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Expression of the Multidrug Resistance Gene (*MDR1*) in Non-small Cell Lung Cancer

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To examine the clinical relevance of P-glycoprotein, encoded by the human multidrug resistance gene (*MDR1*), to multidrug resistance in lung cancer, we examined the expression of *MDR1* in 107 non-small cell lung cancer (NSCLC) specimens and 20 corresponding specimens of normal lung tissues. We also evaluated the relationship between *MDR1* expression and the histopathology and pathological staging of NSCLC. The tumors consisted of 60 adenocarcinomas, 38 squamous cell carcinomas, 8 large cell carcinomas, and 1 adenosquamous carcinoma. *MDR1* expression was semi-quantified by use of the reverse transcriptase-polymerase chain reaction method. We subclassified the NSCLC into 3 grades according to the *MDR1* expression level (−, +, ++). Sixty-one of the 107 tumor specimens (57%) and 18 of the normal lung tissue specimens (90%) expressed various levels of the *MDR1* gene. Only one tumor specimen showed higher *MDR1* expression than the corresponding normal lung tissue. The relationship between pathological stage and *MDR1* expression levels was not significant. These results suggest that the level of *MDR1* expression in lung cells is decreased as cells progress from the normal to the transformed state.

Key words: Multidrug resistance — P-glycoprotein — Lung neoplasm — Polymerase chain reaction

Chemotherapy with various anticancer agents against many solid tumors has steadily improved over the years due to the development of new drugs and well-designed protocols. However, the failure of chemotherapy, due to cellular drug resistance, is still a major problem in lung cancer. Primary lung cancer is divided into two histological groups, SCLC[7] and NSCLC. NSCLC usually shows intrinsic MDR, whereas almost all SCLC respond well to various anticancer agents.

Lung cancer is generally treated by a combination of therapeutic protocols using cisplatin, vinca alkaloids, and VP-16.[1-3] Several types of multidrug resistance have been noted in cell lines resistant to anticancer agents.[4-9] The selection of cells that are resistant to lipophilic compounds (anthracyclines, vinca alkaloids, podophyllotoxins, and colchicine) results in the development of cross-resistance to other related drugs.[4,9] A striking feature of the MDR phenotype is the reduced cellular accumulation of drugs due to an energy-dependent efflux.[5] The drug efflux pump consists of a 170 kDa membrane glycoprotein called P-glycoprotein (P-Gp) encoded by a single gene (*MDR1*), localized on the long arm of chromosome 7.[5] Direct evidence for the role of *MDR1* in MDR has been obtained *in vitro*, using cell lines transfected with this gene.[6] While the lung expresses low levels of P-Gp,[6,10] the clinical relevance of P-Gp in MDR, particularly in lung cancer, remains unknown, despite immunohistochemical and molecular analyses.[10,11]

In this study, we wxamined *MDR1* gene expression in the 107 NSCLC specimens and 20 corresponding normal lung tissue specimens by RT-PCR. We also evaluated the relationship between the *MDR1* expression level and the pathological stage of NSCLC.

MATERIALS AND METHODS

Patients and tumors The specimens were obtained from patients with previously untreated primary lung cancers who underwent surgical resection; the 107 fresh tumor specimens and 20 corresponding samples of adjacent normal lung tissues obtained were rapidly frozen at −80°C. The tumor specimens were not contaminated by normal lung tissues or blood. Total RNA was prepared from the frozen specimens by standard procedures.[12]

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[7] The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; MDR, multidrug resistance; *MDR1*, human multidrug resistance gene 1; P-Gp, P-glycoprotein; RT-PCR, reverse transcriptase-polymerase chain reaction.

Surgical specimens were also processed for routine histopathological analysis. The morphological classification was based on the General Rules for Clinical and Pathological Record of Lung Cancer (Japanese Lung Cancer Society, 1987).[13] The specimens consisted of 60 adenocarcinomas [well differentiated (wd) 24, moderately differentiated (md) 22, poorly differentiated (pd) 14], 38 squamous cell carcinomas (8 wd, 22 md, 8 pd), 8 large cell carcinomas, and 1 adenosquamous cell carcinoma. The tumors were histologically classified by two pathologists. The age distribution of the patients (73 men, 34 women) included one patient below 40 years old, 9 patients between 40 and 49 years, 29 between 50 and 59, 41 between 60 and 69, 25 between 70 and 79, and 2 who were more than 80 years old. Ninety-six patients were staged according to the histopathological findings of surgical specimens (p-TNM); the other 11 patients could not be evaluated since they had had non-curative operations. The pathological stage was also classified according to the General Rules for Clinical and Pathological Record of Lung Cancer (Table I).

RT-PCR Detection of *MDR1* gene expression was determined by reverse transcription of RNA followed by the polymerase chain reaction.[14, 15] Complementary DNA (cDNA) was synthesized with 5 to 1,000 ng of total cellular RNA and 20 pmol of downstream antisense primer in 10 μl of a reaction mixture (50 m*M* Tris-Cl pH 8.3, 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 500 *M* dNTPs) containing 10 units of Moloney murine leukemia virus (MMLV H^-) reverse transcriptase (SuperScript, Bethesda Research Laboratories) at 24°C for 10 min and at 43°C for 60 min.

PCR was conducted with cDNA in a final volume of 100 μl of a reaction mixture (10 m*M* Tris-Cl pH 8.2, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% gelatin) containing 2.5 units of thermostable DNA polymerase (Amplitaq, Perkin-Elmer/Cetus). Each PCR cycle consisted of 2 min of denaturation at 94°C, 2 min of primer annealing at 55°C, and 2 min of extension/synthesis at 72°C. PCR primers (amprimers) were synthesized using a DNA synthesizer (Applied Biosystems, model 391). We prepared an original set of amprimers (#B, sense: AAGCTTAGTACCAAAGAGGCTCTG, residues 2041–2064, antisense: GGCTAGAAACAATAGTGAAAACAA, residues 2260–2283) to evaluate the expression level specific to the human *MDR1* gene. The amprimer sequences (#B) were derived from various exons separated by introns to prevent amplification of genomic DNA. RT-PCR with amprimer #B amplified a 243 bp segment of *MDR1* cDNA. We also used amprimers for β2-microglobulin (β2m) (#D), as reported previously, to evaluate housekeeping gene expression as an internal control.[15]

PCR products separated by electrophoresis through a 3% agarose gel were blotted onto membranes (Zeta Probe, BioRad). Specific PCR products were detected by hybridization with synthetic oligonucleotide probes (AGGATTATGAAGCTAAATTTA, residues 2094–2115, for *MDR1*-#B; GTGTGAACCATGTGACTTTGTCACA, residues 1574–1598, for β2m-#D) labeled with ^{32}P.[16] The relative expression levels of the *MDR1* genes were evaluated by densitometry, using the Interactive Build Analysis System (Carl Zeiss).[17] *MDR1* gene expression levels were calculated by multiplying the mean density by the densitometric area.

Table I. Pathological Stage

Stage I	36	p-T1	26	p-N0	53	p-M0	81
II	8	2	44	1	11	1	15
III	37	3	24	2	31		
IV	15	4	2	3	1		
Unknown[a]	11						

a) Pathological staging could not be evaluated because of non-curative surgical operation. The morphological classification of NSCLC was based on the criteria of the General Rules for Clinical and Pathological Record of Lung Cancer (Japanese Lung Cancer Society, 1987).

RESULTS

***MDR1* gene expression level** We kinetically analyzed the RT-PCR of *MDR1* mRNA in the KB3-1 cell line and in a multidrug-resistant derivative line, KB8-5. The specific

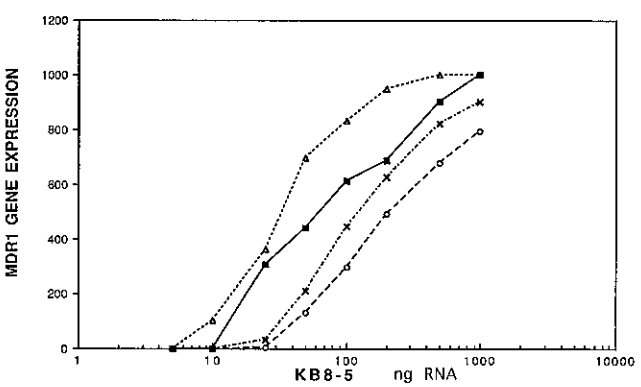

Fig. 1. Kinetics of RT-PCR for semi-quantitation of *MDR1* expression. Complementary DNA was synthesized using a total cellular RNA template (5–1000 ng) from the KB8-5 cell line, the antisense primer, and 10 U of MMLV H^- reverse transcriptase. A specific *MDR1*-cDNA fragment (243 bp) was amplified by PCR with the sense amprimer and 2.5 U of *Taq* DNA polymerase. The expression level of the *MDR1* signal was measured by densitometric scanning. PCR amplification was conducted with 22 (○), 24 (×), 26 (■), or 28 (△) cycles. Southern hybridization was performed using a ^{32}P-labeled *MDR1* specific oligonucleotide probe.

MDR1 products were amplified exponentially by 22, 24, 26, and 28 cycles of PCR (Fig. 1). The *MDR1*-specific RT-PCR product, using more than 0.5 μg of total RNA template from the KB8-5 cell line, reached a plateau at 28 cycles of amplification, whereas with 26 cycles, a linear relationship was maintained using the KB8-5 RNA template up to 1 μg. These results suggested that the initial amount of the RNA template (25 to 1000 ng) could be estimated in different samples before saturation of amplification, after 26 PCR cycles of *MDR1* cDNA (Fig. 2). We used β2m mRNA as an internal control for *MDR1* expression, as previously reported.[15] The ratio of the *MDR1*/β2m PCR product in KB8-5 RNA samples was stable. These findings suggest that *MDR1* gene expression is semiquantifiable after 26 cycles of RT-PCR with 0.5 μg of total RNA. The amounts of *MDR1* mRNA in the samples were evaluated in relation to a standard set of RNA (0.5 μg) prepared from the KB3-1 and KB8-5 cell lines, and processed simultaneously with the test samples.

RT-PCR, using amprimer #B, revealed *MDR1* gene expression in 61/107 (57%) of the tumor specimens and in 18/20 (90%) of the normal lung specimens (Figs. 3 and 4). No *MDR1* expression was observed in the KB3-1 cell line, which was sensitive to the anticancer drugs. The expression levels of *MDR1* were lower than in the KB8-5 cell line. The levels also varied among all 61 tumors. No tumors expressed more *MDR1* gene than KB8-5. NSCLC were subclassified into 3 grades according to *MDR1* expression levels: (++), equivalent to 100 to 500 ng of total cellular RNA of KB8-5 (more than 600 densitometric units); (+), less than 100 ng of KB8-5 RNA (less than 600 densitometric units) and (−), no *MDR1* gene expression.

In the 18 normal lung tissue specimens, *MDR1* expression was marked (++) in 7 patients (Patients No. 1–7), and moderate (+) in 11 (Patients No. 8–18). Seventeen of 18 normal tissue specimens expressed equal or higher *MDR1* levels than their corresponding tumor specimens, whereas only 1 sample (Patient No. 10) expressed less *MDR1* than the corresponding neoplastic tissue (Fig. 3C and Fig. 4B).

Relationship between *MDR1* gene expression and pathological state The 61 NSCLC specimens that expressed *MDR1* were histopathologically classified as follows: 34

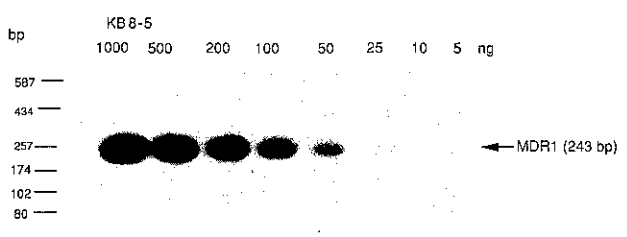

Fig. 2. *MDR1* specific products after 26 cycles of PCR increased in a dose-dependent manner with respect to the initial amount of the KB8-5 RNA template. *MDR1* expression was detected in more than 25 ng of KB8-5 mRNA under these conditions.

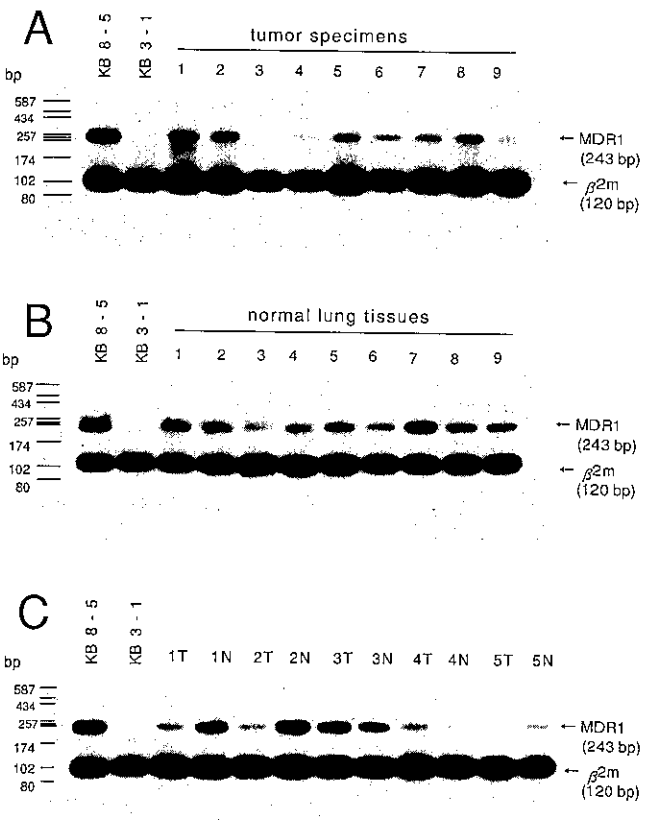

Fig. 3. Gene expression of *MDR1* in clinical specimens. *MDR1* (243 bp) was amplified by 26 cycles of PCR with a cDNA reverse-transcribed from 500 ng of mRNA of tumor xenografts. Arrows indicate the specific PCR products of *MDR1* transcripts hybridized with an oligonucleotide probe. The co-amplified β2m gene PCR products (120 bp) were co-hybridized with the oligonucleotide probe ("Materials and Methods"). DNA size markers (pUC19 DNA digested with *Hae* III) are also shown. (A) *MDR1* expression in primary lung cancer. KB8-5 (MDR epidermoid carcinoma cell line); KB3-1 (drug-sensitive epidermoid carcinoma cell line), lanes 1, 2, 5–8, adenocarcinoma; lanes 3, 4, squamous cell carcinoma; lane 9, large cell carcinoma. (B) *MDR1* expression in normal lung tissues. (C) *MDR1* expression in tumor specimens and their corresponding normal tissues. Five NSCLC (T) and corresponding normal tissue (N) were hybridized. Lane 1, Patient No. 7; lane 2, No. 6; lane 3, No. 3; lane 4, No. 10; lane 5, No. 16.

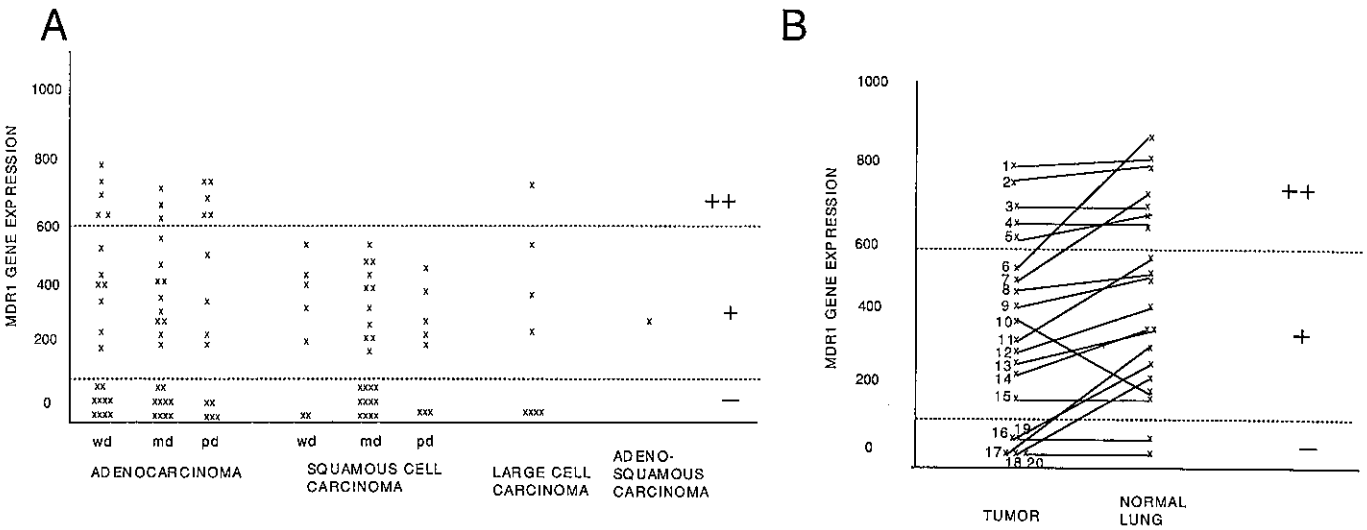

Fig. 4. *MDR1* expression levels in NSCLC (A), and in NSCLC and corresponding normal lung tissues (B). Gene expression levels are shown by densitometric units. NSCLC and normal tissue were subclassified into 3 grades according to the *MDR1* expression levels: (++), equivalent to 100 to 500 ng of total cellular RNA of KB8-5 (more than 600 densitometric units); (+), less than 100 ng of KB8-5 RNA (less than 600 densitometric units) and (−), no *MDR1* expression. Well differentiated, wd; moderately differentiated, md; poorly differentiated, pd. 1–20, Patient number.

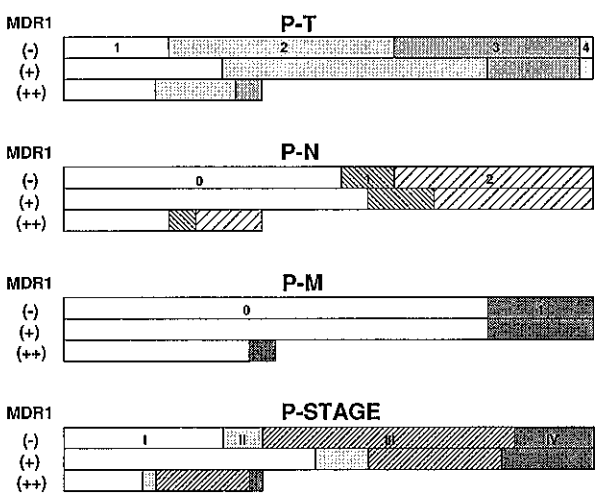

Fig. 5. Pathological TNM scores (P-T, P-N, P-M), stages, and *MDR1* expression. NSCLC were subclassified into 3 grades according to *MDR1* expression levels. (++), more than 600 densitometric units; (+), less than 600 densitometric units; (−), no *MDR1* expression.

adenocarcinomas (wd, 13; md, 12; pd, 9), 22 squamous cell carcinomas (wd, 6; md, 11; pd, 5), 4 large cell carcinomas, and 1 adenosquamous carcinoma (Fig. 4A).

Fifteen NSCLC specimens showed high (++) *MDR1* expression; 14 of those 15 were adenocarcinomas and one was a large cell carcinoma with mucin. These 14 adenocarcinoma specimens that expressed (++) *MDR1* showed various states of differentiation (wd, 6; md, 3; pd, 5). No correlation was observed between *MDR1* gene expression level and the pathological differentiation state.

Pathological staging of 55 of the 61 NSCLC that expressed *MDR1* was carried out. The results were P-T1, 19; T2, 26; T3, 9; T4, 1; and P-N0, 31; N1, 7; N2, 17. We also found P-M0, 46; M1, 9, and P-stage I, 25; stage II, 5; stage III, 17; and stage IV, 8 (Fig. 5). No relationship was observed between *MDR1* gene transcript level and pathological staging.

DISCUSSION

It has been shown that tumors in organs, such as colon, kidney and liver, in which the level of *MDR1* expression is high, generally show marked expression of *MDR1* by Northern blot analysis.[10, 11] However, we did not detect marked expression of *MDR1* in the NSCLC by Northern blot analysis (data not shown). In the present study using highly sensitive RT-PCR, 61 of the 107 NSCLC specimens expressed various levels of *MDR1*, but these levels were all lower than those expressed in the MDR cell line KB8-5. We also evaluated *MDR1* expression in both tumor tissue and the corresponding normal tissue in 20 patients. Normal lung tissues showed various levels of *MDR1* expression. In 19 of 20 patients, NSCLC tumors expressed lower or equal levels of *MDR1* than the corre-

sponding normal lung tissue. These findings suggest that *MDR1* expression in most lung cells is reduced as cells progress from the normal to the transformed states. Another possible explanation for this finding would be clonal expansion of a single cell type in a neoplasm, whereas normal tissue is a mixture of heterogeneous cell types. These findings suggest that the contribution of the *MDR1* gene to the MDR phenotype in NSCLC decreases as the tumor develops. No correlations were noted among *MDR1* gene expression, histological differentiation, and pathological TNM stage.

In the NSCLC, there appears to be no relationship between multidrug resistance and *MDR1* gene expression.[18–22] Previous investigators have used conventional detection methods or methods with low sensitivity (slot blots or Northern blots). Thus, *MDR1* expression in their lung cancer specimens may have been undetectable. Other studies have reported that P-Gp expression was positively related to drug resistance in lung cancer.[23, 24] Holzmayer *et al.*[25] reported a correlation between the clinical course and *MDR1* RNA expression in SCLC, using RT-PCR. They also found that untreated adenocarcinoma expressed high levels of *MDR1* mRNA. We found that 15 NSCLC which had features of adenocarcinoma (glandular structures, mucin production) showed high (++) *MDR1* expression; however, the clinical relevance of this result is not clear. Further studies are required to clarify the relationship between *MDR1* overexpression and intrinsic MDR in NSCLC with adenocarcinomatous features.

In our study, the 107 patients were given postoperative chemotherapy with P-Gp-related drugs (vindesine, VP-16) and or non-P-Gp drugs (cisplatin, carboplatin).[26, 27] The relationship between *MDR1* gene expression and prognosis in these patients was not significant (according to the Kaplan-Meier method, analyzed by the general Wilcoxon test; data not shown). The results would have been influenced by the short experimental period and the administration of the non-P-Gp related drugs.

The overexpression of P-Gp does not completely explain the intrinsic MDR in lung cancer; other drug-resistance mechanisms are thought to exist in lung cancer cell lines. Recently, several investigators have reported non-P-Gp drug-resistance mechanisms such as "atypical MDR," induced by MDR-related protein (MRP) in lung cancer cell lines.[28–30] Further studies of MRP expression in lung cancer are required.

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