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Enhanced Expression of Neuron-specific Enolase (NSE) in Pyothorax-associated Lymphoma (PAL)

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Pyothorax-associated lymphoma (PAL) is a B-cell lymphoma of mostly large cell type developing in the pleural cavity of patients with long-standing pyothorax. Neuron-specific enolase (NSE) is an enolase comprising γ subunit and is located at high levels in neuronal and neuroendocrine cells, together with their neoplasias. Expression of NSE at protein and mRNA levels was examined in PAL and other types (non-PAL) of non-Hodgkin's lymphomas. In PAL, serum levels of NSE were elevated (5.32 to 168.0, mean 42.6 ng/ml) and tended to decrease after incisional biopsy followed by chemotherapy (2.38 to 195.5, mean 34.1 ng/ml). Two cell lines established from two cases of PAL produced and secreted NSE in the culture medium. Immunohistochemistry revealed that the positive rate for NSE staining in PAL (10 of 14 cases, 71.4%) was significantly higher than that in non-PAL cases (6 of 38 cases, 15.8%) ($P<0.01$). RT-PCR analysis showed that the expression levels of NSE mRNA in two cell lines and a biopsy sample from PAL were rather similar to those of the control samples from non-neoplastic lymph nodes. These findings suggest the posttranscriptional regulation of NSE in PAL. Thus, an elevation of serum NSE level in patients with chronic pyothorax may be an indicator of PAL development.

Key words: Pyothorax-associated lymphoma — Neuron-specific enolase — Immunohistochemistry — Quantitative reverse transcription PCR

Enolases are anaerobic glycolytic enzymes catalyzing the interconversion of 2-phosphoglycerate and phosphoenolpyruvate, and are made up of three distinct subunits, α, β, and γ, which form dimers of five types (αα, ββ, γγ, αβ, and αγ).[1, 2)] Because the γ subunit of enolase is mainly located at high levels in neuronal cells and neuroendocrine cells as αγ and γγ forms, they are designated as neuron-specific enolases (NSE).[3)] Since NSE is also present in neurogenic and neuroendocrine tumors, such as small cell carcinoma of lung and neuroblastoma,[4)] measurement of its serum concentration is useful for the diagnosis and follow-up of these tumors. Though the serum concentration of NSE is seldom assayed in patients with malignant lymphoma, there are reports of the elevation of serum concentration of NSE in 17 to 21% of cases with non-Hodgkin's lymphoma (NHL).[5, 6)]

Pyothorax-associated lymphoma (PAL) is a B-cell lymphoma developing in the pleural cavity of patients with over 20 years' history of chronic pyothorax resulting from artificial pneumothorax for the treatment of pulmonary tuberculosis or tuberculous pleuritis.[7, 8)] PAL is one of the Epstein-Barr virus (EBV)-associated lymphomas, and expresses latent genes of EBV including latent membrane proteins and EB nuclear antigens.[9, 10)] The common presenting symptoms of PAL include pain in the chest, back, or shoulder, and respiratory symptoms such as productive cough, often with hemoptysis, fever or dyspnea. Roentgenographic examinations show mass shadows involving the lung in over 30% of PAL patients. These findings might suggest a diagnosis of lung cancer, especially small cell carcinoma after histologic evaluation of biopsy specimens usually showing a proliferation of round cells. Thus, serum levels of NSE are occasionally measured in cases with PAL, and appear to be elevated.

The purpose of this study was to examine whether PAL cells produce and secrete NSE at high levels. First, production of NSE was examined in two cell lines derived from two cases of PAL. Secondly, expression of NSE in the tumor cells was immunohistochemically examined in 14 cases of PAL and 38 non-PAL lymphomas as controls, and its correlation with serum concentrations of NSE was evaluated in the PAL cases. Subsequently, expression of NSE mRNA was examined in two cell lines of PAL and another case of PAL, together with five non-PAL lymphomas of B-cell type.

MATERIALS AND METHODS

Case selection Sixteen cases with PAL and 38 cases with non-PAL lymphoma were examined in this study. Primary sites of the tumor in non-PAL lymphoma were lymph

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nodes in 30 cases, tonsil in two, and thyroid, stomach, intestine, spleen, uterus, and thigh in one each. Two cases of reactive lymphadenitis (17-year-old male and 22-year-old female) were also included as controls for NSE expression. The age of PAL and non-PAL patients on admission ranged from 61 to 79 (median 70) years and 23 to 76 (median 62) years, and the male-to-female ratio was 15:1 and 1:1, respectively. All PAL had stage I disease. Stage of non-PAL cases was stage I in 11 cases, stage II in 12, stage III in six, stage IV in five, and unknown in four. All PAL and non-PAL lymphomas other than gastric, splenic, and uterus ones received incisional or open biopsy for histologic diagnosis, followed by chemotherapy. Lymphoma of stomach, spleen, and uterus was totally resected, followed by chemotherapy. Chemotherapeutic agents included cyclophosphamide, adriamycin, vincristine, and prednisolone in most cases, together with etoposide in a few cases. Concentrations of NSE and/or lactate dehydrogenase (LDH) in the serum of PAL cases before and after chemotherapy are shown in Table I. Histologic specimens obtained were fixed in 10% formalin and routinely processed for paraffin-embedding. Fresh specimens from one case of PAL (case 7) and five of non-PAL lymphomas were snap-frozen in liquid nitrogen and stored at −80°C until use for RNA extraction. Histologic sections, cut at 4 μm, were stained with hematoxylin-eosin and immunoperoxidase procedures, and reviewed by one of authors (K. A.). All lymphomas were classified according to the Revised European American Lymphoma (REAL) classification.[11]

Cell culture and determination of NSE and LDH levels in extracellular medium Two cell lines, OPL-1 and OPL-2, have been established from the biopsy specimens of two PAL cases.[12] The characters of these cell lines were reported in detail previously.[12] Cells were cultured in RPMI1640 medium (Nissui, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (BioWhittaker, Walkersville, MD), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 m*M* glutamine, in a humidified atmosphere with 95% air and 5% CO_2 at 37°C. At various elapsed times after cultivation, extracellular medium was obtained by centrifugation at 400*g* for 5 min and kept at −20°C until use. Concentrations of NSE and LDH in the extracellular medium were determined in triplicate by immunoassay at SRL Laboratory (Tokyo).

Immunohistochemistry Immunohistochemical study for immunophenotyping of lymphomas was carried out using the avidin-biotin-peroxidase complex method. The primary antibodies used in the study, their suppliers, and dilutions were as follows: CD3 (polyclonal) (Dakopatts, Glostrup, Denmark; 1:100), CD43 (MT-1) (Bioscience, Emmenbrucke, Switzerland; 1:50), CD45RO (UCHL1) (Dakopatts; 1:100), CD20 (L26) (Kyowa Medex, Tokyo; 1:50), and MB-1 (Bioscience; 1:50). For detection of NSE, the labeled streptavidin-biotin method (Universal DAKO LSAB kit; DAKO, Carpinteria, CA) with the use of monoclonal antibody for NSE (BBS/NC/VI-H14) (Dakopatts; 1:50) was applied. Sections were treated with 0.1% trypsin solution (Sigma, St. Louis, MO) at 37°C for 30 min before reaction with anti-CD3. When anti-NSE antibody was

Table I. Serum Concentration and Immunohistochemical Detection of Neuron-specific Enolase and Lactate Dehydrogenase in Pyothorax-associated Lymphoma

Case No.	Age	Sex	Serum concentration of NSE (ng/ml)		Immunohistochemistry for NSE	Serum concentration of LDH (IU/liter)	
			Bofore therapy	After therapy		Before therapy	After therapy
1	77	M	7.33	2.44	+	694	359
2	63	M	52.80	50.64	n.d.[b]	2942	3822
3	75	M	8.9	n.a.[a]	n.d.	437	345
4	77	M	9.10	3.01	−	428	257
5	62	F	n.a.	n.a.	+	333	272
6	71	M	n.a.	n.a.	+	409	221
7	73	M	15.44	44.59	+	590	5840
8	68	M	39.9	28.0	+	1781	3868
9	68	M	n.a.	n.a.	+	318	205
10	61	M	n.a.	n.a.	+	47.8	45.6
11	74	M	8.5	8.0	+	269	251
12	73	M	168.0	195.5	+	3625	1963
13	79	M	n.a.	n.a.	+	843	2432
14	69	M	13.61	2.38	−	880	326
15	67	M	5.32	2.70	−	671	563
16	69	M	140.0	4.2	−	4910	343

a) n.a.: not available, *b*) n.d.: not done.

used as the primary antibody, sections rinsed in 10 m*M* citrate buffer (10 m*M* citrate monohydrate in distilled water, pH 6.0) were treated with a microwave oven for 15 min for antigen retrieval.

Total RNA extraction and quantitative RT-PCR for NSE Total RNA was extracted from frozen tissues of one case with PAL (case 7), two cell lines of PAL, OPL-1 and -2, five with non-PAL lymphoma, and two with reactive lymphadenitis, using Trizol reagent (Life Technologies, Rockville, MD). Total RNA was treated with DNase I (Roche, Mannheim, Germany) in the presence of SUPERase In RNase Inhibitor (Ambion, Austin, TX) to remove contaminated DNA before cDNA synthesis. Reverse transcription was carried out with 10 μg of total RNA from each sample in a total of 40 μl of solution containing 200 units of Super Script II RNase H^- Reverse Transcriptase (Life Technologies), 0.5 μM oligo (dT)25 primer, 0.5 μM dNTP, SUPERase In RNase Inhibitor (Ambion), and 10 μM DTT in 1× First Strand Buffer (Life Technologies).

Real-time PCR (TaqMan) analysis was performed with a Perkin-Elmer/Applied Biosystems 7700 Prism (Norwalk, CT). Matching primers and fluorescence probes (see below) were designed for NSE gene according to the Primer Express program provided by Perkin-Elmer/Applied Biosystems. β-Actin primers and probes were obtained from Perkin-Elmer/Applied Biosystems. The PCR reaction was performed in a total volume of 25 μl containing 1× TaqMan buffer, 0.2 m*M* dATP, dCTP, dGTP and 0.4 m*M* dUTP, 0.625 unit of AmpliTaq Gold (Perkin-Elmer/Applied Biosystems), 0.25 unit of Amperase uracil-N-glycosylase (Perkin-Elmer/Applied Biosystems), 5 m*M* $MgCl_2$, and 2 μl of each appropriately diluted sample. The concentrations of the forward and reverse primers were 900 n*M* for NSE and 300 n*M* for β-actin. In both cases, the final probe concentration was adjusted to 150 n*M*. The following primers were used for NSE: (a) forward primer, 5′-TGT CTG CTG CTC AAG GTC AA-3′; (b) reverse primer, 5′-CGA TGA CTC ACC ATG ACC C-3′; and (c) probe, 5′-CAC TGA AGC CAT CCA AGC GTG C-3′.

To quantify the amount of specific mRNA in the samples, a standard curve was constructed with serial dilutions of cDNA from pooled Human Lymph Node Poly A^+ RNA (Clontech, Palo Alto, CA), ranging from 25 fg to 25 ng of poly A^+ RNA. Each sample was normalized with respect to its β-actin content. The pool of normal human lymph node tissues was used as calibrator, and relative amounts of NSE were also normalized to a calibrator. The levels of NSE expression relative to the β-actin gene and the calibrator are given as follows:

$$\frac{NSE^{sample}/\beta\text{-actin}^{sample}}{NSE^{calibrator}/\beta\text{-actin}^{calibrator}}$$

The reproducibility of the quantitative measurements was evaluated by conducting duplicate PCR assessments.

Statistical analysis The significance of differences in serum levels of NSE before and after chemotherapy was evaluated by using Student's *t* test. The significance of differences in frequency of immunoreactivity for NSE between PAL and non-PAL cases was evaluated by using Fisher's exact test.

RESULTS

Histologic findings All PAL were diffuse large B-cell lymphoma (DLBL) with immunoreactivities of $CD20^+$ and/or $MB1^+$, $CD3^-$, $CD43^{-/+}$, $CD45RO^-$. Non-PAL lymphomas were classified as DLBL in 22 cases, follicle center cell lymphoma (FCCL) in 10, peripheral T-cell lymphoma in four, and precursor T-lymphoblastic lymphoma in two. T-cell cases were $CD20^-$, $MB1^-$, $CD3^+$ and/or $CD43^+$, $CD45RO^+$. Nine and four of DLBL in the PAL and non-PAL cases, respectively, showed plasmacytoid differentiation.

Serum levels of NSE and LDH before and after chemotherapy in PAL cases Serum levels of NSE in PAL cases were mildly to moderately elevated (5.32 to 168.0, mean 42.6 ng/ml) before chemotherapy, and tended to decrease after chemotherapy (2.38 to 195.5, mean 34.1 ng/ml), although the difference in values before and after therapy was not significant. Two cases (cases 7 and 12) showed an increase of serum levels in NSE after chemotherapy (Table I).

Serum levels of LDH in PAL cases were moderately elevated (47.8 to 4910, mean 1198.6 IU/liter) before chemotherapy, and tended to decrease after chemotherapy in all (45.6 to 1963, mean 429.2 IU/liter) but four cases (cases 2, 7, 8, and 13).

Detection of NSE in extracellular medium of OPL-1 and -2 In both OPL-1 and -2, NSE levels in the extracellular medium were below the detection limit on days 1 and 2, but showed elevation, 3.73±0.67 ng/ml in OPL-1 and 4.60±0.89 ng/ml in OPL-2, at day 4 (Table II). This indicates that OPL-1 and -2 actually produce and secrete NSE into the extracellular medium. LDH levels in the extracellular medium of both OPL-1 and -2 were also elevated, 87.0±4.0 IU/liter in OPL-1 and 76.3±1.5 IU/liter in OPL-2, at day 4.

Table II. Neuron-specific Enolase Level in Extracellular Medium of OPL-1 and -2

Cell lines	NSE level (ng/ml)			
	Day 0	Day 1	Day 2	Day 4
OPL-1	<2 *a)*	<2 *a)*	<2 *a)*	3.73±0.67
OPL-2	<2 *a)*	<2 *a)*	<2 *a)*	4.60±0.89

a) Detection limit.

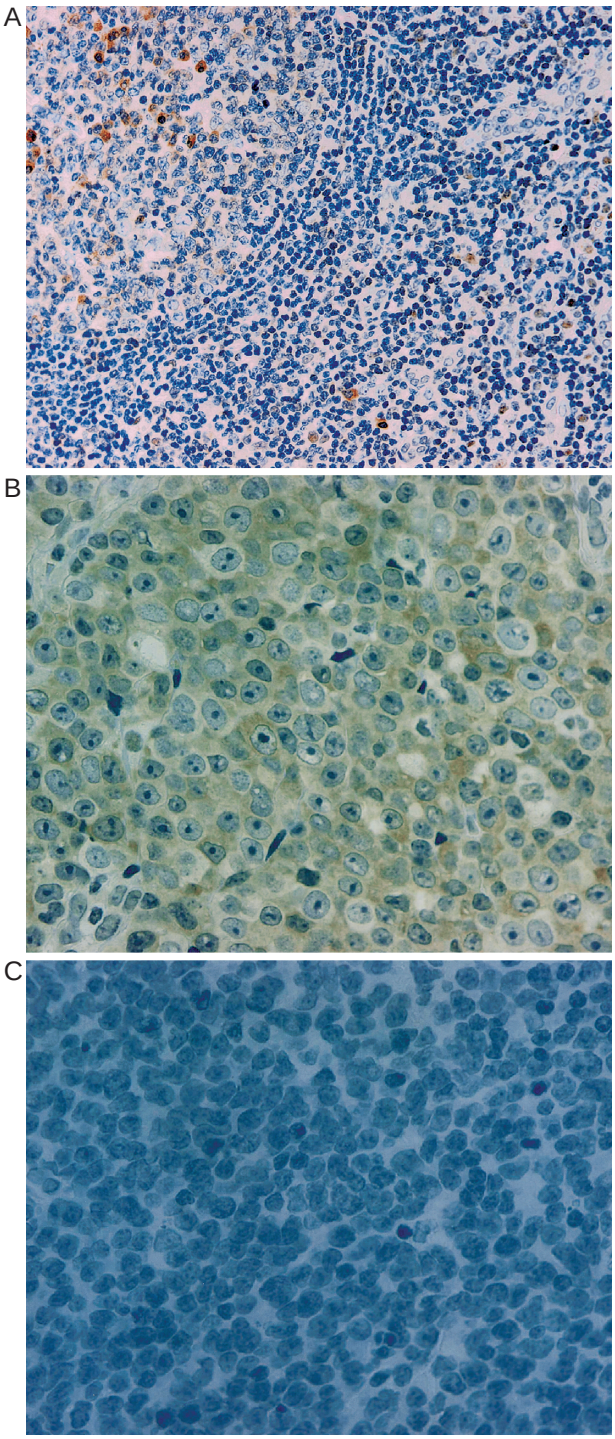

Fig. 1. Immunohistochemical detection of NSE. (A) Reactive lymphadenitis. Most germinal center cells were faintly positive and plasma cells scattered in interfollicular areas and germinal centers were strongly positive (original magnification ×200). (B) PAL case. Most large lymphoid cells showed a diffuse cytoplasmic staining for NSE (×400). (C) Non-PAL case. There were no tumor cells with positive staining (×400).

Immunohistochemical detection of NSE Immunohistochemistry for NSE demonstrated a diffuse intracytoplasmic staining in positive cells. In reactive lymphadenitis, most of the germinal center cells were faintly positive and a few plasma cells scattered in the interfollicular area and germinal centers were strongly positive (Fig. 1A).

Lymphoma cases in which more than 50% of the tumor cells showed a clearly discernible immunoreactivity for NSE were judged as positive. Positive rate for NSE staining in PAL cases, 10 of 14 cases (71.4%), was significantly higher than that in non-PAL lymphoma cases, 6 of 38 cases (15.8%) ($P<0.01$) (Table I). OPL-1 and -2 also expressed NSE. More than 80% of tumor cells were positive in cases with positive reactivity for NSE (Fig. 1B). Intensities of staining in PAL and non-PAL lymphomas were similar. No prominent differences in histology were observed between NSE-positive and -negative cases with PAL. Non-PAL lymphomas with positive immunoreactivities for NSE comprised five cases of DLBL and one of FCCL. None of six T-cell lymphomas was NSE-positive. One DLBL case with positive immunoreactivity showed prominent plasmacytoid differentiation and an elevated level of serum NSE (145.9 ng/ml). The positive rate for NSE in nodal lymphoma, 6 of 30 (20%), was higher than that in extranodal lymphoma, none of 8 (0%), although the difference was not statistically significant. Intensities of NSE staining in immunohistochemistry were not always correlated with serum levels of NSE, which might be due to various factors during sample processing.

Quantitative RT-PCR for NSE The results are summa-

Table III. Quantification of Expression of mRNA of Neuron-specific Enolase

	Histology	Quantitative RT-PCR for NSE	Immunohistochemistry for NSE
PAL cases			
OPL-1	DLBL	1.72	+
OPL-2	DLBL	0.79	+
Case 7	DLBL	1.75	+
Non-PAL cases			
1	DLBL	1.35	–
2	DLBL	0.52	–
3	DLBL	0.37	–
4	FCCL	0.84	–
5	FCCL	0.22	–
Reactive lymphadenitis			
1		0.31	*a*)
2		1.88	*a*)
Normal lymph nodes		1.00	n.d.

a) Most germinal center cells were faintly positive and plasma cells scattered in interfollicular area and germinal center were strongly positive.

rized in Table III. Quantitative RT-PCR showed that the expression of NSE mRNA of PAL was rather similar to that of the control samples derived from lymph nodes of normal healthy individuals. One case of PAL (case 7) showing positive immunoreactivity for NSE expressed NSE mRNA at a slightly higher level (1.75 times compared to the control). The PAL cell line OPL-1 showed mildly increased expression of NSE mRNA, whereas the expression level was slightly decreased in OPL-2.

Non-PAL lymphomas showed similar levels of NSE mRNA expression to the controls in all of five cases. None of these five cases was NSE-positive by immunohistochemistry.

DISCUSSION

Elevated NSE in the serum is a useful marker for diagnosis and monitoring of neuroendocrine tumors including small cell carcinoma of lung, but it was reported that 35% of nonneuroendocrine tumors, including breast cancers, non-small cell lung cancers, pancreatic cancers, multiple myeloma, meningioma, and astrocytoma, also showed elevated serum levels of NSE.[6] As for malignant lymphomas, elevation of serum NSE levels was reported in 17 to 21% of NHL and 6.5 to 23% of Hodgkin's disease.[5, 6] Values of NSE in the serum were reported to be 3.1 ± 0.9 ng/ml in healthy individuals[13] and to range from 5 to 22 (median 10) ng/ml in NHL.[6] Whether serum NSE levels correlated with stage or histology of NHL was not mentioned in the previous reports. In our series of PAL, serum NSE levels were elevated (5.32 to 168, mean 42.6 ng/ml) in most patients. Production and secretion of NSE by the tumor cells were confirmed by our study on the cultured cells of PAL (Table II).

The current immunohistochemical study revealed that the positive rate for NSE was significantly higher in PAL cases (10 of 14, 71.4%) than in non-PAL lymphoma cases (6 of 38, 15.8%) ($P<0.01$). Intensity of staining in PAL was similar to that in non-PAL cases. These findings indicate that accelerated production of NSE is common in PAL, but occasional in non-PAL lymphomas. The serum NSE levels in PAL decreased to various extents after chemotherapy in the majority of cases. The serum NSE levels paralleled the serum LDH levels. These findings suggested the serum NSE levels might reflect the disease activity, although we did not have sufficient information about the relation between tumor volume and the serum levels of LDH and NSE. It might be better to monitor NSE levels at intervals to see the pattern of decrease in cases of PAL.

The reason why expression of NSE is rather common in PAL but not in non-PAL lymphomas is not clear. Previous reports showed that normal B-lymphocytes in the late stage of differentiation were NSE-positive by immunohistochemistry, i.e., plasma cells were strongly positive for NSE, whereas immature B-cells in the lymphoid follicles were negative.[14] T-cell lymphomas in our series were completely NSE-negative. PAL is a DLBL, frequently with immunoblastic features,[7, 8] indicating a relatively late stage of B-cell differentiation. This might be one reason for accelerated production of NSE in PAL cases.

Quantitative RT-PCR analysis for NSE gene revealed that PAL expressed mRNA of NSE at a similar level to reactive lymphadenitis and normal lymphocytes. Because the serum level of NSE is elevated in PAL cases, increased efficiency of posttranscriptional NSE expression is suggested in PAL. In contrast, non-PAL cases showed negative immunohistochemistry although they expressed NSE mRNA at almost equal levels to PAL cases (Table III). These findings also suggest posttranscriptional regulation of NSE.

In conclusion, increased translation of NSE mRNA is suggested to occur in PAL. Thus, elevated serum level of NSE in patients with chronic pyothorax may be a marker of PAL development.

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