RAPID COMMUNICATION

Evaluation by Multivariate Analysis of the Differentiation Inhibitory Factor *nm23* as a Prognostic Factor in Acute Myelogenous Leukemia and Application to Other Hematologic Malignancies

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The differentiation inhibitory factor *nm23* can inhibit the differentiation of murine and human myeloid leukemia cells. We recently reported that *nm23* genes were overexpressed in acute myelogenous leukemia (AML), and a higher level of *nm23-H1* expression was correlated with a poor prognosis in AML, especially in AML-M5 (acute monocytic leukemia). To evaluate the importance of *nm23* expression as a prognostic factor in AML, we compared it with other putative prognostic factors in AML. An analysis of the correlation between *nm23* expression and the clinical parameters of 110 patients with AML demonstrated that increased *nm23-H1* mRNA levels were associated with resistance to initial chemotherapy and with reduced overall survival. Multivariate analysis using Cox's proportional hazard model also showed that elevated *nm23-H1* mRNA levels significantly contributed to the prognosis of patients with AML. Especially in AML-M5, *nm23-H1* status was the most important prognostic factor. Furthermore, to determine whether we can apply the results observed in AML to other hematologic malignancies, we investigated the relative levels of *nm23-H1* and *nm23-H2* transcripts in 149 patients with hematologic neoplasms, including 110 with de novo AML, 9 with de novo acute lymphoblastic leukemia, 14 with myelodysplastic syndrome, 16 with chronic myelogenous leukemia (CML), and 5 normal subjects by the reverse transcriptase-polymerase chain reaction. Expression of *nm23-H1* was significantly higher in all the hematologic neoplasms, except CML in chronic phase, than in normal blood cells. *nm23* may have a prognostic effect in these hematologic malignancies as well as in AML.

ALTHOUGH NORMAL hematopoiesis can be controlled by various positive and negative regulatory molecules, leukemic cells are arrested in less-differentiated stages of development. This suggests that both positive and negative regulators are important for the differentiation of leukemic cells. We previously reported that a nondifferentiating mouse myeloid leukemia cell line produced differentiation inhibiting factors (I-factors). Suppression of the production of these I-factors resulted in nondifferentiating leukemic cells becoming sensitive to differentiation inducers. One of these I-factors was purified as a homologue of *nm23*.[1-3]

The differentiation inhibitory factor *nm23* can inhibit the differentiation of murine and human myeloid leukemia cells, and *nm23* expression is greatly increased during blast formation in normal lymphocytes.[1-4] These findings suggest that *nm23* genes play a role in the growth and differentiation of normal and malignant hematopoietic cells. Few studies have focused on the role of *nm23* in human hematopoietic malignancies.[5] We recently reported that *nm23* genes were overexpressed in acute myelogenous leukemia (AML) and that *nm23-H1* expression was significantly correlated with a poor prognosis in AML, especially in AML-M5.[6] It has been reported that high-grade non-Hodgkin's lymphoma (NHL) and Hodgkin's lymphoma exhibited significantly higher levels of *nm23-H1* expression than low-grade NHL.[7] These studies suggest that *nm23-H1* expression in human hematopoietic malignancies is associated with the aggressiveness of the disease.

In the present study, to evaluate the importance of *nm23* expression as a prognostic factor in AML, we compared the levels of *nm23* expression with other putative prognostic factors in AML by a multivariate analysis. Furthermore, to determine whether we can apply the results observed in AML to other hematologic malignancies, we examined the expression of *nm23-H1* and *-H2* genes in various human hematologic malignancies other than AML.

MATERIALS AND METHODS

Patients' samples. Bone marrow (BM) or peripheral blood (PB) samples were obtained from 149 patients with newly diagnosed hematologic neoplasms, consisting of 110 with de novo AML, including 42 previously reported cases,[6] 9 with de novo acute lymphoblastic leukemia (ALL), 9 with myelodysplastic syndrome (MDS; 1 with chronic myelomonocytic leukemia [CMMoL], 4 with refractory anemia with excess of blasts [RAEB], and 4 with RAEB-T), 5 with MDS overt leukemia, 9 with chronic myelogenous leukemia in the chronic phase (CML-CP), and 7 with CML in blast crisis (CML-BC) with their informed consent at onset, before chemotherapy. De novo AML, ALL, and MDS were classified according to the criteria devised by the French-American-British (FAB) Committee. AML patients were treated with cytosine arabinoside (or behenoyl cytosine arabinoside), daunorubicin, and with or without prednisolone and/or 6-mercaptopurine, and AML-M3 patients were consecutively treated with all-*trans* retinoic acid for remission induction therapy.[8,9] Treated patients were judged to have achieved complete remission (CR) when BM aspirates showed trilineage regeneration with less than 5% blasts by morphology and

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immunocytochemistry in the presence of a normal blood count that persisted for at least 1 month. Patients who died of toxic complication (infection or bleeding) before the time of expected marrow recovery were not evaluated. All other patients were considered nonresponsive (NR). To purify leukemic cells, heparinized PB cells or BM aspirates were mixed with an equal volume of RPMI-1640 medium and centrifuged on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Normal BM and PB cells were obtained from healthy volunteers after obtaining their informed consent. Mononuclear cells from normal BM and PB cells were separated over Ficoll-Hypaque. Total RNA was extracted as described by Chomczynski and Sacchi, using guanidium thiocyanate.[10]

Reverse transcriptase-polymerase chain reaction (RT-PCR). Quantitative RT-PCR was performed using a GeneAmp RNA PCR kit (Takara, Tokyo, Japan), as reported previously.[6] The oligonucleotides used in PCR amplification were as follows: sense strand, 5′-ATGGCCAACTGTGAGC-GTACC-3′; antisense strand, 5′-CATG TATTTCACCAGGCCGGC-3′ for *nm23-H1*; sense strand, 5′-ATGGCCAACCTGGAGCGCACC-3′; antisense strand, 5′-TCCC CACGAATGGTGCCTGGC-3′ for *nm23-H2*; and sense strand, 5′-ACATCGCTCAGACACCATGG-3′; antisense strand, 5′-GTAGTTGAGGTCAATGAAGGG-3′ for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). PCR consisted of 35 cycles for *nm23-H1* and 25 for *nm23-H2* and *gapdh*, with denaturing at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 0.5 minutes. The reaction was performed in a GeneAmp PCR system 9600 (Perkin Elmer, Norwalk, CT). The PCR products were then subjected to 6% polyacrylamide gel electrophoresis, and the dried gel was exposed to imaging plates (Fuji Film Co, Ltd, Tokyo, Japan) at room temperature for 15 to 20 minutes. The results of autoradiography were quantified using a Fuji Bio-Image Analyzer BAS2000 (Fuji Film Co).

Statistical analysis. Differences between groups were evaluated by Mann-Whitney's U-test (nonparametric analysis), and $P < 0.05$ indicated a significant difference. Pearson's correlation coefficient was used to evaluate the correlation between paired values. Survival curves of patients were prepared by the Kaplan-Meier method, and differences between the survival curves were evaluated using the log-rank and generalized Wilcoxon's tests. A multivariate analysis of the prognosis was performed using Cox's proportional hazard model.

RESULTS

Relationship between nm23 expression and clinical data in AML. We examined the levels of *nm23* expression in BM and PB samples from 110 patients with newly diagnosed de novo AML, including 3 M0, 20 M1, 29 M2, 19 M3, 19 M4, 18 M5 (7 M5a and 11 M5b), and 2 M6. To normalize the differences in RNA loading for RT-PCR and in RNA degradation in individual samples, the values of *nm23-H1* and *-H2* gene expression were divided by that of the *gapdh* gene for comparison with the values in erythroleukemia HEL cells, which were defined as 100 (the expression ''Index''). Both *nm23-H1* and *-H2* genes were overexpressed in these 110 AML samples (Table 1). To evaluate the relative importance of *nm23* expression as a prognostic factor in AML, we determined the correlation of *nm23-H1* or *-H2* expression with age, sex, white blood cell (WBC) count, lactate dehydrogenase (LDH) level, surface marker CD7, chromosomal aberration, and the response to initial chemotherapy (Table 2). Increased LDH was correlated with *nm23-H1* ($P = .006$) and *nm23-H2* expression ($P = .038$). $CD7^+$ AML showed higher *nm23-H1* expression than $CD7^-$ AML ($P = .019$). A good response to initial chemotherapy was inversely correlated with *nm23-H1* ($P = .020$). A total of 100 samples were evaluable in terms of the response to treatment. Thirty-two patients failed to achieve remission after the initial chemotherapy. The drug-resistant AML samples expressed a significantly higher *nm23-H1* level (*nm23-H1* index = 144 ± 212) than those from the 68 AML patients who achieved CR (*nm23-H1* index = 97 ± 115). The expression levels of *nm23-H1* and *-H2* in AML-M3 leukemia with t(15;17)

Table 1. Levels of *nm23-H1* and *-H2* Index in Normal and Hematologic Neoplasms

Diagnosis (no.)	*nm23-H1* Index	*P*	*nm23-H2* Index	*P*
Normal (5)	26 ± 26		39 ± 24	
AML (110)	107 ± 147	.003*	95 ± 110	.023*
ALL (9)	87 ± 41	.007*	54 ± 19	.223
MDS (9)	91 ± 81	.011*	58 ± 30	.184
MDS overt leukemia (5)	147 ± 141	.012*	52 ± 34	.569
CML-CP (9)	35 ± 20	.204	42 ± 10	.633
CML-BC (7)	102 ± 34	.006*	83 ± 39	.013*

The mRNA levels were normalized for *gapdh* mRNA. The positive control (the Index = 100) is represented by RNA extracted from the HEL cell line. Values are means ± SD. Analyzed by Mann-Whitney's U-test (*v* normal).

*$P < .05$.

Table 2. Relationship Between *nm23* Expression and Clinical Factors in AML

Clinical Factor (no.)	*nm23-H1* Index	*P*	*nm23-H2* Index	*P*
Age (yr)				
≤50 (53)	101 ± 171		95 ± 119	
>50 (57)	112 ± 123	.275	95 ± 101	.862
Sex				
M (68)	102 ± 87		89 ± 58	
F (42)	115 ± 212	.107	104 ± 162	.216
WBC ($\times 10^9$/L)				
≤50 (79)	103 ± 153		85 ± 102	
>50 (29)	121 ± 136	.266	119 ± 130	.079
LDH				
≥N, ≤5 × N (92)	96 ± 142		84 ± 96	
>5 × N (14)	188 ± 179	.006*	159 ± 175	.038*
CD7				
Negative (70)	105 ± 115		87 ± 94	
Positive (17)	188 ± 279	.019*	141 ± 193	.121
Chromosomal aberration				
− (42)	97 ± 80		85 ± 53	
+ (65)	116 ± 180	.398	100 ± 136	.438
− (42)	97 ± 80		85 ± 53	
t(8;21) (13)	113 ± 108	.898	79 ± 60	.677
t(15;17) (17)	51 ± 29	.010*	63 ± 52	.045*
inv(16) (3)	78 ± 21	.891	89 ± 43	.733
11q23 (3)	81 ± 66	>.999	77 ± 44	>.999
MAKA (6)	300 ± 471	.554	213 ± 317	.513
others (25)	122 ± 148	.866	112 ± 28	.856
Response to initial chemotherapy				
CR (68)	97 ± 115		91 ± 96	
NR (32)	144 ± 212	.020*	110 ± 147	.597

Values are means ± SD. Analyzed by Mann-Whitney's U-test.
Abbreviation: MAKA, major karyotypic abnormalities.
*$P < .05$.

were lower than those in the other AMLs without chromosomal aberrations (*H1*, $P = .010$; *H2*, $P = .045$; Table 2).

Overall survival in AML. One hundred three evaluable AML patients were classified into groups based on age, sex, WBC count, LDH, CD7, chromosomal aberration, and *nm23-H1* and *-H2* expression. Overall survival in each group of patients is shown in Table 3. There were significant differences in the survival time between the patients classified by age, WBC count, LDH level, presence or absence of chromosomal aberration on t(8;21) and t(15;17), and *nm23-H1* and *-H2* expression levels. Cox's proportional hazard model was used to evaluate the relative importance of the putative prognostic factors in Table 3. Of these factors, chromosomal aberration on t(15;17) and t(8;21) had a good prognostic effect. However, if these were excluded from a multivariate analysis of the overall survival of AML patients, *nm23-H1* was the most important factor ($P = .034$, Table 4). Especially in AML-M5, *nm23-H1* mRNA expression significantly contributed to the prognosis, and *nm23-H1* status was the most important prognostic factor ($P = .003$, Table 4 and Fig 1). These results showed that the levels of *nm23* gene expression represent a new prognostic factor for AML.

Application of the expression of the nm23-H1 and -H2 genes to other hematologic neoplasms. To apply *nm23* expression to hematologic neoplasms other than AML, we examined the mRNA levels of the *nm23-H1* and *-H2* genes in hematologic neoplasms, as shown in Table 1 and Fig 2. The expression levels of the *nm23-H1* gene were significantly higher in MDS ($P = .011$), MDS overt leukemia ($P = .012$), AML ($P = .003$), ALL ($P = .007$), and CML-BC ($P = .006$) than in normal blood cells. Figure 3 shows the *nm23-H1* and *-H2* expression levels in all of the cases. Although a statistically significant correlation was observed between the expression levels of *nm23-H1* and *-H2* ($r = .785$, $P < .0001$), the expression levels of the *nm23-H2* gene were significantly higher in only AML ($P = .023$) and CML-BC ($P = .013$) compared with those in normal blood cells. The levels of *nm23-H1* and *-H2* mRNA in CML-CP cells were similar to the average levels in normal blood cells. With respect to the clinical stage of hematologic neoplasms, the expression of both *nm23-H1* and *-H2* was significantly higher in CML-BC than in CML-CP ($P = .001$ and $P = .007$, respectively), as shown in Fig 4. In contrast, the expression of both genes in MDS overt leukemia was similar to the average levels in MDS ($P = .205$ and $P = .548$, respectively; Fig 4). These results indicate that (1) *nm23-H1* is overexpressed in hematologic neoplasms, except CML-CP; (2) this increase in *nm23-H1* is observed in the stages of MDS; and (3) the progression of CML is accompanied by the overexpression of *nm23-H1* and *-H2* mRNA.

Table 3. Correlation of Overall Survival With Clinical and Biologic Features of 103 Patients With AML

Clinical Factor (no.)	Log-Rank (*P*)	g-Wilcoxon (*P*)
Age (yr)		
≤50 (50)		
>50 (53)	.005	<.001
Sex		
M (63)		
F (40)	NS	NS
WBC (×10⁹/L)		
≤50 (74)		
>50 (28)	.004	<.001
LDH		
≥N, ≤5 × N (87)		
>5 × N (14)	.003	<.001
CD7		
Negative (67)		
Positive (17)	NS	NS
Chromosomal aberration		
− (40)		
+ (61)	NS	NS
− (40)		
t(8;21) (13)	.041	NS
t(15;17) (14)	<.005	<.005
inv(16) (3)	NS	NS
11q23 (3)	NS	NS
MAKA (5)	NS	NS
Others (24)	NS	NS
nm23-H1 Index		
≤100 (71)		
>100 (32)	<.001	<.005
nm23-H2 Index		
≤110 (77)		
>110 (26)	NS	<.05

Abbreviation: NS, not significant.

Table 4. Multivariate Analysis of Clinical Factors on Overall AML and AML-M5 Patient Survival

Variable*	All AML (*P*)	AML-M5 (*P*)
Age (≤50/>50 yr)	.037	.218
Sex (M/F)	.145	.926
WBC (+Lz50/>50 × 10⁹/L)	.036	.516
LDH (≥N, ≤5 × N/>5 × N)	.113	.722
nm23-H1 (≤100/>100)	.034	.003
nm23-H2 (≤110/>110)	.691	.396

*Except the factors of chromosomal aberration, t(15;17) and t(8;21).

DISCUSSION

The *nm23* gene was originally identified by the differential hybridization of a cDNA library with total RNA extracted from mildly and highly metastatic melanoma cell lines.[11] Its expression was inversely correlated with the tumor's metastatic potential in experimental rodent cells and in some human tumors.[12] Transfection with *nm23* cDNA reduced the metastatic potential in vivo and the ability of cells to migrate in response to cytokines in vitro.[13-15] Reduced *nm23* expression levels are correlated with increased metastatic potential and an aggressive disease in human breast,[16] hepatocellular,[17] ovarian[18] and gastric carcinoma,[19] and in melanoma.[20] However, an opposite trend is observed in neuroblastoma and pancreatic carcinoma.[21-23] In other tumor types, including colorectal, thyroid, and lung carcinomas, *nm23* expression does not correlate with disease progression.[21] These results suggest a functional difference in *nm23* gene expression in several types of human tumor.[21] In leukemia, as shown in this report, *nm23* gene expression correlated with a poor prognosis in AML and with disease progression of CML. Especially in AML-M5, *nm23-H1* mRNA overexpression was the most important poor prognostic factor (Table 4). These results suggest that there is a connection

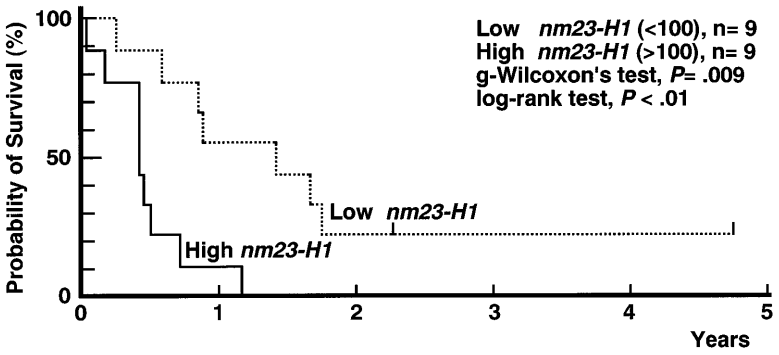

Fig 1. Survival curves of AML-M5 patients. High *nm23-H1* (>100) patients (n = 9, solid line) had a worse prognosis than low *nm23-H1* (100) patients (n = 9, broken line) (generalized Wilcoxon's test, *P* = .009; log-rank test, *P* < .01).

between *nm23* function and malignant phenotypes in leukemia, such as malignant growth, differentiation resistance, and chemotherapy resistance, although it remains unclear why AML-M5 cells exhibit a higher expression of *nm23* nmRNA levels.

We preliminally tried to evaluate the effect of *nm23-H1* mRNA level on prognosis for each of the FAB subtypes, although each group is a small cohorts of patients. For FAB-M5, there were 9 *nm23* low versus 9 *nm23* high; as shown in Fig 1 and Table 4, the elevated *nm23-H1* expression in FAB-M5 significantly contributed to the prognosis, and *nm23-H1* status is the most important prognostic factor. For FAB-M4, there were 12 *nm23* low versus 4 *nm23* high; the elevated *nm23-H1* expression in FAB-M4 significantly predicts a poor response to initial therapy (CR ratios of *nm23* low expression group and *nm23* high group were 83.3% and 25.0%, respectively; $P = .029$, χ^2 test for independence). The correlation between elevated *nm23-H1* expression and poor survival was observed in M4 cases, but the statistical significance has not been observed yet. For FAB-M3, there were 15 *nm23* low versus 0 *nm23* high; theses cases showed the lowest expression levels of *nm23-H1* among FAB subtypes. Because all cases showed *nm23-H1* $\leq$100 (low expression), we could not analyse the effect of the overexpression of *nm23-H1* (>100) on overall survival. The low expression levels of *nm23-H1* in FAB-M3 may be associated with the good prognosis in M3 patients. For FAB-M2, there were 18 *nm23* low versus 9 *nm23* high; the correlation between elevated *nm23-H1* expression and a poor response to initial therapy and poor survival was observed, but the statistical significance was not shown. For FAB-M1, there were 12 *nm23* low versus 8 *nm23* high; the elevated *nm23-H1* expression in these cases did not contribute to the response to initial therapy and the prognosis, although FAB-M1 cases had significantly higher expression levels of *nm23-H1* and *nm23-H2* than did normal subjects. Therefore, further analysis of *nm23-H1* and *nm23-H2* mRNA levels on prognosis for each of FAB subtypes in lager cohorts is required.

nm23-H1 and *-H2* show 88% amino acid sequence homology[24,25] and are located on the same region of chromosome 17q21 in tandem.[26-28] Based on an analysis of the promoter regions of the *nm23-H1* and *-H2* genes, it has been suggested that these *nm23* genes are independently and differentially regulated.[29] However, a statistically significant correlation between the expression levels of *nm23-H1* and *-H2* was observed in AML (Fig 3). Elevated expressions of both *nm23* genes were observed in AML, whereas a poor prognosis and a low percentage of CR in AML were associated only with the *nm23-H1* expression level. Postel et al[30] reported that *nm23-H2* was a transcription factor (PuF) and that one of its targets was the c-myc gene. Overexpression of the c-myc gene has also been reported to inhibit the differentiation of human and mouse leukemia cells.[31-33] However, in our previous experiment, we did not find a correlation between c-myc expression and *nm23-H2* expression in AML.[6] Thus, c-myc expression in AML does not appear to be influenced by the overexpression of *nm23-H2* (PuF).

Drug resistance, either inherent or acquired, is an important cause of treatment failure in hematologic neoplasms. Ferguson et al[34] reported a functional link between *nm23* expression and

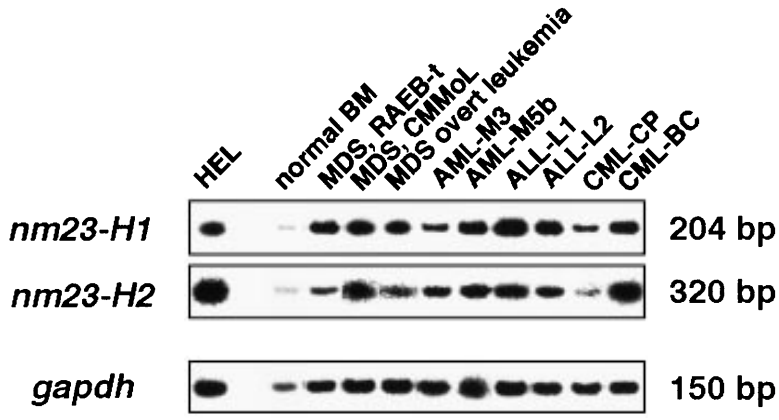

Fig 2. Quantitative RT-PCR analysis of *nm23-H1* and *nm23-H2* mRNA in human normal BM cells, MDS, MDS overt leukemia, AML, ALL, CML-CP, and CML-BC samples. Normal BM cells were from normal volunteers. Neoplasm cells were identified as the mononuclear cell fraction.

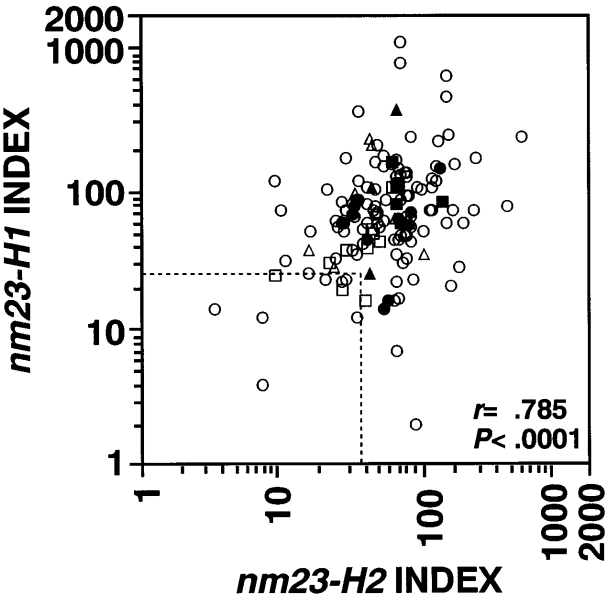

Fig 3. Relationship between the levels of *nm23-H1* and *nm23-H2* in human hematologic neo plasms. (---) Average levels of normal BM cells; (○) AML; (●) ALL; (□) CML-CP; (■) CML-BC; (△) MDS; (▲) MDS overt leukemias

cancer cell sensitivity to the alkylating agent cisplatin. We investigated, *mdr1*, *mrp*, *gst-π*, *topo I*, *topo IIα*, and *topo IIβ* expression in 20 AML patients by RT-PCR, but none of these drug resistance-related genes were associated with *nm23-H1* expression (data not shown). Other mechanisms may be involved in drug resistance. *nm23* genes can modulate differentiation, proliferation (cell cycle), and drug resistance in AML, and these three events are closely linked. Inducing differentiation in leukemic cells is associated with the concomitant block of the cell cycle at the G0/G1 phase. Transforming growth factor-β (TGF-β) is a negative regulator of proliferation, induced growth arrest in the G1 phase of the cell cycle in many cell types, and the loss of the cellular response to this ligand that occurs during oncogenesis in some systems. Transfection of melanoma K-1735 TK and breast carcinoma MDA-MB-435 cells with the *nm23* gene decreases the response to TGF-β.[13,14] We previously isolated a differentiation-resistant mouse myeloid leukemia M1 cells from the parent differentiation-sensitive M1 cells. We then characterized the differentiation-resistant cells and showed that the differentiation-resistant cells had higher leukemogenicity and produced I-factors/nm23 more than the parent differentiation-sensitive M1 cells. The differentiation-resistant M1 cells showed a more progressive potential, which was resistant to not only differentiation-induction but also to growth-suppressive cytokines such as TGF-β.[33,35-37] The role of *nm23* expression in the malignant growth of leukemia cells remains to be clarified, but *nm23* may play key roles in various aspects of hematopoietic cell biology, including differentiation, proliferation (cell cycle), and drug resistance.

MDS expressed higher *nm23-H1* mRNA levels, and this level was similar to that in AML (Table 1 and Fig 4). In this study, MDS included CMMoL, RAEB, and RAEB in transformation (RAEB-T), but excluded RA and RA with ringed sideroblasts (RARS). Although it is necessary to study RA and RARS (low-risk MDS), this study indicated that, if there were at least 11% blasts in marrow, *nm23-H1* expression could be detected at the leukemic level by RT-PCR.

The median survival time in CML is 3 to 4 years. Acute transformation may occur rapidly over weeks or even days. These patients are usually refractory to conventional chemotherapy. These clinical features seem to be associated with the elevation of *nm23* expression (Table 1 and Fig 4). Recently, Venturelli et al[38] isolated a novel cDNA *DR-nm23* that was differentially expressed in a CML blast crisis cDNA library. The sequence of *DR-nm23* is highly similar to that of *nm23*, and *DR-nm23* inhibits granulocyte differentiation and induces apoptosis in 32Dc13 myeloid cells. These results suggest that the *nm23* family, which includes *DR-nm23*, plays an important role in CML blastic crisis as well as in AML.

Cox's proportional hazard model indicated that the chromosomal aberrations t(15;17) and t(8;21) had good prognostic value, but they were limited to just AML-M3 and AML-M2. Because the prognostic evaluation of *nm23* is applicable to all stages of AML, *nm23-H1* status will be the most important prognostic factor. Furthermore, because the overexpression of *nm23-H1* is also observed in other hematologic malignancies, such as ALL and MDS, *nm23* expression could also have prognostic value in these diseases.

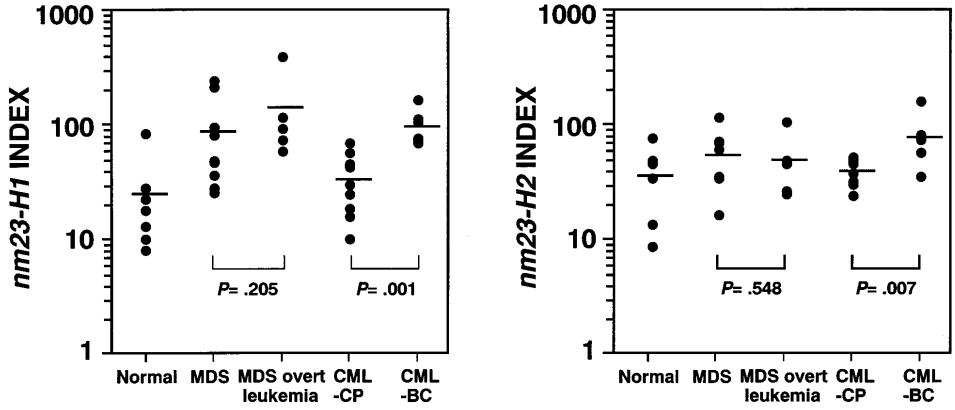

Fig 4. *nm23-H1* and *nm23-H2* expression in MDS in comparison with MDS overt leukemia and in CML-CP in comparison with CML-BC. Analyzed by Mann-Whitney's U-test.

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