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Neural cell adhesion molecule expression and clinical features in small cell lung cancer: a semi-quantitative immunohistochemical approach using an immunogold-silver staining method.

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Abstract

The neural cell adhesion molecule (NCAM) is a family of cell surface sialoglycoproteins mediating homotypic and heterotypic cell-cell adhesion. In tumors, NCAM is supposed to be involved with the malignant features characterized by invasive growth and metastasis. In the present study, we evaluated the correlation between NCAM expression of tumors obtained from small cell lung cancer (SCLC) patients and the clinical outcome. NCAM expression was determined semi-quantitatively by an immunogold-silver staining method using the SCLC cluster 1 monoclonal antibody NCC-LU-243. Of 20 SCLC patients studied, six patients with tumors with high NCAM expression had a poor response to chemotherapy, and a short disease-free ($p = 0.011$) and overall ($p = 0.003$) survival as compared with 14 patients having tumors with low NCAM expression. These findings indicate that the therapeutic outcome of SCLC may be partly predicted by determining the NCAM expression of the tumor.

KEYWORDS: neural cell adhesion molecule, small cell lung cancer, metastatic potential, cluster 1 monoclonal antibodies, immunogold-silver staining method

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Neural Cell Adhesion Molecule Expression and Clinical Features in Small Cell Lung Cancer: A Semi-Quantitative Immunohistochemical Approach Using an Immunogold-Silver Staining Method

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The neural cell adhesion molecule (NCAM) is a family of cell surface sialoglycoproteins mediating homotypic and heterotypic cell-cell adhesion. In tumors, NCAM is supposed to be involved with the malignant features characterized by invasive growth and metastasis. In the present study, we evaluated the correlation between NCAM expression of tumors obtained from small cell lung cancer (SCLC) patients and the clinical outcome. NCAM expression was determined semi-quantitatively by an immunogold-silver staining method using the SCLC cluster 1 monoclonal antibody NCC-LU-243. Of 20 SCLC patients studied, six patients with tumors with high NCAM expression had a poor response to chemotherapy, and a short disease-free ($p = 0.011$) and overall ($p = 0.003$) survival as compared with 14 patients having tumors with low NCAM expression. These findings indicate that the therapeutic outcome of SCLC may be partly predicted by determining the NCAM expression of the tumor.

Key words : neural cell adhesion molecule, small cell lung cancer, metastatic potential, cluster 1 monoclonal antibodies, immunogold-silver staining method

Small cell lung cancer (SCLC) is a highly aggressive tumor and is characterized by neuroendocrine properties, *i.e.*, production of peptide hormones and presence of marker enzymes. In an attempt to analyze biological features of SCLC, a number of monoclonal antibodies (MoAbs) against SCLC have been developed. Such MoAbs were classified at the First International Workshop on Lung Cancer Antigens (1). Recently, the epitope of cluster 1 (SC-1) antigen was demonstrated to be identical to that of neural cell adhesion molecule (NCAM) by a transfection study (2). NCAM is a family of cell surface sialoglycoproteins and plays an important role in homotypic and heterotypic cell-cell adhesion (3, 4). Furthermore, NCAM is supposed to be involved with the invasive growth and metastasis of tumors (5, 6). This implication prompted us to determine if the clinical features of SCLC such as metastases, response to chemo-

therapy, and the patient's survival were related to the NCAM expression of the tumor.

In the present study, we semi-quantitatively determined the level of NCAM expression in SCLC by an immunogold-silver staining (IGS) method and attempted to elucidate the role of NCAM in SCLC.

Materials and Methods

Cell lines and human tissues. The SCLC cell lines, SBC-3 and SBC-7, were established in our laboratory and maintained in RPMI 1640 supplemented with 10 % fetal bovine serum (7). Etoposide-resistant (SBC-7/ETP) and cisplatin-resistant (SBC-7/CDDP) sublines were derived from the parent SBC-7 cells *in vitro* by continuous exposure to increasing concentrations of etoposide and cisplatin, followed by cloning procedures. The SBC-7/ETP cells show continuous growth in the medium containing 0.75 μM etoposide, and the SBC-7/CDDP cells also grow vigorously in the medium containing 1.5 μM cisplatin. All 4 cell lines show

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variant morphological features according to the classification of the National Cancer Institute (8).

Human tumor tissues were obtained from primary lesions by bronchofiberscopy or at postmortem examination. Normal human brain tissues were obtained at postmortem examination as a control. Tissue sections, 2–3 mm in thickness, were fixed in cold acetone, cleared in methylbenzoate and xylene, and then embedded in paraffin. These acetone-fixed paraffin-embedded tissue sections, namely AMeX-processed tissue sections, were kept at 4°C until used (9).

Antibodies. The mouse IgG_{2a} antibody NCC-LU-243 (Nippon Kayaku, Tokyo, Japan) (10, 11), which was classified as SC-1 MoAbs at the International Workshop on Lung Cancer Antigens (1, 12), was used for the IGS method and flow cytometric analysis. All antibodies including NCC-LU-243 were diluted to various concentrations with phosphate buffered saline (PBS, pH 7.2) containing 2% bovine serum albumin and 0.1% NaN₃.

Immunogold-silver staining of SCLC cell lines. Semi-confluent cultured cells were harvested and washed twice with PBS containing 2% bovine serum albumin. Then 1×10^5 cells were incubated with NCC-LU-243 (20 $\mu\text{g}/\text{ml}$) at 37°C for 1h, washed 3 times, and incubated with an anti-mouse goat antibody labeled with 30 nm-colloidal gold ($A_{520} = 0.63$; Ultra, Liverpool, UK) at 37°C for 1h. After washing, the cells were centrifuged on glass-slides at $500 \times g$ for 5 min, and fixed in ethanol containing 4% formalin. To remove aqueous buffer the slides were washed with distilled water for 30 min. The slides were processed for silver enhancement in a dark room for 20 min using a silver staining kit (Ultra) as described by Lucocq and Roth (13). The slides were washed in distilled water, and counterstained with hematoxylin. Observation was made using an epipolarization microscope combined with fluorescence microscope (Olympus, Tokyo, Japan). Gold-silver particles adherent to the cell surface were determined in 100 randomly sampled cells for each cell line, and the level of NCAM expression was expressed as the mean number of gold-silver particles.

Immunogold-silver staining of tissue samples. Sections, 3 μm in thickness, were mounted on glass-slides pretreated with 2% 3-aminopropyltriethoxysilane (Aldrich, Milwaukee, WI) in acetone to prevent dissociation of the sections. According to the method described by Sato *et al.* (9), the sections were deparaffinized with xylene and immediately immersed in acetone. Nonspecific protein binding was blocked with PBS containing 10% fetal bovine serum for 2h. After washing 3 times with PBS containing 0.05% Tween 20, the slides were incubated with NCC-LU-243 (20 $\mu\text{g}/\text{ml}$) at 4°C for 12h, washed 3 times, and then incubated with an anti-mouse goat antibody labeled with colloidal gold ($A_{520} = 0.63$; Ultra) at 37°C for 4h. After fixation with 4% formalin in PBS, the slides were washed, silver-enhanced, and counterstained. Observation was made in the same manner as described above. The mean number of gold-silver particles for 100 randomly sampled cells represented the NCAM level.

Flow cytometric analysis. Fifty thousand cells in culture were incubated with NCC-LU-243 (10 $\mu\text{g}/\text{ml}$) at 37°C for 1h. After

washing 3 times, the cells were incubated with a 1:25 dilution of an anti-mouse fluoresceinated goat antibody (Tago, Burlingame, CA) at 37°C for 1h, washed 3 times, and then analyzed on a fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA). Nonreactive primary antibody (Becton Dickinson) was also used as a negative control. The mean fluorescence intensity of each cell line, adjusted by the negative control, was used to express the level of NCAM expression.

Clinical background of SCLC patients. A total of 29 samples from 20 SCLC patients were analyzed between January 1990 and November 1991. Twenty samples were obtained at diagnosis and 9 were from relapsing tumors.

Of the 20 patients, 15 patients received a 5-drug combination consisting of cyclophosphamide, doxorubicin, vincristine, cisplatin and etoposide (CAV-PVP regimen) (14); 2 received a 4-drug combination of cyclophosphamide, doxorubicin, vincristine and etoposide; 2 received a combination of cisplatin plus etoposide; and the remaining patient received daily oral etoposide. Patients with limited disease (LD) received chest irradiation at a total dose of 50 Gy after achieving a maximal response to chemotherapy, and prophylactic cranial irradiation was given to complete responders. All the 20 patients were evaluated for tumor response and survival. Complete response (CR) was defined as the disappearance of all detectable tumor lesions, and partial response (PR) was defined as a 50% or greater decrease in the sum of all measurable lesions, lasting for at least 4 weeks. At the time of analysis, 10 patients had died of recurrent SCLC and the median follow-up time was 16.2 months.

Statistical analysis. Survival time was calculated from the initiation of chemotherapy by the method of Kaplan and Meier (15). In order to assess the prognostic significance of the NCAM expression in patient tumors, we carried out a univariate analysis using the generalized Wilcoxon test (16) and a multivariate analysis using Cox's proportional hazard model (17). To compare two categorical variables, Fisher's exact probability test was used (18). Probability of correlation coefficient was calculated by Spearman's rank correlation test (19). The difference of mean values between the two groups was evaluated by the Wilcoxon rank sum test (20).

Results

Evaluation of a semi-quantitative application of the immunogold-silver staining method in SCLC cells in culture. Findings on IGS in 4 SCLC cell lines are shown in Fig. 1. NCAM expression, represented by gold-silver particles, appeared on the cell surface, especially at cell-cell adhesion sites. The expression was evident in SBC-7 and SBC-7/ETP cells, but less evident in SBC-7/CDDP cells than in SBC-7 cells. The expression was absent in SBC-3 cells. Flow cytometric analysis also demonstrated NCAM expression in SBC-7, SBC-7/

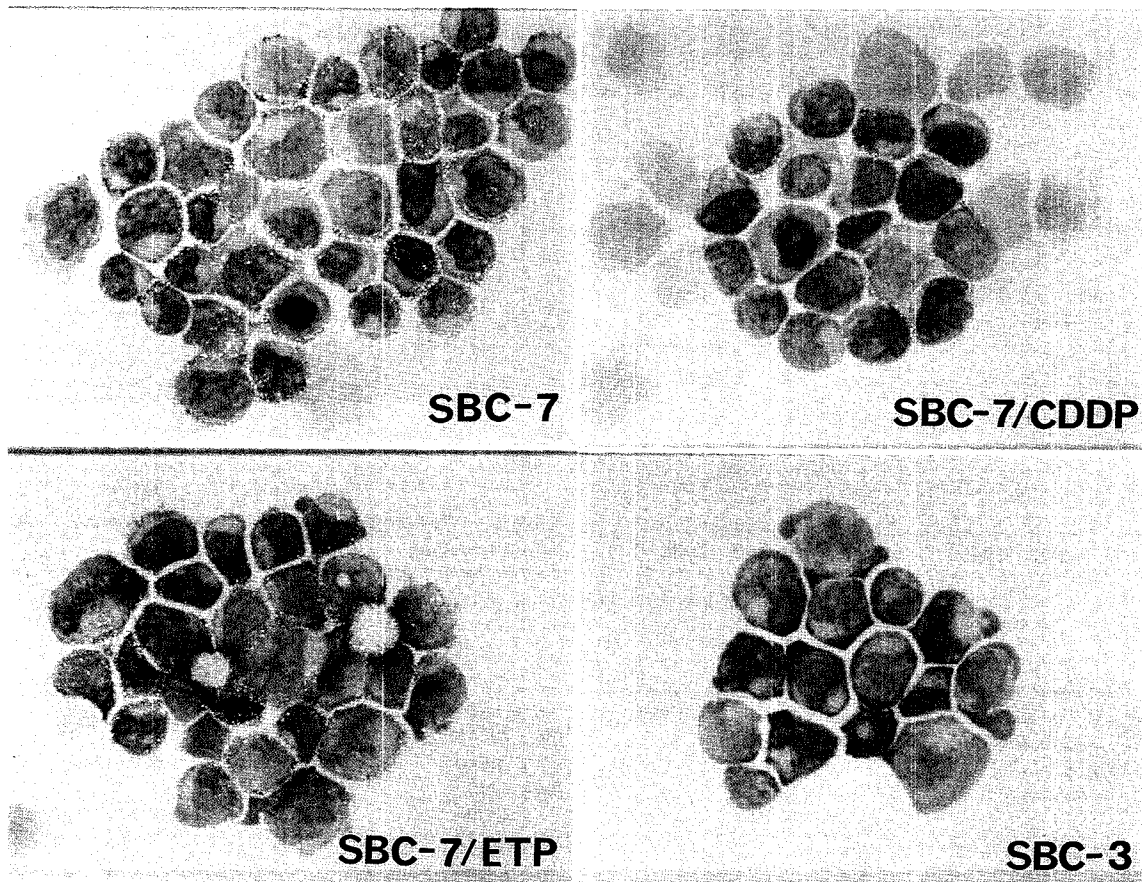


Fig. 1 Immunogold-silver stainings of small cell lung cancer (SCLC) cell lines. The number of gold-silver particles (mean \pm SE) was 71.8 ± 2.8 in SBC-7, 75.8 ± 3.6 in SBC-7/ETP, 28.2 ± 0.9 in SBC-7/CDDP, and 0.05 ± 0.02 in SBC-3. Original magnification: $\times 400$

Table 1 NCAM expression in tissue samples

	No. of tissues examined	Level of NCAM expression ^a
SCLC	29	30.4 ± 3.0^b
Tumor at diagnosis	20	36.5 ± 3.3^c
Relapsing tumor	9	17.1 ± 3.4
NSCLC	5	3.4 ± 1.1
Brain	2	110.9 ± 3.3

a: The level of NCAM expression was expressed as the number of gold-silver particles adherent to a cell, which was counted for 100 randomly sampled cells in each tissue specimen. Values are expressed as mean \pm SE. *b:* $p < 0.01$ compared with NSCLC. *c:* $p < 0.01$ compared with relapsing tumor. Abbreviations: NCAM, neural cell adhesion molecule; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer.

ETP and SBC-7/CDDP cells (Fig. 2). A close correlation between the number of gold-silver particles and the mean fluorescence intensity was evident in these cell lines ($r = 0.983$, $p = 0.021$) (Fig. 3).

Immunogold-silver staining of patient tumors. IGS of a patient tumor is shown in Fig. 4. NCAM expression was present especially on the marginal sites of individual cells. The level of NCAM expression was extremely high in the brain, and significantly higher in SCLC than in non-small cell lung cancer (NSCLC) ($p < 0.01$) (Table 1).

NCAM expression and clinical outcome in SCLC. Twenty tumors obtained at diagnosis showed a significantly higher NCAM expression than 9 tumors

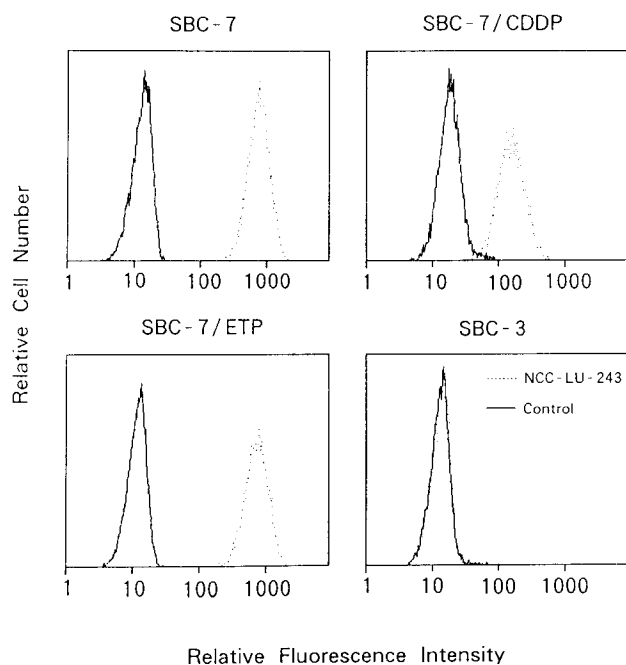


Fig. 2 Flow cytometric analysis of SCLC cell lines. Mean fluorescence intensity, adjusted by the negative control, was 892.5 in SBC-7, 910.7 in SBC-7/ETP, 167.6 in SBC-7/CDDP, and 0.6 in SBC-3. SCLC: See Fig. 1.

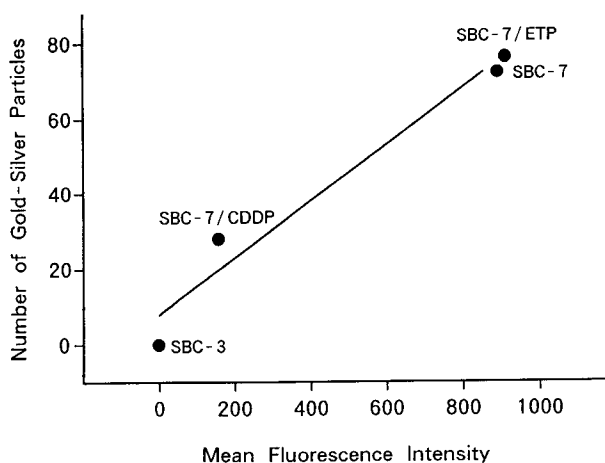


Fig. 3 Relationship between the levels of neural cell adhesion molecule (NCAM) expression in SCLC cell lines determined by the immunogold-silver staining method and by the flow cytometric analysis. A close correlation was found between the results obtained by these methods ($r = 0.983$, $p = 0.021$).



Fig. 4 Immunogold-silver staining of SCLC. Original magnification: $\times 400$

Table 2 Characteristics of 20 SCLC patients according to the level of NCAM expression

	No. of patients whose tumor expressed NCAM with the level of		P-value
	< 40	≥ 40	
No. of patients	14	6	-
Mean NCAM level (range)	28.8 (16.2 - 39.6)	54.3 (44.0 - 70.5)	-
Age: < 70	11	4	
≥ 70	3	2	0.484
Sex: Male	13	5	
Female	1	1	0.521
Weight loss ^a			
Absence	13	5	
Presence	1	1	0.521
Performance status			
0-1	11	4	
2-3	3	2	0.484
Extent of disease			
LD	6	1	
ED	8	5	0.277
Chemotherapy			
CAV-PVP ^b	11	4	
Miscellaneous	3 ^c	2 ^d	0.484
Response			
CR + PR	13 (5) ^e	4 (2) ^e	
NC	1	2	0.202

a: Weight loss was defined as a 10% or greater decrease of body weight during the last 6 months before diagnosis. b: 5-Drug combination chemotherapy consisting of cyclophosphamide, doxorubicin, vincristine, cisplatin and etoposide. c: Two patients received a combination of cyclophosphamide, doxorubicin, vincristine and etoposide, and the remaining patient received daily oral etoposide. d: These patients received cisplatin plus etoposide. e: Numbers in parentheses represent the number of patients achieving CR. Abbreviations: LD, limited disease; ED, extensive disease; CR, complete response; PR, partial response; NC, no change. SCLC, NCAM: See Table 1.

NCAM Expression in Small Cell Lung Cancer

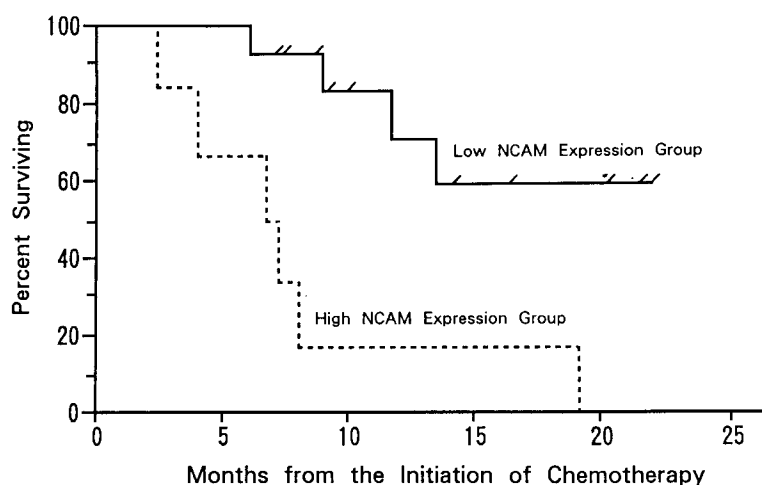


Fig. 5 Survival of 20 SCLC patients according to the level of NCAM expression. A significant difference in prognosis was found between the high NCAM expression group and the low NCAM expression group ($p = 0.003$). SCLC, NCAM: See Figs. 1 and 3.

Table 3 Significance of NCAM level in SCLC determined by multivariate analysis

Variable	Hazard ^a ratio	95 % Confidence interval	P -value
NCAM level (< 40 vs ≥ 40)	7.01	1.21 – 40.80	0.030
Performance status (0 1 vs 2 3)	3.93	0.84 – 18.53	0.083
Extent of disease (LD vs ED)	1.29	0.12 – 14.33	0.948
Weight loss (absence vs presence)	1.07	0.16 – 7.16	0.836

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

obtained after relapse ($p < 0.01$) (Table 1). Among the 20 tumors obtained at diagnosis, those from 13 patients with extensive disease (ED) tended to express higher NCAM than those from 7 LD patients (40.4 ± 4.4 vs 29.1 ± 3.6 , mean \pm SE). Higher NCAM expression was found in tumors accompanying distant metastases: 42.0 ± 6.4 for liver ($n = 4$), 46.6 ± 7.7 for bone ($n = 6$), and 49.4 ± 5.4 for bone marrow metastases ($n = 2$).

The characteristics of 20 patients whose tumors were obtained at diagnosis are shown in Table 2. These patients could be divided into 2 groups according to the level of NCAM expression (< 40 and ≥ 40): Fourteen patients belonged to the low NCAM expression group and 6 to the high NCAM expression group. Between the 2 groups there were no significant differences in age, sex,

presence of weight loss, performance status and chemotherapy regimen. However, the proportion of ED patients in the high NCAM expression group was larger than in the low NCAM expression group.

Of 14 patients in the low NCAM expression group, 13 (93 %) achieved clinical responses including 5 CRs, while only 4 of 6 patients (67 %) in the high expression group achieved responses including 2 CRs. The median time to progression (14.1 months) in the former was significantly longer than that (3.7 months) in the latter ($p = 0.011$). The survival time in the low expression group was also significantly longer than that in the high expression group ($p = 0.003$) (Fig. 5).

The influence of the NCAM level on patient's survival was analyzed by comparing 3 major prognostic variables: performance status, extent of disease and weight loss (21) (Table 3). The NCAM expression level was revealed to be the most significant factor contributing to patient's survival.

Discussion

The SC-1 antigen has recently been reported to be identical to NCAM based on the findings that 3T3 cells transfected with a complementary DNA coding human NCAM became recognizable by SC-1 MoAbs (2), and that the tissue distribution of cells reacting with SC-1

MoAbs was quite similar to that of cells reacting with NCAM antisera (22, 23). NCAM is a family of sialoglycoproteins mediating cell-cell adhesion (3, 4) and is considered to play an important role in ruling cell disposition, movement and differentiation in the embryo (24). Several NCAM isoforms are found in vertebrate species such as mice, chickens and humans. In adult brain tissues NCAM consists of 3 major proteins with molecular weights of 120, 140 and 180 kDa, which are generated from a single copy gene by alternative mRNA splicing and posttranslational modifications (3, 4). NCAM expression in tumors was demonstrated in breast cancer and Ewing's sarcoma as well as in neuroendocrine tumors such as neuroblastoma and SCLC (23, 25).

Because bronchofiberscopy does not yield specimens large enough to analyze NCAM level, the IGS method was employed to determine the level of NCAM expression in SCLC and to evaluate the clinical implications of the molecule. A semi-quantitative approach using an immunogold probe and its application to electron microscopy have been already described by Roth (26). However, the 30 nm-gold probe used in his study is too small to be identified under the light microscope. The silver enhancement method adopted in our study enabled us to enlarge the particles enough to be detected under the light microscope (13). The semi-quantitative application of this method seems to be valid in view of a good correlation between NCAM levels determined by this method and by flow cytometric analysis. Furthermore, NCAM levels in SCLC, NSCLC and brain tissues determined by this method were quite comparable to those determined by radioimmunoassay using tissue extracts (11).

Based on our observation that cisplatin-resistant SBC-7/CDDP cells showed lower NCAM expression than the parent SBC-7 cells, we initially assumed that clinical aggressiveness of SCLC might be partly correlated with the diminished expression of NCAM. This assumption is also supported by the increased metastatic potential in transplanted melanoma and glioma cells with low NCAM expression (27, 28). Nevertheless, the present study revealed that patients with tumors with high NCAM expression showed more aggressive clinical features, *i.e.*, poorer response to chemotherapy, and shorter disease-free and overall survival than those having tumors with low NCAM expression. This is a new finding with regard to NCAM expression of SCLC and its clinical features, although Kibbelaar *et al.* described that in a surgically resected series of NSCLC, patients with posi-

tive NCAM tumors had a shorter disease-free and overall survival than those with negative tumors (5). The reason for such a poor prognosis for patients with tumors with high NCAM expression remains obscure. We reason that the poor prognosis might be attributed a) to a rapid growth property in high NCAM expression tumors as shown in the analysis of disease-free survival, and b) to a large tumor burden caused by an enhanced metastatic potential in high NCAM expression tumors (*e.g.*, frequent metastases of high NCAM expression tumors in this study). The second speculation might seem contradictory, but the adhesive function of NCAM was shown to be reduced by its α -(2,8)-polysialylation (29). Thus, it is not irrational to consider that such a modification of the molecule occurred in SCLC studied. Further studies are required, including a quantitation of the adhesive function of NCAM in SCLC.

In this study, SCLC obtained after relapse showed lower NCAM expression than at diagnosis. This finding seems to be consistent with that observed in our cisplatin-resistant SBC-7/CDDP cells. Doyle *et al.* reported that SCLC cells transformed with *c-myc* and *v-H-ras* oncogenes showed not only a diminished NCAM expression but also a morphology changed toward NSCLC (30). Further, NSCLC components were frequently revealed in a series of SCLC resected after chemotherapy (31, 32). These combined findings seem to indicate that SCLC have a potential to acquire the properties of NSCLC during chemotherapy while retaining their own properties such as early metastasis and rapid growth.

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