Table 1. Treatment schedule

Drug	Dose	Route	Days
Induction			
ATO	0.15 mg/kg	IV (2 h)	1-*
IDA	12 mg/m^2	IV (30 min)	†
MTX, AraC, PSL	15 mg, 40 mg, 10 mg	IT	‡
Consolidation #1			
ATO	0.15 mg/kg	IV (2 h)	1-25
MTX, AraC, PSL	15 mg, 40 mg, 10 mg	IT	‡
Consolidation #2			
ATO	0.15 mg/kg	IV (2 h)	1-25
MTX, AraC, PSL	15 mg, 40 mg, 10 mg	IT	‡
Consolidation #3			
AraC	2 g/m^2, every 12 h	IV (3 h)	1-4
PBSCH			§
Autologous HCT			
Busulfan	1 mg/kg, every 6 h	po	−6, −5, −4
Melphalan	70 mg/m^2	IV (bolus)	−3, −2
PBSCT			0

IT, intrathecally; IV, intravenously; MTX, methotrexate; PBSCH, peripheral blood stem cell harvest; PBSCT, peripheral blood stem cell transplantation; po, by mouth; PSL, prednisolone.

*For induction, ATO was administered until complete remission or for 60 d, whichever was shorter.

†IDA was added for 2 d if the WBC count exceeded 20.0 × 10^9/L before or during the induction therapy, if the combined total count of myeloblasts and promyelocytes in the peripheral blood exceeded 5.0 × 10^9/L before or during the induction therapy, or if an extramedullary myeloid tumor was detected before the induction therapy.

‡Intrathecal injection was given when the platelet count recovered after the end of the courses. PSL could be replaced with 4 mg of dexamethasone.

§PBSCH was performed when the WBC count had recovered.

had previously undergone autologous or allogeneic HCT were not eligible for inclusion. Written informed consent was obtained from all patients prior to registration. The protocol was reviewed and approved by the institutional review board of each of the participating centers and was conducted in accordance with the Declaration of Helsinki. This study is registered at http://www.umin.ac.jp/ctr/ as #C000000302.

Treatments

The treatments used during the study are summarized in Table 1. For remission induction, ATO was administered by a 2-hour infusion at a daily dose of 0.15 mg/kg until CR or a maximum of 60 days. In addition, patients received 12 mg/m^2 of idarubicin (IDA) on days 1 and 2 if 1 or more of the following criteria were met when the treatment was started: (1) the white blood cell (WBC) count exceeded 20.0 × 10^9/L; (2) the combined total count of myeloblasts and promyelocytes in the peripheral blood exceeded 5.0 × 10^9/L; and (3) there was the presence of an extramedullary myeloid tumor. Patients who showed evidence of criteria 1 and/or 2 after the start of induction therapy were given 2 extra doses of 12 mg/m^2 of IDA at that point. Those who achieved CR were scheduled to receive an additional 2 courses of ATO (0.15 mg/kg for 25 days) for consolidation. During ATO administration, a 12-lead electrocardiogram, complete blood cell counts, and chemistry parameters including the electrolytes were monitored at least twice a week, and the serum potassium and magnesium levels were maintained above the lower limits of normal. After the end of each ATO course, central nervous system (CNS) prophylaxis was attained by means of intrathecal injection of methotrexate, AraC, and corticosteroids (3 times in total). Patients with cytological evidence of CNS leukemia received intrathecal injections twice a week simultaneously with ATO, until complete clearance of leukemic cells in the cerebrospinal fluid (CSF) had been achieved. Following the third course of ATO, patients proceeded to PBSC harvest. For this purpose, high-dose AraC was administered at 2 g/m^2 for 3 hours twice daily for 4 days, and granulocyte–colony-stimulating factor was initiated from day 6. Upon recovery, autologous PBSCs were harvested by means of apheresis. Patients who attained a target CD34+ cell dose of 2.0 × 10^6/kg or higher were allocated to undergo autologous HCT unless *PML-RARα* transcripts were detected in PBSCs. The conditioning regimen consisted of busulfan (1 mg/kg orally every 6 hours on days −6 to −4) and melphalan (70 mg/m^2 intravenously on days −3 to −2),[13] whereas unpurged autologous PBSCs were infused on day 0. The study flow is shown in Figure 1.

Assessments and definitions

Hematologic CR was defined as the presence of all of the following: <5% of blasts in the bone marrow, no leukemic blasts in the peripheral blood or extramedullary sites, and recovery of peripheral blood counts. Hematologic relapse was defined as the presence of at least 1 of the following: recurrence of >10% leukemic cells in the bone marrow, recurrence of any leukemic cells in the peripheral blood, or development of extramedullary disease.[3] Molecular relapse was defined as the reappearance of polymerase chain reaction (PCR) positivity for *PML-RARα* in a single bone marrow or peripheral blood sample for this study. Prospective molecular monitoring was performed with the real-time quantitative reverse-transcription PCR (qRT-PCR) assay in a single independent laboratory. The *PML-RARα* levels in bone marrow samples were assessed at enrollment and after each course of therapy. Harvested PBSCs were also subjected to the qRT-PCR assay. The number of transcript copies was normalized by means of glyceraldehyde-3-phosphate dehydrogenase, and then converted into molecules per μg RNA. The threshold for quantification was 50 copies per μg RNA, which corresponds to a sensitivity of 10^{-4}, whereas levels below the threshold were differentiated into "not detected" and "detected but not quantifiable," and PCR negativity was categorized as "not detected."

For posttransplant engraftment, neutrophil engraftment was defined as achievement of a neutrophil count of at least 0.5 × 10^9/L for 2 consecutive days, and platelet engraftment as achievement of a platelet count of at least 30 × 10^9/L independent of transfusions for 2 consecutive days.

Statistical analysis

The primary end point was event-free survival (EFS) at 1 year after registration, which was defined as the time from registration to failure to achieve CR, relapse, death, or last visit, whichever came first. The expected and threshold EFS rates at 1 year were estimated to be 50% and 20%, respectively. The threshold EFS rate of 20% was determined based on historical control data of Japanese patients with relapsed APL who were

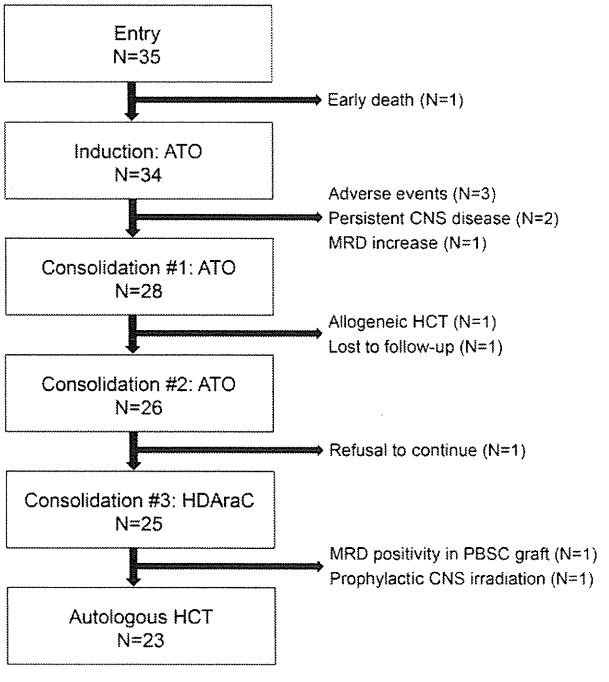

Figure 1. Patient flow diagram. HDAraC, high-dose cytarabine; MRD, minimal residual.

Table 2. Patient characteristics at enrollment

Characteristics	Values
Age in years, median (range)	46 (20-64)
Gender, male/female	23/12
WBC count, x 10^9/L	
Median (range)	2.6 (0.5-18.1)
≤10/>10	34/1
Platelet count, x 10^9/L	
Median (range)	79 (8-260)
≤40/>40	9/26
Performance status, 0/1/2/3	27/6/0/2
Number of prior relapses, 1/2	32/3
Type of relapse, hematologic/molecular	26/9
Interval between primary diagnosis and enrollment in years, median (range)	2.5 (0.8-11.0)

treated with ATRA-based therapy.[14] With a statistical power of 80% and a 1-sided, type I error of 5%, the minimum number of 17 eligible patients required for this study was calculated by means of binomial analysis. Allowing for a premature dropout rate of 15%, we aimed for inclusion of at least 20 patients. Primary end point analysis was performed with the Kaplan-Meier method for the calculation of probability of EFS. The treatment was considered to be effective if the lower limit of the 90% confidence interval (CI) exceeded the threshold EFS (ie, 20%). Overall survival (OS) was defined as the time from registration to death or last visit, and failure-free survival as the time from registration to failure to achieve CR, withdrawal from study, relapse, death, or last visit. Survival estimates and CIs were calculated with the Kaplan-Meier method and Greenwood's formula. The log-rank test was used for group comparison.

Results

Patient characteristics

A total of 35 patients with relapsed APL were enrolled in this study. Patient enrollment was allowed to exceed the originally planned minimum requirement after having ensured it ethical to expand the number of patients. Table 2 summarizes baseline characteristics of the patients. There were 23 males and 12 females, with a median age at enrollment of 46 years (range, 20-64 years). The median interval between primary diagnosis and enrollment was 2.5 years (range, 0.8-11.0 years).

All of the patients had been initially treated with ATRA-based therapy, and most of them in accordance with the protocols of JALSG or modifications thereof.[3,15] Thirty-two patients were in first relapse, and 3 in second relapse, with hematologic relapse accounting for 26, and molecular for 9. None of the patients had received ATO before.

Induction with ATO

ATO was administered to all patients except for 1 who developed intracranial hemorrhage immediately after enrollment and succumbed to early death (unique patient number [UPN] 26). Of the remaining 34 patients who underwent induction therapy, IDA was added for 2 patients on days 1 and 2, and during the induction course for 8 patients as per protocol. None of the patients developed differentiation syndrome. Three patients discontinued the study due to adverse events (grade 3 skin rash [UPN 10], grade 3 QT prolongation [UPN 19], and grade 4 QT prolongation accompanied by frequent ventricular premature contraction [UPN 23]). CSF examination performed at the end of the induction therapy revealed cytological evidence of CNS involvement in 4 patients, 2 of whom discontinued due to persistent CNS disease despite repeated intrathecal injections (UPN 13 and UPN 33). Of the 26 patients with hematologic relapse, 5 were taken off the study as mentioned previously, whereas the other 21 (81%) achieved CR. Of the 9 patients presenting with molecular relapse, 7 proceeded to consolidation therapy, and 2 were withdrawn from the study because of persistent CNS disease (UPN 13) or at the physician's discretion because the *PML-RARα* levels increased significantly after induction therapy (UPN 29).

Consolidation with ATO

During the 2 consolidation courses with ATO, 3 patients were taken off the study: 1 discontinued the protocol after the first consolidation course to receive umbilical cord blood transplantation (UPN 1), 1 was lost to follow-up after completing the first consolidation course (UPN 14), and the other refused to continue for unknown reasons after the second consolidation course (UPN 30). None of the patients discontinued the study because of relapse or adverse events during this phase of the treatment.

High-dose AraC and PBSC harvest

For PBSC harvest, 25 patients were given high-dose AraC as the third consolidation therapy, and all of them attained the target CD34+ cell doses of 2.0×10^6/kg. The median value of the CD34+ cell doses was 6.5×10^6/kg (range, $2.0\text{-}42.2 \times 10^6$/kg). One patient (UPN 18) whose PBSC sample was positive for *PML-RARα* was taken off the study because of ineligibility for autologous HCT as per protocol. One other patient (UPN 3), who had documented CNS leukemia at the end of induction therapy, but whose leukemic cells in the CSF were completely cleared with intrathecal injections, was withdrawn from the protocol at the physician's discretion to undergo prophylactic CNS irradiation. This patient received autologous HCT, but not as part of this study, and subsequently suffered posttransplant relapse in the CNS with fatal outcome. All of the other patients proceeded to autologous HCT. No dropouts due to relapse or adverse events were reported during this phase of the treatment.

Autologous HCT

The remaining 23 patients underwent autologous HCT as per protocol. The median time until engraftment was 12 days (range, 11-39 days) for neutrophils and 15 days (range, 12-136 days) for platelets. Posttransplant relapse occurred in 3 patients after a median duration of 5 months (range, 3-6 months). There was no transplant-related mortality.

Kinetics of the *PML-RARα* transcript levels

The results of the serial qRT-PCR tests during the treatment are summarized in Table 3. Most patients achieved PCR negativity after the first consolidation, but 4 were still positive for *PML-RARα* at this time. The PCR results turned negative after the second and third consolidation in 1 patient each (UPN 25 and 17, respectively). Of the 2 patients who remained positive for *PML-RARα* after the third consolidation, 1 (UPN 18) showed positive and the other (UPN 5) negative PCR test results for PBSCs. The latter underwent autologous HCT with a *PML-RARα*–negative graft but relapsed 5 months after transplantation.

Overall outcome

The probability of EFS was 77% at 1 year, with the 90% CIs ranging from 63% to 86%, thus demonstrating that this study has met its

Table 3. Kinetics of *PML-RARα* transcript levels

UPN	At entry	After induction	After consolidation #1	After consolidation #2	After consolidation #3
1	3000	N	N	Off study	Off study
2	460	N	N	N	N
3	60 000	<50	N	N	N
4	4200	<50	N	N	NA
5	69 000	28 000	760	140	<50
6	32 000	6000	N	N	N
7	15 000	290	N	N	N
8	360 000	<50	N	N	N
9	NA	1000	N	NA	N
10	NA	Off study	Off study	Off study	Off study
11	950	N	NA	N	N
12	64 000	50	N	NA	N
13	10 000	7100	Off study	Off study	Off study
14	120 000	400	NA	Off study	Off study
15	510 000	150	N	N	NA
16	190 000	<50	N	N	NA
17	95 000	1800	110	110	N
18	67 000	1500	480	390	280
19	130 000	Off study	Off study	Off study	Off study
20	450 000	280 000	N	N	N
21	140 000	170	N	N	N
22	26 000	61	N	N	N
23	24 000	Off study	Off study	Off study	Off study
24	730 000	<50	N	N	N
25	1900	2500	<50	N	N
26	440 000	Off study	Off study	Off study	Off study
27	NA	7800	N	N	N
28	NA	2600	N	N	N
29	510	6300	Off study	Off study	Off study
30	45 000	65	N	N	Off study
31	NA	300 000	NA	N	N
32	NA	50	N	N	N
33	180 000	NA	Off study	Off study	Off study
34	20 000	N	N	N	N
35	150 000	10 000	N	N	N

"Off study" indicates that the patient discontinued the study for reasons detailed in the text.

The threshold for quantification was 50 copies per μg RNA, which corresponds to a sensitivity of 10^{-4}. The levels below the threshold were differentiated into "not detected (N)" and "detected but not quantifiable (<50)."

N, not detected; NA, not assessed.

primary end point. Figure 2 shows Kaplan-Meier estimates for EFS and OS. With a median follow-up for surviving patients of 4.9 years (range, 0.3-6.3 years), the 5-year EFS and OS rates were 65% and 77%, respectively. The probability of failure-free survival was estimated to be 59% at 5 years.

Discussion

Current comprehensive practice guidelines have provided recommendations on the management of APL,[10,11] but what the optimal treatments for relapsed APL are remains equivocal. This is primarily because of the lack of prospective studies due to the rarity of relapses in APL, so that we initiated a phase 2 study for patients with relapsed APL in 2005 to evaluate the efficacy and feasibility of a sequential treatment featuring ATO and autologous HCT and enrolled 35 patients from 25 institutions nationwide. The treatment immediately induced molecular remission in a majority of patients, and only 3 patients were taken off the protocol because of adverse events throughout the entire study period, so that 23 patients could receive autologous HCT with a *PML-RARα*–negative PBSC graft. The 5-year probabilities of EFS and OS for the entire cohort were 65% and 75%, respectively. Of note, the EFS curve reached a stable plateau after 2 years from registration. These results have led us to conclude that this sequential treatment is effective and feasible.

ATO is currently the most active agent available for APL. Accumulated evidence has shown that >80% of patients with relapsed APL can achieve CR with ATO monotherapy.[7-9] In addition to high CR rates, the capability of this agent to induce molecular remission is another significant advantage because molecular remission is a prerequisite for long-term disease control in APL and is thus considered an important therapeutic milestone.[10,16] By contrast, ATRA alone is less likely to induce molecular remission, which results in this agent being used generally in combination with intensive chemotherapy rather than as monotherapy.[17] Although high CR rates can be expected for such combined use, this approach is limited by unsustained CR, especially for patients with hematologic relapse, and, more importantly, by quite high toxicity.[18,19] A retrospective study by Thomas et al[18] reported better survival for relapsed APL patients treated with ATO-based therapy than for historical control patients treated with ATRA-based therapy. The favorable safety profile of ATO is also an important advantage, as was seen in our study, where only 3 patients (8%) had to discontinue the protocol because of adverse events during induction therapy. This ratio seems to be only slightly higher than that observed in a US Intergroup study (5%).[8] It is further worth noting that none of our patients developed differentiation syndrome. This contrasts with a high incidence of this complication (25%) in the American study.[8] It can be assumed that the additional use of IDA for cases with high WBC counts may have contributed to reducing the risk of differentiation syndrome in our cohort.

Although the beneficial effect of ATO for induction has been well documented in relapsed APL, it is far less clear what the best consolidation strategy is after achieving CR. Previous studies showed that patients who achieved second or subsequent CR with ATO but did not receive transplantation thereafter had poor outcome; the proportion of those remaining alive and relapse-free ranged from 22% to 37%.[7,17,20] Although some patients may remain in CR without transplantation, overall prognosis is far from satisfactory, and the outcome seems much better for those who receive autologous or allogeneic HCT.[17,20] Owing to its posttransplant graft-versus-leukemia effect, allogeneic HCT is generally considered the most effective treatment of preventing relapse in acute myeloid leukemia.[21] In APL, however, the relapse rate after autologous HCT may be quite low provided the patient is in molecular remission at the time of transplantation.[22-25] Given the lower risk of transplant-related mortality with autologous HCT, the balance of benefits and risks may well favor autologous HCT over allogeneic HCT. For autologous HCT to be successful, it is imperative to reduce the tumor burden substantially at the molecular level before transplantation. For this reason, what constitutes an adequate number of cycles of ATO therapy is a subject of clinical interest. Similar to the observation by the US Intergroup,[8] our study found that 2 courses of ATO therapy induced most patients into molecular remission, although 4 patients remained positive for *PML-RARα* after the second course (ie, consolidation #1). Administration of the third ATO course reduced the transcript levels in 3 of the patients, whereas the level stayed unchanged in the remaining patient. It was possible to administer the third course of ATO because none of the 26 patients who had received this course had to withdraw from the study due to relapse or adverse events. These findings lead us to consider that

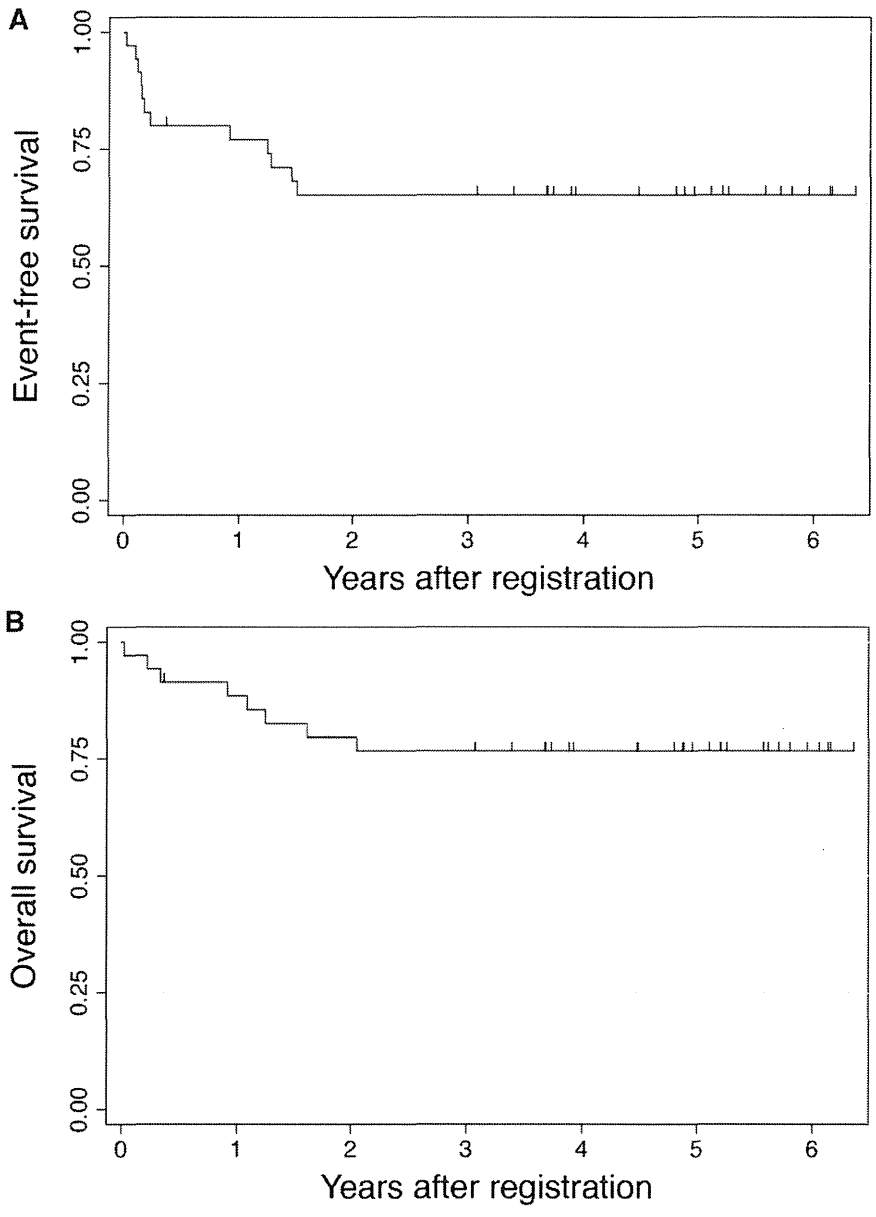

Figure 2. Kaplan-Meier curves for EFS (A) and OS (B). The probabilities of EFS and OS for the entire cohort (N = 35) were 65% and 77% at 5 years, respectively.

administration of a total of 3 courses of ATO before PBSC collection is feasible.

For the PBSC-mobilizing regimen, we chose high-dose AraC, hoping it would produce highly efficient mobilization as well as exert a systemic antileukemic effect. The fact that all the 25 patients undergoing this procedure successfully achieved the target CD34+ cell doses has convinced us of the usefulness of this regimen. In addition, high-dose AraC is known to provide good coverage of the CNS, the most common site of extramedullary involvement in APL.[26,27] Above and beyond our expectations, routine CSF examination at the end of the induction therapy identified 4 patients with cytological evidence of CNS involvement, although they did not show any CNS-related symptoms. This suggests that high-dose AraC may also play a part in protecting against the potential risk of subsequent CNS relapse for these patients.

Except for 1 patient whose PBSC sample was positive for *PML-RARα* and another who was withdrawn from the study to receive off-protocol prophylactic CNS irradiation, all the remaining patients who had undergone PBSC harvest proceeded to autologous HCT without any subsequent transplant-related mortality. This contrasts with a previous prospective study conducted before the advent of ATO, in which a combination of ATRA and intensive chemotherapy was used.[28] In that study, severe toxicity of induction therapy precluded the subsequent conduct of PBSC harvest or autologous HCT for some patients, and nearly 10% of the autografted patients suffered transplant-related mortality. These results highlight the need for active and less toxic therapies that give patients a better chance to proceed to and receive autologous HCT safely. For this reason, ATO can be considered to be an ideal treatment because of its strong antileukemic effect and favorable safety profile.

Although relatively few patients were analyzed in our study, to our knowledge this is the first prospective study to evaluate the use of ATO in conjunction with autologous HCT for relapsed APL. The results presented here provide evidence of the outstanding efficacy and feasibility of the sequential treatment consisting of induction and consolidation with ATO, PBSC harvest after high-dose AraC chemotherapy, and autologous HCT. For patients who are not eligible for this strategy, such as those for whom autologous HCT is

not suitable or whose *PML-RARα* levels do not decrease sufficiently during treatment, other treatment approaches need to be investigated that incorporate, for example, allogeneic HCT,[24,29] gemtuzumab ozogamicin,[30,31] tamibarotene,[32] or novel agents. It is desirable that such studies can be conducted prospectively. Finally, we should remember that the incorporation of ATO into initial therapy is expected to further improve the outcome for newly diagnosed APL,[33,34] which will hopefully lead to reduction in the number of patients who require salvage therapy.

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Authorship

Contribution: M.Y. collected and analyzed data, interpreted results, and drafted the manuscript; M.T., H.F., and A.T. designed the study, collected data, interpreted results, and reviewed the manuscript; K.F., S.F., K.S., M.T., A.O., K.T., and A.M. collected data, interpreted results, and reviewed the manuscript; S.O. contributed to data management, designed the study, collected data, interpreted results, and reviewed the manuscript; Y.M. contributed to data management, interpreted results, and reviewed the manuscript; Y.A. designed the study, analyzed data, interpreted results, and drafted the manuscript; Y.K. designed the study, provided administrative support, interpreted results, and reviewed the manuscript; T.N. provided administrative support, interpreted results, and reviewed the manuscript; and N.E. served as the principal investigator, designed the study, collected and analyzed data, interpreted results, and drafted the manuscript.

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

A complete list of the members of the JALSG appears in "Appendix: study group members."

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Appendix: study group members

The members of the JALSG are Nihon University School of Medicine, Kasukabe Municipal Hospital, Tokyo Metropolitan Komagome Hospital, Tokyo Metropolitan Ohtsuka Hospital, Nagoya University Graduate School of Medicine, Nagoya Ekisaikai Hospital, JA Aichi Showa Hospital, Okazaki City Hospital, Daido Hospital, Yokkaichi Municipal Hospital, Ichinomiya Municipal Hospital, Komaki City Hospital, Toyohashi Municipal Hospital, Ogaki Municipal Hospital, Tosei General Hospital, National Center for Geriatrics and Gerontology, Aichi Cancer Center, Toyota Kosei Hospital, Japanese Red Cross Nagoya First Hospital, Fujita Health University School of Medicine, Mie University Graduate School of Medicine, Suzuka Kaisei Hospital, Takeuchi Hospital, Yamada Red Cross Hospital, JA Suzuka General Hospital, Matsusaka Chuo General Hospital, Kinki University School of Medicine, Osaka Minami Medical Center, Sakai Hospital, Osaka Medical Center for Cancer and Cardiovascular Diseases, Hiroshima Red Cross Hospital & Atomic-Bomb Survivors Hospital, Shikoku Cancer Center, Nagasaki University Graduate School of Biomedical Sciences, Sasebo City General Hospital, Nagasaki Medical Center, Kumamoto University School of Medicine, Kumamoto City Hospital, Kumamoto Shinto General Hospital, Jichi Medical School, Okayama University Hospital, Minami-Okayama Medical Center, Okayama City Hospital, Chugoku Central Hospital, Okayama Medical Center, Okayama Rosai Hospital, Kagawa Rosai Hospital, Gunma University Graduate School of Medicine, Nishi-Gunma National Hospital, Fujioka General Hospital, Fukaya Red Cross Hospital, University of Fukui, Kurashiki Central Hospital, Kanazawa Medical Center, Fukui Red Cross Hospital, Fukui Prefectural Hospital, National Cancer Center Hospital, Saitama Medical School, Hyogo College of Medicine, Osaka National Hospital, Takarazuka Municipal Hospital, Uegahara Hospital, Amagasaki Central Hospital, Kawasaki Medical School, Kochi Health Sciences Center, Chiba University Hospital, Chiba Aoba Municipal Hospital, Funabashi Central Hospital, Saiseikai Narashino Hospital, Oami Hospital, Nara Medical University, Jikei University School of Medicine, Dokkyo University School of Medicine, Nagoya Medical Center, Ohta Nishinouchi Hospital, Kochi Medical School, Shiga University of Medical Science, National Cancer Center East, Anjo Kosei Hospital, St. Marianna University School of Medicine, Yokohama Seibu Hospital, Shinshu University School of Medicine, Nagano Red Cross Hospital, Matsumoto Medical Center Matsumoto Hospital, Showa Inan General Hospital, Tokyo Women's Medical University, Tama-Hokubu Medical Center, Hamamatsu University School of Medicine, Hamamatsu Medical Center, Kagoshima University Hospital, Tochigi Cancer Center, Kanazawa University Graduate School of Medical Science, Keijyu Medical Center, NTT West Kanazawa Hospital, Toyama City Hospital, Ishikawa Central Hospital, JA Takaoka Hospital, Tokyo Medical University, Tokyo Medical University Hachioji Medical Center, Kyorin University School of Medicine, Hokkaido University Graduate School of Medicine, Sapporo Kousei Hospital, Sapporo Aiiku Hospital, Asahikawa City Hospital, Hakodate City Hospital, Hokkaido Cancer Center Hospital, Saiseikai Maebashi Hospital, Nagoya City University Graduate School of Medical Sciences, Enshu General Hospital, Shizuoka Saiseikai General Hospital, Tokai University School of Medicine, Ebina General Hospital, Yamaguchi University School of Medicine, Yamaguchi Prefecture Central Hospital, The University of Tokyo, Osaka City University, Saiseikai Nakatsu Hospital, Osaka University Graduate School of Medicine, University of Tokyo, Niigata University Medical and Dental Hospital, Oita University Faculty of Medicine, Oita Prefectural Hospital, Almeida Memorial Hospital, Kouseiren Tsurumi Hospital, National Kyushu Cancer Center, Kyushu Medical Center, Fukuoka Postal Services Agency Hospital, Aso Iizuka Hospital, Teikyo University School of Medicine, Teikyo University Mizonokuchi Hospital, Sapporo Hokuyu Hospital, Aichi Medical University, Kitasato University Hospital, Yamagata University Faculty of Medicine, Keio University, Aomori Prefectural Central Hospital, Hyogo Cancer Center, Kyoto Prefectural University of Medicine, Kyoto Hospital, Kobe Central Hospital, Matsushita Memorial Hospital, Osaka City General Hospital, National Defense Medical College, Akita University School

of Medicine, NTT Kanto Medical Center, Yokohama City University Hospital, Yokohama City University Medical Center, Kanagawa Cancer Center, Yokosuka City Hospital, Fujisawa City Hospital, Shizuoka Red Cross Hospital, Yamato Municipal Hospital, Saiseikai Yokohama Nanbu Hospital, Tohoku University School of Medicine, Osaki Citizen Hospital, Hiroshima University, Hiroshima-Nishi Medical Center, Kagawa University, Kagawa Prefectural Central Hospital, Sakaide City Hospital, Juntendo University School of Medicine, Kanazawa Medical University, Kobe University Graduate School of Medicine, Imamura Bun-In Hospital, Ehime University School of Medicine, Bokutoh Hospital, Ohtsu Red Cross Hospital, Matsue Red Cross Hospital, Tokyo Medical and Dental University, Yokohama City Minato Red Cross Hospital, Jichi Medical School Omiya Medical Center, Shizuoka Cancer Center Hospital, Ehime Prefectural Central Hospital, International Medical Center of Japan, Kure Medical Center, Nagoya Daini Red Cross Hospital, University of Yamanashi, Heart Life Hospital, Musashino Red Cross Hospital, Saitama Medical School, PL General Hospital, Toyama Prefectural Central Hospital, Shimane Prefectural Central Hospital, Tottori Prefectural Central Hospital, National Disaster Medical Center, Shimane University Hospital, Otemae Hospital, Nakagami Hospital, Tsukuba University Hospital, Mito Medical Center, National Hospital Organization, Tsuchiura Kyodo General Hospital, Hitachi General Hospital, JA Toride Medical Center, Fuchu Hospital, Ibaraki Prefectural Central Hospital, Saga University, Yamanashi Prefectural Central Hospital, Hiroshima City Asa Hospital, Sendai Medical Center, Kurume University of Medicine, Kitakyushu Municipal Medical Center, Kyushu Kosei-Nenkin Hospital, University of Miyazaki Hospital, Hamanomachi Hospital, Kyushu University Graduate School of Medical Sciences, Harasanshin Hospital, Toranomon Hospital, Matsuyama Red Cross Hospital, Fukushima Medical University, Fukuoka University Hospital, Yokohama Municipal Citizen's Hospital, Nagoya City West Medical Center, Takanohara Central Hospital, The University of the Ryukyus, Osaka Red Cross Hospital, and Sapporo Medical University School of Medicine.

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Prognosis of acute myeloid leukemia harboring monosomal karyotype in patients treated with or without allogeneic hematopoietic cell transplantation after achieving complete remission

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ABSTRACT

To evaluate the prognostic impact of monosomal karyotype on post-remission outcome in acute myeloid leukemia, we retrospectively analyzed 2,099 patients who had achieved complete remission. Monosomal karyotype was noted in 73 patients (4%). Of these, the probability of overall survival from first complete remission was 14% at four years, which was significantly lower than that reported in patients without monosomal karyotype, primarily due to a high relapse rate (86%). Monosomal karyotype remained significantly associated with worse overall survival among patients with unfavorable cytogenetics or complex karyotype, and even in patients who underwent allogeneic hematopoietic cell transplantation during first complete remission. These findings confirm that monosomal karyotype has a significantly adverse effect on post-remission outcome in patients with acute myeloid leukemia treated with and without allogeneic hematopoietic cell transplantation in first complete remission, emphasizing the need for the development of alternative therapies for this patient population.

Key words: acute myeloid leukemia, monosomal karyotype, cytogenetics, post-remission therapy, allogeneic hematopoietic cell transplantation.

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease that includes subsets with distinct biological, clinical and prognostic features. It has been well established that cytogenetic abnormalities at diagnosis are associated with the biology of the disease and have important prognostic implications.[1-3] The coexistence of multiple cytogenetic abnormalities designated as complex karyotype (CK) has been recognized as a factor that predicts an extremely unfavorable outcome in AML.[4-7] However, the prognostic significance of CK has recently been challenged by Breems *et al.* who showed that the monosomal karyotype (MK), defined as 2 or more distinct autosomal monosomies or a single autosomal monosomy in the presence of other structural abnormalities, adversely affects the prognosis, and that the overlap of MK with CK is the main contributor to the unfavorable impact of CK.[8] According to Breems *et al.* and reports published subsequently by other groups,[7-10] patients with MK$^+$ AML show low complete remission (CR) rates ranging from 18% to 48% and overall survival (OS) rates of less than 10%. On the other hand, it has been suggested that such a poor outcome may be improved by allogeneic hematopoietic cell transplantation (HCT).[11]

To further clarify the prognosis of patients with MK$^+$ AML, especially regarding outcome after allogeneic HCT during first CR (CR1), we performed a retrospective analysis by using a dataset that included more than 2,000 AML patients in CR. Since failure to achieve CR is obviously associated with a dismal prognosis regardless of the presence or absence

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of MK, the present analysis focused on patients who achieved CR with one or two courses of chemotherapy.

Design and Methods

Patients

For this study, we used a Japanese nationwide database of adult AML patients. Eligible patients were required to be between 16 and 70 years of age, to be diagnosed with AML from 1999 to 2006 according to the World Health Organization (WHO) classification,[12] and to have achieved CR with one or two courses of chemotherapy. We excluded patients with acute promyelocytic leukemia (n=386) and those without pre-treatment cytogenetic results (n=36); this left 2,099 patients available for analysis. This study was approved by the Institutional Review Board at the National Cancer Center Hospital.

Cytogenetic analysis

Cytogenetic analysis was performed on metaphases from samples of bone marrow or blood obtained prior to induction therapy by using standard banding techniques. Karyotypes were determined according to the International System for Human Cytogenetic Nomenclature.[13] An abnormality was considered to be clonal when at least 2 metaphases had the same aberration in the case of either a structural abnormality or an additional chromosome. If there was a monosomy, it had to be present in at least 3 metaphases to be considered significant. Cytogenetics was classified as favorable, intermediate, unfavorable or unknown risk according to the Southwest Oncology Group (SWOG) criteria.[5] Apart from the SWOG classification, the MK status was assessed retrospectively for this study according to the definition proposed by Breems *et al.*[8] Accordingly, patients were divided into 4 cytogenetic subgroups: core binding factor AML (CBF AML), cytogenetically normal AML (CN AML), cytogenetically abnormal non-CBF AML without MK (MK^- AML), and cytogenetically abnormal non-CBF AML with MK (MK^+ AML).

Statistical analysis

A Kaplan-Meier survival analysis was performed to estimate the probabilities of OS and relapse-free survival (RFS). OS was defined as the time from the achievement of first CR (CR1) to death or last visit, and RFS as the time from the achievement of CR1 to relapse, death or last visit. Differences in OS and RFS between groups were compared by means of the log rank test. Cumulative incidences of relapse and non-relapse mortality were calculated with relapse considered as a competing risk for non-relapse mortality, and vice versa. Cox's regression model was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs). All statistical analyses were performed with the SPSS software version 11.0.1 (SPSS, Chicago, IL, USA) and R software version 2.13.0 (The R Foundation for Statistical Computing).

Results and Discussion

The entire cohort consisted of 2,099 AML patients who had achieved CR with one or two courses of chemotherapy, among whom CBF AML, CN AML, MK^- AML and MK^+ AML accounted for 21%, 49%, 27% and 4%, respectively. Table 1 shows the patients' characteristics according to these cytogenetic subgroups. Among the 73 patients with MK^+ AML, 68 (93%) had a cytogenetically unfavorable risk, while the remaining 5 had an unknown risk. In patients younger than 60 years, intensive therapy defined as "3+7" or its equivalent, was given to more than 95% in all of the cytogenetic subgroups. In patients aged 60 years or older, the proportion of those given intensive therapy seemed slightly lower in MK^+ AML but, nevertheless, 75% of them received intensive therapy.

Allogeneic HCT was performed in 32 patients with MK^+ AML, including 15 during CR1, 4 during second CR (CR2) and 13 during other disease phases. The details of patients who underwent allogeneic HCT in CR1 are summarized in the *Online Supplementary Table S1*. The median time from CR1 to transplantation was 93 days (range 14-540 days) for the 15 patients with MK^+ AML, which was significantly shorter than those in the other groups (P=0.011).

Figure 1A compares survival curves from the time of CR1 according to the cytogenetic subgroups. With a median follow up of 4.1 years for surviving patients, the 4-year probabilities of OS were 68% in CBF AML, 58% in CN AML, 46% in MK^- AML and 14% in MK^+ AML, respectively (P<0.001). This significantly inferior OS in MK^+ AML patients can mainly be explained by a high risk of relapse, since the relapse rate was 86% at four years, which was significantly higher than those in the remaining groups (P<0.001). No patient with MK^+ AML survived four years without allogeneic HCT, and the difference in OS was more pronounced when patients undergoing allogeneic HCT were analyzed as censored cases (83%, 66%, 54% and 0% at four years in CBF AML, CN AML, MK^- AML and MK^+ AML, respectively; P<0.001).

Next, we examined whether MK identified a very poor prognostic subset within 2 cytogenetically distinct subpopulations representing poor prognosis, i.e. unfavorable cyto-

Table 1. Patient's characteristics according to cytogenetic subgroup.

	CBF n=437	CN n=1,027	MK^- n=562	MK^+ n=73
Age, years				
Median	45	51	48	53
Range	16-70	16-70	16-70	20-70
Sex				
Male	279 (64%)	576(56%)	311(55%)	47(64%)
Female	158 (36%)	451(44%)	251(45%)	26(36%)
Cytogenetic risk by SWOG				
Favorable	411 (94%)	-	-	-
Intermediate	-	1,027(100%)	64 (11%)	-
Unfavorable	26 (6%)	-	300(53%)	68(93%)
Unknown	-	-	198(35%)	5 (7%)
WBC count, $\times 10^9$/L				
Median	11.2	13.0	8.5	4.4
Range	0.7-281.2	0.4-40.2	0.3-22.3	0.8-408.0
Dysplasia				
Yes	35 (8%)	220(20%)	136(24%)	33(45%)
No	402 (92%)	807(80%)	426(76%)	40(55%)
N. induction courses				
1 course	378 (86%)	825(80%)	419(75%)	56(77%)
2 courses	59 (14%)	202(20%)	143(25%)	17(23%)
Allogeneic HCT				
CR1	32 (7%)	256(25%)	183(33%)	15(21%)
CR2	78 (18%)	106(10%)	57 (10%)	4 (5%)
Other disease phase	66 (15%)	125(12%)	87 (15%)	13(18%)
Not performed	261 (60%)	540(53%)	235(42%)	41(56%)

CBF: core binding factor AML; CN: cytogenetically normal AML; MK-: cytogenetically abnormal non-CBF AML without monosomal karyotype; MK+: cytogenetically abnormal non-CBF AML with monosomal karyotype; SWOG: Southwest Oncology Group; WBC: white blood cell count; HCT: hematopoietic cell transplantation; CR1: first complete remission; CR2: second complete remission.

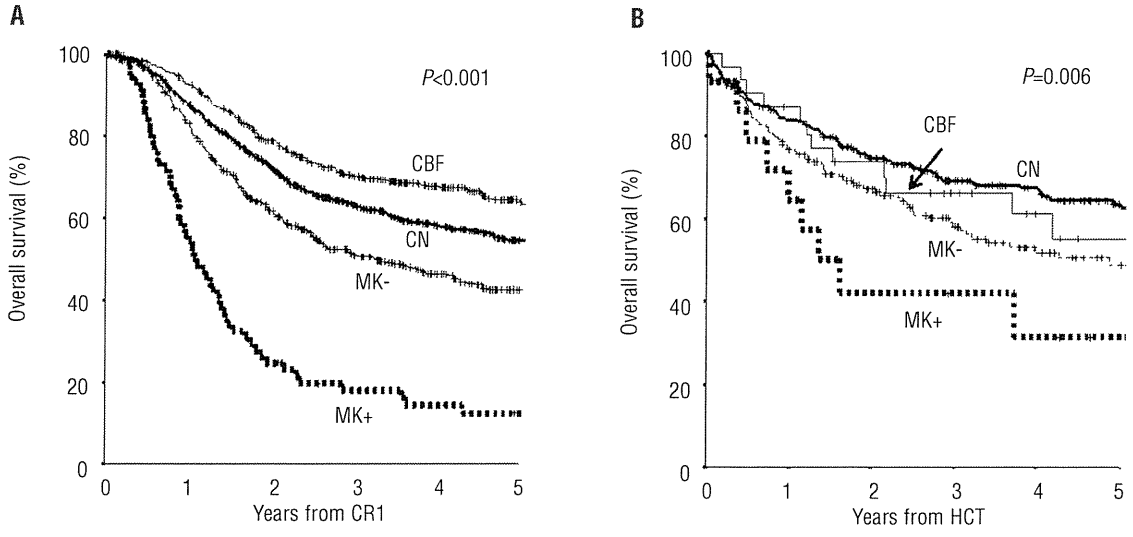

Figure 1. Kaplan-Meier curves for (A) OS after achieving CR1 for the entire cohort, and for (B) OS after allogeneic HCT for patients who underwent allogeneic HCT in CR1, according to the cytogenetic subgroups. CBF represents core binding factor AML; CN: cytogenetically normal AML; MK$^-$, cytogenetically abnormal non-CBF AML without monosomal karyotype; MK$^+$, cytogenetically abnormal non-CBF AML with monosomal karyotype. *P* values are presented for comparisons among the 4 groups.

genetics and CK. MK accounted for 17% of those with unfavorable cytogenetics (68 of 394), and 41% of those with CK (39 of 96). Among patients with unfavorable cytogenetics, there was a statistically significant difference in OS between those with and without MK (16% *vs.* 46% at four years, $P<0.001$; *Online Supplementary Figure S1A*). Similar findings were seen in patients with CK, with 4-year OS rates of 11% and 34% in those with and without MK ($P<0.001$; *Online Supplementary Figure S1B*).

Allogeneic HCT was performed during CR1 in 32 of 437 CBF AML patients (7%), 256 of 1,027 CN AML patients (25%), 183 of 562 MK$^-$ AML patients (33%), and 15 of 73 MK$^+$ AML patients (21%). Figure 1B shows Kaplan-Meier curves for OS after HCT in patients who were transplanted during CR1. These subgroups showed significantly different OS, with 4-year OS rates of 61%, 67%, 52% and 31% in CBF AML, CN AML, MK$^-$ AML, and MK$^+$ AML, respectively ($P=0.006$). A statistically significant difference was observed in terms of post-transplant relapse ($P=0.025$) (*Online Supplementary Table S2*). Non-relapse mortality in patients with MK$^+$ AML appeared to be higher than those in the other groups, but these differences were not statistically significant ($P=0.595$). Table 2 shows results of univariate and multivariate analyses on factors associated with post-transplant OS in patients undergoing allogeneic HCT in CR1. After adjusting for other covariates, MK remained significantly associated with inferior post-transplant OS (HR 3.12; 95% CI, 1.58-6.15; $P=0.001$, with reference to CN AML).

MK is a recently proposed subgroup of cytogenetic abnormalities that confers a very unfavorable prognosis in AML.[8] Reported CR rates have been quite low, ranging between 18 and 48%,[8-10] and this represents a major cause of the poor prognosis. Since patients who fail to achieve CR generally have a very unfavorable prognosis regardless of the presence or absence of MK, we decided to restrict our analysis to patients who had achieved CR. In our patient population, MK was observed in 4%; this was lower than the values reported previously (6-13%).[7-9] The most probable explanation for this could be the fact that our cohort included only patients who had achieved CR, while the other studies included newly diagnosed patients.

Our data clearly demonstrated that MK confers a significantly worse prognosis in patients who have achieved CR. Notably, MK identified patients with a worse prognosis

Table 2. Factors associated with post-transplant OS in patients who underwent allogeneic HCT in CR1.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Cytogenetic subgroup				
CBF	1.14 (0.62-2.09)	0.671	1.17 (0.63-2.15)	0.622
CN	1.00	-	1.00	-
MK$^-$	1.43 (1.05-1.96)	0.023	1.45 (1.06-1.98)	0.021
MK$^+$	2.74 (1.42-5.28)	0.003	3.12 (1.58-6.15)	0.001
Age				
As a numerical variable (1 year older)	1.01 (1.00-1.02)	0.294	1.01 (0.99-1.02)	0.377
Sex				
Male	1.00	-	1.00	-
Female	1.08 (0.81-1.45)	0.597	1.16 (0.86-1.57)	0.327
WBC count				
As a numerical variable (10×10^9/L lower)	1.02 (1.00-1.03)	0.037	1.02 (1.01-1.04)	0.007
Donor				
Related*	1.00	-	1.00	-
Other	1.39 (1.04-1.87)	0.026	1.47 (1.09-1.98)	0.011
Conditioning				
Myeloablative	1.00	-	1.00	-
Reduced-intensity	1.13 (0.81-1.58)	0.465	1.04 (0.70-1.56)	0.846

*HR: hazard ratio; CI: confidence interval; CBF: core binding factor AML; CN: cytogenetically normal AML; MK: cytogenetically abnormal non-CBF AML without monosomal karyotype; MK$^+$: cytogenetically abnormal non-CBF AML with monosomal karyotype; WBC: white blood cell count. *"Related" indicates a matched or 1 antigen-mismatched family donor.*

even among those with unfavorable cytogenetics or those with CK. The detrimental prognostic impact of MK was primarily due to high relapse rates and, importantly, similar results were seen in patients who received allogeneic HCT in CR1. Post-transplant relapse occurred more than 20% more frequently in MK+ AML patients than in those in each of the remaining cytogenetic subgroups. This finding is consistent with published studies.[11,14] Investigators at the University of Minnesota analyzed 134 AML patients, including 17 patients with MK who were allografted in CR1, and showed that the MK classification could significantly predict the risk of post-transplant relapse.[14] A report from the Fred Hutchinson Cancer Research Center described the outcome of 35 patients with MK and 193 patients without MK who underwent allogeneic HCT in CR1, in which the 4-year OS rates were 30 and 65% in those with and without MK.[11] Those results taken together with our present results suggest that allogeneic HCT may be able to improve but not completely override the poor prognosis with MK+ AML. It is widely recognized that allogeneic HCT in CR1 is the treatment of choice for patients with AML at cytogenetically unfavorable risk,[15-17] if they have a suitable donor and are fit enough to undergo the procedure. In this study, allogeneic HCT was given to only 21% of patients with MK+ AML during CR1. This low transplantation rate could partly be due to a short CR1 duration, which likely decreased the chance of receiving allogeneic HCT in CR1. A significantly shorter time to transplantation in our MK+ AML patients might reflect the short duration of their CR1 that precluded an implementation of allogeneic HCT after a relatively long interval after achieving CR. Despite a considerable risk of relapse even after transplantation, it is still conceivable that these cytogenetically very unfavorable patients would benefit from allogeneic HCT. We observed that no patient survived long-term without allogeneic HCT, which is in line with reports from the SWOG study.[9]

Our study has several limitations and the results must, therefore, be interpreted with caution. These limitations include the retrospective nature of the study, and the relatively small number of patients with MK+ AML, especially of those who underwent allogeneic HCT in CR1, leaving room for selection bias or chance effect. However, given that MK+ AML accounted for only 4% of our AML patients in CR, it would be quite impractical to conduct a prospective comparison to assess the role of allogeneic HCT in CR1. Under such conditions, the findings from a large-scale retrospective study could have important implications.

In summary, our data confirm that MK exerts a significantly adverse effect on post-remission outcome in AML patients treated with and without allogeneic HCT in CR1. Although our results suggest that allogeneic HCT is already an available treatment of choice, the development of alternative therapies is warranted for this patient population.

Authorship and Disclosures

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Mutations in the nucleolar phosphoprotein, nucleophosmin, promote the expression of the oncogenic transcription factor MEF/ELF4 in leukemia cells and potentiates transformation

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*Running title: *NPM1 mutations enhance HDM2 expression through MEF/ELF4*

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Background: MEF/ELF4 can function as an oncogene. We demonstrated the role of MEF/ELF4 in acute myeloid leukemia.

Results: NPM1 inhibited the DNA-binding and transcriptional activity of MEF/ELF4 on the HDM2 promoter whereas NPM1 mutant protein enhanced these activities of MEF/ELF4.

Conclusion: MEF/ELF4 activity may be activated by NPM1 mutant protein.

Significance: NPM1 mutant proteins have a role in MEF/ELF4-dependent leukemogenesis.

SUMMARY

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

Introduction

Myeloid ELF1-like factor (MEF/ELF4), a member of the ETS family of transcription factors, is characterized by an 85-amino acid ETS domain that recognizes a core sequence of GGAA or TTCC (1). MEF/ELF4 is

expressed in various normal and malignant hematopoietic cells and regulates the expression of various cytokines (interleukin-3 (1), granulocyte-macrophage colony stimulating factor (1), and interleukin-8 (2) as well as the cytolytic perforin molecule (3), antibacterial peptides lysozyome and human beta-defensin2 (4)), and matrix metalloproteinase (MMP)-9 expression (5). Furthermore, analyses of MEF/ELF4-deficient mice have revealed the essential role of MEF/ELF4 in the development and function of natural killer (NK) cells and NK-T cells (3). Recently, Smith *et al.* have shown that repression of Elf-4 by transcriptional repressor *Gfi1b* is important for the maturation of primary fetal liver erythroid cells (6). MEF/ELF4 also regulates the key aspects of hematopoietic stem cell behavior by controlling movement through the cell cycle from quiescence (G0) to G1 and G1 to S as well as resistance to myelosuppression (7, 8).

MEF/ELF4 is expressed in cancers such as leukemia (9), lymphoma, and ovarian cancer (10). Recently, Totoki *et al.* identified an intra-chromosomal inversion (Xq25) in hepatocellular carcinoma that generated a BCORL1-MEF/ELF4 fusion transcript (11). Experiments in several mouse models have suggested that MEF/ELF4 plays a role in tumorigenesis. For example, models of retrovirus-induced insertional mutagenesis have identified MEF/ELF4 as a gene that is involved in leukemic transformation (12). Sashida *et al.* have shown that overexpression of MEF/ELF4 enhances the expression of Mdm2, leading to decreased p53 expression (13) and enhanced transformation. In experiments with MEF/ELF4-overexpressing cells, they demonstrated that Ets1-induced p16 induction is suppressed, resulting in senescence suppression and tumor promotion.

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

development (19).

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

EXPERIMENTAL PROCEDURES

Cell culture

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

Tandem affinity purification assay

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

eluted with trypsin. The resulting peptides were analyzed with a Procise 49X cLC protein sequencer (Applied Biosystems, Foster City, CA, USA) (20).

In vitro translation

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

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

Immunoprecipitation and immunoblotting

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

Immunoprecipitates were recovered, washed four times with ice-cold co-IP solution (Active Motif), and fractionated by SDS-PAGE. Separated

proteins were transferred to a membrane. After incubation in blocking buffer, membranes were probed with peroxidase-labeled antibodies against FLAG (Sigma), V5 (Invitrogen), or tag (Invitrogen). Detection was achieved with an enhanced chemiluminescence system (ECL Advance Western Blotting Detection Kit, GE Healthcare). Quantification of Western blotting bands was performed by using AE-6982/C/FC and CS Analyzer ver. 3.0 software (ATTO, Tokyo, Japan).

GST and His pull-down assay

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

EMSA

Recombinant proteins GST, GST-NPM1, His, and His-MEF/ELF4 were collected as described above. Nuclear protein from 293T cells transfected with pcDNA/MEF/ELF4, pcDNA/Wt-NPM1, or pcDNA/Mt-A-NPM1 was extracted with an NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. EMSA was performed by using a LightShift Chemiluminescent EMSA Kit (Pierce). Recombinant protein or nuclear extracts were incubated with 20 fmol biotin 3' end-labeled oligonucleotides containing APET (an ETS binding site in the IL-3 promoter that was shown to bind to MEF/ELF4) (1). After electrophoresis, transfer, and crosslinking, the signal was detected by a peroxidase/luminol system (Chemiluminescent Nucleic Acid Detection Module; Pierce). To confirm specificity, a 200-fold excess amount of non-labeled oligonucleotides (APET-competitor) (1) was added. The DNA sequence of the APET oligonucleotide is 5'-CCTCAGTGAGCTGAGTCAGGCTTCCCC TTCCTGCCACAGGG-3'.

RNA interference

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

Luciferase assay

A 0.5μg aliquot of pcDNA/MEF/ELF4, pcDNA/Wt-NPM1, pcDNA/Mt-A-NPM1, pcDNA/Mt-I-NPM1, or pcDNA/Mt-J-NPM1 was transfected into U937, 293T, and COS7 cells seeded in six-well dishes by using Nucleofecten (Qiagen) together with 0.1μg of pGL4 reporter plasmid (pGL4/APET (1), pGL4/ETSm-APET (1), pGL4/HDM2, or pGL4/HDM2mut) and 0.05μg of pLR-Bact vector. PGL4/ETSm-APET contains a mutation in the ETS binding site (ETSm-APET: 5'-CCTCAGTGAGCTGAGTCAGGCTgagCC TcgacGCCACAGGG-3'). pGL4/HDM2 contains a wild-type hdm2 (P2) promoter sequence from bp −82 to −122 (Wt-Ets: CAGGTTGACTCAGCTTTTCCTCTTGAG CTGGTCAAGTTCAG), and pGL4/HDM2mut contains a hdm2 (P2) promoter sequence with a mutated ETS site (Mt-Ets: CAGGTTGACTCAGCTTTTaCTCTTGAG CTGGTCAAGTTCAG) (23). Cell lysates were prepared 48 h after transfection, and luciferase activity was determined by using a Dual-Luciferase Reporter Assay System (Promega).

Anchorage-independent growth assay

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

Immunochemistry

MEF/ELF4 was cloned into the pGFP-C3 vector (Clonetech, Mountain View, CA, USA) (pGFP-MEF/ELF4). 293T cells were transfected with the empty vector, pGFP-MEF/ELF4, pcDNA/V-Wt-NPM1, or pcDNA/V-Mt-A-NPM1. Cells were harvested 3 days after transfection. Cytospin samples were fixed for 15 min in PBS containing 4% paraformaldehyde. Fixed coverslips were washed twice in TBS, permeabilized in 0.5% Triton X-100 for 10 min, and incubated in Image-iT FX Signal Enhancer (Invitrogen) for 30 min. Cells were incubated with primary antibody for 1 h and then washed extensively in TBS before

incubation with Alexa546-conjugated goat anti-mouse-IgG antibody (dilution, 1:2000; Invitrogen) for 1 h. Cells were covered with a drop of ProLong Gold Antifade Reagent with DAPI (Invitrogen). Fluorescent images were obtained by using a confocal laser scanning microscope (LSM 5 Pascal V3.2; Carl Zeiss, Jena, Germany).

ChIP assay

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

Clinical samples

Informed consent for sample collection was obtained according to protocols approved by the International Review Board of Nagasaki University, Nagasaki, Japan (approval number 33-3). Bone-marrow aspirates were collected from 22 AML patients before the initiation of chemotherapy. CD34-positive cells were isolated by using Ficoll density gradient centrifugation and magnetic beads (CD34 Isolation Kit; Miltenyi Biotec, Auburn, CA, USA) to minimize the confounding effect of MEF/ELF4 and NPM1 expression by mature myeloid cells. For the screening of NPM1 mutations, genomic DNA corresponding to exon 12 was amplified by using forward primer 5'-TTAACTCTCTGGTGGTAGAATGAA-3' and reverse primer 5'-CAAGACTATTTGCCATTCCTAAC-3', as reported previously. Amplified products were separated by agarose gel electrophoresis, purified by using a QIAquick gel extraction kit (Qiagen), and directly sequenced by using a DNA sequencer (3100; Applied Biosystems) with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). When mutations were found by direct sequencing, the fragments were cloned into a pTOPO

vector (Invitrogen) and then transfected into the *E. coli* strain DH5A. At least four recombinant colonies were selected, and plasmid DNA samples were prepared by using a QIAprep Spin Miniprep Kit (Qiagen). Cloned fragments were sequenced to confirm the mutation of the *NPM1* gene.

Total RNA was harvested from purified CD34-positive cells by using an RNeasy Minikit (Qiagen). cDNA synthesis was undertaken by using an oligo (dT) primer with a PrimeScript II First-strand cDNA Synthesis Kit (Takara, Shiga, Japan). These cDNA molecules were measured by RQ-PCR with the primers listed in the Methods section of RQ-PCR.

RQ-PCR

RQ-PCR was performed by using a LightCycler TaqMan Master kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Twenty microliters of Universal ProbeLibrary probes (Exiqon, Vedbaek, Denmark) were added in the final reaction. Primers designed by using the Universal ProbeLibrary Assay Design Centre (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) were synthesized by Sigma. PCR amplification was performed by using a LightCycler 350S instrument (Roche). Thermal cycling conditions comprised 2 min at 40°C and 10 min at 95°C, followed by 45 amplification cycles at 95°C for 10 s, 60°C for 30 s, 72°C for 1 s, and then a 40°C cooling cycle for 30 s. Specific primers and probes were as follows: for HDM2, forward 5'-TCTGATAGTATTTCCCTTTCCTTTG-3', reverse 5'-TGTTCACTTACACCAGCATCAA-3', probe 5'-CGCCACTTTTTCTCTGCTGATCCAGG-3'; human MEF/ELF4, forward 5'-TGGAGACTCTCAGGGTCGAAA-3', reverse 5'-AAGCAACGGGATGGATGAT-3', probe 5'-TCACAGCTGGGAACACAGAG-3'; and human G6PDH, forward 5'-AAGCAACGGGATGGATGAT-3', reverse 5'-TCACAGCTGGGAACACAGAG-3', and probe 5'-CGCCACTTTTTCTCTGCTGATCCAGG-3'.

Statistical analyses

Comparisons of patient characteristics between two groups were performed with the Wilcoxon test. The results of *in vivo* experiments are presented as the mean ± SD of three independent experiments and compared by using one-way ANOVA followed by Scheffe's multiple comparison test. A P-value of 0.05 was considered statistically significant.

RESULTS

Identification of MEF/ELF4-binding protein