Acta Med Okayama 47 (4) 243-248 (1993)

MDR1 Gene Expression and Treatment Outcome in Small Cell Lung Cancer: MDR1 Gene Expression as an Independent Prognostic Factor

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We report a preliminary study to determine whether MDR1 gene expression level in small cell lung cancer (SCLC) tumors is a useful predictor of tumor response to chemotherapy and patient survival in association with myc amplification in the tumor. We analyzed 18 patients with SCLC receiving adriamycin and etoposide combination chemotherapy between August 1989 and November 1991; 16 males and 2 females, median age of 68 years, and 7 with limited disease and 11 with extensive disease. MDR1 mRNA expression level and myc family gene amplification were simultaneously determined by polymerase chain reaction using transbronchial biopsy specimens which were obtained at diagnosis. Patients with tumors expressing low MDR1 mRNA responded more favorably to chemotherapy than those with tumors expressing high MDRI mRNA, however, the difference in tumor response was statistically not significant (84.6% *versus* 40%). The overall survival was significantly shorter in the latter than in the former (7.2 months *versus* 11.7 months; $p=0.023$). The survival of the 4 patients with tumor showing myc family gene amplification was almost identical to that of patients with tumors showing no amplification of the gene (8.2 months *versus* 8.8 months; $p=0.73$). Multivariate Cox's regression analysis supports the notion that MDR1 may be a useful independent prognostic factor.

Key words : small cell lung cancer, MDR1 mRNA expression, myc gene amplification, prognostic factor

Intensive combination chemotherapy has resulted in a significant advance in the treatment of small cell lung cancer (SCLC). However, intrinsic and acquired drug resistance remains a major obstacle in the chemotherapy of SCLC. One of the most well-studied types is a resistance to multiple structurally dissimilar hydrophobic chemotherapeutic agents, multidrug resistance (MDR). It is apparent that a major mechanism of MDR in mammalian cells involves increased expression of a 170-kDa plasma membrane glycoprotein, P-glycoprotein (P-gp), which acts as a pump for outward drug transport. With regard to SCLC, some investigators have reported undetectable or very low levels of MDR1 gene expression by conventional analytical techniques (1–3). However, we were able to measure the MDR1 mRNA level in clinical samples of SCLC using reverse transcription-polymerase chain reaction (RT-PCR) in a previous study, and concluded that the MDR1 gene is commonly transcribed at a very low level in SCLC and that MDR1 mRNA levels in tumors that had relapsed after chemotherapy were significantly higher than those in tumors examined at diagnosis (4). Because anthracyclines, vinca alkaloids, and podophyllotoxins, which are commonly affected by MDR1, are among the most active agents for the treatment of SCLC, the overexpression of MDR1 could affect the therapeutic outcome. With this assumption, we tried to determine whether MDR1 plays a role in intrinsic drug resistance in SCLC, whether it predicts response to chemotherapy, and finally whether it is an independent prognostic factor in association with known factors including myc gene amplification.

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Sabjects and Methods

Patients and clinical samples. Between August 1989 and November 1991, prospective 20 untreated patients with SCLC who underwent chemotherapy at the Okayama University Hospital were admitted to this study. Transbronchial biopsy specimens were obtained from the patients when a diagnosis of SCLC was reached. Biopsy specimens were fixed with cold acetone for 24 h, embedded in paraffin (5), and histologically confirmed to contain sufficient tumor cells. All the patients tested received CAV-EP chemotherapy consisting of cyclophosphamide, doxorubicin, vincristine, cisplatin, and etoposide (6).

Preparation of total cellular RNA. RNA was isolated from paraffin-embedded transbronchial biopsy specimens by the modified method reported by Rupp (7). After trimming off excess paraffin and normal tissue from tissue blocks, five 6 μm sections from each block were cut and collected into a 1.5 ml microcentrifuge tube, dewaxed in xylene, and washed with 100 % ethanol followed by complete drying. The tissue was then homogenized in 200 μl of extraction buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 25 mM EDTA, 0.5 % NP-40, 1 mM dithiothreitol, 20 mM vanadyl ribonucleoside complex). Proteinase K (Sigma Chemical Co., MO, USA) was added at a concentration of 0.5 mg/ml. The mixture was incubated at 37 °C for 72 h, then RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform extraction (AGPC) procedure and precipitated mith isopropanol in the presence of 20 μg of glycogen (Boehringer Mannheim Biochemicals, IN, USA) as a carrier. Following precipitation, the samples were treated with DNase I.

Expression of MDR1 mRNA. RNA was reverse-transcribed into cDNA as described previously (8, 9). Then 20 μl of reverse transcription reaction mixture containing 5 μl of total cellular RNA, 1.0 μM random hexadeoxynucleotide primer (Takara Shuzo, Kyoto, Japan), 25 mM Tris-HCl, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol, 1 mM deoxynucleotide triphosphates and 10 units of reverse transcriptase (RAV2, Takara Shuzo, Kyoto, Japan) was incubated at 42 °C for 60 min, and then heated to 95 °C for 5–10 min. MDR1 and β-actin specific sequences were co-amplified in the same vessel containing 100 μl of reaction mixture consisting of heat-treated 20 μl reverse transcriptase reaction mixture, 200 pM MDR1 primers (the sense primer 5′-ACAGGAGATAGGCTGGTTTGA-3′ and the antisense primer 5′-GTTGCCATTGACTGAAAGAAC-3′, which yielded a 126-bp product), 50 pM β-actin primers (the sense primer 5′-TTCTACAATGAGCTGCGTGT-3′ and the antisense primer 5′-GGAGTCCATCACGATGCCAG-3′, which yielded a 198-bp product), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM deoxynucleotide triphosphates and 2.5 units of AmpliTaq (Perkin-Elmer/Cetus, CT, USA). The mixture was amplified for 60 cycles with the BiGene PHC-1 programmable heat block (Techne, Cambridge, UK). Each cycle of PCR included 1 min of denaturation at 94 °C, 1 min of primer annealing at 45 °C and 2 min of extension/synthesis at 72 °C.

Twenty microliters of PCR products was electrophoresed on 3 % NuSieve 3:1 agarose (FMC Bioproducts, ME, USA) gel in TBE buffer. After staining with ethidium bromide, agarose gels were photographed and analyzed by two-dimensional densitometry (300 A Scanning Imager, Molecular Dynamics, CA, USA) to determine the amount of PCR products. The MDR1/β-actin ratio of the PCR-amplified bands was determined as the relative expression of MDR1 mRNA.

Myc family oncogene amplification. DNA was extracted from paraffin-embedded biopsy specimens by the following method. After trimming off excess paraffin and normal tissue, five 6 μm sections were cut and collected into a 1.5 ml microcentrifuge tube, dewaxed in xylene, and washed with 100 % ethanol followed by complete drying. Then, DNA was extracted using phenol and chloroform after proteinase digestion (10). Amplification of myc genes was simply detected based on the PCR (11). In brief, c-myc (3rd exon: the sense primer 5′-CAGAGTCTGGATCACC-TTCT-3′ and the antisense primer 5′-AGGATAGTCCTTCC-GAGTGG-3′, which yielded a 126-bp product), N-myc (2nd exon: the sense primer 5′-ATCATCTGCAAGAACCCAGAC-3′ and the antisense primer 5′-CAGCTCGTTCTCAAGCAGCAT-3′, which yielded a 227-bp product), and L-myc (2nd exon: the sense primer 5′-GATGTTGTGACAGTAGAGAAG-5′ and the antisense primer 5′-CTCTGAAGCCTCTTCTTGGGA-3′, which yielded 179-bp product) specific sequences were co-amplified in the same reaction mixture containing 10 % dimethyl sulfoxide (sterile filtered, Sigma Chemical Co., MO, USA) for 35 cycles. Each cycle of PCR included 1 min of denaturation at 94 °C, 1 min of primer annealing at 55 °C and 2 min of extension/synthesis at 72 °C. The PCR products were electrophoresed on agarose gel and stained with ethidium bromide followed by densitometric scanning. The myc family oncogene was considered to be amplified if the signal from one myc gene specific band was greater than the other two myc gene apecific bands.

Statistical analysis. Correlations between MDR1 mRNA and various prognostic factors were assessed using χ^2-test. Survival was calculated from the beginning of chemotherapy by the Kaplan-Meier method. Survival curves were compared using the generalized Wilcoxon test. Prognostic factors contributing to patient survival were analyzed using Cox's regression method (12).

Results

Expression of the MDR1 gene and alteration in the myc family oncogene. Determination of MDR1 mRNA levels was possible in 18 out of 20 specimens. The isolated RNA of 2 specimens was either extremely degraded or insufficient for RT-PCR analysis. Finally MDR1 mRNA levels in biopsy specimens were determined by RT-PCR in 18 previously untreated patient with SCLC. Expression of MDR1 gene was termed high

Table 1 Patient data with reference to MDR1 mRNA expression and myc amplification

	All	MDR1 mRNA			myc Amplification		
		High	Low	χ^2 test	+	−	χ^2 test
No. of patient analyzed	18	5	13		4	14	
Age (year)							
Median	68	62	68	N.S.	69	68	N.S.
Range	47—84	47—77	61—84		62—77	47—84	
Sex							
Male	16	5	11	N.S.	3	13	N.S.
Femal	2	0	2		1	1	
PS							
0—1	13	3	10	N.S.	4	9	N.S.
2	5	2	3		0	5	
Extent of disease							
LD	7	3	4	N.S.	2	5	N.S.
ED	11	2	9		2	9	

PS: Performance status; LD; Limited disease; ED: Extended disease; N.S.: Not significant

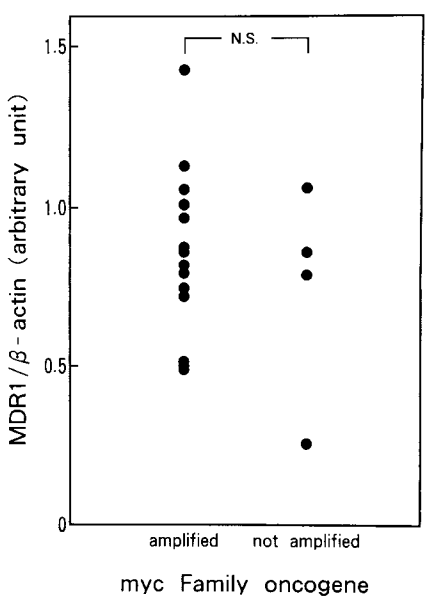


Fig. 1 Correlation between MDR1 expression levels and myc family gene amplification. There were no correlations between myc gene amplification and MDR1 expression levels, although tumors with amplified myc gene showed a tendency for low MDR1 expression. (N.S.: not significant)

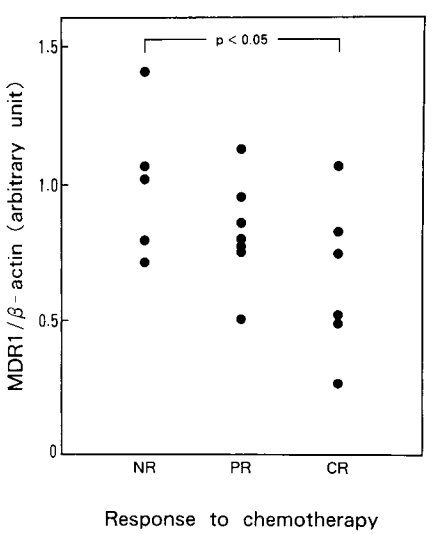


Fig. 2 Correlation between MDR1 expression levels and response to chemotherapy. There appears to be a tendency for lack of response to chemotherapy in association with elevation of MDR1 level in patient tumors. MDR1 expression levels in tumors of non-responders (NR) was significantly higher than those in tumors of complete responders (CR) ($p<0.05$).

when MDR1/β-actin ratio was equal or higher than 1.0, and was termed low when MDR1/β-actin ratio was less than 1.0. Table 1 shows the clinical data of the 18 patients with reference to MDR1 expression and myc amplification. MDR1 mRNA levels were high in 5 patients (27.8 %) and low in 13 patients (72.2 %). Age, sex, performance status (PS), and extent of disease in patients with tumor expressing high MDR1 were comparable with those in patients with tumor expressing low MDR1.

Alteration of any of the myc family oncogenes was detected in 4 patients (22.2 %) (c-myc in 3, and L-myc in 1). Age, sex, PS, and extent of disease in patients with myc gene amplified tumor were also comparable with those with myc gene non-amplified tumor. As shown in Fig. 1, there were no statistically significant correlations between myc gene amplification and MDR1 mRNA

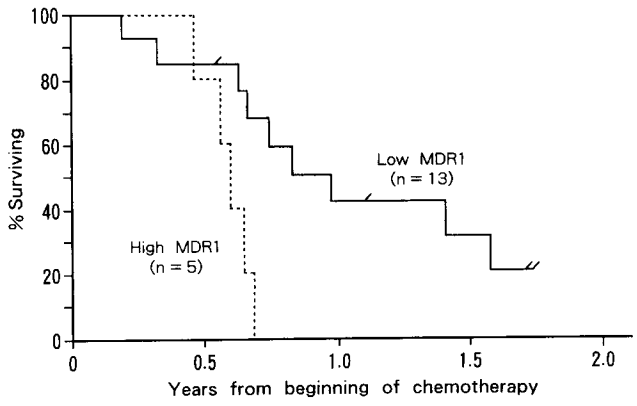
expression level, although tumors with myc amplification showed a tendency for low MDR1 expression.

MDR1 gene expression and clinical outcome. Therapeutic outcome in the 18 patients with reference to MDR1 is shown in Table 2. Eleven out of 13 patients with low MDR1 tumor achieved a major response to CAV-EP; 5 complete responses and 6 partial responses with an overall response rate of 72.2 %, while only 2 (40 %) of 5 patients with high MDR1 tumor responded to the chemotherapy. These results, showing an overall response rate of 72 % for all 18 patients, are consistent with the currently achievable response rates in patients with SCLC receiving chemotherapy (13). The correlation between MDR1 expression and response to chemotherapy is shown in Fig. 2. There appears to be a correlation between the lack of response to chemotherapy and an elevated MDR1 level in tumors (Fig. 2): MDR1 expression levels in non-responders were significantly higher than those in complete responders ($p<0.05$). The median survival for all 18 patients was 8.2 months with a median follow up of 15.3 months; 6.6–34.3 months. The median survival was 7.2 months for the 5 patients with high MDR1 tumor, and 11.7 months for the 13 with low MDR1 tumor (Fig. 3). The difference between the 2 groups was significant ($p=0.023$). In spite of a higher proportion of ED in the low MDR1 group (Table 1), there

Table 2 Response to chemotherapy with referred to MDR1 mRNA levels

	All	MDR1 mRNA	
		High	Low
No. of patients evaluated	18	5	13
Complete remission	6	1	5
Partial remission	7	1	6
No response	5	3	2
Response rate (%)			
All	72.2	40.0[a]	84.6[a]
Limited disease	85.7	[2/3]	[4/4]
Extended disease	63.5	[0/2]	[7/9]
Survival time (months)			
All	8.2 (2.3 -20.8)	7.2 (5.6 -8.2)[b]	11.7 (2.3– 20.8)[b]
Limited disease	8.9 (5.6—20.8)	—	—
Extended disease	8.2 (2.3—20.4)	—	—

a: Not significant, *b*: $p=0.023$, [ ]: Actual number



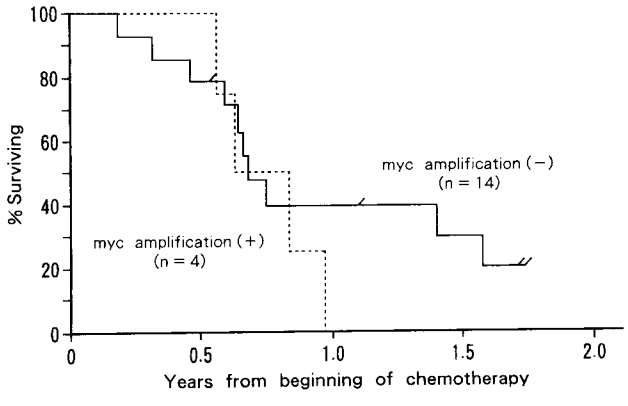


Fig. 3 (left) Actuarial survival of 18 patients with SCLC by levels of MDR1 expression. In spite of a higher proportion of extensive disease in the low MDR1 group, there was a significant difference for survival between patients with high MDR1 tumor and patients with low MDR1 tumor ($p=0.023$).
Fig. 4 (right) Actuarial survival of 18 patients with SCLC by myc family gene amplification. This showed no significant difference for survival between patients with myc amplified-tumor and patients with myc non-amplified tumor.

Table 3 Hazards ratio for the Cox's proprtional hazards model for overall survival

Variables	Hazards ratio	95% Confidential limit	*p* value
MDR1	7.81	1.530-39.8	0.0134
myc	3.18	0.679-14.8	0.1420
Age	2.99	0.852-10.5	0.0870
Performance status	2.65	0.563 12.4	0.2179
Disease extent	1.05	0.302-3.66	0.9378

was a significant difference for survival between patients with high MDR1 expression and patients with low MDR1 expression.

Myc family oncogene amplification and clinical outcome. We further examined the relation of myc family oncogene amplification to clinical outcome in the 18 patients. The response rate was 75 % in the 4 patients with myc family gene amplified tumor, and 72 % in the 14 patients with no myc amplified tumor. Survival of the 4 patients whose tumors showed myc family gene amplification (median, 8.2 months; range, 6.8-11.7 months) was compatible with survival of the 14 patients with myc gene non-amplified tumor (median, 8.8 months; range, 2.3-20.8 months) ($p=0.73$) (Fig. 4).

Multivariate analysis to determine prognostic factors. In order to evaluate MDR1 expression as a prognostic factor in SCLC, we analyzed MDR1 expression with known variables using multivariate Cox's regression analysis. MDR1 expression level, myc family gene amplification, age, PS, and extent of disease were included as variables (Table 3). MDR1 expression level was revealed to be a statistically significant independent prognostic factor for patient survival ($p=0.0134$).

Discussion

In various tumors such as myeloma, leukemia, lymphoma, neuroblastoma, and adrenal, hepatic, renal, colon, and ovarian carcinoma, a number of investigators have reported that biopsies obtained from tumors that relapsed after chemotherapy tended to express a higher level of MDR1 or P-gp than those obtained at the time of initial diagnosis (14). Among such studies indicating that malignant tumor cells in human cancers can obtain an appreciable level of P-gp after chemotherapy, however, only a few investigators have reported the predictive value of P-gp for chemotherapeutic response. Chan *et al.* reported the most convincing data of P-gp for predicting the outcome to therapy in soft-tissue sarcoma and in neuroblastoma in children (15, 16). Waller *et al.* reported that patients with breast cancer with detectable MDR1 gene expression (by slot blot analysis) had a poorer prognosis than those without detectable MDR1 expression (17).

In SCLC, Lai *et al.* reported that no or very low expression of MDR1 or P-gp was detectable and found that there was no correlation between MDR1 gene expression and prior chemotherapy status of patients or tumor response to chemotherapy(2). In our previous and present studies using RT-PCR, however, we confirmed that the MDR1 gene was transcribed in all the SCLC tumors included in the analysis, and that the expression of MDR1 mRNA in tumors obtained from patients with SCLC correlated with treatment outcomes; tumor response and patient survival. Although only a small number of patients was analyzed in this study, it is noteworthy that the level of MDR1 expression was appeared to be an independent prognostic factor in these SCLC patients.

A small number of patients with tumor expressing low MDR1 showed resistance to chemotherapy. Such resistant tumors with low MDR1 mRNA may have MDR1-unrelated mechanisms of drug resistance. Another possible explanation is that MDR1 RT-PCR analytsis, using homogenized tumor samples, does not detect any potential heterogeneity with regard to MDR1 expression within tumor. The *in situ* hybridization technique may be better for the detection of resistant clones within tumors.

Recently some investigators have studied oncogene amplification of c-myc, N-myc, and L-myc in human cancer and their relationship to the clinical presentation and the course of patients. Johnson *et al.* showed that SCLC patients who developed tumor cell lines showing c-myc gene amplification at the time of relapse lived shorter than those who developed unamplified cell lines (1). Funa *et al.*, using *in situ* hybridization, reported that increased expression of N-myc oncogenes in primary biopsies from untreated patients with SCLC was strongly associated with a poor response to chemotherapy, rapid tumor growth, and short survival (18). Nakagawa *et al.* reported that MDR1 mRNA expression was inversely correlated with N-myc expression, and found that neuroblastoma patients whose tumors had a high level of MDR1 expression and a low level of N-myc expression had a significantly better prognosis (19). Nonetheless, in

our series of patients, myc amplification in the tumor did not appear to reflect response to chemotherapy and patient survival. This was also confirmed by the multivariate analysis.

Finally, it appears that patients with tumors with high MDR1 mRNA level are resistant to chemotherapy and have a poor prognosis. These findings need to be confirmed in studies with a larger number of patients.

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Received November 26, 1993; accepted February 8, 1993.