

Transduction of Fas gene or *Bcl-2* antisense RNA sensitizes cultured drug resistant gastric cancer cells to chemotherapeutic drugs

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Abstract

AIM To compare the expression level of *Fas* gene and *Bcl-2* gene in gastric cancer cells SGC7901 and gastric cancer multidrug resistant cells (MDR) SGC7901/VCR, to transduce *Fas* cDNA and *Bcl-2* antisense nucleic acid into SGC7901/VCR cells respectively, and to observe the expression of two genes in transfectants and non-transfectants as well as their drug sensitivity.

METHODS Eukaryotic expression vector pBK-*Fas*cDNA and pDOR-anti *Bcl-2* were constructed and transfected into SGC7901/VCR cells by lipofectamine, respectively. Northern blot and Western blot were used to detect the expression of mRNA and protein in SGC7901/VCR and SGC7901 cells and transfectants, and drug sensitivity of transfectants for VCR, CDDP and 5-FU was analyzed with MTT assay.

RESULTS After gene transfection, 80 for *Fas* and 120 for antisense *Bcl-2* drug-resistant clones were selected from 2×10^5 cells, transfection rate being 0.04% and 0.06%. Two clones of SGC7901 *Fas*/VCR cells and SGC7901 anti *Bcl-2*/VCR cells were randomly selected for further incubation. Hybridization results showed that the expression level of *Fas* mRNA and protein in SGC7901/VCR cells was much lower, but that of *Bcl-2* mRNA and protein was higher than that in SGC7901 cells. The expression of *Fas* mRNA and protein in SGC7901 *Fas*/VCR cells was higher, and of *Bcl-2* mRNA and protein was lower in SGC7901 anti *Bcl-2*/VCR cells than that in non-transfectants. MTT assay showed

that transfectants were more sensitive to VCR, CDDP, 5-FU than non-transfectants.

CONCLUSION *Bcl-2* gene displayed high expression while *Fas* gene had low expression in drug resistant gastric cancer cells. Expression of *Bcl-2* protein was effectively blocked in SGC7901 anti *Bcl-2*/VCR cells by gene transfection. In contrast, the expression of *Fas* mRNA and protein in SGC7901 *Fas*/VCR cells increased. *Fas* gene and *Bcl-2* antisense nucleic acid transfection sensitized drug resistant gastric cancer cells to chemotherapeutic drugs. These results suggest cell apoptosis plays an important role in the mechanism of MDR, and enhancing apoptosis might reverse MDR.

INTRODUCTION

Chemotherapy is one of the major methods in tumor treatment, but it often does not work due to multidrug resistance (MDR). Recent studies indicated that inhibition of cancer cell apoptosis and longer cell life may be one of MDR mechanisms^[1]. So it is assumed that inducing apoptosis might reverse MDR. We transfected *-Fas-* gene and *Bcl-2* antisense nucleic acid into drug resistant gastric cancer cells, and observed the expression of target genes in transfectants and the sensitivity of transfectants to chemotherapeutic agents in order to find the ground for reversing gastric cancer MDR.

MATERIALS AND METHODS

Material

Drug resistant gastric cancer SGC7901/VCR cells and JM109 bacterial strain were kept in our department. Retrovirus vector pDOR-SV40 and expression vector pBK-CMV were generous gifts from Dr. Cui Da-Xiang, Department of Biochemistry, Fourth Military Medical University. The pBluescript *Bcl-2* cDNA plasmid was from our department. The pBluescript *Fas*-cDNA was presented by professor Itoh N, Japan. *Eco* RI, *Bam* HI, *Sal* I, *Xho* I, CIP, RNaseA, T4 DNA ligase, PMSF, Aprotinin, SDS, ABC kit, probe labeled kit and guanidinium isothiocyanate were

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purchased from SABC and Promega, and Lipofectamine from Gibco BRL. MTT, Protease k, DEPC, MOPS, G418, FCM and nitrocellulose filter were products of Sigma. [$\alpha^{32}\text{P}$]dATP was derived from Yahui Ltd, Beijing, primer and oligonucleotides from Sangon, Shanghai, and rat anti-human *Bcl-2* monoclonal antibody and rabbit anti human *Fas* polyclonal antibody from Orient Ltd, Beijing.

Methods

Construction and identification of recombinant vectors

1.9kb *Bcl-2* cDNA was inserted into pBluescript KS at *Eco* RI site and 2.5kb *Fas* cDNA into pBluescript KS at *Xho* I site. According to the construction protocol, primary plasmids were digested by enzyme. The results of agarose gel electrophoresis showed that primary plasmids contained complete *Bcl-2* cDNA and *Fas*-cDNA. We used *Eco* RI and *Bam* HI to cut pBluescript *Bcl-2* cDNA, *Sal* I and *Eco* RI to cut pBluescript *Fas*-cDNA, and obtained 0.622 kb *Bcl-2* cDNA and 1.83 kb *Fas*-cDNA fragments by frozen-thaw methods. Under T4 DNA ligase, cDNA fragments were connected with the corresponding pDOR and pBK-CMV vectors digested by enzyme, the products were transformed into competent cells for routine culture. Ampresistant colonies and mini-prepared plasmids were selected randomly, and recombinant plasmids were identified through enzyme digestion and agarose gel electrophoresis. Three pairs of primers designed according to *Fas*-cDNA sequence were used for PCR amplification of pBK-*Fas*-cDNA, and sequence of PCR product was analyzed to confirm the correctness of open reading frame.

Gene transfection and clone selection

Purified pDOR-anti-*Bcl-2* or pBK-*Fas* cDNA (0.5 μg) diluted in 100 μl RPMI 1640 was mixed with 5 μl lipofectamine, and placed at room temperature for 10min. The mixture was then transfected into 2×10^5 SGC7901/VCR cells. The cells were selected by 500 mg/L G418 24 hours later. At the same time, pDOR or pBK-CMV vector which lacked target genes transfected cells and non-transfectants served as negative controls.

Southern blot and Northern blot

Genomic DNA and total RNA were obtained from the well growing 1×10^7 cells. Genomic DNA digested with *Eco* RI ran agarose gel electrophoresis. The denatured total RNA underwent formaldehyde denaturation and gel electrophoresis. DNA and RNA were transferred to nitrocellulose filter by vacuum aspiration. [$\alpha^{32}\text{P}$]dATP labeled probe was made for prehybridization, hybridization and autoradiography.

Western blot

Half cell lytic protein was used for SDS-PAGE, and comassie brilliant blue stained, the other half was electrotransferred 100v overnight, nitrocellulose filter denatured by SDS and visualized by ABC method.

Drug sensitivity assay

Cells ($10^3 - 10^4$) diluted with 200 μl 10% RPMI 1640 were seeded into 96-well plates, respectively, added cisplatin (1 μg , 10 μg , 100 μg), 5-FU (7 μg , 70 μg , 700 μg), and VCR (0.1 μg , 1.0 μg , 10 μg) according to the clinically established plasma peak concentration. Three days later, 20 μl MTT solution (5g/L) was dropped into plates, the supernant was discarded after 4 hours, and 150 μl DMSO was added to melt crystal. OD value was read at 590nm wavelength.

RESULTS

Construction of eukaryotic expression vector

As shown in Figure 1, 1.9 kb *Bcl-2* cDNA and 2.5kb *Fas*-cDNA were separated from primary plasmids digested with *Eco* RI and *Xho* I, 0.622kb anti-sense *Bcl-2* fragment and 6.5kb vector from recombinant vector pDOR- anti-*Bcl-2* digested by *Eco* RI and *Bam* HI, 1.8 kb *Fas* cDNA and 4.5kb vector from recombinant vector pBK-*Fas*-digested by *Eco* RI and *Sal* I. Electrophoresis of PCR product (Figure 2) indicated that three pairs of primers amplified 500 bp, 850 bp, 700 bp fragments respectively, which were consistent with our expectation.

Establishment of transfectants

When transfected cells were cultured selectively by G418 for 4-5 weeks, resistant clones formed gradually, the number of stable clones reached about 120, with a transfection rate of above 0.05%. In contrast, all non-transfectants died two weeks after G418 selection. Resistant clones were further incubated in the presence of low-dose G418 for 40-50 days. We got two resistant clones, SGC7901 - *Fas* /VCR cells and SGC7901 anti-*Bcl-2*/VCR cells.

Expression of target genes

Northern blot was performed using a single strain cDNA fragment of *Fas* or *Bcl-2* or antisense *Bcl-2* as probes, respectively. The results (Figures 3 and 4) showed that SGC7901 cells had weak signals of *Fas* and strong signal of *Bcl-2*, while SGC7901/VCR had weak signal of *Fas* and very strong signal of *Bcl-2*. The signal of *Fas* became stronger in SGC7901-*Fas*/VCR than that in SGC7901/VCR, which was also dependent on cell number. The signal of antisense *Bcl-2* was stronger in SGC7901 anti-*Bcl-2*/VCR than in SGC7901/VCR and non-transfectants, but the signal of sense *Bcl-2*

was a little weaker than that in non-transfectants. SDS-PAGE and Western blot (Figure 5) showed that a band of Mr 36000-40000 was found in transfectants, but not in non-transfectants, the size of which was consistent with Mr of *Fas*-protein. A band of Mr 25000, which equaled to that of *Bcl-2* was found in non-transfectants, but not in antisense *Bcl-2* transfected cells.

Drug sensitivity assay

The survival rates of transfectants treated with cisplatin or 5-FU were obviously lower than non-transfectants, however, the survival rates of transfectants treated with VCR were only lowered lightly, compared with non-transfectants (Table 1).

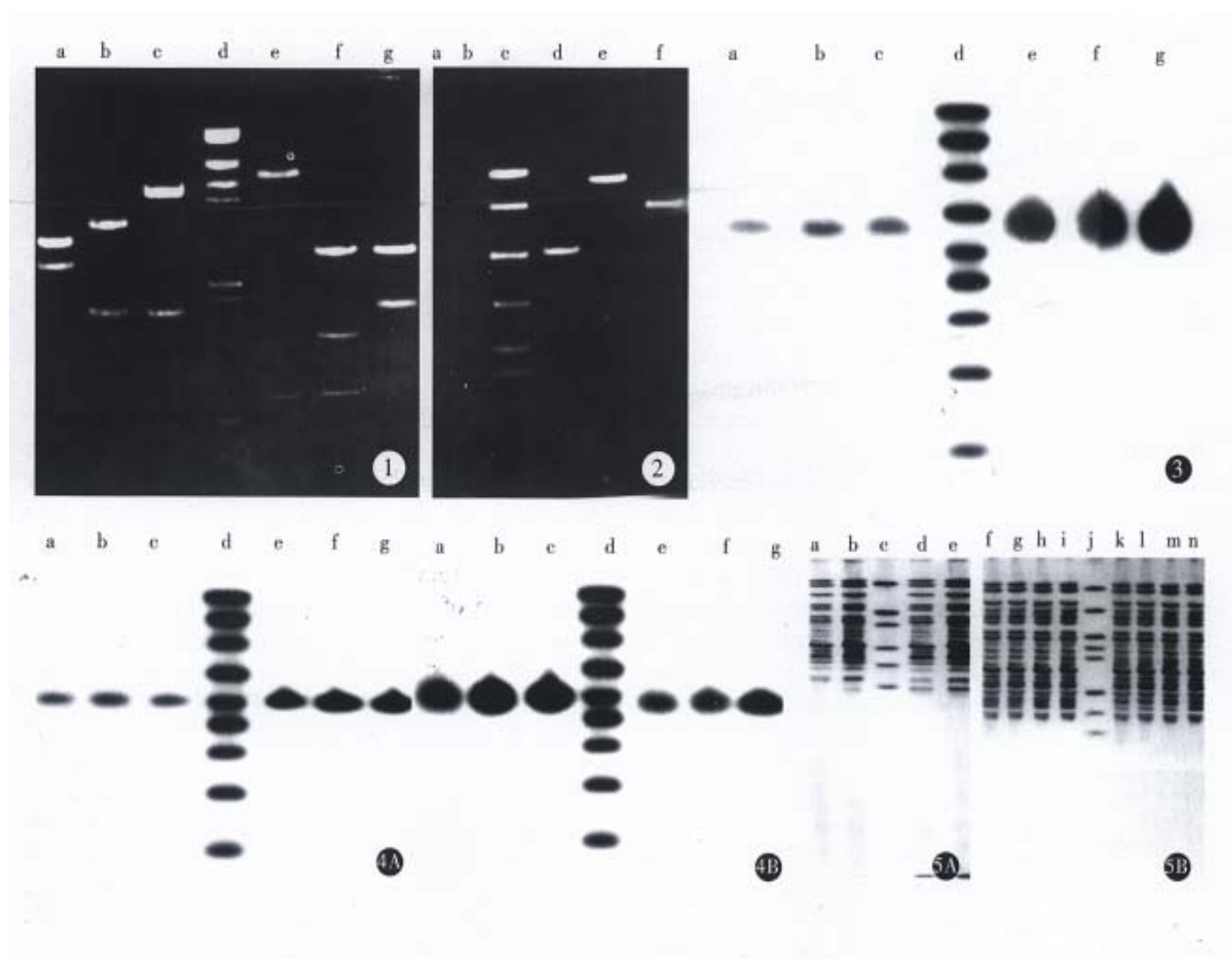


Figure 1 Identification of recombinant vectors digested with enzymes. a. pBluescript *Fas*/*Xho*I, b. pBluescript *Fas*/*Eco*RI+*Sal*I, c. pBK-*Fas*/*Eco*RI+*Sal*I, d. γ DNA Marker/*Hind*III, e. pDOR-*Bcl-2*/*Eco*RI+*Bam*HI, f. pBluescript *Bcl-2*/*Eco*RI+*Bam*HI, g. pBluescript *Bcl-2*/*Eco*RI

Figure 2 Electrophoresis of pBK-*Fas*-PCR product. a. PCR of competent cells as template, b. PCR of empty vector transformed bacterial, c. PCR marker, d. PCR1 of pBK-*Fas* (500bp), e. PCR2 of pBK-*Fas* (850bp), f. PCR3 of pBK-*Fas* (700bp)

Figure 3 Northern blot with *Fas*-cDNA probe. a. SGC7901/VCR/ 3×10^6 cells, b. empty vector transfectant/ 5×10^6 cells, c. SGC7901/ 3×10^6 cells, d. RNA Marker, e. SGC7901 -*Fas*/VCR/ 1×10^6 cells, f. SGC7901 -*Fas*/VCR/ 2×10^6 cells, g. SGC7901 *Fas*/VCR/ 3×10^6 cells.

Figure 4 Northern blot with *Bcl-2* probe. A. antisense probe, B. sense probe

a. SGC7901/ 3×10^6 cells, b. empty vector transfectant/ 2×10^6 cells c. SGC7901/ 3×10^6 cells, d. RNA Marker, e. SGC7901 anti-*Bcl-2*/VCR/ 2×10^6 cells, f. SGC7901 anti-*Bcl-2*/VCR/ 3×10^6 cells, g. SGC7901 anti-*Bcl-2*/VCR/ 4×10^6 cells

Figure 5 SDS-PAGE and Western blot. A. *Fas* transfectants, B. anti-*Bcl-2* transfectants

a. SGC7901/VCR/ 3×10^6 cells, b. SGC7901/ 3×10^6 cells, c. High molecular weight protein marker, d. SGC7901-*Fas*/VCR/ 1×10^6 cells, e. SGC7901-*Fas*/VCR/ 2×10^6 cells, f. SGC7901/ 1×10^6 cells, g. SGC7901/ 2×10^6 cells, h. SGC7901/VCR/ 1×10^6 cells, i. SGC7901/VCR/ 2×10^6 cells, j. Middle molecular weight protein marker, k. SGC7901 anti-*Bcl-2*/VCR/ 5×10^5 cells, l. SGC7901 anti-*Bcl-2*/VCR/ 1×10^6 cells, m. SGC7901 anti-*Bcl-2*/VCR/ 2×10^6 cells, n. SGC7901 anti-*Bcl-2*/VCR/ 3×10^6 cells

Table 1 Survival rates of transfectants and non-transfectants treated with chemotherapeutic drugs (%)

Cells	n	CDDP (mg/L)			5-FU(mg/L)			VCR(mg/L)		
		5	50	500	35	350	3500	0.5	5	50
SGC7901 Fas/VCR	10	45.1 ^a	40.5 ^a	32.0	43.5 ^a	38.6 ^a	30.7 ^a	55.0	46.7 ^a	40.0 ^a
SGC7901 anti-Bcl-2/VCR	10	50.2 ^a	45.1 ^a	40.5	50.1 ^a	44.6 ^a	39.5 ^a	60.3	55.1 ^a	49.2 ^a
SGC7901/VCR	10	70.2	61.3	52.0	71.1	62.2	55.3	72.5	67.4	60.1

In comparison with SGC7901/VCR cells group, ^aP<0.05.

DISCUSSION

Apoptosis and mitosis both are important for keeping the balance of organism *in vivo*, which act in opposite way. Recent studies indicated that inhibition of cancer cell apoptosis and disturbance of apoptosis related genes can increase the anti-apoptosis protein or decrease pro-apoptosis protein, which is one of the mechanisms of tumorigenesis. Therefore, induction of apoptosis may treat tumors. Many chemotherapeutic agents are found to act through damaging DNA or regulating apoptosis related genes *p53*, *c-myc*, *Bcl-2*, *c-H-ras* to trigger apoptosis^[2-4]. But MDR becomes an obstacle in tumor treatment. Recent researches have shown that MDR is the result of many mechanisms, in addition to the changes of Pgp, MRP, LRP, Topo II and GST/GSH, deregulation of apoptosis also produces cross drug resistance of cancer cells^[1,5]. The ability of drug resistant cells repairing damaged DNA and anti-apoptosis has strengthened greatly^[6]. Introduction of *Bcl* xL gene may induce resistance of drug sensitive cells, and overexpression of *Bcl-2* is linearly related to overexpression of Pgp, therefore, it is supposed that the expression level of apoptosis related genes determines the MDR phenomena of cancer cells^[7]. Chinese hamster drug resistant ovarian fibroblastoma LR73/20E cells, which overexpress MDR and Pgp, are not sensitive at all to apoptosis inducing colchicine, but become sensitive to apoptosis after treated with drug resistant reversing agent verapamil^[8]. Apoptosis can not be induced in drug resistant breast cancer MCF-7/ADR cells which overexpress *Bcl-2* mRNA and protein by sugar starvation^[9]. Drug resistant leukemia HL-60/AR, HL-60/VCR, HL-60/TAX1000 cells, overexpressing MRP, Pgp, *Bcl-2*, and *Bcl*-xL, can inhibit the intercellular aggregation of chemotherapeutic agent paclitaxel and prohibit apoptosis. *Bcl-2* and *Bcl*-xL transfected drug sensitive cells can antagonize apoptosis, but can not affect the intercellular aggregation of chemotherapeutic agent paclitaxel^[10]. P53 protein and *Fas* antigen promote cell apoptosis, but they display low expression in drug resistant cells^[1,11]. These results show that high expression of *Bcl-2* and low expression of *Fas* are important for drug resistant cells to antagonize apoptosis and resist

chemotherapeutic agents.

Although antiapoptosis gene *Bcl-2* can not promote cell proliferation, it prolongs cell life. Its inhibition of apoptosis is caused by changing the permeability of Ca²⁺ channel and the expression of ICE^[12], and is related with regulation of Bax, TNF and *Fas*^[13]. Controlling the transcription of *Bcl-2* mRNA by antisense nucleic acid technology can effectively suppress the expression of *Bcl-2* and retard tumor cell growth^[14].

Fas gene product, a type-I membrane protein, belongs to the TNF receptor family. *Fas* molecule on the surface of cell transmits death message into cell through combining *Fas* ligand or anti *Fas* monoclonal antibody, resulting in the death of cells in several hours^[15]. Compared with normal tissues, malignant tumor cells, especially metastatic tumor cells present extremely lower expression of *Fas*, while most benign tumors are similar to their original tissues in the expression of *Fas*. It is hypothesized that malignant tumors might suppress *Fas* expression or lose *Fas* molecule in order to avoid the surveillance of *Fas* ligand. This explains the lack of *Fas* expression in drug resistant cells^[1]. In one word, transduction of proapoptosis *Fas* gene or antisense *Bcl-2* gene into drug resistant cells is obviously helpful in increasing the drug sensitivity. Chen XQ transiently transduced *Bcl-2* antisense RNA into human leukemia cells (CEM), and found that intrinsic *Bcl-2* protein was decreased, and transfected cells were more sensitive to etoposide and a lot of apoptotic bodies and small DNA fragments formed when cells died.

To study the significance of *Fas* and *Bcl-2* protein in drug resistant gastric cancer SGC7901/VCR cells, we constructed the eukaryotic expression vectors pBK *Fas* and pDOR-anti-*Bcl-2* which were respectively transfected into SGC7901/VCR cells. After G418 selection, resistant clones overexpressing *Fas* protein or lacking *Bcl-2* protein were obtained. Molecular hybridization showed drug resistant gastric cancer SGC7901/VCR cells expressed a tiny amount of *Fas* mRNA and protein, but highly expressed *Bcl-2*. Compared with control cells, *Fas* mRNA and protein expression of *Fas* transfected cells was obviously increased. In anti-*Bcl-2* transfected cells, *Bcl-2* mRNA expression was

inhibited slightly, but *Bcl-2* protein was blocked greatly. Drug sensitivity assay demonstrated that transfectants were more sensitive to VCR, CDDP and 5-FU than non-transfectants. It was concluded that suppression of apoptosis play an important role in inducing MDR of tumor cells, and promoting apoptosis could reverse MDR to some extent.

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