Clinical Observations

THE CORRELATION BETWEEN THE EXPRESSION OF MULTIDRUG RESISTANCE RELATED GENE AND CELL APOPTOSIS AND CLINICAL SIGNIFICANCE IN NON-SMALL CELL LUNG CANCER

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ABSTRACT

Objective: To explore the correlation and clinical significance between expression of MDR (multidrug resistance) related gene MRP, MDR_1, C-erbB-2 and cell apoptosis in non-small cell lung cancer (NSCLC). Methods: RT-PCR, Immunohistochemistry were used to examine the expression of mRNA and protein in the MDR and apoptosis related gene. Apoptosis cells were assayed by Terminal deoxynucleotidyl transferase (TdT)- mediated biotin dUTP nick end-labeling (TUNEL). Results: The positive rates of MRP, MDR_1, C-erbB-2, bcl-2, C-myc mRNA in 63 cases NSCLC were 81.0% (51/63), 38.1%(24/63), 47.6%(30/63), 65.1%(41/63), 76.2%(48/63) respectively. Their levels were higher than those of corresponding proteins (74.6%, 34.9%, 46.0%, 61.9%, 71.4%, respectively). The significant association was found between the mRNA level and the protein expression (r =+0.764, $P<0.02$). The C-myc expression in 2 cases adjacent and benign lung tissue were light positive, and another 3 cases were negative. The positive correlation were demonstrated between C-myc and C-erbB-2 (r=+0.547, p=0.001) as well as bcl-2 and C-erbB-2 (r =+0.486, p=0.023) in NSCLC. There is no any correlation among bcl-2, C-myc and MRP or MDR1. There exists inverse correlation between apoptotic index and bcl-2 (r = -0.587, p = 0.017), and no any correlation among apoptotic index and MRP or MDR1 or C-erbB-2 or C-myc. The average apoptotic index were higher in the effective chemotherapy group (27.2± 2.1, 30.5±1.8) than that in the non-effective chemotherapy group (9.4± 1.3, 12.6± 2.4) with adenocarcinoma and squamous cell carcinoma (p =0.01, p=0.004). The positive rates of bcl-2, MRP, C-erbB-2 expression in the effective chemotherapy group (31.8%, 40.9%, 22.7%, respectively) were lower than those in the non-effective chemotherapy group (77.4%, 90.3%, 67.7%, respectively) (p=0.036, p=0.012, p=0.01), but MDR_1 and C-myc expression have no any significant difference (p=0.067, p=0.282). The median survival time in the patients with coexpression of more than three MDR and/or apoptosis related genes are shorter (8.6 months) than that in those patients with coexpression of less than three MDR and/or apoptosis related genes (15.5 months)(p=0.01). Conclusion: The multidrug resistance in NSCLC is not only related to many drug resistance genes, but also involved in cell apoptosis and apoptosis related gene expression. The coexpression of MDR and apoptosis related gene is related to the survival time.

Key words: Lung cancer, Multidrug resistance, Apoptosis, Related gene

The mechanisms about multidrug resistance (MDR) which are studied widely in present include overexpression of multidrug resistance protein such as MDR_1/P-gp, MRP (multidrug resistance related protein), LRP (lung resistance protein), increased detoxification of Glutathione / Glutathione – S – transferase II.[1-3] Recent studies have showed that inhibition of cell apoptosis and overexpression of apoptosis related gene is another reason for the MDR.[4] But there are few reports about the correlation between the expression of MDR related gene and cell apoptosis and their prognostic

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significance in lung cancer tissues. In our experiment, the techniques of RT-PCR, Immunohistochemistry, Terminal deoxynucleotidyl transferase (TdT)-mediated biotin dUTP nick end labeling (TUNEL) of apoptotic cells assay were used to explore the correlation among the expression of MDR and apoptosis related gene in the frozen lung cancer tissues as well as the relation with response to chemotherapy and prognosis.

MATERILAS AND METHODS

Patients and Tumor Samples

Specimens from 63 patients with non-small cell lung cancer untreated with chemotherapy were obtained by biopsing of metastatic lumps outside the thorax or undergoing surgery for primary tumors. The tissues were immediately frozen and stored in liquid nitrogen for use. 21 patients were female and 42 were male. The median age of the patients (at time of biopsy or surgery) was 56.5 years (range 17-74). The tumor-node-metastasis (TNM) stage was 6 in stage I, 12 in stage II, 6 in stage IIIa, 16 in stage IIIb, and 23 in stage IV, respectively the 53 patients were treated with chemotherapy after biopsed or operated surgery (at least 2-3 cycles).The mian chemo-regimens were asplatin based. The response was evaluated according to the WHO's criterion. Those who got to the complete respond (CR) or partial respond (PR) for at least four weeks were effective to chemotherapy. The 22 cases were response to the treatment (13 were adenocarcinoma and 9 were squamous cell carcinomas), 31 cases were no effective. The median following-up duration was 31 months (1-77 months). 38 died during follow-up. All were contrasted with the adjacent and benign lung tissue.

RNA Isolation and RT-PCR

Frozen samples 50mg stored in the fluid nitrogen were homogenized in 100μl 0.01 mol/L PBS buffers. Total RNA was extracted and RT-PCR was performed as previously described.[5] Every target gene primer and the annealing temperature was seen in the Table 1. 10 μl each polymerase chain reaction (PCR) product was separated on a 1.8% agarose gel in TBE at 150 v for 1 h after staining with 0.5 μg/ml ethidium bromide. The amplification product was then scanned by a Molecular Dynamics Densitometer (Sunnyvale, CA) and got A value. Relative quantity of target gene mRNA was calculated by the ratio of A value of target gene and β-acting.

Table 1. Primer sequences of target genes and PCR reaction condition

Target gene	Primer sequence	T	N	Amplification product
MRP	S TCTCTCCCGACATGACCGAGG	55	29	291
	As CCAGGAATATGCCCCGACTTC			
MDR_1	S CCCATCATTGCAATAGCAGG	55	29	167
	As GTTCAAACTTCTGCTCCTAG			
C-erbB-2	S CCCACGTCCGTAGAAAGGTA	55	30	240
	As TGAACAATACCACCCCTGTC			
Bcl-2	S CGACGACTTCTCCCGCCGCTACCGC	58	35	318
	As CCGCTAGCTGGGGCCGTACAGTTCC			
C-myc	S ATTCTCTGCTCTCCTGGAC	55	30	180
	As TCCAGACTCTGACCTTTGCC			
β-actin	S AGCATCCTAGAACTCTGTGC	55	29	400
	As ATTTCGGACCCCTGAACATA			

T: annealing temperature, N: Cycle times

Apoptotic Cells were Evaluated by TUNEL Assay

The apoptotic cells were detected by TUNEL as described.[5] The peripheral blood lymphocytes treated with dexamethzazone was as positive control. The adjacent and benign lung tissues were as negative control. The apoptotic index (percentages of apoptotic cells among tumor cells) was determined by comparing the number of apoptotic cells and viable tumor cells in 5 HPFs.

ImmunohistoChemistry

Monoclonal antibodies of bcl-2, C-myc, C-erbB-2, C_{219}, (1:20 - 1:40) were the products of Santa cruz Company. MRP antibody was constructed and purified by our laboratory(1:50). The samples were stained by SP method.

Statistical Analysis

Correlation among drug resistance and apoptosis related gene such as bcl-2, MRP and C-erbB-2 were determined by Spearman's rank correlation. Kaplan-Meier and long-rank test were used to analyze survival time, and statistical comparisons among different groups were determined by correct x^2-test. $P<0.05$ was considered significant.

RESULTS

Expression of MDR and Apoptosis Related Gene in NSCLC

The positive rates of MRP, MDR1, C-erbB-2, C-myc mRNA in 63 cases NSCLC were 81.0% (51/63), 38.1% (24/63), 47.6%(30/63), 65.1%(41/63), 76.2%(48/63), respectively (Figure 1), and were slightly higher than their corresponding protein levels (74.6%, 34.9%, 46.0%, 61.9%, 71.4%, respectively). The significant correlation was found between the mRNA level and the protein expression ($r \geqslant +0.764$, $P<0.02$). Expression of bcl-2, C-myc and MRP were main in cytoplasm, expression of C-erbB-2, MDR1/P-gp were in cell membrane and cytoplasm (Figure 2). 5 cases adjacent and benign lung tissues were negative in expression of bcl-2, MRP, C-erbB-2 and MDR_1.

Apoptotic cells showed nuclear labeling and were distributed diffusely (Figure 3). There exists inverse correlation between apoptotic index and bcl-2 (expression $r = -0.587$, $p=0.017$), and no any relativity among apoptotic index and MRP or MDR1 or C-erbB-2 or C-myc ($r<0.375$, $P>0.05$).

Expression of MDR and Apoptosis Related Gene and the Chemotherapy Response in NSCLS

The average apoptotic index were higher in the effective chemotherapy group (27.2± 2.1, 30.5± 1.8) than that in the non-effective chemotherapy group (9.4± 1.3, 12.6± 2.4) with adenocarcinoma and squamous cell carcinoma ($p=0.001$, $p=0.004$). The positive rates of bcl-2, MRP, C-erbB-2 expression in the effective chemotherapy group (31.8%, 40.9%, 22.7%, respectively) were lower than those in the non-effective chemotherapy group (77.4%, 90.3%, 67.7%, respectively)($p=0.036$, $p=0.012$, $p=0.01$), but MDR_1 and C-myc expression have no any significant difference between effective and non-effective chemotherapic groups (36.4% and 68.2% versus, 32.3% and 74.2, respective)($P=0.067$, $P=0.282$).

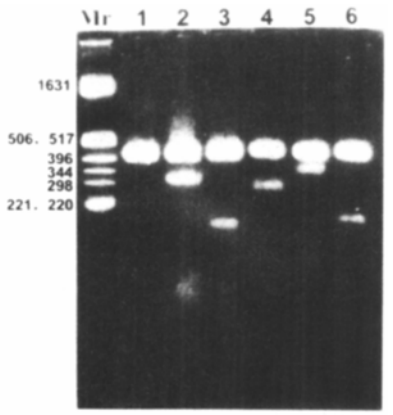
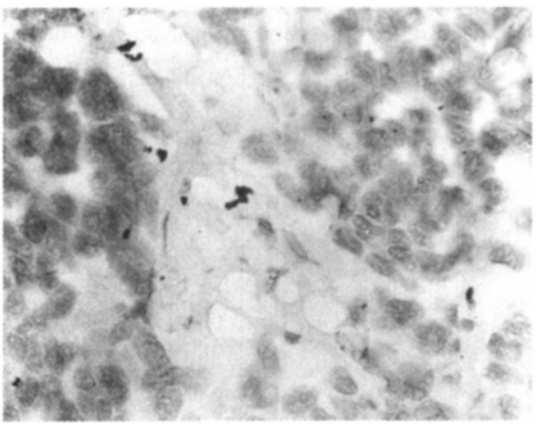
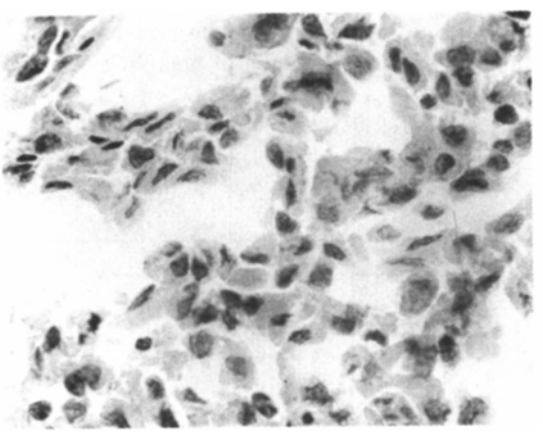


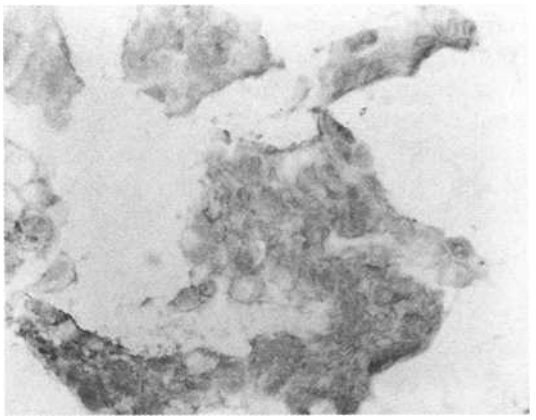
Fig. 1. mRNA expression of multidrug resistance and apoptosis related gene in NSCLC. Mr: PBR322/Hinf I. Lane 1: negative control; Lane 2: MRP; Lane 3: MDR1; Lane 4: C-erbB-2; Lane 5: bcl-2; Lane 6: C-myc Relationship among Apoptotic Index and MDR or Apoptosis Related Gene Expression



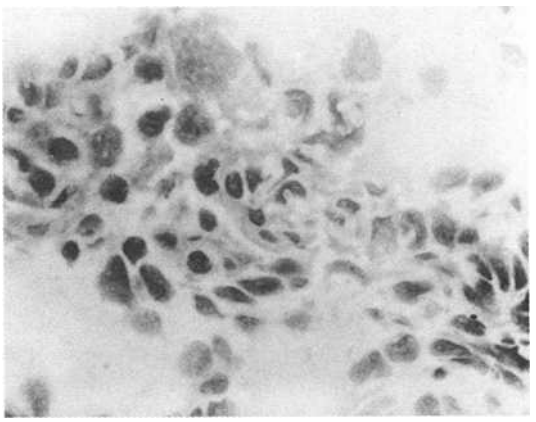
A: bcl-2 positive expression in lung adenocarcinoma



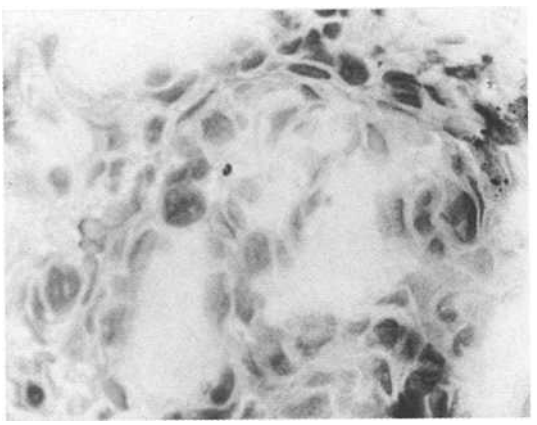
B: C-myc expression in squamous carcinoma.



C: C-erbB-2 expression in lung alveolar cell cancer



D: MDR_1/P-gp expression in squamous carcinoma



E: MRP expression in squamous carcinoma

Fig. 2. Protein expression of multidrug resistance and apoptosis related gene in NSCLC.

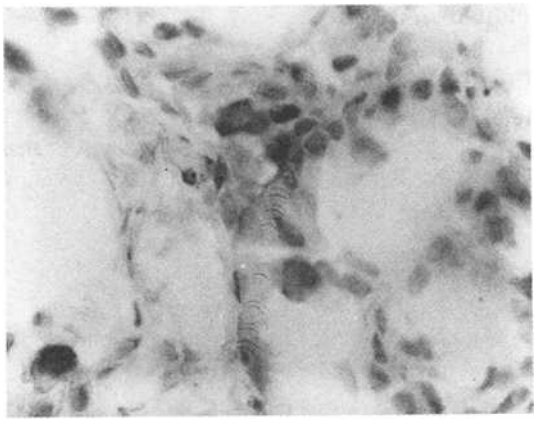


Fig. 3. Apoptotic cells assay in lung adenocarcinoma (TUNEL × 400)

Analyze of Survival Time

Among the above MDR and apoptosis related genes, positive rate of one kind of gene expression was 8.0%(4/50), that of two kinds of gene coexpression was 16.0%(8/50), that of three kinds of genes coexpression was 40.0%(20/50), that of more than three kinds of genes coexpression was 72.0%(36/50). The median survival time in the patients with coexpression of more than three MDR and /or apoptosis related genes are shorter (8.6 months) than that in those patients with coexpression of less than three MDR and/or apoptosis related genes (15.5 months, $P<0.01$).

DISCUSSION

The clinical MDR phenotypes are very complicated. Many molecular mechanisms have been associated with MDR in lung cancer. The traditional MDR mechanisms main include: 1. Enhanced the efflux of drugs from intracellular by transporter proteins or by the transport barrier of drugs between nuclear and cytoplasm to reduce the drug concentration, such as the role of MDR_1, MRP, LRP as well as their coding proteins; 2. Increased detoxification of cytotoxic agents, e.g. by the role of

Table 2. Correlation between chemotherapy response and expression of drug resistance as well as apoptosis and related gene in NSCLC

MDR related gene positive rate(%)	The effective chemotherapy group	The non-effective chemotherapy group
*bc1-2	31.8% (7/22)	77.4% (24/31)
C-myc	68.2% (15/21)	74.2% (23/31)
*MRP	40.9% (9/22)	90.3% (28/31)
MDR_1	36.4% (8/22)	32.3% (10/31)
*C-erbB-2	22.7% (5/22)	67.7% (21/31)

*$P<0.05$

GSTs; 3. Increased the ability of DNA repair, e.g., alterations in quantity and quality of DNA topoisomerase II.[1-3] Whereas the MDR mechanism which cell apoptosis mediate is different from the above.[6,7] This moment drug can be transported into intracellular and damage cell, but due to the overexpression of apoptosis related gene and apoptosis being suppressed, this damage can be converted into invalid signal and result in tumor cells not to die or the mortality to be decreased. Huang[8] reported that the overexpression of MDR and apoptosis related gene coexist in the drug resistant HL-60 cell line and induce MDR. Our study showed MRP, MDR1 C-erbB-2, bcl-2 and C-myc were coexpressed in pre-chemotherapy NSCLC, which demonstrated preliminarily that there are many factors in the clinical MDR of NSCLC. Expression of MRP, C-erbB-2 and bcl-2 have significant differences between the effective and non-effective chemotherapy groups effective group, therefore detection of these three genes in pretreatment patients can be useful to estimate the response to chemotherapy and prognosis.

Although drug resistance of NSCLC was associated with many MDR and apoptosis related gene expression no correlation among bcl-2 and MDR_1 or MRP was found. This further showed that the mechanism which bcl-2 mediates MDR is different from the traditional drug-resistance mechanism. However there are correlation among C-erbB-2 and C-myc or bcl-2, which may be the overexpression of C-erbB-2 coding protein P185 can promote the DNA repair and activate the cycle protein C-myc as well as the apoptosis suppress gene bcl-2, the tumor cell can therefore escape from the apoptosis.[9]

Our experiment assayed the expression of MDR and apoptosis related gene in the tissue of 63 NSCLC patients, results showed that MDR related gene C-erbB-2 is closely related to the positive expression of apoptosis related gene bcl-2, C-myc. The coexpression of three or more MDR and apoptosis related genes has the prognostic significance.

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