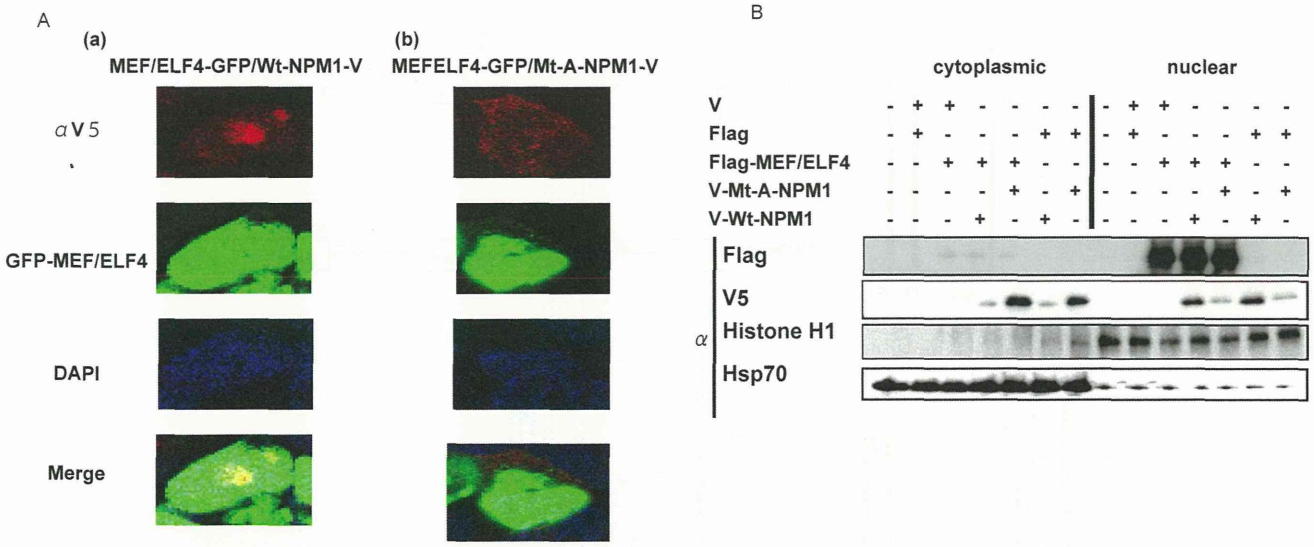

FIGURE 5. **Localization of MEF/ELF4 was unaffected by the mutation of NPM1.** *A*, 293T cells were transfected with the GFP-MEF/ELF4 fusion protein expression vector and pcDNA/V-Wt-NPM1 (*a*) or pcDNA/V-Mt-A-NPM1 (*b*). Forty-eight hours after transfection, cells were fixed and immunofluorescence-stained with anti-V tag antibody. *B*, Western blotting of FLAG-MEF/ELF4 subcellular distribution in 293T cells co-transfected with pFLAG-MEF/ELF4 and pcDNA/V-Wt-NPM1 or pcDNA/V-Mt-A-NPM1. Purity of the subcellular fractions was assessed by blotting with histone H1 (nuclear extraction) and Hsp70 (cytoplasmic extraction).

ELF4 and Wt- or Mt-NPM1 in nuclear and cytoplasmic proteins confirmed the nuclear localization of MEF/ELF4 even with Mt-NPM1 (Fig. 5*B*).

Wt-NPM1 Inhibits, whereas Mt-NPM1 Enhances, the Oncogenic Activity of MEF/ELF4—The overexpression of MEF/ELF4 in NIH3T3 cells increases the growth rate, enhances colony formation in soft agar, and promotes tumor formation in nude mice (10). To determine the effects of the interaction of NPM1 with MEF/ELF4 on cell behavior, we assessed the anchorage-independent growth of NIH3T3 cells after co-transfection of MEF/ELF4 with Wt-NPM1 or Mt-A-NPM1. Compared with NIH3T3 transfected with only MEF/ELF4, Wt-NPM1-coexpressing cells showed reduced anchorage-independent growth, whereas Mt-A-NPM1-coexpressing cells exhibited increased growth (Fig. 6).

MEF/ELF4 Binds to the HDM2 Promoter and Activates Its Expression—In murine cells, MEF/ELF4 binds directly to the Mdm2 promoter, thereby promoting *Mdm2* expression (12). To ascertain whether MEF/ELF4 also directly regulates the promoter activity of *HDM2* (the human analog of Mdm2), we scrutinized the DNA sequence of the *HDM2* gene and found a conserved putative MEF/ELF4 binding site in the P2 promoter (Fig. 7*B*). To establish the association of MEF/ELF4 with the HDM2 promoter, we performed a ChIP assay with nuclear lysates from 293T cells expressing FLAG-MEF/ELF4. Immunoprecipitation with the FLAG antibody (but not with the control IgG) and subsequent PCRs revealed the recruitment of overexpressed MEF/ELF4 to the promoter region of the HDM2 gene (Fig. 7*A*). The luciferase assay revealed that MEF/ELF4 strongly transactivated the wild-type HDM2 promoter (Fig. 7, *B* (*a*) and *C*) and that the effect was abrogated by mutation of the ETS site (−122 to −82) (Fig. 7, *B* (*b*) and *C*). Compared with Wt-NPM1, the expression of Mt-A-NPM1 in 293T cells enhanced the association of MEF/ELF4 with the HDM2 promoter, as detected by ChIP analysis (Fig. 7*D*). Taken together, these findings suggest that Mt-NPM1 up-regulates HDM2 transcription

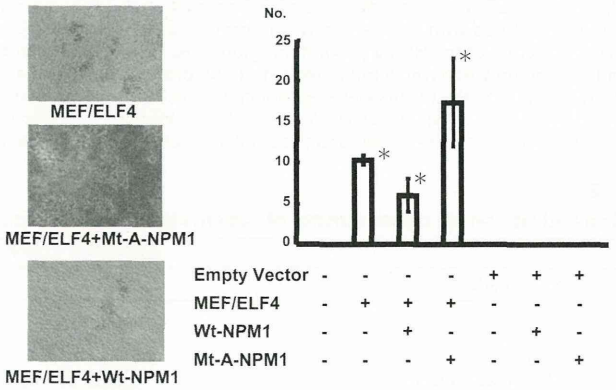

FIGURE 6. **Mt-NPM1 stimulates MEF/ELF4-induced hyperproliferation and transformation.** NIH3T3 cells transfected with various combinations of expression plasmids were plated in soft agar on 60-mm dishes and incubated for 2 weeks. *A*, microscopy of MEF/ELF4-transfected NIH3T3 cells with Wt-NPM1 or Mt-A-NPM1. *B*, the average number of colonies of three independent experiments with S.D. (*error bars*). *, $p < 0.05$.

by increasing the recruitment of MEF/ELF4 to the HDM2 promoter by dislocating Wt-NPM1 that interferes with its binding to the promoter.

Higher Levels of HDM2 mRNA in Clinical Samples from AML Patients with Mt-NPM1 and Higher MEF/ELF4 Expression—To determine the possible clinical relevance of MEF/ELF4, NPM1, and HDM2 in AML patients, we examined the mRNA levels of each in CD34-positive leukemic blasts from 22 AML patients with normal karyotypes. Fourteen patients had Wt-NPM1, and eight patients had Mt-A-NPM1. There was no significant difference between the clinical characteristics of the Wt-NPM1 group and those of the Mt-NPM1 group (Table 1). Samples from the Mt-NPM1 group had significantly higher levels of HDM2 expression as compared with the Wt-NPM1 group ($p = 0.009$) (Fig. 8*A*). In addition, patients with high expression levels of MEF/ELF4 (the MEF/ELF4-H group) had significantly higher HDM2 expression than patients with low expression

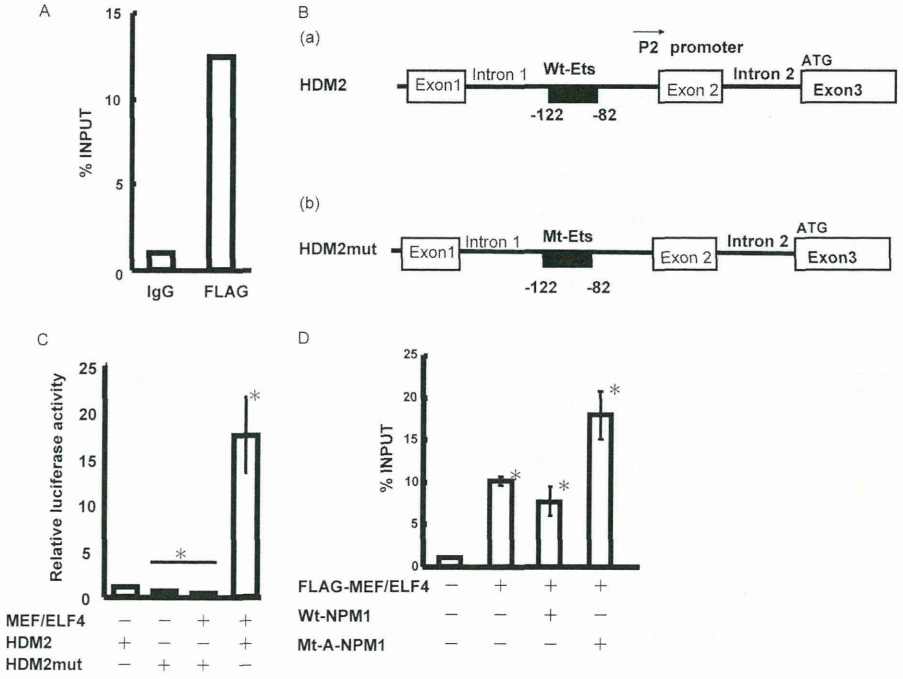

FIGURE 7. **MEF/ELF4 transactivates the HDM2 promoter.** *A*, MEF/ELF4 binds to the HDM2 promoter *in vivo*. FLAG-MEF/ELF4-bound DNA from 293T cells was immunoprecipitated with FLAG antibody or normal mouse IgG. RQ-PCR amplification was performed on the corresponding templates by using primers for HDM2. *B*, structure of the HDM2 promoter region (−82 to −122) (*schematic*). *C*, 293T cells were transfected with HDM2 promoter-driven luciferase reporter plasmid encoding wild-type (*B* (*a*)) or mutant (*B* (*b*)) protein. Luciferase activity by pcDNA alone was assigned a value of 1.0. The analysis was performed in triplicate assays, and the results were reproducible. The results are shown as the mean ± S.D. (*error bars*). *D*, 293T cells were co-transfected with pFLAG/MEF/ELF4 and pcDNA/Wt-NPM1 or pcDNA/Mt-A-NPM1. RQ-PCR amplification was undertaken on corresponding templates using primers for HDM2. The analysis was performed in triplicate assays, and the results were reproducible. The results are shown as the mean ± S.D. *, $p < 0.05$.

TABLE 1

Clinical and laboratory characteristics of patients (ranges shown in parentheses)

	Wt-NPM1	Mt-NPM1	*p*
No. of patients	14	8	
Sex			
Male	5	5	
Female	9	3	0.60
Median age (years)	54.5 (18–78)	62 (44–76)	
FAB classification			
M0	1	0	
M1	2	2	
M2	4	2	
M4	2	2	
M5	2	2	
M6	3	0	0.50
TLD^+	6	4	0.50
Median white blood cell count/μl	7300 (1300–556,000)	47,500 (1700–114,700)	0.10
Median lactate dehydrogenase level	647 (203–5325)	669 (270–2391)	0.07
Median bone marrow cell count/μl	337,000 (9000–738,000)	475,000 (34,900–769,000)	0.10

levels of MEF/ELF4 (the MEF/ELF4-L group) ($p = 0.03$) (Fig. 8*B*).

DISCUSSION

In the present study, we identified NPM1 to be a MEF/ELF4-binding protein. Wt-NPM1 inhibited the function of MEF/ELF4 (*i.e.* DNA binding and transcriptional activities), whereas Mt-NPM1 augmented its function. Some of these effects of Wt-NPM1 and Mt-NPM1 on MEF/ELF4 were reproducible on the HDM2 promoter (one of the target genes of MEF/ELF4), suggesting that HDM2 expression is influenced by NPM1. Furthermore, we found that the expression of Mt-NPM1 in MEF/ELF4-overexpressing NIH3T3 cells resulted in enhanced malignant transformation. We also found that the mRNA level of *HDM2* in primary leukemia cells was higher in patients with NPM1 mutations. Mef/Elf4 directly activates *Mdm2* expression (13). Therefore, NPM1 mutation could enhance HDM2 expression through the increased MEF/ELF4 activity, thereby promoting transformation by inhibiting the p53 pathway.

NPM1 is a multifunctional phosphoprotein that has been implicated in cell proliferation as well as regulation of transcription factors. It appears to repress or stimulate transcription. For example, Wt-NPM1 activates and inhibits p53 function through direct binding (22, 25). Interferon regulatory factor-1 (IRF-1), a transcriptional activator, binds to Wt-NPM1, resulting in the inhibition of DNA binding and transcriptional activity (26). Our findings with Wt-NPM1 and MEF/ELF4 are consistent with these observations. Wt-NPM1 interacts directly with c-Myc and regulates the expression of

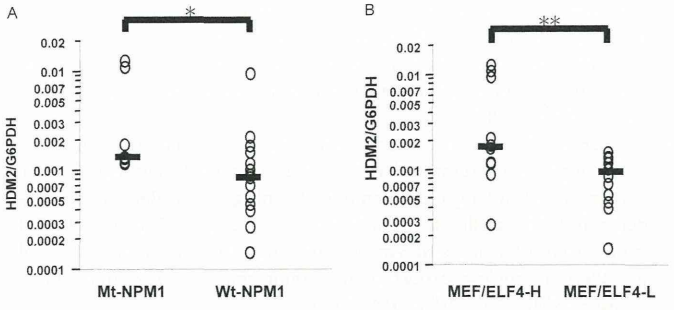

FIGURE 8. **Expression of Mt-NPM1 and higher expression of MEF/ELF4 are associated with the elevated expression of HDM2 in CD34-positive AML cells.** Total RNA isolated from 22 AML patients (CD34-positive leukemia cells) was analyzed for the expression of HDM2 by RQ-PCR. Shown is stratification by the presence of the NPM1 mutation (*A*) and by the level of ELF4/MEF (*B*). These bars were median lines for each group. *, $p < 0.009$ against Wt-NPM1; **, $p < 0.03$ against MEF/ELF4-L, assessed by analysis of variance followed by Scheffe's multiple comparison test.

endogenous c-Myc target genes at the promoter, which enhances c-Myc-induced proliferation and transformation (27). In contrast, the present study suggests that Wt-NPM1 inhibits (whereas Mt-NPM1 facilitates) the transformation induced by MEF/ELF4, suggesting that there is a contradiction in terms of NPM1 function. However, the overexpression of Wt-NPM1 without c-Myc activation has only a small effect on proliferation and has no effect on transformation, so Wt-NPM1 may mainly have a role in c-Myc-driven tumors. Interestingly, c-Myc, IRF-1, and MEF/ELF4 are all regulated during the cell cycle, and the levels of these transcription factors are highest in the G_1 phase (28, 29).

We found that Wt-NPM1 could interfere with the ability of MEF/ELF4 to bind to DNA, resulting in the inhibition of MEF/ELF4-dependent transcriptional activity. The mechanism by which Wt-NPM1 interferes with the DNA binding of MEF/ELF4 is unclear. We previously showed that the 120 amino acids N-terminal to the ETS domain in MEF/ELF4 (residues 87–206) are responsible for its binding to AML1 proteins (30); thus, MEF/ELF4 interacts with other proteins outside the DNA-binding domain. As mentioned above, the association of Wt-NPM1 and IRF-1 inhibits the DNA binding of IRF-1. Narayan *et al.* showed that IRF1 binds directly to Wt-NPM1 through a short linear motif in the nuclear localization sequence outside the DNA-binding domain (31). These results suggest that the inhibition of DNA binding by NPM1 may not be through simple interference with the DNA-binding domain of MEF/ELF4. Determining the protein-binding interface of MEF/ELF4 may help to reveal the mechanism of NPM1-mediated transcriptional regulation.

The heterodimerization domain (residues 186–259) of NPM1 is essential for its interaction with p53 (22), and the c-Myc-binding region is within the NPM1 heterodimerization domain (27). In the case of MEF/ELF4 and NPM1, the N-terminal regions of NPM1 (F1, F2, and F3) could bind to His-MEF/ELF4, implying that the oligomerization domain is important for the interaction.

Recently, it has been shown *in vivo* that NPM1 mutants actively contribute to leukemogenesis by conferring a proliferative advantage in the myeloid lineage. In zebrafish, forced expression of mutant NPM1 causes an increase in PU.1-positive primitive early myeloid cells (32). Furthermore, in a transgenic mouse expressing the human NPM1 mutant, although spontaneous AML was not found, myeloproliferation occurred in the bone marrow and spleen (33). Moreover, Vassiliou *et al.* (34) showed that activation of a humanized mouse NPM1 mutant knock-in allele in mouse hematopoietic stem cells caused overexpression of the *Hox* gene, enhanced self-renewal, and expanded myelopoiesis, resulting in delayed onset AML in one-third of the mice. Taken together, these data suggest that NPM1 mutations initiate leukemia by activating a set of proliferative pathways. Mt-NPM1 enhances the transcriptional activity of MEF/ELF4, so the up-regulation of HDM2 and subsequent down-regulation of p53 may also have a role in leukemogenesis.

In vitro transfection studies and immunohistochemical observations in samples from AML patients have demonstrated that NPM1 mutants recruit Wt-NPM1 from the nucleolus and delocalize it to the nucleoplasm and cytoplasm (18) and that aberrant NPM1 accumulation in the cytoplasm may have a critical role in leukemogenesis. While Wt-NPM1 protein co-localizes with tumor suppressor p19ARF in the nucleolus, Mt-NPM1 delocalizes p19ARF from the nucleolus to the cytoplasm, which results in reduced p19ARF activities (*e.g.* Mdm2 and $p21^{cip1}$ induction, stimulation of NPM1) (35). Furthermore, by using OCI/AML3 human leukemia cells where mutant NPM1 is localized in the cytoplasm, Bhat *et al.* (36) have recently shown that NPM1-co-localizing nuclear transcription factor, FOXM1 (forkhead box M1), disappears from the cytoplasm following transient NPM1 knockdown. These data suggest that NPM1 may determine the intracellular localization of interacting transcription factors. However, in our experiments, Mt-NPM1 did not interact with MEF/ELF4 *in vivo*, and the subcellular distribution of MEF/ELF4 was not affected by the presence of Mt-NPM1. It seems that Mt-NPM1 binds and dislocates Wt-NPM1 into the cytoplasm of leukemia cells, which eventually leads to uncontrolled transactivation of MEF/ELF4. Wt-NPM1 knockdown with siRNA against NPM1 also enhanced MEF/ELF4 activity (Fig. 3*E*), suggesting that the depletion of an MEF/ELF4 inhibitor (*i.e.* Wt-NPM1) in the nucleus is responsible for the transactivation of MEF/ELF4. Taken together, it is likely that NPM1 mutants exert oncogenic functions at least in part through the up-regulation of the activities of oncogenic transcription factors, such as MEF/ELF4. The correlation between NPM1 mutations and the elevated expression of HDM2 in primary leukemia cells seems to support this theory.

In patients with AML, NPM1 mutations are mutually exclusive of recurrent genetic abnormalities. It can be speculated that the enhanced MEF/ELF4-HDM2-p53 pathway induced by NPM1 mutations may participate in leukemia development, especially in patients with a normal karyotype. The transactivation of MEF/ELF4 by E2F1 is inhibited by p53 (37), suggesting that p53 suppression induced by NPM1 mutation could lead to the activation of E2F1, resulting in the enhanced expression of MEF/ELF4. Our previous data showing the elevated expression of MEF/ELF4 in AML cells with a normal karyotype compared with that of AML cells carrying t(8;21) and t(15;17) seem to support this hypothesis.

Our results suggest a new role for NPM1 and MEF/ELF4 in leukemia development.

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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

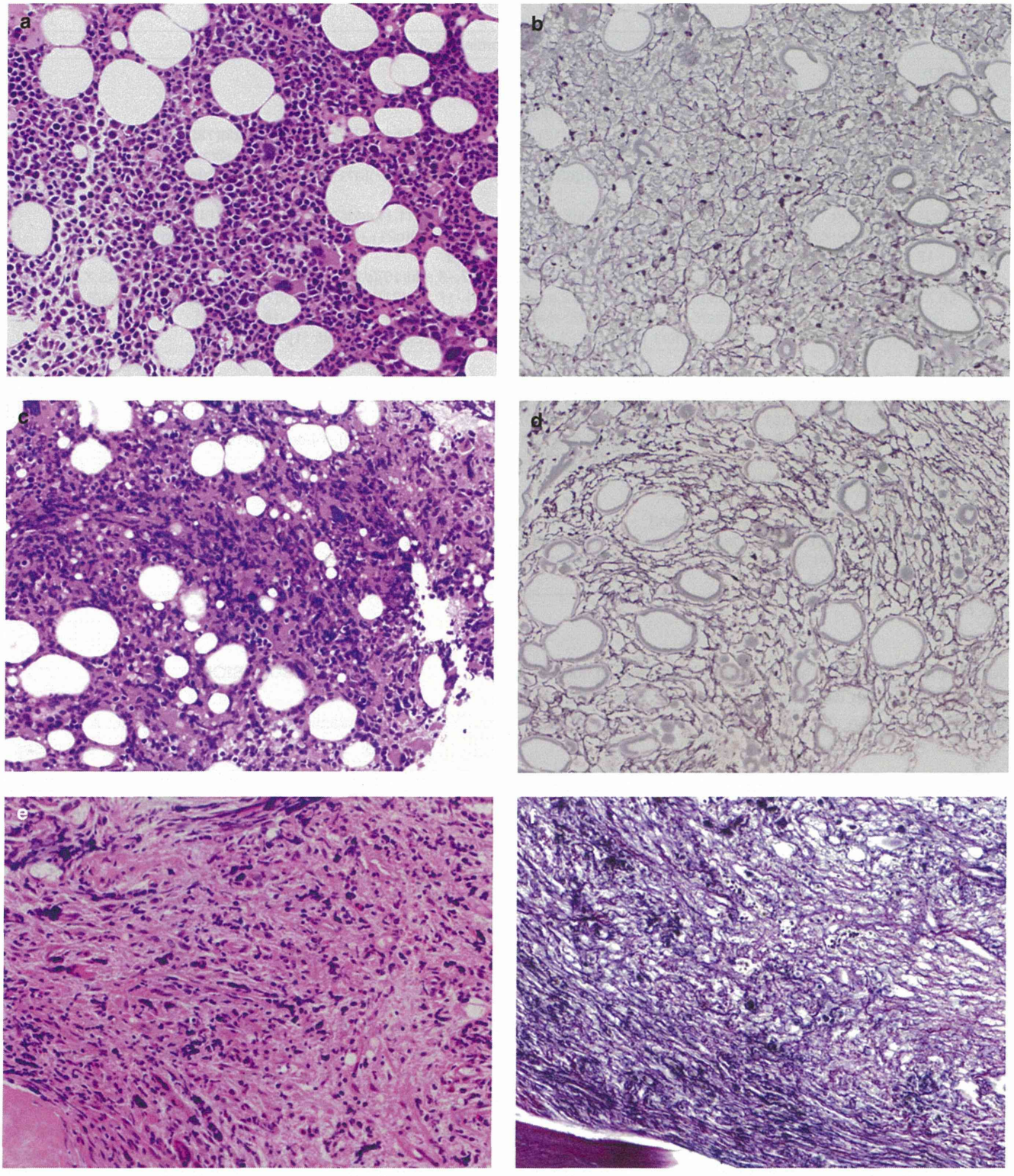

Figure 1 Histopathological features of (**a-b**) a representative case of acute megalaryoblastic leukemia (AMGL) with mild fibrosis (**a**, H&E), (**b**, silver reticulin stain), (**c-d**) a representative case of AMGL with moderate fibrosis (**c**, H&E), (**d**, silver reticulin stain) and (**e-f**)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

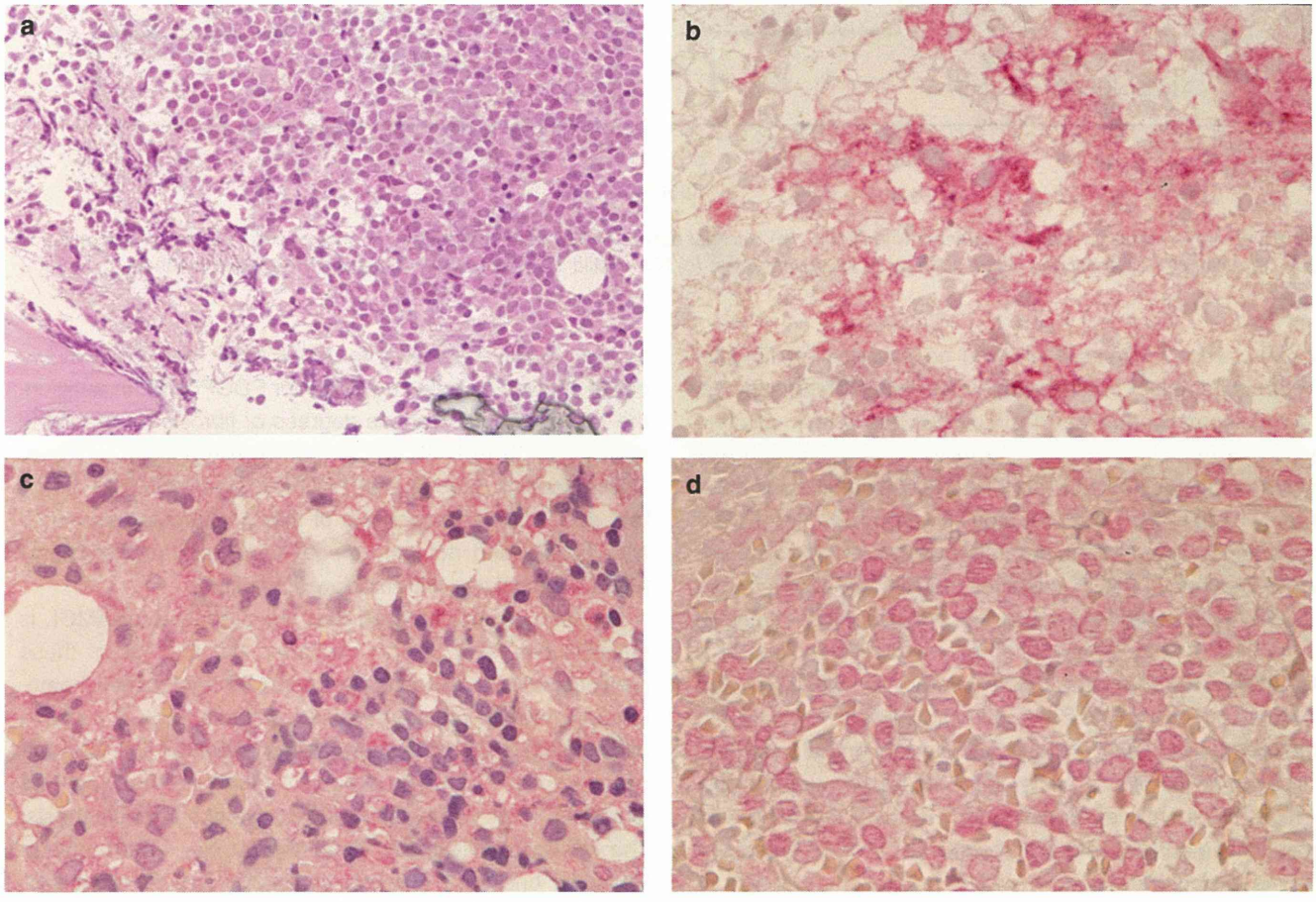

Figure 2 Immunohistochemistry. A representative case of AMGL with mild fibrosis. (**a**) H&E, (**b**) von Willebrand factor, (**c**) CD41, (**d**) platelet-derived growth factor (PDGF) (-BB).

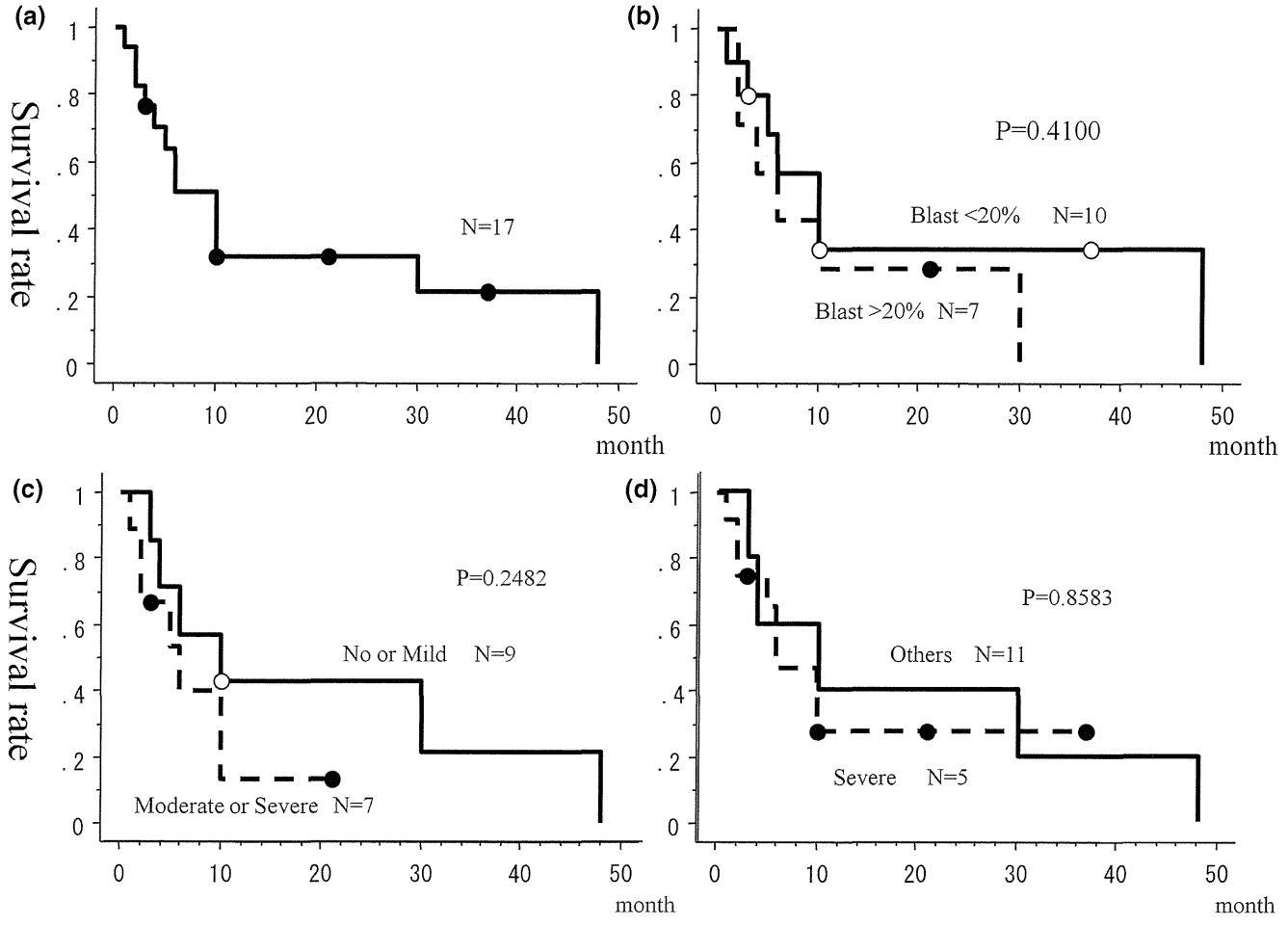

Figure 3 (**a**) Survival curves of 17 patients with AMGL. (**b**) Survival curves according to the proportion of blast cells among peripheral blood cells. Survival curves according to the severity of marrow fibrosis: (**c**) No or Mild VS Moderate or Severe, (**d**) Severe VS Others.

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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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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Romidepsin Overcomes Cell Adhesion-Mediated Drug Resistance in Multiple Myeloma Cells

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Multiple myeloma (MM) is a malignant hematopoietic disease that remains incurable. Therapeutic strategies for this disease have been rapidly progressing based on the development of new drugs, including proteasome inhibitors, immunomodulatory agents, antibodies and small molecular compounds such as histone deacetylase inhibitors (HDIs); however, drug resistance remains a major challenge [1]. It is well known that cell adhesion-mediated drug resistance (CAM-DR) occurs when MM cells interact with stromal cells [2]. Specifically, MM cells express surface adhesion receptor molecules which bind with corresponding ligands on stromal cells. Such interaction results in protection of MM cells from the cytotoxic effects of anti-myeloma drugs. We previously found that MM cells express various adhesion molecules, including CD29 (β1-integrin), CD49d (α4-integrin, a subunit of VLA-4), CD54 (intercellular adhesion molecule-1), CD138 (syndecan-1), CD184 (CXC chemokine receptor-4), and CD44. Furthermore, among them CD49d was crucial for CAM-DR to conventional anti-myeloma drugs such as bortezomib and dexamethasone [3]. Thus, it is of great importance to suppress CD49d expression to overcome CAM-DR.

HDI- and DNA-methylating agents show anti-tumor activity by epigenetically re-expressing various genes [4, 5]. These effects might ultimately affect the expression and function of various intracellular molecules, including transcription factors. We therefore hypothesized that these agents influence the expression levels of adhesion molecules in MM cells. To verify this hypothesis, we examined the effect of the HDI romidepsin and DNA-methylating agent azacitidine on the expression levels of CD49d and two other representative adhesion molecules, CD29 and CD138, by flow cytometry analyses in two human MM cell lines, RPMI8226 and U266. Surprisingly and importantly, romidepsin repressed the expression levels of CD49d with statistical significance in both cell lines (fig. 1a, b). Levels of *CD49d* mRNA also markedly decreased after addition of romidepsin, suggesting that romidepsin suppresses *CD49d* expression at the mRNA level (fig. 1c). In contrast, romidepsin had no significant effect on the expression levels of CD29 and CD138 (fig. 1a, b). In RPMI8226 cells, azacitidine also repressed CD49d as well as CD138 (fig. 1a). However, in U266 cells it had no influence on all three adhesion molecules tested, including CD49d (fig. 1b). Since azacitidine failed to disrupt DNA methyltransferases, which are its main targets, in U266 cells [unpubl. data], it is possible that azacitidine had no effect on the pathophysiology of these cells.

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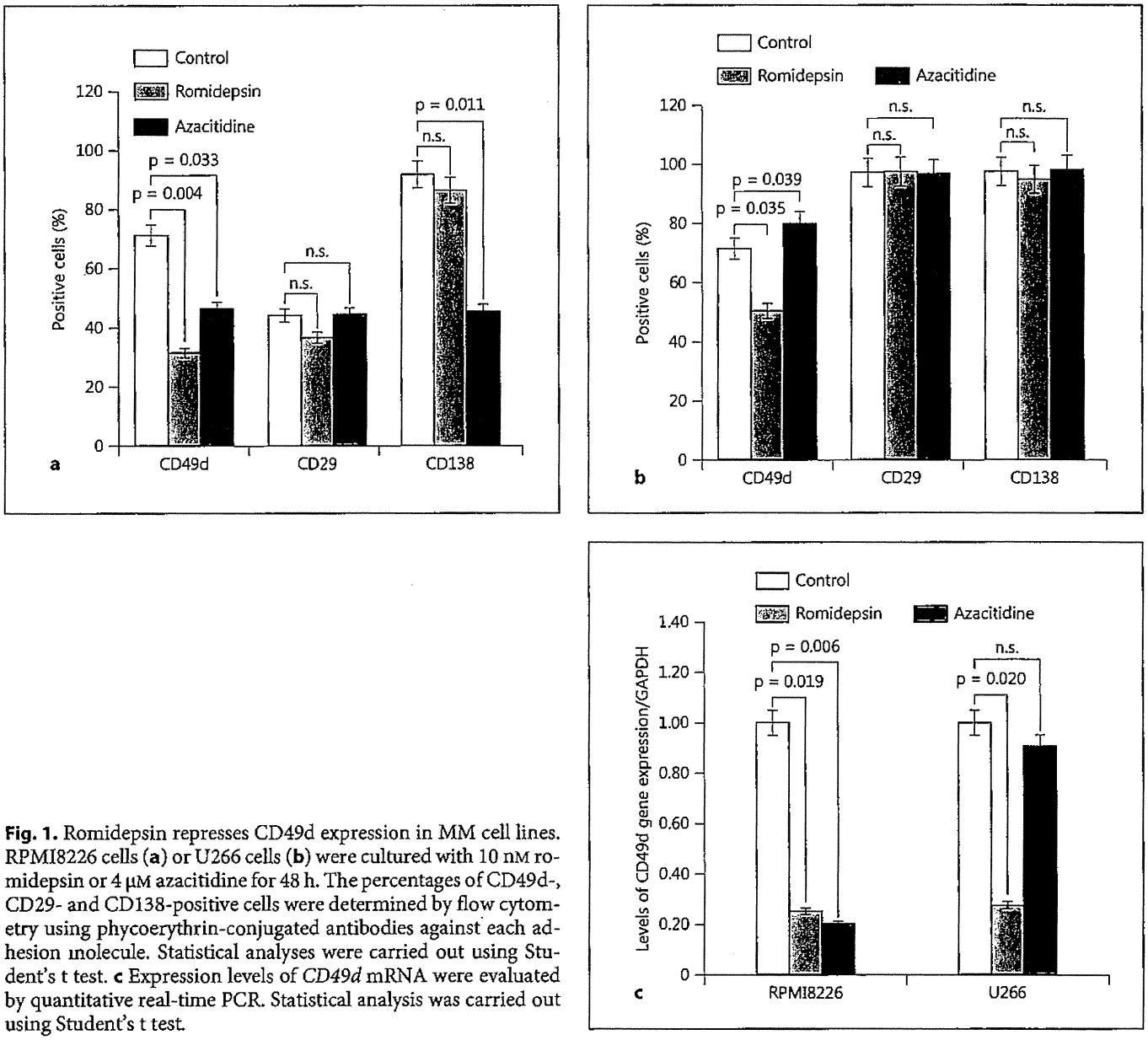
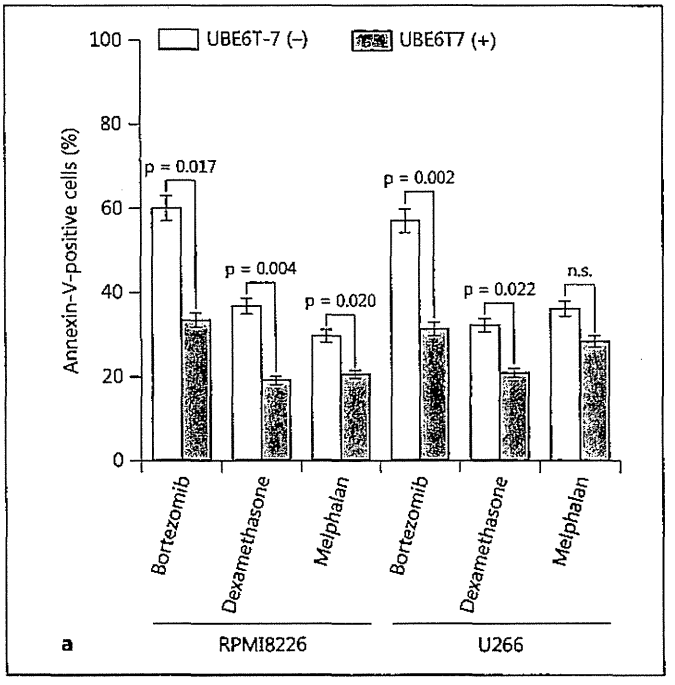


Fig. 1. Romidepsin represses CD49d expression in MM cell lines. RPMI8226 cells (**a**) or U266 cells (**b**) were cultured with 10 nM romidepsin or 4 μM azacitidine for 48 h. The percentages of CD49d-, CD29- and CD138-positive cells were determined by flow cytometry using phycoerythrin-conjugated antibodies against each adhesion molecule. Statistical analyses were carried out using Student's t test. **c** Expression levels of *CD49d* mRNA were evaluated by quantitative real-time PCR. Statistical analysis was carried out using Student's t test.

To clarify whether romidepsin-mediated suppression of *CD49d* expression results in abrogation of CAM-DR to other drugs, we assessed CAM-DR to bortezomib, melphalan and dexamethasone, which are key drugs for MM therapy, using the co-culture system as described previously [3]. In the absence of the stromal cell line UBE6T-7, bortezomib, melphalan and dexamethasone significantly increased the percentage of annexin-V-positive cells, suggesting that these agents effectively induce apoptosis. However, when MM cells were co-cultured with UBE6T-7 cells, induction of apoptosis was suppressed (fig. 2a). We then assessed the reversal of CAM-DR, which was defined as the ratio of annexin-V-positive MM cells in the presence of UBE6T-7 cells to those in the absence of UBE6T-7 cells [3]. As expected, pretreatment of MM cells with romidepsin significantly increased the reversal of CAM-DR to bortezomib, melphalan and dexamethasone in both cell lines (fig. 2b), suggesting that romidepsin effectively overcomes CAM-DR. Consistent with the findings that azacitidine repressed *CD49d* expression in RPMI8226 cells, azacitidine induced the reversal of CAM-DR to these drugs with statistical significance (fig. 2b). In contrast,



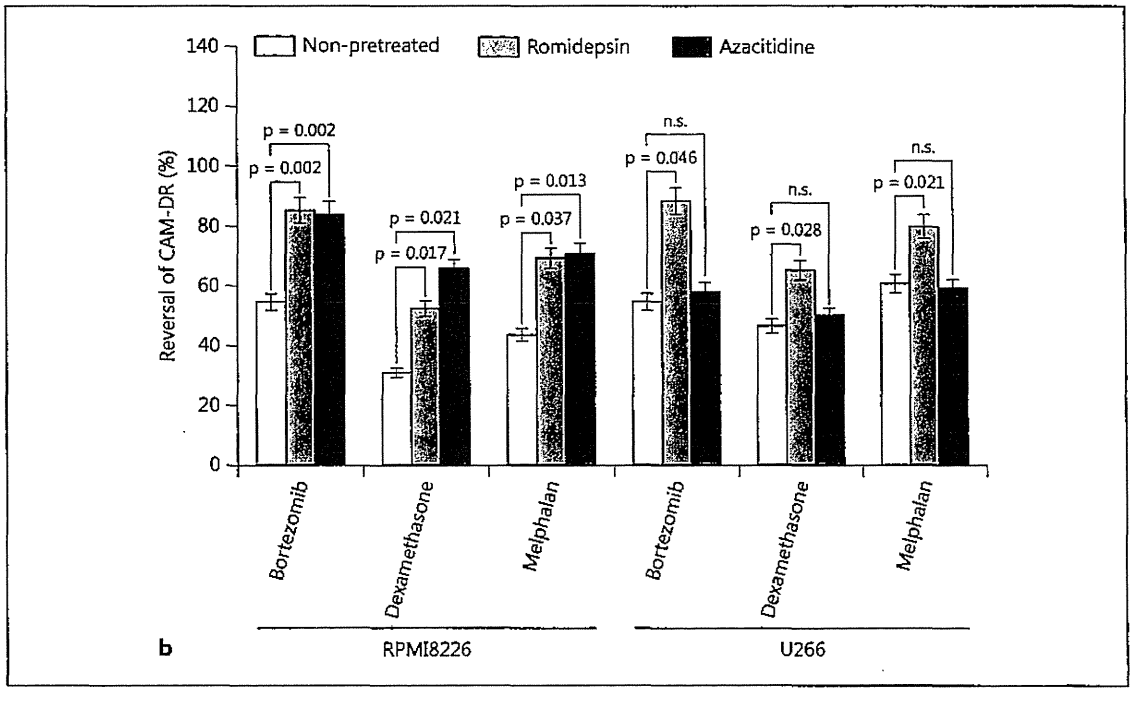


Fig. 2. Romidepsin abrogates CAM-DR to conventional anti-MM drugs. **a** Cells were cultured with 2 nM bortezomib, 10 μM melphalan or 1 μM dexamethasone in the presence or absence of UBE6T-7 cells for 48 h. MM cells were then harvested and stained with annexin-V-FITC. **b** Cells were pretreated with 10 nM romidepsin or 4 μM azacitidine for 24 h and subsequently cultured with 2 nM bortezomib, 10 μM melphalan or 1 μM dexamethasone in the presence or absence of UBE6T-7 cells for a further 48 h. Reversal of CAM-DR was determined as the ratio (%) of annexin-V-positive MM cells in the presence of UBE6T-7 cells to those in the absence of UBE6T-7 cells. Statistical analysis was carried out using Student's t test.

azacitidine had no effect on CAM-DR in U266 cells, the CD49d levels of which were not suppressed by azacitidine.

Histone deacetylases (HDACs) are a class of enzymes that lyse acetyl groups within histones, thus affecting DNA gene expression. They also affect the acetylation status of non-histone proteins such as heat shock protein 90 and α-tubulin, which are involved in the pathophysiology of MM cells. Romidepsin mainly inhibits HDAC1, HDAC2 and HDAC6 [6]. Since HDAC1 and HDAC2 belong to class 1 HDACs, which target histone proteins, it is possible that romidepsin-mediated restoration of gene expressions affects the expression or function of tran-

scription factors that regulate *CD49d* gene expression. Since there are potential binding sites for ETS and WT1 transcription factors on the *CD49d* promoter region [7, 8], it is of great interest to clarify whether romidepsin affects activities of these factors. It is also important to confirm that the *CD49d* expression of MM cells reduces in patients treated with romidepsin.

Interestingly, azacitidine failed to suppress *CD49d* expression, showing no effect on CAM-DR in U266 cells; whereas, just like romidepsin, it abrogated CAM-DR in RPMI8226 cells. U266 are azacitidine-resistant cells, in which its demethylating activity is negated [unpubl. data]; therefore, these results also suggest that restoration of gene expressions due to an improvement of epigenetic status is mainly involved in the repression of *CD49d* in MM cells.

Previous preclinical studies have shown that the combinations of HDIs and bortezomib or other anti-myeloma drugs enhance anti-tumor effects in MM cells [9]. Furthermore, clinical trials have also shown a certain clinical efficacy of combinations of HDIs and other drugs [10, 11]. Combination therapies are promising strategies for the treatment of MM. Interestingly, overexpression of HDAC1 is involved in resistance to bortezomib, and romidepsin overcomes this resistance [12]. The fact that romidepsin overcomes CAM-DR to bortezomib, melphalan and dexamethasone provides additional rationale for an advantage of the combination therapy.

In conclusion, our findings suggest that potentiations of romidepsin and other anti-myeloma drugs, such as bortezomib, melphalan and dexamethasone, are potential therapies for MM in view of overcoming CAM-DR, which is critical for improving the efficacy of anti-myeloma therapy, and might be essential for establishing novel therapeutic strategies to enhance patient outcome or possibly cure the disease.

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