

Elevated plasma level of differentiation inhibitory factor nm23-H1 protein correlates with risk factors for myelodysplastic syndrome

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We measured plasma nm23-H1 level (nm23-H1), a differentiation inhibitory factor, by an enzyme-linked immunosorbent assay (ELISA) in patients with aplastic anemia (AA) and myelodysplastic syndrome (MDS). The nm23-H1 in AA was not significantly elevated when compared to normal subjects (6.66 ± 1.20 ng/ml vs 5.13 ± 0.81 ng/ml; *P* = 0.274). In contrast, MDS patients had significantly high levels of nm23-H1 compared not only to normal subjects (11.16 ± 1.42 vs 5.13 ± 0.81 ng/ml; *P* = 0.0004) but also to those of the AA group (11.16 ± 1.42 ng/ml vs 6.66 ± 1.20 ng/ml; *P* = 0.018). In the MDS group of patients, no significant difference was observed in the nm23-H1 levels between patients with refractory anemia (RA) and RA with excess blasts (RAEB)/RAEB in transformation (10.71 ± 1.61 ng/ml vs 9.24 ± 2.66 ng/ml; *P* = 0.672). Of the patients with RA, patients with low risk according to the International Prognostic Scoring System (IPSS) had significantly low levels of nm23-H1 compared to those of IPSS INT-1 level cases (6.40 ± 1.36 ng/ml vs 13.05 ± 2.50 ng/ml; *P* = 0.0028), suggesting that nm23-H1 may be useful as a prognostic marker for MDS, especially in low risk patients.

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Introduction

Myelodysplastic syndrome (MDS) is a disorder characterized by clonal proliferation of abnormal hematopoietic stem cells that results in ineffective hematopoiesis and dysplastic features^{1,2} and functional abnormalities^{3,4} of blood cells. The diagnosis of MDS patients with cytopenias and minimum dysplasia is sometimes difficult. To date, the difference between aplastic anemia (AA) and refractory anemia (RA) is still unclear.⁵ Analyzing chromosomal findings in bone marrow cells at the beginning of the clinical course is sometimes not sufficient.⁶ Although many trials to distinguish AA and RA have been made,^{7–9} few have been successful in clinics.

A differentiation inhibitory factor purified from conditioned medium of a differentiation-resistant mouse myeloid leukemia cell line was found to be identical to the nm23 protein.^{10–16} The nm23 proteins are involved in tumor metastasis regulation and have nucleoside diphosphate kinase enzyme activity that catalyzes the ATP-dependent synthesis of nucleoside diphosphates.^{17–24} The expression of nm23-H1 has been reported to have an inverse relationship with metastatic potential and in various malignancies.¹⁸ The inhibition of differentiation may be associated with the aggressive behavior of leukemia. A high expression of nm23-H1 has been detected in bone marrow CD34⁺ cells, while decreased expression was noted in more differentiated cell types.²⁵ In patients with acute myeloid leukemia (AML), nm23-H1 mRNA levels correlated with poor

prognosis, resistance to chemotherapy and reduced overall survival.^{13,15} In addition, expression of nm23-H1 mRNA was significantly higher in other hematological malignancies than in normal volunteers.¹⁵ An enzyme-linked immunosorbent assay (ELISA) was also established to detect plasma nm23-H1 protein levels. Elevated plasma levels of nm23-H1 protein were also demonstrated to predict poor outcome of advanced non-Hodgkin's lymphoma (NHL)²⁶ and AML.²⁷

It could be speculated that the plasma levels of nm23-H1 protein might be higher in patients with the hematological potential to develop to leukemia. Usually AA does not develop to leukemia, while some cases of MDS, even of RA, develop to acute leukemia. The development to leukemia or high risk MDS is occasionally seen after long follow-up in patients with the initial diagnosis of AA accompanied by G-CSF treatment.^{28,29} The cases with disease progression might have the potential for development even in the early stage of the disorder and the nm23-H1 family might be an indicator of the potential of disease progression even at an early stage. In this study, we studied the difference of plasma nm23-H1 levels in patients with AA and MDS in an attempt to clarify the clinical implications for diagnosis and prediction of disease progression. In MDS patients, patients with RA were mostly chosen, since the aim of this study was to evaluate a diagnostic tool for patients with cytopenias only.

Materials and methods

Blood

Venous blood was drawn in sodium heparin from 25 patients with AA, age range from 19 to 74 years (median 44), 42 with MDS, age range from 22 to 82 years (median 60), and 40 healthy volunteers, age range from 20 to 50. Informed consent was obtained prior to drawing blood from all patients and volunteers. Newly diagnosed patients during 1999 and 2000 and patients diagnosed earlier and followed without any therapeutic medication were also included in this study. Patients with any infection or any past severe complications were not included in the study in order to exclude the unknown influence of agents to treat those complications. The subtypes of patients according to the FAB criteria were 31 with RA, three with RA with excess of blasts (RAEB), five with RAEB in transformation (RAEBt), two with RA with ringed sideroblasts (RARS) and one with chronic myelomonocytic leukemia (CMML). There were 10 men and 15 women with AA and 24 men and 18 women with MDS. All healthy volunteers were screened to exclude the presence of disease or use of medication.

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ELISA for human nm23-H1

The protein level of human nm23-H1 in plasma was measured by ELISA assay as previously described.^{26,27} We counted 96-well plates (Costar 3369; Corning, Corning, NY, USA) with monoclonal anti-nm23-H1 antibody (Seikagaku Kogyo Co, Tokyo, Japan), washed four times with phosphate-buffered saline (PBS), and incubated with 25% Block Ace (Dainihon Seiyaku, Osaka, Japan). Plasma samples were diluted two-fold with PBS, and 50 μ l aliquots were then added to the wells. After incubation at room temperature for 1 h, the wells were washed with PBS containing 0.05% Tween 20 (T-PBS). The samples were then incubated with polyclonal anti-nm23-H1 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), washed four times with T-PBS, and incubated with alkaline phosphate-conjugated anti-rabbit IgG (Bio-Rad Lab, Hercules, CA, USA). After washing four times with T-PBS, alkaline phosphatase activity was detected using diethanolamine as a substrate and an alkaline phosphatase-detection kit (Bio-Rad). The absorbance was measured at 405 nm with a correction wavelength of 630 nm using a microplate reader. Recombinant nm23-H1-GST protein was used as a standard.^{11,30} To avoid the effects of hemolysis, we determined free hemoglobin in the same plasma samples as previously described.³¹ Samples with values greater than 120 μ g/ml were not used for analysis.

Statistical analysis

Data were expressed as means \pm standard error of the mean (s.e.m.). Student's *t*-test and Mann-Whitney *U* test were used and a *P* value of 0.05 or less was considered significant. Fisher's exact probability test was also used to analyze distribution of patients according to nm23-H1 levels. Kaplan-Meier plots were drawn to analyze the prognosis of patients.

Results

We measured nm23-H1 protein in plasma from patients with AA and MDS (Table 1 and Figure 1). There was no significant difference in the value between groups in relation to either age or gender (data not shown). The nm23-H1 in AA was not significantly elevated when compared to normal subjects (6.66 ± 1.20 ng/ml vs 5.13 ± 0.81 ng/ml; *P* = 0.274, Table 1). In contrast, MDS patients had significantly high levels of nm23-H1 compared not only to normal subjects (11.16 ± 1.42

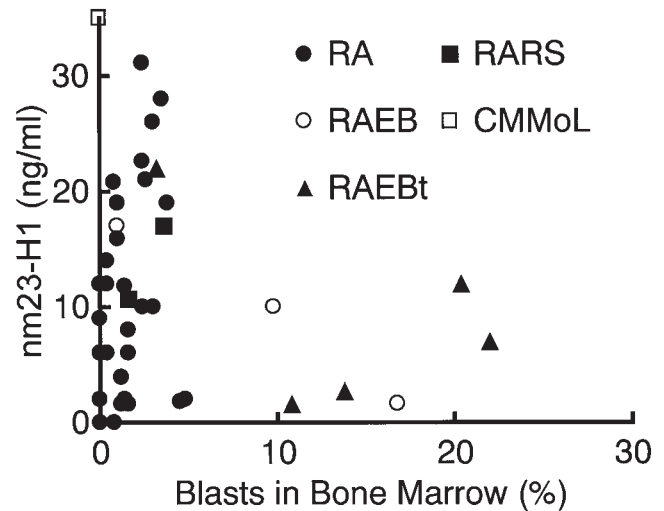


Figure 1 The relationship of nm23-H1 levels and blast cell count in bone marrow in patients with MDS. The nm23-H1 levels in plasma from RA (solid dots), RARS (solid squares), RAEB (circles), RAEBt (solid triangles) and CMMoL (outlined squares) are indicated.

ng/ml vs 5.13 ± 0.81 ng/ml; *P* = 0.0004) but also to those of AA patients (11.16 ± 1.42 ng/ml vs 6.66 ± 1.20 ng/ml; *P* = 0.018, Table 1).

In the MDS patients, we failed to demonstrate a significant difference in nm23-H1 between RA and other types of MDS according to FAB criteria (Table 1). When we analyzed RAEB and RAEBt together, no significant difference was observed in the nm23-H1 levels between patients with RA and RAEB/RAEBt (10.71 ± 1.61 ng/ml vs 9.24 ± 2.66 ng/ml; *P* = 0.672). In contrast, RA patients (*n* = 31) had significantly higher levels of nm23-H1 than normal subjects (10.71 ± 1.61 ng/ml vs 5.13 ± 0.81 ng/ml; *P* = 0.003) and AA patients (10.71 ± 1.61 ng/ml vs 6.66 ± 1.20 ng/ml; *P* = 0.049, Table 1). The value of 15.85 ± 2.99 ng/ml (*n* = 13) in the MDS patients with chromosomal anomaly was significantly higher than the value of 8.95 ± 1.47 ng/ml (*n* = 28) in the MDS patients with normal karyotypes. Among 13 patients with chromosomal anomaly, five belonged to the poor risk group and eight were included in the intermediate risk group of karyotypes according to the International Prognostic Scoring System (IPSS) score.

When the MDS patients were subdivided based on IPSS, the nm23-H1 of 7.55 ± 1.43 ng/ml in the low group (*n* = 13) was significantly lower than the value of 14.42 ± 2.33 ng/ml in the INT-1 group (*n* = 21) (*P* = 0.017). There were no significant differences in the nm23-H1 levels among the INT-1, INT-2 and high groups. When we analyzed the INT-1, INT-2 and high groups together (*n* = 29), value in the low risk group was also significantly lower (*P* = 0.033, Figure 2a). The nm23-H1 levels of 10.29 ± 2.31 ng/ml (*n* = 16) in patients with normal karyotypes in the INT-1, INT-2 and high groups was not significantly different from the value of 15.85 ± 2.31 ng/ml (*n* = 13) in patients with chromosomal anomaly in the INT-1, INT-2 and high groups (*P* = 0.146). The nm23-H1 level in the low group was not significantly different from the value in AA, while the value in the INT-1 group was significantly higher than the value in AA (*P* = 0.009). The value in the INT-1, INT-2 and high group was also significantly higher than the value in AA (*P* = 0.006, Figure 2a).

We then tried to analyze the correlation between patients with only RA and other groups. We analyzed 31 patients diagnosed as RA in 42 MDS patients. According to the IPSS cri-

Table 1 The nm23-H1 levels in plasma from normal volunteers, AA and subtypes of MDS according to the FAB criteria

	<i>n</i>	nm23-H1	<i>P</i> value if significant	
			vs control	vs AA
Control	40	5.13 ± 0.18		
Aplastic anemia	25	6.66 ± 1.20		
Myelodysplastic syndrome	42	11.16 ± 1.42	0.0004	0.032
RA	31	10.71 ± 1.61	0.003	0.049
RARS	2	13.85		
RAEB	3	9.53 ± 4.45		
RAEBt	5	9.00 ± 3.72		
CMMoL	1	35.00		

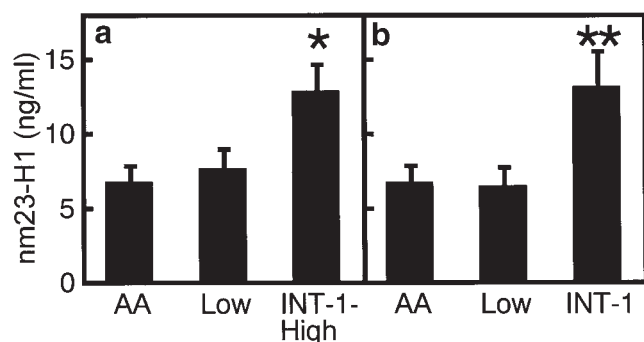


Figure 2 The nm23-H1 levels in plasma from AA and subgroups of MDS according to IPSS when INT-1, INT-2 and high were analyzed together (a) and the nm23-H1 levels in plasma from AA and subgroups of IPSS in RA patients (b). *The value in INT-1, INT-2, and high was significantly higher than the value in AA ($P = 0.009$) or low ($P = 0.033$). **The value in INT-1 was significantly higher than the value in AA ($P = 0.031$) or low ($P = 0.028$).

teria, there were 11 patients in the low group, 18 with INT-1 and two with INT-2 among RA patients. Among these, 13 patients with INT-1 were categorized only by cytopenia, while five with INT-1 and both patients in INT-2 revealed chromosomal anomaly. There were no high group patients with RA. The nm23-H1 of 6.40 ± 1.36 ng/ml in the low group ($n = 11$) was significantly lower than the value of 13.05 ± 2.50 ng/ml in the INT-1 group ($n = 17$) ($P = 0.028$, Figure 2b). Taking the groups with INT-1 and INT-2 together ($n = 20$), the value in the low group was also significantly lower than the value of 13.10 ± 2.24 ng/ml in INT-1 and INT-2 ($P = 0.016$). The value of 6.40 ± 1.36 ng/ml in the low group was not significantly different from that of 6.66 ± 1.20 ng/ml in AA, while the value in the INT-1 group was significantly higher than the value in AA ($P = 0.031$, Figure 2b). The value in INT-1 and INT-2 together was also significantly higher than the value in AA ($P = 0.017$). When we did the analysis in smaller groups according to the IPSS, we failed to demonstrate differences in the protein level (data not shown).

We also analyzed these data using Fisher's exact probability test. The nm23-H1 protein level in healthy volunteers was 5.13 ± 5.13 ng/ml (mean \pm standard deviation). Values higher than mean plus one standard deviation of 10.26 ng/ml were considered as high-level nm23-H1. Only 4/25 AA patients had high nm23-H1 levels, while 18/42 MDS patients had nm23-H1 levels of ≥ 10.26 ng/ml ($P = 0.021$, Table 2). When

Table 2 Number of patients with AA and MDS in whom plasma nm23-H1 protein levels were high or not high

	nm23-H1		P value if significant
	High	Not high	
Aplastic anemia	4	21	
Myelodysplastic syndrome	18	24	0.021
RA	13	18	0.034
RARS	2	0	
RAEB	1	2	
RAEBt	2	3	
CMMoL	1	0	

The higher level was defined as more than the mean plus standard deviation in healthy volunteers. Numbers of all MDS patients or subtypes of them vs numbers of AA patients were analyzed by Fisher's exact probability test.

we analyzed RA patients only, significance was obtained in 13/31 RA patients with high nm23-H1 levels ($P = 0.034$, Table 2). We also tried to analyze survivals in these patients. No significant differences among patients separated by the nm23-H1 values were noted using a Kaplan–Meier curve (data not shown), perhaps because the observation period was too short. Among 26 RA patients with more than 1 year follow-up, three patients developed to RAEB or RAEBt. The value of 22.00 ± 2.09 ng/ml in these three patients was higher than the value of 10.10 ± 1.86 ng/ml in the other 23 patients analyzed by the Mann–Whitney U test ($P = 0.044$).

Discussion

The nm23 proteins have been characterized as a differentiation inhibitory factor for acute leukemia.^{10,11,14} Overexpression of the nm23-H1 and nm23-H2 transcripts in AML cells has been previously described.^{13,15} In addition, high nm23-H1 expression level has been correlated with the poor prognosis of AML.^{12,13,15} Elevated plasma nm23-H1 protein levels have also been associated with prognosis in aggressive NHL²⁶ and AML.²⁷ It can be speculated that elevated plasma nm23-H1 protein levels may be seen in some MDS patients. Our interest is whether we can distinguish the potential to develop acute leukemia in the early stage of MDS.

In the present study, we demonstrated elevated plasma nm23-H1 levels in MDS and RA as compared to AA (Table 1 and Figure 1). The numbers of patients with elevated levels of plasma nm23-H1 protein in MDS or RA were also greater than in AA (Table 2). As is already known, some patients with RA develop to RAEB, RAEBt or post-MDS AML. It is not rare that abnormal chromosomal findings become present long after the stable state with normal chromosomes in bone marrow.⁶ In some patients with MDS, dysplasia in hematological cells is not obvious in the early stage. The diagnosis of patients with cytopenia in peripheral blood and hypoplastic features, normal chromosomes and minimal dysplasia in bone marrow cells is still difficult. On the other hand, susceptibility to infection is sometimes life-threatening for patients with AA and MDS.^{3,4} For these patients, G-CSF is sometimes necessary, in addition to antibiotics in cases of severe infection and for prevention of these infections. Treatment with G-CSF in patients with AA sometimes induces a poor prognostic chromosomal anomaly of monosomy 7 in bone marrow cells.^{28,29} For these patients, effective laboratory methods are urged by hematologists to differentiate diagnosis of RA and AA or to predict their prognosis for progression of disease.

We can speculate that RA patients with the potential to develop leukemia may express relatively higher nm23-H1 protein levels than those with a low potential of disease progression or AA, which usually does not progress to leukemia. IPSS is now recognized as a standard method to predict the prognosis of patients with MDS. Here, we analyzed differences in nm23-H1 protein levels according to IPSS. Plasma nm23-H1 in patients in the low risk group was significantly lower than in patients with INT-1 in RA or whole MDS. These data are compatible with the hypothesis that RA patients having the potential to develop leukemia may express relatively higher nm23-H1 protein levels than RA with a low potential of disease progression. Although IPSS includes not only disease progression but also MDS-related death, with infection or bleeding, these data suggest that nm23-H1 could be considered as one of the clinical markers to predict prognosis. We must still remember that nm23-H1 in the low risk group

was not higher than that in the AA group. This means that there is still a problem in distinguishing patients with minimal dysplasia in the low risk group and patients with AA. Many hematologists have attempted to provide one clear diagnostic tool for differential diagnosis of these disorders,^{5,7,8} but no one has been successful. Given the present situation it seems best to have as many tools as possible. Here, we propose the nm23-H1 protein as a possible diagnostic marker for MDS, which may be useful to distinguish MDS and AA. The nm23-H1 protein could also be a possible prognostic marker for patients with MDS, since we demonstrated a correlation between nm23-H1 protein and IPSS.

Recently, much attention has focused on the role of nm23 as a contributing mechanism to many hematological malignancies.^{10,11,14} An increased level of nm23-H1 contributes to potential leukemic progression.²⁷ This report may provide evidence of a clinical role for nm23-H1 measurement in patients with AA or MDS. It may also be a valuable marker to choose appropriate therapy for individual patients.

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