Table 2 Patients' characteristics

	Donor	No-donor	*p*
Total number	73	92	
Age			
Median (range)	37 (16–50)	36 (15–50)	0.60[a]
15–35 years	33	46	
36–50 years	40	46	0.54[b]
Sex			
M/F	44/29	45/47	0.15[b]
WBC at diagnosis (10^9/l) (range)	3.8 (0.05–36.8)	5.1 (0.14–45.0)	0.16[a]
MPO positivity of blasts (range)	30 (0–100)	50 (0–100)	0.18[a]
FAB classification			
M0	4	6	
M1	18	25	
M2	22	24	
M4	20	23	
M5	7	14	
M6	1	0	
M7	1	0	0.67[b]
Performance status			
0–1	66	84	
2–3	7	8	0.70[b]
Risk classification by JALSG scoring system			
Intermediate	64	84	
Poor	9	8	0.45[b]
Cytogenetics			
t(8;21) or inv(16)	4	4	0.74[b]
Chemotherapy group			
Group A	38	42	
Group B	30	47	0.28[c]
Not randomized	5	3	
Allogeneic transplant			
During CR1	38	11	
		9 from UD	
		1 from MUD	
		1 from MRD	
After relapse	18	31	
No transplant	17	50	

UD HLA-matched unrelated donor, *MUD* HLA-mismatched unrelated donor, *MRD* HLA-mismatched related donor, *WBC* white blood count, *MPO* myeloperoxidase

[a] Mann–Whitney test

[b] Chi-square test

[c] Chi-square test excluding non-randomized

patients in the donor group and four in the no-donor group died of transplant-related causes during CR1. The lower RR in the donor group resulted in a significantly better DFS compared with the no-donor group (39 vs. 19%, respectively, HR, 0.63; 95% CI, 0.44–0.92; $P = 0.016$; Table 3; Fig. 3). The significant superiority of DFS in the donor group translated into a higher OS rate, but the difference in OS between the two groups did not reach statistical significance (46 vs. 29%, HR, 0.70; 95% CI, 0.47–1.06; $p = 0.088$; Table 3; Fig. 4).

The donor/no-donor analysis was performed on the intention-to-treat principal, which may underestimate the beneficial effect of allo-HSCT probably because of low compliance of transplantation. The 8-year DFS and OS of the recipients actually transplanted during CR1 ($n = 38$) in the donor group were significantly better than those of the patients not transplanted in the no-donor group ($n = 50$); 58 versus 27%, HR, 0.36; 95% CI, 0.20–0.66; $p < 0.001$, and 61 versus 24%, HR, 0.36; 95% CI, 0.19–0.68; $p = 0.001$, respectively.

Table 3 Effects of donor availability on outcome in donor and no-donor groups

Outcome	Donor			No-donor			*p*	HR (95% CI)
	n	No. of events	Probability of outcome at 8 years ±SE (%)	*n*	No. of events	Probability of outcome at 8 years ±SE (%)		
All patients	73			92				
RR		36	52 ± 6		67	77 ± 5	0.008	0.58 (0.39–0.88)
TRM		7	16 ± 6		7	17 ± 7	0.959	0.97 (0.34–2.80)
DFS		44	39 ± 6		74	19 ± 4	0.016	0.63 (0.44–0.92)
OS		37	46 ± 7		61	29 ± 6	0.088	0.70 (0.47–1.06)
Age ≤35	33			46				
RR		17	52 ± 9		31	70 ± 7	0.309	0.74 (0.41–1.33)
TRM		2	12 ± 8		3	15 ± 8	0.785	0.78 (0.13–4.71)
DFS		20	39 ± 9		34	26 ± 7	0.366	0.78 (0.45–1.35)
OS		18	42 ± 10		27	35 ± 9	0.860	0.95 (0.52–1.72)
Age >35	40			46				
RR		19	52 ± 9		36	85 ± 6	0.006	0.46 (0.26–0.81)
TRM		5	19 ± 8		4	19 ± 11	0.962	1.03 (0.27–3.92)
DFS		24	39 ± 8		40	12 ± 5	0.012	0.52 (0.31–0.87)
OS		19	49 ± 9		34	24 ± 7	0.031	0.54 (0.31–0.95)

RR relapse rate, *DFS* disease-free survival, *TRM* treatment-related mortality, *OS* overall survival

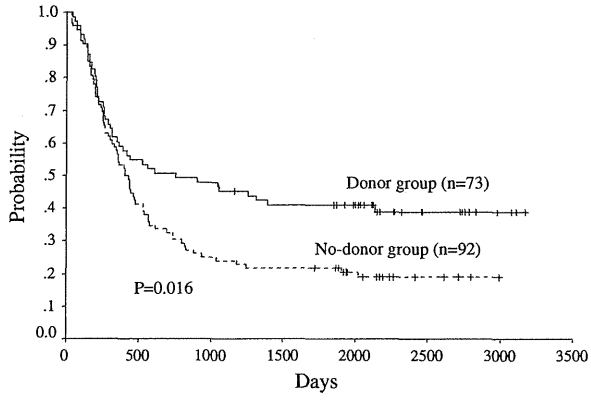

Fig. 3 Disease-free survival in donor and no-donor groups

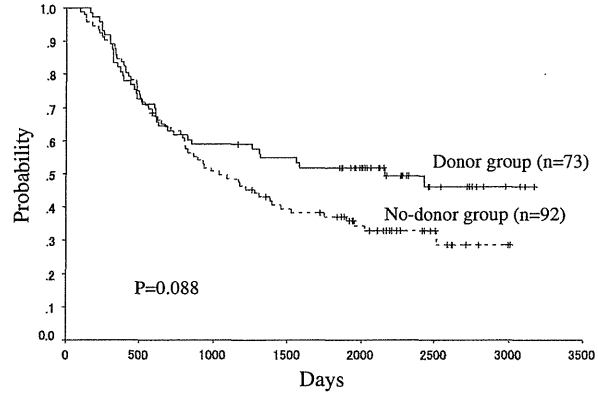

Fig. 4 Overall survival in donor and no-donor groups

3.6 Subset analysis according to patient age

The OS of the patients younger than 35 years of age were comparable between the donor and the no-donor groups (Fig. 5a). However, the OS of the patients aged >35 in the donor group was significantly better compared with the no-donor group (49 vs. 24%, respectively, HR, 0.54; 95% CI, 0.31–0.95; $p = 0.031$; Table 3; Fig. 5b). The RR, TRM, DFS and OS in the donor group were comparable between the two age categories (Table 3; Fig. 5c). In contrast, OS and DFS were marginally worse in the no-donor group of patients aged >35 than ≤35 years (Table 3; Fig. 5d). The distribution of the cytogenetic profile, risk by the JALSG scoring system, myeloperoxidase positivity of blasts, WBC count, FAB classification and performance status at diagnosis did not significantly differ between the two age categories in the no-donor group (data not shown).

4 Discussion

Many clinical trials have compared allo-HSCT with chemotherapy as a post-remission therapy for the patients with AML during CR1. Most of these targeted all patients in CR1 as a single population without prospective stratification by the prognostic factors. Thus, patients were simply assigned into the allo-HSCT or the chemotherapy groups according to donor availability [7, 10, 17, 18]. Here, we

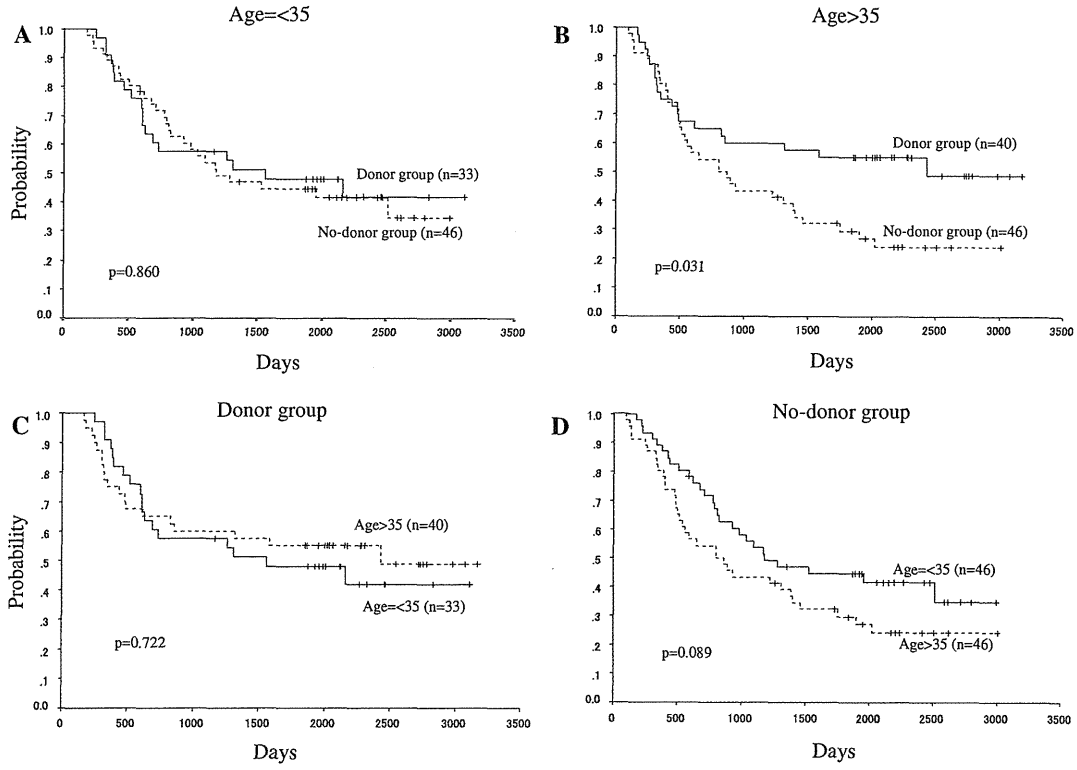

Fig. 5 Overall survival of patients according to age (**a** and **b** ≤35 and >35 years, respectively) and donor availability (**c** and **d**, donor and no-donor groups, respectively)

prospectively compared the effectiveness of allo-HSCT with chemotherapy among patients who were stratified into intermediate or poor risk groups according to JALSG scoring, which constitutes a new means of predicting the prognosis of AML. When this study was planned, as the availability of the cytogenetic study was expected to be variable, and the JALSG scoring system was revealed to be useful to stratify the patients, we adopted a scoring system to select the intermediate and poor risk patients. In contrary to our expectation, cytogenetic studies were performed in 99.2% of the registered patients and the results were available in 97% of the patients. Of 330 CR patients younger than 50 years old, cytogenetic studies disclosed that 97 had good prognostic chromosomal abnormalities, i.e., t(8;21) or inv(16). The OS was significantly better among patients with than without good prognostic cytogenetic profiles (70 vs. 47% at 5 years, with HR, 0.51; 95% CI, 0.34–0.77; $p = 0.001$; Fig. 2b). According to JALSG scoring, 87, 10 and 0 patients with good prognostic cytogenetic abnormalities corresponded to the good, intermediate and poor risk groups, respectively. More good risk patients were selected using this scoring system than by that using karyotype of AML cells alone and about 10% of patients who might be classified into the good risk group by cytogenetic profiles entered the comparison groups by the JALSG scoring system. The JALSG scoring system, which resembles the index used in the Bordeaux Grenoble Marseille Toulouse (BGMT) intergroup study [18], obviously separated patients with a good prognosis who should be excluded from the transplantation trials.

Allo-HSCT prevents AML relapse through intensive cytoreduction using high-dose chemoradiotherapy and graft-versus-leukemia effects. However, previous trials have not always shown advantages of this strategy on the survival of AML patients in CR1. Some studies have not found a benefit of allo-HSCT either on DFS or OS [7, 8], and some showed an advantage only on DFS [10, 17] compared with chemotherapy/auto-transplantation. Retrospective subgroup analysis and meta-analysis have shown a better OS in the donor group [10, 13, 19, 20], demonstrating the importance of limiting the indication of allo-HSCT for only the patients with an intermediate or poor risk.

The following issues should be considered regarding the prospective comparison of allo-HSCT with chemotherapy: assignment of patients according to sibling donor availability [21], low compliance of allo-HSCT for patients in the donor group, and allo-HSCT performed in the no-donor

group from unrelated donors. We could compare the effectiveness of treatment strategies using the intention-to-treat analysis. However, the intrinsic issues of this type of trial and recent advances in alternative stem cell sources will cause difficulties with future prospective comparison of allo-HSCT and chemotherapy using a similar study design.

Although the comparison was performed among patients in the intermediate and poor risk groups, the benefit of allo-HSCT was not significant in OS. Low compliance of allo-HSCT during CR1 in the donor group (52% in the current trial) and allo-HSCT in the no-donor group (total 45%; 11% during CR1) appeared to make the efficacy of allo-HSCT underestimated, especially with regard to OS. However, survival was significantly better among older patients in the donor group (Table 3; Fig. 5b), which seemed to contradict previous findings [19]. Age usually adversely affects allo-HSCT outcome, but it was not associated with the decrease of OS in the donor group in the present study (Table 3; Fig. 5c). Low incidence of TRM probably allowed the powerful anti-leukemic effect of allo-HSCT to function properly, indicating the advantage of allo-HSCT especially among older patients with leukemia that was more resistant to chemotherapy than that among younger patients [1] shown in the no-donor group (Fig. 5d), and caused a contrary result from HOVON/SAKK study. The recent reduction in TRM seemed to contribute much to these results as suggested by others [22, 23]. Different population of the cohorts selected by JALSG scoring and by cytogenetic profiles might also have influenced the present findings.

Molecular markers can be very useful for selecting patients who will most likely benefit from allo-HSCT during CR1 among those with a normal karyotype, which comprises the largest group of patients with AML [24]. The overall safety of allo-HSCT obviously needs improvement, and also patients with chemotherapy-resistant AML who could benefit from allo-HSCT should be identified. Thus, stratification of patients with AML should be improved using a combination of leukemic cell karyotype and, genetic markers and also other clinical findings.

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ORIGINAL ARTICLE

Randomized trial of response-oriented individualized versus fixed-schedule induction chemotherapy with idarubicin and cytarabine in adult acute myeloid leukemia: the JALSG AML95 study

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Abstract A multicenter, prospective, randomized study was conducted to compare a response-oriented individualized remission induction therapy with a standard fixed-schedule induction therapy, using idarubicin (IDR) and cytarabine (Ara-C), in adult patients with acute myeloid leukemia (AML). Newly diagnosed patients with AML of age less than 65 were randomly assigned to receive either of the two schedules. Both groups received IDR (12 mg/m^2) for 3 days and Ara-C (100 mg/m^2) for 7 days. In the individualized group, if the bone marrow on day 8 did not become hypocellular with less than 15% blasts, patients received additional IDR for one more day and Ara-C for 2 or 3 more days. Patients achieving complete remission (CR) received the same post-remission therapy. The CR rate was 79.4% for the individualized group ($n = 209$) and 81.9% for the fixed group ($n = 221$) ($p = 0.598$). At a median follow-up of 81 months, 7-year predicted overall survival was 37% for the individualized group and 39% for

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the fixed group ($p = 0.496$), and 7-year predicted event-free survival was 22% for the individualized group and 23% for the fixed group ($p = 0.546$). Thus, the present study could not demonstrate any advantage of a response-oriented individualized induction therapy over a fixed-schedule induction therapy in this protocol setting.

Keywords Acute myeloid leukemia · Response-oriented individualized induction therapy · Idarubicin · Cytarabine

1 Introduction

In Japan, a response-oriented individualized induction therapy has been used for adult acute myeloid leukemia (AML) since the reporting of the success of DCMP two-step therapy using daunorubicin (DNR), cytarabine (Ara-C), 6-mercaptopurine (6MP) and prednisolone (PSL), by Uzuka et al. in the mid 1970s [1]. They reported a complete remission (CR) rate of more than 80% in adult AML, which is currently not surprisingly high but was remarkable in the mid 1970s even for a single institutional study. A subsequent multi-institutional study conducted at the Koseisho Leukemia Study Group using this DCMP two-step protocol could not replicate the high CR rate, but a subset analysis revealed the first-step alone could induce almost the same CR rate as the two-step therapy [2]. Accordingly, a response-oriented individualized induction therapy, the BHAC-DMP therapy, using enocitabine (BHAC), Ara-C, 6MP, and PSL, was developed, and Ohno et al. [3] reported more than 80% CR in adult AML by a single institutional study.

The multi-institutional AML87 study conducted by the Japan Adult Leukemia Study Group (JALSG) confirmed the high CR rate of response-oriented individualized BHAC-DMP therapy in adult AML, reporting an 80% CR rate [4]. Subsequent JALSG studies, AML89 [5] and AML92 [6], also employed the response-oriented individualized induction therapy, and reported 81 and 77% CR rates, respectively, in adult patients of age less than 65 years with non-M3 type AML. These CR rates were around 10% higher than those reported from cooperative study groups in the USA and Europe, where fixed-schedule induction therapies are used [7]. Therefore, even though the necessity for a randomized study was seriously discussed among JALSG members, it was not possible to find any fixed-schedule regimen worth comparing with the present individualized therapy.

In the above 3 JALSG studies, DNR was used as one of the key drugs. However, in the late 1980s, a new DNR analogue, idarubicin (IDR), was introduced clinically, and in the early 1990s, one single [8] and 2 multi-institutional studies [9, 10] reported that IDR plus Ara-C regimens could produce 70–80% CR rates in adult AML by fixed-schedule therapy, which were significantly higher than the 58–59% CR rates of DNR plus Ara-C regimens.

Consequently, after IDR had been approved in Japan in 1995, a randomized study using IDR and Ara-C was conducted, comparing a response-oriented individualized induction therapy with a fixed-schedule therapy in previously untreated adult patients with AML.

2 Patients and methods

2.1 Patients

From August 1995 to December 1997, 437 newly diagnosed adult patients with AML, aged 15–64 years, were consecutively registered from 79 institutions, which participated in JALSG. The enrolled number of patients per hospital varied from 1 to 23 with median number of 4, and about 60% of patients were registered from major hospitals listed in the institutions of the authors.

AML was diagnosed by the French-American-British (FAB) classification at each institution. Peripheral blood and bone marrow smears from all registered patients were sent to Nagasaki University and examined by May-Giemsa, peroxidase, and esterase staining. Then, diagnosis was reevaluated by the central review committee. FAB-M3 was not registered in this study. Eligibility criteria included adequate functioning of the liver (serum bilirubin level < 2.0 mg/dL), kidney (serum creatinine < 2.0 mg/dL), heart, and lungs, and an Eastern Cooperative Oncology Group performance status between 0 and 3. Patients were not eligible if they had prediagnosed myelodysplastic syndrome (MDS), but were eligible if they had no definite diagnosis of MDS, as confirmed by bone marrow histological analysis even when they had a previous history of

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hematological abnormality. Cytogenetic analyses were performed at either laboratories in participating hospitals or authorized commercial laboratories according to standard methods of G-banding. Cytogenetic abnormalities were grouped by standard criteria and classified according to the MRC classification [11]. The protocol was approved by institutional review board of each hospital. Informed consent was obtained from all patients before registration.

2.2 Treatment regimens

Patients were assigned randomly to receive either a response-oriented individualized induction therapy or a fixed-schedule induction therapy, using a centralized telephone procedure. All patients received IDR (12 mg/m^2/day, intravenously) from days 1 to 3 and Ara-C (100 mg/m^2/day, by 24-h continuous infusion) from days 1 to 7. Examination of bone marrow on the day 8 was evaluated at each participating hospital and the decision was made by the attending physician in charge of the hospital. In the individualized group, bone marrow aspiration was performed on day 8, and if the marrow was not severely hypoplastic and had more than 15% blasts, additional IDR was given on day 8 and Ara-C on days 8 to 10, or if the marrow was severely hypoplastic and had more than 15% blasts, additional IDR was given on day 8 and Ara-C on days 8 and 9. If patients suffered from documented infection on day 8, cancellation of additional chemotherapy was permitted according to the judgment of the attending physician (Fig. 1). The main aim of the individualized therapy was to give highly intensive but not too toxic doses of anti-leukemia drugs, especially IDR, to make the bone marrow severely hypoplastic, reduce the percentage of blasts to less than 5% within 10 days and obtain CR by the first course of induction therapy. In the fixed-schedule group (fixed group), patients did not receive additional doses regardless of their marrow status at day 8. If patients did not achieve CR by the first course, the same induction therapy was repeated at approximately 3- to 4-week intervals. If patients did not achieve CR with two courses, they were judged as failure cases.

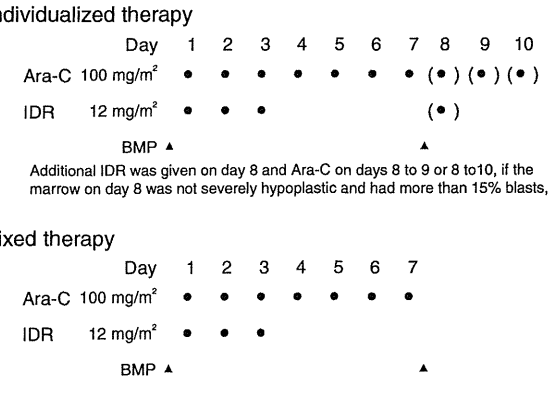

Fig. 1 Treatment scheme of induction therapy

All patients in both groups who had achieved CR planned to receive the same 3 courses of consolidation therapy. The first course consisted of mitoxantrone (MIT; 7 mg/m^2 by 30-min infusion on days 1–3) and Ara-C (200 mg/m^2 by 24-h continuous infusion on days 1–5). The second consisted of BHAC (200 mg/m^2 by 3-h infusion on days 1–7), DNR (50 mg/m^2 intravenously on days 1–3), 6MP (70 mg/m^2 orally on days 1–7), and etoposide (ETP; 100 mg/m^2 by 1-h infusion on days 1–5). The third consisted of BHAC (200 mg/m^2 on days 1–7) and aclarubicin (ACR; 14 mg/m^2 intravenously on days 1–7). Each consolidation course was given as soon as possible after WBC and platelet counts had recovered to more than 3,000/μL and 100,000/μL, respectively. Intrathecal methotrexate (15 mg), Ara-C (40 mg), and PSL (10 mg) were given after the second consolidation therapy for the prophylaxis of central nervous system leukemia.

After the completion of consolidation therapy, all patients planned to receive 6 courses of maintenance/intensification therapy every 2 months. The first course consisted of BHAC (170 mg/m^2 on days 1–5), DNR (40 mg/m^2 on days 1 and 4), and 6MP (70 mg/m^2 on days 1–7). The second consisted of BHAC (170 mg/m^2 on days 1–5) and MIT (5 mg/m^2 on days 1 and 2). The third consisted of BHAC (170 mg/m^2 on days 1–5), ETP (80 mg/m^2 on days 1, 3, and 5), and vindesine (2 mg/m^2 intravenously on days 1 and 8). The fourth consisted of BHAC (170 mg/m^2 on days 1–5), ACR (14 mg/m^2 on days 1–4) and 6MP (70 mg/m^2 on days 1–7), the fifth was the same as the first, and the sixth was the same as the third. Each course was given at 2-month intervals.

Best supportive care, including administration of antibiotics and platelet transfusion from blood cell separators, was given if indicated. When patients had life-threatening infections during neutropenia, the use of granulocyte colony-stimulating factor was permitted.

2.3 Response criteria and statistical analysis

CR was defined as the presence of all of the following: less than 5% of blasts in bone marrow, no leukemic blasts in peripheral blood, recovery of peripheral neutrophil counts over 1,000/μL and platelet counts over 100,000/μL, and no evidence of extramedullary leukemia. CR had to continue for at least 4 weeks, but the date of CR was defined as the first day when these criteria were fulfilled. Relapse was defined as the presence of at least one of the following: recurrence of more than 10% leukemic cells in bone marrow, any leukemic cells in peripheral blood, and appearance of extramedullary leukemia.

Overall survival (OS) was calculated from the first day of induction therapy to death by any cause and censored at the last follow-up. Event-free survival (EFS) was computed from the first day of induction therapy to relapse or death by any cause and censored at the last follow-up, and the survival time of patients who did not achieve CR was defined as 0 days. Relapse-free survival (RFS) for patients who achieved CR was measured from the date of CR to relapse or death by any cause and censored at the last follow-up. Patients who underwent allogeneic bone marrow transplantation (BMT) were censored at the date of BMT or not censored according to the object of the analysis. Kaplan–Meier product-limit estimates were used to determine OS, EFS, and RFS. To test factors to predict CR, χ^2 test and Wilcoxon rank-sum test were used for univariate analysis and the multiple logistic regression model for multivariate analysis. For comparison of OS, EFS, and RFS, the log-rank test was used for univariate analysis and Cox's proportional hazard model for multivariate analysis. JMP software (SAS Institute Inc., Cary, NC, USA) was used for the analysis; p values less than 0.05 (two-sided) were considered statistically significant. Analysis was done on an intent-to-treat basis.

3 Results

3.1 Patient population and characteristics

Of 437 patients registered, 7 patients were judged as ineligible by the central review committee because of other diseases: one refractory anemia with excess of blast, 5 mixed-lineage leukemia, and one acute lymphoblastic leukemia (ALL), with 430 patients considered evaluable. Two hundred nine patients received the individualized therapy and 221 the fixed-schedule therapy. Pretreatment characteristics are presented in Table 1. There were no major imbalances between the two randomized groups. Overall, the median age was 44 years, and 154 patients (36%) were of age 50 years or older. Cytogenetic analysis was reported in 414 patients (96%), and the cytogenetic prognostic groups were equally distributed in both arms.

3.2 Overall treatment results

Of 430 evaluable patients, 347 (80.7%) achieved CR. Of 209 patients in the individualized group, 166 (79.4%) achieved CR, and of 221 in the fixed group, 181 (81.9%) obtained CR ($p = 0.516$) (Table 2). CR rates related to FAB classification, age, and cytogenetics are shown in Table 2, and there were no statistically significant differences between the two groups. In the individualized group, of 41 patients with favorable chromosomes, 39 (95%) achieved CR, of 133 with intermediate chromosomes, 109 (79%) achieved CR, and of 28 with adverse chromosomes, 17 (61%) achieved CR. In the fixed group, of 49 patients with favorable chromosomes,

Table 1 Pretreatment characteristics

	Individualized group ($n = 209$)	Fixed group ($n = 221$)
Median age (range)	44 years (15–64)	44 years (15–64)
PS 0	34.9%	38.5%
PS 1	42.6%	45.2%
PS 2	14.4%	9.5%
PS 3	8.1%	6.8%
Leukocyte counts > 50,000/μL	17.7%*	29.9%*
Peroxidase positivity ≥ 50%	62.8%	64.2%
Presence of Auer body (%)	37.5%	46.1%
Presence of trilineage dysplasia	25.4%	21.2%
LDH ≥ 500 IU/L	65.9%	69.1%
Cytogenetics		
Favorable	19.6%	22.2%
Intermediate	63.6%	59.7%
Adverse	13.4%	14.0%
Unknown	3.3%	4.1%

* $p < 0.05$

Table 2 CR rates related to FAB classification, age, and cytogenetics

	All cases		Individualized group		Fixed group	
	No.	CR (%)	No.	CR (%)	No.	CR (%)
FAB						
M 0	16	62.5	8	62.5	8	62.5
M 1	80	85.0	41	85.4	39	84.6
M 2	192	82.3	95	77.9	97	86.6
M 4	108	78.7	55	80.0	53	77.4
M 5	20	90.0	5	100.0	15	86.7
M 6	8	50.0	2	50.0	6	50.0
M 7	6	66.7	3	66.7	3	66.7
Age						
15–19	40	90.0	19	100.0	21	81.0
20–29	65	78.5	29	75.9	36	80.6
30–39	71	81.7	41	75.6	30	90.0
40–49	100	83.0	45	77.8	55	87.3
50–59	105	77.1	53	79.2	52	75.0
60–64	49	77.6	22	77.3	27	77.8
Cytogenetics						
Favorable	90	93.3	41	95.1	49	91.8
Intermediate	265	80.8	133	78.9	132	82.6
Adverse	59	62.7	28	60.7	31	64.5
Unknown	16	75.0	7	71.4	9	77.8
Total	430	80.7	209	79.4	221	81.9

45 (92%) achieved CR, of 132 with intermediate chromosomes, 109 (83%) achieved CR, and of 31 with adverse chromosomes, 20 (65%) achieved CR.

In the individualized group, 149 patients (71%) achieved CR after the first course, and 79 (38%) patients who had received additional chemotherapy during the first course, 56 (71%) achieved CR. In the fixed group, 159 (72%) achieved CR after the first course (Table 3; Fig. 2). CR rates between patients who had equal to or more than 15% of blasts in bone marrow on day 8 and those had less than 15% were not significantly different in the individualized group (75 and 63%, respectively; $p = 0.09$), but were significantly different in the fixed group (81 and 56%, respectively; $p < 0.001$).

Myelosuppression judged by the nadir of leukocyte counts and the period of leukocyte count less than 1,000/μL after the first course of induction therapy was significantly more severe in the individualized group, as shown in Table 4. Early death within 30 days occurred in 10 (4.8%) in the individualized group and 4 (1.8%) in the fixed group ($p = 0.105$). There were no statistically significant differences in the distribution or frequency of complications between the two groups.

Significant favorable prognostic features for the achievement of CR were cytogenetic risk group (favorable or intermediate), blast peroxidase positivity of 50% or more, and pretreatment LDH value of less than 500 IU/L. These features were independent by the logistic regression analysis and not different between the two groups.

All courses of consolidation therapy were administered to 72% of patients in the individualized group and 80% in the fixed group ($p = 0.087$), and all courses of maintenance therapy were administered to 36 and 41% ($p = 0.365$), respectively. The most common reason for these cancellations was relapse in both groups (34 and 42 patients, respectively). The second common reason was BMT in the first remission (22 and 12 patients, respectively).

At a median follow-up of 81 months, 23 patients underwent BMT in the first remission, 29 after relapse and 4 without remission in the individualized group, and 15, 32 and 7 patients, respectively, in the fixed group. If patients who underwent BMT were censored at the date of transplantation to decrease the influence of BMT, 7-year predicted OS was 37% for the individualized group and 39% for the fixed group ($p = 0.496$) (Fig. 3a), and 7-year predicted EFS was 22 and 23%, respectively ($p = 0.546$) (Fig. 3b). If patients who underwent BMT were not censored, 7-year predicted OS was 35 and 35%, respectively

Table 3 Effect of individualized induction therapy

	Patients (%)	CR after first course	
		n	%
Individualized group	209	149	71
Additional chemotherapy −	130 (62)	93	72
Additional chemotherapy +	79 (38)	56	71
Fixed group	221	159	72

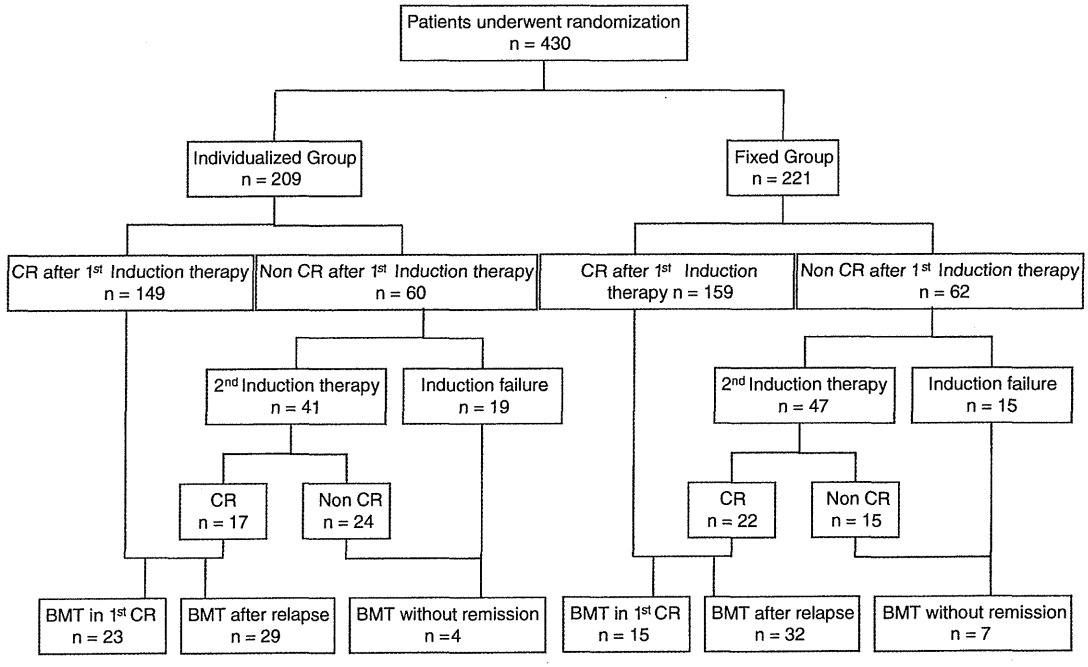

Fig. 2 Flow diagram: study design and outcome

Table 4 Comparison of treatment outcome

	Individualized group ($n = 209$)	Fixed group ($n = 221$)	p
CR rate (%)	79.4	81.9	0.516
After the first course	71.3	71.9	
After the second course	8.1	10.0	
Marrow blasts at day 8	12.9 ± 17.8%	11.1 ± 18.4%	0.021
Nadir of WBC[a]	328 ± 205/μL	394 ± 215/μL	0.0002
Period of WBC < 1,000/μL[a]	19.6 ± 9.8 days	17.8 ± 8.5 days	0.024
Days to CR[a]	38.9 ± 17.5	38.5 ± 16.2	0.802
Days till the consolidation therapy	49 ± 22	46 ± 18	0.157
Early death rate			
Within 30 days	4.8%	1.8%	0.105
Between 30 and 60 days	0.9%	1.4%	
Overall survival at 7 years	37%	39%	0.496
Event-free survival at 7 years	22%	23%	0.546

Data with ± denotes mean ± standard deviation

[a] After the initial course of induction therapy

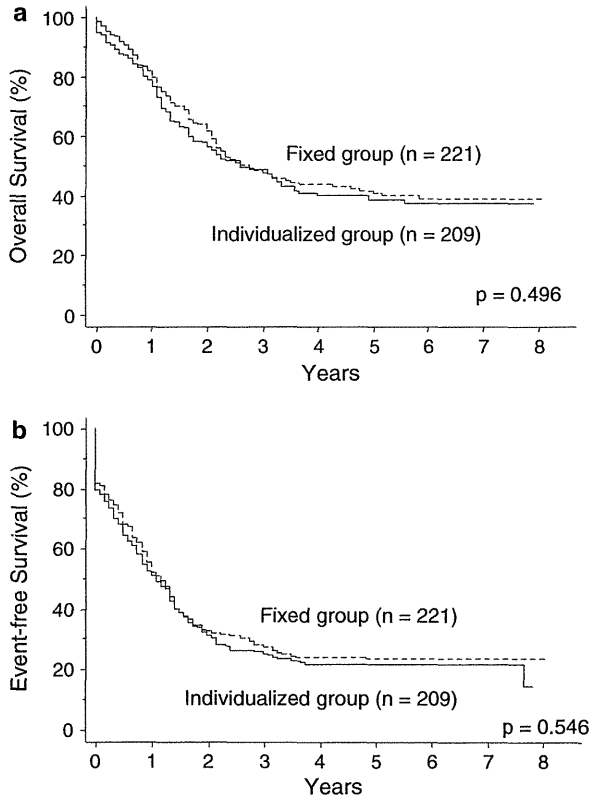

Fig. 3 Overall survival (**a**) and event-free survival (**b**). Predicted 7-year OS was 37% for the individualized group ($n = 209$) (*solid line*) and 39% for the fixed group ($n = 221$) (*dotted line*) ($p = 0.496$), and EFS was 22% for the individualized group (*solid line*) and 23% for the fixed group (*dotted line*) ($p = 0.546$)

($p = 0.840$), and 7-year predicted EFS was 23 and 24%, respectively ($p = 0.717$). Significant adverse prognostic features for OS were absence of Auer body, cytogenetic risk group (adverse), and age more than 30 years, and those for EFS were blast peroxidase positivity less than 50%, cytogenetic risk group (adverse), pretreatment LDH value equal or more than 500 IU/L, and FAB classification (M0, M6, or M7). When patients who underwent BMT were censored, RFS of CR patients was 27% for the individualized group and 29% for the fixed group ($p = 0.712$). Significant adverse prognostic features for RFS of CR patients were cytogenetic risk group (adverse) and FAB classification (M0, M6, or M7). There were no significant differences in these prognostic features between the two groups. However, among patients of age 50 years or older, the individualized group had significantly lower RFS (17%) than the fixed group (34%, $p = 0.026$), but there was no such difference of RFS (34 and 25%, respectively, $p = 0.194$) among patients of age less than 50 years.

4 Discussion

Most drug therapies are generally carried out in a response-oriented and individualized manner. Physicians adjust the dosage and treatment period depending on the response of patient's disease to the administered drugs. The reason why cancer chemotherapy is generally carried out by fixed dosage and period is because myelosuppression, the most important toxic effect of cytotoxic drugs, appears 7–10 days after the discontinuation of drugs. Myelosuppression is usually judged by leukocyte or platelet counts in the peripheral blood. However, if it is judged by bone marrow itself it is possible to obtain information on myelosuppression directly and earlier. Although the present individualized therapy requires frequent bone marrow aspirations and a prompt decision by attending physicians, well-trained hematology oncologists have little difficulty in

making such a decision. In addition, the present protocol states that, if the decision is difficult due to equivocal findings, additional drugs should be given.

It was considered that the higher CR rates of previous JALSG studies for adult AML: AML87 [4], AML89 [5] and AML92 [6], were due to response-oriented individualized therapy, giving highly intensive but not too toxic doses of anti-leukemia drugs, especially IDR, to make the bone marrow severely hypoplastic, reduce the percentage of blasts to less than 5% within 10 days, and aim to obtain CR by the first course of induction therapy. For example, in the AML89 study, the primary objective of which was to compare Ara-C with BHAC in remission induction therapy, 130 (82%) of 159 patients in the DNR + Ara-C + 6MP + PSL group achieved CR by this individualized induction therapy [5]. It is clear that without a prospective randomized study, one cannot argue whether the individual therapy is superior to a standard fixed-schedule remission induction therapy. However, it is noteworthy, that in the 3 randomized studies in the USA mentioned in Sect. 1, which compared IDR plus Ara-C with DNR plus Ara-C, the fixed-schedule therapy with DNR plus Ara-C resulted in merely 57–58% CR rates, while IDA plus Ara-C regimens produced 70–80% CR rates [8–10].

Disappointingly, the present study could not demonstrate that response-oriented individualized therapy was superior to the fixed-schedule therapy. Both regimens resulted in almost the same CR rates: 79 and 82%, respectively. Actually, both therapies produced very good CR rates. The results were interpreted as follows: IDR is a good but very powerful drug, therefore, additional IDR and Ara-C on day 8 or later may not be necessary and gave too much myelosuppression. In fact, in the individualized group, leukocytopenia was significantly more severe and its duration was significantly longer, and early death within 30 days tended to occur more frequently. From the present study it is suggested that response-oriented individualized therapy could be successful in cases where DNR is used as a key drug. Usui et al. [12] reported that the optimal dose of DNR in the induction therapy for newly diagnosed adult AML was approximately 280 mg/m^2 (40 mg/m^2 for 7 days), which was more than its conventional dose of 40–60 mg/m^2 for 3 days.

It is very interesting that among patients of age 50 years or older, the individualized group had significantly lower RFS than the fixed group, but there was no such difference in younger patients. However, we cannot clearly explain the real reason of this observation. There may be potential sources of bias in our subset analysis of clinical data that have many confounding factors. Therefore, we must be cautious in drawing a conclusion from this observation.

So far, CR rates around 80% for newly diagnosed adults of age less than 65 years with non-M3 AML seems to be the upper limit by currently available anti-leukemia drugs in multi-institutional studies [7]. To increase the CR rates and improve treatment outcomes, novel drugs other than cytotoxic ones such as all-*trans* retinoic acid (ATRA) for acute promyelocytic leukemia (APL) are needed. With ATRA in combination with conventional cytotoxic drugs such as IDR and Ara-C, CR rates around 95% and more than 80% overall survival for APL with PML/RARα can be obtained [13, 14]. The remarkable success of molecule targeting therapy with ATRA against APL as well as imatinib mesylate against chronic myeloid leukemia [15] and Philadelphia chromosome-positive ALL [16] with BCR/ABL is a good example. Specific molecule targeting therapy should be developed against pathogenic molecules responsible for leukemogenesis. Meanwhile, it is necessary to explore separate treatment regimens for prognostically different subtypes of AML with conventionally available modalities in order to increase the cure rate of adult leukemia.

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ONCOGENOMICS

Array-based genomic resequencing of human leukemia

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To identify oncogenes in leukemias, we performed large-scale resequencing of the leukemia genome using DNA sequence arrays that determine ~9 Mbp of sequence corresponding to the exons or exon–intron boundaries of 5648 protein-coding genes. Hybridization of genomic DNA from CD34-positive blasts of acute myeloid leukemia ($n=19$) or myeloproliferative disorder ($n=1$) with the arrays identified 9148 nonsynonymous nucleotide changes. Subsequent analysis showed that most of these changes were also present in the genomic DNA of the paired controls, with 11 somatic changes identified only in the leukemic blasts. One of these latter changes results in a Met-to-Ile substitution at amino-acid position 511 of Janus kinase 3 (JAK3), and the JAK3(M511I) protein exhibited transforming potential both *in vitro* and *in vivo*. Further screening for *JAK3* mutations showed novel and known transforming changes in a total of 9 out of 286 cases of leukemia. Our experiments also showed a somatic change responsible for an Arg-to-His substitution at amino-acid position 882 of DNA methyltransferase 3A, which resulted in a loss of DNA methylation activity of >50%. Our data have thus shown a unique profile of gene mutations in human leukemia.

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Keywords: resequencing; AML; JAK3; DNMT3A

Introduction

Leukemias are clonal disorders of hematopoietic stem cells or immature progenitors. Several subtypes of leukemia are associated with disease-specific karyotype anomalies in the malignant blasts. Most cases of acute promyelocytic leukemia a subtype of acute myeloid leukemia (AML), for instance, are associated with a t(15;17) chromosomal rearrangement that results in the production of the PML-RARA fusion-type oncoprotein (Tallman and Altman, 2008). Similarly, another subtype of AML is associated with a t(8;21) rearrangement, resulting in the production of the oncogenic RUNX1-CBFA2T1 protein (Nimer and Moore, 2004).

The karyotype of leukemic blasts is an important determinant of the long-term prognosis of affected individuals. AML with t(15;17), t(8;21) or inv(16) rearrangements thus constitutes a subgroup of leukemias with a 'favorable' karyotype, with a 5-year survival rate of >60%, whereas AML with an 'adverse' karyotype (monosomy 7, monosomy 5 or complex anomalies) has a 5-year survival rate of only <15% (Grimwade *et al.*, 1998). The prognosis of AML with a normal karyotype (constituting ~50% of all AML cases) is substantially worse than that with a favorable karyotype, with a 5-year survival rate of 24% (Byrd *et al.*, 2002), indicating that blasts with a normal karyotype may contain transforming genes generated as a result of (1) sequence alterations, (2) epigenetic abnormalities or (3) small chromosomal rearrangements not detectable by the G-banding technique. Indeed, several genes, including *NPM1* and *KIT*, have been found to be mutated and activated in AML blasts with a normal karyotype (Schlenk *et al.*, 2008).

The identification of transforming genes in AML will require large-scale resequencing of the blast genome. Although a new generation of sequencing technologies is now available, whole-genome resequencing of many samples remains a demanding task (Bentley *et al.*, 2008; Wheeler *et al.*, 2008). Although DNA microarray-based sequencing is suitable for analysis of multiple samples, currently available platforms are limited in the number of nucleotides that each array is able to probe. To overcome such limitations, we have now applied the extra-large arrays ('wafers') manufactured by Perlegen Sciences (Mountain View, CA, USA) (originally developed for typing of

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single-nucleotide polymorphisms) (Patil *et al.*, 2001) to resequencing of the human genome. Our two-step analysis of human leukemia specimens ($n=20$) has identified a novel transforming mutation in the gene for Janus kinase 3 (JAK3) and a hypomorphic mutation in that for DNA methyltransferase 3A (DNMT3A).

Results

Sequencing strategy

Oligonucleotide probes on the sequencing wafer for the first phase of our study were designed to detect nonsynonymous nucleotide changes in the coding exons of the genome. Intronic sequences (GT in the splicing donor sequence AG-GT and AG in the splicing acceptor sequence AG-G) adjacent to coding exons were also interrogated with the wafer to capture splicing anomalies. Genes examined by the wafer included those known to be mutated in cancer and reported in the catalog of somatic mutations in cancer (COSMIC, http://www.sanger.ac.uk/genetics/cgp/cosmic) as of September 2006 ($n=338$) and those related to the regulation of DNA repair ($n=419$), chromatin structure ($n=299$), redox regulation ($n=102$), epigenetic regulation ($n=44$), cell signaling ($n=2490$), protein kinases ($n=314$), gene transcription ($n=797$), cell cycle ($n=297$), apoptosis ($n=312$), DNA replication ($n=144$) or other functions ($n=92$) (Figure 1a). A total of 5648 genes were thus analyzed with the wafer.

To efficiently isolate oncogenes generated by point mutation using our sequencing array, we selected leukemic blasts with a karyotype characterized by few chromosome anomalies and by few copy number variations of chromosomes, as determined by comparative genomic hybridization with single-nucleotide polymorphism-typing arrays (Supplementary Figure S1). We isolated 15 cases of *de novo* AML, 4 cases of AML that developed from myelodysplastic syndrome, and 1 case of myeloproliferative disorder negative for the JAK2(V617F) and MPL(W515L) mutations (Kralovics *et al.*, 2005; Pikman *et al.*, 2006) (Supplementary Table S1).

From each of these 20 individuals enrolled in the study, we purified immature blasts positive for the surface expression of CD34 (leukemic fraction) as well as a paired control fraction of mature T cells positive for the surface expression of CD4. Although monocytes-macrophages may also express a low level of CD4 at the cell surface, our magnetic bead-based purification system preferentially enriched mature T cells with a high level of CD4 expression; contamination of the mature T-cell fraction with monocytes-macrophages was judged to be <9% by flow cytometry (Supplementary Figure S2).

Given the potential presence of substantial numbers of unreported single-nucleotide polymorphisms in the human genome, we adopted a two-step analysis to select somatic changes (Figure 1b). In phase I, genomic DNA was isolated from the $CD34^+$ fraction, subjected to mid-range PCR amplification and hybridized with the wafer to examine ~9 Mbp of nucleotide sequence. In phase II, we constructed a smaller wafer to investigate only the

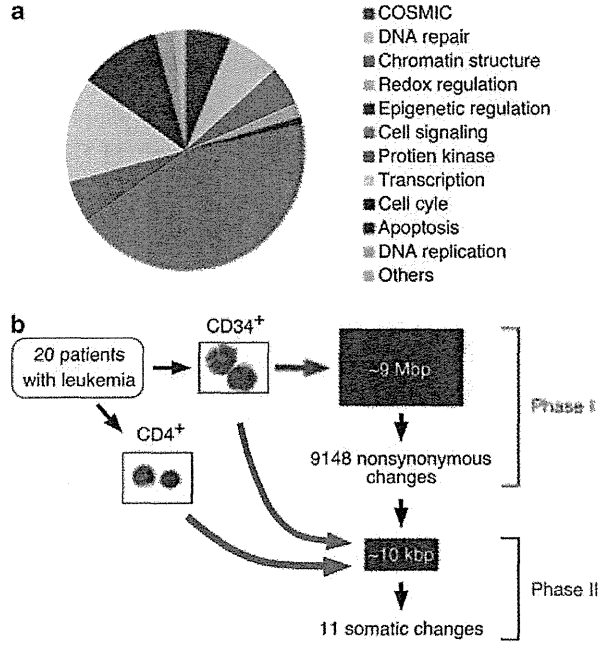

Figure 1 Resequencing of the leukemia genome with wafers. (**a**) Genes interrogated by the phase I wafer ($n=5648$) included those listed in the COSMIC database and those categorized on the basis of function of the encoded protein as indicated. (**b**) $CD34^+$ and $CD4^+$ cell fractions were purified from individuals with leukemia ($n=20$). Genomic DNA of the former fractions was assayed with the phase I wafer including ~9 Mbp of sequence, resulting in the isolation of 9148 nonsynonymous nucleotide changes in 3403 independent genes. The phase II wafer was then constructed to analyze these 9148 changes and was hybridized with genomic DNA from both $CD34^+$ and $CD4^+$ fractions separately. Only 11 mutations were found to be present in the former fraction but not in the latter.

nucleotides shown to be changed in phase I relative to the human reference sequence. Genomic DNA isolated from leukemic blasts and paired control fractions was then analyzed individually with the phase II wafer. We assumed that a nucleotide change was a germline polymorphism if it was observed in both leukemic and control fractions of the same individual, and that it was a somatic mutation if it was observed in the former fraction but not in the latter.

Identification of the JAK3(M511I) mutation

Screening of the leukemic blasts of the 20 individuals for point mutations in phase I yielded 9148 nonsynonymous changes among 3403 independent genes, a frequency similar to that observed in other large-scale resequencing studies performed with capillary sequencers (Sjoblom *et al.*, 2006; Greenman *et al.*, 2007). However, analysis of $CD4^+$ fractions showed that most of these sequence changes were also present in the paired control genome, leaving only 11 nonsynonymous somatic mutations in 11 genes (Supplementary Table S2). Such small number of somatic mutations is in a good agreement with the eight somatic mutations found in AML through whole-genome resequencing using the

Illumina Genome Analyser (Illumina, San Diego, CA, USA) (Ley *et al.*, 2008). All of our 11 somatic changes were confirmed by analysis of both genomic DNA and cDNA of the corresponding specimens with a capillary sequencer (data not shown). These data thus support the necessity of examining paired noncancerous specimens to pinpoint somatic changes in the cancer genome.

One of the gene mutations found only in the $CD34^+$ fractions results in a Met-to-Ile change at amino-acid position 511 of JAK3. A heterozygous *JAK3* mutation responsible for the amino-acid change was confirmed in both genomic DNA and cDNA from the $CD34^+$ fraction, but not in those from the corresponding $CD4^+$ fraction of patient ID JM07 (Supplementary Figure S3), who had *de novo* AML (M1 subtype) and a normal karyotype (Supplementary Table S1). In contrast to JAK2, activating mutations in which are preferentially associated with myeloproliferative disorder, several gain-of-function mutations (such as I87T, P132T, Q501H, A572V, R657Q and V722I) of JAK3 have recently been associated with acute megakaryoblastic leukemia of children (Walters *et al.*, 2006; Sato *et al.*, 2008). Other JAK3 mutations (such as A573V and A593T) were also identified in the same disorder, and an M576L substitution was detected in an adult with acute megakaryocytic leukemia (AML, M7 subtype) (Kiyoi *et al.*, 2007), although the transforming potential of these changes remains unknown.

Given that the M511I mutant of JAK3 has not previously been described and that the relevance of JAK3 to the pathogenesis of adult AML has not been extensively investigated, we first focused on the function of JAK3(M511I). The M511 residue is located in the linker region between the Src homology 2 (SH2) domain and the pseudokinase domain of JAK3 (Figure 2a). The transforming mutation Q501H that is associated with juvenile acute megakaryoblastic leukemia (Sato *et al.*, 2008) is also located in this region. Given that JAK3 is abundant in and has an essential role in the development of lymphocytes (Russell *et al.*, 1995), we examined the expression level of *JAK3* in AML blasts. The gene was expressed at a high level in most AML specimens ($n = 52$), with its expression level being greater than that of *JAK2* in all but three cases (Supplementary Figure S3).

To examine the transforming potential of JAK3(M511I), we introduced the mutant or wild-type protein into the interleukin-3 (IL-3)-dependent mouse cell line 32D (Greenberger *et al.*, 1983). Although 32D cells forced to express wild-type JAK3 underwent rapid apoptosis after withdrawal of IL-3, those expressing JAK3(M511I) continued to grow even in the absence of IL-3, although at a reduced rate compared with that of cells expressing the artificially generated, highly transforming mutant JAK3(V674A) (Choi *et al.*, 2007) (Supplementary Figure S3). 32D cells differentiate into terminal granulocytes in the presence of granulocyte colony-stimulating factor. However, cells expressing the M511I or V674A mutant of JAK3 maintained an exponential rate of growth, without any sign of differentiation, in the presence of granulocyte colony-stimulating factor (Figure 2b, Supplementary Figure S3), supporting the notion that the M511I mutant has transforming potential.

To directly examine the leukemogenic activity of JAK3(M511I), we generated a recombinant retrovirus encoding this mutant and used it to infect murine hematopoietic stem cells. Reconstitution of the bone marrow of lethally irradiated mice with such infected cells resulted in marked lymphocytosis in peripheral blood and enlargement of the spleen in the recipient animals (Figure 2c). The cells in the peripheral blood, spleen and bone marrow of the recipients responsible for these phenotypes manifested a medium-sized, blastic morphology, and flow cytometric analysis revealed them to be $CD8^+$ T cells (Figure 2c). The clonal nature of these proliferating T cells was further confirmed by Southern blot analysis (Supplementary Figure S4), indicative of the development of T-cell acute lymphoblastic leukemia in the recipient mice.

To assess the prevalence of *JAK3* mutations in adult leukemia, we further examined the nucleotide sequence of the entire coding region of *JAK3* cDNA in an additional 266 specimens of leukemic blasts. The coding region of *JAK3* cDNA was successfully amplified by PCR from 83 specimens. We could further identify 4 distinct *JAK3* sequence changes in 8 of these 83 samples: 1 case with G62S, 4 cases with Q501H, 2 cases with R657Q and 1 case with R918C (Figure 2a). Taking into account the 20 cases evaluated in the phase I analysis, we thus identified a total of 9 cases with a mutant form of JAK3 (3.1%) among 286 cases of leukemia (Supplementary Table S3). Our identification of known transforming JAK3 mutants (Q501H and R657Q) originally associated with acute megakaryoblastic leukemia prompted us to determine the prevalence of these two changes in another cohort of AML ($n = 148$), revealing two cases with JAK3(Q501H) and one case with JAK3(R657Q). In addition, analysis of a hematopoietic cell line (KCL22) (Kubonishi and Miyoshi, 1983) established from a patient with chronic myeloid leukemia in BC revealed yet another mutation (L1017M) of JAK3 (Figure 2a).

To directly compare the transforming potential of these various JAK3 mutants, we introduced each protein into the IL-3-dependent mouse B-cell line BA/F3 and examined the growth properties of the resulting transfectants. Whereas all cells expressing the JAK3 mutants proliferated in a similar manner in the presence of IL-3 (data not shown), culture without IL-3 revealed marked differences in the transforming potential among the mutants. JAK3(M511I) was the most efficient oncokinase, with a transforming activity similar to that of JAK3(V674A). The frequent mutants JAK3(Q501H) and JAK3(R657Q) exhibited weaker but still pronounced transforming potential, whereas the remaining mutants (G62S, R918C and L1017M) showed an even lower potential (Figure 2d).

Somatic mutations of DNMT3A

Another somatic mutation identified in the phase II data set was a heterozygous change in *DNMT3A* that results

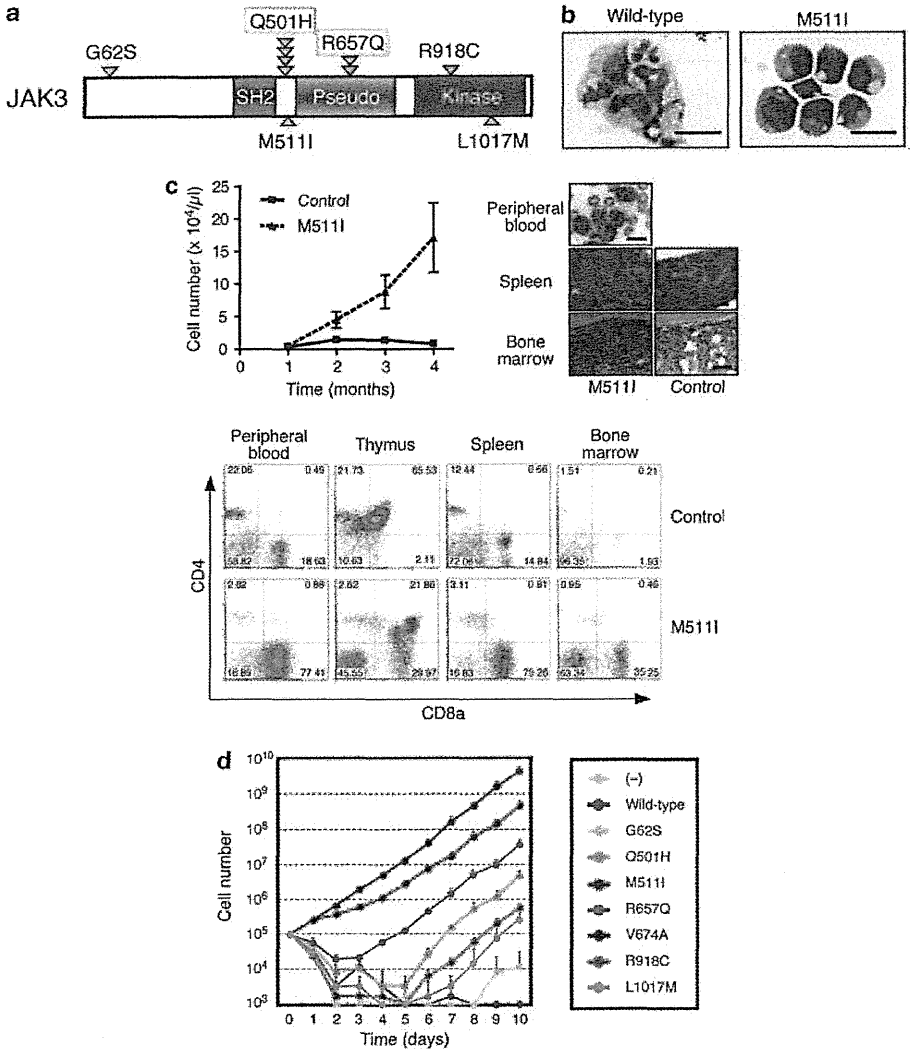

Figure 2 Identification of JAK3 mutants in leukemia. (**a**) Amino-acid substitutions detected in this study are shown relative to the domain organization of JAK3. The mutations M511I (one case) and Q501H (four cases) are located in the linker region between the SH2 and pseudokinase domains of JAK3, whereas G62S (one case), R657Q (two cases) and R918C (one case) are located in the amino-terminal region, the pseudokinase domain and the kinase domain, respectively. The KCL22 cell line also harbors an L1017M mutation within the kinase domain of JAK3. Previously known activating mutations of JAK3 (Q501H and R657Q) are indicated by red rectangles. (**b**) Mouse 32D cells expressing wild-type human JAK3 or the JAK3(M511I) mutant were incubated with G-CSF (0.5 ng/ml) for 14 days, stained with Wright–Giemsa solution and examined by light microscopy. Scale bars, 20 μm. (**c**) C57BL/6 mice were irradiated and then injected intravenously with syngeneic $CD34^-KSL$ hematopoietic stem cells infected with a retrovirus encoding JAK3(M511I) or the corresponding empty virus (control). The number of white blood cells in peripheral blood was counted at the indicated times thereafter; data are means ± s.d. for 10 mice in each group (upper left panel). Peripheral blood, spleen and bone marrow isolated from recipient mice 3 months after cell injection were stained with the Wright–Giemsa solution (peripheral blood) or hematoxylin–eosin (spleen and bone marrow) and were then examined by light microscopy (upper right panel); scale bars represent 10, 200 and 50 μm, respectively. Mononuclear cells isolated from peripheral blood, thymus, spleen and bone marrow of recipient mice 3 months after cell injection were subjected to flow cytometric analysis of surface expression of CD4 and CD8a (lower panel). (**d**) Control BA/F3 cells (–) or those expressing the indicated JAK3 mutants were cultured without IL-3 for the indicated times, after which the cell number was determined. Data are means + s.d. of triplicates from a representative experiment.

in an R882H substitution in the encoded protein (Figure 3a, Supplementary Figure S5). DNMT3A, together with DNMT3B, has an essential role in *de novo* methylation of the human genome (Okano *et al.*, 1999), and an aberrant methylation profile (hypermethylation of CpG islands and hypomethylation of other regions) is a hallmark of cancer cells (El-Osta, 2004). Despite a direct linkage between such methylation changes and silencing of tumor-suppressor genes in cancer, the molecular mechanism responsible for such abnormal methylation remains unknown. Our data thus provide the first evidence of somatic mutation of a DNA methyltransferase gene in cancer cells. Mutations in the catalytic domain of DNMT3B have been shown to be

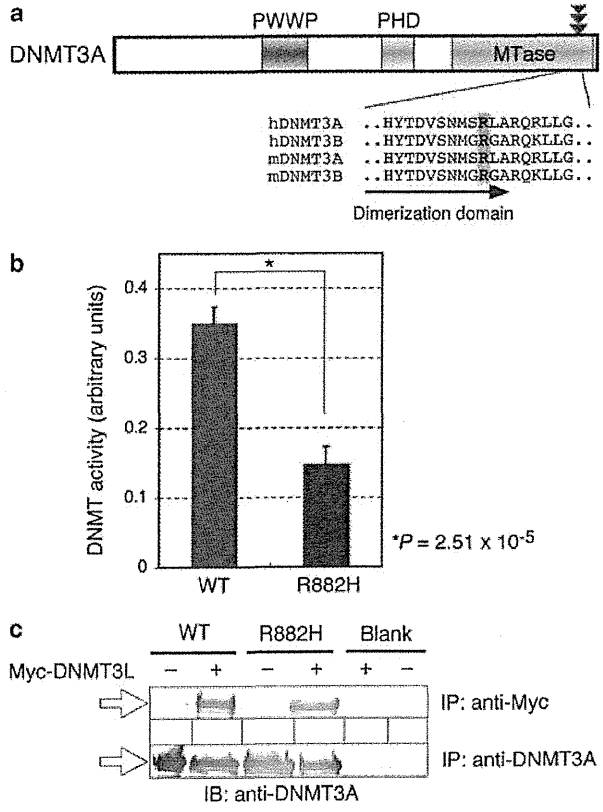

Figure 3 Identification of a DNMT3A mutant in leukemia. (**a**) Domain organization of human DNMT3A showing that the R882 residue found to be mutated in leukemia is conserved among human (h) and mouse (m) members of the DNMT3 family. DNMT3A contains a tetrapeptide PWWP domain, polybromo homology domain (PHD) and methyltransferase (MTase) domain. The R882 residue is located in the homodimerization region present within the MTase domain. (**b**) Wild-type (WT) and R882H forms of DNMT3A were expressed in and purified from insect cells and then subjected to an *in vitro* assay of methyltransferase activity. Data are means + s.d. of triplicates from a representative experiment. The *P*-value was determined by Student's *t*-test. (**c**) Lysates of HEK293 cells expressing Myc epitope-tagged DNMT3L and wild-type or R882H forms of DNMT3A, as indicated, were subjected to immunoprecipitation (IP) with antibodies to Myc or to DNMT3A, and the resulting precipitates were subjected to immunoblot analysis (IB) with antibodies to DNMT3A. The position of DNMT3A (wild-type or mutant) is indicated by an open arrow.

responsible for a hereditary syndrome characterized by ICF (immunodeficiency, instability of the centromeric region of chromosomes and facial anomalies) in humans (Ehrlich, 2003). One of the mutation sites of DNMT3B (R823) associated with the ICF syndrome corresponds to the residue of DNMT3A (R882) shown to be mutated in this study.

The R882 residue of DNMT3A is considered to participate in the homodimerization and activation of the protein (Jia *et al.*, 2007) (Figure 3a). To determine whether the R882H mutation affects the catalytic activity of DNMT3A, we expressed mutant and wild-type proteins separately in insect cells, purified them to near homogeneity and subjected them to an *in vitro* assay of methyltransferase activity with a synthetic substrate (Suetake *et al.*, 2003). The catalytic activity of DNMT3A(R882H) was <50% of that of the wild-type protein (Figure 3b). DNMT3L acts as a coactivator for the methyltransferase activity of DNMT3A or DNMT3B through its association with the latter proteins (Jia *et al.*, 2007). The R882H mutation did not affect the interaction of DNMT3A with DNMT3L in transfected mammalian cells (Figure 3c) or its sensitivity to DNMT3L as examined by the *in vitro* assay of methyltransferase activity (data not shown). These data thus suggested that the R882H mutation directly inhibits the enzymatic activity of DNMT3A.

Screening of another cohort of leukemia cases ($n = 54$) for mutant forms of *DNMT3A* revealed another two patients with a mutation of the same amino acid (R882H in one patient and R882C in the other) (Supplementary Table S4). Therefore, we identified a total of 3 cases with an R882 mutation (4.1%) among 74 cases of leukemia. Screening for mutations of *DNMT3B* failed to detect any somatic changes in the same individuals (data not shown), suggesting that *DNMT3A* is a preferential target in leukemia.

Multistep transformation in leukemia

Although >99% of nucleotide changes in the phase I data were also observed in the paired $CD4^+$ cells, it is unlikely that all of these changes are actually germline polymorphisms because they include established oncogenic mutations. They thus include 190 nucleotide changes previously described in cancer cells (Supplementary Table S5), such as those giving rise to NRAS(Q61H) in patient ID JM17 and to FLT3(D835Y) in patient ID JM08 (Figure 4a). Given that both NRAS(Q61H) and FLT3(D835Y) are well-characterized oncoproteins (Yamamoto *et al.*, 2001), it is unlikely that these individuals harbored such nucleotide changes in the germ line. There are at least two possible explanations for these findings. First, it is possible that purification of the $CD4^+$ fraction was not efficient, with the result that this fraction was contaminated by $CD34^+$ cells. However, the *CD4* expression ratio for the $CD4^+$ and $CD34^+$ fractions of each individual was ⩾17.1 (median = 40.1) (Figure 4b), and contamination of the $CD4^+$ fraction with $CD34^+$ cells at such a level would not likely produce detectable changes in Sanger sequencing outputs (compare, for instance, the signal intensities of the normal and mutant alleles in Figure 4a).

Furthermore, although CD4 expression has been occasionally observed in AML blasts (Schwonzen *et al.*, 2007), quantitation of *CD4* and *CD34* mRNA within our purified $CD34^+$ fractions failed to detect a significant level of the former message in the blasts (Supplementary Figure S6). Therefore, it is unlikely that contamination of $CD4^+$ leukemic blasts within the purified, control $CD4^+$ fraction substantially affected the sequencing results in our phase II experiment.

Rather, it is more likely that leukemia may develop in a stepwise manner with a substantial time interval

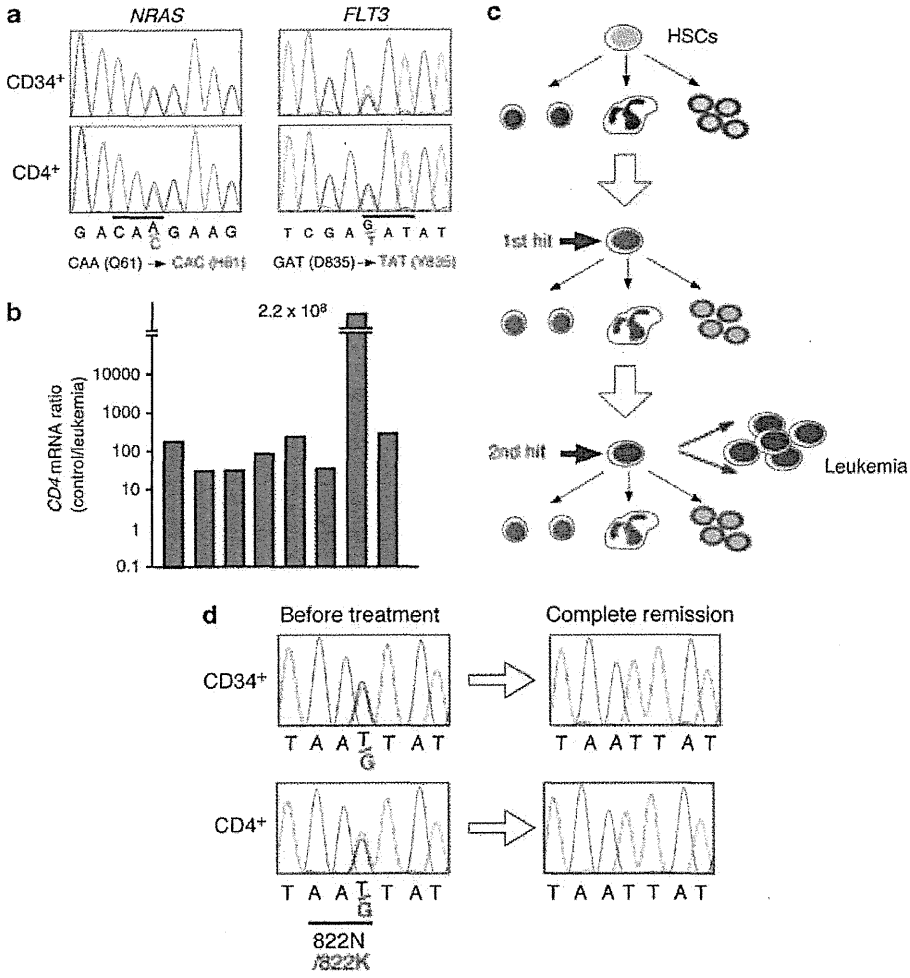

Figure 4 Proposed stepwise nature of leukemogenesis. (**a**) Sequencing electrophoretograms for the regions surrounding codon 61 of *NRAS* or codon 835 of *FLT3* in genomic DNA from the CD34$^+$ and CD4$^+$ fractions of patient IDs JM17 and JM08, respectively. Heterozygous nucleotide changes that give rise to NRAS(Q61H) or FLT3(D835Y) were detected in both fractions of the corresponding patients. (**b**) The amount of *CD4* mRNA in the CD4$^+$ (control) and CD34$^+$ (leukemia) fractions of leukemia patients (with a substantial amount of control *GAPDH* mRNA) was quantitated by reverse transcription and real-time PCR analysis and expressed as the control/leukemia ratio. (**c**) Hematopoietic stem cells (HSCs) give rise to a wide range of mature blood cells. Even after the first hit (mutation) of the genome, HSCs retain their full differentiation capacity, and therefore produce differentiated cells harboring this first hit. After the second hit, the affected cell fraction undergoes full transformation to leukemia. (**d**) Sequencing electrophoretograms for the genome of CD34$^+$ and CD4$^+$ fractions from patient ID JM03 showing a heterozygous mutation for KIT(N822K) before chemotherapy but not after.

between steps (Figure 4c). If a first hit occurs in the genome of hematopoietic stem (or progenitor) cells and if such a somatic change does not result directly in the generation of full-blown leukemia, the preleukemic clones may give rise to terminally differentiated blood cells (including CD4$^+$ cells). After a certain period, a second (or possibly a third) hit occurs in the immature cells and triggers the rapid growth of leukemic clones without differentiation. In such a scenario, terminally differentiated 'normal' cells may still harbor the first hit in their genome.

Support for this latter possibility was provided by patient ID JM03, who had AML (M2 subtype) with a t(8;21) chromosome anomaly. Before chemotherapy, the genomic DNA of both CD34$^+$ and CD4$^+$ fractions from this patient harbored a heterozygous mutation of *KIT* that results in the production of a constitutively activated mutant protein, KIT(N822K) (Shimada *et al.*, 2006) (Figure 4d). The same change was also detected in cDNA prepared from the CD34$^+$ fraction (data not shown). Leukemic blasts in this patient were sensitive to standard chemotherapeutic regimens, and the patient underwent complete remission. Examination of CD34$^+$ and CD4$^+$ fractions obtained during the remission period revealed that the N822K codon change was no longer detectable not only in the CD34$^+$ fraction but also in the CD4$^+$ fraction (Figure 4d). These data thus support the scenario shown in Figure 4c: The N822K

change represents the first hit and was present in differentiated blood cells, and the corresponding pre-leukemic clones were simultaneously eradicated together with the leukemic clones by chemotherapy.

On the other hand, as shown in Supplementary Tables S1 and S2, a heterozygous mutation for NRAS(G12S) was found only in the $CD34^+$ fraction, but not in the $CD4^+$ fraction of the patient ID JM16. Conventional chemotherapy for this patient eradicated the leukemic blasts carrying the mutation (Supplementary Figure S7), also confirming that a successful treatment results in the disappearance of cells with a (possible) 'second hit'.

Our hypothesis of the stepwise leukemogenesis is also consistent with the previous detection of the *RUNX1-CBFA2T1* oncogene in differentiated blood cells (Kwong *et al.*, 1996; Miyamoto *et al.*, 1996, 2000).

Discussion

Our large-scale genomic resequencing of human leukemia specimens with DNA microarrays has identified recurrent nucleotide changes responsible for the generation of JAK3 and DNMT3A mutants. Whereas JAK3 mutants were unexpectedly found in adult AML, their transforming ability, and possibly their contribution to leukemogenesis, varied substantially. However, our bone marrow transplantation experiments showed that at least one of these JAK3 mutants (M511I) directly participates in the development of leukemia. Identification of the M511I mutation of JAK3 in the leukemic fraction but not in the control fraction of patient ID JM07 suggests that this mutation may be the second hit triggering AML. Given that the blasts of this patient had a normal karyotype, it is likely that the first hit is present in the genome of both fractions. Karyotyping of other patients with *JAK3* mutations showed a total of three cases with a normal karyotype, one case with t(8;21), and one case with a numerical anomaly of several chromosomes (Supplementary Table S3), suggesting that *JAK3* mutations may be preferentially associated with leukemia with a normal karyotype.

Although JAK3(M511I) was identified in AML, our bone marrow transplantation experiments with hematopoietic stem cells expressing this mutant yielded T-cell acute lymphoblastic leukemia. In contrast to human leukemia, in which JAK3 changes may constitute a second hit (probably in progenitor cells), JAK3(M511I) may have been expressed in all hematopoietic cells of the recipient mice. JAK3(M511I) thus likely triggered leukemia within a T-cell fraction the intracellular context of which is optimized for JAK3 signaling.

It has been frequently observed that transgenic mouse or bone marrow transplantation experiments for leukemic oncogenes do not accurately recapitulate the original leukemia subtypes (Wong and Witte, 2001). Transgenic mice expressing $p210^{BCR\text{-}ABL1}$, for instance, usually develop T-cell lymphoma or acute lymphoblastic leukemia, not chronic myeloid leukemia. Furthermore, bone marrow transplantation with hematopoietic progenitor cells expressing $p210^{BCR\text{-}ABL1}$ often leads to development of lymphoma, AML, acute lymphoblastic leukemia or macrophage tumors. Generation of malignancy in such systems may, thus, be elaborately influenced by mouse strains, promoter fragments for artificial expression and/or cell types to be used for gene transduction.

Our detection of recurrent DNMT3A hypomorphic mutations in leukemia clones may indicate the presence of an abnormal methylation profile in the genome of such blasts. However, given the limited amount of the specimens available, we were able to investigate microsatellite stability only at certain loci (Koinuma *et al.*, 2005), revealing no apparent microsatellite instability (data not shown). We also generated BA/F3 cells expressing wild-type or R882H forms of DNMT3A to compare the methylation status of some CpG islands in the genome; again, we detected no discernable differences between the two cell preparations (data not shown). However, given that BA/F3 cells contained two copies of wild-type *Dnmt3a* in addition to multiple copies of mutant *DNMT3A*, whereas the leukemic blasts likely harbor one copy each of the wild-type and mutant *DNMT3A* alleles, the clinical relevance of the R882 mutant requires further examination under the latter condition. Cell proliferation/differentiation is indeed influenced substantially by the copy number of *DNMT3* genes (Okano *et al.*, 1999; Ehrlich, 2003).

Our observations indicate the importance of preparing paired normal fractions in large-scale resequencing projects, but they also reveal a difficulty in the preparation of *bona fide* 'normal' fractions in the case of leukemic disorders. Our data thus indicate that nonleukemic blood cells may harbor early genomic hits, rendering them inappropriate as controls. Furthermore, a substantial proportion of fingernail DNA was recently shown to be derived from donor cells among recipients of allogeneic stem cell transplants (Imanishi *et al.*, 2007), indicating that nonblood cells may contain DNA derived from transplanted cells. Therefore, it is possible that buccal, fingernail or even hair cells may not be suitable as normal cell controls. In contrast to solid tumors, for which blood cells are appropriate as paired normal fractions, leukemic disorders require that caution be taken to discriminate somatic nucleotide changes from germline polymorphisms.

Materials and methods

Wafer sequencing

$CD34^+$ and $CD4^+$ fractions were isolated from leukemic individuals using CD34microbeads and CD4microbeads, respectively, and a MidiMACS separator (Miltenyi Biotec, Gladbach, Germany). All clinical specimens were obtained with written informed consent, and the study was approved by the ethics committees of both the Jichi Medical University and the Nagasaki University. DNA sequencing wafers were designed and processed at Perlegen Sciences. Genes to be interrogated on the wafers were selected from the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene)