Basic Investigation

EXPRESSION AND REVERSION OF DRUG RESISTANCE-AND APOPTOSIS-RELATED GENES OF A DDP-RESISTANT LUNG ADENOCARCINOMA CELL LINE

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ABSTRACT

Objective: To investigate the co-expression of drug resistance- and apoptosis-related genes of cisplatin (CDDP)-selected lung adenocarcinoma cell line A_{549}^{DDP} for compared to the parental cell line A_{549}, and reverse of drug resistance by antisense s-oligodeoxynucleotides (S-ODNs) of differentially expressed genes. Methods: Sense and antisense S-ODN were transferred into A_{549}^{DDP} cells by lipofectin. The expression of drug resistance and apoptosis related genes was examined by RT-PCR, immunocytochemistry and flow cytometry, respectively. Apoptostic cells were identified by DNA electrophoresis and terminal deoxynucleotidyl transferase (TdT)-mediated biotin dUTP nick end-labeling(TUNEL). Drug resistance of tumor cells was detected by a cell viability (MTT) assay. Results: The expression of bcl-2 was positive and that of multidrug resistance-associated protein (MRP) at mRNA and protein level was increased in A_{549}^{DDP} compared to A_{549} cells. MDR1, c-myc and topoisomeras II (TOPO II) were similarly co-expressed in two cell lines. Both cell lines were negative for c-erbB-2 expression. In A_{549}^{DDP} cells, the expression of bcl-2 and MRP was significantly inhibited by their respective antisense S-ODNs. Antisense S-ODNs could also decrease significantly drug resistance of A_{549}^{DDP} cells to CDDP by promoting cell apoptosis. Conclusion: Both intrinsic and acquired drug resistance were involved in co-expression of multiple MDR-related genes in lung adenocarcinoma. Co-operation of bcl-2 and MRP genes appeared to play an important action to confer the resistance of A_{549}^{DDP} cells to CDDP. Their antisense S-ODNs are responsible for the decrease of drug resistance of this cell line by promoting apoptosis.

Key words: Lung neoplasm, A_{549} and A_{549}^{DDP} cell lines, Apoptosis, Antisense oligoxynucleotide, Drug resistance-gene

Resistance of cancer cells to cytotoxic chemotherapy is a common problem in patients with cancer and a major obstacle to effective treatment of disseminated neoplasm. Several molecular mechanisms have been associated with multidrug resistance (MDR) in experimental tumor models. These include 1) enhanced efflux of drug by transporter proteins such as P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP) and human major vault protein (LRP) which might play an important role in vesicular sequestration of drug;[1-3] 2) alterations of drug targets such as DNA topoisomeras II (TOPO II);[4,5] and 3) increased detoxification of compounds, for instance, by the glutathione (GSH) system.[5,6] Recently, a growing family of anti-apoptosis gene products has been showed to modulate drug cytotoxicity by regulating drug-induced apoptosis, for example, overexpression of bcl-2, down-regulation of bax, overexpression of c-myc and mutation or deletion of p53, which have all been demonstrated to inhibit apoptosis and reduce sensitivity of tumor cell to a variety of anticancer agents.[7-9] Although the co-existence and the significance of Pgp, MRP, TOPO II-MDR and apoptosis-MDR have been documented in leukemia cells.[10] but not in non small cell lung cancer (NSCLC) cells. The objective of the present study was to investigate whether multidrug resistance-and apoptosis-related genes could co-exist in CDDP-

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resistant lung adenocarcinoma cell line A_{549}^{DDP} and its parental cell line A_{549} , detect differentially expressed genes between the two cell lines, and investigate the possibility to reverse drug resistance with antisense S-oligodeoxynucleotides (S-ONDs) of the differentially expressed genes.

MATERIALS AND METHODS

Primers and S-Oligodexynecleotide

The primers of MRP, bcl-2, c-myc, c-erbB-2, TOPO II, MDR_1 for RT-PCR were as follows;

MRP: 5'-TCTCTCCCGACATGACCGAGG-3',
5'-CCAGGAATATGCCCCGACTTC-3',
MDR_1: 5'-CCCATCATTGCAATAGCAGG-3',
5'-GTTCAAACTTCTGCTCCTAG-3',
TOPO II: 5'-CTTGTACTGCAGACCCACA-3',
5'ATAATAGAATCAAGGGAATTCCCAAA CTCGA-3' ,
bcl-2: 5'-CGACGACTTCTCCCGCCGCTACCGC-3',
5'-CCGCTAGCTGGGGCCGTACAGTTCC-3',
c-erbB-2: 5'-CCCACGTCCGTAGAAAGGTA-3',
5'-TGAACAATACCACCCCTGTC-3',
β-actin: 5'-AGCATCCTAGAACTCTGTGC-3',
5'-ATTTCGGACCCCTGAACAATA-3'

The product length were 290, 167, 223, 318, 180 and 240bp, respectively. β-actin was as a positive control for the RT-PCR primers, and the length was 400bp.

The MRP and bcl-2 antisense S-ODNs used in this study were complementary to the coding region and translation initiation site of mRNA,[11,12] respectively. They were all synthesized using an Applied Biosystems 392 DNA Synthe Sizer. In addition, their sense S-ODNs were also prepared. The sequences were:

Bcl-2 sense: 5'-CAGCGTGCGCCATCCTTCCC-3'
antisense: 5'-GGGAAGGATGGCGCACGCACGCTG-3'
MRP sense: 5'-ACGACAAGCACGGGGGCGGC-3' anti-sense: 5'-TGC-TGTTCGTGCCCCCGCCG-3'

Cell Culture and Treatment

A_{549}^{DDP} was a cisplatin-resistant human lung adenocarcinoma cell line which was established in our laboratory by stepwise increasing concentration of cisplatin to 100 μmol/L. The 16.5 times drug resistant A_{549}^{DDP} cell and its parental cell A_{549} were cultured in Dulbeceo Medium (DMEM) supplemented with 10% fetal calf serum in a 5% CO_2 and 95% air incubator at 37°C, which were negative for mycoplasma contamination. In addition, SCLC cell line was also cultured using above method.

The cells were cultured during logarithmic grow in 24-well plates until 60%~70% confluent and then were washed twice with serum-free DMEM medium before the addition of lipofectin/S-ODN complexes, Lipofectin (GIBCO/BRL) (working concentration 2 μg/ml) and S-ODN (20 μmol/L) were allowed to form complexes in serum-free DMED medium, at room temperature for 15 min after gentle mixing. The cells were incubated with lipofectin/S-ODN at 37°C for 6h, washed once with DMEM with 10% fetal calf serum and incubated in fresh DMEM with 10% fetal calf serum until harvested. The trial was designed to divide into seven groups: bcl-2 sense and antisense, MRP sense and antisense, bcl-2+MRP sense and antisense and untreated group (the substitution of normal culture medium for lipofectin/S-ODN).

RNA Isolation and RT-PCR

Total RNA was isolated from 2.5 × 10^6 cells by guanidinium isothiocyanate (GITC)-acid phenl extraction as described.[13] The precipitated RNA pellet was dissolved in 10 Aμl free-RNase waters. The RNA from 1.25×10^6 cells was reverse transcribed (RT) in a total volume of 50 μl using, 10U/μl of Superscript reverse transcriptase (GIBCO), 1U/μl RNasin (Sigma), 2.5μmol/L random primer (Sigma), 1mmol/L of each dNTP (Sigma), 10mmol/L DTT (GIBCO), 1×first strand buffer (50mmol/L Tris-HC1 PH8.0 75mmol/L KC1, 3mmol/L $MgC1_2$) freshly diluted from 5×stock (GIBCO BRL) at 37°c for 60 min. Aliquots (8μl) of RT products were used for PCR amplification in a volume of 50μl containing 1mmol/L $MgC1_2$, 0.12mmol/L of each dNTP, 1× buffer (20mmol/L Tris-HC1 PH8.0, 50mmol/L KC1), IU of Taq polymerase (GIBCO BRL) and 1μmol/L of both the upstream and downstream primers and β-actin PCR primers. Amplification was carried out in a pre-denaturation at 94°C for 5 min, then 29–30 cycles of denaturation at 94°C for 30 second, annealing at 55°C for 1 min and extension at 72°C for 1 min and then a final extension at 72°C for 5 min. Each PCR product was separated on a 1.8% agarose gel in TBE at 150V for 1h after staining with 0.5 μg/ml ethidium bromide. The negative was then scanned by a Molecular Dynamics Densitometer (Sunnyvale, CA) and got A value. The mRNA relative quantity of the target gene mRNA was calculated by the ratio of A value of target gene and β-actin.

Eastimation of Target Gene Protein by ICH and FCM

Immunohistochemistry (ICH): Expression of bcl-2, c-myc, c-erbB-2, MDR_1 or MRP proteins was detected by Strepavidin-peroxidase (SP) method using monoclonal antibody bcl-2, C-myc, C-erbB-2, C_{219} (Santa Cruz, diluted 1: 20 to 1: 40) and MRP_{507} (manufactured in our Lab, diluted 1: 50).

FCM: Flow cytometric analysis was also performed to measure the percentage of the positive cells of the drug resistance- and apoptosis-related protein as described.[11] Briefly, cells that have been treated by S-ODNs were harvested by centrifugation and washed in PBS PH7.4, permeated with 0.5ml of ice-cold 70% ethanol and incubated for at least 1h at 4°C, washed twice in PBS, incubated for 45 min at 4°C with monoclonal antibody as above, and then washed twice in PBS, incubated for 30 min at 4°C with FITC-conjugated monoclonal mouse IgG antibody, and washed twice in cold PBS and analyzed immediately using the FACSCAN. Flow Cytometer, Consort 30 and Lysis software (Becton Dickinson, Oxford, UK). The FITC-conjugated IgG was replaced by PBS as a negative control.

MTT Assay

To detect drug resistance, the cells were transferred to a 96-well microplate in 1×10^4 cells/well. The medium whjich contained different concentrations of cisplatin (0, 20, 40, 80, 100, 200 μmol/L) was frequently replaced every 4–6h for about 2 days until the cells in control well were confluent. The MTT assay was performed as reported by Huang et al.[10] IC_{50} was defined as the concentration of cytotoxic drug that caused 50% inhibition of cell growth compared to untreated control. The relative drug resistance was determined by comparing the IC_{50} of transfected cells and untransfected cells.

Assessment of Apoptosis and Proliferation

***DNA fragmentation assay*:** The pattern of DNA cleavage was analyzed by agarose gel electrophoresis. In briefly, cells (1×10^6) were lyzed with 0.5 ml lysis buffer (0.5% Nonidet P-40, 20 mmol/L EDTA, 50 mmol/L Tris-HCl, PH7.5), followed by the addition of RNase A (Sigma) at a final concentration of 50 mg/L and 1% SDS, and incubated for 1h at 37°C, treated with 50 μg/ml proteinase K for 1h at 56°C. After the addition of 4 μl loading buffer, 20 μl samples in each lane were subjected to electrophoresis on a 1.2% agarose at 50V for 2.5~3h, and DNA was stained with ethidium bromide.

***Apoptosis index*:** This was measured by terminal deoxynucleotidyl transferase (TdT)-mediated biotin dUTP nick end-labeling (TUNEL). Sections were fixed in 4% paraformaldehyde at room temperature for 15 min and then the following incubation steps were carried out: blocking of endogenous peroxidase by placement in 1% hydrogen peroxide, addition of 0.3 μmol/L biotin-16-dUTP (Boehringer) plus 10 units/ml terminal transferase enzyme (Boehringer) at 37°C for 1h, the addition of horseradish peroxidase-conjugate antibody (Boehringer) (1: 200) for 30 min at room temperature, development with 0.1% diaminobenzidine/0.01% H_2O_2 (Sigma) and counterstaining with haematoxylin. Two control sections were include: A negative control without DNA polymerase I, and a positive control of peripheral blood lymphocytes treated by dexamethasone.

Detection of Proliferative Cell

SP method was used to detect Ki-67 protein expression in the proliferating cells.

Statistical Analysis

Statistical comparisons among different groups were determined by a two-tailed t-test assuming equal variance.

RESUTLS

Expression of MDR-related Genes in A_{549}^{DDP} Cell Lines

The exprtession of bcl-2 mRNA and protein was positive (0.49± 0.03, 45.5±1.3%) and that of MRP was increased in A_{549}^{DDP} cells (0.89± 0.02, 82.4± 2.1%) compared to A_{549} cells (bcl-2: negative. MRP mRNA and protein levels: 0.37±0.03, 35.8±1.1%, respectively) ($P<0.05$). MDR1, c-myc and TOPO II were similarly co-expressed in the two cell lines ($P>0.05$). Both cell lines were negative for c-erbB-2 expression (Figure 1, 2). Flow cytometry method further testified that the expression of MRP protein was 2.3-fold higher in A_{549}^{DDP} than in A_{549}, and bcl-2 protein was positive in A_{549}^{DDP} but negative in A_{549} (Table 1). No significant difference of the expression of c-myc, C_{219} or c-erbB-2 protein was observed between the two cell lines ($P>0.05$)(Table 2).

Effects of S-ODN on MDR-related mRNA and Protein Level in A_{549}^{DDP} Cell Line

Subsequent experiment was solely focused on.

Table 1. The levels of MDR-and apoptosis related proteins by detected by FCM in A_{549}^{DDP} cells treated with S-ODN ($\bar{x}\pm s$)

Group	Bcl-2	c-myc	MRP	P-gP	c-cerbB-2
A_{549} cell	Negative	59.1± 1.2	35.8± 1.1	33.2± 2.1	Negative
Untransfected	45.5± 1.3	52.1± 3.5	82.4± 2.1	31.2± 2.3	
Sense bcl-2	42.4± 2.1	53.5± 2.4	85.7± 1.2	34.1± 1.7	Negative
Antisense bcl-2	*17.7± 1.1	*30.2±1.3	80.7± 1.6	30.4± 1.5	Negative
Sense MRP	41.1± 2.5	51.3± 2.7	82.8± 1.2	28.5± 1.4	Negative
Antisense MRP	44.2± 1.2	29.2± 1.1	38.2± 1.3	32.6± 2.1	Negative
Sense bcl-2+MRP	40.9± 2.3	56.4± 3.2	80.1± 1.5	31.7± 1.4	Negative
Anstisense bcl-2+MRP	*15.2±1.1	*20.7± 2.3	*31.3± 2.1	26.2± 2.5	Negative

*$P<0.05$

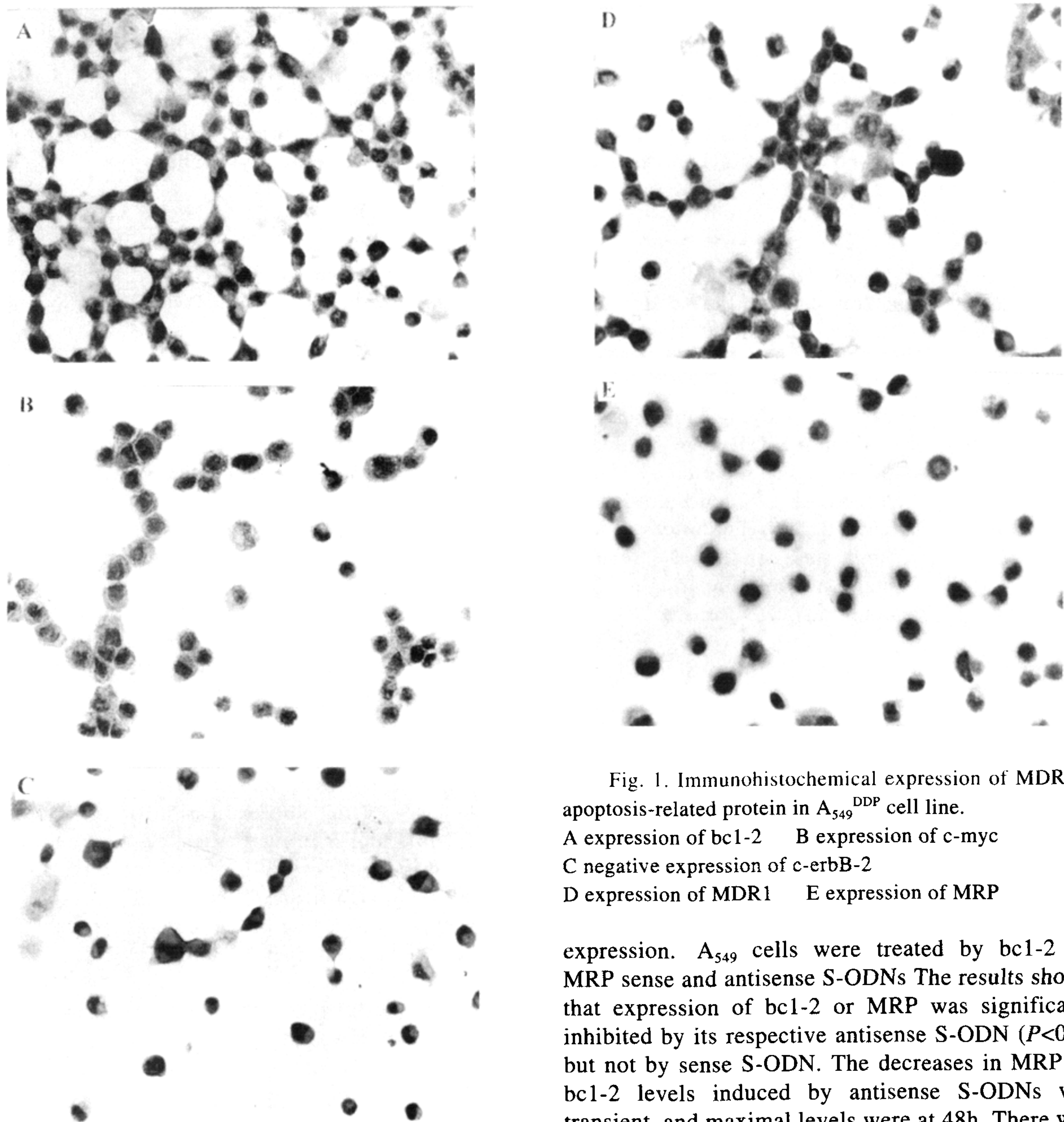

Fig. 1. Immunohistochemical expression of MDR-and apoptosis-related protein in A_{549}^{DDP} cell line.
A expression of bcl-2 B expression of c-myc
C negative expression of c-erbB-2
D expression of MDR1 E expression of MRP

expression. A_{549} cells were treated by bcl-2 and MRP sense and antisense S-ODNs The results showed that expression of bcl-2 or MRP was significantly inhibited by its respective antisense S-ODN ($P<0.05$) but not by sense S-ODN. The decreases in MRP and bcl-2 levels induced by antisense S-ODNs were transient, and maximal levels were at 48h. There was

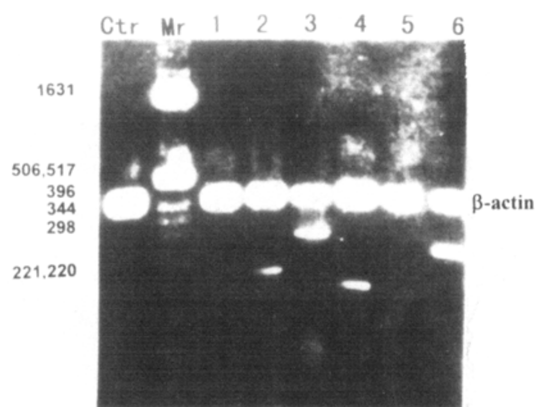

Fig. 2a. RT-PCR analysis of expressions of MDR-and apoptosis-related genes in the A_{549} cell line. Ctr: Control group (untreated by S-ODN). Mr: Marker, PBR322/Hinf 1. Lane 1: bcl-2. Lane 2: c-myc. Lane 3: MRP. Lane 4: MDR1. Lane 5: c-erbB-2. Lane: TOPO II.

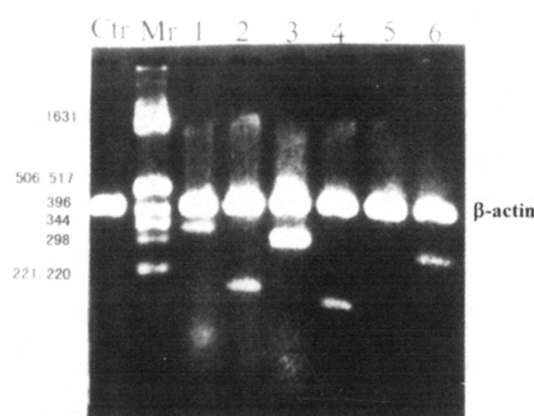

Fig. 2b. RT-PCR analysis of expressions of MDR-and apoptosis-related genes in the A_{549}^{DDP} cell line. Ctr: Control group (untreated by S-ODN). Mr: Marker, PBR322/Hinf I. Lane 1: bcl-2. Lane 2: c-myc. Lane 3: MRP. Lane 4: MDR1. Lane 5: c-erbB-2. Lane: TOPO II.

Table 2. Apoptosis index and proliferative cell rate in A_{549}^{DDP} cells treated by 100 μmol/L CDDP for 48h after treated with S-ODN ($\bar{x} \pm s$)

Cells	Apoptosis Index	Ki-67
Untransfected	10.6± 0.8	74.3± 1.8
Bcl-2 sesnse	15.6± 1.1	69.4± 2.4
Bcl-2 antisense	*28.4± 2.7	58.3± 3.1
MRP sense	11.8± 0.6	70.1± 3.6
MRP antisense	*23.4± 1.4	53.1± 1.6
Bcl-2+MRP sense	14.3± 0.9	54.8± 2.3
Bcl-2+MRP antisense	*35.2± 1.2	*41.3± 1.8

*$P<0.05$

not a notable alteration of the expression of MDR c-myc and TOPO II in sense, antisense and untreated groups ($P>0.05$). Similar decreased levels of bcl-2 and MRP mRNA were found between the cells co-treated and treated alone by MRP or/and bcl-2 antisense S-ODN. However, the decrease of c-myc mRNA in the cells co-treated by antisense S-ODNs was more remarkable than in the cells treated alone (Figure 3)

Effects of S-ODNs on Drug Resistance in A_{549}^{DDP}

Drug resistance of A_{549} cell line was 3.5 times as many as small cell lung cancer (SCLC) cells. In untreated A_{549}^{DDP} cells, the degree of resisstance to cisplatin calculated from IC_{50} was 16.5 times. After A_{549}^{DDP} cells were treated by bcl-2 or/and MRP antisense S-ODN, their drug resistance cut down to 6.5 times, 7.2 times and 4.8 times, respectively ($P<0.05$), but the drug resistance in the sense-treated groups was similar to 16.5 times.

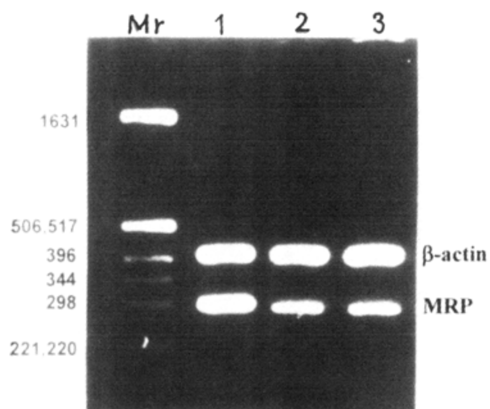

Fig. 3a. RT-PCR analysis of bcl-2 Mrna expression in the cells treated by antisense S-ODN. M: molecular weight marker (PBR322/Hinf I). Lane 1: The cells untreated. Lane 2: The cells treated with bcl-2 antisense. Lane 3: the cells co-treated with bcl-2 and MRP antisense.

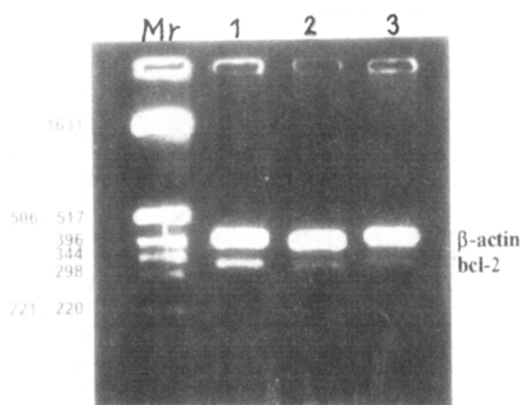

Fig. 3b. RT-PCR analysis of MRP mRNA expression in the cells treated by antisense S-ODN. M: molecular weight marker (PBR322/Hinf I). Lane 1: The cells untreated. Lane 2: The cells treated with bcl-2 antisense. Lane 3: The cells co-treated with bcl-2 and MRP antisense.

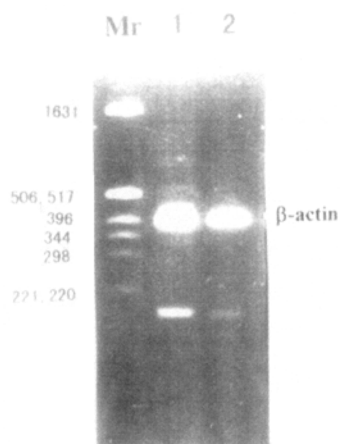

Fig. 3c. RT-PCR analysis of c-myc mRNA expression in the cells treated by antisense S-ODN. M: molecular wseight marker (PBR322/Hinf I). Lane 1: The cells treated with bcl-2 antisense. Lane 2: the cells c0-treated with bcl-2 and MRP antisense.

Effects of S-ODN on Apoptosis and Proliferation in A_{549}^{DDP} Cells

Though a dose and concentration-dependent increase in apoptosis cells and the decrease in Ki-67 positive cells were found in each group, no statistically significant difference (P>0.05) and a typical ladder of apoptosis-related were observed between antisense-, sense-treated and untreated group, except for the group exposed to 100 μmol/L cisplatin for 48h after co-treated with bcl-2 and MRP antisense S-ODN (P<0.05)(Figure 4). When the cells of each groups were exposed to cisplatin for 72h, most cells collapsed and remain cells had a number of granules, and few Ki-67 positive and apoptotic cells existed.

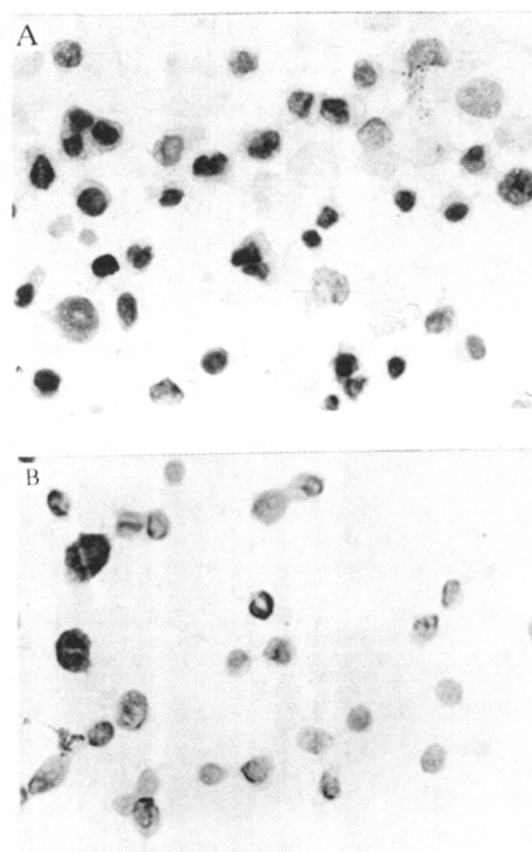

Fig. 4. Detection of apoptostic cell stained by TUNEL technique in the A_{549}^{DDP} cells. A: untransfected cells. B: bcl-2 antisense-transfected cells. (×400)

DNA Ladder and Apoptosis Index

Apoptotic cells in TUNEL analysis showed the nuclear positive and had usually small nucleus, with the fragmented chromatin being distributed throughout the nucleus (Figure 4). Though a dose and concentration-dependent increase in apoptosis cell and decrease in Ki-67 positive cells were observed in each group, no statistically significant difference (P>0.05) and a typical ladder of apoptosis-related were found between antisense-, sense-treated and untreated group, except for the group exposed to 100 μ mol/L cisplatin for 48h after co-treated with bcl-2 and MRP antisense S-ODN (P<0.05) (Figure 5). When the cells of these groups were exposed to cisplatin for 72h, the most of the cells colapsed and remain cells had a number of granules, and few Ki-67 positive cells.

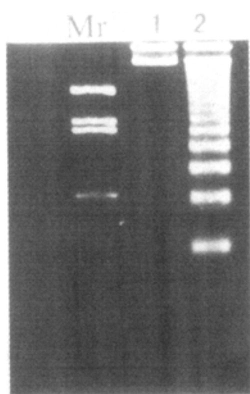

Fig 5. Detection of DNA ladder in a 1.8% agarose gel by DNA fragmentation assay. Mr: Molecular weight marker. Lane 1: the cells transfected by bcl-2 antisense. Lane 2: The cells co-transfected by bcl-2 and MRP antisense.

DISCUSSION

Recent studies have indicated the clinical drug resistance to chemotherapy is multifactorial or/and heterogeneous in most tumors.[9] Any single mechanism fails to interpret satisfactorily intrinsic and extrinsic drug resistance of lung cancer. The present study showed that MDR_1, MRP, TOPO II and c-myc were co-expressed in A_{549} cell line, whose drug resistance was 3.5 times as many as SCLC cell line lacking of the expressions of MDR_1, MRP and TOPO II. The results supported that the intrinsic drug resistance in A_{549} cell line might be associated with cooperation of MDR related genes above-mentioned. Whereas c-erbB-2, confirmed to link with the intrinsic resistance in NSCLC, breast cancer, etc.,[14,15] was not found to mediate the drug resistance of A_{549} cell line.

A_{549}^{DDP} cells were found to have the positive bcl-2 and 2.3-fold higher MRP expression compared to A_{549} cells. Our previous study have demonstrated the increase of GSH/GSTs content was concerned with the resistance of A_{549}^{DDP} cells. Therefore, it was inferred that the co-operation of bcl-2, MRP and GSH/GSTs might contribute to the acquired resistance in A_{549}^{DDP} cells. Recent studies have showed that the over-expression of bcl-2 protein inhibits apoptosis by a variety of antioxideant functions and decreases the susceptibility of tumor cells to most anti-tumor drug, which contributes to multidrug resistance.[16,17] MRP could act as a GS-X pump which transports drugs conjugated with GSH, and confer the multidrug resistance phenotype of A_{549}^{DDP} cells.[18]

Effects of bcl-2 and MRP antisense S-ODN on drug resistance of A_{549}^{DDP} cell line, by inhibiting the expression of bcl-2 and MRP, respectively, further demonstrated that the overexpression of bcl-2 and MRP genes were responsible for the acquired resistance of A_{549}^{DDP} cells to cisplatin. Consequently, the use of antisense ODNs provides an attractive approach to abolish or reverse drug resistance. Though the dose and concentration-dependent increase in apoptosis or decrease in proliferation were detected in each group cells, statistically significant changes were only observed in the cells exposed to 100 μmol/L cisplantin for 48h after treated by bcl-2 or/and MRP antisense S-ODN, in which typical DNA ladder could also be detected. The results testified that effectively inhibited in the expression of the bcl-2 and MRP might reduce the resistance of A_{549}^{DDP} cells to cisplatin by promote cell apoptosis and inhibiting their proliferation.

In conclusion, both intrinsic and extrinsic drug resistance were involved in co-expression of multiple drug resistance- and apoptosis genes. Co-operations of bcl-2 and MRP genes appear to play an important action to confer the resistance of A_{549}^{DDP} cells to CDDP. Their antisense S-ODNs are responsible for the decrease of drug reisstance of this cell line by promoting apoptosis.

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