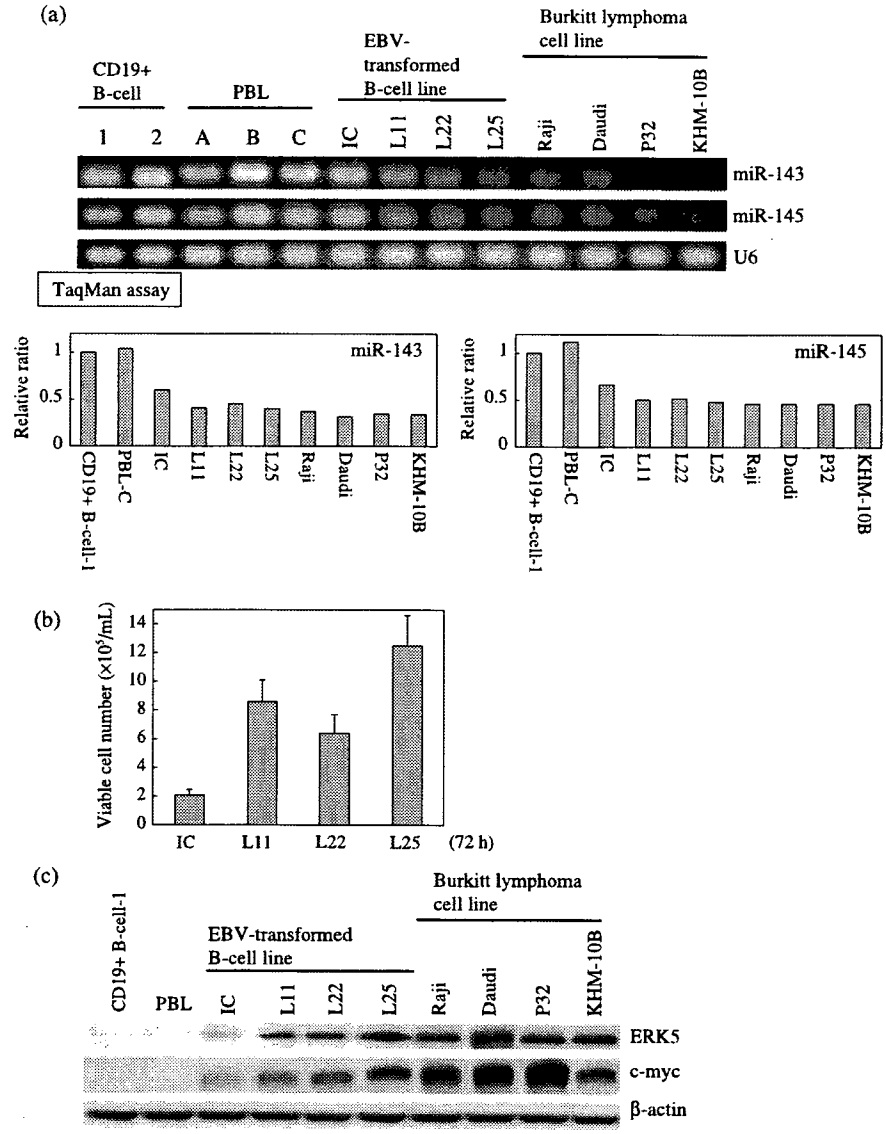

Fig. 2. Expression of microRNAs (miRNAs)-143 and -145 and cell growth in B-cell cultured cell lines. (a) Evaluation of expression of miRNAs-143 and -145 in human Epstein-Barr virus (EBV)-transformed B-cell lines and Burkitt lymphoma cell lines by use of semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and TaqMan MicroRNA assays using real-time PCR. U6 was used as an internal standard. $CD19^+$ B-cell-1 was designated as 1 in quantitative RT-PCR by TaqMan miRNA assays using real-time PCR. The results of real-time PCR were expressed as the mean values of two independent experiments. (b) Steady-state cell growth of EBV-transformed B-cell lines at 72 h after seeding at the concentration of 1×10^5/mL (c) Western blot analysis of ERK5 and c-myc in control and B-cell lines in the same samples as in (b), and in human Burkitt lymphoma cell lines. β-actin was used as an internal control.

(Fig. 3a). In order to further confirm that miR-143 or -145 has a growth inhibition, we carried out a transfection experiment using mature miR-143 (miR-143 m) or -145 (miR-145 m) (Fig. 3b). In both experiments, a dose-dependent growth inhibition by the introduction of mature miR-143 or -145, which results were very similar to those obtained when the transfection was carried out with precursor miR-143 or -145. These findings indicate that miR-143 and -145 negatively contribute to cell growth, because the compensation of miR-143 or -145 by the transfection induced growth inhibition and that the perturbation of processing of miR-143 or -145 in the nucleus including transcription and microprocessor of Drosha and DG8 could cause the cells to reduce their expression of both.

Genomic status and epigenetic change in miR-143 and -145 loci on 5q32. In order to examine the chromosomal aberrations of miRs-143 and -145 loci on 5q32, we carried out genomic PCR on the B-cell lines by using a primer pair covering both loci.[34] As shown in Fig. 4a, more than one allele was confirmed to be present in all EBV-transformed and Burkitt lymphoma cell lines, as in the placenta, used as the positive control. Furthermore, the treatment with 2–10 μM 5-Aza, which completely reduced the level of DNMT-1, did not upregulate the expression of miRNAs-143 and -145 at all in L25 cells (Fig. 4b); nor did that with TSA. Thus, it appears that some genomic aberration or epigenetic change did not cause the low expression of miR-143 and -145 in the cells.

MicroRNA-143 targets the *ERK-5* mRNAs. Previously, we determined that one of the target genes of miR-143 was *ERK5* in the human colon cancer cell line DLD-1.[34] ERK5 is a recently characterized MAPK, which is most similar to the well-studied ERK1/2 subfamily, but uses distinct mechanisms to elicit responses. The physiological importance of this signaling cascade is underscored by the early embryonic death caused by the targeted deletion of the *erk5* or the *mek5* genes in mice.[36] In Fig. 2c, the level of ERK5 expression in Burkitt lymphoma cell lines was approximately several times higher than that in the other B-cells including $CD19^+$ B-cells and EBV transformed B-cell lines except L25, where the pattern was very similar to that of c-myc. We clearly demonstrated that the expression levels between miR-143 and ERK5 were inversely correlated in regard to cell growth in EBV-transformed cell lines (Fig. 2). Based on these results, ERK-5 was shown to be a growth-related MAPK even in B-cells. In order to further confirm that the target mRNA of miR-143 is *ERK5*, we examined the expression of ERK5 and the mRNA in the RAJI cells transfected with precursor miR-143 (Fig. 3). Expectedly the protein expression levels were significantly decreased in a dose-dependent

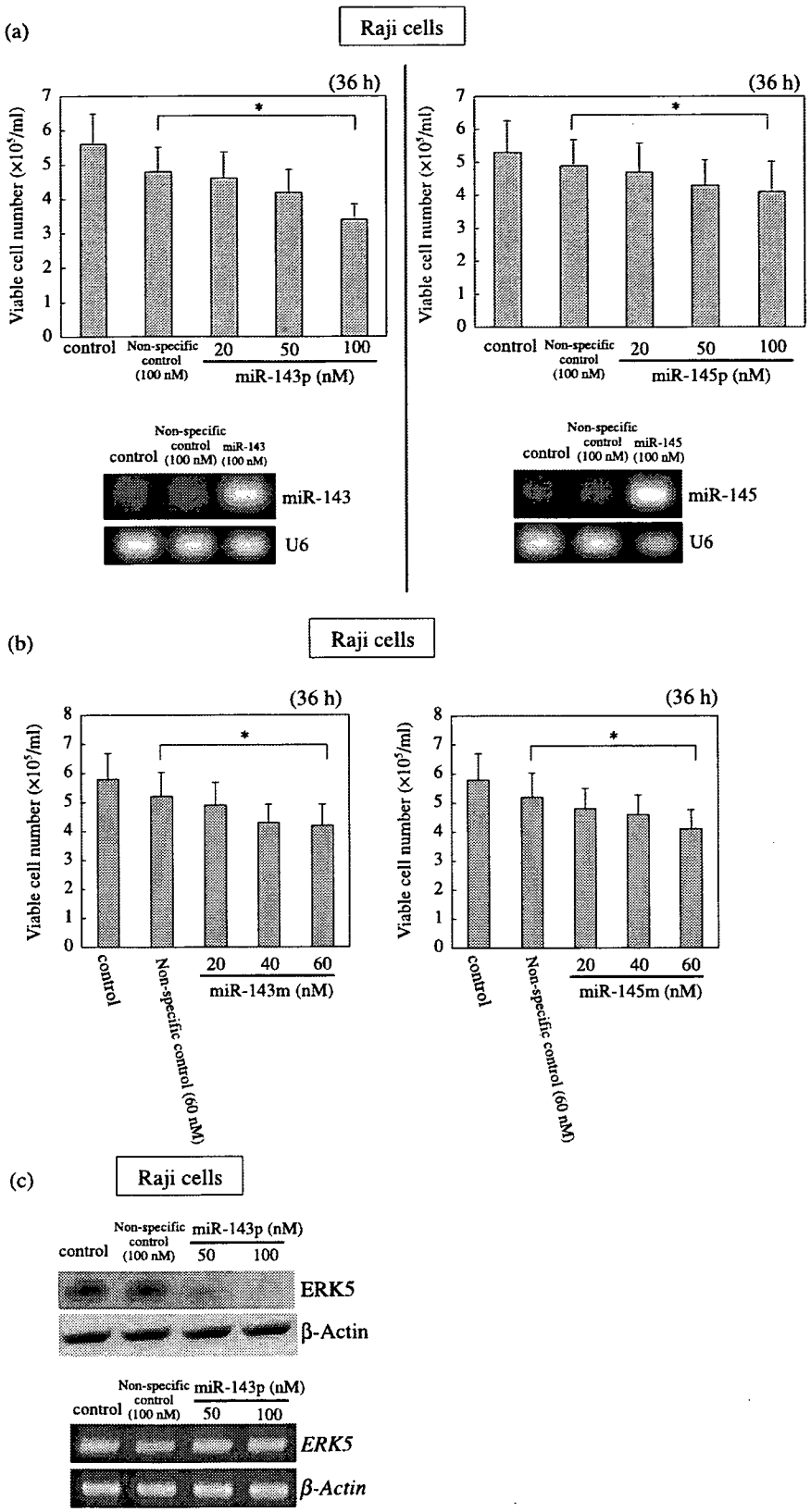

Fig. 3. Effect of transfection of human Burkitt lymphoma Raji cells with either precursor (a) or mature type (b) of microRNAs (miRNAs)-143 and -145, and ERK5 expression in the cells transfected with the precursor miR-143 (c). (a, b) Number of viable transfected or control cells at 36 h after transfection is shown. Data are presented as the mean ± standard deviation (SD) of three different experiments, each carried out in duplicate. Levels of miRNAs-143 and -145 in Raji cells at 36 h after the transfection of the cells with miR-143 or -145 precursor miRNAs at 100 nM are shown in (a). U6 was used as an internal standard. The difference between the non-specific control and 100 (a) or 60 (b) nM treatment was significant ($*P < 0.01$). (c) Expression levels of ERK5 protein and the mRNA at 36 h after the transfection of Raji cells with miR-143 precursor miRNAs, as evaluated by western blot analysis (upper panel) and by quantitative RT-PCR (lower panel), respectively. β-actin was used as an internal standard. Control cells were incubated in medium containing transfection reagent alone.

manner, whereas the mRNA levels were almost unchanged (Fig. 3c). Thus, these findings and the previous results in colon cancer[34] altogether indicate that miR-143 could target the *ERK5* gene even in B-cells.

Discussion

In the current study, we demonstrated that the expression levels of miRs-143 and -145 were significantly reduced in the B-cell

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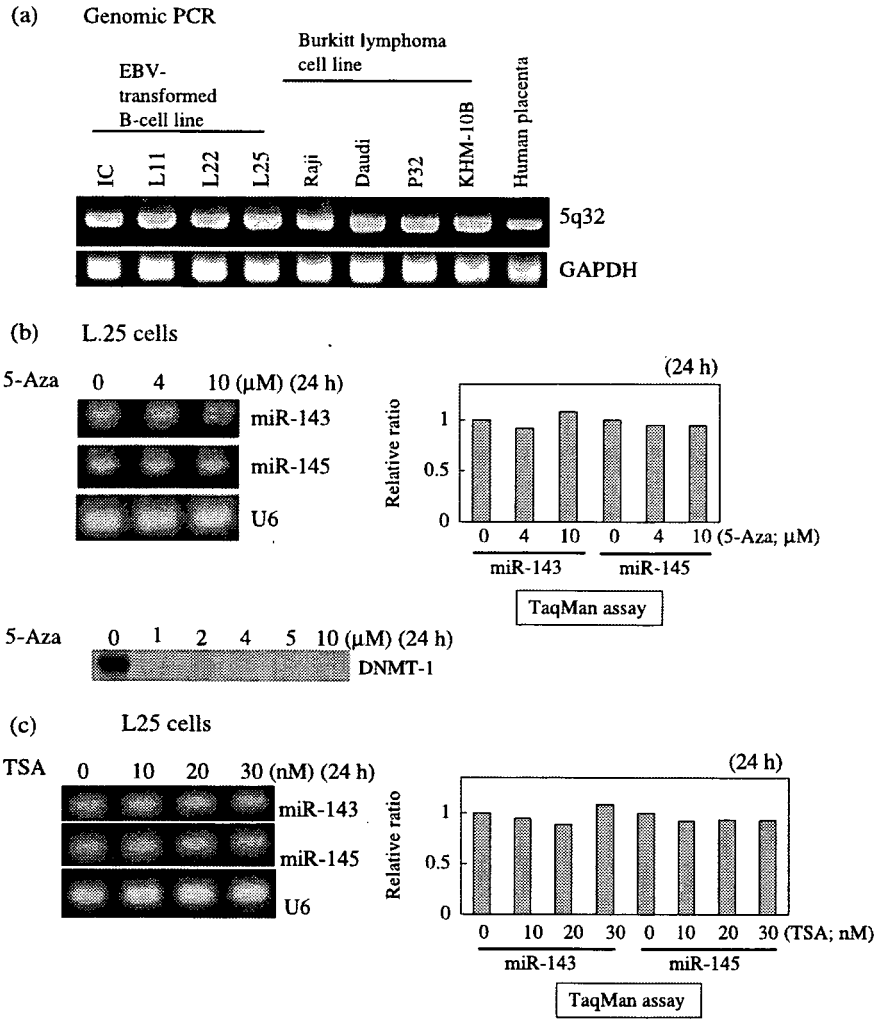

Fig. 4. Confirmation of the presence of the genomic loci of microRNAs (miRNAs)-143 and -145 at chromosome 5q32 by genomic polymerase chain reaction (PCR) (a) and expression of the miRNAs in Epstein-Barr virus (EBV)-transformed L25 cells after 24-h treatment with 5-Aza-2′-deoxycytidine (5-Aza, b) or tricostatin A (TSA, c). (a) The primers amplified the DNA fragment including the loci of miRs-143 and -145. Placental DNA was used as a positive control. The genomic locus of glyceraldehyde phosphate dehydrogenase (*GAPDH*) was used as an internal control. (b, c) The expression of the miRNAs in L25 cells after the treatment with either agent at the indicated concentrations is shown. The value for the control cells is designated as 1 in quantitative reverse transcription (RT)-PCR by TaqMan miRNA assays using a real-time PCR. The results of real-time PCR are expressed as the mean values of two independent experiments. U6 was used as an internal standard. Results of Western blot analysis of DNA methyltransferase (DNMT)-1 are also given in (b).

malignancies tested and further that the expression levels were inversely related to the cell growth in the EBV-transformed B-cell lines, which have no genomic aberrations. The introduction of miR-143 or -145 precursors or mature types into the Raji cells led to a significant growth inhibition. Such data suggest that miRs-143 and -145 could negatively contribute to cell growth by targeting growth-related genes.

The increased expression of miR-143 by the transfection with precursor miR-143 into Raji cells lowered the protein expression levels of ERK5, whereas the mRNA level of *ERK5* remained unchanged, which suggests that *ERK5* is one of the target genes of miR-143, as shown in previous reports by us,[34] and Esau *et al.*[37]

Recent studies indicate that proteins involved in miRNA biogenesis, including Drosha and double-stranded-RNA-binding protein DGCR8, Dicer 1, Argonaute 2, and RISC, may also participate in the complex interactions that regulate miRNA expression, together with additional mechanisms that regulate miRNA at the epigenetic, transcriptional level. Since the transfection of Raji cells with the precursor miR-143 or -145 exhibited growth inhibition, transcription and/or processing by Drosha and DGCR8 microprocessor in the nucleus was possibly perturbed, which is true in the case of human colon cancer DLD-1 cells.[34] Furthermore, we did not obtain any data that indicated an inappropriate expression caused by genomic aberration or epigenetic change such as methylated DNA and histone. Recently, miR-223 was shown to be upregulated by the retinoic acid-induced replacement of NFI-A with C/EBP α, resulting in promotion of human granulocyte differentiation.[38] As miR-223 repressed NFI-A translation, the upregulation of miR-223 by C/EBP α and granulopoiesis was further accelerated through positive feedback. Therefore, the machinery involved in the transcription step of miRs-143 and -145, whose primary miRNAs are most likely identical, because their genomic loci are located within a 1.8-kb span, should be clarified for achieving a fully comprehensive view of the processes operating in carcinogenesis.

Rescued expression of downregulated or functionally-deficient miRNAs and/or inhibitors of overexpressed miRNAs may contribute to rebalanced the expression of large gene clusters implicated in oncogenesis, tumor progression, and cell death. One of the target genes for miR-143 was presently shown to be *ERK5* MAPK, which was also shown to be targeted in DLD-1 cells by us,[34] and in adipocytes by others.[37] Also, we suggest that miR-145 may target *MAP3K3* and *MAPK4K4* and that other potential targets for miR-145, which have oncogenic functions, are *MYCN*, *FOS*, *YES*, and *FLI* (http://microrna.sanger.ac.uk/) and cell-cycle promoters such as *cyclin D2* and *L1*. Particularly, the MAPK signal via ERK5 contains c-myc in the downstream of ERK5.[39] Thus, the reduced expression of both miRNAs directly or indirectly affects cell fate such as growth, survival, and death signals; that is, low-expression of miR-143 and -145 possibly by perturbed transcription and/or microprocessing, could result in an unbalanced signaling cascade including MAPK, which would lead to a sustained cell proliferation.

To our knowledge, upregulation of mir-155 from BIC RNA in B-cell lymphomas,[40] and downregulation of miR-14 and -15, which originated from the deletion of a region of chromosome

13q13.4,[28] have been reported in B-cell malignancies. Furthermore, the target gene of miR-15 and -16 was shown to be the antiapoptotic gene *BCL-2*.[29] Therefore, the genomic deletion of 13q13.4 causes an inappropriate expression of miR-15 and -16 that targets antiapoptotic *BCL-2* mRNAs, which negatively works against cell death. We found that the level of miR-15a was reduced in seven of 13 CLLs; however, the level of decrease was not as great as in the case of miRNAs-143 or -145. Our results suggested that the incidence of the 13q13.4 deletion in Japanese CLLs may be lower than that in Caucasian CLLs.[28] Recently, the same group also evaluated the miRNA expression profiles of 41 samples of CLL, and found 25 genes (of 161 analyzed) to have a unique miRNA expression signature.[41] However, miR-143 or -145 was not found to be significant with respect to the miRNA expression profile, although it is not clear whether miR-143 and -145 were examined. Previously, we reported that the expression of miRs-143 and 145 was strongly related to tumorigenesis in colon cancer,[34] because more than 80% of the cases were shown to have low-expression compared with non-cancerous tissues. In addition, all of the malignant cell lines tested were shown to be downregulated for these miRs.[34]

Thus, miRs-143 and -145 play pivotal roles in the pathogenesis of B-cell malignancies and could be plausible biomarkers to differentiate B-cell malignant cells from normal B-cells, being even better ones than miRs-15a and -16 in Japanese CLLs. Also, we propose miR-143 or -145 as a candidate for the development of RNA medicine against cancer. Further detailed study to decipher the transcription machinery of miR-143 and -145 including their promoter region on 5q32 and their binding sites in the 3′ UTR of *ERK5* will be needed for a better understanding of the carcinogenesis involving miRNAs.

Acknowledgments

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A randomized study with or without intensified maintenance chemotherapy in patients with acute promyelocytic leukemia who have become negative for *PML-RARα* transcript after consolidation therapy: The Japan Adult Leukemia Study Group (JALSG) APL97 study

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To examine the efficacy of intensified maintenance chemotherapy, we conducted a prospective multicenter trial in adult patients with newly diagnosed acute promyelocytic leukemia treated with all-*trans* retinoic acid and chemotherapy. Of the 302 registered, 283 patients were assessable and 267 (94%) achieved complete remission. Predicted 6-year overall survival in all assessable patients and disease-free survival in patients who achieved complete remission were 83.9% and 68.5%, respectively. A total of 175 patients negative for *PML-RARα* at the end of consolidation were randomly assigned to receive either intensified maintenance chemotherapy (n = 89) or observation (n = 86). Predicted 6-year disease-free survival was 79.8% for the observation group and 63.1% for the chemotherapy group, showing no statistically significant difference between the 2 groups ($P = .20$). Predicted 6-year survival of patients assigned to the observation was 98.8%, which was significantly higher than 86.2% in those allocated to the intensified maintenance ($P = .014$). These results indicate that the intensified maintenance chemotherapy did not improve disease-free survival, but rather conferred a significantly poorer chance of survival in acute promyelocytic leukemia patients who have become negative for the *PML-RARα* fusion transcript after 3 courses of intensive consolidation therapy. (Blood. 2007;110:59-66)

Introduction

The use of all-*trans* retinoic acid (ATRA) has markedly improved the therapeutic outcome in patients with acute promyelocytic leukemia (APL).[1-3] However, most patients treated with ATRA alone after achievement of complete remission (CR) eventually relapse, indicating that postremission chemotherapy is essential to obtain long-term survival.[2,3] Noncross-resistance between ATRA and chemotherapeutic drugs has contributed to not only a high CR rate but also a decrease in the relapse rate, leading to a significant improvement in disease-free survival (DFS) and overall survival (OS) rates.[4-11] Despite the impact of ATRA in the treatment of APL, approximately 10% to 30% of patients who were given intensive chemotherapy after achievement of CR still experienced relapse in several cooperative group studies.[5,7-12]

Before the introduction of ATRA in the treatment of APL, the efficacy of maintenance chemotherapy had been observed in patients with APL.[13,14] In our previous study, the Japan Adult Leukemia Study Group (JALSG) APL92 study, patients with newly diagnosed APL received intensified maintenance therapy according to an earlier result of the AML87 study, which was performed before the use of ATRA.[5] The AML87 study showed a significantly better DFS in patients who received 12 courses of intensified maintenance chemotherapy compared with those administered 4 courses of the same chemotherapy.[15] However, it is not clear whether maintenance chemotherapy actually prevents relapse in APL patients treated with ATRA and chemotherapy, especially after they have become negative for the *PML-RARα* transcript at the end of intensive consolidation chemotherapy. If short-term therapy without maintenance shows DFS rates identical to those for long-term therapy with maintenance, it would be beneficial for patients' quality of life as well as for medical costs. To determine the value of intensified maintenance chemotherapy, this study was

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designed to compare DFS and survival in previously untreated APL patients who had become negative for *PML-RARα* transcript after 3 courses of intensive consolidation and were randomly allocated to either intensified maintenance chemotherapy or observation.

Patients and methods

Eligibility

Adult patients with previously untreated de novo APL were consecutively registered in the JALSG APL97 study. Eligible criteria were a diagnosis of APL with t(15;17) and/or the *PML-RARα* fusion gene; age from 15 to 70 years; Eastern Cooperative Oncology Group performance status between 0 and 3; and sufficient function of the heart (no severe abnormalities detected on ECGs and echocardiographs), lung ($PaO_2 > 60$ mm Hg or $SpO_2 > 93\%$), liver (serum bilirubin level < 2.0 mg/dL), and kidney (serum creatinine level < 2.0 mg/dL). The study was approved by the Institutional Review Boards at each participating institution. Written informed consent was obtained from all patients before registration in accordance with the Declaration of Helsinki.

Assessment of disease

Morphologic diagnosis of APL was made according to the French-American-British classification and the bone marrow smears were centrally reviewed at the JALSG pathology committee. The diagnosis was confirmed by the presence of t(15;17) and/or the *PML-RARα* fusion gene. Bone marrow samples were obtained at diagnosis, after induction therapy, after each cycle of consolidation chemotherapy, and periodically during maintenance chemotherapy. The *PML-RARα* fusion gene was amplified using bone marrow samples obtained at diagnosis and after consolidation therapy by reverse-transcriptase polymerase chain reaction analysis.[16,17] The detection limit of *PML-RARα* fusion transcript in this assay was 10^{-4}.

Treatment regimens

Induction therapy. Treatment was started as soon as a morphologic diagnosis of APL had been made. For remission induction therapy, patients received 45 mg/m²/d of ATRA orally divided into 3 doses given after meals daily until the day before the start of the first consolidation therapy. If patients had leukocyte counts below 3.0×10^9/L and APL cells below 10^9/L at the start of therapy, they were treated with ATRA alone (group A). ATRA at the same dosage combined with idarubicin (12 mg/m²/d by 30-minute intravenous infusion on days 1 and 2) plus cytarabine (Ara-C) (80 mg/m²/d by continuous intravenous infusion on days 1 through 5) was given to patients with initial leukocyte counts between 3.0×10^9/L and 10.0×10^9/L, and those with leukocyte counts below 3.0×10^9/L and APL cells above 10^9/L (group B). Patients with initial leukocyte counts of 10.0×10^9/L or more received idarubicin (12 mg/m² on days 1 to 3) plus Ara-C (100 mg/m² on days 1 to 5) in addition to ATRA (group C). During treatment with ATRA, if blast and promyelocyte counts in the peripheral blood were more than 10^9/L, an additional cycle of chemotherapy consisting of idarubicin (12 mg/m² for 2 days) and Ara-C (80 mg/m² for 5 days) was given. Patients in groups A and B who received an additional cycle of chemotherapy during induction were designated as groups AD and BD, respectively.

For prevention of bleeding, patients received transfusions of platelets and fresh frozen plasma to maintain platelet counts above 30×10^9/L or more and plasma fibrinogen level above 4.4 μmol/L (150 mg/dL) or more, respectively. If coagulation studies were abnormal, prophylactic use of heparin and/or other antifibrinolysis agents (dalteparin, gabexate mesilate, or nafamostat mesilate) was recommended. When retinoic acid (RA) syndrome occurred, ATRA was discontinued and 20 mg/kg of methylprednisolone was administered by 1-hour intravenous infusions for at least 3 days. RA syndrome was diagnosed in patients with unexplained fever, respiratory distress, weight gain, interstitial pulmonary infiltrate, and pleural or pericardial effusions, as previously described.[18-20] After resolution of the syndrome, ATRA was resumed at the same dosage.

Consolidation therapy. After achieving CR, patients received 3 courses of consolidation chemotherapy. The first consolidation consisted of mitoxantrone (7 mg/m²) by 30-minute intravenous infusion on days 1 to 3, and Ara-C (200 mg/m²) by continuous infusion on days 1 to 5. The second consolidation contained Ara-C (140 mg/m²) for 5 days, etoposide (100 mg/m²) by 1-hour intravenous infusion for 5 days, and daunorubicin (50 mg/m²) by 30-minute infusion on days 1 through 3. The third consolidation consisted of Ara-C (140 mg/m²) for 5 days and idarubicin (12 mg/m²) for 3 days. Each consolidation course was given after recovery from the previous course, when polymorphonuclear cells were 1.5×10^9/L or more and platelets were 100×10^9/L or more. All patients received an intrathecal administration of methotrexate (MTX)(15 mg), Ara-C (40 mg), and prednisolone (10 mg) at the end of the second consolidation therapy.

Intensified maintenance chemotherapy. After completion of consolidation therapy, patients negative for the *PML-RARα* transcript were randomly allocated either to receive 6 courses of intensified maintenance chemotherapy every 6 weeks or to observation. Randomization was stratified by age and initial leukocyte count, both of which were prognostic factors for DFS in the JALSG APL92 study.[21] The first course of intensified maintenance therapy consisted of behenoyl Ara-C (BHAC) (170 mg/m², 2-hour infusion, days 1 through 5), daunorubicin (30 mg/m², 30-minute infusion, days 1 and 4) and mercaptopurine (6MP; 70 mg/m², orally, days 1 through 7). The second consisted of BHAC and mitoxantrone (5 mg/m², 30-minute infusion, days 1 and 2). The third consisted of BHAC, etoposide (80 mg/m², 1-hour infusion, days 1, 3, and 5), and vindesine (2 mg/m², bolus infusion, days 1 and 8). The fourth consisted of BHAC, aclarubicin (14 mg/m², 30-minute infusion, days 1 through 4), and 6MP. The fifth and sixth courses were the same as the first and third, respectively. Patients who were positive for the *PML-RARα* fusion transcript at the end of consolidation chemotherapy received late ATRA therapy (45 mg/m²/day, orally after meals for 4 weeks) followed by maintenance therapy. These patients were also scheduled to receive allogeneic hematopoietic stem cell transplantation (HSCT) if there was a human leukocyte antigen-identical donor.

Definition and study end points

Hematologic response was evaluated by standard criteria generally used for chemotherapy.[22,23] CR was defined as less than 5% of blasts and promyelocytes with normal erythropoiesis, thrombopoiesis, and granulopoiesis in the bone marrow, and neutrophil counts of more than 1.5×10^9/L and platelet counts of more than 100×10^9/L in the peripheral blood. Hematologic relapse was defined as the presence of more than 10% blasts plus abnormal promyelocytes in the marrow or the presence of any those cells in the peripheral blood or extramedullary sites. In addition, molecular relapse detected by the reverse-transcriptase polymerase chain reaction analysis of *PML-RARα* was also considered as a relapse event.

The primary end point of this study was survival and DFS of patients in CR who had become negative for the *PML-RARα* fusion transcript after the consolidation therapy and who were registered in the randomized study of the maintenance chemotherapy. OS for all patients was calculated from the first day of therapy to death or last visit. DFS for patients who had achieved CR was measured from the date of CR to relapse, death from any cause, or last visit. Survival and DFS in patients who were randomized to either observation or maintenance chemotherapy groups were measured from the date of random assignment to the same end points of these mentioned.

Statistical analyses

Baseline characteristics of the 2 randomized groups were compared using the chi-square test or Fisher exact test for categorical data, and the Wilcoxon rank-sum test for continuous data. Probabilities of survival and DFS were estimated using the Kaplan-Meier method and compared by the log-rank test. The follow-ups on these patients were updated on September 30, 2004. Patients who were lost to follow-up or were still alive at the time of data cutoff were censored at the last date they were known to be alive. Patients who underwent HSCT were also censored at the date of HSCT. Factors affecting survival and DFS were analyzed by the use of the Cox regression model to estimate a hazard ratio with 95% confidence intervals (CI). All analyses were performed according to the intent-to-treat principle.

All statistical tests were 2-sided, and the significance level was set at .05. Statistical analyses were performed using SAS 8.2 (SAS Institute Japan, Tokyo, Japan).

Results

Patient characteristics

Between May 1997 and June 2002, 302 patients from 92 institutes participating in the JALSG were consecutively enrolled in the study. Of these, 19 were excluded because 4 were misdiagnosed, 2 were not consistent with the eligibility criteria, 7 were negative for t(15;17) or *PML-RARα*, and 6 had no test for t(15;17) or *PML-RARα*. Early death was not excluded, although 5 patients died of hemorrhage within 7 days. The characteristics of the 283 evaluable patients are listed in Table 1. Ages ranged from 15 to 70 years, with a median of 48 years. Eighteen patients (6%) had a variant form of French-American-British morphology (M3v). The median leukocyte count was 1.7×10^9/L (range, 0.03 to 257×10^9/L) on admission. One hundred fifty-one patients started on ATRA alone during induction, and in 66 of these, chemotherapy was later added because of increased blasts and promyelocytes according to the protocol (groups A and AD; Table 1). One hundred twenty-five patients received both ATRA and chemotherapy from the beginning of therapy (groups B and C), and in 4 of group B an additional cycle of chemotherapy was later added because of increased blasts and promyelocytes (group BD).

Treatment outcome. Of the 283 evaluable patients, 267 (94.3%) had CR at a median of 42 days (range, 14 to 98) after the start of therapy. During induction therapy, 60 (21%) patients showed signs of RA syndrome and 2 died of the syndrome. In addition, 65 (23%) patients developed organ bleeding, and 9 patients had fatal bleeding, including 5 early deaths within 7 days (Table 2). Thus, early death caused by bleeding was a major cause of induction failure. Although one patient had resistant leukemia, this patient received ATRA for only 16 days because of RA syndrome. Of the 267 patients who achieved CR, 258 (97%) completed the first course of consolidation, 250 (94%) completed the second, and 235 (88%) patients completed the third (Table 2 and Figure 1). After the consolidation, 5 patients underwent allogeneic HSCT at their first CR and 30 patients underwent HSCT after relapse. At a median follow-up of 64 months (range, 27 to 88 months), 60 (22%) of the 267 patients had relapsed and 18 had died. A further 16 (6%) patients died in CR, and 10 of those died of infection during myelosuppression after consolidation therapy (Table 2). The predicted 6-year OS rate in all 283 assessable patients was 83.9% (95% confidence interval [CI], 79.2% to 88.6%; Figure 2A). The predicted 6-year DFS rate in 265 CR cases was 68.5% (95% CI, 62.1% to 74.9%; Figure 2B).

Randomized study with or without intensified maintenance therapy. Among the 235 patients who completed 3 courses of consolidation and were evaluated for minimal residual disease, 5 (2.1%) were positive for the *PML-RARα* fusion transcript. Three of these subsequently relapsed and another patient received allogeneic HSCT. However, 230 patients (97.9%) showed no *PML-RARα* transcript in the bone marrow cells at the end of consolidation. A total of 55 patients negative for *PML-RARα* were not included in the randomized study for a variety of reasons. Of these, 33 patients refused the randomization because 20 did not want to receive

Table 1. Clinical features of patients at diagnosis

Parameters	Total		Maintenance chemotherapy		Observation		P*
	No. (%)	Median (range)	No. (%)	Median (range)	No. (%)	Median (range)	
No. of patients	283		89		86		
Sex							
Male	158 (56)		53 (60)		47 (55)		.51
Female	125 (44)		36 (40)		39 (45)		
Age, years		48 (15-70)		49 (15-70)		46 (16-67)	.70
15-29	49 (17)		17 (19)		15 (17)		
30-49	106 (37)		32 (36)		34 (40)		.88
50-70	128 (45)		40 (45)		37 (43)		
FAB Morphology							
M3	265 (93)		80 (90)		82 (95)		.25
M3v	18 (6)		9 (10)		4 (5)		
Leukocyte count, $\times 10^9$/L		1.7 (0.03-257)		1.9 (0.03-152)		2.1 (0.1-98)	.95
Less than 3.0	174 (61)		50 (56)		47 (55)		
3.0-10.0	58 (20)		21 (24)		20 (23)		.95
10.0 or higher	51 (18)		18 (20)		19 (22)		
Platelet count, $\times 10^9$/L		30 (2-238)		31 (4-230)		23 (2-238)	.10
Less than 10	39 (14)		8 (9)		16 (19)		
10-40	140 (49)		47 (53)		41 (48)		.18
40 or higher	104 (37)		34 (38)		29 (34)		
Induction therapy#							
Group A	85 (30)		29 (33)		29 (34)		1.0
Group AD	66 (23)		17 (19)		16 (19)		
Group B+BD	73 (26)		23 (26)		22 (26)		
Group C	52 (18)		20 (22)		19 (22)		
Unknown	7 (2)		NA		NA		

*Baseline characteristics of the two randomized groups were compared with Chi-squqre test or Wilcoxon rank-sum test.

Patients in Group A were treated with ATRA alone; patients in Groups B and C were treated with ATRA plus idarubicin and cytarabine. Patients in Groups A and B who received an additional cycle of chemotherapy due to increased leukemic cells during induction were designated as Groups AD and BD, respectively. Four patients were in Group BD.

NA indicates not applicable.

Table 2. Events occurring during the induction and consolidation therapy

	Induction	Consolidation 1	Consolidation 2	Consolidation 3
No. of registered patients	283	267	258	250
Death during treatment	13	0	4	6
Infection	1	0	4	6
Bleeding	9	0	0	0
RA syndrome	2	0	0	0
Other	1	0	0	0
Going off study by toxicity	0	3	0	0
Lost to follow-up	2	6	3	7
Relapse	0	0	1	2
Refractory	1	0	0	0
Stem cell transplantation	0	0	0	0
No. of completed patients	267	258	250	235

further therapy and 13 wanted to receive additional chemotherapy; another 13 had residual toxicity from the consolidation and were considered as lack of tolerance to subsequent therapy (10 myelosuppression, 2 general fungal infection, and 1 heart disease); lost to follow-up in 3 patients; and unknown causes or no report in 6 patients. There was no significant difference in the 6-year DFS between 175 patients included in the randomized study (70.8%; 95% CI, 62.7% to 78.8%) and 55 patients not included (76.7%; 95% CI, 65.1 to 88.3%; $P = .87$). The 6-year OS was 92.1% (95% CI, 87.2% to 97.1%) in the patients enrolled in the randomized study and 93.1% (95% CI, 85.3% to 100%) in the patients not enrolled ($P = .97$).

A total of 175 patients who were negative for *PML-RARα* at the end of consolidation were randomly assigned to either observation (n = 86) or intensified maintenance chemotherapy (n = 89; Figure 1). Median interval from the recovery of myelosuppression after the third course of consolidation to the randomization was 20 days in both the maintenance and observation groups ($P = .35$). More than 90% of patients were allocated to either intensified maintenance chemotherapy or observation groups within 2 months after the consolidation. There was no significant difference between the 2 groups in patient profiles, including sex, age, French-American-British morphology, initial leukocyte count, platelet count, and induction therapy (Table 1).

At a median follow-up time of 49 months (range, 24 to 81 months) after randomization, there were 25 (28%) relapses and 13 (15%) deaths among the 89 patients who were allocated to the intensified maintenance chemotherapy. Of the 86 patients who were assigned to the observation, 17 (20%) relapsed and 3 (3%) died. There was no therapy-related mortality during the intensified maintenance chemotherapy. All but 2 patients in the maintenance group died after relapse. In the chemotherapy group, one patient developed therapy-related myelodysplastic syndrome and another developed acute myeloid leukemia during their first CR of APL. By contrast, none of patients in the observation group developed therapy-related leukemia and all 3 patients died after relapse. A second CR was achieved in 13 of 24 (54%) in the chemotherapy group and 13 of 17 (76%) in the observation group ($P = .19$). The predicted 6-year DFS rates were 63.1% (95% CI, 50.2 to 76.0%) for

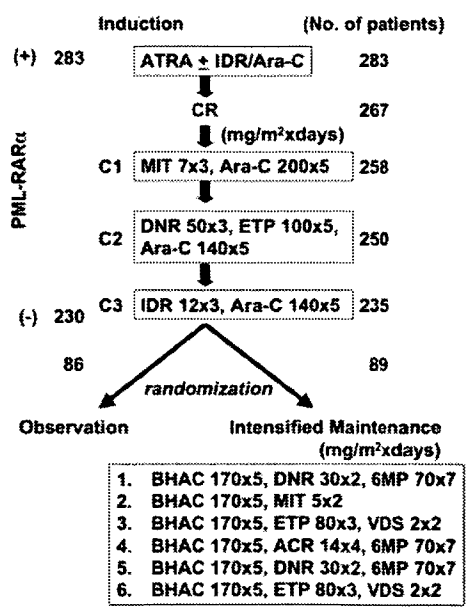

Figure 1. Study design. The number of patients who completed each step is indicated. C1, C2, and C3 were consolidation courses 1, 2, and 3. A total of 283 patients had t(15;17) and/or the *PML-RARα* transcript at the time of diagnosis, and 230 patients were negative for *PML-RARα* at the end of 3 courses of consolidation therapy. After completion of consolidation therapy, 175 patients who showed absence of *PML-RARα* transcript were randomized either to receive 6 courses of intensified maintenance chemotherapy (n = 89) or to observation (n = 86).

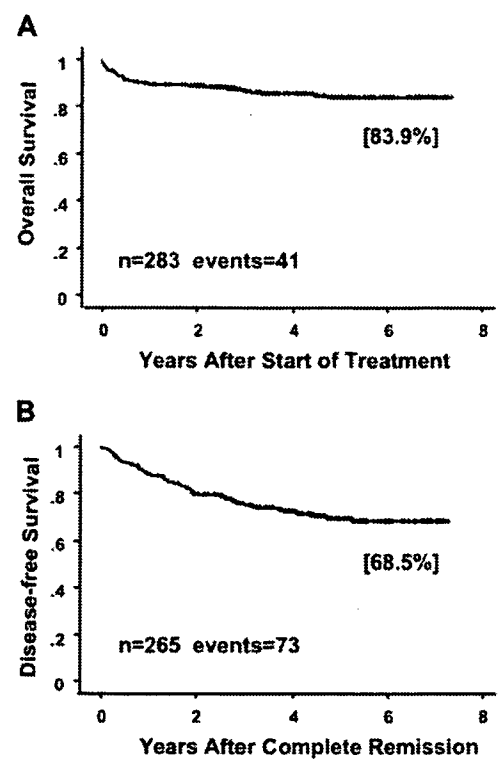

Figure 2. Overall survival and disease-free survival in patients enrolled in the JALSG APL97 study. Overall survival (A) in all assessable patients and disease-free survival (B) in patients who achieved CR are estimated by the Kaplan-Meier method.

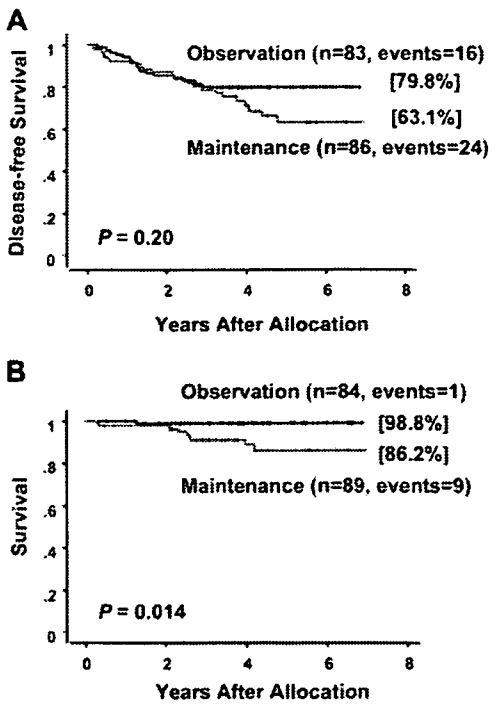

Figure 3. Disease-free survival and survival of randomized patients in the maintenance phase. Disease-free survival (A) and survival (B) are estimated from the date of randomization.

patients assigned to the maintenance chemotherapy and 79.8% (95% CI, 71.0 to 88.7%) for patients assigned to the observation (Figure 3A). No statistically significant difference in DFS was observed in patients treated with or without the maintenance chemotherapy ($P = .20$). In the chemotherapy group, 8 patients showed late relapses occurring after at least 3 years of continuous CR, whereas no patients in the observation group showed a late relapse (Figure 3A; $P = .006$). Univariate analysis showed that an initial leukocyte count of more than 10.0×10^9/L and induction group C trended to be unfavorable prognostic factors for DFS (Table 3). The predicted 6-year survival in the observation group was 98.8% (95% CI, 96.3 to 100%), which was significantly higher than 86.2% (95% CI, 77.3 to 95.0%) in the intensified maintenance group ($P = .014$; Figure 3B). Univariate analysis revealed that induction group C and maintenance chemotherapy were significant unfavorable prognostic factors for survival (Table 4). Patients with initial leukocyte counts above 10.0×10^9/L showed a trend toward unfavorable DFS and survival, although this cohort was small (Figure 4A,B).

Discussion

The present randomized study demonstrated that intermittent intensified maintenance chemotherapy did not improve DFS, but rather worsened survival in patients with newly diagnosed APL who had become negative for the *PML-RARα* fusion transcript at the end of consolidation therapy.

In this study, ATRA and chemotherapy resulted in a high CR rate, improved OS, and DFS in patients with previously untreated APL. In our previous APL92 study, in which ATRA was used for the first time in the JALSG studies to newly diagnosed APL, the combination of ATRA plus chemotherapy induced CR in 333 of 369 (90%) assessable patients.[24] The 6-year OS rate of all evaluable patients and the 6-year DFS rate of CR cases in the APL92 study were 65% and 59%, respectively. In both APL92 and APL97 studies, patients received ATRA only in the induction phase. Therefore, the improvement of OS and DFS in the present study can mostly be attributed to the intensification of chemotherapy during induction and consolidation. In the present study, idarubicin and Ara-C were used instead of daunorubicin and BHAC in the induction, and one of the anthracyclines in combination with Ara-C was given in each consolidation.[25] Thus, the OS and DFS appear to depend on the intensities of chemotherapy in the treatments of APL. The high sensitivity of APL to anthracyclines is well-documented by several cooperative groups.[26,27] In addition, there was a hypothesis that an anthracycline alone may be as effective as combinations of anthracycline and Ara-C.[8,27,28] However, the interim analysis of the European APL2000 study showed

Table 3. Effects of factors on disease-free survival

Parameters	No. of patients	No. of relapses	Univariate analysis HR (95%CI)	P*
Sex				
Female	100	16	1	
Male	75	26	1.4 (0.7-2.7)	.29
Age, years				
15-50	98	24	1	
50-70	77	18	0.9 (0.5-1.7)	.75
Leukocyte count, × 10^9/L				
Less than 10.0	138	29	1	
10.0 or higher	37	13	1.7 (0.9-3.5)	.12
Platelet count, × 10^9/L				
Less than 40	112	25	1	
40 or higher	63	17	1.4 (0.7-2.6)	.32
Induction therapy				
Group A	58	10	1	
Group AD	33	6	1.0 (0.3-2.9)	.97
Group B, BD	45	12	1.8 (0.8-4.1)	.17
Group C	39	14	2.3 (1.0-5.3)	.05
Maintenance chemotherapy				
No maintenance	86	17	1	
Maintenance	89	25	1.5 (0.8-2.8)	.20

*Factors affected on disease-free survival were analyzed by the Cox hazard regression model.

Table 4. Effects of factors on survival

Parameters	No. of patients	No. of deaths	Univariate analysis HR (95%CI)	P*
Sex				
Female	100	12	1	
Male	75	4	2.1 (0.6-8.3)	.27
Age, years				
15-50	98	8	1	
50-70	77	8	2.6 (0.7-10)	.16
Leukocyte count, × 10^9/L				
Less than 10.0	138	11	1	
10.0 or higher	37	5	2.8 (0.8-10)	.11
Platelet count, × 10^9/L				
Less than 40	112	12	1	
40 or higher	63	4	0.2 (0.03-1.8)	.16
Induction therapy				
Group A	58	1	1	
Group AD	33	4	3.8 (0.3-41)	.28
Group B, BD	45	5	2.8 (0.3-31)	.4
Group C	39	6	8.9 (1.0-76)	.05
Maintenance chemotherapy				
No maintenance	86	3	1	
Maintenance	89	13	8.6 (1.1-68)	.04

*Factors affected on survival were analyzed by the Cox hazard regression model.

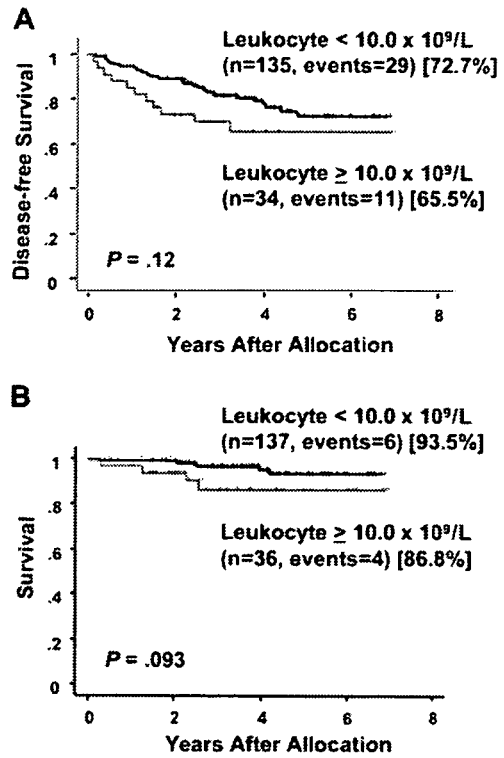

Figure 4. Disease-free survival and survival by initial leukocyte count. Disease-free survival (A) and survival (B) in patients with initial leukocyte counts above or below 10.0 × 10^9/L are estimated from the date of randomization.

the efficacy of Ara-C in induction and consolidation even in patients with leukocyte counts of less than 10.0 × 10^9/L.[29] Therefore, despite significant improvement of therapeutic outcome in APL, concern still exists regarding which is the best chemotherapeutic strategy for APL.

PML-RARα generated by t(15;17) provides the most clinically relevant information in patients with APL.[2,3,16,17] A number of patients who achieve molecular remission assessed by reverse-transcriptase polymerase chain reaction for *PML-RARα* after consolidation are predicted to obtain a long-term survival.[7,30] However, detection of *PML-RARα* identifies patients at risk for relapse after consolidation. In addition, treatment of patients at the time of molecular relapse provides a survival advantage compared with treatment at overt hematologic relapse.[31] In this study, 5 of 235 (2.1%) patients showed the *PML-RARα* fusion transcript after the consolidation therapy, and 3 of these relapsed subsequently. In contrast, 97.9% of patients were negative for *PML-RARα* transcript. In the GIMEMA-AIEOP study, 646 of 664 (97.3%) patients were negative for the *PML-RARα* fusion transcript at the end of consolidation.[32] Because approximately half of APL patients are molecularly positive after induction,[7,9] elimination of *PML-RARα* positive cells might be associated with intensive consolidation chemotherapy.

Our present results showed no benefit of moderately intensive and intermittent chemotherapy in the maintenance phase. This result is consistent with an earlier GIMEMA study before the availability of ATRA, in which patients randomized to maintenance therapy with low-dose 6MP and MTX did not have better outcomes than those randomized to the observation.[27] However, the North American Intergroup trial showed a benefit for ATRA in both induction and maintenance therapy.[6,12] In addition, the European APL93 study revealed that maintenance therapy with a combination of low-dose chemotherapy (6MP and MTX) and intermittent ATRA reduced the incidence of relapse.[10] However, the recent GIMEMA-AIEOP study documented no difference in DFS in patients treated with maintenance consisting of either ATRA, 6MP/MTX, ATRA plus 6MP/MTX, or observation.[32] Therefore, the role of maintenance chemotherapy in the treatment of APL remains to be determined. Because intensified maintenance chemotherapy in this study is apparently different from the continuous maintenance with low-dose 6MP and MTX, comparison with other studies of maintenance is difficult. It is very likely that the usefulness of maintenance therapy depends on the intensity of chemotherapy delivered during induction and consolidation phases. In the US Intergroup and European APL93 studies, patients were treated with only 2 cycles of consolidation,[6,10] whereas patients received 3 cycles of consolidation both in the GIMEMA-AIEOP and our studies.[32] Recently, we did not find a benefit for intensified maintenance therapy in patients with acute myeloid leukemia other than APL treated with intensive consolidation therapy.[33] Our present study confirms that there is no beneficial effect of intensified maintenance chemotherapy in previously untreated APL patients who have become negative for the *PML-RARα* fusion transcript at the end of consolidation. In addition, there was a trend toward better DFS in patients with no maintenance chemotherapy. Patients in the maintenance chemotherapy group showed a significant number of late relapses occurring after at least 3 years of continuous CR compared with the observation group. This was a quite unexpected finding for us. Although the limited number of patients prohibits a robust conclusion, we speculated that intensified maintenance chemotherapy may impair potential immune surveillance to eradicate minimal residual leukemic cells in patients with molecularly undetectable residual leukemia. Further studies are required to investigate whether ATRA has a role in maintenance. The current JALSG APL204 study compares the efficacy of ATRA versus tamibarotene (Am80) in the maintenance phase.

It is interesting to note that patients assigned to the observation group showed a significantly better survival than those randomized to the maintenance group. Because the difference in DFS was not statistically significant and there was no chemotherapy-related death in the latter group, the difference in survival is thought to result from the difference in the second CR rates and CR durations. Although APL cells usually lack p-glycoprotein expression, multidrug resistance is generally acquired by the use of antileukemic agents.[34] As the chemotherapy in the maintenance phase of this study mainly consisted of one of the anthracyclines and BHAC, accumulation of chemotherapeutic agents in patients in the maintenance group may induce drug resistance to additional chemotherapy. In addition, accumulated chemotherapy may induce an overall increased toxicity and lack of tolerance to subsequent therapy after relapse. Furthermore, it is of note that 2 patients in the maintenance group died of therapy-related leukemia in the first CR of APL. Occurrence of therapy-related leukemia in patients treated for APL is an emerging problem.[35] Chemotherapeutic agents in the maintenance phase seem to increase the risk of therapy-related leukemia.

Although APL has become the most curable subtype of acute leukemia in adults, approximately 20% of patients still die of the disease because of early death or relapse.[2,3] One of the unfavorable prognostic factors for DFS and survival, in the present study as well as in our previous and other studies, was high initial leukocyte count.[9,21,36] In this study, the stratification by intensities of chemotherapy in the induction phase failed to improve DFS in patients with high initial leukocyte count (group C). Thus, patients with

high leukocyte count will require an alternative approach to obtain long-term survival. Use of arsenic trioxide, Am80, and/or gemtuzumab ozogamicin during the front-line therapy may improve DFS and OS in these patients at high risk.[37-41]

In conclusion, we did not find any beneficial effect of intensified maintenance chemotherapy in patients negative for *PML-RARα* at the end of consolidation chemotherapy. On the contrary, intensified maintenance chemotherapy unexpectedly conferred a significantly poor survival as well as an increased risk of therapy-related leukemia in these patients.

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Authorship

Contribution: N.A., S.O., T.N., and R.O. participated in the study design, analysis of the experiments, and writing of the manuscript. Y.K., H.K., M.O., Y.K., M.T., K.H., M.M., K.S., T.K., M.N., M.T., F.Y., A.T., and Y.K. were significant clinical contributors to the trial and have reviewed the manuscript. M.I. collected the data and performed statistical analysis.

A complete list of the Japan Adult Leukemia Study Group is provided in Document S1 as a data supplement to the online version of this article.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Concurrent transcriptional deregulation of AML1/RUNX1 and GATA factors by the AML1-TRPS1 chimeric gene in t(8;21)(q24;q22) acute myeloid leukemia

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Brief report

Concurrent transcriptional deregulation of AML1/RUNX1 and GATA factors by the *AML1-TRPS1* chimeric gene in t(8;21)(q24;q22) acute myeloid leukemia

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The Runt domain transcription factor AML1/RUNX1 is essential for the generation of hematopoietic stem cells and is the most frequent target of chromosomal translocations associated with leukemia. Here, we present a new *AML1* translocation found in a patient with acute myeloid leukemia M4 with t(8;21)(q24;q22) at the time of relapse. This translocation generated an in-frame chimeric gene consisting of the N-terminal portion of *AML1*, retaining the Runt domain, fused to the entire length of *TRPS1* on the C-terminus. *TRPS1* encodes a putative multitype zinc finger (ZF) protein containing 9 C2H2 type ZFs and 1 GATA type ZF. AML1-TRPS1 stimulated proliferation of hematopoietic colony-forming cells and repressed the transcriptional activity of AML1 and GATA-1 by 2 different mechanisms: competition at their cognate DNA-binding sites and physical sequestrations of AML1 and GATA-1, suggesting that simultaneous deregulation of AML1 and GATA factors constitutes a basis for leukemogenesis. (Blood. 2007;109:4023-4027)

Introduction

AML1/RUNX1 encodes the DNA-binding α subunit of the heterodimeric transcription factor PEBP2/CBF, which interacts with the partner β subunit (PEBP2β/CBFβ) through its evolutionarily conserved Runt domain.[1] *AML1* is one of the most frequently mutated genes in human leukemia,[2-4] and was originally identified as a gene on chromosome 21 involved in t(8;21)(q22:q22).[5] To date, 11 *AML1*-related translocations are known that produce chimeric proteins such as AML1-MTG8/ETO in t(8;21), AML1-EVI1 in t(3;21), and TEL-AML1 in t(12;21).[2,6,7] All these AML1 chimeric proteins retain the Runt domain and inhibit transcriptional activity of wild-type AML1 in a dominant-negative manner.[2-4] However, the functional contributions of partner moieties in leukemogenesis remain largely undetermined. Here, we report a new *AML1* translocation, t(8;21)(q24;q22), in a patient with acute myeloid leukemia (AML), and present results from functional analyses of the chimeric protein AML1-TRPS1.

Patient, materials, and methods

Patient profile

A 56-year-old Japanese man was diagnosed with AML M4 with a normal karyotype in October 1997. He was treated with idarubicin and cytarabine, followed by postremission therapy according to the Japan Adult Leukemia Study Group (JALSG) AML97.[8] In July 1999, his marrow showed 55.6% blasts with t(8;21)(q24;q22) at relapse. Bone marrow cells at diagnosis and relapse showed the similar morphology and immunophenotypes positive for CD13, CD33, CD4, and HLA-DR, but negative for CD34, which were consistent with AML M4.[9] The study was approved by the Institutional Review Board of Kumamoto University School of Medicine, Japan, and informed consent was obtained from the patient, according to the Declaration of Helsinki.

Molecular cloning

The fusion partner gene was cloned by long-distance 3′ rapid amplification of cDNA ends (RACE) using the SMART RACE kit (Clontech Labs, Mountain View, CA).

Plasmid constructions

The cloned fusion gene *AML1-TRPS1* was inserted into the pEF-Bos expression plasmid[10] or MIG retroviral vector.[11] Mutant constructs were generated by polymerase chain reaction (PCR)–based site-directed mutagenesis.

EMSA

Electrophoretic mobility shift assays (EMSAs) were performed using biotin-labeled probes containing the AML1[12] or GATA factor-binding sites.[13] The specificity of the probes are shown in Figure S1, available on the *Blood* website (see the Supplemental Figure link at the top of the online article). Whole-cell extracts of COS7 cells (1×10^6) transfected with pEF-Bos expression vectors were subjected to the assay.

Transcription assay

The luciferase reporter constructs pBXH2-LTR-luc and pRBGP3-MαP were used to assay the transcriptional activities mediated by AML1 and GATA-1, respectively. pBXH2-LTR-luc contains the long terminal repeat (LTR) of the BXH2 retrovirus[11] that includes a functional AML1 site, while

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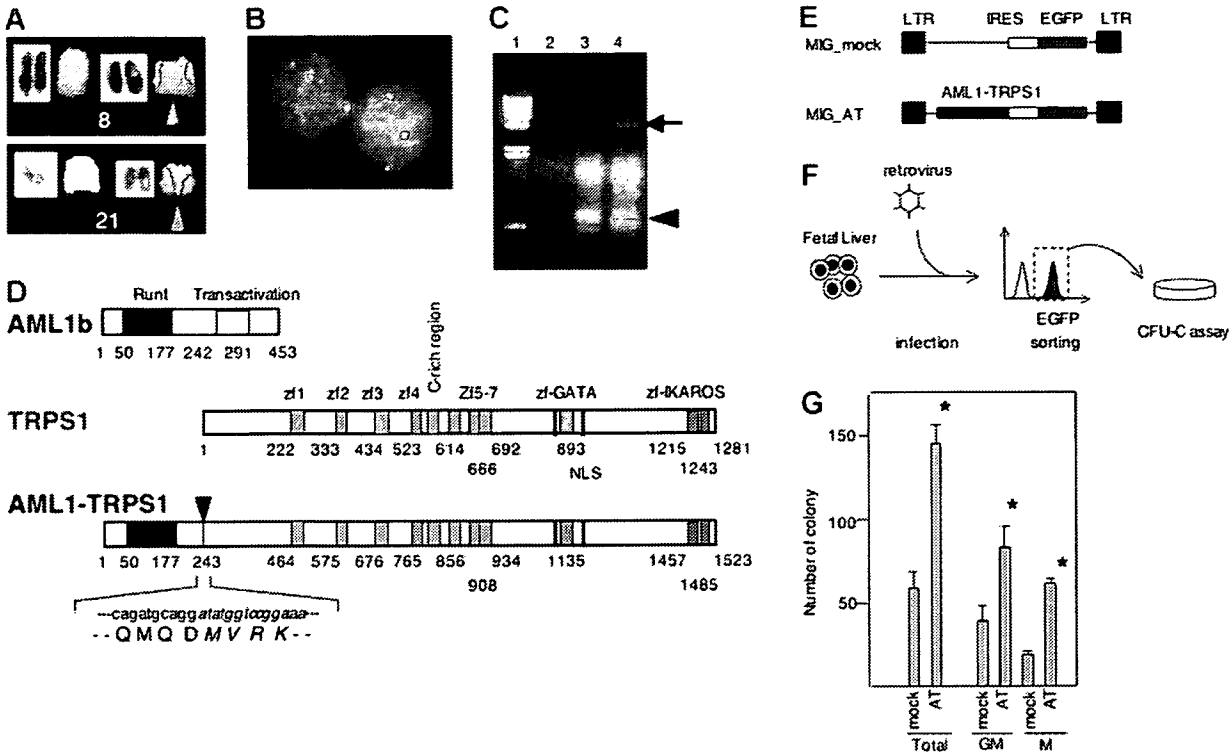

Figure 1. Cloning of the *AML1-TRPS1* that stimulates proliferation. (A) Spectral karyotyping (SKY) showed the balance translocation between chromosomes 8 and 21 (arrowheads). Representative SKY of chromosomes 8 and 21 is indicated as reverse DAPI (left) and SKY (right). (B) Fluorescence in situ hybridization using cosmid clones covering the whole *AML1* gene showed that t(8;21)(q24;q22) involved the *AML1* gene. Nearly half (55 [48.2%] of 114) of the cells had 3 signals of the *AML1* gene. Images were acquired within an SD200 spectral imaging system (Applied Spectral Imaging, Migdal Haemek, Israel) attached to an Optiphot-2 epifluorescence microscope (Nikon, Tokyo, Japan) through a Splan Apo 100×/1.4 NA oil objective lens (Olympus, Tokyo, Japan) and analyzed with SkyView software (Applied Spectral Imaging). (C) The fusion partner gene with AML1 was cloned by long-distance 3′ RACE with 5′ primers on the *AML1* exon 5 (AML1S5: 5′-cacagtggatgggccccgagaacctcg-3′) or exon 6 (AML1S6: 5′-tgcggcgcacagccatgagggtcagc-3′). The arrowhead indicates AML1a; the arrow shows the fusion gene. Lane 1, λ/*Hin*dIII DNA marker; lane 2, Human placental DNA; lane 3, AML cells (AML1S5); lane 4, AML cells (AML1S6). (D) N-terminal portion of *AML1* retaining the Runt domain fused to the whole of *TRPS1*. An in-frame fusion gene consists of exon 6 of *AML1* and *TRPS1*. In addition, sequencing analyses of subclones showed another transcript between exon 5 of *AML1* and *TRPS1*, indicating skipping of exon 6 of *AML1* and generating another in-frame fusion gene. The reciprocal TRPS1-AML1 transcript was not detected in the presenting case. (E) Structures of retrovirus constructs for control (MIG_mock) or AML1-TRPS1 (MIG_AT). AML1-TRPS1 was inserted into the indicated position of the plasmid MIG with internal ribosomal entry site (IRES) and the enhanced green fluorescent protein (EGFP) gene. (F) Schematic depiction of the CFU-C assay. Fetal liver cells from embryonic day–14.5 (E14.5) mouse embryo were infected with the MIG vector. EGFP-positive cells were sorted and subjected to the CFU-C assay, supplemented with interleukin-3, stem cell factor, erythropoietin, and granulocyte colony-stimulating factor, as described previously.[11] (G) The number of all kinds of colony (total), granulocyte-macrophage (GM), and macrophage (M) are shown. Error bars indicate standard deviations for 3 independent experiments. Differences between AML1-TRPS1 (AT) and control (mock) transfectants were statistically significant (*$P < .001$, unpaired student *t* test) in all 3 categories.

the mouse α-1 globin gene promoter in pRBGP3-MαP[14] contains a functional GATA site. The specificity of AML1 and GATA site-dependent activation of each reporter is shown in Figure S2.

Retroviral transduction and CFU-C assay

Retroviral transduction, sorting by FACS Vantage (Becton Dickinson, San Jose, CA), and colony-forming unit-culture (CFU-C) assay were performed as previously described.[11]

Immunoprecipitation

Immunoprecipitation of FLAG-tagged fusion proteins was performed according to the manufacturer's protocol (Sigma, St Louis, MO).

Results and discussion

Spectral karyotyping of the marrow cells at relapse confirmed a balanced translocation between chromosomes 8 and 21 (Figure 1A). Fluorescence in situ hybridization using cosmid clones containing the *AML1* gene showed that this late-appearing translocation involved the *AML1* gene (Figure 1B).[15] We cloned the fusion partner gene with *AML1* exon 6 by using 3′ RACE (Figure 1C). The t(8;21)(q24;q22) generated an in-frame chimeric gene that encoded a fusion protein consisting of the N-terminal portion of AML1, retaining the Runt domain but lacking its C-terminal transactivation domain, fused to the entire TRPS1/GC79 (Figure 1D). *TRPS1* on chromosome 8q24.1 encodes a nuclear transcription factor with 10 zinc finger (ZF) domains, including a single GATA-type ZF.[13,16,17] The gene is widely expressed in human tissues, including prostate, testis, ovary, kidney, lung, mammary gland, and hematopoietic cells.[16,17] *TRPS1* has been identified as a disease gene for tricho-rhino-phalangeal syndrome (TRPS) type I and type III (MIM190350 and MIM190351), which is a dominantly inherited disease characterized by craniofacial and skeletal abnormalities.[16]

To investigate whether the fusion gene *AML1-TRPS1* affects proliferation and differentiation of hematopoietic cells, we introduced AML1-TRPS1 into mouse fetal liver cells and carried out a CFU-C assay (Figure 1E-F). As shown in Figure 1G, *AML1-TRPS1* transfectants gave rise to a higher number of colonies than did the control transfectants, indicating that AML1-TRPS1 stimulated the proliferation of immature hematopoietic cells.

Since AML1-TRPS1 contains the Runt and GATA ZF domains, we evaluated the DNA-binding ability of AML1-TRPS1 to both

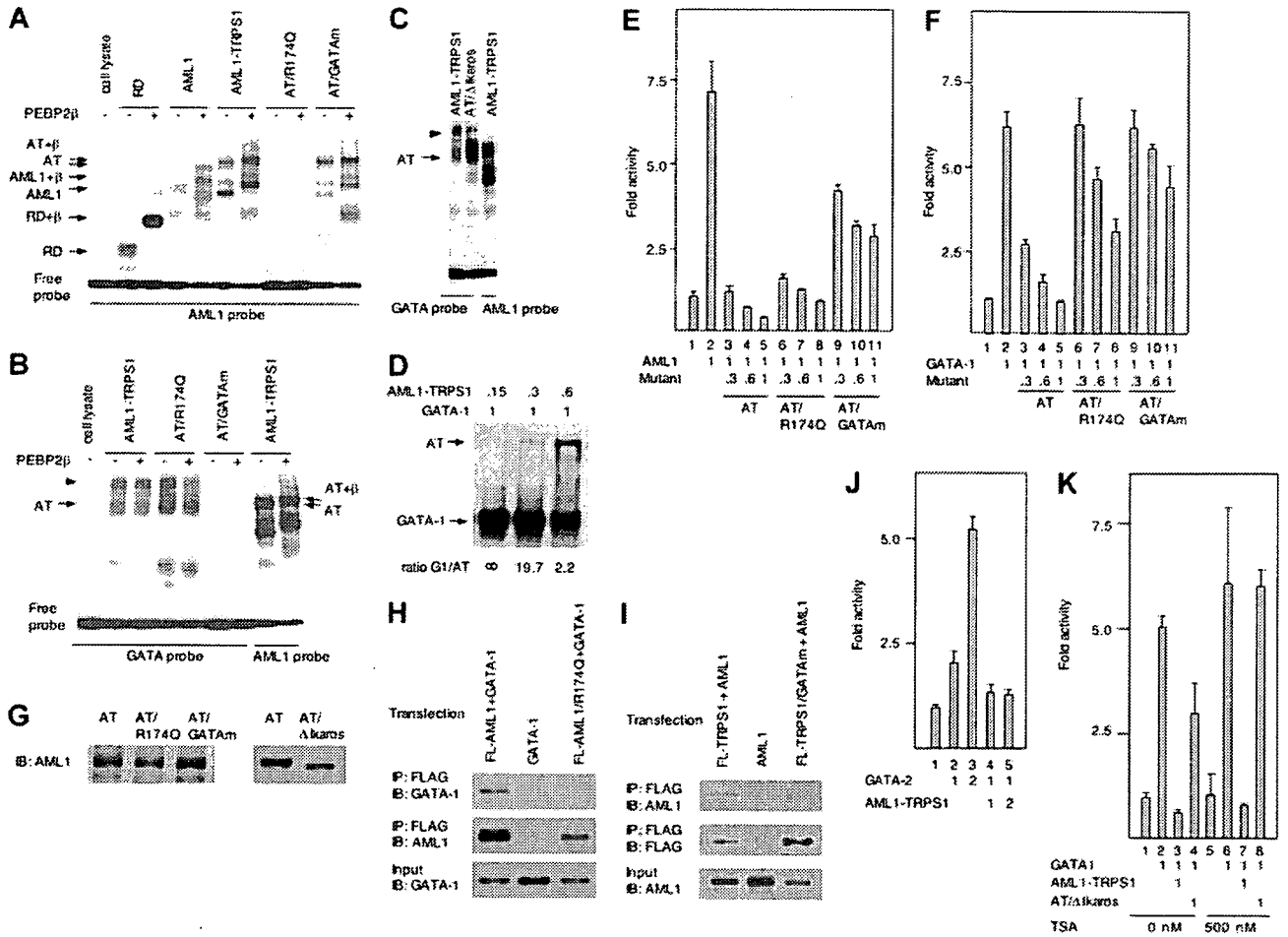

Figure 2. Functional analysis of AML1-TRPS1. (A-B) AML1-TRPS1 and their mutants were subjected to EMSA in the presence (+) or absence (−) of PEBP2β/CBFβ. RD and AT indicate the Runt domain and AML1-TRPS1, respectively. RD and AML1 served as positive controls. The position of the indicated factor or complex with DNA is shown. Mutant constructs of AML1-TRPS1 were generated by PCR-based mutagenesis, using the following primers: AT/R174Q, 5′-gtggatgggccccaagaacctcgaagaca-3′; and AT/GATAm, 5′-ggatatgtaggcaacgcgggtggcctctacc -3′. (C) AML1-TRPS1 seems to form a homodimer through the Ikaros-like ZF domain. The deletion of Ikaros-like ZF (AT/ΔIKAROS) resulted in a decrease in the upper band (arrowhead), which might indicate the homodimer of AML1-TRPS1, and an increase in the lower band (AT, AML1-TRPS1 monomer). The AT/ΔIKAROS mutant was made by PCR-based mutagenesis using 5′-gaagtactcaagatgaactttcaacataatgtgrgcactgtggc-3′ as a primer. (D) A fixed amount of GATA-1 (1 U) and increasing relative amounts (0.15, 0.3 and 0.6 U) of AML1-TRPS1 were subjected to EMSA. Relative amounts of expressed proteins were evaluated by Western blotting. (E-F) Wild-type AML1, GATA-1, AML1-TRPS1, or its mutants were cotransfected with pBXH2-LTR-luc or pRBGP3-MαP reporter at varying relative doses, as indicated, into NIH3T3 cells by the nonliposomal transfection reagent FuGENE6 (Roche Applied Science, Basel, Switzerland). Luciferase activities are expressed as fold changes relative to the control transfected with the backbone expression vector alone. The total input of plasmid DNAs was kept constant (0.6 μg) by supplementing appropriate amounts of the backbone pEF-Bos plasmid so as to avoid potential artifacts due to uneven overall DNA dosages. Error bars indicate standard deviations for 3 independent experiments. (G) Expression levels of AML1-TRPS1 and its mutants were comparable with each other. COS7 cells were transfected with expression plasmids for the indicated genes, and whole-cell extracts were prepared 48 hours after transfection and subjected to Western blotting using rabbit polyclonal anti-AML1 antibody (Active Motif, Carlsbad, CA). (H-I) The Runt domain of AML1 physically interacted with GATA-1 (H), while GATA ZF of TRPS1 interacted with AML1 (I) in immunoprecipitation (IP) assays. COS7 cells were cotransfected with pEF-Bos expression vectors for FLAG-tagged AML1 (FL-AML1), FLAG-tagged AML1 mutant R174Q (FL-AML1/R174Q), and GATA-1, or FLAG-tagged TRPS1 (FL-TRPS1), FLAG-tagged TRPS1 GATA ZF mutant (FL-TRPS1/GATAm), and AML1, as indicated. Cell lysates were immunoprecipitated using anti-FLAG antibody (M2 monoclonal antibody; Sigma). Immunoprecipitates were detected by immunoblotting (IB) using rat monoclonal anti-GATA1 antibody (N6; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-AML1 antibody (Active Motif), or anti-FLAG antibody. (J) AML1-TRPS1 inhibits GATA-2–mediated transcription. GATA-2 and AML1-TRPS1 were cotransfected with MαP reporter at the indicated doses, into HL-60 cells. Luciferase activity is expressed as fold changes relative to the control. (K) HDAC inhibitor TSA did not greatly affect the repression mediated by intact AML1-TRPS1, but dramatically relieved that of AT/Δ Ikaros. GATA-1, AML1-TRPS1, or AT/ΔIKAROS were cotransfected into NIH3T3 cells with the MαP reporter at the indicated doses. Cells were treated with TSA (500 nM) for 36 hours from 12 hours after transfection. Luciferase activity is expressed as fold changes relative to the control. The results represent 3 independent experiments.

AML1 and GATA sites. AML1-TRPS1 bound to the AML1 site and showed heterodimerization activity with the PEBP2β subunit (Figure 2A). AML1-TRPS1 also showed DNA-binding to the GATA site (Figure 2B). However, it did not form a heterodimer with the β subunit at this site. It is interesting to note that AML1-TRPS1 shifted as a doublet of bands with the GATA probe, and the upper band (arrowhead in Figure 2B) was much slower than that of AML1-TRPS1/PEBP2β complex bound to the AML1 probe (right end lane). Since the Ikaros-like ZF in the C-terminal TRPS1 moiety is known to serve as a domain to form homo- or heterodimers of factors containing this motif,[18] the slower migrating band might indicate the formation of homodimer by AML1-TRPS1. In fact, the deletion of the Ikaros-like ZF resulted in a significant decrease in the intensity of the upper band, accompanied by an increase in that of the lower band (Figure 2C).

To assess the effect of AML1-TRPS1 on transcription, we next performed a reporter assay. While AML1 and GATA-1 activated BXH2-LTR and MαP reporters, respectively, AML1-TRPS1 inhibited transactivation activity of both AML1 and GATA-1 in a dose-dependent manner (Figure 2E-F; bars 3-5). AT/R174Q has an amino acid substitution in the Runt domain and resulted in the loss of AML1 site-binding activity (Figure 2A).[19] AT/R174Q showed reduced ability to suppress wild-type AML1 in cotransfection assays (Figure 2E; bars 6-8 vs 3-5), suggesting that AML1-TRPS1

inhibits wild-type AML1 transactivation through DNA binding of its Runt domain. Likewise, AT/GATAm lost its DNA-binding function for the GATA site (Figure 2B) due to changes in 2 amino acids that contribute to its structural integrity,[13] and demonstrated weaker inhibition of wild-type GATA-1 transactivation than did AML1-TRPS1 (Figure 2F; bars 9-11 vs 3-5). As the DNA-binding affinity of AML1-TRPS1 was comparable with that of GATA-1 (Figure 2D), this competition for GATA-binding sites is considered to play a significant role in transcriptional suppression mediated by AML1-TRPS1. However, these DNA-binding mutants still retained significant repressive activity (compare bars 2, 5, and 8 in Figure 2E and bars 2, 5, and 11 in Figure 2F), and AT/R174Q and AT/GATAm even showed decreased suppression in the MαP and BXH2-LTR reporter assays, respectively, in a dose-dependent manner (bars 6-8 in Figure 2F and bars 9-11 in Figure 2E). Since AML1 is known to physically interact with GATA-1 through its Runt domain,[20-22] the chimera may also bind to GATA-1. As expected, in an immunoprecipitation experiment, wild-type AML1 interacted with GATA-1, while the AML1 R174Q mutant did not (Figure 2H). On the other hand, GATA ZF in GATA-1 is one of the domains responsible for the interaction with the Runt domain.[21] It is therefore plausible that GATA ZF in TRPS1 is also capable of interacting with AML1. Indeed, we demonstrated that wild-type TRPS1 bound to AML1, whereas the GATA ZF mutant of TRPS1 did not (Figure 2I). Taken together, we postulate that AML1-TRPS1 compromises AML1 and GATA-1 functions through 2 distinct mechanisms: (1) as a dominant-negative protein competing for their cognate binding sites; and (2) via the physical sequestration of both AML1 and GATA-1 proteins. Collectively, these 2 functions may enable AML1-TRPS1 to simultaneously disrupt AML1 and GATA factor-driven genetic programs. Since AML1-TRPS1 showed the inhibition of GATA-2 mediated transactivation (Figure 2J), the inhibitory mechanism may be extended further to other GATA factors.

In an attempt to further investigate the AML1-TRPS1–mediated transcriptional inhibitory mechanism, we carried out a reporter assay in the presence of histone deacetylase (HDAC) inhibitor trichostatin A (TSA). TSA did not affect greatly the repression mediated by intact AML1-TRPS1 (Figure 2K; bar 3 vs bar 7), but dramatically relieved that of AT/Δ Ikaros (Figure 2K; bar 4 vs bar 8), suggesting that the dimerization of AML1-TRPS1 through Ikaros-like ZF confers potent recruitment of corepressors such as HDAC. AML1-TRPS1 seems to serve as another example confirming the emerging hypothesis that most of the chimeric proteins possess the property of forming a dimer (or a multimer), thereby leading to aberrant transcriptional regulation.[2,23]

Although there is only 1 additional reported patient with AML with t(8;21)(q24;q22) probably carrying AML1-TRPS1,[6,7] a similar fusion, AML1-FOG2, that represses both AML1- and GATA-1–mediated transactivation, was found in a patient with myelodysplastic syndrome.[24] Moreover, the most pervasive fusion gene in AML, *AML1-MTG8*, has also been shown to repress GATA-1 function in addition to its dominant-negative effect on AML1.[20,25] Collectively, the concurrent transcriptional deregulation of AML1 and GATA factors in leukemia seems relatively common. This novel chimeric gene *AML1-TRPS1* could serve as a tool for elucidating the details of the interplay between AML1 and GATA factors and its disruption in leukemogenesis.

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Authorship

Author contributions: N.A. and M. Yanagida contributed equally to this study. N.A. participated in the study design, molecular cloning, and writing of the manuscript. M. Yanagida performed the molecular analyses and wrote the manuscript. L.H. and M. Yamamoto provided vital analysis tools. K.S., H.M., and Y.I. contributed to the writing of the manuscript and discussions on the experimental design. M.O. participated in the study design, analysis of the experiments, and writing of the manuscript. All authors have reviewed the manuscript.

Conflict-of-interest statement: All authors declare no competing financial interests.

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Case Report

Oral Melphalan, Dexamethasone, and Thalidomide for the Treatment of Refractory Multiple Myeloma

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Abstract

We present a patient with refractory multiple myeloma who showed a good response to a combination therapy with oral melphalan, dexamethasone, and thalidomide (MDT). A 48-year-old woman with myeloma refractory to thalidomide, dexamethasone, and clarithromycin received 6 mg melphalan for 4 days every 6 weeks in combination with thalidomide (100 mg daily) and dexamethasone (5 mg daily for 2 days every week). Four months after the initiation of MDT therapy, a 78% reduction of monoclonal protein was achieved. Although the efficacy of oral MDT combination therapy in elderly patients with newly diagnosed myeloma has been reported, the present data demonstrate the effectiveness of MDT therapy for refractory myeloma and warrant further exploration with this MDT regimen to treat myeloma.

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Key words: Multiple myeloma; Melphalan; Dexamethasone; Thalidomide

1. Introduction

Although high-dose chemotherapy followed by autologous hematopoietic stem cell transplantation (HSCT) extends overall survival in comparison with standard treatments [1,2], multiple myeloma (MM) remains an incurable disease, and new treatments are urgently needed. Thalidomide is active in the treatment of refractory MM [3]. In addition, an approach using melphalan and prednisolone (MP) in combination with thalidomide (MPT) has been increasingly adopted as a first-line treatment for elderly patients with previously untreated MM [4,5]. We present a MM patient who had disease refractory to thalidomide and dexamethasone and who showed a good response to melphalan, dexamethasone, and thalidomide (MDT) therapy, which is essentially similar to MPT.

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2. Case Report

A 48-year-old Japanese woman was referred to our hospital in January 2004 because of a 2-month history of back pain with gait disturbance. Magnetic resonance imaging revealed compression fracture of the spine (T9-T12 and L1-L4). The blood cell count showed anemia (hemoglobin, 8.3 g/dL) and slight leukopenia (leukocyte count, 2.9×10^9/L with a differential of 45% neutrophils and 53% lymphocytes). Biochemical results were as follows: total protein, 9.5 g/dL; albumin, 3.5 g/dL; M protein in the γ region, 3.89 g/dL (Figure 1A); lactate dehydrogenase, 158 U/L; creatinine, 1.05 mg/dL; β_2-microglobulin, 2.94 mg/dL; immunoglobulin G (IgG), 3652 mg/dL; IgA, 6 mg/dL; and IgM, 19 mg/dL. Immunoelectrophoresis of serum and urine samples revealed M protein of the IgG κ type and Bence Jones protein (κ type), respectively. Bone marrow aspiration from the iliac crest produced a dry tap, and plasma cell clustering was identified in a smear of a sample obtained from the tip of the aspiration needle. We could not perform a bone marrow biopsy to determine whether the patient had myelofibrosis. A diagnosis of symptomatic MM, Durie-Salmon stage IIIA, was made on the basis of these findings.

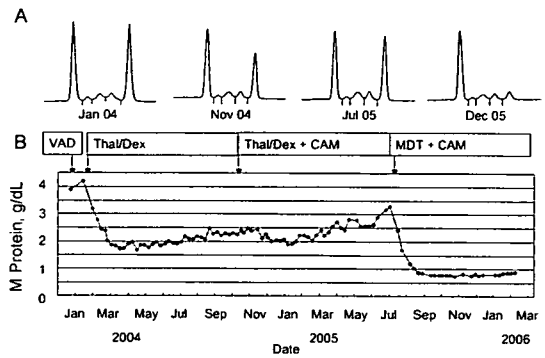

Figure 1. Electrophoresis of serum proteins (A) and changes in serum M protein levels (B) in the patient. VAD indicates vincristine, doxorubicin, and dexamethasone; Thal, thalidomide; Dex, dexamethasone; MDT, melphalan, dexamethasone, and thalidomide; CAM, clarithromycin.

The patient gave informed consent and received VAD chemotherapy consisting of continuous infusion of 0.4 mg vincristine, 10 mg doxorubicin, and 24 mg dexamethasone per day for 4 days as a therapy preceding high-dose melphalan supported by autologous HSCT. Intravenous pamidronate was administered every 8 weeks. One month later, collection of peripheral blood stem cells could not be performed because of her leukopenia and hypoplastic marrow. Because the M protein level actually increased from 3.89 g/dL to 4.20 g/dL after 1 course of VAD, we judged that there was no response to VAD therapy. We therefore started combination therapy with thalidomide (100 mg every day) and dexamethasone (5 mg/day for 2 days/week) after we obtained informed consent. We subsequently observed improvement of the lumbago and a decrease in the serum M protein concentration, which reached 1.7 g/dL in April 2004 (Figure 1B). The patient's serum M protein concentration gradually increased, however, and reached 2.5 g/dL in October 2004. In addition, a subcutaneous injection of granulocyte colony-stimulating factor was required to maintain neutrophil counts at greater than 1.0×10^9/L. On the basis of the published literature [6] and our experiences, we administered clarithromycin at a dosage of 400 mg every day beginning in October 2004, in addition to thalidomide and dexamethasone. We subsequently observed a decrease in the M protein concentration, which reached 1.9 g/dL in January 2005. This response was only transient, however, and the M protein level gradually increased again. We could not increase the daily thalidomide dose to 200 mg because that dose induced leukopenia and intolerable sleepiness. In July 2005, the patient's M protein concentration increased to 3.29 g/dL (Figure 1A), and her back pain worsened because of a new compression fracture of the spine. After providing informed consent, the patient received 6 mg of melphalan for 4 days every 6 weeks in combination with thalidomide and dexamethasone. In addition, 100 mg/day of aspirin was given, because the combination of thalidomide with cytotoxic therapy has been associated with an increased risk of deep vein thrombosis [4]. Surprisingly, the patient's serum M protein level decreased to 1.2 g/dL (64% reduction) 1 month later. Her M protein level declined further to 0.74 g/dL (78% reduction) after 4 months (Figure 1A). Since then, the patient's M protein concentration has remained below 1 g/dL for more than 7 months with the aid of 5 intermittent courses of MDT given approximately every 6 weeks (Figure 1B). The MDT regimen was well tolerated; the only toxicity was constipation. After the initiation of MDT therapy, the leukocyte count gradually increased, indicating recovery of the bone marrow microenvironment with this therapy.

3. Discussion

Despite modern treatment modalities, including high-dose chemotherapy with autologous HSCT rescue, MM remains an incurable disease for the majority of patients, and survival times for those with relapsed or refractory disease seldom exceed 2 years [1]. In relapsed or refractory MM, thalidomide monotherapy produces response rates between 32% and 66% [3], whereas the combination of thalidomide with dexamethasone induces partial-response rates of 41% to 55% [7]. Although the precise mechanisms behind the action of thalidomide remain to be determined, the accumulating evidence has established a role for thalidomide in the treatment of MM. We have presented a patient with MM refractory to thalidomide and dexamethasone in whom MDT therapy showed good efficacy.

We did not choose high-dose melphalan as an initial therapy in the present case because the patient's hypocellular marrow precluded the collection of a sufficient number of stem cells for subsequent HSCT. Standard MP therapy also was not chosen because of its cytotoxicity and consequent decrease in stem cells, which should be reserved for HSCT as a future therapeutic option. We thus chose a combination of thalidomide with dexamethasone as the first-line therapy. The thalidomide dosage was set at 100 mg/day to avoid the neutropenia that this drug might cause. Because evidence is emerging that a clinical response can be achieved at lower thalidomide doses (50 mg) with minimal long-term toxic effects [8], dose escalation was not undertaken.

Combination therapy of thalidomide with dexamethasone and cytotoxic agents attempts to take advantage of their additive or synergistic activities. When thalidomide is used in combination with alkylating agents, the response rate increases to approximately 70%. The addition of cyclophosphamide to thalidomide and dexamethasone has produced higher response rates and induced longer remissions [7,9]. Despite the evidence of efficacy, these regimens are associated with moderate toxicity and mortality. Recently, Kyriakou et al [10] reported a high response rate to a well-tolerated oral regimen incorporating daily thalidomide, weekly cyclophosphamide, and monthly pulses of dexamethasone for relapsed or refractory MM patients. It is of note that this regimen can be used successfully in patients with primary refractory disease, those who relapse after autologous HSCT, and those with renal failure. In addition, the feasibility and efficacy of MPT therapy, which includes another alkylating agent, melphalan, have been established in elderly patients with newly diagnosed MM [4]. In the