

Expression of nm23-H1 Is Associated with Poor Prognosis in Peripheral T-Cell Lymphoma, Not Otherwise Specified

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Abstract

Purpose: We examined whether nm23-H1 is a prognostic factor of peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS).

Experimental Design: We studied 102 consecutive, untreated PTCL-NOS patients from 1998 to 2008. The expression of nm23-H1 and TIA-1 was studied by immunohistochemistry.

Results: nm23-H1 was positive in 44.1% and TIA-1 in 78.4% of the PTCL-NOS patients. nm23-H1 expression was not correlated with age, performance status (PS), lactate dehydrogenase (LDH) level, or stage but was significantly correlated with the prognostic index for T-cell lymphoma. The serum nm23-H1 level was 43.44 ng/mL in the cytoplasmic nm23-H1 strongly positive, 24.32 ng/mL in the cytoplasmic nm23-H1 moderately positive, and 13.64 ng/mL in the cytoplasmic nm23-H1-negative patients. The nm23-H1-positive group had significantly shorter overall survival (OS). TIA-1 had no prognostic impact on 5-year OS rates. OS was significantly shorter in patients with the following clinicopathologic features: age 60 or more years, PS of 2 to 4, LDH level greater than normal, bone marrow involvement, or nm23-H1-positive lymphoma. Multivariate analysis confirmed nm23-H1 expression to be an independent prognostic factor.

Conclusions: The nm23-H1 protein may be an important prognostic factor in PTCL-NOS. Because our results suggested that nm23-H1 is produced by lymphoma cells, we expect to see the development of new treatments targeting nm23 overexpression. *Clin Cancer Res*; 17(9); 2893–9. ©2011 AACR.

Introduction

Peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), is relatively rare, accounting for only 10% to 15% of non-Hodgkin's lymphomas (1, 2), and has heterogeneous clinical, histologic, immunophenotypic, cytogenetic, and molecular features. The international prognostic index (IPI; ref. 3) is generally used as a prognostic factor. The IPI may be used in PTCL-NOS patients for risk stratification to identify patients for clinical trials. Recently, Gallamini and colleagues (4) analyzed patients with PTCL-NOS and proposed a new prognostic index for T-cell lymphoma (PIT) including age, performance status (PS), lactate dehydrogenase (LDH) level, and bone marrow (BM) involvement. This PIT model could identify 4 groups of patients with different outcomes and had overall superior predictive capacity compared with the IPI.

nm23-H1 was originally identified as a protein that was expressed at a lower level in metastatic cancer cells. The *nm23* genes play critical roles in cellular proliferation, differentiation, oncogenesis, and tumor metastasis. We previously established an ELISA technique for determination of the serum level of nm23-H1 protein (5, 6) and reported that the serum level of nm23-H1 in patients with aggressive lymphoma was significantly higher than that in healthy controls and that a high nm23-H1 level was associated with poor prognosis in aggressive lymphoma (5). In our previous immunohistochemical study on cytoplasmic nm23-H1 expression in diffuse large B-cell lymphoma (DLBCL), we found that patients with positive cytoplasmic staining had significantly poorer prognosis than patients with negative staining (7).

In this study, our first purpose was to examine nm23-H1 expression in PTCL-NOS to evaluate whether lymphoma cells produce the protein. Our second purpose was to examine the clinical significance of cytotoxic molecules, such as TIA-1, and nm23-H1 expression in PTCL-NOS.

Patients and Methods

Patients

We studied 102 consecutive, untreated PTCL-NOS patients who were managed by the Adult Lymphoma Treatment Study Group (ALTSG) in Japan from 1998 to 2008. All patients were newly diagnosed, were

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doi: 10.1158/1078-0432.CCR-10-2999

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Translational Relevance

There have been few reports on biological prognostic factors of peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS). Therefore, there are few indexes that can be used to decide whether to carry out autologous hematopoietic stem cell transplantation (AH SCT) after high-dose chemotherapy or only chemotherapies. Therefore, a study on nm23-H1 expression is important when treatment strategies such as AH SCT for nm23-H1 overexpression in PTCL-NOS are being considered.

In addition, there are a few effective treatments of PTCL-NOS at present. Therefore, for PTCL-NOS with high nm23-H1 expression, the development of treatments that target nm23-H1 and neighboring molecules is needed.

previously untreated, and received anthracycline-containing combination chemotherapy. Briefly, the CycLOBEAP [cyclophosphamide (CPA), vincristine (VCR), bleomycin, etoposide, doxorubicin (DXR), prednisone (PDN)] regimen (8) was primarily administered to younger patients with PTCL-NOS (≤ 69 years old) and the CHOP (CPA, DXR, VCR, PDN) regimen was primarily administered to older patients with PTCL-NOS (>70 years old). The median follow-up time was 76 months (range: 28–122 months).

Morphologic and immunophenotypic studies

Histologic analysis of the materials from each patient was carried out independently by 6 pathologists from the ALTSG. Lymphomas were classified according to the 4th World Health Organization (WHO) classification by the committee pathologists (9).

The expression of nm23-H1 and TIA-1 by lymphoma cells was examined immunohistochemically on formalin-fixed, paraffin-embedded sections by the avidin–biotin–peroxidase complex method (7, 10). Monoclonal antibodies to nm23-H1 (clone 37.6, Novocastra Laboratories, Ltd; clone H1-229, Seikagaku Corporation) were used as primary reagents. Working dilutions of these antibodies and tissue distribution of positive cells were checked using lymph nodes with reactive hyperplasia. Consequently, clone 37.6 among these antibodies gave satisfactory staining at a range of 1:50 to 1:200 dilutions. Monoclonal antibodies to TIA-1 (Immunotech) were used to detect cytotoxic cell-associated proteins.

The following categories were defined for TIA-1 expression: negative ($<30\%$ positively stained tumor cells) and positive ($>30\%$ positively stained tumor cells). The following categories were defined for nm23-H1 expression: negative ($<30\%$ positively stained tumor cells), low-to-medium positive (30%–50% positively stained tumor cells), and strongly positive ($>50\%$ positively stained tumor cells).

The rates of cells positive for nm23-H1 or TIA-1 per proliferating fraction were determined by 6 hematopathol-

ogists who constituted the ALTSG Pathology Review Board (5 are acknowledged and the remaining, H.N., is one of the authors). The rates were first determined independently by each pathologist, and the results were later discussed and verified by all of the pathologists by using a multiheaded microscope and TV monitors at the time of the ALTSG Central Review Meeting for the diagnosis of lymphoma cases.

ELISA for determination of the serum nm23-H1 level

We previously established an ELISA procedure to determine the nm23-H1 protein level in the serum (5). Briefly, 96-well plates (Corning Co.) were coated with a monoclonal anti-nm23-H1 antibody (Seikagakukougyo Co.), washed 4 times with PBS, and incubated with 25% Block Ace solution (Dainihon Seiyaku). Serum samples were diluted 2-fold with PBS and 50- μ L aliquots were added to the wells. After incubation at room temperature for 1 hour, the wells were washed 4 times with PBS containing 0.05% Tween 20 (T-PBS). Samples were then incubated at room temperature for 1 hour with a polyclonal rabbit anti-nm23-H1 antibody (Santa Cruz Biotechnologies Inc.), washed 4 times with T-PBS, and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (BioRad Lab). After 4 washes with T-PBS, alkaline phosphatase activity was detected with diethanolamine as a substrate and an alkaline phosphatase detection kit (BioRad Lab). The reaction was stopped with 50 μ L of 0.4N NaOH. Absorbance was measured at 405 to 415 nm with a correction wavelength of 620 to 630 nm by using a microplate reader.

Statistical analyses

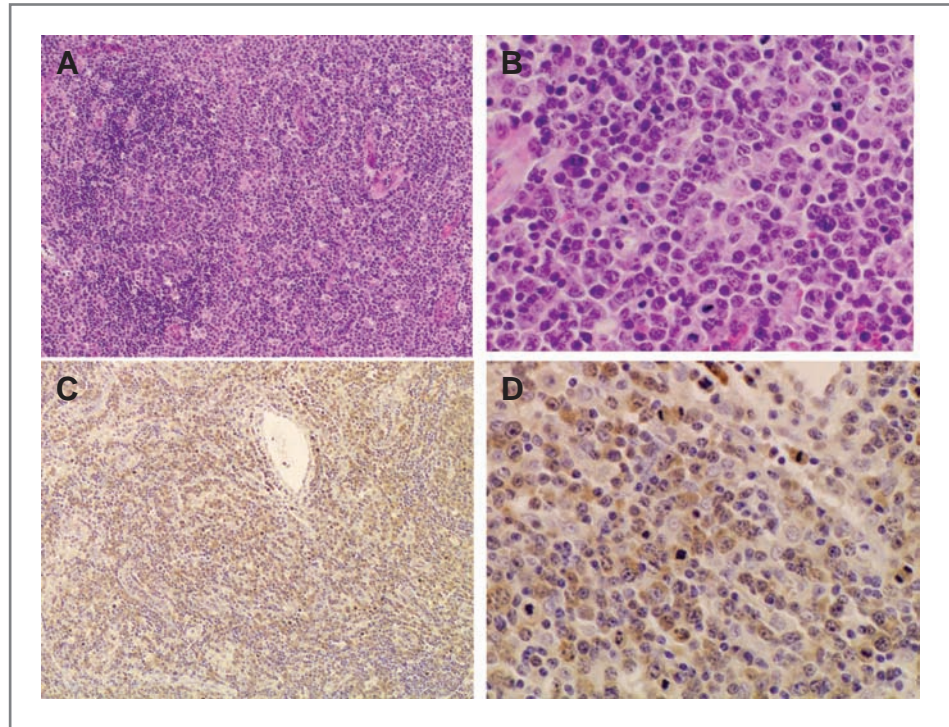
Differences in characteristics between the 2 groups were examined by the χ^2 test, Fisher's exact test, and the Mann-Whitney *U* test, and $P < 0.05$ was taken to indicate significance. Judgment criteria used for the analysis were progression-free survival (PFS) and overall survival (OS). Progression was defined as progression of the lymphoma in nonresponding patients and in partial response patients; a relapse in complete response (CR) patients; or death from any cause without progression. PFS was calculated as the duration from the date of beginning chemotherapy to the date of progression or relapse or to the date of the last contact. Survival analysis was carried out according to the Kaplan–Meier method. The statistical significance of the differences in survival was determined by the log-rank test. Multivariate analysis of the prognosis was carried out by using Cox's proportional-hazards regression model. All statistical analyses were carried out with SAS software (version 9; SAS Institute).

Results

nm23-H1 and TIA-1 expression in mature T-cell lymphoma

Eighty-four (45.7%) of 184 patients with mature T-cell lymphoma in the ALTSG from 1998 to 2008 were positive for nm23-H1 (Fig. 1). In 53 patients (28.8%), more than

Figure 1. Hematoxylin and eosin staining (A and B) and immunohistochemical staining for nm23 (C, D) of PTCL-NOS. The nodal structure is largely obliterated by a diffuse process with occasional remaining atrophic lymph follicles on the left (A). The proliferating cells are medium-sized to large and have a considerable amount of cytoplasm (B). They are diffusely positive for nm23 (C), and cytoplasmic staining is evident at this higher magnification (D). A and B, H&E stain; C and D, immunoperoxidase stain with hematoxylin counterstain. A and C, $\times 10$; B and D, $\times 40$.



70% of the lymphoma cells expressed nm23-H1, whereas in the remaining 31 patients (16.8%), 30% to 69% of the lymphoma cells were stained. The frequencies of positivity for nm23-H1 expression according to lymphoma subtype were 45 (44%) of 102 patients with PTCL-NOS, 20 (38%) of 52 patients with angioimmunoblastic T-cell lymphoma (AITL), and 19 (63%) of 30 patients with anaplastic large cell lymphoma (ALCL). A significantly higher proportion of ALCL cases were positive for nm23-H1 expression than were PTCL-NOS ($P = 0.031$) and AITL cases ($P = 0.015$). On the other hand, 41 (22%) of 184 patients with mature T-cell lymphoma were positive for TIA-1. The frequencies of positivity for TIA-1 according to lymphoma subtype were 22 (21.6%) of 102 patients with PTCL-NOS, 15 (28.8%) of 52 patients with AITL, and 4 (13.3%) of 30 patients with ALCL. As for the expression of TIA-1, there was no significant difference in positivity of TIA-1 among the 3 T-cell lymphoma subtypes.

Relationship between nm23-H1 or TIA-1 expression and clinical characteristics in patients with PTCL-NOS

The relationship between nm23-H1 or TIA-1 expression and clinicopathologic factors was investigated in the 102 patients with PTCL-NOS (Table 1). There were no correlations between nm23-H1 expression and age, PS, serum LDH level, stage, or BM involvement, but a significantly higher percentage of patients with a high PIT score showed nm23-H1 expression ($P = 0.005$). The nm23-H1-positive rate among the patients who achieved CR was 34.8%, whereas the nm23-H1-positive rate among those who failed to do so was 61.1%, the difference being statistically

significant ($P = 0.004$). Thus, a close relationship between cytoplasmic nm23-H1 expression and therapeutic responsiveness was found. Similarly, there were no correlations between TIA-1 expression and age, PS, serum LDH level, stage, BM involvement, PIT score, and CR rate.

Survival in patients with PTCL-NOS

Among the 102 PTCL-NOS patients, the 5-year OS and PFS rates were 51.5% and 35.7%, respectively. The patients were divided into 3 groups according to the level of nm23-H1 expression. The 5-year OS rate of the patients with negative ($n = 57$), low-to-medium positive ($n = 15$), and strongly positive ($n = 30$) nm23-H1 expression was 71.3%, 31.7%, and 24.1%, respectively, and there was a correlation among the 3 groups by the log-rank test ($P = 0.0004$; Fig. 2A). PFS was examined in a similar manner. The 5-year PFS rate among patients with negative, low-to-medium positive, and strongly positive staining was 57.7%, 12.7%, and 8.5%, respectively. The log-rank test revealed a significant correlation among the 3 groups ($P = 0.0003$; Fig. 2B). The patients were divided into 2 groups according to the presence or absence of TIA-1 expression. The 5-year OS rate of the TIA-1-negative and -positive groups was 40.5% ($n = 80$) and 51.6% ($n = 22$), respectively ($P = 0.37$), showing no significant difference (Fig. 3A). The 5-year PFS rate of the TIA-1-negative and -positive groups was 40.5% ($n = 80$) and 51.8% ($n = 22$), respectively ($P = 0.32$), showing no significant difference (Fig. 3B).

When the patients were classified according to the PIT, the 5-year OS rate of groups 1 and 2 ($n = 43$) was 56.5% and that of groups 3 and 4 ($n = 59$) was 40.2%, showing no

Table 1. Correlation of nm23-H1 with TIA-1 expression and prognostic characteristics in patients with PTCL-NOS

| Characteristic | Number of patients | nm23-H1(+), ^a n (%) | P | TIA-1(+), ^b n (%) | P |
|--------------------|--------------------|--------------------------------|-------|------------------------------|------|
| Age, y | | | | | |
| ≤60 | 46 | 21 (45.7) | 0.78 | 11 (23.9) | 0.59 |
| >60 | 56 | 24 (42.9) | | 11 (19.6) | |
| WHO PS | | | | | |
| 0, 1 | 64 | 26 (40.6) | 0.36 | 13 (20.3) | 0.67 |
| 2-4 | 38 | 19 (50) | | 9 (23.6) | |
| Serum LDH level | | | | | |
| Normal | 22 | 10 (45.5) | 0.89 | 5 (22.7) | 0.89 |
| >Normal | 80 | 35 (43.8) | | 17 (21.2) | |
| Ann Arbor stage | | | | | |
| I, II | 24 | 12 (50) | 0.51 | 6 (25) | 0.64 |
| III, IV | 78 | 33 (42.3) | | 16 (20.5) | |
| BM involvement | | | | | |
| Absent | 81 | 33 (40.7) | 0.18 | 19 (23.4) | 0.38 |
| Present | 21 | 12 (57.1) | | 3 (14.3) | |
| PIT | | | | | |
| Groups 1/2 | 43 | 15 (34.9) | 0.005 | 11 (23.9) | 0.39 |
| Groups 3/4 | 59 | 30 (50.8) | | 11 (19.6) | |
| Therapeutic effect | | | | | |
| CR | 66 | 23 (34.8) | 0.004 | 18 (27.3) | 0.06 |
| Non-CR | 36 | 22 (61.1) | | 4 (11.1) | |

^an = 45.^bn = 22.

significant difference ($P = 0.055$). The 5-year PFS rate of groups 1 and 2 ($n = 43$) was 55.1% and that of groups 3 and 4 ($n = 59$) was 37.3%, showing no significant difference ($P = 0.09$). Further evaluation among the patients according to the IPI showed that the 5-year OS rate in patients with L-I, H-I, or H risk was 57%, 52%, and 28%, respectively ($P = 0.08$), and the 5-year PFS rate in patients with L-I, H-I, or H risk was 54%, 50%, and 26%, respectively ($P = 0.14$; data not shown).

CycloBEAP therapy was administered to 48 PTCL-NOS patients. The 5-year OS rate was 68% and 5-year PFS rate

was 56%. On the other hand, CHOP therapy was administered to 54 patients. The 5-year OS rate was 38%, and the 5-year PFS rate was 31%. In both therapies, nm23-H1 overexpression of greater than 50% tumor cells stained was significantly associated with poor prognosis.

Significance of nm23-H1 expression in the OS and PFS of patients in various PIT groups

We evaluated the significance of nm23-H1 expression in PTCL-NOS among patients classified according to the PIT. In groups 1 and 2, OS (Fig. 4A) was worse among patients

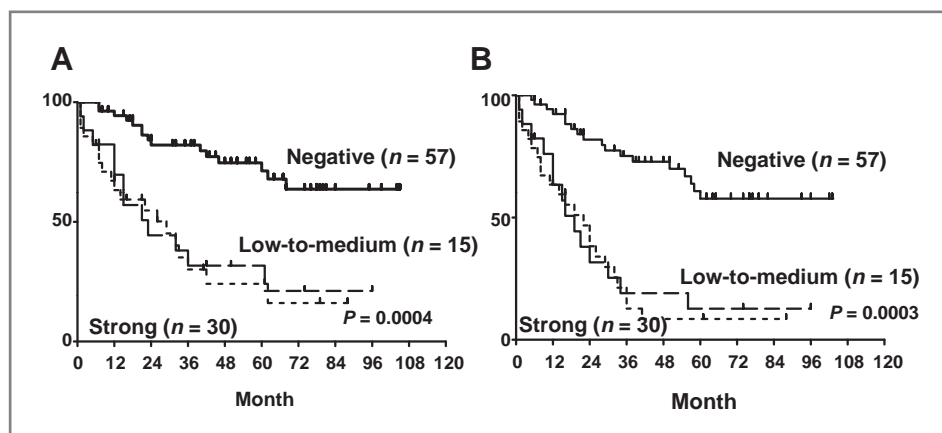
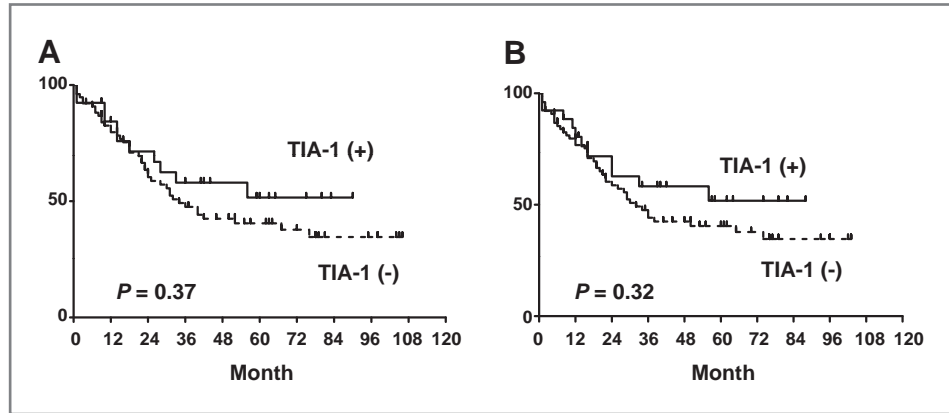


Figure 2. OS (A) and PFS (B) curves of patients with PTCL-NOS according to the expression of cytoplasmic nm23-H1.

Figure 3. OS (A) and PFS (B) curves of patients with PTCL-NOS according to the expression of TIA-1.

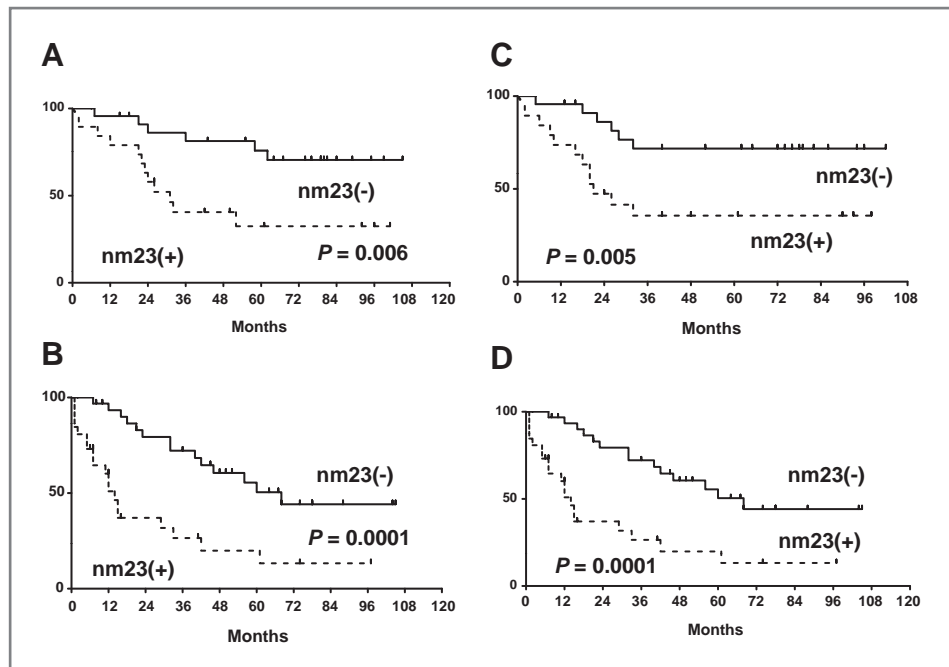


who were positive for nm23-H1 ($n = 19$, 5-year OS 32.4%) than among those who were negative for nm23-H1 ($n = 24$, 5-year survival 75.9%; $P = 0.006$), indicating that the therapeutic outcome was worse with nm23-H1 expression. The prognosis of patients who were positive for nm23-H1 was also poor in groups 3 and 4 ($P = 0.0001$; Fig. 4B). We examined the PFS with nm23-H1 expression levels and obtained the following results: in groups 1 and 2, 19 patients were positive for nm23-H1 (5-year PFS 35.5%) and 24 patients were negative for nm23-H1 (5-year PFS 71.7%; Fig. 4C); in groups 3 and 4, 26 patients were positive for nm23-H1 (5-year PFS 19.8%) and 32 patients were negative for nm23-H1 (5-year PFS 50.4%; Fig. 4D). The PFS prognosis of patients with high nm23-H1 was poor in all of these risk groups ($P = 0.0001$). Therefore, we might be able to predict the therapeutic outcome in each PIT risk group of PTCL-NOS by using nm23-H1 expression at diagnosis.

Comparison between serum nm23-H1 level and cytoplasmic nm23-H1 expression in PTCL-NOS

The serum nm23-H1 level in 30 patients with cytoplasmic nm23-H1 strongly positive PTCL-NOS was 43.44 ± 21.48 ng/mL (mean \pm SD), that in 15 patients with cytoplasmic nm23-H1 low-to-medium positive PTCL-NOS was 24.32 ± 22.11 ng/mL, and that in 57 patients with cytoplasmic nm23-H1 negative PTCL-NOS was 13.64 ± 11.62 ng/mL. Although there was no significant difference in the serum nm23-H1 level between the patients with negative staining and those with low-to-medium positive staining ($P = 0.058$), there were significant differences between the patients with strongly positive staining and those with moderately positive staining ($P = 0.029$) and also between those with strongly positive staining and those with negative staining ($P = 0.0001$; Fig. 5). Therefore, this result suggests that a portion of the serum nm23-H1 had been produced directly by lymphoma cells.

Figure 4. OS (A and B) and PFS (C and D) curves of patients with PTCL-NOS according to the PITA. A and C, groups 1 and 2; B, D, groups 3 and 4.



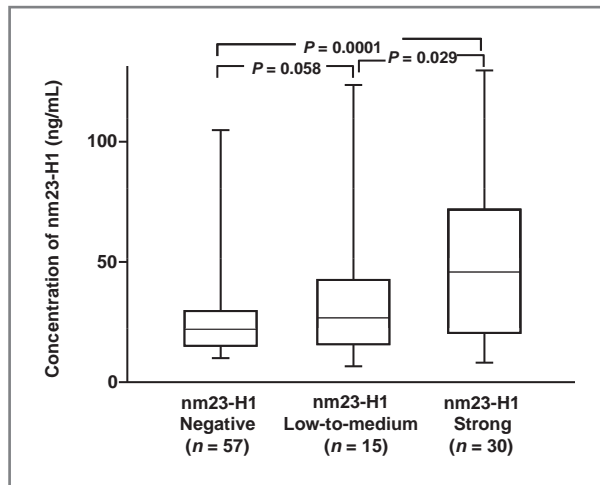


Figure 5. Relationship between the serum nm23-H1 level and cytoplasmic nm23-H1 expression in patients with PTCL-NOS.

Univariate and multivariate analyses of OS and PFS in patients with PTCL-NOS

OS was significantly shorter in patients with the following clinicopathologic features: age more than 60 years, PS of 2 to 4, LDH level greater than normal, BM involvement, and nm23-H1-positive lymphoma. PFS was significantly shorter in patients with the following clinicopathologic features: age more than 60 years, LDH level greater than normal, BM involvement, and nm23-H1-positive PTCL-NOS. Multivariate analysis with these individual factors showed nm23-H1 expression (HR, 6.09, 95% CI, 2.85–17.21; $P = 0.0001$) and BM involvement (HR, 3.31, 95% CI, 0.23–12.87; $P = 0.032$) to be significant and independent prognostic factors among the 102 PTCL-NOS patients (Table 2).

Discussion

PTCL-NOS is more aggressive and has a poorer prognosis than DLBCL (1). The treatment strategy for PTCL-NOS at present is generally determined by the IPI and PIT. By using the IPI and PIT, a fairly accurate prognostication could be made and hence it would be possible to make a stratified treatment plan for each patient. However, IPI and PIT are

Table 2. Multivariate analysis of prognostic factors in patients with PTCL-NOS

| Covariate | Covariate subgroup | HR | 95% CI | P |
|-----------------|--------------------|------|------------|--------|
| nm23-H1 | Positive | 6.09 | 2.85–17.21 | 0.0001 |
| BM involvement | Present | 3.31 | 0.23–12.87 | 0.032 |
| Serum LDH level | >Normal | 1.51 | 0.85–4.41 | 0.45 |
| PS | 2–4 | 1.46 | 0.76–6.62 | 0.56 |
| Age, y | ≥60 | 2.12 | 0.60–10.24 | 0.63 |

markers based primarily on clinical findings and laboratory data. In the present study, when the patients were classified according to the PIT, the 5-year OS and PFS rates of groups 1 and 2 and those of groups 3 and 4 showed no significant differences. Therefore, treatment strategies stratified by a new prognostic factor are necessary. Recently, prognostic factors for PTCL-NOS based on the biological characteristics of tumor cells have been identified. TIA-1 is a 15-kD cytotoxic granule-associated protein that is structurally related to the TNF receptor family; TIA-1 induced apoptotic cell death when introduced into permeabilized target cells (11). Cytotoxic cell-associated molecules, such as TIA-1, are primarily expressed in cytotoxic T cells and natural killer cells. TIA-1 moves to the cytoplasm through the perforin-formed pore and activates apoptosis-associated protein. It was originally recognized as a protein that directly induces apoptosis of target cells and is present in azurophilic cytoplasmic granules of T lymphocytes. TIA-1, however, is expressed in cytotoxic T cells, regardless of their activation status, and in myeloid cells (12). Asano and colleagues (13) identified that expression of cytotoxic antigens in T-cell lymphoma cells may represent a poor prognostic factor in PTCL-NOS. In the present study, TIA-1 was expressed in 22% of PTCL-NOS tumors and there were no significant differences in OS and PFS between the TIA-1-positive group and the TIA-1-negative group.

On the other hand, the *nm23* gene was initially identified as a putative metastasis suppressor gene on the basis of its reduced expression in certain highly metastatic cell lines and tumors (14). The level of nm23-H1 expression was inversely correlated with the metastatic potential of tumors in experimental rodent cells and in human tumors, such as ovarian, breast, and cervical tumors, and melanomas (15). On the contrary, the opposite trend has been reported in thyroid carcinoma, neuroblastoma, and non-Hodgkin's lymphoma, although the mechanism of this discrepancy is unknown (5–7, 16). We previously reported cytoplasmic nm23-H1 expression in DLBCLs (7) and Hodgkin's lymphomas (10) and found that the serum and cytoplasmic nm23-H1 levels were significant prognostic factors in both DLBCLs and Hodgkin's lymphomas. In the present study, cytoplasmic nm23-H1 expression in lymphoma cells was analyzed in 102 patients with PTCL-NOS. nm23-H1 expression was not correlated with age, PS, LDH level, or stage but was significantly correlated with PIT. Next, we examined the relationship between nm23-H1 expression and OS and PFS. Patients with nm23-H1-positive PTCL-NOS had significantly shorter OS and PFS than those with nm23-H1-negative PTCL-NOS. OS was significantly shorter in patients with the following clinicopathologic features: age more than 60 years, PS of 2 to 4, LDH level greater than normal, BM involvement, or nm23-H1-positive PTCL-NOS. Multivariate analysis confirmed nm23-H1 expression to be an independent prognostic factor.

nm23-H1 binding protein is a latent protein encoded by the Epstein-Barr virus (EBV) nuclear antigen 3C (EBNA 3C). EBV is a common human virus that is associated with a number of human cancers, including Burkitt's

lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and PTCL-NOS (17). It is positive in approximately 30% of patients with PTCL-NOS, although its role in pathogenesis is unknown (18). EBNA 3C, a transcription factor, interacts with nm23-H1, which results in enhanced transactivating activity (17). In the present study, EBER was positive in 10 of the 26 PTCL-NOS patients in whom EBER ISH was carried out and nm23-H1 was positive in 5 of the 10 patients (data not shown). The number of PTCL-NOS patients will increase in the future and the role of EBER needs to be examined. On the other hand, nm23-H1 binding proteins include the T-cell lymphoma invasion and metastasis 1, a guanine nucleotide exchange factor for Rac1 (19). Rac1 was not examined in this study, but it is thought to be an important factor when the relationship between nm23-H1 and PTCL-NOS was examined.

On the other hand, we previously transplanted a human B-lymphocytic lymphoma cell line that overexpressed nm23-H1 into immunosuppressed nude mice and examined the tumor size and serum human nm23-H1 protein levels. nm23-H1 protein was detected in the serum of nude mice depending on the tumor size. These results strongly suggest that serum nm23-H1 protein is directly produced by lymphoma cells and its level depends on the total mass of malignant cells overexpressing nm23-H1 (20).

In conclusion, the nm23-H1 protein may be an important prognostic factor in PTCL-NOS. Because our results suggested that nm23-H1 is produced by lymphoma cells, we expect to see the development of new treatment options targeting nm23 overexpression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Drs. S. Nakamura, Oshima, Tamaru, N. Nakamura, and Yoshino for carrying out pathologic diagnosis.

Grant Support

This study was supported in part by a grant from the Ministry of Health and Welfare, and Grants-in-Aid for Scientific Research (C) and Cancer Research from The Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

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Received November 18, 2010; revised February 12, 2011; accepted March 7, 2011; published OnlineFirst April 8, 2011.

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