian-Ming Wang, Bao-Lai Xiao, Jian-Wei Zheng, Hai-Bing Chen, Sheng-Quan Zou

Jian-Ming Wang, Bao-Lai Xiao, Jian-Wei Zheng, Sheng-Quan Zou, Department of General Surgery of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Hai-Bing Chen, Department of Material, Wuhan University of Technology, Wuhan 430030, Hubei Province, China
Supported by the Hi-Tech Research and Development Program of China, NO.2002AA214061

Correspondence to: Sheng-Quan Zou, Professor, Department of General Surgery, Tongji Medical College of Huazhong University of Science and Technology, Hangkong Road, Wuhan 430030, Hubei Province, China. wjm18jgm@yahoo.com.cn
Telephone: +86-27-83663814 Fax: +86-27-83662398
Received: 2006-12-25 Accepted: 2007-02-08

the expression of *bax* and *caspase 3* genes.

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Key words: Hepatocellular carcinoma; Magnetic nanoparticles; Fluorouracil; *Bcl-2*; *Bax*; *Caspase 3*

Wang JM, Xiao BL, Zheng JW, Chen HB, Zou SQ. Effect of targeted magnetic nanoparticles containing 5-FU on expression of *bcl-2*, *bax* and *caspase 3* in nude mice with transplanted human liver cancer. *World J Gastroenterol* 2007; 13(23): 3171-3175

<http://www.wjgnet.com/1007-9327/13/3171.asp>

Abstract

AIM: To investigate the anti-tumor effect and mechanisms of magnetic nanoparticles targeting hepatocellular carcinoma.

METHODS: Human hepatocellular carcinoma was induced in nude mice, and the mice were randomly divided into group A receiving normal saline, group B receiving magnetic nanoparticles containing 5-fluorouracil (5-FU), group C receiving 5-FU, and group D receiving magnetic nanoparticles containing 5-FU with a magnetic field built in tumor tissues. The tumor volume was measured on the day before treatment and 1, 4, 7, 10 and 13 d after treatment. Tumor tissues were isolated for examination of the expression of *bcl-2*, *bax* and *caspase 3* by immunohistochemical method, reverse transcription polymerase chain reaction and Western blotting.

RESULTS: The tumor volume was markedly lower in groups C and D than in groups A and B (group C or D *vs* group A or B, $P < 0.01$). The volume was markedly lower in group D than in group C ($P < 0.05$). The expression of protein and mRNA of *bcl-2* was markedly lower in groups C and D than in groups A and B (group C or D *vs* group A or B, $P < 0.01$), and was markedly lower in group D than in group C ($P < 0.01$). The expression of *bax* and *caspase 3* in groups C and D was significantly increased, compared with that in groups A and B ($P < 0.01$).

CONCLUSION: The targeted magnetic nanoparticles containing 5-FU can improve the chemotherapeutic effect of 5-FU against hepatocellular carcinoma by decreasing the expression of *bcl-2* gene, and increasing

INTRODUCTION

Targeted magnetic chemotherapy can selectively focus chemotherapeutics on tumor tissue when a magnetic field is used to confine the magnetic drug carrier to the target site^[1-3]. Targeted drug delivery is an attractive field in tumor therapy^[4]. In our experiment, nanochemotherapy was used in nude mice with transplanted human liver cancer and the anti-tumor effect and mechanism of targeted magnetic nanoparticles were studied by examining the expression of *bcl-2*, *bax* and *caspase 3* in tumor tissue.

MATERIALS AND METHODS

Chemicals and reagents

Magnetic nanoparticles containing 5-fluorouracil (5-FU, 10.1% \pm 1.2%) were prepared by the Pharmaceutical Department of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Magnetic metal rack for medication was prepared by Department of Material, Wuhan University of Technology (Wuhan, China). Culture media (RPMI-1640) were obtained from Gibco Co., Ltd, USA. Calf serum was purchased from Hangzhou Sijiqing Co., Ltd, China. Rabbit polyclonal antibodies against human *bcl-2*, *bax*, *caspase 3* and β -actin protein, and goat anti-rabbit polyclonal antibody were purchased from Wuhan Boster Biological Technology Co., Ltd, China.

Cell line and preparation

Cells of HepG2, a primary human hepatocellular

carcinoma cell line, were provided by the Surgical Laboratory of Tongji Hospital. All media were supplemented with 100 mL/L, heat-inactivated calf serum, penicillin G (100 IU/mL), and streptomycin (100 µg/mL). The cells were incubated as a monolayer in RPMI 1640 at 37°C in a humidified atmosphere containing 50 mL/L CO₂.

Animals

Thirty-two BALB/C male nude mice weighing 17-20 g at 3-5 wk of age, provided by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, were used in this study. The study protocol was approved by the Animal Ethics Committee of Huazhong University of Science and Technology.

Tumor induction in nude mice

HepG2 cells were grown in monolayer culture, harvested and adjusted to 5×10^7 cells/mL. For subcutaneous tumor formation, 0.2 mL of cell solution was injected subcutaneously into the right back of each nude mouse. After 14 d, when the volume of tumor was about 130 mm³, the mice were randomly divided into group A receiving normal saline, group B receiving magnetic nanoparticles containing 250 mg/kg 5-FU, group C receiving 25 mg/kg 5-FU, group D receiving magnetic nanoparticles containing 250 mg/kg 5-FU, with a magnetic field at 300 gauss built in tumor tissues. Each group was treated with the same volume (0.2 mL) by vena caudalis injection, once a day for 5 d.

Tumor assessment

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

Immunohistochemistry

Bcl-2, *bax* and *caspase 3* were detected with immunohistochemical (SABC) method. In brief, tumor specimens were incubated with 3 mL/L hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity, washed in phosphate buffered saline (PBS) and incubated in 100 mL/L normal goat serum for 20 min to reduce nonspecific antibody binding. Specimens were then incubated with rabbit polyclonal antibodies at a dilution of 1:100 against human *bcl-2*, *bax* or *caspase 3* overnight at 4°C, followed by three washes with PBS, then incubated with biotinylated goat anti-rabbit polyclonal antibody at a dilution of 1:100 for 30 min followed by 3 washes. Slides were treated with streptavidin peroxidase reagent for 30 min at a dilution of 1:100 and washed 3 times with PBS. Finally, slides were incubated in PBS containing diaminobenzidine and 10 L/L hydrogen peroxide for 10 min, counterstained with hematine and mounted. The rabbit antibody was replaced by PBS as a blank. Five high power fields of each section were selected randomly and

input to the HM IAS-2000 analysis system for staining intensity (average absorbance) analysis.

Reverse transcription polymerase chain reaction (RT-PCR)

The tumor mass was isolated and total RNA was extracted with TRIzol (GIBCO, Gran Island, NY, USA). The sequence of primers (primer 5.0, Shanghai Sangon Biological Engineering & Technology and Service Co.Ltd, Shanghai, China) used is: *bcl-2* (455 bp): sense: 5'-GGCACTGTCTTGACCCAC-3' and antisense: 5'-TCATAAACCCCTGCTTGCTG-3'; *bax* (121 bp): sense: 5'-AATGCCCCGTTCATCTCAG-3' and antisense: 5'-GGGACATCAGTCGCTTCA-3'; *caspase 3* (445 bp): sense: 5'-CACAATAGCACCCATCCG-3' and antisense: 5'-GGGACATCAGTCGCTTCA-3'; β -actin (550 bp): sense: 5'-GGTGGGGCGCCC CAGGCACCA-3' and antisense: 5'-GCTCCTTAATGT CACGCACGA-3'. The following PCR conditions were used: initiation at 94°C for 4 min, then 35 cycles of denaturing at 94°C for 45 s, annealing at 48°C (*bcl-2*, and *bax* for 45 s, at 50°C (*caspase 3*) for 45 s or at 52°C (β -actin) for 45 s, extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining using Gel-Pro analyzer. The bands were quantitated by densitometry and the gene expression was represented as the ratio of target mRNA in comparison to that of β -actin.

Western blot analysis

Western blotting was used for the detection of *bcl-2*, *bax* and *caspase 3* proteins. On day 20 after treatment, 0.1 g tumor tissue was collected from each mouse and homogenized. Tissues were lysed in a lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% deoxycholic acid, 1% NP40, 0.1% SDS, 1 mmol/L PMSF, and 100 µg/mL leupeptin. Protein concentration was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, California, USA). A 50 µg sample of protein was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Rabbit anti-*bcl-2* antibody (1:500), rabbit anti-*bax* antibody (1:500), rabbit anti-*caspase 3* antibody (1:500) or rabbit anti- β -actin antibody (1:500) was used respectively as the primary antibody. Horseradish peroxidase-conjugated anti-rabbit antibody (1:1000) was used to probe for *bcl-2*, *bax*, *caspase 3* or β -actin as the secondary antibody. The band was detected using the enhanced chemiluminescence (ECL) detection system. The protein expression was represented as the optical density ratio of the interest protein in comparison to that of β -actin.

Statistical analysis

Data were presented as mean \pm SD. Repetitive analyses of variance (ANOVA) were performed for comparison of tumor volume among treatment groups. One-way ANOVA was performed for comparison of the expression levels of *bcl-2*, *bax* and *caspase 3* among groups. All data were analyzed by SPSS 12.0, and $P < 0.05$ was considered statistically significant.

Table 1 Comparison of gross tumor volume (mm³, mean \pm SD)

Group	n	0	1d	4d	7d	10d	13d
A	8	131.9 \pm 4.3	261.0 \pm 29.7	362.0 \pm 52.9	513.1 \pm 67.3	643.9 \pm 83.4	776.1 \pm 116.2
B	8	133.7 \pm 8.3	253.0 \pm 27.8	341.5 \pm 34.6	473.4 \pm 53.2	600.2 \pm 72.4	764.7 \pm 100.6
C ^a	8	134.4 \pm 4.7	228.7 \pm 26.7	294.8 \pm 31.6	364.9 \pm 34.5	416.9 \pm 64.4	465.0 \pm 91.1
D ^{a,b}	8	135.8 \pm 6.8	213.0 \pm 18.1	261.5 \pm 22.0	315.5 \pm 27.6	337.2 \pm 32.6	367.8 \pm 44.0

^a $P < 0.01$ vs groups A and B; ^b $P < 0.05$ vs group C.

Table 2 Intensity of staining of bcl-2, bax and caspase 3 proteins (mean \pm SD)

Group	n	Bcl-2	Bax	Caspase 3
A	8	0.1185 \pm 0.0111	0.1031 \pm 0.0119	0.0806 \pm 0.0073
B	8	0.1161 \pm 0.0094	0.1090 \pm 0.0141	0.1067 \pm 0.0075
C	8	0.0981 \pm 0.0121 ^a	0.1375 \pm 0.0084 ^b	0.1284 \pm 0.0074 ^b
D	8	0.0768 \pm 0.0119 ^{a,b}	0.1587 \pm 0.0086 ^{a,b}	0.1468 \pm 0.0080 ^{a,b}

^a $P < 0.01$ vs groups A and B; ^b $P < 0.01$ vs group C.

RESULTS

All models of nude mice with transplanted hepatocellular carcinoma were established 14 d after subcutaneous injection, with an average volume of 130 mm³. The rate of subcutaneous tumor formation was 100%. There was no significant difference in the average tumor volume between the groups of A and B. The average tumor volume was smaller in groups C and D than in groups A and B ($P < 0.01$). Also, the volume was smaller in group D than in group C ($P < 0.05$) (Table 1).

Expression of bcl-2, bax and caspase 3 proteins

The expression of *bcl-2*, *bax* and *caspase 3* proteins observed under microscope was positive. The distribution of the Buffy particles was diffuse. The expression of *bcl-2* protein was markedly decreased, while that of *bax* and *caspase 3* proteins was markedly increased in groups C and D, in comparison with groups A and B ($P < 0.01$). The expression of *bcl-2* protein was markedly decreased whereas that of *bax* and *caspase 3* proteins was markedly increased in group D compared with group C ($P < 0.01$) (Table 2, Figure 1).

mRNA expression in bcl-2, bax and caspase 3 by PCR

The mRNA expression in *bcl-2* was lower in groups C and D than in groups A and B ($P < 0.01$), and was lower in group D than in group C ($P < 0.01$). The mRNA expression in *bax* and *caspase 3* was higher in groups C and D than in groups A and B ($P < 0.01$), and was markedly higher in group D than in group C ($P < 0.01$, Table 3, Figure 2A).

Western blot analysis

Western blot was used for examining the expression of *bcl-2*, *bax* and *caspase 3* proteins. The expression of *bcl-2* was lower in groups C and D than in groups A and B ($P < 0.01$), and was markedly lower in group D than in group C ($P < 0.01$). The expression of *bax* and *caspase 3* proteins was higher in groups C and D than in groups A and B

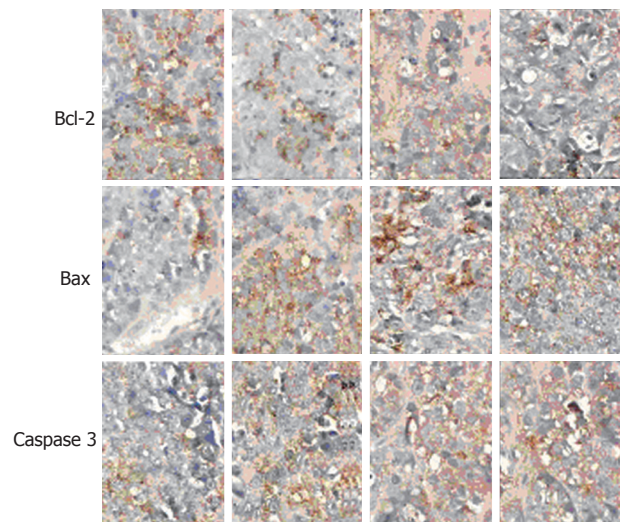


Figure 1 Expression of Bcl-2, Bax and Caspase 3 proteins in 4 groups, SABC $\times 400$.

($P < 0.01$), and markedly higher in group D than in group C ($P < 0.01$, Table 4, Figure 2B).

DISCUSSION

Hepatocellular carcinoma (HCC) is a serious problem in developing countries, accounting for 81% of the total cases in the world and 54% of the total cases in China^[5]. Even 5 years after curative resection of small HCC, the recurrent rate is as high as 40%-60%^[6,7]. Furthermore, these tumors are quite resistant to radiotherapy and chemotherapy^[8]. No effective postoperative adjuvant chemotherapeutic agent is available so far.

At present, the main chemotherapeutics of HCC are 5-FU, cis-diaminedichloroplatinum (CDDP), adriamycin (ADM), mitomycin (MMC)^[9-11]. The newly developed antineoplastic agents, such as capecitabine^[12], are expected to increase the therapeutic effect on liver cancer. However, the cost of treatment is expensive and the therapeutic effect is uncertain, indicating that more clinical data and trials are needed. Patients with HCC have complications of liver cirrhosis and chronic hepatitis. Since almost all chemotherapeutics have side effects, the tolerance of patients to chemotherapy is usually poor^[13]. Magnetic drug-coated nanoparticles provide a new method for the treatment of cancer^[14]. Under the guidance of magnetic fields to the tumor, the target distribution of magnetic nanoparticles containing drugs can increase the therapeutic effect on the tumor by increasing the concentration of drugs, both in tumor tissue and in tumor cell embolism in tumor blood vessels^[15,16], and by

Table 3 mRNA expression in *bcl-2*, *bax* and *caspase 3* (mean \pm SD)

Group	Bcl-2	Bax	Caspase 3
A	0.557 \pm 0.064	0.136 \pm 0.012	0.134 \pm 0.012
B	0.524 \pm 0.049	0.187 \pm 0.019	0.226 \pm 0.028
C	0.275 \pm 0.025 ^a	0.681 \pm 0.057 ^a	0.425 \pm 0.036 ^a
D	0.154 \pm 0.014 ^{a,b}	0.821 \pm 0.065 ^{a,b}	0.557 \pm 0.039 ^{a,b}

^a*P* < 0.01 vs group A; ^b*P* < 0.01 vs group C.**Table 4** Expression of *bcl-2*, *bax* and *caspase 3* proteins (mean \pm SD)

Group	Bcl-2	Bax	Caspase3
A	0.415 \pm 0.052	0.086 \pm 0.012	0.062 \pm 0.006
B	0.402 \pm 0.048	0.182 \pm 0.028	0.145 \pm 0.017
C	0.216 \pm 0.033 ^a	0.382 \pm 0.044 ^a	0.409 \pm 0.024 ^a
D	0.156 \pm 0.018 ^{a,b}	0.508 \pm 0.058 ^{a,b}	0.628 \pm 0.033 ^{a,b}

^a*P* < 0.01 vs group A; ^b*P* < 0.01 vs group C.

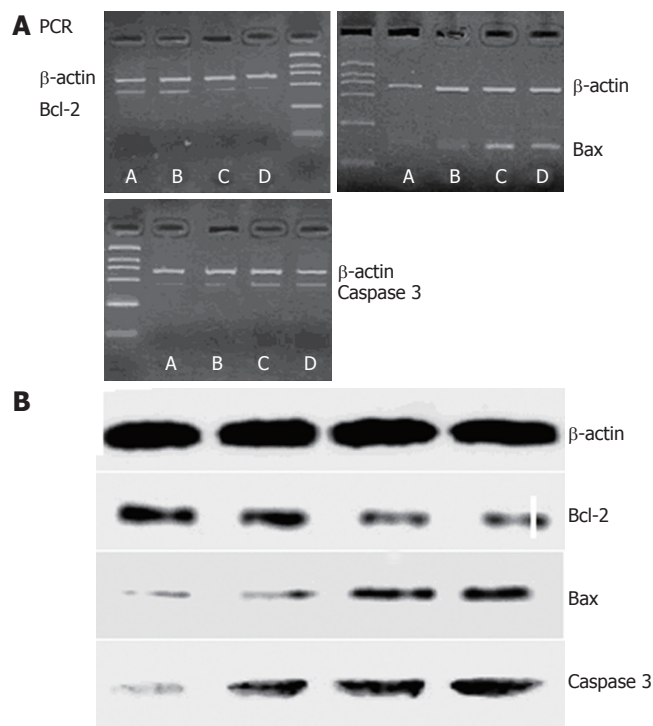
reducing their side effects^[17,18].

In the present study, magnetic nanoparticles containing 5-FU with a magnetic field built in tumor tissues could selectively act on the transplanted liver cancer in nude mice, indicating that targeted therapy with magnetic nanoparticles containing 5-FU inhibits the growth of hepatocellular carcinoma (*P* < 0.05).

Experimental studies have shown that the antitumor effect of 5-FU, CDDP and MMC is correlated to the induction of apoptotic cells^[19]. Apoptosis is regulated by some genes or other factors^[20]. Escape from apoptotic signals often accompanies tumor progression. Genes controlling apoptosis can be divided into suppressor gene or trigger gene. *Bcl-2*, *bcl-X_L* and *mcl-1* are the main suppressor genes of apoptosis, and *bcl-2* is the most important. *Bax*, *bcl-X_S*, *p53* and *c-myc* are the main trigger genes of apoptosis^[21,22]. Human *bcl-2* gene, located on chromosome 18, is a proto-oncogene in the hematopoietic system. *Bcl-2* gene suppresses apoptosis induced by many other factors^[23,24] and *bax* gene encodes a 21 kD protein, and has 21% homology with *bcl-2* gene. Over-expression of *bax* gene can inhibit the function of *bcl-2* and promote apoptosis^[25]. Caspases are crucial mediators for apoptosis. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. Caspase-3 may be the best understood mammalian caspases in terms of its specificity and role in apoptosis^[26,27].

Our study showed that the targeted therapy with magnetic nanoparticles containing 5-FU could decrease the expression of *bcl-2*, and increase the expression of *bax* and *caspase 3* (*P* < 0.01), indicating that this therapy can inhibit the growth of hepatocellular carcinoma and up-regulate apoptosis of liver cancer cells by decreasing the expression of *bcl-2* gene and increasing the expression of *bax* and *caspase 3* genes.

The targeted therapy with magnetic nanoparticles containing 5-FU can selectively make 5-FU focus on tumor tissue, with a high concentration in tumor tissues. Also, concentrated nanoparticles can lead to embolism in tumor

**Figure 2** mRNA expression in *Bcl-2*, *Bax* and *Caspase 3* determined by PCR (A) and Western blotting (B).

blood vessels, ischemia and anoxiaemia in tumor tissues, and a higher sensitivity of chemotherapy. Moreover, this therapy could decrease the expression of *bcl-2* gene, increase the expression of *bax* and *caspase 3* genes, suggesting that it up-regulates apoptosis of liver cancer cells and inhibits the growth of hepatocellular carcinoma.

In conclusion, targeted magnetic nanoparticles containing 5-FU can improve the chemotherapeutic effect of 5-FU on hepatocellular carcinoma by decreasing the expression of *bcl-2* gene and increasing the expression of *bax* and *caspase 3* genes.

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