Serum Pseudouridine as a Biochemical Marker in Small Cell Lung Cancer

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ABSTRACT

The serum level of pseudouridine, primarily a degradation product of tRNA, was determined by high-performance liquid chromatography in 24 patients with small cell lung cancer (SCLC), 13 patients with non-SCLC with advanced stages, 15 patients with pulmonary infectious diseases, and 18 healthy controls. The mean serum pseudouridine concentration was significantly higher in the patients with SCLC (4.75 ± 1.76 (SD) nmol/ml) than that in the patients with pulmonary infectious diseases (3.39 ± 1.38 nmol/ml) or in healthy controls (2.21 ± 0.78 nmol/ml). The mean serum pseudouridine concentration in the patients with non-SCLC (4.07 ± 0.95 nmol/ml) was significantly higher than that in healthy controls but not statistically different from that in the patients with pulmonary infectious diseases. The serum pseudouridine level was elevated above the mean value plus 2 SD for the healthy subjects (3.77 nmol/ml) in 66.7% of all patients with SCLC including 3 of 8 (37.5%) with limited disease and 13 of 16 (81.3%) with extensive disease, and 53.8% of the patients with non-SCLC. Serum carcinoembryonic antigen was elevated (>5 ng/ml) in 29.2% and serum neuron-specific enolase (>10 ng/ml) in 58.3% of the cases with SCLC. In the patients with SCLC followed up during chemotherapy, serum pseudouridine levels changed considerably parallel with the changes in the clinical response. These findings indicate that serum pseudouridine may be a useful biochemical marker in the patients with SCLC.

INTRODUCTION

Modified nucleosides have been thought to be derived predominantly from the enzymatic degradation of tRNA and have been shown to be excreted in abnormally large amounts in the urine of patients with various malignant diseases (1-8) and tumor-bearing animals (9). Many studies have demonstrated the usefulness of urinary modified nucleosides as diagnostic and monitoring markers in various cancer patients (10-14). A higher turnover rate of tRNA has been found in tumor tissue and this has been proposed as a mechanism by which the elevated levels of these modified nucleosides are caused (15). Of these modified nucleosides, the concentration of pseudouridine is elevated most frequently and significantly (16).

Recently, a sensitive analytical method to measure serum pseudouridine levels was developed by Colonna et al. (17), essentially based on that described by Gehrke et al. (18), which includes the purification of serum samples by boronate affinity gel, followed by reverse-phase high-performance liquid chromatography. Several reports have proposed the usefulness of serum pseudouridine as a biochemical marker in patients with several types of cancers (16, 19) and in animals bearing lymphoma (20).

This article will report on the usefulness of serum pseudouridine as a tumor marker in patients with SCLC,2 which comprises about one-fifth of all lung cancer cases and has a tendency to progress rapidly and to metastasize early and extensively (21). At the same time, we examined the additive usefulness of this nucleoside level in serum when combined with serum NSE which have been clinically used most frequently as a marker.

MATERIALS AND METHODS

Patients. Twenty-four patients (18 males and 6 females) with SCLC were investigated in this study. The mean age of the patients was 65 years, ranging from 42 to 79 years. Diagnosis of SCLC was made on the basis of cytological or histological examination in all cases. The stage of the disease was established in compliance with the general rules of the Japan Lung Cancer Society into limited or extensive disease (22) by the usual clinical, laboratory, and radiological findings including bone marrow aspiration, radionuclide bone and liver-spleen scan, and abdominal, chest, and brain computer-assisted tomographic scan. Limited disease was defined as disease confined to one hemithorax including ipsilateral supraclavicular lymph nodes. Extensive disease was defined as disease beyond this extent. Cases with pleural effusion were classified as extensive disease.

Thirteen patients with NSCLC of advanced stages (3 with squamous cell carcinoma, 8 with adenocarcinoma, 2 with large cell carcinoma) and 15 patients with pulmonary infectious diseases (4 with bacterial pneumonia, 2 with pulmonary suppuration, 3 with pulmonary tuberculosis, 6 with chronic obstructive pulmonary diseases with infection) were also investigated in this study. Serum samples were collected before any treatment and then serially during treatment and were kept frozen at -20°C until analysis. The serum pseudouridine concentration of 18 healthy controls was determined for comparison (normal value).

Sample Preparation and Analytical Procedure. Serum pseudouridine was purified and determined as described by Colonna et al. Briefly, 0.5 ml of serum was diluted with 3 ml of distilled water and centrifuged for 60 min at 1000 × g through a Centriflo CF 25 membrane cone (Amicon Corp., Lexington, MA). This filtrate was buffered with 0.3 ml of 2.5 M CH₃COONH₄, pH 9.5, and then applied to phenylboronate affinity gel, Affi-Gel 601 (Bio-Rad Laboratories, Richmond, CA), which had been equilibrated with 0.25 M CH₃COONH₄, pH 8.8. The ribonucleosides were eluted with 10 ml of 2.5 M CH₃COONH₄, pH 8.8. The ribonucleosides were then redissolved in 0.5 ml of water.

Fifty μl of this solution were injected into a C₁₈-μBondapak column (Waters Associates, Milford, MA) in a high-performance liquid chromatograph (Shimadzu liquid chromatograph, Model LC-5A; Shimadzu Co., Ltd., Kyoto, Japan), equipped with an absorbance detector (Shimadzu UV spectrophotometer, Model SPD-2A; Shimadzu Co., Ltd.). Peak area and retention time were measured at 254 nm on a C-RIB Chromatopac (Shimadzu Co., Ltd.), with 2'-deoxyuridine as an internal standard. Elution was performed at a flow rate of 1 ml/min with 0.01 M NH₄H₂PO₄ (pH 5.1) containing 6% methanol. Identification of serum pseudouridine was done by comparing retention time and absorbance ratio (A₂₅₄/A₃₂₀) to those of authentic pseudouridine (Sigma Chemical Co., St. Louis, MO).

Chemotherapy and Clinical Response Categories. Patients with SCLC received a chemotherapeutic regimen consisting of either cyclophosphamide, aclacinomycin, vincristine, and etoposide or doxorubicin, etoposide, and cisplatin.

CR was defined as the disappearance of all measurable lesions. PR was defined as a decrease of ≥50% in the mathematical product of the two largest perpendicular diameters of measurable lesions. Stable disease was defined as a decrease of <50% or an increase of ≥25% in the mathematical product of the two largest perpendicular diameters of measurable lesions. PD was defined as an increase of >25% in the mathematical product of the longest perpendicular diameters of measurable lesions and/or the occurrence of the new lesions.

Determination of Serum CEA and NSE. Serum CEA and NSE were determined with commercially available radioimmunoassay kits (CEA,

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2 The abbreviations used are: SCLC, small cell lung cancer; CEA, carcinoembryonic antigen; NSE, neuron-specific enolase; CR, complete response; PR, partial response; PD, progressive disease; NSCLC, non-SCLC.
RESULTS

Among 24 patients with SCLC, 16 had extensive disease (7 with 1 metastatic site, 5 with 2, and 4 with 3 or more) and 8 had limited disease.

The mean serum pseudouridine concentration in 24 patients with SCLC was $4.75 \pm 1.76$ (SD) nmol/ml which was significantly higher than that of the patients with pulmonary infectious diseases, $3.39 \pm 1.38$ ($P < 0.01$), and that of healthy controls, $2.21 \pm 0.78$ nmol/ml ($P < 0.001$) (Fig. 1). The mean serum pseudouridine concentration in 13 patients with NSCLC was $4.07 \pm 0.95$ nmol/ml which was significantly higher than that of healthy controls but not statistically different from that of the patients with pulmonary infectious disease. There was also no significant difference in mean serum pseudouridine concentration between the patients with SCLC and those with NSCLC. Among cases with pulmonary infectious disease, only one who suffered from pulmonary abscess had an extremely high serum pseudouridine level despite the benign nature of the disease. There was no significant difference in mean serum pseudouridine level between SCLC patients with limited disease and those with extensive disease; however, among the patients with extensive disease, 9 with 2 or more metastatic sites had significantly higher serum pseudouridine concentration than 7 with only 1 metastatic site ($P < 0.05$) (Fig. 2). The positivity rate in the patients with extensive disease was higher than in the patients with limited disease. Moreover, all 9 patients with 2 or more metastatic sites had elevated serum pseudouridine levels while 4 of 7 with 1 metastatic site had elevated levels.

In total, 16 cases (66.7%) of the patients with SCLC and 7 (53.8%) of the patients with NSCLC showed serum pseudouridine levels exceeding the mean + 2 SD of the healthy controls (3.77 nmol/ml) (Table 1). Table 1 also shows the positivity rates of serum CEA (cutoff level, 5 ng/ml) and serum NSE (cutoff level, 10 ng/ml). Serum CEA and NSE were positive in 29.2 and 58.3% of cases with SCLC, respectively. The mean serum CEA and NSE in SCLC patients with extensive disease were both statistically higher than those in the patients with limited disease (data not shown). In 10 SCLC patients with negative NSE, 6 had positive serum pseudouridine levels, and thus when serum pseudouridine and serum NSE were used in combination, 20 patients (83.3%) had elevated levels of either each marker or both.

The changes in serum pseudouridine level during chemotherapy are shown in Fig. 3 with respect to each response category. In 18 patients who gained CR or PR, 13 patients showed pretreatment serum pseudouridine levels exceeding the mean + 2 SD of the controls. Twelve of these 13 patients showed decreased serum pseudouridine levels after successful treatment. In Fig. 3, right, are shown the changes in serum pseudouridine levels in the patients showing PD. Ten of 12 PD
SERUM PSEUDOURIDINE IN SMALL CELL LUNG CANCER

Fig. 3. Changes in serum pseudouridine level with respect to each response category in patients with SCLC.

Fig. 4. Changes in serum CEA level with respect to each response category in patients with SCLC.

Fig. 5. Changes in serum NSE level with respect to each response category in patients with SCLC.

cases showed increased serum pseudouridine levels during the worsening of the disease.

Fig. 4 shows the changes in serum CEA level during the course of the disease. Only 6 of 15 patients who achieved CR or PR had high pretreatment serum CEA levels, but all these patients showed decreased serum CEA levels after treatment, while in 9 patients who failed to respond to treatment, only 2 had parallel changes in serum CEA level with clinical response category.

Fig. 5 shows the changes in serum NSE level during the course of the disease. The changes in serum NSE level were almost parallel to those of the clinical response categories except for one case showing PR and 2 cases showing PD.

DISCUSSION

The urinary level of pseudouridine, one of the modified nucleosides derived predominantly from tRNA, has been proposed as a useful biochemical marker in patients with various malignant diseases (10–14). However, the frequency of elevation of urinary pseudouridine is different among various malignant diseases according to their proliferative growth characteristics (12). Because SCLC has a tendency to progress rapidly and metastasize early and extensively, the determination of urinary pseudouridine has been proposed as a useful marker to determine the extent of disease or quantitate the response to chemotherapy (11, 12).

The pseudouridine level in serum has been reported to be much lower than that in urine (17, 19), but in spite of the low level, it seems to be of value to estimate serum pseudouridine levels because it would be less affected by exogenous factors than that of urine. The sensitive analytical method developed by Colonna et al. (17) made it possible to measure serum pseudouridine level precisely. Recently, Russo et al. (20) reported, using this method, that in animal systems in which lymphoma developed spontaneously, an increase in serum pseudouridine level preceded the appearance of a neoplastic disease and correlated with the tumor burden. Few reports have been made until the present, however, on the usefulness of serum pseudouridine as a tumor marker in a clinical setting.

In the present study, the mean serum pseudouridine concentration in patients with SCLC was significantly higher than that in patients with pulmonary infectious diseases or the healthy controls. Furthermore, 16 of 24 SCLC cases (66.7%) showed a serum pseudouridine level higher than the normal mean value + 2 SD (3.77 nmol/ml). Although the positivity rate was higher in the patients with extensive disease than with limited disease, there was no significant difference in mean serum pseudouridine level between these two stages. The reason for this is unknown, but the limited number of cases in this study might be a factor. However, in the patients with extensive disease, those with one metastatic site showed significantly lower serum pseudouridine levels than those with two or more metastatic sites and all the patients with two or more metastatic sites had elevated serum pseudouridine levels, while 57% with one metastatic site did. These results suggest that serum pseudouridine and tumor burden are related. Among 15 patients with pulmonary infectious diseases, only 1 patient suffering
from pulmonary abscess had extremely high serum pseudouridine levels before treatment. Having recovered with the administration of antibiotics, the patient was discharged without thorough examination of other organs and could not be followed up thereafter. Thus we cannot rule out the possibility of underlying malignant disease.

Serum CEA is known to be elevated in some patients with SCLC and is used as a marker for the diagnosis and monitoring of the disease course (23, 24). In this study, CEA was positive in 29.2% of the patients with SCLC, the percentage being somewhat lower than that of previous reports (11, 12, 23, 24). In the limited number of cases examined, the positivity rate for serum pseudouridine was higher than that for serum CEA.

Many studies have demonstrated that a large number of patients with SCLC had high pretreatment serum NSE levels and it reflects the tumor extent and treatment response well. Thus, this enzyme has been used as a most reliable biochemical marker in patients with SCLC (25, 26). The positivity rate for pretreatment serum NSE in this study was 58.3%. This percentage was slightly lower than that in the previous studies.

Among these three markers studied, serum pseudouridine and serum NSE had a considerably high positivity rate, so we examined the usefulness of the combination assay of these two markers. In 10 NSE-negative SCLC cases, 6 had positive serum pseudouridine levels; in total 83.3% of our SCLC cases could be detected by the combination of these 2 markers.

In evaluating the efficacy of serum pseudouridine, CEA, and NSE concentrations for monitoring the clinical response to chemotherapy, the changes in serum pseudouridine level considerably paralleled the clinical response, either CR or PR, and PD. Almost all patients with high pretreatment serum pseudouridine levels showed decreased levels after successful treatment. Pretreatment serum pseudouridine levels of the cases with discordant changes were near or within normal range in all cases. Five patients whose pretreatment serum CEA levels were above cutoff level showed markedly decreased levels after successful treatment, while only 2 patients who failed to respond to treatment showed elevated serum CEA levels. It appears that serum CEA is a more useful marker, in cases of tumor regression for those patients whose pretreatment level is high, than in cases of tumor recurrence. Conversely, serum NSE seems to be more useful indicating cases with tumor progression, rather than the cases with tumor regression, partly because the positivity rate for pretreatment serum NSE level was lower in this study than in previous studies (25, 26).

In summary, serum pseudouridine is not a specific marker for SCLC, but it relates to the tumor burden and reflects the clinical status of the patients. In the limited number of cases examined, the positivity rate for serum pseudouridine concentration was higher than that for serum CEA and NSE. When serum pseudouridine and NSE are combined, the positivity rate is further elevated above that obtained with either single marker.

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