EXPRESSION AND PROGNOSTIC SIGNIFICANCE OF MULTIDRUG RESISTANCE ASSOCIATED PROTEIN (MRP) GENE IN NON-SMALL CELL LUNG CANCER BY IN SITE HYBRIDIZATION*

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ABSTRACT

Objective: To study on the effect of MRP gene overexpression on prognosis of patients with non-small lung cancer (NSCLC). Methods: Paraffin-embedded tissues from 47 cases of NSCLC who had undergone radical tumor resection were examined for expression of MRP gene mRNA by *in situ* hybridization using labelled digoxigenin probes combined with immunohistochemistry. All the patients were retrospectively followed-up. Results: All of the 47 lung cancer specimens were found to have overexpression of MRP gene mRNA. It was significantly correlated with patients' survival time, response to chemotherapy, recurrence or metastases after surgery, but was not correlated with histology, tumor size, node status, TNM stage, degree of differentiation, age and sex. Conclusion: Overexpression of MRP gene is a marker of prognostic significance in patients with NSCLC.

Key words: Lung neoplasms, Multi-drug resistance, MRP gene, Prognosis

It has been a hot spot in molecular biology to research the mechanism of multi-drug resistance and its reverse.[1] In the present study, we had examined MRP gene mRNA overexpression on the paraffin-embedded tissues that suffering from NSCLC using *in situ* hybridization labeled with digoxigenin probes combined with immunohistochemistry. All the patients were retrospectively followed-up. To research the relationship between MRP gene overexpression on NSCLC and the histology, tumor size, node status, degree of differentiation, TNM stage, age, sex and the survival time, response to chemotherapy, recurrence or metastasis after surgery.

Received April 9, 2000, accepted June, 19 2000

This work was supported by the Natural Science Foundation of Shanghai, China (96ZB14043).

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MATERIALS AND METHODS

Clinical Materials

Samples of the paraffin-embedded tissues of 47 patients suffering from NSCLC were collected from the Department of Thoracic Surgery, Xinhua Hospital, Shanghai Second Medical University. All of the patients had been diagnosed and treated with radical resection. Among them, there were *35* males and 12 females. Age distribution was as follows: <55 years 13 cases, 55–65 years 20 cases and >65 years 14 cases. The pathological types included squamous cell carcinoma 24 cases, and adenocarcinoma 23 cases. TNM stages were classified: 27 cases into stage I, 5 stage II, 9 stage IIIa, 6 stage IIIb. As to the degree of differentiation, 11 cases were found highly differentiated, 21 moderately, 15 low. Thirty-five cases received chemotherapy after the operation.

Preparation of Probes

Two antisense oligoprobes, each 30 nucleotides in length, were synthesized by Shanghai Bioengineering Center, Chinese Academy of Science, according to the report by Thomas et al.[2] Probe A (CAAGCTGGCGCT-GCCCGACACTGAGGTTCT) was complementary to the sequence of bases (4508–4479) corresponding to amino acids situated close to the nucleotide binding site in the 3'-terminal half of MRP. Probe B (CAGACA-GGTTCACGCCCTTCTCGCCAAT-CT) was comple-

mentary to the sequence of bases (2504–2475) corresponding to amino acids situated close to the nucleotide binding site in the 5'-terminat half of MRP.

Label of Probes

Two antisense oligoprobes were labeled with digoxigenin using reagent kits provided by Boehringer Mannheim Corp.

Tissue Section

Serial sections were taken from routine formalin-fixed, paraffin-embedded blocks from lung cancer and adjacent normal lung tissues. All sections were mounted on slides.

In situ Hybridization

The *in situ* hybridization technique was used according to Thomas et al..[2]

Analysis of Results

The positive cells were detected by the presence of Cambridge blue grains inside the cells under optic microscopy, and their was obtained by cell counting. The percentage ratio of positive grain cellular area was deduced by the computer assistant scan.

Statistical Analysis

The results were statistically analyzed by using the computer SAS system. *P* value less than 0.05 was considered significant.

RESULTS

Relationship between MRP Gene mRNA Expression Level and the Pathological Type, Tumor Size, Node Metastasis, TNM Stage, Degree of Differentiation, Age, Sex

The major lung cancer cells showed a strong hybridization signal, but all normal lung tissues from areas adjacent to the cancer just showed a weak to negative hybridization signal and the expression of MRP gene mRNA in lung cancer was remarkably higher than that in normal lung tissues from areas adjacent to the cancer ($P<0.01$). No significant differences between the levels of MRP gene mRNA overexpression and the pathology type, tumor size, node metastasis, TNM stage, degree of differentiation, age, sex could be found.

Relationship between MRP Gene mRNA Expression and the Survival Time after Operation in Patients with Lung Cancer

Results from the two probes showed that there were significant differences between the MRP gene mRNA expression and the postoperative survival time (Table 1). When the positive percentages were compared among the 1st, 2nd, 3rd year, they were significant ($P<0.01$ or $P<0.05$); the higher the levels of MRP gene mRNA expression, the shorter the survival time. Meanwhile the statistical results showed that the detection of 5'-terminal was better than that of 3'-terminal ($P<0.05$).

Table 1. Relationship between MRP gene mRNA expression and survival time ($\bar{x}\pm s$, %)

Survival time	N	Probes	Positive percentage
<1st year	11	3'-terminal	2.54±0.24
		5'-terminal	3.03±0.26*
<2nd year	14	3'-terminal	2.07±0.12
		5'-terminal	2.80±0.27**
<3rd year	9	3'-terminal	1.83±0.14
		5'-terminal	1.99±0.10*
>3rd year	13	3'-terminal	1.54±0.18◊
		5'-terminal	1.66±0.19*◊

Compared with the same parameter of the other groups: ◊$P<0.05$;
Compared with 3'-terminal between the same group: *$P<0.05$, **$P<0.01$

Effect of MRP Gene mBNA Expression Level on Chemotherapy

The overexpression of MRP gene mRNA seriously affects the effectiveness of the chemotherapy of lung cancer patients after surgery ($P<0.01$ or $P<0.05$)(Table 2).

Relationship between the MRP Gene mRNA

Expression and the Recurrence or Metastasis in 1 Year after Surgery

As shown by statistical analysis, the expression levels of MRP gene mRNA with recurrence or metastasis in 1 year after surgery were higher than those with no recurrence or metastasis 3 years after surgery ($P<0.01$) (Table 3).

Table 2. Effect of MRP gene mRNA expression level on chemotherapy ($\bar{x}\pm s$, %)

Survival time	n	Probes	Positive percentage
<1st year	8	3'-terminal	2.54±0.27
		5'-terminal	3.30±0.24**
<2nd year	8	3'-terminal	2.09±0.13
		5'-terminal	2.75±0.31**
<3th year	8	3'-terminal	183±0. 14
		5'-terminal	1.98±0.10*
<3th year	11	3'-terminal	1.52±0.18◊
		5'-terminal	1.64±0.18*◊

Compared with the same parameter of the other groups: ◊$P<0.05$;
Compared with 3'-terminal between the same group: *$P<0.05$, **$P<0.01$

Table 3. Relationship between the MRP gene mRNA expression and recurrence or metastasis($\bar{x}\pm s$, %)

Classification	n	Positive percentage
in 1 year#		
3'termina	12	2.36±0.33*
5'-terminal		3.05±0.45◊
in 3 year##		
3'termina	11	1.55±0.23
5'-terminal		1.90±0.41

#Recurrence or metastasis in 1 year, ##No recurrence or metastasis in 3 year; *Compared with in 3 year, $P<0.01$; Compared with in 3 year, $P<0.01$

DISCUSSION

Cole and his colleagues in 1992[3] discovered MRP gene and its product Pgp-190. It belongs to ATP-energy-dependent pump molecule and is believed to act as a drug efflux pump, driving the chemotherapeutic drug into the extracellular space in a contradictory concentrative-difference mode to reduce the accumulation of cytotoxic drug so as to produce multi-drug resistance in tumor cells. It was located in chromosome 16P13. 1. Sorensen et al.[4] using immunohistochemistry detecting the MRP gene mRNA and Pgp-190 in small-cell and non-small-cell lung cancer showed pretreatment levels of MRP gene appeared to play an important role in determining the degree of the sensitivity of primary lung cancer to drugs, and the multi-drug resistance phenotypes increased. The MRP gene mRNA and Pgp-190 expression were changed when treatment with drugs. The results indicated that there was primary (intrinsic) MRP gene mRNA expression in the lung cancer cells and MRP gene chemotherapy drugs, which cause multi-drug resistance could activate mRNA. Recently, investigation in other solid tumor showed that MRP gene not only affected the effects of chemotherapy, but also the survival time of the cancer patient. Murry et al.[5] reported that high levels of MRP gene mRNA expression in patients with neuroblastoma correlated closely to low chemotherapeutic effect and poor prognosis. In the present study, from paraffin-embedded tissues in 47 cases suffering from NSCLC treated with radical resection, we investigated MRP gene mRNA expression by means of *in situ* hybridization using labeled digoxigenin probes combined with immuno-histochemistry. The results showed that in NSCLC, MRP gene mRNA was expressed with markedly variable intensity and the levels of MRP gene mRNA expression significantly correlated with survival time, effect of chemotherapy, recurrence and metastasis after surgery. But it appeared to be independent with histology, tumor size, node status, TNM stage, and degree of differentiation, age and sex. Berger et al.[6] used 15 NSCLC cell lines, not stimulated by anti-tumor drugs, to study the multi-drug resistance of lung cancer cells and showed there were markedly different levels of MRP gene mRNA expression in these unstimulated NSCLC cell lines. The expressed levels in two of these NSCLC cell lines were higher than those NSCLC cell lines stimulated by anti-tumor drugs. The MRP gene mRNA expression levels had significant correlation with resistance of VP-16, VBL, but not to CDDP, bleomycin and showed the MRP gene mRNA and Pgp-190 played an important role in transporting anti-tumor precursor drugs. Furthermore, we detected the MRP gene mRNA in 3'-terminal and 5'-terminal and the results suggested that the

detecting effect of the 5'-tenninal was better than 3'-terminal.

In the research of the correlation between the MRP gene mRNA expression in lung cancer and drug resistance, the methods routinely used by all laboratories were polymerase chain reaction (PCR) analysis, northern blot analysis, southern blot analysis and western blot analysis, which can just be used *in vitro* to detected the MRP gene mRNA expression. Clinically, owing to the complexity of the lung cancer samples, these tissue cells may have markedly different expression levels of MRP gene mRNA which may easily lead to false positive and reduce the accuracy of the results. We used the *in situ* hybridization with labeled digoxigenin probes combined with immunohistochemistry to detect the expression of MRP gene mRNA of paraffin-embedded tissues from non-small cell lung cancer. The *in situ* hybridization method can directly analysis the MRP gene mRNA expression of lung cancer cells from complex tissues by direct vision and at the same time, does not pickup nucleic acid. It is extremely sensitive to very low target sequence in tissues and can keep the integrity of organic and cellular morphology; therefore it is reliable and scientific.

To sum up, by using *in situ* hybridization with labeled digoxigenin probes combined with immuno-histochemistry to detect the expression of MRP gene mRNA of paraffin-embedded tissues from non-small cell lung cancer is more suitable in clinical practice than other routine laboratory methods. The results suggest that detection of the MRP gene mRNA expression may be regarded as a means to forecast the prognosis of the patients with lung cancer and to adopt proposal of chemotherapy.

REFERENCES

[1] Endicott JA, Ling V. The biochemistry of P-glycorprotein-mediated multi-drug resistance. Ann Rev Biochem 1989; 58:137.

[2] Thomas GA, Barrand MA, Stewart S, et al. Expression of the multidrug resistance-associated protein (MRP) gene in human lung tumors and normal tissue as determined by *in situ* hybridization. Eur J Cancer 1994; 30:1705.

[3] Cole SPC, Bhardwaj G, Gerlach JH, et al. Overexpression of a transporter gene in a multidrug resistance human lung cancer cell line. Science 1992; 258:1650.

[4] Sorensen KM, Jensen PB, Nielsen BS, et al. Immunohistochemical detection of DNA topoisomerase IIa, P-glycoprotein and, multidrug resistance protein (MRP) in small-cell and non-small-cell lung cancer. Br J Cancer 1998; 77: 1469.

[5] Berger W, Elbling L, Hauptmann E, et at. Expression of the multidrug resistance-associated protein (MRP) and chemoresistance of the human non-small-cell lung cancer cells. Int Cancer 1997; 73:84.

[6] Murray DN, Boraow SB, Marshall GM, et al. Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma. N Eng J Med 1996; 334:231.