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The MDR1 gene and Its Transcript P-glycoprotein Expressions in Lung Cancer

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To determine the clinical significance of the MDR1 gene and its transcript P-glycoprotein (PGP) expression in lung cancer, ninety-one surgical lung cancer samples and normal lung tissues were analyzed. They consisted of 85 NSCLC (84 untreated and one treated) and 6 SCLC (three untreated and three treated). MDR1 messenger RNA (mRNA) levels in all surgical samples were determined by Northern blotting and compared with anticancer drug-sensitive and -resistant cell lines. The transcript PGP was detected in frozen sections by immunohistochemical staining. There was a strong correlation between the levels of MDR1 mRNA and PGP expression in the tumors. No normal lung tissues expressed the MDR1 gene in Northern blots. Fifteen percent (13/87) of the untreated tumors were positive for the MDR1 gene at low levels, which did not relate to any pathologic factors such as histologic type, tumor extensions, and differentiation grade. All three treated SCLC expressed high levels of the MDR1 gene, although all three untreated SCLC did not. Furthermore, only SCLC negative for MDR1 gene tended to respond to chemotherapy. The MDR1 gene is thought to be related to multidrug resistance in SCLC. A prospective study in a large number of patients should be attempted to clarify the clinical significance of the MDR1 gene in the treatment of SCLC.

Key Words: Multidrug Resistance, Lung Cancer, MDR1 Gene, P-glycoprotein

ABBREVIATIONS
MDR=multidrug resistance; PGP=P-glycoprotein; NSCLC=non-small-cell lung cancer; SCLC=small-cell lung cancer; mRNA=messenger RNA

Lung cancer is one of the most common human cancers in the world, the rate of which is increasing. Among human cancers, its mortality is comparatively high, despite extensive investigations and intensive chemotherapy.[1] Multidrug resistance (MDR) against anticancer drugs is the most serious problem encountered in treating lung cancer as well as other cancers.[1,2] The MDR1 gene encoding P-glycoprotein (PGP), acting as an energy-dependent drug efflux pump, is the molecular basis for drug resistance.[3-5] P-glycoprotein-transported drugs (or MDR1-related drugs) are lipophillic compounds such as anthracycline antibiotics, vinca alkaloids, and podophyllotoxins[2], which are key drugs in cancer chemotherapy.

The MDR1 gene and its transcript PGP are expressed in many normal tissues and tumors at various levels.[6-9] Tumors that originate from tissues, which express essentially high levels of MDR1 gene, tend to express high levels of the MDR1 gene.[6,7] Normal lung tissue and most lung tumors express low levels of MDR1 gene, compared with the colon, kidney, and adrenal gland.[10,11] However, the role of the MDR1 gene in acquired drug resistance or heterogenous MDR1 expression in solid tumors including lung cancer have not yet been clarified.

We examined the expression of the MDR1 gene and its transcript PGP, by Northern blotting and immunohistochemical staining, in surgical lung cancer samples to determine their clinical significance in lung cancer.

MATERIALS AND METHODS

Tumor samples

Ninety-one surgical lung cancer samples were obtained from Nagasaki University Hospital and its satellite hospitals, and immediately frozen in liquid nitrogen and stored at -70℃ until processing. From several lung cancer patients, normal lung tissues were obtained simultaneously. Some of the tumor samples were obtained for accurate diagnosis rather than for surgical treatment. The patients consisted of 62 males and 29 females, and the mean age was 64 years ranging from 34 to 81. The histologic types were 53 adenocarcinoma, 29 squamous cell carcinoma, 3 large cell carcinoma, and 6 SCLC. Four of them, one adenocarcinoma and three SCLC, had received MDR1-related anticancer drugs before surgery.

The histologic type and grade of differentiation were classified according to World Health Organization histologic classification.[12] The postsurgical pathologic

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stage, including computed tomography, magnetic resonance imaging, and radionuclide scan, was determined according to the International Union Against Cancer staging system. [13]

Cell lines

The KB-3-1 cell line is a drug-sensitive parental line originated from HeLa cells. The drug-resistant KB-8-5 and KB-C1 cell lines, which express high levels of MDR1 mRNA without gene amplification, were derived from KB-3-1 by continuous exposure to colchicine. [14] They are 3 and 160 times as resistant to doxorubicin, and 6 and 96 times as resistant to vinblastine as the parental line, respectively. [6,15] All KB cell lines were provided by Dr. Shinichi Akiyama (Kagoshima University, Kagoshima, Japan).

Northern blot analysis

Total RNA was isolated from tissues and culture cells using guanidium thiocyanate method. [16] RNA (10 μg per lane) was analyzed by standard Northern blot hybridization [17], in which the human MDR1 probe was the 2.2-kbp EcoRI-Pst I fragment from clone pMDRA1 that originated from a human adrenal gland. [18] The probe was provided by Dr. Kazumitsu Ueda (Kyoto University, Kyoto, Japan). Autoradiographs were visualized using an Imaging Plate type BAS-III (Fuji Photo Film Co., Kanagawa, Japan) for about three hours. All membranes were rehybridized in a similar manner with a beta-actin probe.

The MDR1 mRNA levels of all samples were determined by scanning the autographic bands and comparing them with beta-actin mRNA, using a Bio-Image Analyzer BAS2000(Fuji Photo Film Co., Kanagawa, Japan). The latter was a control for the amount of RNA on the membranes. The levels were recorded on a scale of "-" to "++", where "-"(negative) corresponded to the level in the KB-3-1 cell line,"+"(low level) to an intermediate level between KB-3-1 and KB-8-5, and "++"(high level) to a level higher than that in KB-8-5.

Immunohistochemical Staining for PGP

Frozen sections of normal lung tissues and the tumors were prepared and processed by standard procedures. [19] The sections were incubated overnight at 4℃ with the C219 mouse monoclonal antibody (Centocor, Malvern, U.S.A.) that recognizes the epitope in the cytoplasmic domain of PGP. [20] Subsequently, they were incubated with goat antimouse IgG conjugated horseradish peroxidase (Vector Laboratories, Burlingame, U.S.A.), and stained with 3,3 diaminobenzydine tetrahydrochloride. For every staining, KB-8-5 and KB-3-1 cells prepared by a cytocentrifuge were used as positive and negative controls, respectively. The results were separately evaluated by two pathologists.

Data Analysis

All results were statistically evaluated by the chisquared test. A two-tailed $P<0.05$ was considered to indicate statistical significance.

RESULTS

Figure 1 shows a Northern blot of the MDR1 gene in the control KB cell lines, the normal lung tissues, and the tumors, which were compared with one adrenocortical adenoma, and one renal cell carcinoma. In the KB lines, the MDR1 mRNA expression levels correlated with the degree of resistance to anticancer drugs. Among three types of tumor, adrenocortical adenoma expressed the highest level of MDR1 mRNA. The lung tumors expressed various levels of MDR1 mRNA ranging from negative to high, whereas it was undetectable in normal lung tissues.

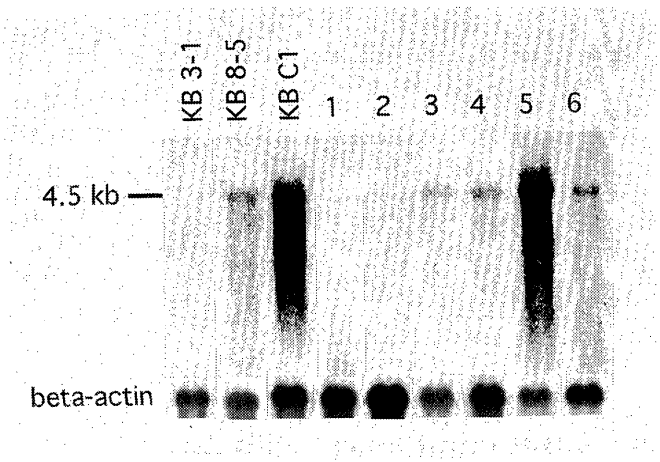

Figure 1. Northern blots of MDR1 gene expression in lung cancer. A 10 μg of total RNA from cell lines and tumor samples was used in each lane. Lane 1, normal lung tissue; lane 2, lung adenocarcinoma (negative); lane 3, large-cell carcinoma (low level); lane 4, small-cell carcinoma (high level); lane 5, adrenal tumor, lane 6 ; renal cell carcinoma. Hybridization with a beta-actin probe provided comparable amounts of RNA loaded in each lane. KB-3-1=drug-sensitive parental cell line; KB-8-5 and KB-C1=drug-resistant KB cell lines.

Table 1 shows the MDR1 gene expressionin 87 previously untreated lung cancers. Fifteen percent (13/87), which were all NSCLC, were positive for the MDR1 gene, and all three SCLC were negative. Only one adenocarcinoma of 13 positives was at a high level, and the remaining twelve at low levels, as shown in Table 3. There was no statistical difference between MDR1 gene expression and histologic type.

Table 2 shows the relationship between MDR1 gene

Table 1. Incidence of MDR1 Gene Expression in Lung Cancer*

Histology	No. of patients	Positive(%) †
Non-small-cell carcinoma	84	13(15)
adenocarcinoma	52	8(15)
squamous carcinoma	29	4(14)
large-cell carcinoma	3	1(33)
Small-cell carcinoma	3	0(0)
Tatal	87	13(15)

* None of the patients had received anticancer drugs.
† No statistival correlation between MDR1 gene expression and histologic type.

Table 2. MDR1 Gene Expression and Pathologic Factors in NSCLC*

Factors	No.of patients	Positive (%) †
p-Stage (n=80)		
I	30	5(17)
II	12	2(17)
III A	22	4(18)
III B	6	1(16)
IV	10	1(10)
T-factor (n=80)		
T1	25	3(12)
T2	38	9(24)
T3	11	0(0)
T4	6	1(17)
N-factor (n=80)		
N0	45	5(11)
N1	11	3(27)
N2	24	5(21)
Differentiation (n=80)		
well	29	5(17)
moderately	27	3(11)
poorly	14	4(29)
unknown	10	-

* None of the patients had received anticancer drugs.
† No statistical correlations between MDR1 gene expression and any factors.

expression and pathologic factors in 80 untreated NSCLC, in which the pathologic stages were determined. There were no statistical correlations between MDR1 gene expression and pathologic factors such as pathologic stage, T factor, N factor, and differentiation grade. Even within each histologic type of NSCLC, no statistical differences among factors were seen.

Table 3 shows all sixteen MDR1 gene positives in this study. Three SCLC had received anticancer drugs and expressed high levels of the MDR1 gene, although a treated adenocarcinoma was negative. Patient No.14 had been treated with a CAV (cyclophosphamide, doxorubicin, and vincristine) regimen before surgery, and did not respond to chemotherapy at recurrence. The other two SCLC had been treated with cisplatin and VP-16 for neo-adjuvant chemotherapy, and patient No.15 expressed the highest level of MDR1 gene among these positives.

Table 3. Lung Cancer Patients Positive for the MDR1 Gene

Patient No.	Sex	Age	Histology	Defferentiation	p-TNM	MDR1level*
1	m	55	adeno	poorly	220	+
2	m	58	adeno	well	100	+
3	m	62	adeno	well	220	+
4	f	46	adeno	well	100	+
5	m	62	adeno	poorly	200	+
6	m	55	adeno	moderately	210	+
7	f	52	adeno	well	220	+
8	f	70	adeno	moderately	100	++
9	m	71	squamous	well	410	+
10	m	73	squamous	moderately	200	+
11	m	59	squamous	poorly	200	+
12	f	75	squamous	poorly	220	+
13	f	64	large	-	221	+
14 †	m	59	small	-	401	++
15 †	m	61	small	-	320	++
16 †	m	58	small	-	220	++

* A low level (+) of MDR1 gene expression corresponds to an intermediate level between KB-3-1 and KB-8-5, and a high level (++) to a level higher than that in KB-8-5.(see Materials and Methods in text)
† Patients had received MDR1-related anticancer drugs.

Table 4. MDR1 Gene Expression and Response to Chemotherapy*

Response	NSCLC (n = 9)	SCLC (n= 3)
Non-responder / MDR1 positive	2/2(100%)	1/1(100%)
responder / MDR1 negative	1/7(14%)	2/2(100%)

* Patients received MDR1-related anticancer drugs at recurrence or after surgery. The responder was a patient whose tumor was reduced by over 50% after chemotherapy.

The control cell lines and some of the tumors expressing low and high levels of MDR1 gene in Northern blots, were stained with C219 antibody (Figure 2). In normal tissue only bronchial epithelium was positively stained, and the cancer cells in the tumor were heterogeneously positive as shown in Figures 2C and 2D. The cancer cells in patient No.15 with SCLC were heterogeneously and strongly positive (Figure 2D). The level of MDR1 gene expression in the Northern blots correlated with those of PGP expression in the tumors.

Table 4 shows the relationship between MDR1 gene expression and the response to chemotherapy. Subsequent responses to chemotherapy at recurrence or after surgery were evaluated in twelve patients. Six NSCLC negative for the MDR1 gene and two positive NSCLC did not respond to MDR1-related drugs, and only one negative NSCLC responded. One positive SCLC, treated previously, did not respond to chemotherapy, although two negative SCLC untreated did.

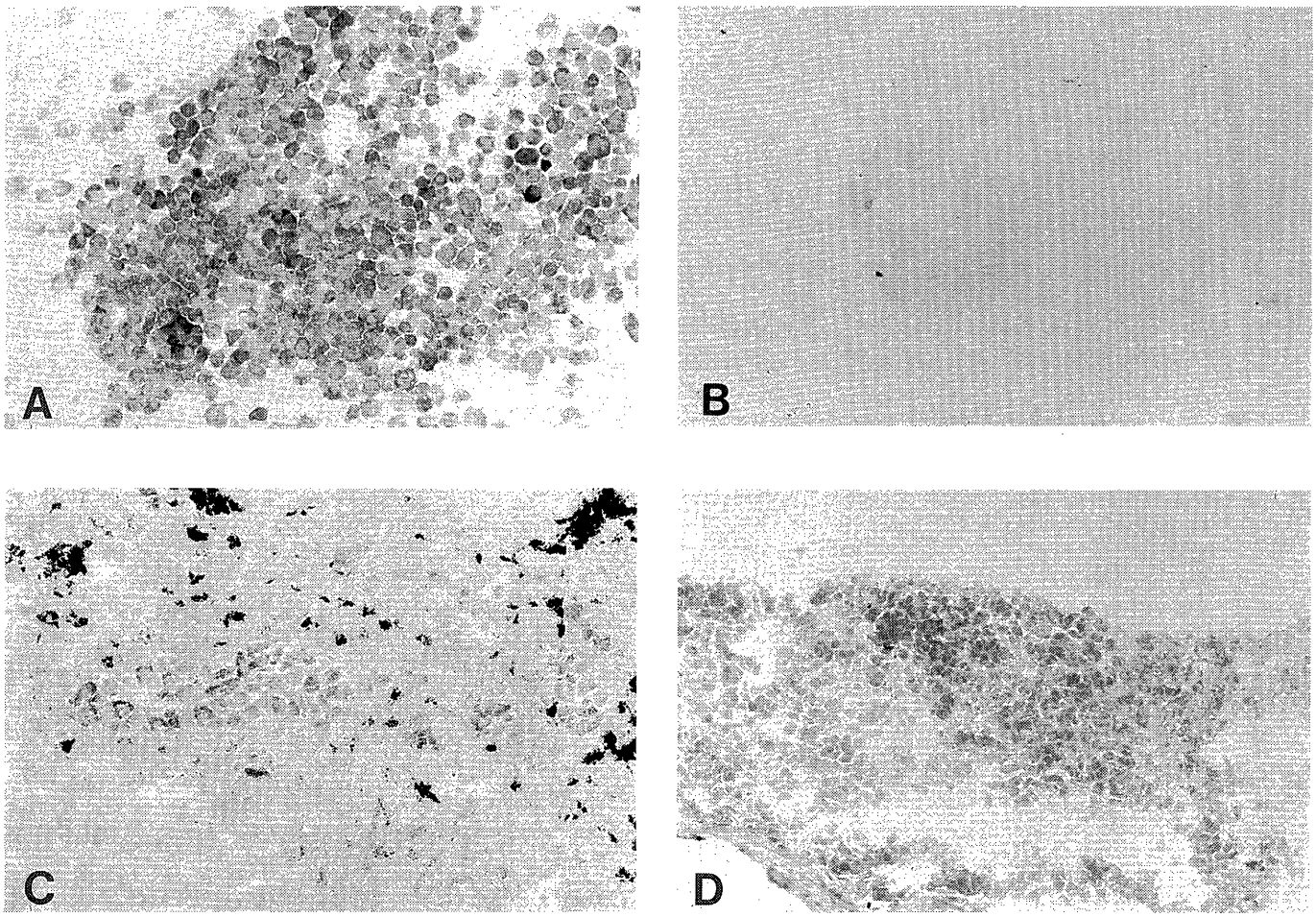

Figure 2. Immunohistochemical staining of P-glycoprotein (PGP) in control cell lines and lung cancer.(A) KB-8-5 cells of the positive control are positively stained in the cytoplasm.(B) KB-3-1 of negative control.(C) In the adenocarcinoma, some of the cancer cells are positively stained, and some of them are negative (lower right).(D) In patient No.15 with SCLC, most cancer cells are strongly stained (upper), and some of them are negative or weakly stained (left).
(original magnification: A, x200; B, x200; C, x200; D,x300)

DISCUSSION

Multidrug resistance is one of the most serious problems in the treatment of lung, as well as other human cancers .[1,2] The MDR1 gene, one molecular basis for drug resistance, and its transcript PGP, have been extensively examined in normal human tissues and tumors.[6-9] Tumors originating from tissues which express essentially high levels of MDR1 gene tend to express high levels of the MDR1 gene, such as colon, kidney, adrenal gland, and pancreatic tumors.[6,7] Normal lung tissue and most lung tumors reportedly express low levels of the MDR1 gene and PGP.[8-11] However, the role of the MDR1 gene in acquired drug resistance or heterogeneous MDR1 gene expression in solid tumors including lung cancer have not been sufficiently clarified. All the treated SCLC in this study expressed high levels of the MDR1 gene, although only fourteen percent (12/87) of the untreated tumors expressed low levels.

The level of the MDR1 gene in clinical samples is determined by Northern or slot-blot analysis, polymerase chain reaction (PCR), RNAse protection assay, and in situ hybridization.[6,7,11,21] Western blotting, immunohistochemical staining, and flow cytometry are used to detect PGP.[8,9,22] These methods vary considerably in their sensitivity, specificity, and quantitation.[22] At least two methods should be used, because evaluation of the MDR1 gene or PGP expression depends on the detection method. To detect MDR1 gene expression in individual cancer cells is quite important, because cells expressing the MDR1 gene survive and relapse, despite intensive chemotherapy with MDR1-related drugs. Northern blots and immunohistochemical staining in frozen sections were compared in this study. The levels of MDR1 gene expression determined by Northern blotting correlated with those of PGP expressed

heterogenously in the tumors (Figure 2).

Only fifteen percent (13/84) of the untreated NSCLC were positive for the MDR1 gene, one of which was highly positive and the others marginally so, regardless of any pathologic factors (Table 3). The MDR1 gene is unlikely to relate to drug resistance in NSCLC[10,23,24], because the response rate to chemotherapy is low, ranging from 20 to 55%, despite the low level of MDR1 gene expression[1,25], suggesting the existence of other MDR mechanisms.[26] Six out of seven negative NSCLC for MDR1 gene in this study did not respond to chemotherapy with MDR1-related drugs. However, the role of the MDR1 gene in SCLC remains controversial, apart from other drug resistance mechanisms.[10,26-29] The results (Tables 2 and 4), suggested that the MDR1 gene is associated with drug resistance in SCLC. The MDR1 gene in lung cancer should be examined separately in NSCLC and in SCLC, because their biological and clinical behavior differs.[30]

Most cancer cells are heterogeneous with regard to immunogenicity, growth rates, and sensitivity to anticancer drugs, including MDR1 gene expression.[23,31] The MDR1 gene in SCLC has been studied in cell lines established from treated and untreated clinical samples.[7,10,27,28] Whereas our results from clinical samples were similar to those of previous studies using untreated SCLC, they were different from those using treated SCLC.[10,27] The possibility remains that the cell lines established from treated SCLC were MDR1 gene negative clones.

Drug-sensitive cancer cell lines in vitro can acquire resistance to several cytotoxic drugs[5,14,26], although it is unclear whether the clinical dose of anticancer drugs induces acquired resistance in MDR1 gene-negative cancer cells. Those that acquired MDR1 related-resistance might heve biological aggressiveness and metastatic potential.[32,33] To detect MDR1 gene expression in SCLC following intensive chemotherapy would be important in predicting prognosis and attempting new clinical trials. MDR1 gene expression is reportedly a negative prognostic factor in hematolymphoid malignancies, such as acute myeloid leukemia (AML), lymphoma, and multiple myeloma.[34] Several clinical trials, using chemosensitizing agents, for these cancers have been attempted to reverse the MDR.[34,35] For SCLC which do not respond completely to chemotherapy, such trials might be worthwhile in the future.

In conclusion, the MDR1 gene is thought to be related to multidrug resistance in SCLC. A prospective study of a large number of patients should be attempted to clarify the clinical significance of MDR1 gene in the treatment of SCLC.

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