relapse did not differ significantly between those who underwent allogeneic HCT in CR2 and those who did not undergo allogeneic HCT after achieving CR2 (70% *versus* 78% at 3 years after relapse, P=0.415, Figure 4A). For patients with t(8;21), overall survival probabilities were generally inferior to those of patients with inv(16) (allogeneic HCT in CR2, 64%; no allogeneic HCT after CR2, 53%; allogeneic HCT in non-CR2, 32%; no achievement of CR2 without salvage allogeneic HCT, 0%, Figure 4B). Also in this group of patients, there was no significant difference in overall survival between patients who underwent allogeneic HCT in CR2 and those who did not undergo allogeneic HCT after CR2 (P=0.600). Allogeneic HCT in a disease status other than CR2 provided significantly better survival than no achievement of CR2 without salvage allogeneic HCT (P<0.001).

Among patients with intermediate-risk acute myeloid leukemia, overall survival in those who did not undergo allogeneic HCT after they had achieved CR2 was significantly worse than that in patients who did undergo allogeneic HCT in CR2 (58% *versus* 19% at 3 years from relapse, P<0.001, Figure 4C). We performed subset analyses according to relapse-free interval (≥ 1 year *versus* < 1 year) and the number of courses of remission induction therapy (1 course or 2 courses) among intermediate-risk patients. The performance of allogeneic HCT in CR2 was associated with significantly better overall survival than no allogeneic HCT after the achievement of CR2 or allogeneic HCT in a disease status other than CR2 in all subgroups other than those who required two courses of remission induction chemotherapy (*Online Supplementary Figure S1*). Allogeneic HCT in non-CR2 provided a comparable or better overall survival than no allogeneic HCT after CR2, and a significantly better overall survival than no remission/no allogeneic HCT.

Among patients with unfavorable-risk acute myeloid leukemia, only selected patients who underwent allogeneic HCT in CR2 had an improved overall survival (allogeneic HCT in CR2, 67%; no allogeneic HCT after CR2, 35%; allogeneic HCT in non-CR2, 13%; no achievement of CR2 without salvage allogeneic HCT, 0%, Figure 4D).

Prognostic factors after first relapse

A multivariate analysis showed that the achievement of CR2, salvage allogeneic HCT, a longer relapse-free interval from CR1, a more favorable cytogenetic risk and a single course of induction therapy to achieve CR1 were significantly associated with improved overall survival after relapse (Table 2). Since CR2 was shown to be an important step toward an improved prognosis after the first relapse, we also performed a multivariate analysis to identify factors that may be associated with the likelihood of the achievement of CR2. Except for age, these already-known prognostic factors were found to independently predict the achievement of CR2 with a relatively higher risk ratio in relapse-free interval.

Discussion

In this study, we investigated the prognosis of 1,015 patients with acute myeloid leukemia who relapsed after being treated with conventional chemotherapy during CR1. The independent prognostic factors we identified were achievement of CR2, performance of salvage allogeneic HCT, a relapse-free interval of 1 year or longer, a more favorable cytogenetic risk and achievement of CR1 after a single course of remission induction chemotherapy. Although the outcome of patients who underwent allogeneic HCT after a first relapse were inferior to that of patients transplanted in CR1, we found that a comparable outcome was achieved when allogeneic HCT was successfully performed in CR2. We also found that the outcome according to the treatment strategy after the first relapse varied depending on the patients' cytogenetic risk.

The global overall survival of the 1,015 relapsed patients was 30% at 3 years after the first relapse. The overall survival differed significantly according to factors that have been reported to be prognostic at diagnosis or after relapse. Breems *et al.* presented a prognostic score to predict the outcome of patients with acute myeloid leukemia after first relapse, including patients who relapsed after allogeneic HCT in CR1.[12] They indicated that a longer relapse-free interval, a favorable cytogenetic risk, and younger age were favorable prognostic factors and that the performance of allogeneic HCT before first relapse unfavorably influenced the outcome after relapse. Armistead *et al.* showed that allogeneic HCT was effective in patients with refractory or recurrent acute myeloid leukemia who were stratified into diverse subgroups according to age, relapse-free interval and cytogenetics.[13] In our study, achievement of CR2, performance of salvage allogeneic HCT, a longer relapse-free interval, more favorable cytogenetic characteristics and achievement of CR1 after a single course of remission induction chemotherapy were independent prognostic factors in patients who relapsed after conventional chemotherapy. Our database only consisted of information from patients who successfully achieved CR1 and subsequently relapsed after treatment with chemotherapy alone, which may be one of the reasons why we found slightly different prognostic factors from these found in prior studies. Salvage chemotherapy obtained a CR2 in half of the patients, which was consistent with the previously reported probability.[5]

We found that, overall, allogeneic HCT after first relapse provides an inferior overall survival compared to allogeneic HCT in CR1. This result did not change when we excluded patients who relapsed early after they had achieved CR1. The outcome after salvage allogeneic HCT was significantly affected by the disease status at the time of transplantation. Patients who underwent salvage allogeneic HCT in a disease state other than CR2 had a significantly worse overall survival than those who received the transplant in CR2. Patients who never achieved CR2 may include not only those who received chemotherapy but also those who never received chemotherapy after relapse. Nevertheless, our results may indicate that immediate salvage allogeneic HCT after relapse without an effort to induce CR2 by giving remission induction chemotherapy does not improve the prognosis.

Achievement of CR2 was shown to be a crucial step for an improved outcome after relapse. Additionally, one of the intriguing facts we found was that patients who underwent allogeneic HCT in CR2 had an overall survival that was comparable to that in patients who underwent allogeneic HCT in CR1. For patients who do not have a definite indication for allogeneic HCT in CR1, the likelihood of successfully receiving an allogeneic transplant in CR2 if they relapsed would be invaluable information. However, among the available prognostic factors that are

generally used to predict the ultimate prognosis of acute myeloid leukemia at diagnosis, all of the factors except for age were shown to be independent factors that predicted the achievement of CR2. As a result, it was difficult to clearly define candidates for allogeneic HCT, not in CR1, but rather in CR2 using already-known prognostic factors. These results may suggest the need for further information on how parameters such as WT-1 or other molecular markers behave in acute myeloid leukemia after relapse.

We also investigated the advantage of additional allogeneic HCT after the achievement of CR2 as well as the effectiveness of allogeneic HCT if CR2 was not achieved or sustained. The outcomes were analyzed based on stratification according to cytogenetic risk. We found that the outcome of patients with core-binding factor acute myeloid leukemia who did not undergo additional allogeneic HCT after they had achieved CR2 was comparable to that of patients who did undergo allogeneic HCT in CR2. Over 80% of the patients with inv(16) achieved CR2 with a comparable overall survival regardless of additional allogeneic HCT. Considering the likelihood of the achievement of CR2 and the favorable outcome thereafter, we think that patients with inv(16) may not be indicated for prompt allogeneic HCT in CR2 under close monitoring. Acute myeloid leukemia with t(8;21) has been reported to have a worse prognosis than that with inv(16), as also confirmed in this study.[13-15] Although we did not find a significant improvement in outcome with additional allogeneic HCT after the achievement of CR2 among patients with t(8;21), some patients may have an improved outcome if they are consolidated with allogeneic HCT even after they achieve CR2. Since we do not have detailed information on the chemotherapy after the first relapse or minimal residual disease monitoring, the true indications for allogeneic HCT after the achievement of CR2 in patients with core binding factor acute myeloid leukemia need to be investigated more closely. A molecular profile such as c-Kit mutation may provide more potent prognostic factors.[16-18]

For patients with intermediate-risk acute myeloid leukemia, there was a significant difference in overall survival between those who underwent allogeneic HCT in CR2 and those who did not after they had achieved CR2. Although the molecular profile at diagnosis has been reported to have an effect on the prognosis of patients with intermediate-risk acute myeloid leukemia,[19-21] how these parameters predict the outcome of relapsed acute myeloid leukemia remains to be clarified. Based on our current understanding, consolidation with allogeneic HCT after the achievement of CR2 should be suggested for patients with intermediate-risk acute myeloid leukemia.

Among patients with unfavorable-risk acute myeloid leukemia, only one third achieved CR2. Although allogeneic HCT in CR2 provided an improved outcome after relapse, only 15% of all the patients with unfavorable-risk acute myeloid leukemia who relapsed had a successful HCT in CR2. Since survival is less likely after the first relapse, patients with unfavorable-risk acute myeloid leukemia should be promptly prepared for allogeneic HCT in CR1, as has been demonstrated in many prior studies.[2,4,22-25]

Our results may be susceptible to the disadvantages of any retrospective study, such as the heterogeneity in the treatment strategies chosen at the discretion of physicians. The performance of allogeneic HCT after relapse may include several inherent selection biases such as unfavorable features in those who did not have a chance to undergo transplantation because of disease progression or comorbidity. Our database also lacked detailed information on chemotherapy treatment after achievement of CR1 or after relapse. However, the results we obtained from this large database containing clinical information on patients who were treated with chemotherapy alone or salvage allogeneic HCT after relapse should provide valuable information on this issue which is difficult to evaluate in a prospective, randomized manner.

In summary, using a large amount of retrospectively collected data, we showed that both the achievement of CR2 and the application of salvage allogeneic HCT after relapse are crucial factors in improving the outcome after first relapse. Our results also suggest that the optimal treatment strategy after relapse may differ based on the risk of the disease. Further studies on molecular profiles are needed to stratify the prognosis and treatment strategies for acute myeloid leukemia after first relapse.

Authorship and Disclosures

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Trough plasma concentration of imatinib reflects BCR–ABL kinase inhibitory activity and clinical response in chronic-phase chronic myeloid leukemia: A report from the BINGO study

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Pharmacokinetic (PK) factors have been suggested to be involved in the unfavorable clinical responses of chronic myeloid leukemia (CML) patients treated with imatinib. The purpose of this study was to clarify prognostic implications of PK factors in CML patients treated with imatinib. The plasma trough (C_{min}) level of imatinib and serum α_1-acid glycoprotein (AGP) level were measured on two different days in 65 CML patients treated with imatinib for more than 12 months. We further examined whether the C_{min} level of imatinib actually reflects inhibitory activity against BCR–ABL kinase using the plasma inhibitory activity (PIA) assay. Since the differences of five patients were statistically rejected by the Smirnov–Grubbs' test, we excluded them for further analysis. The C_{min} level was strongly associated with the achievement of MMR at the 12th month, and ROC analysis demonstrated C_{min} levels and their discrimination potential for major molecular response (MMR) with the best sensitivity (63.2%) and specificity (68.2%) at a C_{min} threshold of 974 ng/mL. The α_1-Acid glycoprotein (AGP) level was within the normal range in 57 of 60 patients, indicating little impact of AGP on our study. There was a weak correlation between PIA against phospho (P)-BCR–ABL and the C_{min} level of imatinib ($r^2 = 0.2501$, $P = 0.0007$), and patient plasma containing >974 ng/mL imatinib sufficiently inhibited P-BCR-ABL. These results collectively indicated that maintaining ~1000 ng/mL of C_{min} was clinically and biologically important for the optimal response in CML patients treated with imatinib. A prospective intervention study is required to establish PK-based management in CML patients treated with imatinib. (*Cancer Sci* 2010; 101: 2186–2192)

Imatinib mesylate is a selective BCR–ABL kinase inhibitor, and has demonstrated excellent efficacy in the treatment of chronic myeloid leukemia (CML), particularly in the chronic phase (CP).[1] The international randomized study of interferon and STI571 (IRIS) trial and several subsequent clinical studies have confirmed that imatinib is the first-line agent for the treatment of newly diagnosed CML patients.[2–5] Although imatinib reveals a high complete cytogenetic response (CCyR) and major molecular response (MMR) rates in CML patients, a variable time to achieve CCyR and MMR in individual patients has been observed. Furthermore, the accumulation of clinical information in imatinib-treated patients has disclosed patients with treatment failure or suboptimal response.[6–8] Previous studies demonstrated several mechanisms of imatinib resistance, such as mutation or amplification of the *BCR–ABL* gene, up-regulation of P-glycoprotein, activation of SRC family kinases, and pharmacokinetic (PK) variability.[5,9–11]

Pharmacokinetic (PK) studies of imatinib in healthy volunteers and CML patients have demonstrated that orally administered imatinib is well absorbed, and has 98% bioavailability, irrespective of the dosage.[12,13] However, recent studies revealed that the trough plasma concentration level (C_{min}) of imatinib varied in individual patients even at the steady state, and that the C_{min} was associated with the clinical response and adverse effects.[14–16] Therefore, the measurement of C_{min} is thought to be useful for deciding on a dose escalation to improve the response or a dose reduction to prevent adverse effects during treatment with imatinib.

On the other hand, *in vitro* studies indicate that imatinib is approximately 95% bound to plasma proteins, mainly albumin and α_1-acid glycoprotein (AGP).[15] α_1-Acid glycoprotein (AGP) is a serum acute phase reactant, and is elevated in a variety of physiological and pathological conditions as well as CML, especially in the advanced phase.[17] α_1-Acid glycoprotein (AGP) is predominantly synthesized in the liver, and is negatively charged at physiological pH. Although human serum albumin is largely responsible for the binding of acidic acids, AGP mainly binds to basic and neutral drugs. Notably, AGP tightly binds imatinib, resulting in a decreased free and active plasma fraction of imatinib. It has been reported that AGP exerts a significant effect on the intracellular distribution of imatinib in CML patients, resulting in the resistance;[18,19] however, another study revealed that AGP purified from the plasma of CML patients did not mediate significant *in vitro* resistance to imatinib.[17] Since it currently requires great efforts to measure a free or active plasma fraction and an intracellular concentration of kinase inhibitors, the plasma inhibitory activity (PIA), which is determined by the inhibitory activity of plasma obtained from a patient treated with a kinase inhibitor against the target kinase, is reportedly an

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alternative surrogate marker for evaluating the active fraction level of kinase inhibitors.[20]

In this study, we measured the plasma C_{min} level of imatinib and serum AGP level on two different days in 65 CML patients who continued to be in CP and were treated with only imatinib for more than 12 months; the correlation among the C_{min} level of imatinib, AGP level, and clinical response; and the stability of the C_{min} level of imatinib was examined in individual patients. We further examined whether the C_{min} level of imatinib actually reflects the inhibitory activity against BCR–ABL kinase by means of the PIA assay.

Materials and Methods

Patients. Sixty-five CML patients were registered for the blood level testing of imatinib in the Nagoya (BINGO) study. The registration criteria was being in CP, no history of an accelerated phase or blast crisis, administration of imatinib for more than 12 months, and no history of treatment with anti-CML drugs other than imatinib. We confirmed that no patients took drugs or dietary supplements, or grapefruit juice, which may affect the metabolism of imatinib, within 14 days before peripheral blood (PB) sampling. We also confirmed that no patients had a history of infection and external injury, which may increase the serum AGP level, within 14 days before PB sampling.

Informed consent was obtained from all patients, and this study was approved by the Ethics Committees of the participating hospitals.

Evaluations of clinical response and adverse effects. Cytogenetic response to imatinib was examined by conventional cytogenetic analysis of the bone marrow (BM) metaphase. In some patients, fluorescent *in situ* hybridization (FISH) analysis for the *BCR–ABL* fusion gene was performed. To assess the molecular response, the *BCR–ABL* transcript level was quantitated by real-time reverse transcriptase-mediated PCR as previously described.[21] Briefly, total RNA was extracted from the BM or PB sample, and a cDNA was synthesized from each RNA using a random primer. The *BCR–ABL* transcript level was quantitated using a real-time fluorescence detection method. The expression level of the *GAPDH* gene was used for the internal control.

Complete cytogenetic response (CCyR) was defined as no Philadelphia chromosome-positive cells in BM. Major molecular response (MMR) was defined as a more than 3-log reduction in the *BCR–ABL* transcript level or <100 copy/μg RNA of the *BCR–ABL* transcript.

Adverse effects of imatinib were evaluated according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE, Bethesda, MD, USA) version 3.0.

Measurement of the trough plasma imatinib concentration and serum AGP level. Peripheral blood (PB) was obtained from the patients within 24 ± 2 h from the last imatinib administration twice on different days. The daily dose of imatinib and the dosing schedule were not changed within 14 days before PB sampling. Plasma and serum were immediately separated at 4°C by centrifugation and preserved at −80°C until measurements. The plasma imatinib concentration was measured at the Toray research Center (Tokyo, Japan) using liquid chromatograpy-tandem mass spectrometry (API 4000; PE-Sciex, Toronto, Canada) as previously reported.[22] The internal standard, imatinib mesylate, was provided by Novartis Pharma (Basel, Switzerland), and the assay system was approved by Novartis Pharma.

The serum AGP level was measured at BML (Tokyo, Japan) by nephelometry. Normal plasma and serum were obtained from PB of the authors, and used as a control.

Plasma inhibitory activity (PIA). The BCR–ABL (p210)-expressing the Ba/F3 (BCR/ABL-Ba/F3) cell line was a kind gift from Professor Martin Ruthardt, and was maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen). Imatinib was a kind gift from Novartis Pharma. BCR/ABL-Ba/F3 cells (2×10^6) were incubated with 1 mL plasma obtained from patients at 37°C for 3 h. At this condition, we confirmed that the viability of BCR/ABL-Ba/F3 cells was not affected by the incubation with human plasma from healthy donors. The cells were washed twice with ice-cold PBS and then cell pellets were suspended with lysis buffer. An equal amount of whole cell lysates was separated by SDS-PAGE, and electroblotted onto Immobilon PVDF membranes (Millipore, Bedford, MA, USA). Immunoblotting was performed with anti-phospho (P)-ABL and anti-P-STAT5 antibodies (Cell Signaling, Danvers, MA, USA). Signals were developed using an ECL system (GE Healthcare, Uppsala, Sweden). The membranes were incubated with stripping buffer, and then reprobed with anti-ABL (BD Pharmingen, Franklin Lakes, NJ, USA) and anti-STAT5 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Each band density corresponding to P-BCR–ABL and total BCR–ABL protein was determined by using ImageJ software (National Cancer Institute, Bethesda, MD, USA). The band density corresponding to P-BCR–ABL obtained from cells incubated with normal plasma was used as each baseline. The band density of P-BCR–ABL in each sample was adjusted by the band density of total BCR–ABL. The PIA for a given patient's plasma was calculated by expressing the density of the P-BCR–ABL band as a percent decrease from the density of the baseline.

Statistical analysis. Differences in continuous variables were analyzed with Student's *t*-test or the Mann–Whitney *U*-test for the distribution between two groups. Frequencies were analyzed using Fisher's exact test for 2×2 tables or Pearson's chi-squared test for larger tables. Linear regression was used for the analysis of correlation between two continuous variables. Receiver–operator curve (ROC) analysis was performed to assess the discrimination potential of C_{min} of imatinib for MMR. The Smirnov–Grubbs' test was used for evaluating the outlier. These statistical analyses were performed with the STATA9 software (Stata, College Station, TX, USA). For all analyses, *P*-values were two-tailed, and a *P*-value of <0.05 was considered significant.

Results

Individual differences of trough imatinib concentration. We measured the C_{min} of imatinib twice on different days in 65 patients. The median duration of two sampling points was 28 days (range, 12–90 days). The mean difference of the C_{min} level between two points was 183 ± 176 ng/mL, while five patients showed >400 ng/mL of difference between two points (Fig. 1a). We could not find any factors which would have affected the imatinib C_{min} level, such as the last administration time and the administration of other drugs, in any patients; however, we excluded these five patients for further analysis, because their differences were statistically rejected by the Smirnov–Grubbs' test.

Characteristics of patients. Characteristics and clinical response to imatinib of 60 patients are shown in Table 1. The median age was 56 years (range, 26–82 years). The median duration of imatinib administration was 1025 days (range, 370–2434 days). The daily imatinib dose at the sample collection was 200 mg in four patients, 300 mg in nine, 400 mg in 46, and 600 mg in one. In all patients, complete hematological response was achieved before the 12th month and persisted until registration for this study. Major molecular response (MMR), CCyR without MMR, and partial cytogenetic response (PCyR) at the 12th month were observed in 38 (63.3%), 21 (35.0%), and one (1.7%) patients, respectively. The distribution of clinical response according to the daily imatinib dose was not

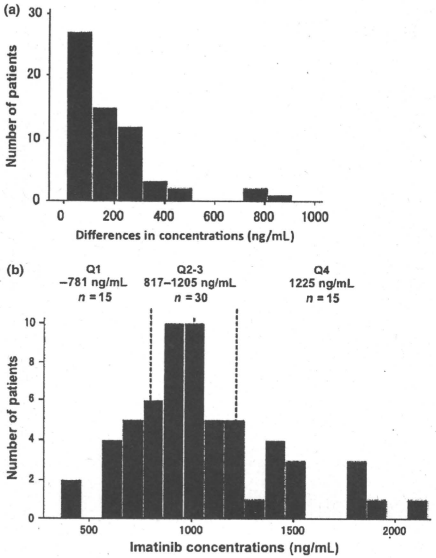

Fig. 1. Trough plasma imatinib level in steady-state chronic myeloid leukemia (CML) patients. (a) Individual differences in C_{min} level between two sampling points. Five patients showed >400 ng/mL of difference between two points, and their differences were statistically rejected by the Smirnov–Grubbs' test. (b) Distribution of C_{min} level in 60 CML patients treated with imatinib for more than 12 months. Vertical dashed lines represent the 25th, 50th, and 75th percentile.

statistically different. Complete cytogenetic response (CCyR) without MMR and MMR as the best response during imatinib treatment were observed in eight (13.3%) and 52 (86.7%) patients, respectively. The distribution of the best response according to the daily imatinib dose was also not statistically different. There were no ≥Grade 3 adverse effects in any patients.

Trough imatinib concentration. The average C_{min} of two measurements in each patient was used for further analysis. The median C_{min} of imatinib was 1003 ng/mL (range, 361–2150 ng/mL). When patients were divided into quartile groups (Q1–4) based on their C_{min} levels, the average of C_{min} level of the lowest quartile (Q1) was 718 ng/mL and the highest quartile (Q4) was 1495 ng/mL (Fig. 1b). The C_{min} of imatinib was not associated with the age ($r^2 = 0.0227$, $P = 0.0547$), the body weight of patients ($r^2 = 0.0217$, $P = 0.4431$), the body surface area (BSA) ($r^2 < 0.0001$, $P = 0.8765$), and the daily imatinib dose and C_{min} ($r^2 = 0.1348$, $P = 0.1011$) (Fig. 2). No significant correlation between the C_{min} level of imatinib and adverse effects was observed.

Correlation of trough imatinib concentration with clinical responses. Thirty-eight patients achieved MMR at the 12th month (63.3%): six (40.0%) in Q1, 17 (56.7%) in Q2–3, and 15 (100%) in Q4 ($P = 0.002$) (Table 2). The C_{min} levels of imatinib among patients who achieved MMR at the 12th month ($n = 38$) were significantly higher than those for patients who did not achieve MMR ($n = 22$): the median values were 1092.5 ng/mL (range, 562–2150 ng/mL) and 853 ng/mL (range, 361–1205 ng/mL), respectively ($P = 0.0022$, Mann–Whitney *U*-test) (Fig. 3a). Concentration-effect ROC analysis showed the discrimination potential of imatinib C_{min} levels for MMR (Fig. 3b). The area under the ROC (AUC) was 0.7386 with the best sensitivity (63.2%) and specificity (68.2%) at a C_{min} threshold of 974 ng/mL. As shown in Table 3, when patients were divided into two groups based on the C_{min} threshold of 974 ng/mL, MMR at the 12th month was highly achieved in the patients whose C_{min} was >974 ng/mL compared to those ≤974 ng/mL: 24 of 31 patients (77.4%) and 14 of 29 patients (48.3%) achieved MMR, respectively ($P = 0.019$).

We also examined the correlation of the imatinib C_{min} level with MMR as the best response during imatinib treatment. The C_{min} levels of imatinib among patients who achieved MMR during treatment ($n = 52$) were higher than those for patients who did not achieve MMR ($n = 8$): the median values were 1025 ng/mL (range, 562–2150 ng/mL) and 748.5 ng/mL (range, 361–1205 ng/mL), respectively ($P = 0.0037$, Mann–Whitney *U*-test) (Fig. 3c). Concentration-effect ROC analysis showed the dis-

Table 1. Clinical characteristics of patients

	Total	Daily dose of imatinib			
		200 mg	300 mg	400 mg	600 mg
Number	60	4	9	46	1
Male/female	35/25	1/3	4/5	29/17	1/0
Age, years (median)	26–82 (56)	58–82 (71)	42–81 (69)	26–76 (50)	31 (31)
Duration of imatinib, days (median)	328–2434 (1025)	701–1726 (1251)	384–1959 (1366)	328–2434 (901)	1626 (1626)
C_{min} of imatinib, ng/mL (median)	361–2150 (1003)	361–1020 (747)	781–1395 (954)	450–1875 (1005)	2150 (2150)
AGP, mg/dL (median)	44–106 (72)	68–93 (70)	58–104 (72)	44–106 (72)	105 (105)
Response at 12 months (%)					
PCyR	1 (1.7)	0 (0)	0 (0)	1 (2.2)	0 (0)
CCyR without MMR	21 (35.0)	3 (75.0)	6 (66.7)	12 (26.1)	0 (0)
MMR	38 (63.3)	1 (25.0)	3 (33.3)	33 (71.7)	1 (100)
Best response during imatinib treatment (%)					
CCyR without MMR	8 (13.3)	1 (25.0)	2 (22.2)	5 (10.9)	0 (0)
MMR	52 (86.7)	3 (75.0)	7 (77.8)	41 (89.1)	1 (100)

Clinical characteristics of chronic myeloid leukemia (CML) patients treated with imatinib for more than 12 months are shown. There was no significant difference in the clinical response among the daily doses of imatinib. AGP, α1-acid glycoprotein; CCyR, complete cytogenetic response; MMR, major molecular response; PCyR, partial cytogenetic response.

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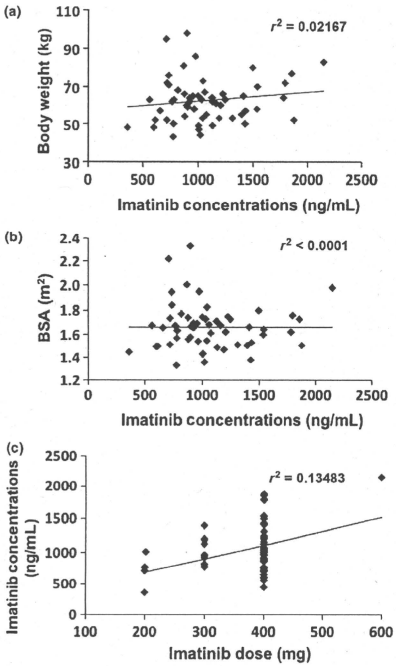

Fig. 2. C_{min} levels by body weight, body surface area (BSA), and imatinib daily dose. C_{min} levels were not correlated with body weight (a), BSA (b), or imatinib daily dose (c).

crimination potential of imatinib C_{min} levels for MMR during treatment (Fig. 3d). The AUC was 0.8209 with the best sensitivity (80.8%) and specificity (75.0%) at a C_{min} threshold of 817 ng/mL. As shown in Table 3, when patients were divided into two groups based on the C_{min} threshold of 817 ng/mL, MMR during treatment was highly achieved in patients whose C_{min} was >817 ng/mL compared to those ≤817 ng/mL: 44 of 42 patients (95.5%) and 10 of 16 patients (62.5%) achieved MMR, respectively (P = 0.001).

Serum AGP level in CML patients treated with imatinib. The serum AGP level was measured twice at the same time when PB was collected for the measurement of plasma imatinib concentration. Since there was no significant difference of serum AGP level in each patient between two sampling points, the average of the two sampling points was used for the AGP level in each patient. The normal range of serum AGP was confirmed to be 42–93 mg/dL in the healthy Japanese population by BML. Although the AGP levels in CML patients treated with imatinib varied from 44 to 106 mg/dL (median, 72 mg/dL), they were within normal limit in 57 of 60 patients (95.0%). There was no significant difference in serum AGP levels among the daily imatinib doses (Table 1).

Plasma inhibitory activity (PIA). Plasma inhibitory activity (PIA) was examined in 51 patients using plasma obtained at the

Table 2. Clinical response according to the trough imatinib concentration level

	Quartile 1	Quartiles 2 and 3	Quartile 4
Number	15	30	15
C_{min} of imatinib, ng/mL (median)	361–781 (718)	817–1205 (1003)	1225–2150 (1495)
Response at 12 months (%)			
PCyR	1 (6.7)	0 (0)	0 (0)
CCyR without MMR	8 (53.3)	13 (43.3)	0 (0)
MMR	6 (40.0)	17 (56.7)	15 (100)
Best response during imatinib treatment (%)			
CCyR without MMR	5 (33.3)	3 (10.0)	0 (0)
MMR	10 (66.7)	27 (90.0)	15 (100)

Clinical response according to the quartile of C_{min} level is shown. In the lowest quartile (Q1), the MMR achievement rate was significantly lower than in other quartiles. CCyR, complete cytogenetic response; MMR, major molecular response; PCyR, partial cytogenetic response.

time of sampling for the measurement of imatinib C_{min} levels. We first validated our PIA assay system by evaluating dephosphorylation levels of P-BCR–ABL and P-STAT5 using six patients' plasma. As shown in Figure 4(a), 1000 ng/mL imatinib apparently reduced the phosphorylation levels of BCR–ABL and STAT5 in the liquid culture system without human plasma, while the inhibitory activity of patient's plasma, even that which contained an equivalent concentration of imatinib, was lower than in the liquid culture system. This result indicated that the binding of imatinib to plasma proteins reduced the free or active fraction of imatinib, resulting in lower inhibitory activity than in the conventional *in vitro* assay.

Since PIA against P-BCR–ABL and P-STAT5 were observed as the same level in each patient, we examined the correlation between C_{min} level of imatinib and PIA against P-BCR–ABL in all patients. The median PIA against P-BCR–ABL was 62% (range, 26–80%). There was a weak correlation between PIA against P-BCR–ABL and the C_{min} level of imatinib (r^2 = 0.2501, P = 0.0007) (Fig. 4b); however, western blot analysis showed that PIA activities apparently increased when the plasma contained >1000 ng/mL imatinib (Fig. 4a). Since ROC analysis showed that the efficient C_{min} threshold for achieving MMR at the 12th month was 974 ng/mL, we divided patients into two groups based on the threshold. In 27 patients whose C_{min} of imatinib was >974 ng/mL, 19 patients (70.4%) showed >62% PIA against P-BCR–ABL, which was the median PIA. In contrast, seven of 24 patients (29.2%), whose C_{min} of imatinib was ≤974 ng/mL, showed more than 62% of PIA (P = 0.003) (Table 4). These results indicated that patient plasma containing imatinib over the efficient threshold for the optimal response could actually inhibit the BCR–ABL activity.

Discussion

Although the excellent efficacy of imatinib against CML has been well established, a suboptimal response or treatment failure is observed in some CML patients. Several factors have been suggested to be involved in these unfavorable clinical responses. Among them, PK-related factors, which affect exposure to imatinib, are the most important as a host-dependent factors for treatment with imatinib, because several studies have demonstrated that the steady-state C_{min} level of imatinib correlated with both cytogenetic and molecular responses.[23] Previous reports demonstrated that the C_{min} level of imatinib varied

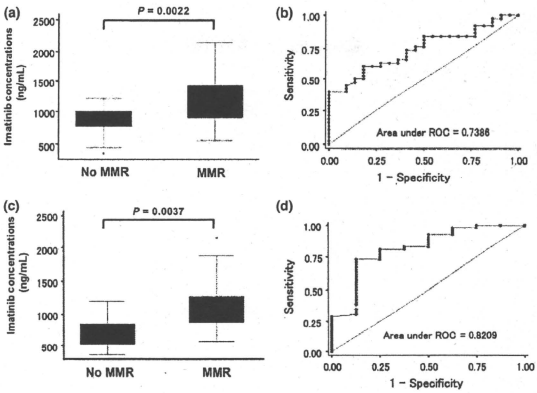

Fig. 3. C_{min} threshold for major molecular response (MMR). (a) C_{min} levels of imatinib among patients who achieved MMR at the 12th month (n = 38) were significantly higher than in patients who did not achieve MMR (n = 22); the median values were 1092.5 ng/mL and 853 ng/mL, respectively (P = 0.0022, Mann–Whitney U-test). (b) Concentration-effect ROC analysis of the discrimination potential of imatinib C_{min} levels for MMR at the 12th month. The area under the ROC (AUC) was 0.7386 with the best sensitivity (63.2%) and specificity (68.2%) at a C_{min} threshold of 974 ng/mL. (c) C_{min} levels of imatinib among patients who achieved MMR during treatment (n = 52) were higher than in patients who did not achieve MMR (n = 8); the median values were 1025 ng/mL and 748.5 ng/mL, respectively (P = 0.0037, Mann–Whitney U-test). (d) Concentration-effect ROC analysis of the discrimination potential of imatinib C_{min} levels for MMR during treatment. The AUC was 0.8209 with the best sensitivity (80.8%) and specificity (75.0%) at a C_{min} threshold of 817 ng/mL.

greatly even in patients administrated with the same daily dose. For example, Larson *et al.*[14] reported that the C_{min} level of imatinib for the 400 mg/day dose ranged from 153 to 3910 ng/mL in the International Randomized Study of Interferon and STI571 (IRIS) study; Picard *et al.*[15] reported that it ranged from 184 to 2947 ng/mL in the French study; and Sakai *et al.*[16] reported that it ranged from 582 to 2420 ng/mL in Japanese patients in the Nagasaki CML study. Several reasons, such as incomplete adherence to imatinib therapy, the demographic factors of patients, differential absorption from the gastrointestinal tract, variability of metabolizing enzyme activity, and differential binding of imatinib to AGP, have been suggested to affect the large variation in the C_{min} level.[13,19,24–27]

Adherence to imatinib therapy should be the first concern when evaluating the clinical significance of the C_{min} level, because it has been reported that only 41% of patients were >90% compliant with imatinib over 14 months of treatment.[23] In addition, it is also important whether blood sampling was accurately performed in the trough state. To avoid the effects of adherence factors on the C_{min} level, we confirmed that all patients continued to take imatinib as prescribed, and that neither the daily dose nor the dosing-schedule had been changed within 14 days before blood sampling; however, the C_{min} level of imatinib for the 400 mg/day dose varied from 328 to 2463 ng/mL in our study. Since previous studies measured the C_{min} level of imatinib only once in each patient, individual variation during treatment was not considered. For the first time, we report individual variation of the C_{min} level by measuring twice on different days. It was notable that five of 65 patients revealed >400 ng/mL difference between the two points. Since the differences of these five patients were statistically rejected, we excluded them for further analysis. However, we could not completely determine these individual differences as outliers, because we could not find any factors which may have affected the C_{min} level of imatinib, such as the last administration time and the administration of other drugs, in any patients. When these five patients were included in the analysis, the C_{min} levels of imatinib among patients who achieved MMR at the 12th month (n = 40) were also significantly higher than those for patients who did not achieve MMR (n = 25): the median values were 1092.5 ng/mL (range, 562–2150 ng/mL) and 896 ng/mL (range, 361–1475 ng/mL), respectively (P = 0.0119, Mann–Whitney U-test), and ROC analysis demonstrated C_{min} levels and their discrimination potential for MMR with the best sensitivity (62.5%) and specificity (64.0%) at a C_{min} threshold of 1002 ng/mL. These results collectively indicated that the C_{min} level is actually associated with the clinical response in CML patients treated with imatinib; however, repeated measurements are required to amend the daily dose based on the C_{min} level.

Table 3. MMR achievement according to the imatinib trough level threshold

At the 12th month (P = 0.019)			
	C_{min} of imatinib (ng/mL)		Total (%)
	≤974	>974	
MMR (%)	14 (48.3)	24 (77.4)	38 (63.3)
No MMR (%)	15 (51.7)	7 (22.6)	22 (26.7)
Best response during treatment (P = 0.001)			
	C_{min} of imatinib (ng/mL)		Total (%)
	≤817	>817	
MMR (%)	10 (62.5)	42 (95.5)	52 (86.7)
No MMR (%)	6 (37.5)	2 (4.5)	8 (13.3)

MMR, major molecular response.

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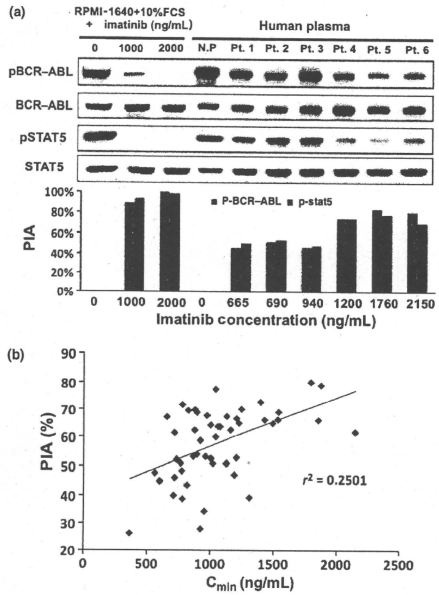

Fig. 4. Plasma inhibitory activity (PIA). (a) In the conventional *in vitro* inhibitory assay without human plasma, 1000 ng/mL imatinib sufficiently reduced the phosphorylation levels of BCR–ABL and STAT5, although the inhibitory activity of patients' plasma was lower than that of the conventional assay at an equivalent concentration of imatinib. However, PIA apparently increased when the plasma contained >1000 ng/mL imatinib. N.P. indicates normal health donor plasma. Pt. 1 to Pt. 6 were plasma from CML patients treated with imatinib. (b) There was a weak correlation between PIA against P-BCR–ABL and the C_{min} level of imatinib (r^2 = 0.2501, P = 0.0007).

Table 4. Correlation of plasma imatinib level with PIA

C_{min} of imatinib	Total	PIA against P-BCR–ABL	
		<62%	≥62%
≤974 ng/mL	24	17 (70.8%)	7 (29.2%)
>974 ng/mL	27	8 (29.6%)	19 (70.4%)
Total	51	25	26

Patient plasma containing >974 ng/mL imatinib revealed higher plasma inhibitory activity (PIA) than those containing ≤974 ng/mL (P = 0.003).

In this study, we measured the C_{min} level of imatinib in the steady-state after at least 12 months of treatment with imatinib, irrespective of the daily dose, and evaluated its clinical significance retrospectively. Therefore, our results should be carefully interpreted for the clinical application of PK-based dosage optimization. In our analysis, the C_{min} level of imatinib was not significantly correlated with age, sex, body weight, and BSA; and this was anticipated because all patients were being prescribed the optimal dose of imatinib according to their tolerability. In addition, the administration of the optimal dose of imatinib might result in no relationship between the adverse effects and C_{min} level. However, the median age of the patient group treated with 200 mg or 300 mg imatinib was older than that of the 400 mg or 600 mg group, indicating the lower tolerability of older patients to standard-dose imatinib. Since ≥Grade 3 adverse effects were not found in all patients at the registration to this study, we should further compare the C_{min} levels in individual patients with or without ≥Grade 3 adverse effects to evaluate the relationship between the adverse effects and C_{min} level.

Since our study was retrospectively conducted in patients who were in CP without a history of an accelerated phase or blast crisis, and administrated only imatinib for more than 12 months, most patients who could not achieve optimal response before the 12th month or who were imatinib therapy intolerant, were avoided during study registration, resulting in a higher MMR rate at the 12th month in this study than previous reports.[3,28,29] However, the C_{min} threshold of 974 ng/mL for achieving MMR at the 12th month was almost the same as that of 1002 ng/mL in a previous report.[15] Furthermore, subanalysis of the IRIS study also demonstrated that the mean C_{min} of imatinib in patients who achieved CCyR was 1009 ng/mL.[14] These results collectively suggest that maintaining the C_{min} of imatinib above ~1000 ng/mL may be important for achieving the optimal response in CML patients; however, it is difficult to identify the definitive threshold of the C_{min} level for clinical use, because the C_{min} threshold for achieving MMR as the best response was 817 ng/mL in our analysis. The first analysis of the IRIS study indicated that achieving MMR at the 12th month predicted a better progression-free survival,[30] while the recent updated analysis showed that MMR predicted for a better 6-year event-free survival at the 18th month but not at the 12th month.[31] Unfortunately, we could not determine the C_{min} threshold for achieving MMR at the 18th month, because all patients were not evaluated the molecular response at the 18th month. However, since some studies demonstrated the value of MMR at an earlier period of imatinib therapy,[32] we believe that maintaining the C_{min} of imatinib ~1000 ng/mL is a target point for the patients with CML-CP.

It has been suggested that plasma concentration does not necessarily reflect the free or active form fraction of imatinib, leading to the discrepancy in evaluating clinical response based on the C_{min} level. Since a high AGP level reportedly reduces the free or active form fraction of imatinib, the AGP level should be considered when evaluating the correlation between the C_{min} level and clinical response. However, in our study, the AGP level in most patients in the steady-state of imatinib therapy was within the normal range, indicating little impact of AGP on our analysis. In addition to AGP, it has been reported that imatinib binds several plasma proteins, such as albumin, γ-globulin, high density lipoprotein, and low density lipoprotein, while it has not been fully clarified how this binding affects the free or active fraction of imatinib. Plasma inhibitory activity (PIA) is reported to be an alternative surrogate marker reflecting the actual inhibitory effect *in vivo*.[20] Notably, our study demonstrated that patient plasma containing >974 ng/mL concentration of imatinib sufficiently inhibited P-BCR–ABL and P-STAT5, indicating that the C_{min} threshold determined by clinical analysis had been proved biologically.

In conclusion, we confirmed that maintaining ~1000 ng/mL of C_{min} level was clinically and biologically important for the optimal response in CML patients treated with imatinib. On the other hand, we also demonstrated individual variation of the C_{min} level even in the steady-state of imatinib therapy, indicating the importance of repeated measurement of the C_{min} level. Although the C_{min} level of imatinib reflects the clinical response, it remains unclear whether dose adjustment based on

the C_{min} level would provide clinical benefits to CML patients, such as increasing the optimal response and decreasing adverse effects. A prospective intervention study is necessary to establish PK-based management in CML patients treated with imatinib.

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Disclosure Statement

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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 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.

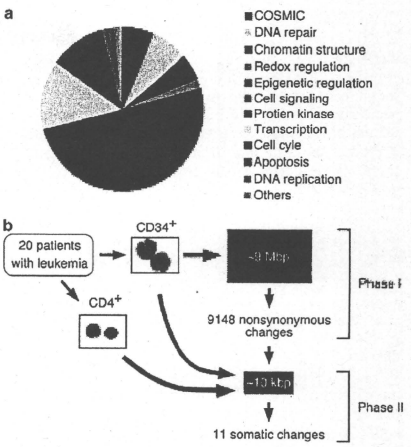

Figure 1 Resequencing of the leukemia genome with wafers. (**a**) Genes interrogated by the phase I wafer ($n=5648$) included those listed in the COSMIC database and those categorized on the basis of function of the encoded protein as indicated. (**b**) CD34$^+$ and CD4$^+$ cell fractions were purified 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.

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

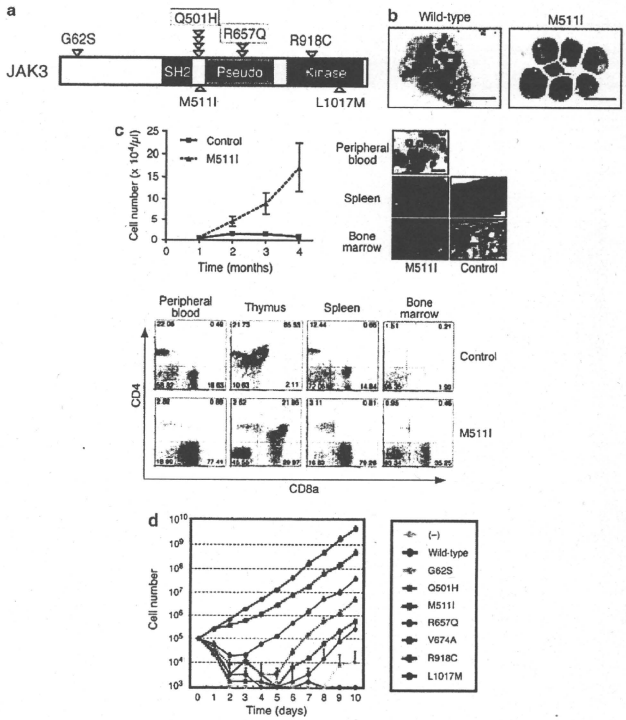

Figure 2 Identification of JAK3 mutants in leukemia. (**a**) Amino-acid substitutions detected in this study are shown relative to the domain organization of JAK3. The mutations M511I (one case) and Q501H (four cases) are located in the linker region between the SH2 and pseudokinase domains of JAK3, 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

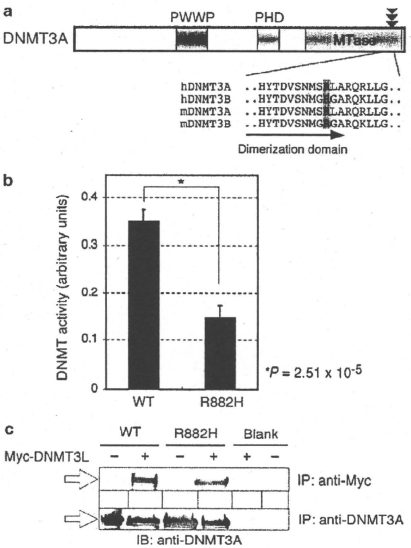

Figure 3 Identification of a DNMT3A mutant in leukemia. (**a**) Domain organization of human DNMT3A showing that the R882 residue found to be mutated in leukemia is conserved among human (h) and mouse (m) members of the DNMT3 family. DNMT3A contains a tetrapeptide PWWP domain, polybromo homology domain (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 $\geq$17.1 (median = 40.1) (Figure 4b), and contamination of the CD4$^+$ 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

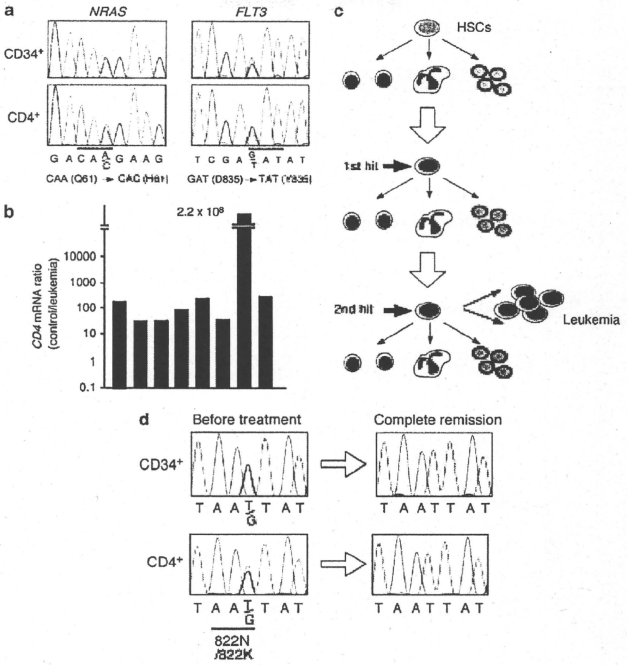

Figure 4 Proposed stepwise nature of leukemogenesis. (**a**) Sequencing electrophoretograms for the regions surrounding codon 61 of *NRAS* or codon 835 of *FLT3* in genomic DNA from the CD34+ and CD4+ fractions of patient IDs JM17 and JM08, respectively. Heterozygous nucleotide changes that give rise 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)

by searching with various keywords characteristic to each subcategory (such as DNA repair, regulation of chromatin structure, etc.), followed by manual inspection. The final gene list for the wafers is shown in Supplementary Table S6. Construction of the wafers, quality control analysis and data processing are described in Supplementary Text.

JAK3 analysis

Complementary DNAs for JAK3 mutants were generated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and ligated into the pMX retroviral vector (Onishi *et al.*, 1996). Ecotropic recombinant retroviruses encoding each mutant were produced in BOSC23 cells transfected with the corresponding pMX-based plasmid and were used to infect BA/F3 or 32D cells as described previously (Choi *et al.*, 2007). Both types of cell were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (both from Life Technologies, Carlsbad, CA, USA) and mouse IL-3 (Sigma, St Loius, MO, USA) at 10 Units/ml; differentiation of 32D cells was induced by culture in the presence of serum and mouse granulocyte colony-stimulating factor (Sigma) at 0.5 ng/ml. A concentrated preparation of a retrovirus with a VSV-G envelope and encoding both JAK3(M511I) and enhanced green fluorescent protein was used to infect $CD34^-$ c-Kit^+ $Sca\text{-}1^+$ Lineage-$marker^-$ ($CD34^-KSL$) hematopoietic stem cells isolated from the bone marrow of C57BL/6 mice, and the infected cells were transplanted into lethally irradiated mice congenic for the *Ly5* locus (Iwama *et al.*, 2004). *CD4*, *JAK2* and *JAK3* mRNAs were quantitated by reverse transcription and real-time PCR analysis using an ABI7900HT system (Life Technologies) and with the primers 5′-CTGGAATCCAACATCAAGGTTCTG-3′ and 5′-AATTGTAGAGGAGGCGAACAGGAG-3′ for CD4, 5′-CTCCAGAATCACTGACAGAGAGCA-3′ and 5′-CCAC TCGAAGAGCTAGATCCCTAA-3′ for *JAK2* and 5′-GAGC TCTTCACCTACTGCGACAAA-3′ and 5′-AGCTATGAAA AGGACAGGGAGTGG-3′ for JAK3; the cDNA for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was also amplified with the primers 5′-GTCAGTGGTGGACC TGACCT-3′ and 5′-TGAGCTTGACAAAGTGGTCG-3′. The relative abundance of the cDNAs of interest was calculated from the threshold cycle (C_T) for each cDNA and that for *GAPDH* cDNA.

DNMT3A analysis

Recombinant His_6-tagged DNMT3A or DNMT3A(R882H) was expressed in SF9 cells using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA), and each protein was purified by stepwise column chromatography as described previously (Suetake *et al.*, 2003). The enzymatic activity of each protein was assayed with -*S*-adenosyl-L-methionine (GE Healthcare, Waukesha, WI, USA) and dIdC or dGdC as substrates (Suetake *et al.*, 2003). The association between Myc epitope-tagged human DNMT3L and wild-type or R882H forms of human DNMT3A in transfected HEK293 cells was examined by immunoprecipitation and immunoblot analyses.

Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

The clinical characteristics of CD7$^+$ CD56$^+$ acute myeloid leukemias other than M0

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Abstract Immunological phenotyping of acute leukemia have provided enormous and important information for the classification and lineage determination of leukemia. Forty-nine patients with CD7$^+$ CD56$^+$ acute myeloid leukemia (AML) were analyzed. There were 17 patients of M0, which corresponded to myeloid/NK cell precursor acute leukemia, and 32 patients of AML other than M0 (9 each for M1 and M2, one for M3, 3 for M4, 4 for M5 and 6 for M7). Age distribution was similar between these two groups, but CD7$^+$ CD56$^+$ M0 showed significant male predominance than CD7$^+$ CD56$^+$ M1–M7 (M:F = 15:2 vs. 15:17, $P = 0.006$). The disease localization and the hematological manifestations were different, showing fewer white blood cell counts and circulating leukemic blasts, less anemia, less thrombocytopenia and more frequent extramedullary involvement in M0 group. The prognosis was poor in both groups, and there was no statistical difference. These findings suggest that extramedullary involvement of myeloid/NK cell precursor acute

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