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LETTER TO THE EDITOR

Correlation between the low marrow blast cutpoint and WHO classification for myelodysplastic syndromes

To the Editor:

The most widely used prognostic classification system used for myelodysplastic syndromes (MDS) is the International Prognostic Scoring System (IPSS) (1). Greenberg *et al.* (2) recently proposed a Revised IPSS (IPSS-R). Bone marrow (BM) cytogenetics, BM blast percentages, and cytopenias remained the basis of this new system. A low marrow blast cutpoint (2%) was added to novel components of the IPSS-R. In the IPSS-R, the <5% marrow blast category was split between 0– ≤ 2% and >2–<5%. The World Health Organization (WHO) classification-based Prognostic Scoring System (WPSS) proposed by Malcovati *et al.* (3) was published long before the IPSS-R adopted the split of blasts <5% into two groups. The WHO category according to the WHO classification 3rd edition (4) is included in components of the WPSS, but is not included in IPSS-R components. The low BM blast cutpoint (2%) of IPSS-R is not included in criteria of the WHO category. To clarify the correlation between the low marrow blast cutpoint and WHO category, we compared marrow blast percentages of refractory anemia of the WHO category (WHO-RA) and refractory cytopenia with multilineage dysplasia (RCMD).

Data set of our previous study (5) was used for the present analysis. The database consisted of primary untreated MDS patients with refractory anemia according to the French–American–British classification. Patients with MDS associated with isolated del (5q) were excluded from the present analysis. WHO-RA and RCMD patients totaled 238 cases (Japanese 96 cases, German 142 cases) and 448 cases (Japanese 32 cases, German 416 cases), respectively. U.G. and Y.M., who are coauthors of the present analysis, are coauthors of the IPSS-R report. Therefore, some patients of the present analysis may have been included in the IPSS-R report. However, in the IPSS-R report, there is no mention of analysis of the WHO category. Definition of blast cells by Goasguen *et al.* (6) was used in this study. This definition was adopted in consensus proposals of International Working Group on Morphology of MDS (IWGM-MDS) (7). Continuous data were compared using the nonparametric Mann–Whitney test, and proportions were compared using the chi-square test. The present analysis was approved by the Institutional Review Board of Saitama International Medical Center, Saitama Medical University.

Definition of blast cells by Goasguen *et al.* is simple. In fact, the distinction between blasts and promyelocytes was easy. In addition, we held two times of joint review meetings for making cytomorphologic database. Therefore, we believe that the reliability of the blast percentage is high in present study. In IPSS-R study, it was reported that the split between 0– ≤ 2% and >2–<5% was reproducible within the various databases from the different institutions (2). The BM blast percentage of RCMD patients was higher than that of WHO-RA patients ($P = 0.0011$). The frequency of patients with BM blast >2–<5% in RCMD was higher than that in WHO-RA ($P = 0.0022$) (Table 1). It was reported that RCMD patients had a more unfavorable prognosis than WHO-RA patients (3, 4). Therefore, it seems that the low marrow blast cutpoint (2%) may have prognostic significance.

Table 1 Correlation between WHO classification and bone marrow blast percentages

	No. of Patients	BM blasts (%), mean ± SD	*P*-value	No. of Patients with BM blast >2–<5%	*P*-value
WHO-RA	238	1.8 ± 1.1	0.0011	67 (28.2%)	0.0022
RCMD	448	2.2 ± 1.3		179 (40.0%)	

BM, bone marrow; WHO-RA, refractory anemia according to the WHO classification 3rd edition; RCMD; refractory cytopenia with multilineage dysplasia according to the WHO classification 3rd edition.

Conflict-of-interest disclosure

The authors declare no conflict of interests.

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Original Article

Clinicopathological features of acute megakaryoblastic leukaemia: Relationship between fibrosis and platelet-derived growth factor

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Acute megakaryoblastic leukaemia (AMGL) is an uncommon disease with poor prognosis. Histopathologically, AMGL cases show variable degree of fibrosis and the presence of uniform blasts or mature dysplastic megakaryocytes. Here we examined 18 cases of AMGL, including idiopathic (n = 9) and secondary (n = 9) cases. Fourteen cases were males and four were females, ranging in age from 14 to 87 years (median, 58). All cases had anaemia, but leukocyte and platelet counts varied. Blast cells were detected in the peripheral blood of 14 cases. Fourteen of 16 cases showed chromosomal abnormalities. The median survival was 6 months (range, 1–48 months). Survival rates did not correlate with the severity of fibrosis, proportion of blast cells and cause of AMGL. Nine of the 11 cases examined immunohistochemically were positive for platelet-derived growth factor (PDGF)(-BB), especially megakaryoblasts and a few fibroblasts. The PDGF-positive cases showed various degrees of fibrosis, while the negative cases showed no evidence of fibrosis. Our results confirmed the poor prognosis of patients with AMGL, irrespective of the degrees of fibrosis, and demonstrated that PDGF could play an important role in the pathogenesis of marrow fibrosis.

Key words: acute megakaryoblastic leukaemia, PDGF(-BB)

Acute megakaryoblastic leukaemia (AMGL) is an acute leukaemia in which ≥50% of the blasts are of megakaryocyte lineage. AMGL occurs in both adults and children but is an uncommon disease comprising approximately 3–5% of cases of acute myelocytic leukaemia (AML). Patients with AMGL present with pancytopenia, especially thrombocytopenia, although some may have thrombocytosis. Dysplastic features in the neutrophils and platelets may be present. Organomegaly, e.g. hepatosplenomegaly, is usually infrequent. Morphologically, although AMGL may be associated with fibrosis, the histopathology of the biopsy varies from cases with a uniform population of poorly differentiated blasts to a mixture of poorly differentiated blasts and maturing dysplastic megakaryocytes; and a variable degree of reticulin fibrosis may be present.[1]

Platelet-derived growth factor (PDGF) is the major serum mitogen for cells of mesenchymal origin in humans.[2] PDGF, which is stored in α-granules of human platelets, is synthesized in megakaryocytes as well as in macrophages and some other cells and tissues.[3] The biologically active protein is a dimmer composed of two related polypeptides designated A and B. The protein exists in either a heterodimer AB or homodimer AA or BB.[4] All three forms have been observed in vivo and each possesses biological activity in vitro with PDGF AA being intrinsically less active than PDGF BB.[5] PDGF BB has been identified as the human homologue of the *v-sis* oncogene product and as such has been designated *c-sis*.[6,7] The *v-sis* transforming domain is 89 amino acids in length and is identical in sequence to PDGF BB.[8] The PDGF protein has been implicated both directly as well as indirectly in several pathological states including neoplasia, arthritis, arteriosclerosis and bone marrow sclerosis.[2]

In this study, we examined 18 cases of AMGL, including idiopathic and secondary cases to determine the role of PDGF in the pathogenesis of marrow fibrosis. For this purpose, we analysed the prognosis of patients with AMGL and correlated it with reticulin fibrosis, which was examined by immunohistochemistry of PDGF(-BB).

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MATERIALS AND METHODS

Patients

Eighteen patients (14 males and 4 females, age, range, 14 to 87 years, median, 58 years) were studied from cases filed at the Department of Pathology, Kurume University, Japan, between 1990 and 2010 (Table 1). Haematological and clinical data were obtained at the time of the study. Diagnosis of AMGL was established by standard procedure. The phenotype of blast cells was determined based on morphology, immunohistochemical staining and/or flow cytometry analysis. The grade of marrow fibrosis was evaluated by reviewing the biopsy specimens. For the assessment of marrow fibrosis, paraffin sections were stained with Gomori's silver impregnation technique, and fibrosis was assessed following the European consensus guidelines.[9] Specimens were graded as follows: in detail, no fibrosis was defined as the presence of single scattered reticulin fibers; mild fibrosis was defined as the presence of a loose meshwork of thin reticulin fibers with many intersections; moderate fibrosis was defined as the presence of a dense and diffuse increase in reticulin forming extensive intersections and focal thick collagen fibers; and severe fibrosis was defined as the presence of dense reticulin fibers intermingled with bundles of collagen and associated with endophytic bone formation. This study was carried out in accordance with the Helsinki Declaration as revised in 1989 and with the ethical guidelines of the participating hospitals.

Immunohistochemistry

Immunohistochemical staining was performed using bone marrow paraffin-embedded sections in order to establish the diagnosis of AMGL. The antibodies used were CD41 (Immunotech, Marseille, France), von Willebrand factor (vWF) (Dako, Glostrup, Denmark), CD34 (Immunotech) and PDGF(-BB) (Genzyme, Minneapolis, MN, USA).

Flow cytometry

Flow cytometry was performed in order to establish the diagnosis of AMGL. Peripheral blood mononuclear cells (PBMC) were analysed with monoclonal antibodies (mAb) for CD13 (My7, Coulter Clone; Hialeah, FL), CD14 (My4, Coulter Clone), CD33 (My9, Coulter Clone), CD34 (Becton Dickinson, San Jose, CA, USA) and CD41 (Immunotech), using a FACScan analyser (Becton-Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis

Statistical analysis of the Kaplan-Meier survival curves (log-rank test by StatView version 5.0 (Abacus Concept, Inc., Berkeley, CA, USA)) was performed in the present study. A *P*-value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Clinical data

Haemoglobin levels ranged from 4.0 to 10.1 g/dL. Total leukocyte counts ranged from 1.12×10^9 to 48.3×10^9/L. Blastic cells were detected in the peripheral blood of 14 cases and comprised 2.5 to 100% of leukocytes. Platelet counts ranged from 1.0×10^9 to 2010×10^9/L. Six cases progressed from myelodysplastic syndrome (MDS) and one case was from chronic myeloid leukaemia (CML) and one case was from essential thrombocythemia (ET) and one cases from chronic idiopathic myelofibrosis (CMF).

Fourteen of 16 cases were found to have chromosomal abnormalities. In addition, 6 of the 14 cases had chromosomal abnormalities of –5. Four of the 6 cases also had chromosomal abnormality of –7. Sixteen cases were treated with aggressive chemotherapy, three cases received allogenic peripheral blood stem cell transplant (allo-PBSCT) and one case received allogenic cord blood stem cell transplant (allo-CBSCT). Three cases achieved complete remission (CR), but one relapsed. Thirteen cases, including three that received allo-PBSCT, died between 1 and 48 months after diagnosis. The median survival was 6 months (range, 1–48 months) (Tables 1,2).

Histopathological analysis, including fibrosis and PDGF(-BB) expression

Seventeen of the 18 cases could be morphologically classified. Five cases showed normoplastic marrow, one case showed hypoplastic marrow and 11 cases showed hyperplastic marrow, and all had various densities of neoplastic megakaryoblasts. Fibrosis was observed in 13 cases; which was of mild degree in five, moderate in two and severe in six cases. Four cases showed no evidence of fibrosis (three of these were idiopathic cases) (Fig. 1).

We examined the expression of PDGF(-BB) by immunohistochemistry in 11 cases. The megakaryoblasts and a few fibroblasts in 9 of the 11 cases showed immunoreactivity for PDGF(-BB), while the other two cases were negative. In

Table 1 Clinical data of 18 cases

Case no	Sex	Age (yr)	Type	Peripheral blood cells RBC ($x10^{12}$/L)	Hb (g/dL)	WBC ($x10^9$/L)	Blasts (%)	Plt ($x10^9$/L)	Performance status	Therapy Chemotherapy	Radiation therapy	Others	Complete remission	Survival (month)
1	M	55	Idiopathic	18.2	5.9	21.1	34	64	2	Yes	No	Allo PBSCT	No	2
2	M	14	Idiopathic	36.7	10.1	4	0	1	3	Yes	No	No	No	6
3	M	50	MDS	17.5	6.3	2.1	0	14	2	Yes	No	No	No	(ND)
4	M	58	MDS	14.6	4	1.12	2	10	1	Yes	No	No	No	1
5	M	58	MDS	14.6	4.7	1.5	0	10	2	Yes	Yes	Allo PBSCT	No	3
6	F	64	Idiopathic	32.7	9.8	6.2	35	77	1	Yes	No	No	No	2
7	M	20	Idiopathic	28.8	8.8	48.3	100	59	2	Yes	No	Allo CBSCT	Yes	21+
8	M	78	Idiopathic	30.8	8	3.3	0	16	2	Yes	No	No	No	10
9	F	40	ET	35.4	9.5	30.32	9	2010	2	Yes	No	No	No	10
10	M	72	CMF	18.5	5.4	20.9	60	12	4	Yes	No	No	No	6
11	M	31	MDS	21.4	6.6	3.16	56	47	3	Yes	No	Allo PBSCT	No	4
12	M	58	Idiopathic	17.4	6.4	1.6	5	6	2	Yes	No	No	No	5
13	M	54	Idiopathic	22.3	8.6	24.2	5	393	1	No	No	No	No	48
14	M	63	MDS	(ND)	6.8	2.4	29	74	2	Yes	No	No	No	30
15	M	73	Idiopathic	23.7	8.2	2.1	39	22	1	Yes	No	No	Yes	10
16	M	60	CML	17.4	6.3	21.5	2.5	421	1	Yes	No	No	No	10+
17	F	87	MDS	20.8	6.4	3.2	8	283	1	No	No	No	No	3+
18	F	81	Idiopathic	25.9	9.4	1.2	18	82	1	Yes	No	No	Yes	37+

Allo PBSCT, allogenic peripheral stem cell transplant; CMF, chronic idiopathic myelofibrosis; CML chronic myeloid leukaemia, ; ET, essential thrombocythemia; MDS, myelodysplastic syndrome; ND, not done.

Table 2 Chromosomal analysis

Case no	Karyotype according to G-bands
1	(ND)
2	46,XY
3	45,XY,+der(1q7p),**-5,-7,**-22,-22,12p+,13p+,15p+,+2mar [9]/46,XY[1]
4	47,XY,-3,-4,**-5,-7**,-9,-13,+7mar [1]/44,XY,-3,**-5,-7**,-9,add(12) (p11.2),-13,-21,+4mar [1]/43,XY,-3,**-5,-7**,add(12) (p11.2),-13,-14,add(19)(q13),-22,+3mar [1]/43,XY,-3,**-5,-7**,add(12) (p11.2),-13,add(19)(q13),-20,-22,+3mar [1]
5	54,XY,add(1)(p13),+add(1)(q21),+add(2)(p23),add(5)(q31).+6,add(7)(p11.1),add(7)(q11.2),-12,der(14)(14qter-14p11::?::12q1? -12qter),add(15)(p11.1),-19,+7mar [4]/54,XY,add(1)(p13),+add(1)(q21),+add(2)(p23),**-5**,add(5)(q31).+6,add(7)(p11.1),add(7)(q11.2),-12,der(14) (14qter-14p11::?::12q1?-12qter),add(15)(p11.1),-19,+8mar[2]/53,XY,add(1)(q21),+2,+6,add(7)(p11.2),add(7)(q11.2), -12,der(14)(14qter-14p11::?::12q1?-12qter),add(15)(p11.1),-19,+6mar [1]/56,XY,add(1)(p13),+add(1)(q21),+add(2)(p23),add(5)(q31),+6,**-7,-7**,+8,add(10)(p11.1),add(14)(p11.1),-19,+mar [1], 46,XX [8]
6	46,XX,add(1)(q21),**-5**,del(7)(q?),add(8)(q11),-10,-11,-12,add(14)(p11),-17,add(21)(q22),+der(?)t(?;11)(?;q13),+mar1,+mar2,+mar3, +mar4[1]/45,idem,-18,-mar4,+mar5 [10]/45,idem,-18,-mar3,-mar4,+mar5,+mar6 [2]/46,idem,-18,+mar2[1]
7	46,XY,add(1)(q11),-18,add(18)(q21),+mar1 [2]/46,XY,+3,-6,-10,-11,-14,add(22)(q11),+3mar [1]/46,XY [7]
8	(ND)
9	46,XX,-20,+der(20)t(1,20)(q21,p11),t(17,22)(q25,q11) [12]
10	46,XY,t(3,8)(q26,q24),t(11,12)(q13,p13) [18]/46,XY [2]
11	47,XY,+8,der,(15)t(1,15)(q12,q26) [1]/47,idem,add(3)(q12-13) [19]
12	36-39,X,-Y,add(4)(q34),**-5,-7**,-9,-11,add(11)(q24),-13,der(13)t(13;?1;?)(p10;q?11-q44;?),-14,der(14)t(14;?;14)(p10;?;q32),-16, add(17)(q22),-18.-19,-19,-20,der(21)t(?17;21)(q?12;p11),+22,+mar[cp5] [5]/46,XY [15]
13	46,XY,t(3,11)(q23,q21) [19]/46,XY [1]
14	46,XY [20]
15	46,XY,inv(9)(p11q13) [20] (*normal variant)
16	46,XY,t(9,22)(q34,q11) [2]/46,idem,del(5)(q_?),der(11)add(11)(q21),add(17)(q11) [18]
17	45-48,XX,del(3)(q21),del(5)(q13q?33),**-5**,add(7)(q11.2),-11,-12,add(12)(p?13),-13,-18,-19,add(20)(q?11,2).+mar2,+mar3 [20]
18	47,XX [6]/46,XX [14]

ND, not done.

addition, the former group showed various degrees of fibrosis (from mild to severe) while the latter showed no evidence of fibrosis (Table 3) (Fig. 2).

Survival rates

The survival rate of the 17 patients who could be evaluated is shown in Fig. 3a. We compared the survival rates based on a cut-off value of proportion of blastic cells of 20% (Fig. 3b). The number of patients with blastic cells ≥20% was seven, while in 10 patients the proportion of blast cells was <20%. The results showed no significant difference in the survival rate between the two groups. We also compared the survival rates of 16 patients based on the severity of bone marrow fibrosis (Fig. 3c); seven patients had mild or no evidence of marrow fibrosis, while nine had moderate or severe fibrosis. There was no significant difference in the survival rate between the two groups. We also compared the survival rate of patients with severe marrow fibrosis (n = 5) with that of cases with no, mild or moderate marrow fibrosis (n = 11). There was also no significant difference in the survival rate between the two groups. Finally, we compared the survival rate of patients with idiopathic AMGL (n = 9) with that of patients with secondary AMGL (n = 8). There was no significant difference in survival rates between the two groups.

DISCUSSION

In this study, we investigated 18 cases of AMGL, a relatively large number of this uncommon disease that comprises approximately 3–5% of all cases of AML.[1] The clinical and pathological features of AMGL were evaluated.

Previous studies reported that AMGL includes not only idiopathic cases, but also the so-called secondary AMGL, such as those that progress from MDS,[10–13] CML,[14–21] ET[22–28] and CMF.[14,15,29–32] In this study, six cases progressed from MDS, one case from CML, one case from ET and one case from CMF. However, there was no significant difference in survival rate between idiopathic and secondary cases.

With regard to chromosomal analysis of AMGL, previous studies reported that in children and particularly infants under 1 year of age, there may be an association with a t(1;22)(p13;q13).[1,33] In addition, the *c-sis* gene, which encodes the B chain of PDGF,[34] is on 22q13.[35] Others demonstrated an association between AMGL and abnormalities of –5, –7, –5q and –7q.[36] In the present study, 14 of 16 cases had chromosomal abnormalities and, six of these 14 had chromosomal abnormality of –5 and four of these six had chromosomal abnormality of –7 but no chromosomal abnormality of 22q13. These findings were similar to those reported previously.

Histopathologically, fibrosis was observed in 13 cases; five had mild fibrosis, two moderate fibrosis and six had severe

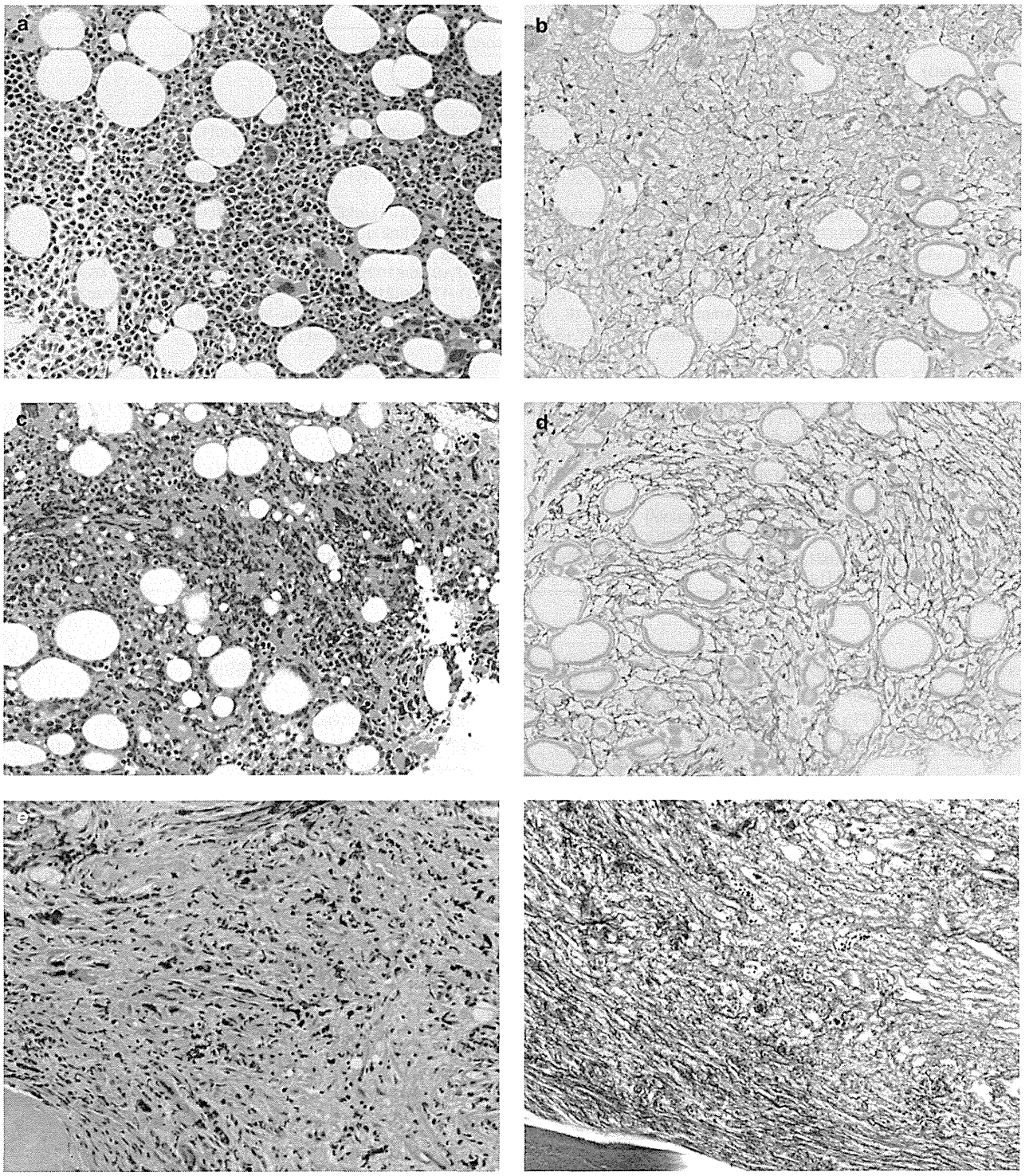

Figure 1 Histopathological features of (**a-b**) a representative case of acute megalaryoblastic leukemia (AMGL) with mild fibrosis (**a**, H&E), (**b**, silver reticulin stain), (**c-d**) a representative case of AMGL with moderate fibrosis (**c**, H&E), (**d**, silver reticulin stain) and (**e-f**) a representative case of AMGL with severe fibrosis (**e**, H&E), (**f**, silver reticulin stain).

Table 3 Results of bone marrow analysis, immunohistochemistry and flow cytometric analysis

Case no	Bone marrow		Immunohistochemistry				Flow cytometric analysis				
	Fibrosis	Cellularity (%)	CD41	vWF	CD34	PDGF (-BB)	CD13	CD14	CD33	CD34	CD41a
						megakaryoblasts					
1	no	90	*+	*+	ND	–	–	–	–	–	ND
2	no	90	–	+	ND	ND	–	ND	***+	ND	***+
3	severe	70	+	+	ND	**+	***+	ND	+	***+	ND
4	mild	90	+	+	–	+	+	–	+	+	+
5	severe	90	ND	+	–	+	+	–	+	–	–
6	mild	50	+	+	*+	ND	+	ND	+	+	+
7	mild	90	–	+	+	+	+	ND	+	+	ND
8	severe	70	+	+	+	+	+	–	+	+	–
9	no	90	ND	+	ND	–	+	–	+	ND	ND
10	moderate	60	+	+	ND	+	+	ND	+	+	+
11	severe	50	+	+	ND	+	+	ND	+	+	ND
12	mild	70	+	+	ND	+	+	***+	+	–	+
13	severe	60	+	+	+	ND	+	–	+	+	+
14	severe	80	ND	+	ND	ND	+	–	+	ND	ND
15	no	20	–	+	+	ND	+	–	+	+	+
16	moderate	90	ND	ND	ND	+	+	–	+	+	+
17	mild	40	ND	+	+	ND	ND	ND	ND	ND	ND
18	ND	ND	ND	ND	ND	ND	+	–	+	–	+

*+: ≥50% positive; **+: ≥30% positive; ***+: ≥20% positive.
PDGF, platelet-derived growth factor; vWF, von Willebrand factor.
ND, not done.

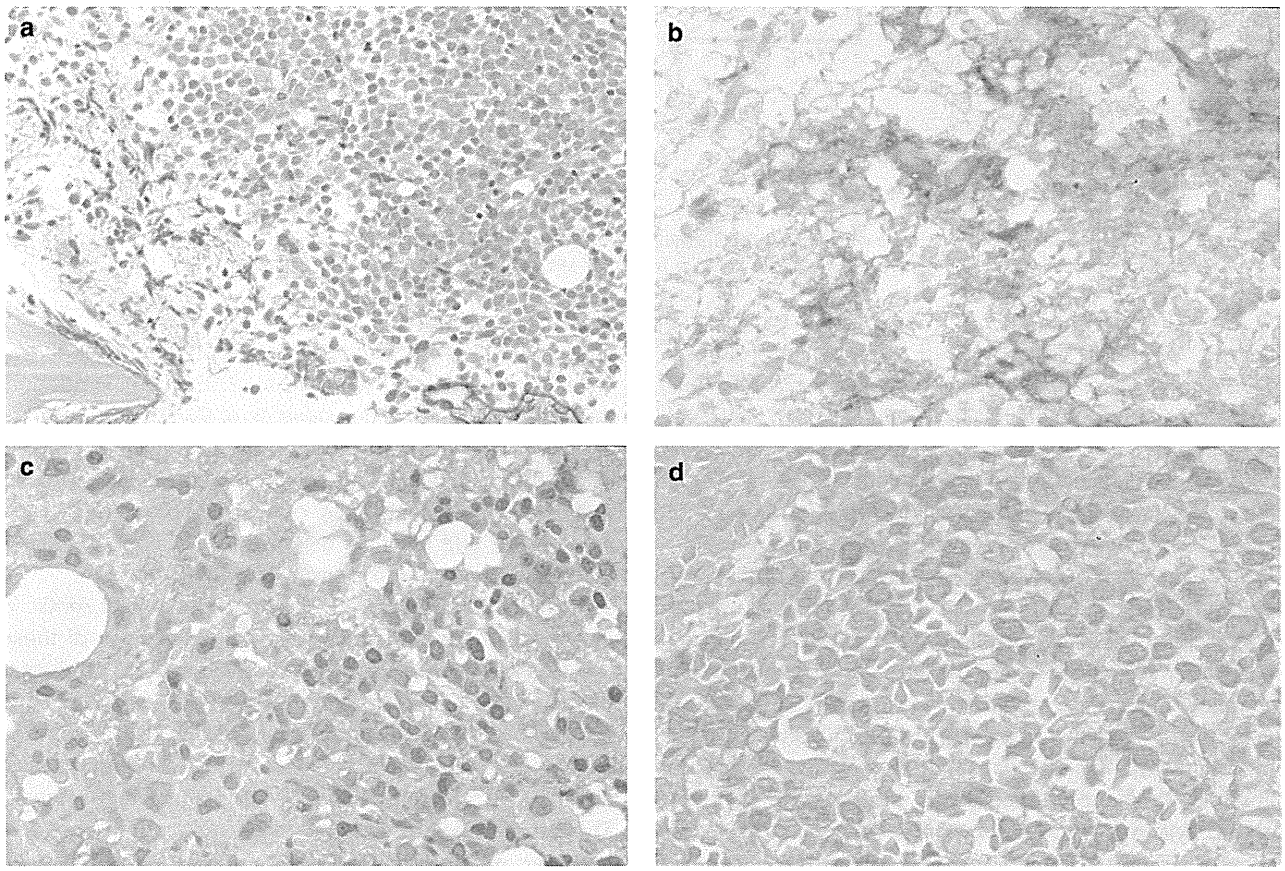

Figure 2 Immunohistochemistry. A representative case of AMGL with mild fibrosis. (**a**) H&E, (**b**) von Willebrand factor, (**c**) CD41, (**d**) platelet-derived growth factor (PDGF) (-BB).

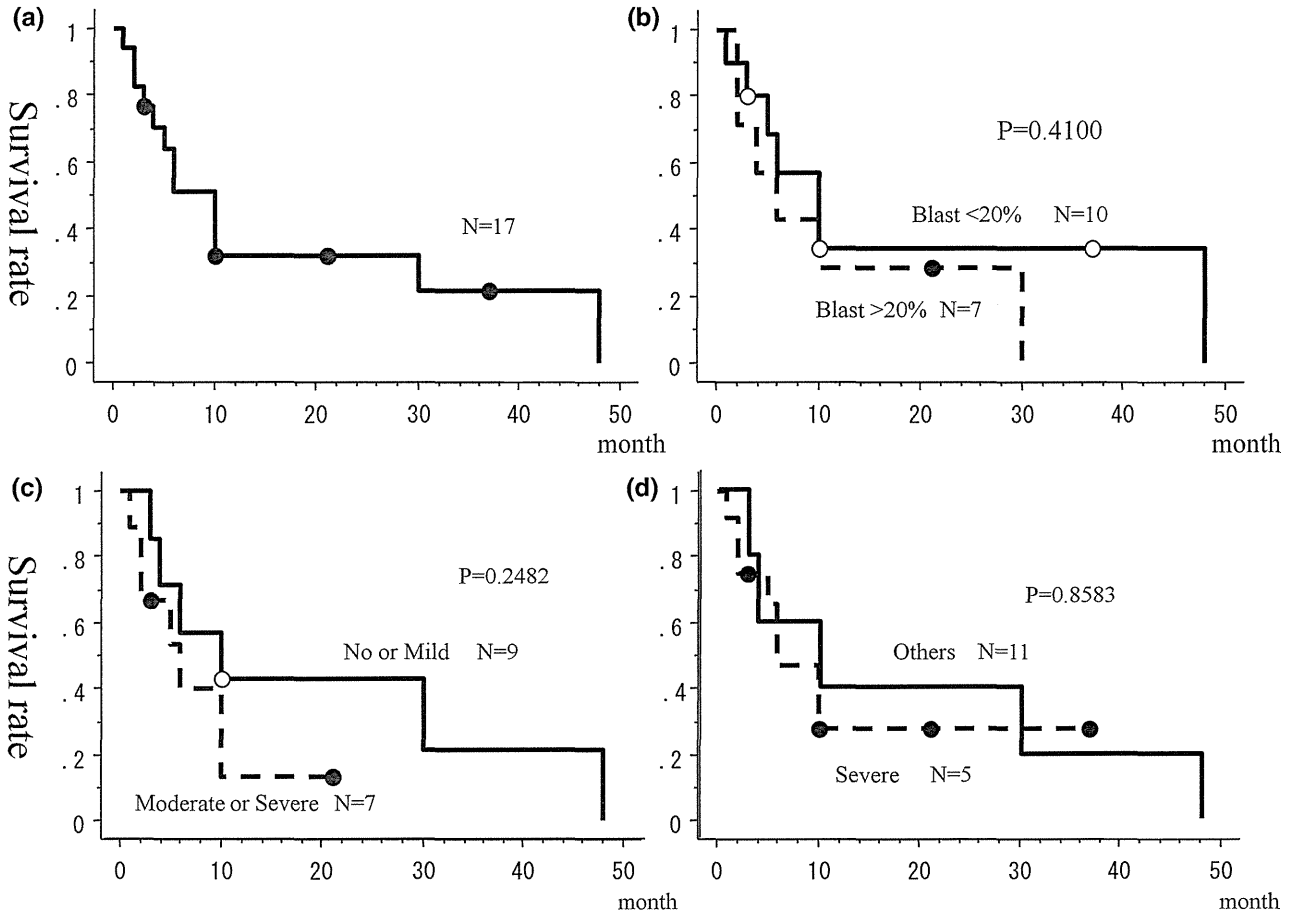

Figure 3 (**a**) Survival curves of 17 patients with AMGL. (**b**) Survival curves according to the proportion of blast cells among peripheral blood cells. Survival curves according to the severity of marrow fibrosis: (**c**) No or Mild VS Moderate or Severe, (**d**) Severe VS Others.

fibrosis, while four cases showed no evidence of fibrosis. PDGF is the major serum mitogen for cells of mesenchymal origin in humans, and the PDGF protein has been implicated both directly as well as indirectly in several pathological states including neoplasia, arthritis, arteriosclerosis and bone marrow sclerosis.[2] Several studies indicated that PDGF plays an important role in the pathogenesis of marrow fibrosis. For example, PDGF is associated with the blastic phase of CML; PDGF mRNA is expressed in blast cells as demonstrated by reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, PDGF protein is produced and secreted by blast cells as determined by direct bioassays.[3] In that report, five cases, including a case with granulocytic and megakaryocytic blastic crisis, of 10 cases showed marrow fibrosis. Furthermore, three cases, including the case with granulocytic and megakaryocytic blastic crisis, of these five showed a significant amount of PDGF secretion (>0.2 ng/mL/10^6 cells). In the present study, we examined the expression of PDGF(-BB) by immunohistochemistry in 11 cases. Megakaryoblasts and a few fibroblasts in nine of the 11 cases showed positive reactivity for PDGF(-BB), and those in two cases showed negative reactivity for PDGF(-BB). In addition, the former showed various degrees of fibrosis (from mild to severe) while the latter showed no evidence of fibrosis. These findings suggest that PDGF(-BB) may play an important role in the pathogenesis of marrow fibrosis associated with AMGL, and not only megakaryoblasts but fibroblasts may be associated with marrow fibrosis.

Previous reports showed that the prognosis of AMGL is usually poor.[1] Our results confirmed the findings of these previous studies. Sixteen cases received aggressive chemotherapy, three cases were treated with allo-PBSCT and one case with allo-CBSCT. Three cases achieved CR, but one case relapsed. Thirteen cases, including three that received allo-PBSCT, died between 1 and 48 months after diagnosis. The median survival was 6 months (range, 1–48 months). However, one case (20-year-old, male) that received allo-CBSCT achieved CR and is still alive 21 months later with no evidence of relapse. This limited result indicates that intensive therapies, such as allo-CBSCT, are required for the

treatment of AMGL. We also compared the survival rates based on various parameters, such as the proportion of neoplastic megakaryocytes in the peripheral blood and the severity of marrow fibrosis, but no definite differences in survival rates were found between the groups, probably due to the small number of cases. However, taking rarity of the disease into consideration, this study including detailed clinicopathologic data of as many as 18 patients in a single institute would be of value for reference. Further studies of large case analysis are necessary to define the clinicopathological features of AMGL.

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Mutations in the Nucleolar Phosphoprotein, Nucleophosmin, Promote the Expression of the Oncogenic Transcription Factor MEF/ELF4 in Leukemia Cells and Potentiates Transformation*

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Background: MEF/ELF4 can function as an oncogene. We demonstrated the role of MEF/ELF4 in acute myeloid leukemia.
Results: NPM1 inhibited the DNA binding and transcriptional activity of MEF/ELF4 on the HDM2 promoter, whereas NPM1 mutant protein enhanced these activities of MEF/ELF4.
Conclusion: MEF/ELF4 activity may be activated by NPM1 mutant protein.
Significance: NPM1 mutant proteins have a role in MEF/ELF4-dependent leukemogenesis.

Myeloid ELF1-like factor (MEF/ELF4), a member of the ETS transcription factors, can function as an oncogene in murine cancer models and is overexpressed in various human cancers. Here, we report a mechanism by which MEF/ELF4 may be activated by a common leukemia-associated mutation in the nucleophosmin gene. By using a tandem affinity purification assay, we found that MEF/ELF4 interacts with multifactorial protein nucleophosmin (NPM1). Coimmunoprecipitation and GST pull-down experiments demonstrated that MEF/ELF4 directly forms a complex with NPM1 and also identified the region of NPM1 that is responsible for this interaction. Functional analyses showed that wild-type NPM1 inhibited the DNA binding and transcriptional activity of MEF/ELF4 on the HDM2 promoter, whereas NPM1 mutant protein (Mt-NPM1) enhanced these activities of MEF/ELF4. Induction of Mt-NPM1 into MEF/ELF4-overexpressing NIH3T3 cells facilitated malignant transformation. In addition, clinical leukemia samples with NPM1 mutations had higher human MDM2 (HDM2) mRNA expression. Our data suggest that enhanced HDM2 expression induced by mutant NPM1 may have a role in MEF/ELF4-dependent leukemogenesis.

Myeloid ELF1-like factor (MEF/ELF4), a member of the ETS family of transcription factors, is characterized by an 85-amino acid ETS domain that recognizes a core sequence of GGAA or TTCC (1). MEF/ELF4 is expressed in various normal and malignant hematopoietic cells and regulates the expression of various cytokines (interleukin-3 (1), granulocyte-macrophage colony-stimulating factor (1), and interleukin-8 (2) as well as the cytolytic perforin molecule (3) and antibacterial peptides lysozyome and human β-defensin2 (4)) and matrix metalloproteinase-9 expression (5). Furthermore, analyses of MEF/ELF4-deficient mice have revealed the essential role of MEF/ELF4 in the development and function of NK (natural killer) cells and NK-T cells (3). Recently, Smith *et al.* (6) have shown that repression of Elf-4 by transcriptional repressor *Gfi1b* is important for the maturation of primary fetal liver erythroid cells. MEF/ELF4 also regulates the key aspects of hematopoietic stem cell behavior by controlling movement through the cell cycle from quiescence (G_0) to G_1 and from G_1 to S as well as resistance to myelosuppression (7, 8).

MEF/ELF4 is expressed in cancers such as leukemia (9), lymphoma, and ovarian cancer (10). Recently, Totoki *et al.* (11) identified an intrachromosomal inversion (Xq25) in hepatocellular carcinoma that generated a BCORL1-MEF/ELF4 fusion transcript. Experiments in several mouse models have suggested that MEF/ELF4 plays a role in tumorigenesis. For example, models of retrovirus-induced insertional mutagenesis have identified *MEF/ELF4* as a gene that is involved in leukemic transformation (12). Sashida *et al.* (13) have shown that overexpression of MEF/ELF4 enhances the expression of Mdm2,

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leading to decreased p53 expression and enhanced transformation. In experiments with MEF/ELF4-overexpressing cells, they demonstrated that Ets1-induced p16 induction is suppressed, resulting in senescence suppression and tumor promotion.

Nucleophosmin (NPM1) is a nucleolar phosphoprotein (14) and a frequent target of genetic alterations in hematopoietic malignancies. *NPM1* gene mutations have been found in ~60% of adult patients who have acute myeloid leukemia (AML)[2] and a normal karyotype (15). These mutations lead to the aberrant cytoplasmic expression of NPM1 ($NPMc^+$) due to nucleotide gain at the C terminus (16, 17), which results in the loss of tryptophan residues essential for nucleolar localization and the gain of a new nuclear export signal (18). Increased NPM1 export into the cytoplasm probably perturbs multiple cellular pathways by delocalizing the proteins that interact with NPM1. By using a transgenic mouse model expressing the human $NPMc^+$ mutation, it has been shown that $NPMc^+$ confers a proliferative advantage in the myeloid lineage, suggesting that NPM1 mutations can participate in leukemia development (19).

In the present study, we found that wild-type NPM1 (Wt-NPM1) down-regulates, whereas mutated NPM1 (Mt-NPM1) up-regulates, the transcriptional activity of MEF/ELF4 on the human MDM2 (HDM2) promoter. The expression of Mt-NPM1 in MEF/ELF4-overexpressing NIH3T3 cells resulted in enhanced malignant transformation. We also found that *HDM2* mRNA expression in primary AML cells with *NPM1* mutations is significantly higher compared with AML cells without *NPM1* mutations. Taken together, our data suggest that *NPM1* mutations may promote transformation by enhancing the oncogenic functions of MEF/ELF4.

EXPERIMENTAL PROCEDURES

Cell Culture—293T cells (CRL-11268, ATCC (Manassas, VA)) were maintained at 37 °C in DMEM (Invitrogen) with bovine calf serum. U937 cells (CRL-1593.2, ATCC) were maintained with 10% (v/v) FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Fisher). NIH3T3 cells (CRL-1658, ATCC) were maintained under identical conditions with 10% (*v/v*) FBS and grown in RPMI 1640 (Fisher) with 10% FCS (HyClone, Logan, UT), 100 units/ml penicillin G, and 100 μg/ml streptomycin. COS7 cells (CRL-1651, ATCC) were cultured in DMEM (Invitrogen) containing 10% FCS.

Tandem Affinity Purification Assay—The cDNA of MEF/ELF4 was inserted into InterPlay N-terminal mammalian TAP vector (pTAP/MEF/ELF4, Stratagene (San Diego, CA)) comprising two affinity tags (immunoglobulin G (IgG)-binding domain and calmodulin-binding peptide) separated by the cleavage site of tobacco etch virus protease (20). 293T cells were transfected with pTAP or pTAP/MEF/ELF4 plasmids in a 10-cm dish. Transfected cells were collected and lysed in a solution containing 100 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 0.1% Nonidet P-40. The lysate was centrifuged at 15,000 rpm for 30 min at 4 °C. The resulting supernatant was incubated for 2 h at 4 °C with IgG-Sepharose 6 Fast Flow (GE Healthcare), after which the resin was washed and incubated with tobacco etch virus protease for 2 h at 16 °C. Purification on calmodulin affinity resin (Stratagene) was performed according to the manufacturer's instructions. Purified proteins were precipitated with trichloroacetic acid, resolved with 1× sample buffer and subjected to SDS-PAGE. Gels were stained with Coomassie Blue, and protein bands were cut out. Proteins were eluted with trypsin. The resulting peptides were analyzed with a Procise 49X cLC protein sequencer (Applied Biosystems, Foster City, CA) (20).

In Vitro Translation—The cDNA molecules of Wt-NPM1 and Mt-NPM1 (21) were inserted into the pTnT vector (pTnT-NPM, Promega (Madison, WI)) for *in vitro* translation. NPM1 protein (biotin-NPM1) was *in vitro*-translated with pTnT-NPM1 and labeled with biotinylated lysine (Transcend tRNA, Promega) by using the TNT Quick Coupled transcription/translation system (Promega). The cDNA of MEF/ELF4 was inserted into pET-3a (Novagen, VWR (Lisbon, Portugal)), which allows the introduction of a His tag into the N terminus of MEF/ELF4 (pET/MEF/ELF4). Overexpression of the recombinant protein (His-MEF/ELF4) was achieved in *Escherichia coli* BL21Gold (DE3) cells (Stratagene) transformed with the constructed plasmid pET/MEF/ELF4. His-MEF/ELF4 was isolated from cells broken in lysis buffer (STE buffer) with sonication and centrifuged at 15,000 × *g* for 10 min at 4 °C (1).

Biotin-NPM1 was incubated with His-MEF/ELF4 or His (as a control) proteins at 4 °C for 1 h. The mixture was loaded onto His spin traps (GE Healthcare) and eluted with 500 mM imidazole at pH 7.4. After SDS-PAGE and electroblotting, biotin-NPM1 in purified samples was detected by using the Transcend non-radioactive translation detection system (Promega).

Immunoprecipitation and Immunoblotting—MEF/ELF4 was cloned into p3xFLAG-CMV (Sigma) (FLAG-MEF/ELF4) from PCR products generated from pcDNA/MEF/ELF4 (1). Wt-NPM1 and Mt-A-NPM1 were cloned into pcDNA3.1/V5-His (pcDNA/V-Wt-NPM1 and Mt-A-NPM1, respectively) (Invitrogen) from PCR products generated from pcDNA/Wt-NPM1 and pcDNA/Mt-A (21). 293T cells were transfected with each plasmid by using Effectene transfection reagent (Qiagen, Berlin, Germany). After 48 h, cells were lysed by using the Universal Magnetic co-immunoprecipitation kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions for nuclear extraction. Lysates were centrifuged at 15,000 rpm for 10 min at 4 °C to remove the resin. The resulting supernatants were incubated for 4 h at 4 °C with 5 μg of antibodies against FLAG (Sigma), 5 μg of antibodies against V5 (Invitrogen), or normal mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Immunoprecipitates were recovered, washed four times with ice-cold co-immunoprecipitation solution (Active Motif), and fractionated by SDS-PAGE. Separated proteins were transferred to a membrane. After incubation in blocking buffer, membranes were probed with peroxidase-labeled antibodies against FLAG (Sigma), V5 (Invitrogen), or tag (Invitrogen). Detection was achieved with an enhanced chemiluminescence system (ECL Advance Western blotting detection kit, GE Healthcare). Quantification of Western blotting bands was per-

[2] The abbreviations used are: AML, acute myeloid leukemia; Wt-NPM1, wild type NPM1; Mt-NPM1, mutant NPM1; RQ-PCR, quantitative reverse transcription-polymerase chain reaction.

formed by using AE-6982/C/FC and CS Analyzer version 3.0 software (ATTO, Tokyo, Japan).

GST and His Pull-down Assay—Fusion protein of GST and Wt-NPM1 (GST-NPM1) and GST-NPM1 deletion mutant constructs (Fig. 1*C*) were generated by PCR with pcDNA/Wt-NPM1 as a template. PCR products were cloned in-frame into bacterial expression vector pGEX-T4. Plasmids that express GST fusion protein (GST-NPM1, GST-NPM1 deletion mutants) and His-MEF/ELF4 protein (pET/MEF/ELF4) or their controls were transfected into *E. coli*. Bacterial pellets were lysed in 1 ml of phosphate-buffered saline (PBS) with sonication. His-MEF/ELF4 or His alone was incubated with an equivalent amount of GST, GST-Wt-NPM1, or GST-Wt-NPM1 deletion mutants for 1 h at 4 °C. Proteins were purified by using GST columns (MicroSpin GST Purification Module, GE Healthcare) or His columns. Bound proteins were analyzed by using SDS-PAGE/immunoblot.

EMSA—Recombinant proteins GST, GST-NPM1, His, and His-MEF/ELF4 were collected as described above. Nuclear protein from 293T cells transfected with pcDNA/MEF/ELF4, pcDNA/Wt-NPM1, or pcDNA/Mt-A-NPM1 was extracted with the NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's instructions. EMSA was performed by using the LightShift chemiluminescent EMSA kit (Pierce). Recombinant protein or nuclear extracts were incubated with 20 fmol of biotin 3′-end-labeled oligonucleotides containing APET (an ETS binding site in the IL-3 promoter that was shown to bind to MEF/ELF4) (1). After electrophoresis, transfer, and cross-linking, the signal was detected by a peroxidase/luminol system (chemiluminescent nucleic acid detection module, Pierce). To confirm specificity, a 200-fold excess amount of non-labeled oligonucleotides (APET competitor) (1) was added. The DNA sequence of the APET oligonucleotide is 5′-CCTCAGTGAGCTGAGTCAGG-CTTCCCCTTCCTGCCACAGGG-3′.

RNA Interference—siRNA for NPM1 was transfected into 293T cells by using the GeneClip U1 hairpin cloning system (Promega) according to the manufacturer's instructions. The siRNA sequence-targeting NPM1 gene corresponded to nucleotides 103–125 of the coding region relative to the first nucleotide of the start codon, as described previously (22).

Luciferase Assay—A 0.5-μg aliquot of pcDNA/MEF/ELF4, pcDNA/Wt-NPM1, pcDNA/Mt-A-NPM1, pcDNA/Mt-I-NPM1, or pcDNA/Mt-J-NPM1 was transfected into U937, 293T, and COS7 cells seeded in 6-well dishes by using Nucleofectin (Qiagen) together with 0.1 μg of pGL4 reporter plasmid (pGL4/APET (1), pGL4/ETSm-APET (1), pGL4/HDM2, or pGL4/HDM2mut) and 0.05 μg of pLR-Bact vector. pGL4/ETSm-APET contains a mutation in the ETS binding site (ETSm-APET, 5′-CCTCAGTGAGCTGAGTCAGGCTgagC-CTcgacGCCACAGGG-3′). pGL4/HDM2 contains a wild-type hdm2 (P2) promoter sequence from bp −82 to −122 (Wt-Ets, CAGGTTGACTCAGCTTTTCCTCTTGAGCTGGTCAAG-TTCAG), and pGL4/HDM2mut contains an hdm2 (P2) promoter sequence with a mutated ETS site (Mt-Ets, CAGGTTG-ACTCAGCTTTTaCTCTTGAGCTGGTCAAGTTCAG) (23). Cell lysates were prepared 48 h after transfection, and luciferase activity was determined by using the Dual-Luciferase reporter assay system (Promega).

Anchorage-independent Growth Assay—NIH3T3 cells were plated on 24-well dishes in soft agar containing DMEM supplemented with 10% FCS after they were transfected with various combinations of empty vector, pcDNA/MEF/ELF4, pcDNA/Wt-NPM1, or pcDNA/Mt-A-NPM1 and cultured for 2 weeks. Images were taken with a Leica DM IRBE inverted microscope (Leica Microsystems GmbH, Mannheim, Germany) with a ×10 objective lens.

Immunochemistry—MEF/ELF4 was cloned into the pGFP-C3 vector (Clontech, Mountain View, CA) (pGFP-MEF/ELF4). 293T cells were transfected with the empty vector, pGFP-MEF/ELF4, pcDNA/V-Wt-NPM1, or pcDNA/V-Mt-A-NPM1. Cells were harvested 3 days after transfection. Cytospin samples were fixed for 15 min in PBS containing 4% paraformaldehyde. Fixed coverslips were washed twice in TBS, permeabilized in 0.5% Triton X-100 for 10 min, and incubated in Image-iT FX signal enhancer (Invitrogen) for 30 min. Cells were incubated with primary antibody for 1 h and then washed extensively in TBS before incubation with Alexa546-conjugated goat anti-mouse-IgG antibody (dilution 1:2000; Invitrogen) for 1 h. Cells were covered with a drop of ProLong Gold Antifade Reagent with DAPI (Invitrogen). Fluorescent images were obtained by using a confocal laser-scanning microscope (LSM 5 Pascal V3.2, Carl Zeiss).

ChIP Assay—293T cells were transfected with empty vector, pcDNA/MEF-FLAG, pcDNA/Wt-NPM1, or pcDNA/Mt-A-NPM1 by using a nucleofection kit (Qiagen). After 48 h of culture at 26 °C, cells were fixed by the addition of 1% formaldehyde in PBS for 10 min. Chromatin isolation and shearing were performed by using the OneDay ChIP kit (Diagenode, Liege, Belgium) and Shearing-ChIP kit (Diagenode) according to the manufacturer's instructions. Immunoprecipitation reactions were performed with anti-FLAG monoclonal antibody (Sigma) or isotype control IgG (BD Biosciences). Samples were analyzed by quantitative reverse transcription-polymerase chain reaction (RQ-PCR) by using the LightCycler DNA Master SYBR Green I kit (Roche Applied Science) as specified by the manufacturer. The primer sequences for the HDM2 promoter were 5′-GAACGCTGCGCGTAGTCTGG-3′ (forward) and 5′-ACTGC-AGTTTCGGAACGTGT-3′ (reverse).

Clinical Samples—Informed consent for sample collection was obtained according to protocols approved by the International Review Board of Nagasaki University, Nagasaki, Japan (approval number 33-3). Bone marrow aspirates were collected from 22 AML patients before the initiation of chemotherapy. CD34-positive cells were isolated by using Ficoll density gradient centrifugation and magnetic beads (CD34 Isolation Kit, Miltenyi Biotec, Auburn, CA) to minimize the confounding effect of MEF/ELF4 and NPM1 expression by mature myeloid cells. For the screening of *NPM1* mutations, genomic DNA corresponding to exon 12 was amplified by using forward primer 5′-TTAACTCTCTGGTGGTAGAATGAA-3′ and reverse primer 5′-CAAGACTATTTGCCATTCCTAAC-3′, as reported previously. Amplified products were separated by agarose gel electrophoresis, purified by using a QIAquick gel extraction kit (Qiagen), and directly sequenced by using a DNA sequencer