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Enzyme-linked Immunosorbent Assay of Pro-gastrin-releasing Peptide for Small Cell Lung Cancer Patients in Comparison with Neuron-specific Enolase Measurement

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Our previous study demonstrated that pro-gastrin-releasing peptide(31–98), or ProGRP, is a specific tumor marker in patients with small cell lung carcinoma (SCLC). Using a newly developed, highly sensitive enzyme-linked immunosorbent assay (ELISA) for ProGRP, we analyzed 1,446 samples including those obtained from 478 lung cancer patients to evaluate the clinical usefulness of this ELISA. Several properties indicated that ProGRP is a useful tumor marker for SCLC. First, ProGRP was specifically elevated in SCLC patients. In non-SCLC patients and patients with non-tumorous lung diseases, its serum level was very rarely elevated. Secondly, ProGRP was a reliable marker, in terms of the marked elevation of serum ProGRP levels in SCLC patients. Thirdly, serum ProGRP levels were elevated in SCLC patients even at a relatively early stage of this disease. Fourthly, changes in the serum ProGRP level showed an excellent correlation with the therapeutic responses in SCLC patients. Neuron-specific enolase (NSE) is accepted as a tumor marker of SCLC patients. With the aim of comparing ProGRP and NSE as tumor markers for SCLC patients, we measured serum NSE levels in all samples collected in the present study. We found that ProGRP was superior to NSE in terms of sensitivity, specificity and reliability. Therefore, we consider that ProGRP can play a major role as a clinical tumor marker for SCLC patients.

Key words: Gastrin-releasing peptide — Pro-gastrin-releasing peptide(31–98) — Small cell lung carcinoma — ELISA — Tumor marker

Small cell lung carcinoma (SCLC) is one of the subtypes of lung cancer. Since this cancer arises in the hilar parts of the lung, its diagnosis by plain chest X-ray is rather difficult. Moreover, this cancer is always treated with combined chemotherapy and radiotherapy, because it has a tendency to metastasize at an early stage of the disease. Thus, a reliable tumor marker could yield valuable information for the diagnosis and treatment of these patients. We previously reported that gastrin-releasing peptide (GRP) is frequently produced by SCLC cells,[1] and suggested that the determination of plasma GRP levels could serve as a useful tumor marker for SCLC patients[2]; however, instability of GRP in blood made it impossible to develop a clinically applicable system for the measurement of GRP. Recently, with the aid of a molecular biology technique, we succeeded in developing a radioimmunoassay (RIA) for ProGRP(31–98), a region common to three types of human ProGRP; this RIA enabled us to measure serum ProGRP(31–98) without extraction, and it was shown that the determination of serum ProGRP(31–98) levels could serve as a reliable tumor marker in SCLC patients.[3] For more convenient clinical application of this assay system, we developed a highly sensitive enzyme-linked immunosorbent assay (ELISA) for ProGRP(31–98).[4] The performance study revealed several advantages of this ELISA over RIA: (1) This system is non-isotopic, and no special apparatus is required. (2) Only 0.05 ml of non-extracted serum is needed. (3) The results are obtained in only 2 h. (4) Basal levels could be detected in all normal subjects. (5) More than 2,000 samples could be examined in one day per person. In the present study, we have analyzed 1,446 samples including those obtained from 478 lung cancer patients to evaluate the ELISA. Moreover, we measured serum neuron-specific enolase (NSE) levels in all of these samples to compare the clinical usefulness of these two tumor markers.

MATERIALS AND METHODS

Materials The ELISA system for ProGRP(31–98) and its performance data were reported previously.[4] For measurement of serum NSE levels, an NSE ELISA kit

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was purchased from Eiken Chemicals (Tokyo), and used according to the manufacturer's instructions. Both assays were performed by technicians who had no clinical information on the serum samples.

Subjects Serum samples were obtained from 127 consecutive patients with previously untreated SCLC. In these patients, designation of the stage of disease as limited disease (LD) or extensive disease (ED) was decided according to the Veterans' Administration Lung Study Group criteria.[5] The responses to therapy were evaluated by means of imaging diagnostic techniques including plain and tomographic chest X-ray and computed tomographic scanning, and then the overall response to systemic chemotherapy was analyzed according to the World Health Organization criteria.[6] Serum samples were also obtained from 844 normal control subjects, 124 patients with non-tumorous lung diseases and 351 patients with non-SCLC. The non-tumorous lung diseases were pneumonia, pulmonary suppuration, interstitial pneumonia, pulmonary mycotic diseases, pulmonary tuberculosis, pulmonary emphysema, bronchial asthma, chronic bronchitis, bronchiectasis, pulmonary sequestration and sarcoidosis. The non-SCLC patients consisted of 202 with adenocarcinoma, 122 with squamous cell carcinoma and 27 with large cell carcinoma. Venous blood samples were drawn into tubes and centrifuged at 1,500*g* for 10 min. After centrifugation, the serum samples were stored at −20°C until assay. Since hemolysis interfered with the serum NSE assay, samples showing hemolysis were not included in this study. All samples were collected during the period from 1986 to 1994.

Statistical analyses For evaluating serum ProGRP and NSE as aids to the diagnosis of SCLC, we calculated sensitivity, specificity, efficiency, predictive value of positive results and predictive value of negative results, expressing the fractions as percentages.[7] The equations used were reported previously.[3]

Student's *t* test was used to determine the statistical significance of differences in the mean serum ProGRP and NSE levels between LD and ED patients with SCLC.

Furthermore, since we evaluated the same samples with the two ELISAs for ProGRP and NSE, the diagnostic accuracy of these two tumor markers was evaluated by receiver operating characteristics (ROC) analysis,[8] which is now recognized to be useful for evaluating the relative accuracy of two different assays. To draw an ROC curve, true positive rates calculated at various cutoff values are plotted on the vertical axis and false positive rates are plotted on the horizontal axis. When two ROC curves were drawn, the areas under the two curves were calculated, and then statistically analyzed by the z-score test.[9,10] The difference was considered to be significant when the *P* value was less than 0.05. ROC analyses were performed in various combinations, as shown in Table I.

RESULTS

Serum ProGRP(31–98) and NSE levels in normal subjects Immunoreactive ProGRP(31–98) analyzed by the ELISA was expressed as "ProGRP" in the present study. Serum ProGRP and NSE levels in normal subjects were distributed in a gaussian pattern after logarithmic transformation (Fig. 1). Mean levels of serum ProGRP and NSE in normal subjects were 14 pg/ml and 2.8 ng/ml, respectively. Based on these data and results of ROC analyses, we tentatively set the cutoff values for ProGRP and NSE at the mean+3SD of 50 pg/ml and 8.1 ng/ml, respectively.

Serum ProGRP(31–98) and NSE levels in patients with various lung diseases Serum ProGRP and NSE levels and the frequencies of their elevation in normal subjects and patients with various lung diseases are shown in Fig. 2 with the cutoff values.

Table I. Results of Comparison of ProGRP and NSE by ROC Analysis Based on Area under Curve for Differential Diagnosis of SCLC

Subjects	SCLC		
	LD+ED (n=127)	LD (n=58)	ED (n=69)
Normal subjects (n=844)	NS[a]	<0.05	NS
Patients with non-tumorous lung diseases (n=124)	NS	<0.05	NS
Patients with non-SCLC (n=351)	<0.05[b]	<0.01[c]	NS
Patients with non-tumorous lung diseases +non-SCLC (n=475)	<0.05	<0.01	NS
Normal subjects+Patients with non-tumorous lung diseases+non-SCLC (n=1319)	NS	<0.05	NS

a) No significant difference between ProGRP and NSE.
b) ProGRP significantly better than NSE ($P<0.05$).
c) ProGRP significantly better than NSE ($P<0.01$).

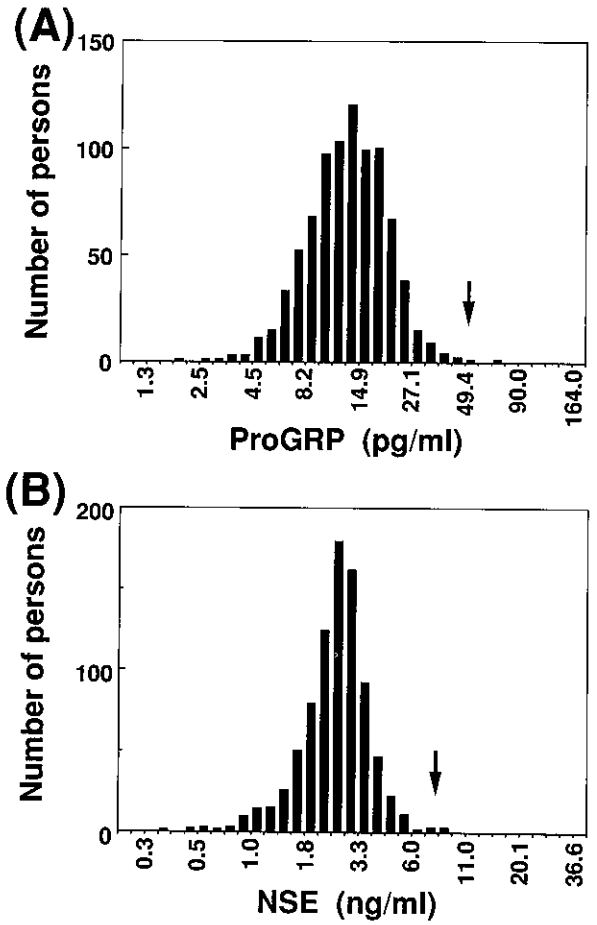

Fig. 1. Serum ProGRP (A) and NSE (B) levels and their distribution in normal subjects. Distribution patterns were gaussian after logarithmic transformation in these two assays. Cutoff values were tentatively set as the mean+3SD. Arrows indicate cutoff values.

In the case of ProGRP, in only less than 1.6% of patients with non-tumorous lung diseases, squamous cell carcinoma, adenocarcinoma and large cell carcinoma, was ProGRP elevated above the cutoff value. On the other hand, serum ProGRP levels were elevated in 80 of the 127 untreated SCLC patients (63.0%). The sensitivity, specificity, efficiency, predictive value of positive results and predictive value of negative results, calculated between SCLC and non-SCLC patients, are summarized in Table II.

In the case of NSE, none of the patients with non-tumorous lung diseases had elevated levels, but higher positive rates ranging from 5.9 to 22.2% were observed in patients with non-SCLC. In SCLC patients, serum NSE levels were elevated at a rate of 62.2%. The sensitivity, specificity, efficiency, predictive value of positive results and predictive value of negative results, calculated between SCLC and non-SCLC patients, are summarized in Table II; all of these values were inferior to those for ProGRP.

Moreover, the differences in serum levels of ProGRP between normal subjects and SCLC patients were quite large, when compared to those of NSE. The ratio of the mean levels in patients/the cutoff value and the ratio of the mean levels in patients/the mean levels in normal subjects were calculated for ProGRP and NSE (Table III). In SCLC patients, both of the ratios for ProGRP are markedly higher than those for NSE, indicating that ProGRP is a more reliable tumor marker than NSE.

Serum ProGRP and NSE levels and stage of disease in SCLC patients The relationship between serum ProGRP and NSE levels and stage of the disease in these 127 SCLC patients was investigated (Fig. 3).

In the 58 patients with LD, 33 had elevated serum ProGRP levels (56.9%); the mean ProGRP level in all LD patients was 540 pg/ml. In the 69 patients with ED, 47 had elevated levels (68.1%); the mean ProGRP level in all ED patients was 1,500 pg/ml. The frequency of ProGRP elevation was not significantly different, but there was a significant difference in ProGRP levels between the two groups ($P<0.01$).

Regarding NSE, the 25 LD patients had elevated levels (43.1%); the mean NSE level in all LD patients was 15 ng/ml. In the 69 ED patients, 54 had elevated levels (78.3%), and the mean NSE level in all ED patients was 28 ng/ml. There was a significant difference in the frequency of elevation as well as the NSE levels between the two groups ($P<0.01$).

Changes in serum ProGRP and NSE levels following treatment Changes in serum ProGRP and NSE levels following treatment are shown in Fig. 4. In the 11 patients who achieved a complete response (CR), 8 had elevated ProGRP levels, which decreased to the normal range when the tumor disappeared and remained in the normal range for one month, when the patients were judged to have achieved CR. In the 18 patients of the partial response (PR) group (50% reduction in the sum of the products of the perpendicular diameters of all measurable tumors, and, after one month, the clinical response was judged as PR), 14 had elevated ProGRP levels, in 8 of whom (57.1%) the levels had decreased to the normal range when the patients achieved PR. In the remaining 6 patients in that group (42.9%), the serum ProGRP levels had decreased, but were still elevated when these patients achieved PR. In all 9 patients with progressive disease (PD), serum ProGRP levels had increased at the time of the PD judgment (25% or more increase in tumor size or the appearance of a new lesion).

With regard to NSE, in the CR group, 6 of the 11 SCLC patients had elevated NSE levels, which decreased

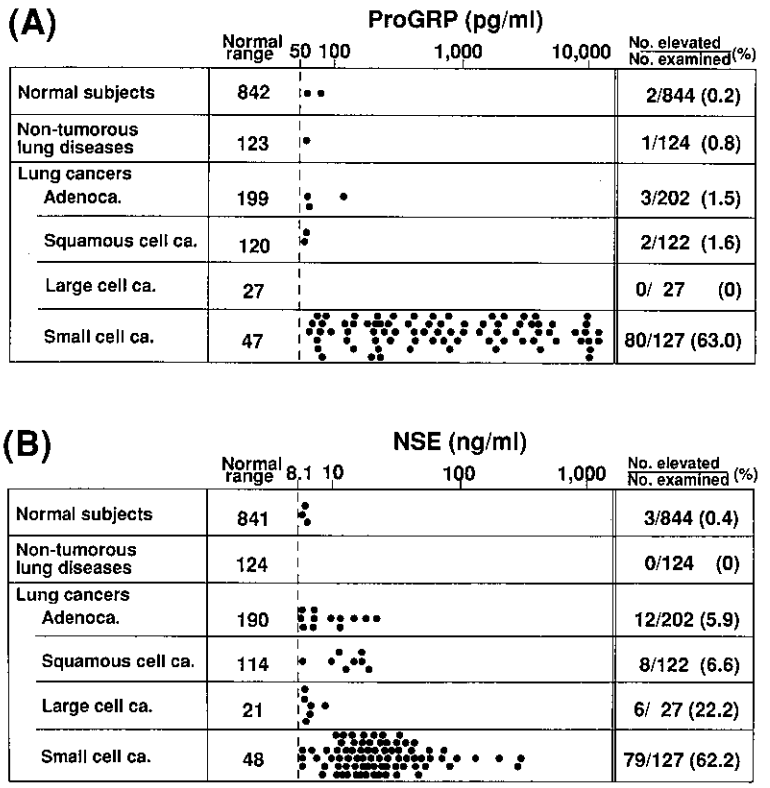

Fig. 2. Serum ProGRP (A) and NSE (B) levels and frequencies of their elevation in normal subjects and patients with non-tumorous lung diseases and lung cancers. Numerals below the normal range indicate the number of samples within the normal range.

Table II. Evaluation of the Ability of Serum ProGRP and NSE Levels to Predict the Diagnosis of SCLC

Values analyzed[a)]	ProGRP	NSE
Sensitivity	63[b)]	62
Specificity	99	93
Efficiency	89	85
Predictive value of positive results	94	75
Predictive value of negative results	88	87

a) Analyzed between SCLC and non-SCLC patients.
b) Expressing the fractions as percentages.

to the normal range when the tumor disappeared, and remained in the normal range when the patients were judged to have achieved CR. In the PR group, 11 of the 14 had elevated ProGRP levels, which decreased to the normal range in all except for one, whose level increased. In the 9 PD patients, serum NSE levels were increased at the time of the PD judgment. Although similar tendencies were observed in the cases of ProGRP and NSE, differences in serum ProGRP levels between those before treatment and at restaging in the CR, PR and PD groups were markedly greater than those of serum NSE.

ROC analyses The results of comparison of ProGRP and NSE by ROC analysis for the differential diagnosis of SCLC are summarized in Table I. ProGRP always had a tendency to be superior to NSE in all comparisons, and ProGRP was significantly better than NSE in several of them. Typical ROC curves for comparison of ProGRP and NSE in terms of the following comparisons are shown in Fig. 5; these are between total cases of SCLC and non-tumorous lung diseases plus non-SCLC, and between LD cases of SCLC and non-tumorous lung diseases plus non-SCLC, demonstrating that ProGRP is superior to NSE in both comparisons.

DISCUSSION

By using a newly developed ProGRP ELISA, we confirmed our previous suggestion that the determination of serum ProGRP levels could be an important clinical aid in diagnosis and treatment of SCLC.[3)] Several properties indicated that ProGRP is a very useful tumor marker for SCLC. First, ProGRP is specifically elevated in SCLC patients. In non-SCLC patients and patients with non-tumorous lung diseases, its serum level is very rarely elevated, resulting in the very high specificity rate of 99% for SCLC. Secondly, ProGRP is a reliable tumor marker for SCLC patients, in terms of the marked difference in serum ProGRP levels between SCLC patients and normal subjects, patients with non-tumorous lung dis-

Table III. Comparison of ProGRP and NSE in Terms of the Mean Levels in Patients, the Cutoff Values and the Mean Levels in Normal Subjects

Subjects	ProGRP		NSE	
	Mean/cutoff[a)]	Mean/normal[b)]	Mean/cutoff	Mean/normal
Patients with non-tumorous lung diseases	0.3	1.1	0.3	1.0
Patients with lung cancers				
Adenoca.	0.3	1.2	0.5	1.4
Squamous cell ca.	0.3	1.1	0.5	1.5
Large cell ca.	0.3	1.1	0.9	2.6
Small cell ca. (LD+ED)	21.0	75.1	2.8	8.0
Small cell ca. (LD)	10.9	38.8	1.9	5.5
Small cell ca. (ED)	29.6	105.6	3.5	10.1

a) Ratio of the mean levels in patients/the cutoff value.
b) Ratio of the mean levels in patients/the mean levels in normal subjects.

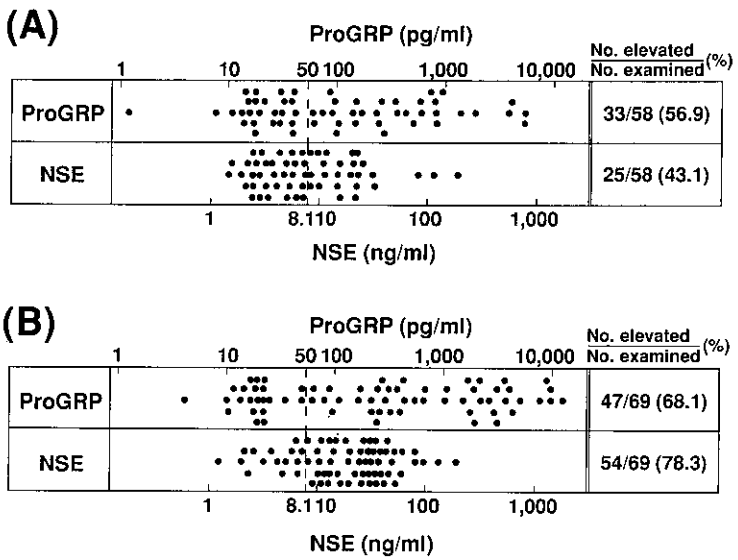

Fig. 3. Serum ProGRP and NSE levels and frequencies of their elevation in SCLC patients with LD (A) and with ED (B).

eases or non-SCLC patients. As shown in Table III, the mean serum ProGRP level in SCLC patients was 21.0- and 75.1-fold higher than the cutoff value and the mean level of normal subjects, respectively, which is superior to other tumor markers. Thirdly, ProGRP can aid diagnosis at a relatively early stage of this disease. Serum ProGRP levels were elevated in SCLC patients with LD as well as those with ED at almost the same frequency, and the mean serum ProGRP level in LD patients was still 10.9-fold greater than the cutoff value. Taking all of these observations together, we consider that ProGRP determination may help general physicians as well as specialists in respiratory diseases or lung cancer to make the diagnosis of SCLC. For instance, when a patient is a smoker, has an abnormal chest X-ray image compatible with a lung mass or inflammation and has elevated ProGRP levels, it is possible to diagnose SCLC with a certainty of 94% based on the predictive value of positive results in ProGRP determination. This allows the patient to be treated as a probable SCLC patient as early as possible. Moreover, serum ProGRP determination could be useful as a strategy for mass-screening of SCLC in smokers, since serum ProGRP levels were frequently elevated at the relatively early stage of SCLC. Further studies will be required to confirm this speculation.

The superiority of ProGRP as a tumor marker for SCLC patients is dependent on the following characteristics of this molecule. First of all, our previous studies demonstrated that GRP, and possibly ProGRP, are produced by SCLC cells at the highest frequency and in the

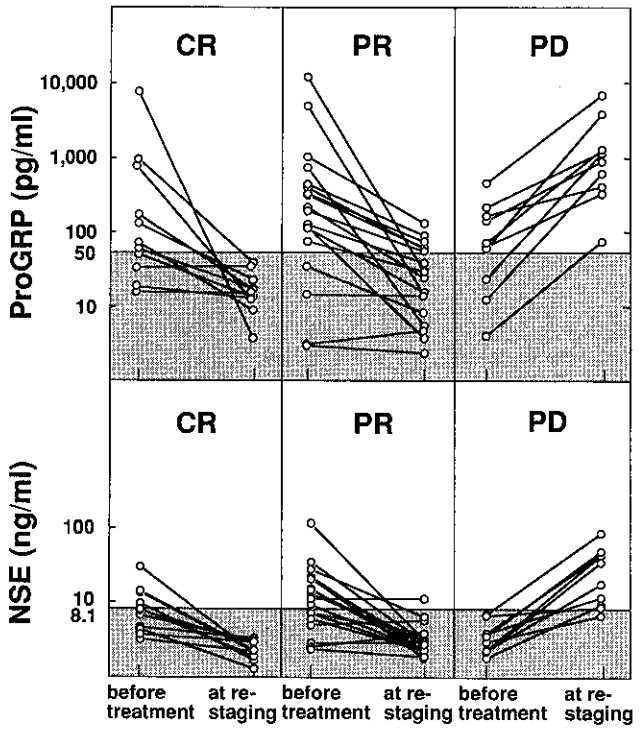

Fig. 4. Serum ProGRP (upper panel) and NSE (lower panel) levels before and after treatment in SCLC patients. CR, complete response; PR, partial response; PD, progressive disease.

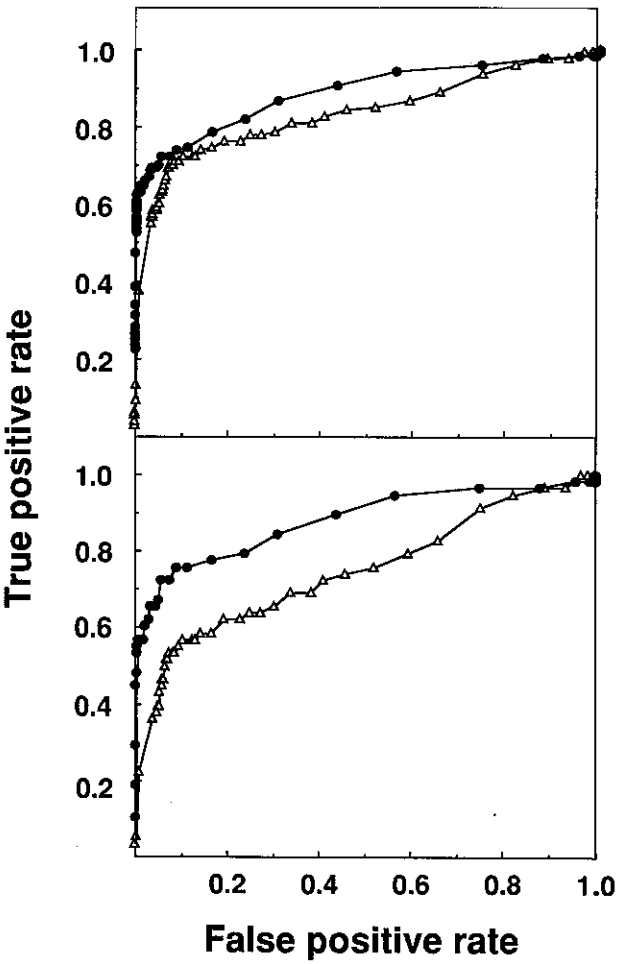

Fig. 5. Typical ROC curves for comparison between total cases of SCLC and non-tumorous lung diseases plus non-SCLC (upper panel), and between LD cases of SCLC and non-tumorous lung diseases plus non-SCLC (lower panel). ●, ProGRP; △, NSE.

greatest amount, and that they are rarely produced by non-SCLC cells.[11, 12] This may account for the very high specificity for SCLC patients. Secondly, GRP and ProGRP have the property of being produced and then actively secreted into the blood, since they possess the characteristics of hormones. In addition, in adulthood, GRP is known to be present only in nervous system tissues and a small number of pulmonary neuroendocrine cells; this accounts for the very low serum concentration in normal subjects. Thirdly, ProGRP is remarkably stable in the blood. Our preliminary studies revealed that GRP immunoreactivity decreased to 74 and 31% of the initial level after incubation with serum for 1 and 6 h, respectively, but in the case of recombinant ProGRP(31–98) its immunoreactivity decreased to 93 and 92%, respectively, after a similar treatment, suggesting that molecules in the blood detected by ProGRP ELISA were stable. This may explain the marked difference between the mean serum ProGRP level and the mean plasma GRP level in SCLC patients; the former was 76-fold higher than the latter (unpublished data). All of these characteristics of ProGRP may explain its sensitivity, specificity, reliability and apparent clinical usefulness, even in SCLC patients at a relatively early stage of the disease.

The present study also demonstrated that serum ProGRP determination is a useful tool for monitoring therapeutic effects in SCLC patients. In patients with CR, serum ProGRP levels decreased to the normal range. In patients with PR, serum levels also decreased but not always to the normal range. In patients with PD, the levels increased. Using ProGRP RIA, we reached the same conclusion,[3] but the present data on ELISA offer a more reliable estimation of the therapeutic effects, since the normal range could be determined with the present ELISA. Furthermore, serial determination of the serum ProGRP levels in SCLC patients indicated that the determination of serum ProGRP levels could serve to detect

recurrence of the disease in SCLC patients earlier, which would allow earlier treatment (data not shown).

NSE has been approved as a tumor marker in SCLC patients in Japan as well as in European countries. With the aim of comparing ProGRP and NSE as tumor markers for SCLC patients, we measured serum NSE levels in all samples collected in the present study. Serum NSE was detectable in all of the normal subjects, and the mean+3SD was 8.1 ng/ml, which was regarded as the tentative cutoff value, consistent with that for ProGRP. With this criterion, the rate of positive NSE in SCLC patients was 62.2%, almost equal to that of ProGRP. However, the present study revealed several advantages of ProGRP as a tumor marker in SCLC patients. First, the frequency of serum NSE elevation in patients with non-SCLC ranged from 5.9 to 22.2%, indicating higher false-positive rates than those obtained with serum ProGRP determination. ROC analysis also confirmed this observation. Therefore, it is reasonable to postulate that ProGRP is a more specific tumor marker for SCLC patients than NSE. Secondly, the levels of ProGRP in SCLC patients were markedly higher than the cutoff value, when compared with those of NSE (Table III). This observation was not reflected in the ROC analysis, but caused the reliability of ProGRP to be higher than that of NSE. Thirdly, the rate of positive ProGRP in SCLC patients with a rather early stage of the disease was higher than that of NSE. Of LD patients, elevated ProGRP and NSE levels were observed in 56.9 and 43.1%, respectively. Furthermore, the mean ProGRP and NSE levels in SCLC patients with LD was 10.9- and 1.9-fold higher than the cutoff values, respectively. In a previous study on NSE, similar results for SCLC patients with LD were found by Carney *et al.*; the mean NSE value for normal subjects was 5.2 ng/ml, and those of SCLC patients with LD was 13.8 ng/ml, demonstrating that serum NSE levels in SCLC patients with LD was only 2.7-fold higher than the mean value of normal subjects.[13] ROC analysis of the present data confirmed that, in SCLC patients with LD, ProGRP gave a better performance in all the comparisons examined than NSE. Fourthly, in comparison with NSE, the difference in serum ProGRP levels before and after the treatment was greater, indicating that ProGRP is a more reliable tumor marker than NSE in terms of monitoring therapeutic effects in SCLC patients. All of these observations indicated that ProGRP is superior to NSE as a tumor marker for SCLC patients, but it should be kept in mind that the sensitivity of ProGRP was 63%, and that in a small number of ProGRP-negative SCLC patients the NSE level was elevated. Therefore, it is possible that NSE could serve as a tumor marker in the small number of ProGRP-negative SCLC patients.

In conclusion, the present study revealed that ProGRP is a specific, reliable and sensitive tumor marker for the diagnosis and treatment of SCLC patients, being superior to NSE. Therefore, we consider that ProGRP can play a major clinical role as a tumor marker for SCLC patients.

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