

• LIVER CANCER •

## CIK cells from patients with HCC possess strong cytotoxicity to multidrug-resistant cell line Bel-7402/R

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### Abstract

**AIM:** To investigate the cytotoxicity of the cytokine-induced killer (CIK) cells from the post-operation patients with primary hepatocellular carcinoma (HCC) to multidrug-resistant (MDR) cell of HCC both *in vitro* and *in vivo*.

**METHODS:** A drug-resistant cell line was established by culturing human HCC cell line Bel-7402 in complete RPMI 1640 medium with increasing concentrations of adriamycin from 10 to 2 000 nmol/L. CIK cells were obtained by inducing the peripheral blood mononuclear cells with rhIFN- $\gamma$ , monoclonal anti-CD3 antibody, rhIL-1 $\alpha$  as well as rhIL-2, which were added into the culture. To detect the cytotoxicity of the CIK cells from HCC patients, the Bel-7402/R was taken as target (T) cells and CIK cells as effect (E) cells. Cytotoxic test was performed and measured by MTT. As to *in vivo* test, CIK cells were transfused into patients with HCC. The tumor specimens of the patients were obtained and immunohistochemistry was carried out to detect CD3, CD45, CD45RO as well as CD68.

**RESULTS:** A MDR 1 HCC cell line Bel-7402/R was established. Its MDR1 mRNA overexpressed which was shown by RT-PCR; the P-glycoprotein expression increased from 1.32% of parent cells to 54%. CIK cells expanded vigorously by more than 70-fold and the CD3+CD56+ increased by more than 600-fold after 3-wk incubation on average. The cytotoxicity of CIK from HCC patients to Bel-7402/R was about 50% and to L-02 below 10% ( $t = 8.87$ ,  $P < 0.01$ ), the same as that of CIK from normal individuals. Each of the 17 patients received  $1.5 \times 10^{10}$  of CIK cell transfusion. No side effects were observed. After CIK treatment, the tumor tissue nodules formed and a large amount of lymphocytes infiltrated in the liver cancer tissue and CD3, CD45, CD45RO, and CD68 increased greatly which was shown by immunohistochemistry.

**CONCLUSION:** A stable MDR1 HCC cell line has been established which could recover from liquid nitrogen and

CIK from HCC patients has strong cytotoxicity to MDR HCC cell. CIK adoptive immunotherapy is safe and has no side effects. Receivers improved their immunity to tumor evidently. CIK treatment may be a better choice for HCC patients after operation to prevent the recurrence, especially when tumors have developed drug resistance.

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**Key words:** Hepatocellular carcinoma; Cytokine-induced killer; Cytotoxicity; Multidrug resistance; P-glycoprotein

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors with a high mortality especially in China because of wide prevalence of HBV infection. Surgical operation is still the most effective and widely performed treatment, but the high frequency of recurrence after operation dramatically decrease the cure rate of HCC. To prevent recurrence of HCC, many measures have been taken such as regular chemotherapy after operation, intrahepatic arterial infusion of anticancer agents, transcatheter arterial embolization and recently, biotherapy<sup>[1-3]</sup> such as DC<sup>[4]</sup> cells and cytokine-induced killer (CIK) cells<sup>[5]</sup>. However, many patients' prognosis is poor and the 5-year survival rate usually is below 15-30%. Besides other reasons, drug resistance of the tumors induced in the chemotherapy plays an important role, which in turn protects the tumors against the chemical agents<sup>[6]</sup>. Among the many known biological mechanisms of drug resistance, multidrug resistance (MDR) is of particular interest<sup>[7,8]</sup>. MDR is associated with amplification or overexpression of the MDR1 gene and the expression of a transmembrane glycoprotein of 170 ku termed P-glycoprotein (Pgp). As a drug pump, it excludes the chemical drugs out of tumor cells and causes treatment failure.

CIK cells are generated according to a novel protocol<sup>[9]</sup> and several authors<sup>[10-13]</sup> have demonstrated that CIK possesses versatile anti-tumor advantages including easier generation from T cells, stronger cytotoxicity to tumor as well as more enhanced *in vivo* proliferation potency while compared with other adoptive immunotherapies. Shi *et al*,

and Wang *et al*, demonstrated that CIK treatment for HCC is effective and also safe. Now we show in this paper that multi-drug resistant HCC cell line Bel-7402/R, established by us, is also sensitive to CIK from HCC patients and positive pathological and immunohistochemical results were observed in one case whose tumor sample was verified as positive MDR1 expression by RT-PCR.

## MATERIALS AND METHODS

### Patients and controls

Seventeen HCC patients post-operation were enrolled in the study, including 16 cases of male and 1 female, aged 36-58 years with an average of 48.8 years. A written informed consent was obtained before CIK treatment which was certified by the Health Bureau of Hubei Province, China. Six of the patients were chosen randomly to take part in the *in vitro* test and six normal people from hospital staff were selected as normal controls.

### Cell lines and culture conditions

All cell lines were grown<sup>[14]</sup> in complete medium (CM) namely RPMI 1640 (Gibco) with 100 mL/L calf serum, 25 mmol/L hepes, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 50  $\mu$ mol/L 2-mercaptoethanol (2-ME, all from Sigma). Some other agents were added according to research needs. Human HCC cell line Bel-7402 was a generous present from Professor Guanxin Shen, Department of Immunology, Tongji Medical School, Huazhong Science and Technology University. HCC cell line SMMC-7721 and embryo liver cell line L-02 were provided by the China Center for Type Culture Collection. Drug-resistance cell lines Bel-7402/R as well as SMMC-7721/R were induced by serially increasing adriamycin (ADM, Sigma) from 10 to 2 000 nmol/L in the culture.

### CIK induction

CIK cells were prepared as described according to Schmidt-Wolf<sup>[9]</sup>. Briefly, for *in vivo* treatment peripheral blood mononuclear cells (PBMCs) were collected by CS-3000 Plus blood cell separator (Baxter, USA) or for *in vitro* test prepared by ficoll separation from post-operation patients with HCC (HCC group) or from volunteers as control group (normal group). The cells were grown in CM and 1 000 U/mL rhIFN- $\gamma$  was added on d 0, after 24 h of incubation, 50  $\mu$ g/L mouse monoclonal antibody (mAb) against CD3, 100 U/mL rhIL-1 $\alpha$  (all from R&D Systems) and 1 000 U/mL rhIL-2 (Sunshine Pharma. Co., Ltd, China) were added. Cell density was about 1 $\times$ 10<sup>6</sup>/mL and fresh CM with IL-2 was replaced every 3 d. Cell phenotypes were identified by FCM on d 0, 7, 14, 25. Cytotoxicities to target were determined after d 14. Cells were transfused back to patients between d 14 and 21 based on the cell number.

### RT-PCR

Primers for MDR1 RT-PCR were designed based on the sequence in GenBank (NM000927), forward primer 5'-CAGGAGATAGGCTGGTTTGATG T-3' and backward

primer 5'-TTAGCTTCCAACCACG TGTAATC-3'. PCR was performed according to molecular cloning protocol, briefly as follows: after the collection of cultured cells or mincing of HCC tissue into small pieces, RNA was extracted by TRIzol reagent. Extracted RNA was solved in diethyl pyrocarbonate-treated water (DEPC water). RT-PCR was performed in 50  $\mu$ L of mixture containing: 1  $\mu$ L of extracted RNA, 1  $\mu$ L of 10  $\mu$ mol/L of both forward and backward primers (synthesized by Sangon, Shanghai, China), 5  $\mu$ L of 10 $\times$  buffer (100 mmol/L of Tris-HCl, pH 8.0, 500 nmol/L of KCl, 0.1% gelatin, 1% Triton X-100), 1  $\mu$ L of 10 mmol/L dNTP, 4  $\mu$ L of 25 mmol/L MgCl<sub>2</sub>, 2.5 U of ribonuclease inhibitor, 200 U of M-MLV RT enzyme, 2 U of Taq polymerase, and DEPC water was added to the final volume of 50  $\mu$ L. The reaction mixture was kept at 42  $^{\circ}$ C for 30 min to synthesize first cDNA, and at 94  $^{\circ}$ C $\times$ 3 min to denature DNA followed by 35 cycles of amplification consisting of 30 s of denaturing DNA at 94  $^{\circ}$ C, 30 s of annealing DNA at 58  $^{\circ}$ C and 30 s of extending DNA at 72  $^{\circ}$ C.  $\beta$ -actin served as control.

### Flow cytometry analysis

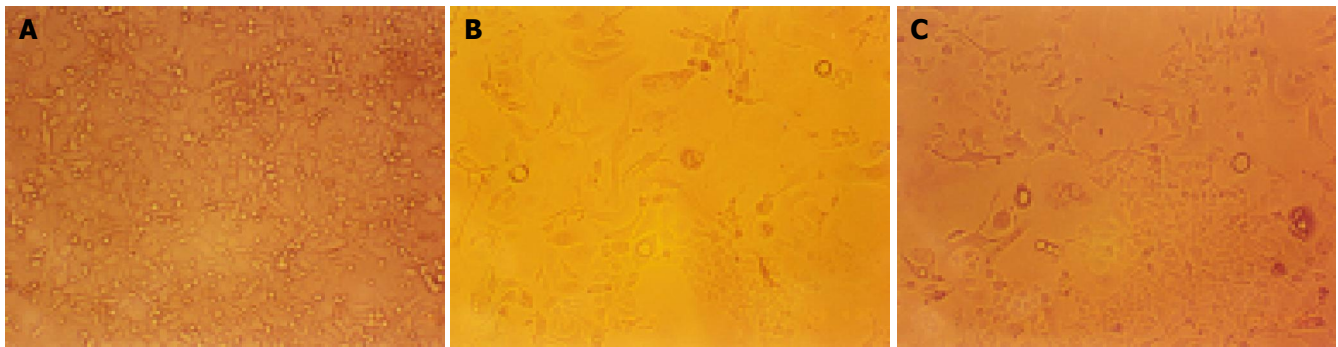
All samples were pretreated before analyzed by FCM analyzer (Coulter: EPICX XL). For cell phenotype identification, cells were incubated for 30 min with mAb against CD3, CD4, CD56, coupled to FITC or PE. For the detection of Pgp, cells were fixed with 4% paraformaldehyde for 10 min, incubated with mouse anti-human p-170 for 30 min at room temperature, and stained with goat antimouse IgG coupled to FITC. For Pgp function determination<sup>[15]</sup>, cells were incubated with Rhodamine 123 (Sigma) for 1 h before being trypsinized and washed.

### Drug-resistance test

One hundred microliters of each parent or drug-resistant cells were distributed in 96-well culture microplates at 1 $\times$ 10<sup>6</sup>/mL density in every well. ADM was added after a serial dilution from 40  $\mu$ mol/L to 36.25 nmol/L. A triplicate of same samples was placed to control variation. Cells were incubated at 37  $^{\circ}$ C with 50 mL/L CO<sub>2</sub> for 48 h prior to the addition of 10  $\mu$ L of MTT<sup>[18,20]</sup> (5 g/L, Sigma). After another 4 h of incubation, the plate was centrifuged at 3 000 r/min for 5 min, medium was discarded, 150  $\mu$ L of DMSO was added to each well. Optical absorbance value was read at 570 nm by a microreader (BioRad 550, USA). Cell survival ratio (CSR) was calculated according to the formula: CSR =  $A_{570 \text{ experiment}}/A_{570 \text{ control}} \times 100\%$ . The 50% inhibition concentration (IC<sub>50</sub>) was determined by concentration-CSR curve.

### Cytotoxicity test

One hundred microliters of target cells were seeded in a microplate at a 1 $\times$ 10<sup>5</sup>/mL density and 0.1 mL of effect cells at a 1 $\times$ 10<sup>6</sup>/mL density were mixed into each well (to reduce the influence of effect cell number, we deliberately made the E:T = 10:1). After 4 h of incubation, 10  $\mu$ L of MTT was added. And following treatments were the same as in drug-resistant test. Cytotoxicity rate (CR) was calculated by the following formula: CR =  $\{1 - (A_{570 \text{ experiment}} - A_{570 \text{ E control}})/A_{570 \text{ T control}}\} \times 100\%$ .



**Figure 1** Morphological change of Bel-7402 during induction of drug-resistance. A: Parent cells; B: morphology change during culture; C: most of the cells died.

Some "normal" cell islands appeared.

### Immunohistochemistry

Specimens were HE stained and observed under a microscope. Cell markers were demonstrated by immunohistochemistry according to S-P protocol on slides with mAb against CD3, CD45, CD45RO, CD68 (Neomarker) as first antibody, goat anti-mouse antibody as second antibody.

### Statistical analysis

Analysis was performed by using Student's *t* test, the data were expressed as mean $\pm$ SD and  $P < 0.05$  was considered statistically significant.

## RESULTS

### Generation of CIK from PBMCs

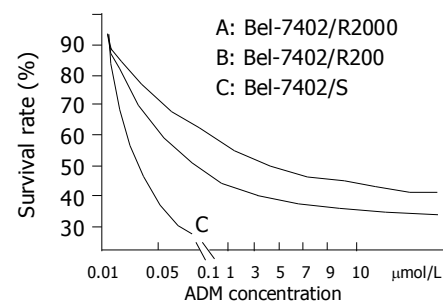
The proliferation and phenotype of the PBMCs after CIK induction varied according to individual HCC patients. The cell number increase was more than 70-fold on average ( $n = 17$ ) after 25-d incubation. CD3+CD56+ cells increased greatly by about 60-fold to more than 1 400-fold with an average of more than 600-fold (Table 1).

### Induction of drug-resistant HCC cells

Bel-7402 HCC cell line under went drug-resistant induction test beginning with 10 nmol/L of ADM in the medium increased gradually to 200 or 2 000 nmol/L. Cells showed a series of changes (Figure 1) such as enlarged, polynuclear, discontinuous in plasma membrane (Figure 1B). Most of them died then some "normal" cell islands (Figure 1C) appeared, the same as the parent cell (Figure 1A). We designated the induced cells which survived in 200 nmol/L of ADM as Bel-7402/R-200, and those survived in 2 000 nmol/L of ADM, as Bel-7402/R-2000. After both grew in 2 mmol/L of ADM for 1 h, most of the former died and most of the latter still survived, indicating there might be some difference

between the two. When MTT test was done to the parent cell and both drug-resistant cells, they showed different IC<sub>50</sub> with ADM. IC<sub>50</sub> of the parent Bel-7402 was 42 nmol/L, and of the induced cells, 2 000 and 8 000 nmol/L respectively (Figure 2). RT-PCR showed MDR1 overexpressed in induced cells and the parent cells generally gave negative results. Intracellular concentration of Rhodamine 123 was also determined by FCM (Figure 3), since Rhodamine 123 produces fluorescence itself and would be pumped out of cells if the Pgp existed. Bel-7402/R cells kept only 25.1%, 28.4% of Rhodamine 123 intracellularly each and the parent Bel-7402, 77.7%. Stained with anti-P-170 mAb (Figure 4), and determined by FCM, we found that only 1.32% of parent cells expressed P-170 and Bel-7402/R increased to 47%, 54% respectively.

Different from other reports, although it showed similar characters to Bel-7402/R in drug-resistance, the SMMC-7721/R only endured a smaller range of drug concentration (below 100 nmol/L of ADM) and it was difficult to recover from liquid nitrogen keeping.

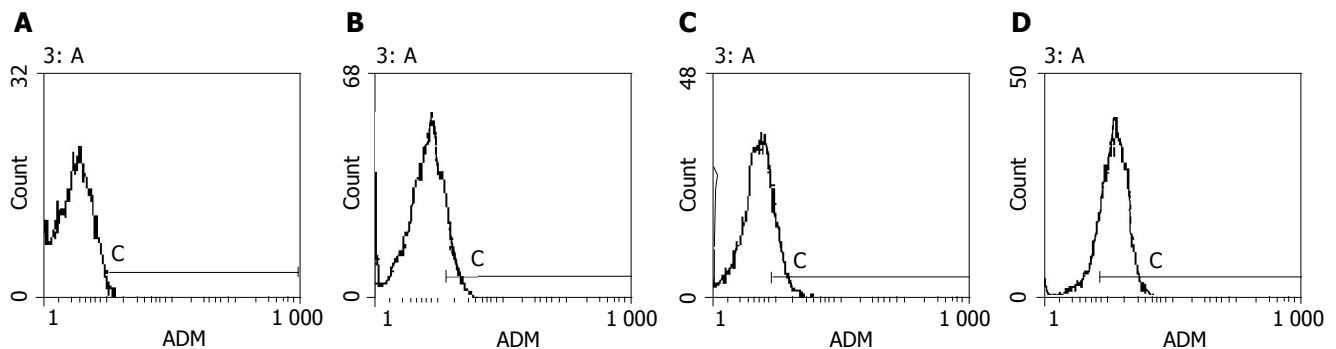


**Figure 2** Survival-ADM concentration curves of three cells. A: Bel-7402/R-2000; B: Bel-7402/R-200; C: Bel-7402/S.

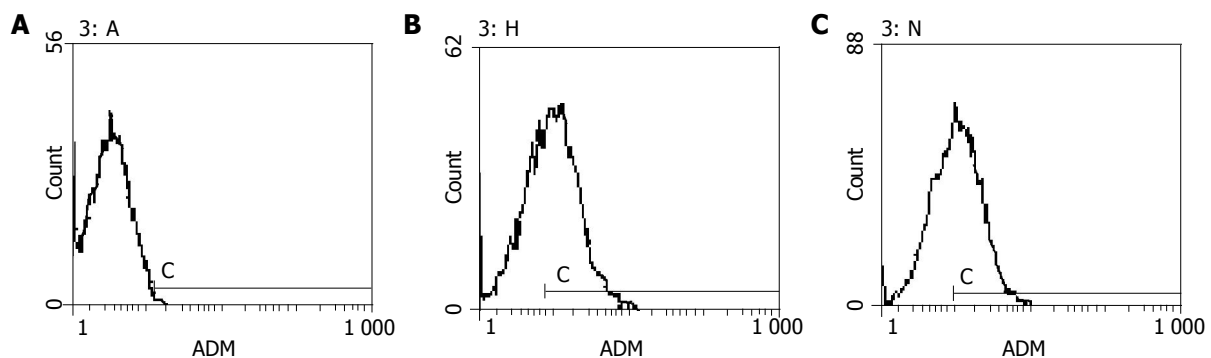
**Table 1** Determination of CD3CD56 of CIK cells ( $n = 6$ )

CD3+(%)				CD3+CD56+(%)			
D 1		D 25		D 1		D 25	
Normal	HCC	Normal	HCC	Normal	HCC	Normal	HCC
59.5 $\pm$ 7.8 <sup>a</sup>	45.1 $\pm$ 12.1 <sup>a</sup>	93.3 $\pm$ 6.5	89.4 $\pm$ 5.1	3.2 $\pm$ 1.6	2.4 $\pm$ 2.1	20.6 $\pm$ 13.4	19.1 $\pm$ 11.7

<sup>a</sup> $P < 0.05$  vs others.



**Figure 3** Rhodamine 123 excluding test detected by FCM. **A:** Control; **B:** Bel7402/r-2000; **C:** Bel7402/r-200; **D:** Bel7402/S.



**Figure 4** Indirect immunofluorescence on Pgp detected by FCM. **A:** Bel7402/s; **B:** Bel7402/r-200; **C:** Bel7402/r-2000.

### Cytotoxicity of CIK to drug-resistant HCC cells

Shi *et al*, and Wang *et al*, have proven the CIK cells from HCC patients have stronger cytotoxicity to HCC cells and here we found that CIK both from normal persons and HCC patients had similar cytotoxicity to drug-resistant HCC cells (Figure 5) at different E:T ratios, so we chose E:T = 10:1 to avoid bias produced by effect cell number in the test. The experiment wells contained  $10^4$  target cells mixed with  $10^5$  effect cells. Effect control (E control) included  $10^5$  effect cells only and target control (T control),  $10^4$  target cells only. Cytotoxicity of CIK from HCC patients to Bel-7402/S was 33-68% with an average of 50.7%. While to Bel-7402/R it was 37-64% with an average of 52.3% ( $P>0.05$ ), the same as normal group. Cytotoxicity CIK from HCC patients to L-02, an embryo liver cell line, was under 10%, indicating no side effect to normal liver cells (Tables 2 and 3).

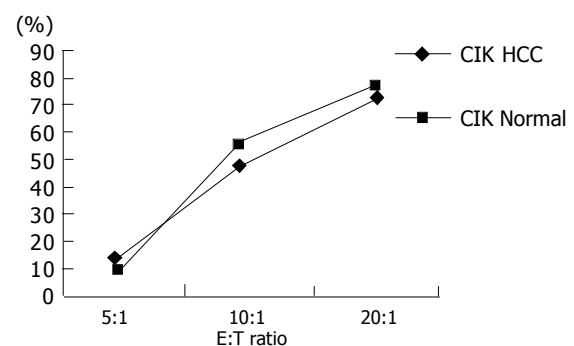
**Table 2** Results of cytotoxicity of CIK to target cells

Patient Number	A value (rate of cytotoxicity)			
	7402/s <sup>b</sup> (%)	7402/R <sup>d</sup> (%)	L-02 <sup>bd</sup> (%)	E control
1	0.802 (33)	0.743 (49)	1.011 (12)	0.485
2	0.913 (48)	0.895 (47)	1.201 (4)	0.627
3	0.681 (46)	0.599 (57)	0.888 (14)	0.379
4	0.630 (68)	0.602 (60)	0.959 (7)	0.401
5	0.711 (58)	0.802 (37)	1.001 (12)	0.479
6	0.987 (51)	0.903 (64)	1.302 (2)	0.718
T control	0.554	0.508	0.595	

<sup>t</sup><sub>d</sub> = 8.70, <sup>b</sup> $P<0.01$  <sup>t</sup><sub>i</sub> = 8.87, <sup>d</sup> $P<0.01$ .

**Table 3** Average of cytotoxicity and significance of difference ( $n = 6$ )

	7 402/s	7 402/R
HCC	50.7	52.3
Normal	57.1	54.7

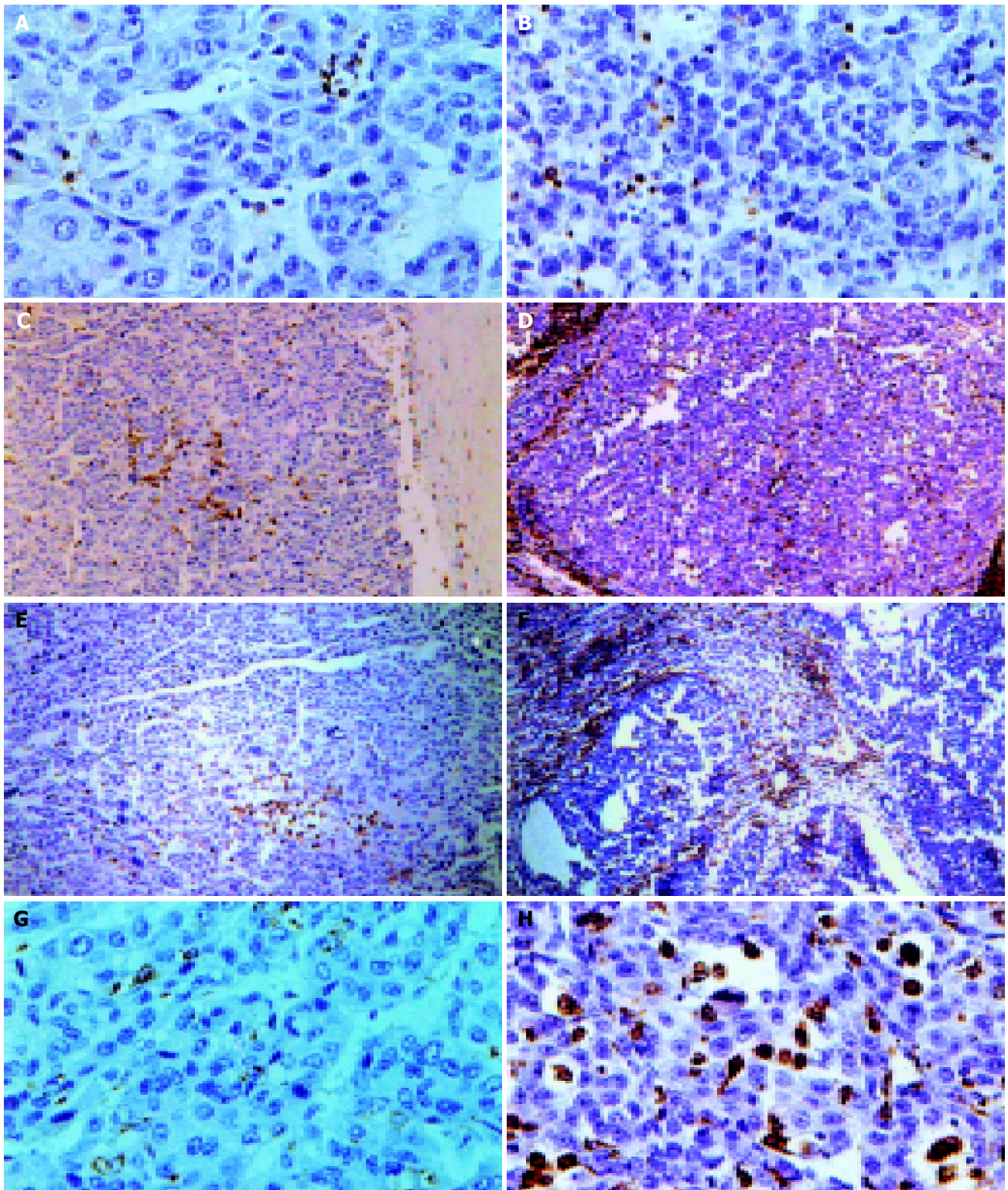


**Figure 5** Cytotoxicity of CIK to Bel-7402 at different E:T ratios.

### In vivo effect of CIK

Seventeen cases of recurrent HCC patients post-operation were recruited into our study, most of them with relapsed bulk tumors and no effective treatment. The results showed that CIK treatment for HCC was safe, effective, without side effects (data reported elsewhere). Here we only focus on one case whose tumor had been demonstrated MDR1 overexpression by RT-PCR and immunohistochemistry assay





**Figure 6** Immunohistochemistry results of the tumor specimen before and after CIK treatment, demonstrated that a large amount of lymphocytes with CD3, CD45, CD45RO, CD68 markers infiltrated in tumor after treatment. **A:** Immunohistochemistry with anti-CD3 to the specimen before treatment; **B:** with anti-CD3 to the specimen after treatment; **C:** immunohistochemistry with anti-

CD45 to the specimen before treatment; **D:** with anti-CD45 to the specimen after treatment; **E:** immunohistochemistry with anti-CD45RO to the specimen before treatment; **F:** with anti-CD45RO to the specimen after treatment; **G:** immunohistochemistry with anti-CD68 to the specimen before treatment; **H:** with anti-CD68 to the specimen after treatment.

before and after operation was carried out. The patient was a male, 56 years old, relapsed about one year after liver tumor resection with pneumal metastasis and cancer embolus in portal vein, and received CIK treatment. After transfused  $1.3 \times 10^{10}$  CIK cells, the patient had improvement

of nausea, vomiting and the windy abdomen, as well as his appetite. His ascites abated too. After another 3 mo a second operation was performed and the tumor sample underwent pathological and immunohistochemical assay. Compared with the specimen before CIK treatment the tumor became

nodular with a large amount of lymphocytes infiltration after treatment (Figure 6). CD3, CD45, CD45RO, CD68 positive cells increased vigorously (Table 4 and Figure 6) to 41, 283, 44, 77/HPF each from 0.1, 15, 3, 8/HPF before CIK treatment indicating the anti-tumor immunity was enhanced significantly since CD3, CD45 represent T cells, CD45RO is the marker of T memory subset, while CD68 is the marker of mono/macrophage.

**Table 4** Comparison of immune cells in immunohistochemistry

	CD45	CD3	CD45RO	CD68
1	15.4 cells/HPF	0.15 cells/HPF	2.9 cells/HPF	8.0 cells/HPF
2	283 cells/HPF	41.3 cells/HPF	44.1 cells/HPF	77.7 cells/HPF
x-fold	18.4	275	15.2	9.7

1: before treatment. 2: after treatment. HPF: high power field.

## DISCUSSION

Other than transgene models, several drug-resistant HCC cell lines induced by ADM have been established<sup>[17-19]</sup>. In our laboratory, we found that Bel-7402 is easier and has a wider range in drug-dose to induce drug-resistance than smmc-7721, as the latter is difficult to grow in >100 nmol/L of ADM and to recovered from liquid nitrogen preservation. Bel-7402/R still keep its resistance even revived from the liquid nitrogen and so it is easy to use. Our research demonstrated that MDR1 gene expression is one of important mechanisms of drug-resistance in our Bel-7402/R. At the same time, that Bel-7402/R-2000 was more resistant to the drug than Bel-7402/R200 reminded us that there should be some other reasons to be disclosed.

A number of studies<sup>[20,21]</sup> have illuminated that CIK possessed much stronger cytotoxicity than LAK to several kinds of cancer such as leukemia, renal cell carcinoma, melanoma<sup>[22]</sup>, and proved that dendritic cells increased in serum of host after transfusion of CIK to HCC patients. Here we demonstrated CIK cells from HCC patients could equally kill drug-resistant HCC cell line as those from normal. One case of *in vivo* study also gave same results. Immunohistochemistry of specimens revealed large amounts of lymphocytes infiltrating into tumor; CD3+, CD45RO+, CD68+ cells were recruited there in great numbers. These observations may imply activation of anti-tumor immunity since CD45RO+ represents T memory subset while CD68+ is the marker of monocyte/macrophage<sup>[23]</sup>. Anti-CD3Ab can activate CD45RO+ T subset<sup>[24]</sup>, so we deduced that the increase of CD45RO+ cells in the tumor locals may come from CIK.

CIK cell killing drug-resistant HCC cell line is of much benefit to HCC treatment. HCC is easy to induce drug-resistance. Because of tendency of metastasis and recurrence of HCC, post-operation chemotherapy is necessary. Since p-170 pumps drugs out of the cells, chemotherapy is not only useless to the drug-resistant tumor but produces the side effects to the host as well. CIK would be the better choice to kill the drug-resistant tumor. We suggest to perform CIK treatment to eradicate the remaining tumor cells after bulk tumor loads are removed by operation.

It is interesting that while CIK kills the tumor cells vigorously it has little effect on L-02 cells. Similar results were reported by Schmidt-Wolf *et al*<sup>[25]</sup>. They found that despite high cytotoxic activity of CIK against lymphoma cells, they had little toxicity against a subset of normal human hematopoietic progenitor cells. Some difference between Bel-7402 and L-02 has been reported recently by Shao *et al*<sup>[26]</sup>, that LAPT4B, a newly found HCC related antigen, was expressed in Bel-7402 but not in L-02. A polyclonal antibody against LAPT4B has been produced. Monoclonal antibody against it is in process of production. It may be helpful to solve the problem.

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