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Abstract

In an attempt to elucidate the tumor properties relating to responsiveness to chemotherapy, we examined immunohistochemically the expression of P-glycoprotein (P-gp) and carcinoembryonic antigen (CEA) in small cell lung cancer (SCLC) tumors. Tumor specimens from 33 patients were obtained at the time of diagnosis and relapse. Four patients expressed P-gp in their initial tumors, and 7 others did in recurrent tumors. The overall response rate to chemotherapy of the initial tumors was 75% for P-gp-positive initial tumors and 86% for P-gp-negative tumors, whereas the disease-free and overall survival times were significantly shorter in the former than the latter. Three patients showed CEA in their initial tumors, and 5 others did in recurrent tumors. The patients with CEA-positive initial tumors tended to relapse earlier than those with CEA-negative tumors. In addition, recurrent tumors expressing CEA were resistant to salvage chemotherapy. A clear correlation between CEA expression by tumors and the CEA level in the serum was observed at diagnosis as well as at relapse. These findings indicate that P-gp and/or CEA expression by a tumor and elevated CEA level in the serum may predict refractoriness of the tumor to chemotherapy.

KEYWORDS: small cell lung cancer, immunohistochemistry, drug resistance, P-glycoprotein, carcinoembryonic antigen

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Immunohistochemical Detection of P-Glycoprotein and Carcinoembryonic Antigen in Small Cell Lung Cancer: With Reference to Predictability of Response to Chemotherapy

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In an attempt to elucidate the tumor properties relating to responsiveness to chemotherapy, we examined immunohistochemically the expression of P-glycoprotein (P-gp) and carcinoembryonic antigen (CEA) in small cell lung cancer (SCLC) tumors. Tumor specimens from 33 patients were obtained at the time of diagnosis and relapse. Four patients expressed P-gp in their initial tumors, and 7 others did in recurrent tumors. The overall response rate to chemotherapy of the initial tumors was 75% for P-gp-positive initial tumors and 86% for P-gp-negative tumors, whereas the disease-free and overall survival times were significantly shorter in the former than the latter. Three patients showed CEA in their initial tumors, and 5 others did in recurrent tumors. The patients with CEA-positive initial tumors tended to relapse earlier than those with CEA-negative tumors. In addition, recurrent tumors expressing CEA were resistant to salvage chemotherapy. A clear correlation between CEA expression by tumors and the CEA level in the serum was observed at diagnosis as well as at relapse. These findings indicate that P-gp and/or CEA expression by a tumor and elevated CEA level in the serum may predict refractoriness of the tumor to chemotherapy.

Key words : **small cell lung cancer, immunohistochemistry, drug resistance, P-glycoprotein, carcinoembryonic antigen**

Small cell lung cancer (SCLC) has properties that are clearly different from other histologic types of lung cancer. Among other things, the resistance of SCLC to drug therapy is of particular importance. This tumor shows wide diversity in its responsiveness to drug therapy, resulting in a wide range of patient's survival. The biological properties inherent to the tumor have been assumed to relate to its responsiveness to drug therapy and the patient's prognosis. These biological properties include the histologic subtype of SCLC (1, 2), morphology in culture (3, 4), overexpression of some oncogenes (5) and expression of some cell adhesion molecules (6).

Aside from the aforementioned factors, P-glycoprotein (P-gp) has been identified as a pump molecule that transports drugs outside the cells (7). In cultured tumor cells, increased expression of P-gp has been shown to result in multidrug resistance to structurally dissimilar drugs with different mechanisms of action (7). P-gp-associated multidrug resistance has also been observed in a variety of malignancies of man, but it has scarcely been observed in SCLC (7-10). Carcinoembryonic antigen (CEA), one of the most studied tumor antigens, has recently been demonstrated to belong to the immunoglobulin supergene family and is assumed to function as an intercellular adhesion molecule (11-13). In the present study, we found that a substantial proportion

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of patients with relapsing SCLC presented an elevated serum CEA level while their neuron-specific enolase (NSE) levels in the serum remained normal.

Based on the assumption that expression of P-gp and CEA in SCLC tumors may correlate with the chemotherapy outcome, we examined immunohistochemically their expression in tumor tissue specimens which were obtained at the time of diagnosis and relapse. In addition, we analyzed the correlation of CEA and NSE expression by SCLC tumors and the concentrations of these markers in the patient's sera.

Subjects and Methods

Patients. This retrospective study employed tumor tissue specimens obtained from 33 patients with SCLC. They were part of a series of 206 patients who had been enrolled in 2 protocols of chemotherapy conducted between April 1981 and December 1990 (14). All the patients underwent a series of examinations for staging, which included fiberoptic bronchoscopy, chest plain radiographs, computed tomography of the cest, brain and upper abdomen, radionuclide bone scan, iliac crest bone marrow biopsy, complete blood count and blood chemistry tests. Based on the staging, the patients were divided into limited disease (LD)

Table 1 Characteristics of 33 small cell lung cancer (SCLC) patients

Patient No.	Sex	Age (years)	PS	Extent of disease	Induction			Salvage CT
					CT	Chest RT	Response	
1	M	58	1	L D	CAV-PVP	+	C R	—
2	M	58	0	L D	CAV-PVP	−	C R	SM5887
3	M	71	2	L D	COMP-VAN	+	C R	COMP
4	M	67	0	L D	CAV-PVP	+	C R	IFX + VDS + MTX + ETP
5	F	65	0	L D	CAV-PVP	+	P R	PVP
6	M	58	1	L D	CAV-PVP	+	C R	CAV-PVP, CPT11
7	F	60	0	L D	COMP-VAN	+	P R	PVP
8	M	76	0	L D	COMP-VAN	+	P R	CDDP + CQ
9	M	39	1	L D	CAV-PVP	+	C R	CPT11, CAV-PVP
10	M	64	1	L D	CAV-PVP	+	C R	CDDP + ETP
11	M	62	1	L D	COMP-VAN	−	C R	PVP
12	M	63	1	L D	CAV-PVP	+	C R	—
13	M	56	0	L D	CAV-PVP	+	N C	IFX + CDDP + VDS
14	M	63	3	L D	COMP-VAN	+	C R	—
15	M	74	2	L D	CAV-PVP	+	P R	—
16	M	61	1	L D	COMP-VAN	+	N C	—
17	M	72	2	L D	COMP-VAN	−	P R	—
18	M	63	3	L D	CAV-PVP	−	N C	—
19	M	69	1	E D	CAV-PVP	+	P R	navelbine, CBDCA + ETP
20	F	68	0	E D	CAV-PVP	−	C R	CDDP + CBDCA, lonidamine
21	M	65	0	E D	CAV-PVP	−	P R	CAV-PVP, CPT11
22	F	69	1	E D	CAV-PVP	−	C R	254S
23	M	74	1	E D	CAV-PVP	−	P R	PVP
24	M	77	1	E D	CAV-PVP	+	C R	IFX + VCR + ETP
25	M	65	1	E D	CAV-PVP	−	P R	CAV-PVP
26	F	48	1	E D	CAV-PVP	+	P R	CBDCA + ETP
27	M	80	1	E D	COMP-VAN	−	C R	—
28	F	65	1	E D	CAV-PVP	−	P R	CBDCA + ETP
29	M	56	2	E D	COMP-VAN	−	N C	—
30	M	61	1	E D	CAV-PVP	−	P R	—
31	M	47	2	E D	CAV-PVP	+	P R	—
32	M	57	0	E D	CAV-PVP	−	P R	—
33	M	63	2	E D	CAV-PVP	−	N C	—

PS, performance status; CT, chemotherapy; RT, radiotherapy; LD, limited disease; ED, extensive disease; CR, complete response; PR, partial response; NC, no change

Abbreviations for drug therapy: COMP-VAN, cyclical alternating chemotherapy consisting of cyclophosphamide, vincristine, methotrexate and procarbazine (COMP), and etoposide, doxorubicin and nimustine (VAN); CAV-PVP, hybrid or sequential combination of cyclophosphamide, doxorubicin and vincristine (CAV), and cisplatin and etoposide (PVP); PVP, cisplatin plus etoposide; IFX, ifosfamide; VDS, vindesine; MTX, methotrexate; ETP, etoposide; CDDP, cisplatin; CQ, carboquone; CBDCA, carboplatin; SM5887, an analog of doxorubicin; CPT11, an analog of camptothecin; 254S, an analog of cisplatin

confined to the ipsilateral hemithorax, or extensive disease (ED) with metastasis. The criteria for the response to the chemotherapy defined a complete response (CR) as the disappearance of all tumor lesions, and a partial response (PR) as 50 % or more reduction in the sum of all measurable lesions, lasting for at least 4 weeks.

The details of the characteristics of the 33 patients are shown in Table 1. They received either COMP-VAN (15) or CAV-PAP regimen (16), and the majority of LD cases received radiation therapy as a part of the treatment protocol. The results were 14 patients with CR and 14 with PR, while the remaining 5 had stable disease. All the patients, however, finally experienced relapse or progressive disease. Twenty patients underwent salvage chemotherapy, but the remaining 13 were ineligible to receive it, mainly due to their poor medical condition.

Immunohistochemical detection of P-gp, NSE and CEA expression in SCLC tumors. Seventy-five tumors from the 33 patients were studied. These tumors were preserved as formalin-fixed paraffin-embedded blocks, and they consisted of 33 tumors biopsied at initial diagnosis (initial tumors) and 42 tumors at relapse or progression (recurrent tumors) obtained by biopsy or postmortem examination. Fifty-seven specimens were collected from primary lesions, and the remaining 18 were from metastatic lesions.

The immunoperoxidase method was used for the detection of P-gp, NSE and CEA. Briefly, 4.5-μm thick sections were deparaffinized with xylene and ethanol, and then exposed to 0.3 % hydrogen peroxide in methanol to reduce endogenous peroxidase activity. Nonspecific protein binding was blocked with 10 % fetal calf serum (FCS) in phosphate-buffered saline, and the sections were incubated with each primary antibody at 4°C overnight. Mouse monoclonal antibodies specific for P-gp (C219; Centocor; Malvern, USA; 10 μg/ml) and CEA (Oxoid; Basing-stoke, UK; 2 μg/ml) and rabbit anti-NSE serum (Chemicon; Temecula, USA; a 1:300 dilution) were used as the primary antibodies, and detection was performed using an ABC kit (Nichirei; Tokyo, Japan).

The criterion for positive staining was defined as the presence of 10 % or more positive-staining tumor cells in each section, after correcting for each negative and positive control. A sample of each tumor section incubated with 10 % FCS alone was used as the matched negative control. A doxorubicin-resistant human SCLC cell line SBC-3/ADM showing overexpression of P-gp (17, 18), human brain tissue and human colon carcinoma tissue were used as positive controls for P-gp, NSE and CEA, respectively.

NSE and CEA concentrations in the serum. The relationship between NSE and CEA expression by the tumor and the serum NSE and CEA levels was evaluated in 24 patients. The serm NSE level was measured with an RIA kit (Pharmacia; Uppsala, Sweden), and the CEA level was measured with an EIA kit (Boehringer-Mannheim; Mannheim, Germany).

Statistical analysis. The disease-free time and overall survival time from the beginning of chemotherapy were calculated by the method of Kaplan and Meier (19). We assessed the prognostic significance of P-gp and CEA expression by the patient's tumors by means of a univariate analysis of the generalized Wilcoxon test (20) or a multivariate analysis using Cox's proportional hazard model (21). For comparison of the proportion of categorical variables, Fisher's exact probability test was used (22).

Results

P-gp expression by SCLC tumors and treatment outcome. As shown in Table 2, four (12 %) of the 33 patients showed P-gp in the initial tumor, and an additional 7 (11 in total, 33 %) expressed this protein at relapse or progression. The expression rate was significantly increased at relapse compared with at diagnosis ($p = 0.038$). Four of the patients in stage ED had a P-gp-positive tumor at diagnosis (Patient Nos. 20, 27, 32 and 33 in Fig. 1) and were still positive at recurrence. Another 7 patients (5 with LD and 2 with ED) showed a P-gp-positive tumor at recurrence (Patient Nos. 1, 3, 4, 16, 17, 22 and 31 in Fig. 1).

The relationship between P-gp expression by the initial tumor and the result of the treatment was analyzed by comparing the differences in the response rate, disease-free survival and overall survival between the 4 patients with a P-gp-positive initial tumor and the remaining 29 patients with a negative initial tumor (Table 3). All patients in the former group were ED cases, whereas the latter group consisted of 18 patients with LD and 11 with ED. No significant differences were found in relation to the age, sex, performance status (PS), weight loss or chemotherapy regimen between the 2 groups. The overall response rate was 75 % for the former and 86 % for the latter. The CR rate was also comparable for the 2 groups: 50 % for the former and 41 % for the latter. The median disease-freee survival time was shorter in the P-gp-positive group (5.1 months) than in the P-gp-negative group (9.4 months)($p = 0.041$), and the data were similar

Table 2 Immunohistochemical detection of P-gp, NSE and CEA in small cell lung cancer tumors

Tumor	No. of patients examined	No. (%) of patients with positive expression by tumor		
		P-gp	NSE	CEA
Initial tumor	33	4(12)[a]	29(88)	3(9)
Recurrent tumor	33	11(33)[a]	30(91)	8(24)

a: $p = 0.038$. P-gp, P-glycoprotein; NSE, neuron-specific enolase; CEA, carcinoembryonic antigen.

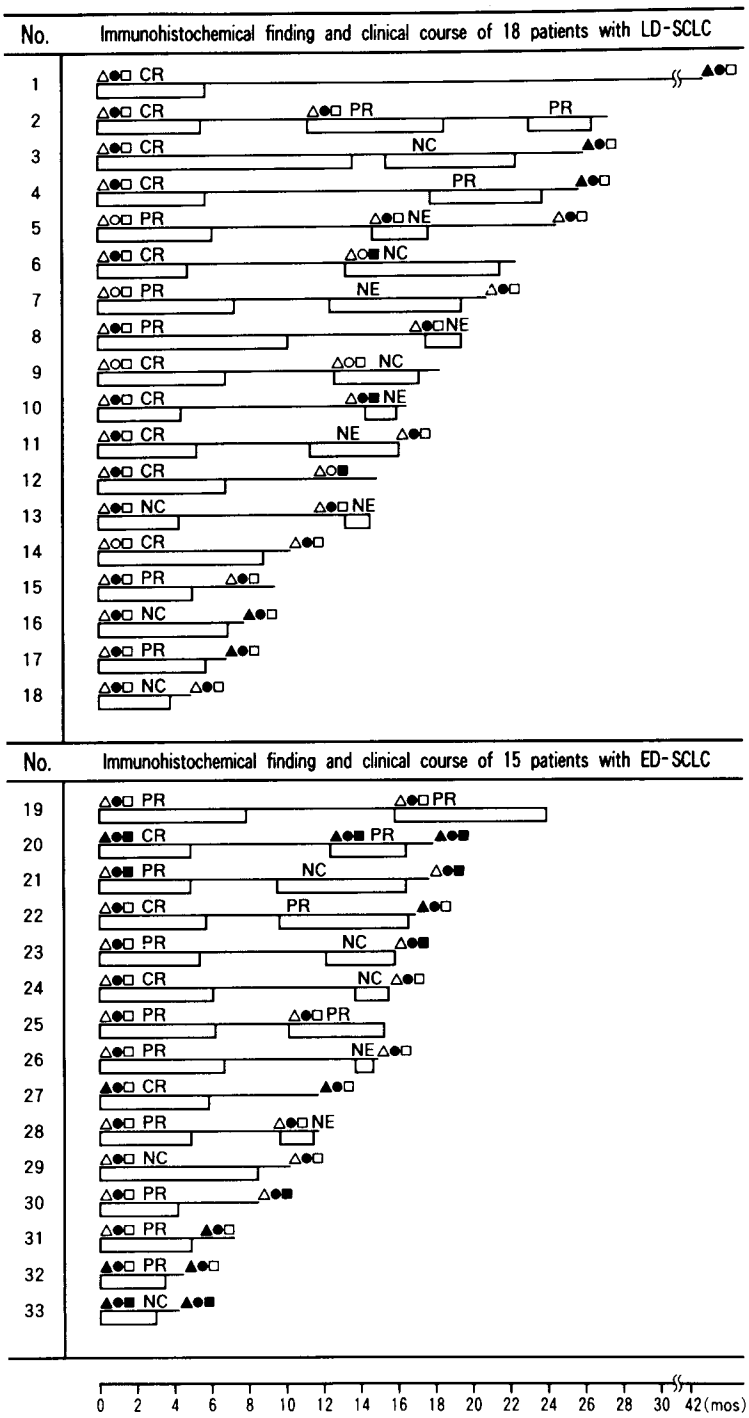

Fig. 1 Results of immunohistochemistry and clinical course of 33 small cell lung cancer (SCLC) patients according to the extent of disease. All the patients except Patient No. 19, who was still alive at the time of analysis, had died of recurrent SCLC. The box below the duration of survival represents the duration of treatment. The abbreviations and marks used are: LD, limited disease; ED, extensive disease; CR, complete response; PR, partial response; NC, no change; NE, nonevaluable; ▲, positive for P-glycoprotein (P-gp); ●, positive for neuron-specific enolase (NSE); ■, positive for carcinoembryonic antigen (CEA); △, negative for P-gp; ○, negative for NSE; □, negative for CEA.

even when only the ED cases were compared (5.1 months *vs* 9.2 months)(Fig. 2). The median survival time was 8.1 months in the former and 16.0 months in the latter, and these values were also similar when only the ED cases were compared (8.1 months *vs* 15.3 months).

In an attempt to corroborate the significance of P-gp expression to the outcome, a multivariate analysis was carried out including the PS, extent of disease and weight loss (Tables 4 and 5). The prognostic significance of P-gp was confirmed in relation to the disease-free survival ($p = 0.030$) and overall survival ($p = 0.043$). Especially, for the disease-free survival, P-gp expression was the most important factor, as indicated by the highest hazard ratio.

NSE and CEA expression by SCLC tumors and treatment outcome. Of the 33 patients, 29 (88 %) had an NSE-positive tumor at diagnosis and 30 (91 %) were positive at relapse or progression (Table 2). Three patients having an NSE-negative initial tumor developed an NSE-positive tumor at relapse (Patient Nos. 5, 7 and 14 in Fig. 1). Conversely, 2 patients having an NSE-positive initial tumor had become negative at recurrence (Patient Nos. 6 and 12 in Fig. 1). Three patients (9 %) expressed CEA in their initial tumors and 8 (24 %) in recurrent tumors (Table 2). All patients having a CEA-positive initial tumor were in stage ED (Patient Nos. 20, 21 and 33 in Fig. 1), and their tumors still expressed CEA at relapse. Five patients (3 with LD and 2 with ED) newly expressed CEA at relapse (Patient Nos. 6, 10, 12, 23, and 30 in Fig. 1).

The relationship between NSE and CEA expression by the tumors and the serum concentrations of these markers was subsequently analyzed (Fig. 3). There were neither uniform changes in the serum NSE levels between the 2 points of time examined, nor any correlation between NSE expression by the tumors and the serum NSE level. On the other hand, there was a close relationship between CEA expression by the tumors and the serum CEA level. First, 2 of 3 patients having a CEA-positive tumor at initial diagnosis showed an abnormal serum CEA level (> 10 ng/ml); second, all 4 patients showing an elevated serum CEA level at initial diagnosis presented an even higher CEA level at relapse; and third, all 8 patients having a CEA-positive tumor at relapse showed high CEA levels (> 20 ng/ml).

In relation to CEA expression by the initial tumors, there were no differences in the response rate or overall survival between 3 patients with a CEA-positive tumor and 30 patients with a CEA-negative tumor. The disease-free survival was shorter in the former than the latter, but statistical significance was not detected (median of 7.7

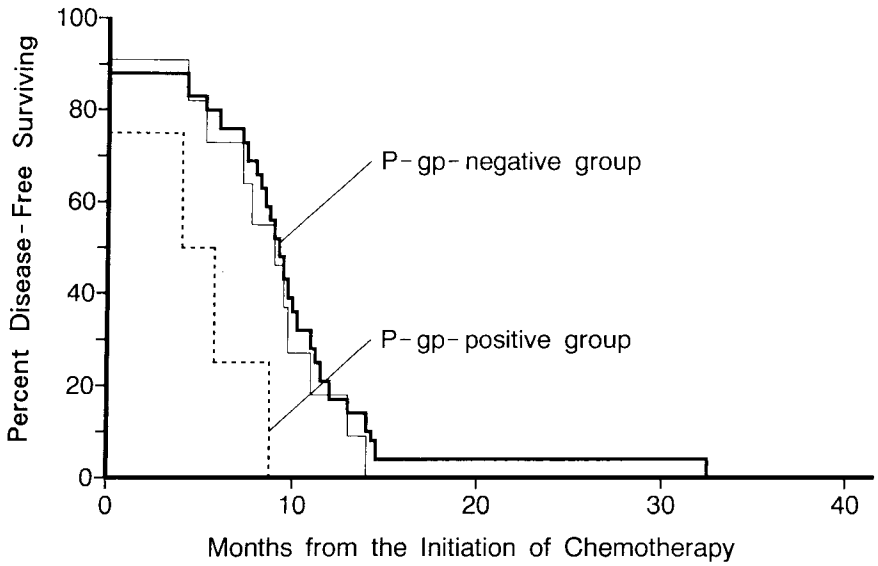

Fig. 2 Disease-free survival of 33 SCLC patients according to P-gp expression by initial tumor. A significant difference was shown between the P-gp-positive and P-gp-negative groups ($p = 0.041$). The disease-free survival of 11 ED patients with a P-gp-negative tumor is shown by the fine line as a reference. Abbreviations: See Fig. 1.

Table 3 Comparison of characteristics of 33 SCLC patients according to P-gp expression by initial tumor

Condition	No. of patients with		P-value
	P-gp-positive tumor	P-gp-negative tumor	
Total (median age; range)	4 (66; 57-80)	29 (63; 39-77)	—
Sex			
Male	3	25	0.500
Female	1	4	
PS			
0-1	3	22	0.691
2-3	1	7	
Weight loss			
No	3	23	0.635
Yes	1	6	
Extent of disease			
LD	0	18	0.033
ED	4	11	
Chemotherapy			
COMP-VAN	1	8	0.705
CAV-PVP	3	21	
Response to chemotherapy			
CR + PR	3(2)[a]	25(12)[a]	0.500
NC	1	4	

a: Numbers in parentheses represent the number of patients achieving CR. Abbreviations: See Tables 1 and 2.

Table 4 Prognostic significance of P-gp expression for disease-free survival of SCLC patients

Variable	Hazard ratio[a]	95% confidence interval	P-value
P-gp expression (no *vs* yes)	4.28	1.15-15.93	0.030
PS (0-1 *vs* 2-3)	2.77	1.03- 7.44	0.043
Extent of disease (LD *vs* ED)	1.85	0.81- 4.23	0.141
Weight loss (absence *vs* presence)	1.77	0.67- 4.67	0.252

a: Hazard ratio represents the ratio of risk of death between the two categories of each variable. Abbreviations: See Tables 1 and 2.

Table 5 Prognostic significance of P-gp expression for overall survival of SCLC patients

Variable	Hazard ratio[a]	95% confidence interval	P-value
PS (0-1 *vs* 2-3)	6.73	2.14-21.16	0.001
P-gp expression (no *vs* yes)	4.14	1.05-16.38	0.043
Weight loss (absence *vs* presence)	2.82	0.88- 8.99	0.080
Extent of disease (LD *vs* ED)	1.95	0.82- 4.60	0.130

a: Hazard ratio represents the same as in Table 4. Abbreviations: See Tables 1 and 2.

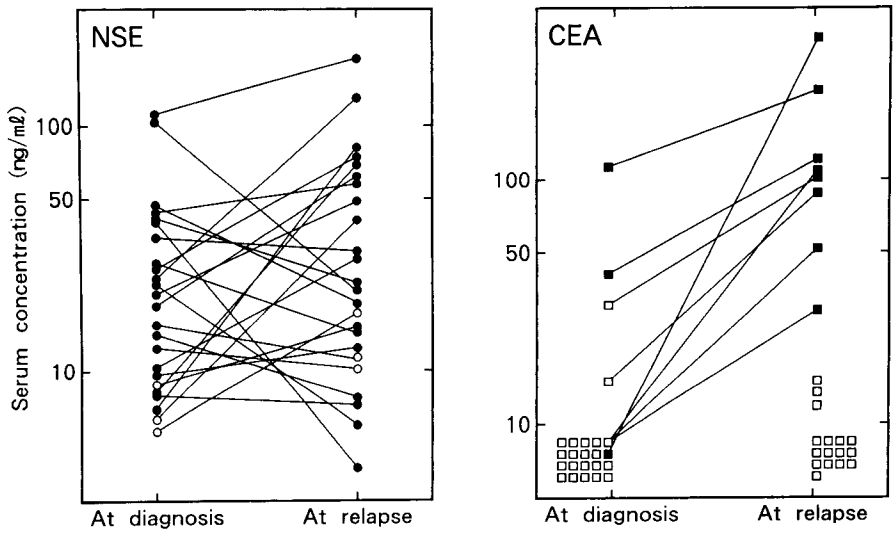

Fig. 3 Relationship between NSE and CEA expression by tumors and their serum levels at diagnosis and at relapse. The symbols and abbriviations are the same as used in Fig. 1.

Table 6 Response to salvage chemotherapy and characteristics of 20 recurrent SCLC patients according to CEA expression by recurrent tumor

Condition	No. of patients with	
	CEA-positive tumor	CEA-negative tumor
No. received salvage chemotherapy	5	15
No. with nonevaluable disease	1	7
No. evaluated for response	4	8
Response to induction chemotherapy		
CR	2	6
PR	2	2
PS		
0-1	3	5
2-3	1	3
Location of tumors		
Primary complex	4	7
Liver	3	4
Bone	1	3
Bone marrow	1	0
Abdominal nodes	0	3
Response to salvage chemotherapy		
PR	1(25)[a]	5(63)[a]
NC	3	3
Median survival time (months)[b]	5.1	5.5

a: Numbers in parentheses represent percent of patients achieving PR.
b: Survival time was calculated from the initiation of salvage chemotherapy.
Abbreviations: See Tables 1 and 2.

months *vs* 9.4 months, $p = 0.132$).

Finally, we analyzed the relationship between the effect of salvage chemotherapy and CEA expression by the recurrent tumors. Among 20 patients who received salvage chemotherapy (Tables 1 and 6), 4 patients with a CEA-positive tumor and 8 with a negative tumor were evaluable. Six having a nonevaluable lesion and 2 who died within 1 month were excluded from the analysis. No differences in the PS or tumor location were found between the 2 groups. However, the response rate was lower in the CEA-positive group than in the CEA-negative group (25 % *vs* 63 %), whereas the survival was quite comparable for the 2 groups (median of 5.1 months *vs* 5.5 months).

Discussion

SCLC is one of the tumors most sensitive to chemotherapy. Current treatment strategies for SCLC have achieved CR rates ranging from 35 % to 45 %. However, more than 90 % of those who respond eventually relapse within a few years, and long-term survival remains at less than 10 % (23). The development of drug resistance by the tumor is considered to be mainly responsible for this discouraging result, and overcoming this resistance is regarded as essential for improving the present status of chemotherapy of SCLC.

P-gp, encoded by the *MDR1* gene, is an efflux pump that confers multidrug-resistance to tumor cells (7). This resistance is shown to be clinically involved in various tumors such as soft tissue sarcoma in childhood and acute leukemia (8, 9), but it has not been confirmed in SCLC (10). The findings were contradictory in a study comparing P-gp expression by two SCLC cell lines before and after chemotherapy (24). In our present series of 33 patients, P-gp expression by the tumors was clearly demonstrated using an immunohistochemical technique. P-gp expression was detected only in ED patients for the initial tumors, and the positive rate increased significantly in the recurrent tumors. These findings suggest that P-gp expression may be innately increased with progression of the tumor and/or secondarily induced by chemotherapy. Patients with a P-gp-positive initial tumor showed a shorter disease-free survival time and reduced overall survival time than those with a P-gp-negative tumor. The difference was more significant when only ED patients were compared. In addition, P-gp expression was confirmed to be a negative prognostic factor by a multivariate analysis. Attempting to reverse the P-gp-associated multidrug resistance of tumor cells, a number of clinical trials using drugs such as verapamil have been reported. Unfortunately, they uniformly resulted in only a small degree of enhancement of the anticancer drug activity, for a short period of time (25, 26). A new approach is thus necessary.

CEA is a tumor marker of a wide variety of malignancies, including SCLC, in which significant elevation of the serum level is shown by 27 % to 66 % of patients. Serial observation is useful in monitoring the response to chemotherapy and predicting recurrence of a tumor (27-30). Patients with a normal level of serum CEA at initial diagnosis show a significant elevation at recurrence (27). In addition, patients with an increased level show a poor response to chemotherapy and a short survival time (28). In this series, CEA was detected only in ED patients at diagnosis, but the number of patients presenting a CEA-positive tumor had increased at the time of relapse. Moreover, we observed a very interesting finding in 2 patients (Patient Nos. 6 and 12 in Fig. 1): their NSE-expressing initial tumors had become negative at recur-

rence, whereas conversely, initially CEA-negative tumors became positive. SCLC is known to have potential to differentiate toward non-small cell lung cancer (NSCLC) tumors (31, 32); indeed, this was demonstrated in a series of SCLC tumors resected after chemotherapy (33, 34). The development of CEA expression by tumors may be attributed to the emergence of an NSCLC tumor. However, in our present series, such a component could not be detected by examination using light microscopy. Therefore, a CEA-positive clone may develop during chemotherapy. CEA-positive initial tumors showed a tendency to relapse earlier, and CEA-positive recurrent tumors showed a poor response to salvage chemotherapy. However, the CEA expression did not correlate with the survival time. Recent studies have indicated that CEA is a cell adhesion molecule and may be involved in intercellular recognition and binding in colon tumors (11, 12). Further study is necessary to elucidate the role of CEA expression by SCLC tumors, including its possible function as a cell adhesion molecule.

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