Miki were significantly ($p < 0.05$) lower in cells with these abnormal mitosis/nuclear morphology to compare with those without these abnormalities (Fig. 4G).

Discussion

Here, we identified a microdeletion cluster among JMML patients within 120 kb in 7q21.3 subband. This cluster contains three poorly characterized genes: *Miki* (*LOC253012*), *Samd9*, and *Samd9L*. Since single gene deletion of *Samd9* or *Miki* was proved by two-independent methods (mCGH and qPCR) in patient #1 or #8, respectively, we prefer to consider that three genes, rather than one of them, are candidates for myeloid tumor suppressors on 7q. Three genes are also deleted in adult MDS and AML either as a part of large deletions or single gene loss (Fig. 2C).

Among systems detecting microdeletions, SNP-array hybridization becomes the first choice for primary screening [4]. However, because SNPs tend to cluster within introns and intergenic spaces, SNP-array may not always be the best. For instance, although there are nine SNP probes in this microdeletion cluster in Genome-Wide SNP6.0 system (Affymetrix), no probes can detect *Samd9* gene deletion (Fig. 2A, bottom). In addition, only one probe (A-866741) locates to coding region, casting doubt on the potential of SNP-array to detect small deletions in the critical genes. Application of the short probe-based mCGH to samples containing few copy number abnormalities (such as JMML) would be a good alternative of SNP-array.

In myeloid tumors, −7/7q− has been most implicated in pathogenesis of MDS, which is characterized by myelodysplasia (morphological abnormality in hematopoietic progenitors) [2]. Myelodysplasia includes abnormal nuclear morphologies, such as bi-, tri-, or multi-nucleated cells and abnormal mitoses involving lagging chromosomes, multi-polar mitoses or so-called colchicine-mitosis (chromosome scattering similar to colchicine-treated cells). Despite the fact that these features are routinely observed, underlying molecular mechanisms are largely unknown. Our findings (Fig. 4E–G) raised a possibility that attenuated expression of Miki plays important roles in such abnormal mitosis/nuclear morphology, although detailed mechanisms remained to be established.

Samd9 and Samd9L are related proteins with 60% amino acid identity. Recently, point mutations of Samd9 were reported as a causative gene alterations in Normophosphatemic Familial Tumoral Calcinosis, a rare autosomal recessive disorder in five families of Jewish-Yemenite origin [16,17]. In addition, downregulation of Samd9 was reported to be implicated in aggressive fibromatosis [18], suggesting that Samd9 could be a tumor suppressor. However, Samd9/Samd9L does not show significant homology to any other genes and no biological functions were elucidated. We overexpressed or downregulated Samd9 or Samd9L in various cells and found no prominent phenotype, possibly because functional redundancy of these two proteins. Because there is only *Samd9L* gene in mouse genome [18], *Samd9L*-deficient mice would show unambiguous phenotypes. Indeed, currently we are accumulating phenotypes from *Samd9L*-deficient mice that support our hypothesis that Samd9/Samd9L are myeloid tumor suppressors.

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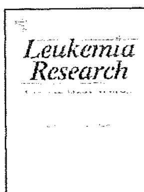

Level of DNA topoisomerase IIα mRNA predicts the treatment response of relapsed acute leukemic patients

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ABSTRACT

The DNA topoisomerase IIα (Topo IIα) is known as a target enzyme for many chemotherapeutic agents. We investigated the Topo IIα mRNA expression by real-time RT-PCR in 37 paired samples at diagnosis and at relapse of acute leukemic patients in relation to drug sensitivity and clinical outcome. The Topo IIα levels in leukemic blasts at relapse were significantly higher than that at diagnosis, especially in ALL. The increase in the Topo IIα level at relapse was significant in cases which could not achieve a second remission, but not significant in cases which achieved a second remission. These results suggest that the change of Topo IIα expression in leukemic blasts at relapse may predict therapeutic responsiveness.
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1. Introduction

In acute leukemia, although clinical outcomes have been improved due to recent advances in chemotherapy, relapses are still preventing the long-term survival of patients. The mechanisms of relapse and the accompanying resistance of leukemic blast cells to anti-leukemic treatment have not been fully clarified. The multidrug resistance (MDR) phenomenon has been shown to be composed of many factors including the *mdr1 gene*/P-glycoprotein (P-gp), DNA topoisomerases (Topo), lung resistance-related protein, multidrug resistance-associated protein, glutathione-S-transferase and so on [1,2].

Topo are essential nuclear DNA-binding enzymes that control and regulate cellular functions such as DNA replication, repair, gene transcription and cell division during cell proliferation [3]. Topo II is the only enzyme able to cleave and religate double-stranded DNA, and plays a major role in the remodeling of chromatin during mitosis in cell cycles [4,5]. In human cells, two distinct isoenzymes of Topo II have been identified based on differences in molecular weight, pattern of expression, protein structure and function. Topo IIα has been shown to have a critical role in drug sensitivity, while Topo IIβ has not [6–8]. Topo IIα has been known as the main target for a variety of anti-leukemic agents including anthracyclines and epipodophyllotoxins. The cytotoxicity of Topo IIα targeting drugs is thought to be a consequence of the stabilization of the Topo II–DNA complexes, which leaves DNA breaks and leads to apoptosis [9], though the exact mechanisms remain elusive. The expression of Topo IIα also varies in different phases of the cell cycle [10–12]. There are some reports on Topo IIα gene expression of leukemic blasts at relapse in comparison to that at onset from clinical samples, but a controversy remains whether quantitative alteration of Topo IIα occurs in relapsing acute leukemia [7,13–15].

To investigate the significance of Topo II in relapse of leukemia and relapse related drug resistance, we have examined Topo IIα mRNA relative levels with real-time quantitative reverse transcriptase-polymerase chain reaction (real-time RT-PCR), which is a sensitive and highly reliable method for detecting the enzyme quantitatively, in paired samples at diagnosis and at relapse of adult acute leukemic patients. We have also evaluated the correlation between the Topo IIα, Topo IIβ mRNA level, drug sensitivity to the Topo IIα-mediated agent daunorubicin (DNR), and the clinical therapeutic response in these patients.

2. Materials and methods

2.1. Cell line

The human myelogenous leukemic cell line K562, which is known to express Topo IIα [16], was used to make standard curves for real-time RT-PCR in this study. The cells were cultured in RPMI1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; Life Technologies, Grand Island, NY). When the leukemic cells were in a logarithmic proliferation phase, they

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were collected and washed with phosphate-buffered saline (PBS), and stored at −80 C until use.

2.2. Patients and chemotherapy

Thirty-seven patients were studied after written informed consent was given both at diagnosis and at the time of the first relapse; 19 were *de novo* acute myelocytic leukemia (AML), 4 were AML from myelodysplastic syndrome (AML-MDS), and 14 were acute lymphocytic leukemia (ALL). The median age of the patients was 51 years (range 16–71 years). All samples were taken before chemotherapy both at diagnosis and at relapse. The characteristics of patients are summarized in Table 1. The diagnosis of *de novo* AML and its subtypes was determined according to the French–American–British classification [17]. All patients received chemotherapy including Topo IIα inhibitors at diagnosis, primarily in accordance with the Japan Adult Leukemia Study Group (JALSG) protocols [18–27]. The response to treatment was assessed after two courses of induction chemotherapy. Complete remission (CR) was judged when the normocellular bone marrow showed less than 5% leukemic blast cells and the peripheral blood counts recovered to a normal level. The period of time from when the patients achieved CR to relapse varied from 9 to 48 months. At relapse, 18 out of 23 AML and 13 out of 14 ALL patients received the re-induction treatment which, except for 8 patients, included Topo IIα inhibitors. The other six patients did not receive any treatment due to their poor performance status at relapse. The second CR was only achieved in 7 out of the 18 AML and 4 out of the 13 ALL patients.

2.3. Separation of leukemic blast cells

Mononuclear cells were separated through Ficoll-Conray density gradient centrifugation (density 1.077 g/ml) from bone marrow and/or peripheral blood samples at the initial diagnosis and at the relapse of the disease. The leukemic blast cells

Table 1
Clinical data and Topo IIα mRNA levels in paired blast cells samples of acute leukemia.

Patients			Treatment regimen		Clinical outcome		Topo IIα mRNA level (×10 [illegible])	
No.	Type	Age/Sex	Diagnosis	Relapse	Diagnosis	Relapse	Diagnosis	Relapse
1	AML M1	51/M	IDA, AraC[a]	NT	CR	-	1.9	2.0
2	AML M1	29/F	IDA, AraC[a]	DNR, AraC	CR	CR	9.6	43.7
3	AML M1	62/F	IDA, AraC[a]	DNR, AraC	CR	NR	17.1	204.7
4	AML M1	63/F	DNR, BH-AraC, 6-MP, PSL[b]	MIT, BH-AraC, VP-16	CR	CR	24.1	51.2
5	AML M2	68/F	DNR, BH-AraC, 6-MP, PSL[b]	NT	CR	-	28.2	6.2
6	AML M2	65/M	DNR, BH-AraC, 6-MP, PSL[b]	MIT, BH-AraC, VP-16	CR	CR	210.3	163.5
7	AML M2	43/M	DNR, BH-AraC, 6-MP, PSL[b]	ACR, AraC	CR	NR	40.0	2.5
8	AML M2	52/M	DNR, BH-AraC, 6-MP, PSL[b]	IDA, AraC	CR	NR	0.0	33.9
9	AML M2	57/F	IDA, AraC[a]	HD-AraC	CR	NR	4.8	59.7
10	AML M2	57/F	IDA, AraC[a]	HD-AraC	CR	CR	8.8	8.4
11	AML M2	40/F	DNR, AraC[c]	MIT, BH-AraC, VP-16	CR	CR	22.6	37.4
12	AML M3	37/M	IDA, AraC, ATRA[d]	As_2O_3	CR	CR	140.8	133.5
13	AML M4	21/M	IDA, AraC[a]	MIT, HD-AraC	CR	NR	10.1	66.8
14	AML M4	43/M	IDA, AraC[a]	HD-AraC	CR	CR	116.9	40.9
15	AML M4	57/F	IDA, AraC[a]	MIT, BH-AraC, VP-16	CR	NR	71.4	98.7
16	AML M4	50/F	IDA, AraC[a]	VCR, PSL	CR	NR	72.7	168.9
17	AML M5a	47/M	IDA, AraC[a]	DNR, HD-AraC	CR	NR	22.1	47.9
18	AML M5a	54/M	IDA, AraC[a]	MIT, AraC	CR	NR	0.9	1.0
19	AML M5a	65/M	IDA, AraC[a]	DNR, HD-AraC	CR	NR	13.1	2.2
20	AML-MDS	69/F	IDA, AraC[e]	NT	CR		29.1	8.4
21	AML-MDS	50/M	IDA, AraC[e]	MIT, BH-AraC, VP-16	CR	NR	7.1	27.6
22	AML-MDS	65/F	IDA, AraC[e]	NT	CR		95.9	41.2
23	AML-MDS	71/M	IDA, AraC[e]	NT	CR		7.1	58.1
24	ALL	55/F	VCR, ADM, CPM, MTX, PSL[f]	VCR, DNR, MTX, AraC, L-asp, 6-MP, PSL[h]	CR	NR	23.8	103.9
25	ALL	21/M	VCR, ADM, CPM, MTX, PSL[f]	MIT, HD-AraC	CR	NR	45.7	137.9
26	ALL	46/M	VCR, ADM, CPM, MTX, PSL[f]	VCR, PSL	CR	NR	16.4	64.4
27	ALL	32/F	VCR, ADM, CPM, MTX, PSL[f]	VCR, DNR, MTX, AraC, L-asp, 6-MP, PSL[h]	CR	NR	11.2	74.0
28	ALL	32/M	VCR, ADM, CPM, MTX, PSL[f]	NT	CR		32.5	141.3
29	ALL	16/M	VCR, ADM, CPM, MIT, PSL[f]	VCR, ADR, CPM, L-asp, PSL[i]	CR	CR	53.1	16.7
30	ALL	25/M	VCR, ADM, CPM, MTX, PSL[f]	HD-AraC	CR	NR	12.6	9.6
31	ALL	31/F	VCR, ADM, CPM, MTX, PSL[f]	VCR, DNR, CPM, L-asp, PSL[g]	CR	NR	26.9	53.4
32	ALL	66/F	VCR, ADM, CPM, MTX, PSL[f]	VCR, PSL	CR	NR	20.2	19.9
33	ALL	65/F	VCR, ADM, CPM, MTX, PSL[f]	VCR, DNR, L-asp, PSL	CR	NR	15.3	11.6
34	ALL	57/F	VCR, ADM, CPM, MTX, PSL[f]	DNR, HD-AraC	CR	CR	60.9	86.5
35	ALL	32/F	VCR, ADM, CPM, MTX, PSL[f]	VCR, DNR, ADM, CPM, DEX[g]	CR	CR	26.5	27.8
36	ALL Ph1^{+}	39/M	VCR, DNR, CPM, STI571, PSL[g]	VCR, ADM, CPM, MTX, DEX, AraC, STI571[j]	CR	CR	41.2	105.7
37	ALL Ph1^{+}	63/F	VCR, DNR, CPM, STI571, PSL[g]	VCR, DNR, PSL[j]	CR	NR	21.2	34.5

Abbreviations: ACR, aclarubicin; ADM, doxorubicin; AraC, cytarabine; ATRA, tretinoin; BH-AraC, behenoylcytarabine; CPM, cyclophosphamide; DNR, daunorubicin; DEX, dexamethasone; HD-AraC, high-dose AraC; IDA, idarubicin; L-asp, L-asparaginase; 6-MP, 6-Mercaptopurine; MIT, mitoxantrone; MTX, methotrexate; PSL, prednisolone; VCR, vincristine; VP-16, etoposide; STI571, imatinib mesilate; CR, complete remission; NR, no response; NT, no treatment.

[a] Okamoto et al. [18].
[b] Miyawaki et al. [19].
[c] Ohtake et al. [20].
[d] Asou et al. [21].
[e] Ohtake et al. [22].
[f] Slater et al. [23].
[g] Towatari et al. [24].
[h] Hoelzer et al. [25].
[i] Takeuchi et al. [26].
[j] Martino et al. [27].

thus prepared were suspended in α-modified Eagle's minimal essential medium (α-MEM; Invitrogen), and were either used immediately or cryopreserved in liquid nitrogen with 10% dimethylsulphoxide and 50% FCS until use, or 1 × 10⁷ cells were frozen after washing with PBS buffer until RNA extraction. The presence of more than 80% leukemic blast cells in each sample was confirmed by a cytospin preparation.

2.4. Topo IIα and Topo IIβ mRNA levels by real-time RT-PCR

Total RNA was isolated by a guanidinium thiocyanate-phenol chloroform extraction method using ISOGEN reagent (Nippongene, Tokyo, Japan). After being checked by agarose gel electrophoresis, 1 μg of total RNA was converted to single-stranded cDNA using a random primer and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Each resulting cDNA sample was adjusted to the same density within a standard curve range with nuclease-free water and stored at −20 °C until use.

Measurement of mRNA levels of Topo IIα and Topo IIβ were based on the TaqMan probe method using an ABI PRISM 7700 sequence detector system (Applied Biosystems) as previously described [28]. The real-time amplification reaction was performed in a total volume of 25 μl with a concentration of 300 nM for the primers and 200 nM for the probes. After adding 2.5 μl of cDNA and 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), the paired samples from initial diagnosis and relapse were amplified in triplicates in each experiment according to the standard protocol. Under this assay, serially diluted cDNA prepared from the cell line K562 was used as a standard curve for Topo IIα, Topo IIβ and an internal control gene, human β-actin (TaqMan Endogeneous Control Kit, Applied Biosystems). All final measurements were normalized with the Topo IIα or Topo IIβ mRNA/β-actin average value for each sample. The CV (sd/mean) value of the triplicates for each sample was within 5% in all experiments. The Topo IIα and Topo IIβ primer pairs and probes were made as follows [29]: for Topo IIα, the forward primer was 5′-AGT CGC TTT CAG GGT TCT TGA G-3′, the probe was 5′-CCC TTC ACG ACC GTC ACC ATG GA-3′, and the reverse primer was 5′-TTT CAT TTA CAG GCT GCA ATG G-3′; for Topo IIβ, the forward primer was 5′-GTG GGA ATG TTG GGA GCA-3′, the probe was 5′-TCT ACA AAG CTT AAC AAT CAA GCC CGT TTC ATT TT-3′, and the reverse primer was 5′-CAA ATC TTT CTT TGA CCT ATT CTC TAT AGT A-3′.

2.5. Drug sensitivity of leukemic blast cells

For AML blast cells, the sensitivity to DNR was determined by leukemic blast colony formation according to the method previously reported [30,31].

The sensitivity of ALL leukemic blast cells to DNR was determined with an MTT assay as described by Legrand et al. [32] with a slight modification, using HCl-isopropanol to dissolve the formazan crystals. The absorbance was measured at 570 nm with a microplate reader (Bio-Rad Lab, Hercules, CA). For every DNR concentration 6 wells were counted in all experiments. The cellular sensitivity to DNR was determined by calculating the DNR concentration which was the point where the dose of DNR reduced the colony numbers or inhibited the growth to 50% of control cells (D_{50} value).

2.6. Cell cycle in leukemic blast cells

The DNA content of individual cell nuclei was determined using the cell cycle assay [33] as follows: 2 × 10⁶ blast cells were separated ($n = 29$), fixed with 70% (v/v) ethanol at −20 °C, suspended in PBS solution with 0.25 mg/ml of RNase (Sigma Chemical Co., St. Louis, MO), and incubated for 30 min at 37 °C. After incubation with propidium iodide (50 μg/ml, Sigma) for 30 min at 4 °C in the dark, fluorescence intensity was measured by an EPICS-XL system II flow cytometer (Beckman Coulter, Miami, FL). Cell cycle distribution was analyzed with the *MultiCycle for Windows (WinCycle)* software program (version 3.0; Phoenix Flow Systems, San Diego, CA).

2.7. Statistical analyses

The significant differences and correlations between the expression values of groups of untreated and relapsed acute leukemia patients were analyzed with the Wilcoxon signed-rank test and regression analysis.

3. Results

3.1. Expression of Topo IIα and Topo IIβ mRNA

The amplification curves with real-time quantitative RT-PCR and the standard curves, for Topo IIα and the internal control human β-actin, are shown in Fig. 1A and B. The amplification plots for Topo IIβ were also obtained (data not shown). The relative quantity Topo IIα mRNA expression level in every sample was measured under the high efficiency conditions of PCR amplification. We compared the individual values of quantitative RT-PCR obtained from 37 patients both at initial diagnosis and at relapse (Table 1). The level of Topo IIα mRNA expression (arbitrary units) at relapse was significantly higher than at diagnosis ($P = 0.016$). When AML and ALL samples were analyzed separately, the increased Topo IIα mRNA level was significant in the ALL samples ($P = 0.022$), but not in the AML samples. We also found a difference in Topo IIα mRNA levels between the group which achieved a second CR and the group which did

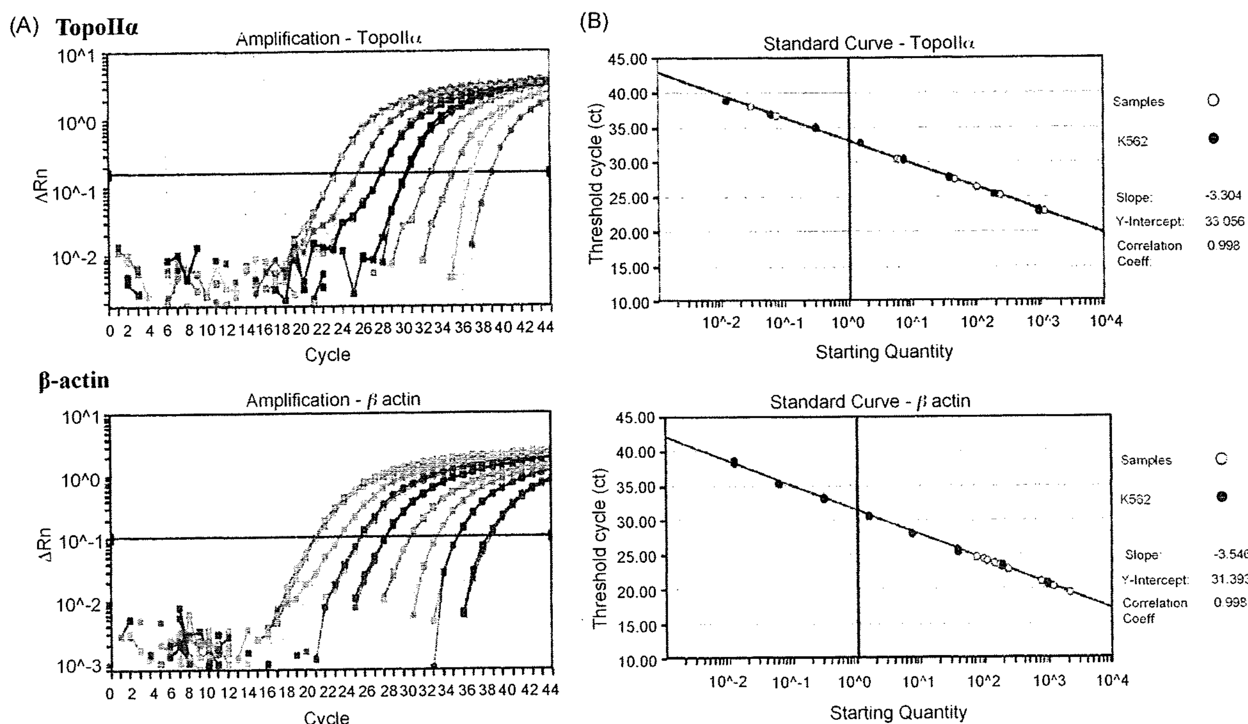

Fig. 1. Real-time quantitative RT-PCR amplification plots for Topo IIα and homozygous human β-actin. (A) The amplification curves from cell line K562 showing a dilution series of cDNA. (B) Representative standard curves for K562 (black cycle) and leukemic samples (cases no. 9–16, white cycle).

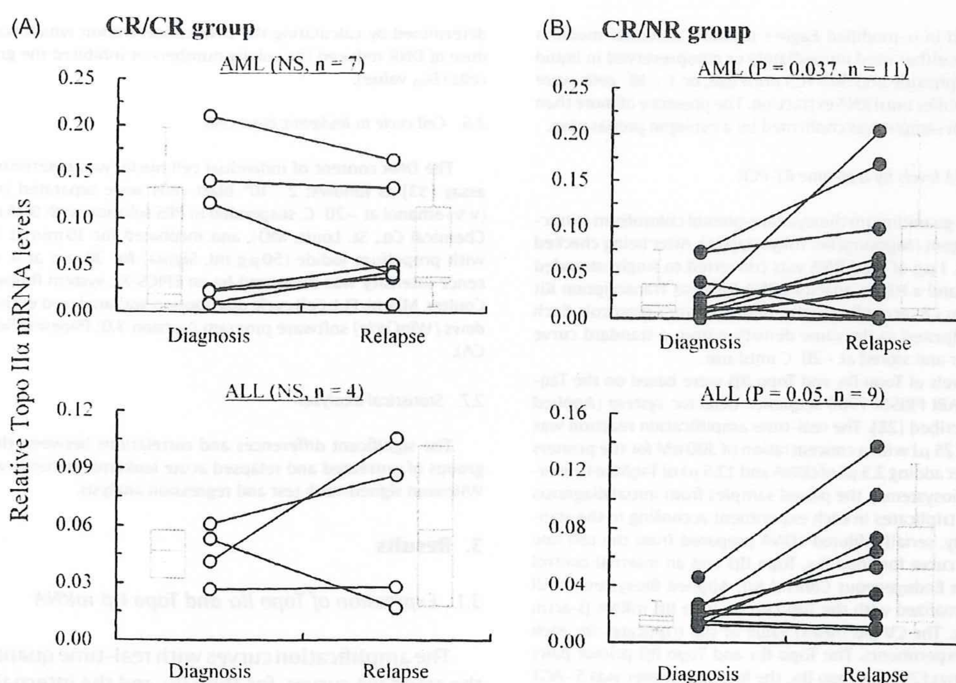

Fig. 2. Relative Topo IIα mRNA expression levels in leukemic blast cells at diagnosis and relapse. We divided the patients into two groups according to the outcome of treatment both in AML and ALL patients. (A) CR/CR group: patients who achieved CR after re-induction treatment. (B) CR/NR group: patients who did not achieve CR. (○) CR/CR patients; (●) CR/NR patients. The large boxes represent the 25th and 75th percentiles, and the lines inside the boxes represent the median. Upper and lower bars represent the 10th and 90th percentiles of the samples studied.

not. Fig. 2 shows the change of the Topo IIα mRNA levels in samples from patients who could achieve CR after relapse (CR/CR group) and from patients who could not (CR/NR group). When divided into two groups according to the clinical responsiveness, the increase in Topo IIα mRNA levels was recognized in the CR/NR group (AML group, $P=0.037$; ALL group, $P=0.050$; total, $P=0.003$) and not in the CR/CR group in AML and ALL patients respectively. The level of Topo IIβ mRNA expression was also measured in 34 cases; the median were 0.167 (10th and 90th percentiles, 0.05 and 0.441) at diagnosis and 0.175 (0.079 and 0.584) at relapse, and there was no significant difference of the expression level between the initial diagnosis and at relapse in the CR/CR or CR/NR group, in neither AML nor ALL patients (data not shown). There was no correlation between Topo IIα and Topo IIβ expression level.

3.2. Drug sensitivity

In determining the DNR sensitivity, enough cells were available for 8 out of 23 AML and 5 out of 14 ALL cases. As shown in Table 2, the change in the median D_{50} value for the AML samples increased significantly at relapse, from 1.38×10^{-7} to 3.25×10^{-7} mol, but not significantly for the ALL samples. The median D_{50} value for the whole CR/NR group increased from 2.0×10^{-7} mol (10th and 90th percentiles, 0.73×10^{-7} and 3.8×10^{-7} mol) at initial diagnosis to 3.0×10^{-7} mol (0.94×10^{-7} and 9.0×10^{-7} mol) at relapse ($P=0.049$, n = 9). There was no correlation between the level of Topo IIα mRNA or Topo IIβ mRNA and drug sensitivity.

Table 2
The D_{50} values of DNR at diagnosis and at relapse in AML and ALL patients.

Patients	Diagnosis ($\times10^{-7}$ mol) Median (range)	Relapse ($\times10^{-7}$ mol) Median (range)	P value
AML ($n=8$)	1.38 (0.61–5.25)	3.25 (1.24–8.08)	0.018
ALL ($n=5$)	3.00 (0.70–4.00)	7.00 (0.90–10.0)	0.079
Total ($n=13$)	1.80 (0.67–4.40)	3.50 (0.98–10.0)	0.006

Range: represents the 10th and 90th percentiles of the samples studied.

3.3. Cell cycle

No statistically significant difference was observed for the change of the ratio of S-phase cells at diagnosis and at relapse for the total of 29 paired samples. When analyzing the AML samples as a group there also was no statistically significant difference (Fig. 3A), but for the ALL samples the median percentage of S-phase cells increased significantly at relapse from 4.1% to 7.1% ($P=0.011$, $n=13$), as shown in Fig. 3B. When analyzed by the result of re-induction treatment we found a statistically significant increase for the whole CR/NR group ($P=0.024$, $n=17$), but this significant increase of S-phase cells was observed only for the ALL CR/NR samples ($P=0.015$, $n=9$) and not for the AML samples.

There was no correlation between the Topo IIα mRNA expression level and the median percentage of cell cycle S-phase cells both at presentation and at relapse ($n=29$). Also there was not correlation observed between the Topo IIβ mRNA expression level and the median percentage of cell cycle S-phase cells.

4. Discussion

In the present study we demonstrated that the expression levels of Topo IIα mRNA significantly increased in relapsed patients' samples compared to those at diagnosis using the real-time RT-PCR technique. Expression levels of Topo IIβ mRNA did not increase at relapse, and this is in agreement with previous reports [34,35]. There are few reports which examined the Topo IIα levels in paired samples from the same case taken at diagnosis and at relapse, though the importance of paired sample analysis was discussed by Valkov and Sullivan [2]. Gekeler et al. [36] reported that the Topo IIα mRNA expression was high at relapse in 18 paired samples cases with ALL, using a slot-blot or Northern-blot hybridization method. In another study, Kaufmann et al. [13] has shown that the expression of Topo IIα was rather consistent in paired AML samples by Western blot analysis, though their samples might include refractory cases. Galmarini et al. [15] also reported the Topo IIα level did

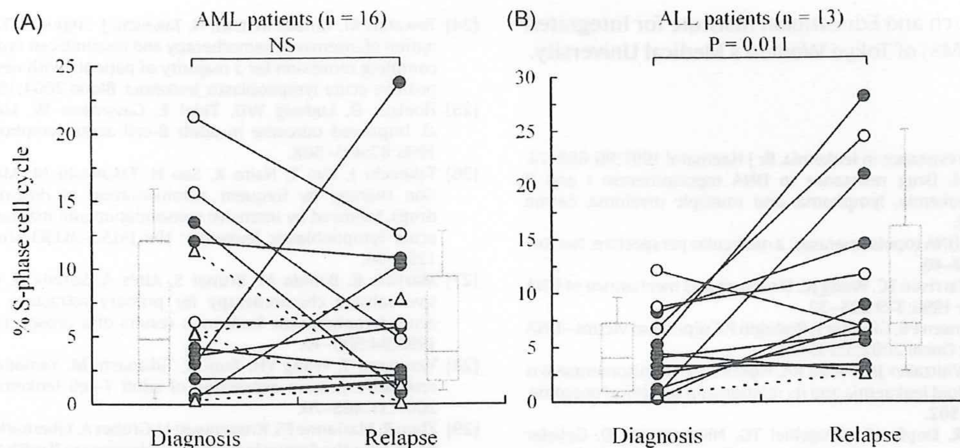

Fig. 3. Changes in the S-phase of the cell cycle of leukemic blast cells in AML and ALL patients at diagnosis and at relapse. (○) CR/CR patients; (●) CR/NR patients; (□) CR/NT patients (no treatment at relapse). The large boxes represent the 25th and 75th percentiles, and the lines inside the boxes represent the median. Upper and lower bars represent the 10th and 90th percentiles of the samples studied.

not change significantly in 31 paired AML samples using RT-PCR. Our results clarified that the increase of Topo IIα mRNA expression at relapse was a reflection of the significant increase in ALL, but not in AML, which is in agreement with previous reports [13,15,36].

There are a few contradicting reports on the Topo IIα level at diagnosis and clinical outcome [37,38], but there has been no report on a change in Topo IIα levels during the clinical course and the response to chemotherapy in paired samples of acute leukemic patients. Our results clearly showed the relationship between the leukemia responsiveness to re-induction treatment and the change of Topo IIα mRNA level. In the cases of failed re-induction therapy, the Topo IIα mRNA level was significantly increased at relapse. In the cases of successful re-induction therapy after relapse the Topo IIα level had not changed significantly. Less Topo IIα expression and Topo IIα activity associated with cellular drug resistance to Topo IIα inhibitors have been reported in some cell lines [39,40]. We also observed that the expression of Topo IIα in K562ADM, which is a drug-resistant subline of K562, was clearly lower than in K562 in our study with real-time RT-PCR (data not shown). There are several points that may be considered for the discrepancy between cell lines and clinical samples. First, it may be that high levels of Topo IIα repair DNA breakages induced by chemotherapeutic agents more easily, since Topo IIα is reported not only to cleave double-stranded DNA, but also to repair DNA breakages [1,41]. Galmarini et al. [15] also commented on this possibility in their report which described the correlation between the poorer clinical outcome of acute leukemia and higher Topo IIα expression. DNA repair is also known to be one of the mechanisms in drug resistance. Second, it is possible that Topo IIα is likely to reflect the aggressiveness of leukemia, which shows a wide variation between individuals, but helps to understand the time course of the disease. Topo IIα is reported to be a reliable indicator of tumor aggressiveness in patients with soft tissue scarcoma and breast carcinoma [42,43]. Third, the drug resistance is different by orders of magnitude between cell lines and clinical samples [30,31], and the differences in the proliferation rates and the level of Topo IIα expression between these are distinct features. The difference we detected in clinical samples looks small compared to the changes reported with cell lines. Due to the reasons above, the present results with clinical samples might not be concordant with the results with cell lines.

Topo IIα is known to be related to the cell cycle in ALL, and tumor aggressiveness is partly reflected in the S-phase cell population [36,44]. The increased Topo IIα level at relapse may indicate that the leukemia became more aggressive at relapse compared to at diagnosis, and it might predict the poorer outcome. On the other hand, Uggla et al. [45] have reported that the expression of Topo IIα is more connected to the G0/G1 phase than the S-phase using 22 AML cases out of 25 leukemia patients. Although the S-phase cell population at relapse significantly increased in ALL blast cells, which is consistent with previous reports [36], we did not recognize a correlation with Topo IIα level and cell cycle. The present study revealed contrasting differences between AML and ALL; in the AML cases at relapse Topo IIα mRNA levels and the cell cycle remained stable, whereas in the ALL cases Topo IIα mRNA levels and the S-phase population increased at relapse.

In this study, the drug sensitivity of AML at relapse is significantly lower than at the diagnosis. In ALL, the drug sensitivity at relapse is lower than that at diagnosis, with marginal significance ($P = 0.079$), which may be due to the small number of ALL samples. Our previous report showed a significant decrease of sensitivity both in AML and ALL [46]. Klumper et al. [14] also reported that the drug sensitivity of DNR became low at relapse in childhood ALL. There is no report about the relationship of the Topo IIα mRNA level with real-time RT-PCR and drug sensitivity in paired samples according to our knowledge. We could not find a correlation between the degree of Topo IIα mRNA expression and DNR sensitivity of leukemic cells at diagnosis nor at relapse in the samples that we examined. However, we found the expression of Topo IIα at relapse increased in 7 of 13 samples in which the DNR sensitivity (D_{50}) at relapse became lower than at diagnosis. One factor may be the small number of cases examined for drug sensitivity. Kaufmann et al. [13] also reported that the Topo IIα level in Western blotting did not correspond with drug resistance for 13 paired samples. As their results and ours both dealt with a small number of samples, it may be difficult to draw a conclusion on the correlation between the Topo IIα mRNA expression at relapse and drug sensitivity.

In conclusion, the leukemia cases with increased Topo IIα mRNA levels at relapse tend to fail to respond to re-induction treatment. A change in the Topo IIα mRNA level in the clinical course could predict the responsiveness to treatment. Topo IIα may be one of many mechanisms which participate in MDR at relapse in acute leukemia, but larger studies will be required to assess the role of Topo IIα in relation to MDR in paired leukemia samples.

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ORIGINAL ARTICLE

Phase 1/2 clinical study of dasatinib in Japanese patients with chronic myeloid leukemia or Philadelphia chromosome-positive acute lymphoblastic leukemia

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Abstract A phase 1/2 study was conducted to assess the safety and efficacy of dasatinib in Japanese patients with chronic myelogenous leukemia (CML) or Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph^+ ALL) resistant or intolerant to imatinib. In phase 1, 18 patients with chronic phase (CP) CML were treated with dasatinib 50, 70, or 90 mg twice daily to evaluate safety. Dasatinib $\leq$ 90 mg twice daily was well tolerated. In phase 2, dasatinib 70 mg was given twice daily to CP-CML patients for 24 weeks and to CML patients in accelerated phase (AP)/blast crisis (BC) or Ph^+ ALL for 12 weeks. In the CP-CML group ($n = 30$) complete hematologic response was 90% and major cytogenetic response (MCyR) 53%. In the AP/BC-CML group ($n = 11$) major hematologic response (MaHR) was 64% and MCyR 27%, whereas in the Ph^+ ALL group ($n = 13$) MaHR was 38% and MCyR 54%. Dasatinib was well tolerated and most of the nonhematologic toxicities were mild or moderate. Dasatinib therapy resulted in high rates of hematologic and cytogenetic response, suggesting that dasatinib is promising as a

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new treatment for Japanese CML and Ph+ ALL patients resistant or intolerant to imatinib.

Keywords CML · Ph+ ALL · Dasatinib · Imatinib resistant · Imatinib intolerant

1 Introduction

Chronic myeloid leukemia (CML) is a disease attributable to abnormalities of hematopoietic stem cells involving uncontrolled proliferation of cells originating from the bone marrow. The Philadelphia (Ph) chromosome is formed by translocation between chromosomes 9 and 22. The *BCR-ABL* fusion gene on this chromosome produces BCR-ABL, which constitutively activates ABL tyrosine kinase and is thus responsible for CML and 20–30% of adult patients with acute lymphoblastic leukemia (ALL) [1]. Imatinib (Glivec®) is a selective BCR-ABL inhibitor effective against CML and Ph-positive (Ph+) ALL. Currently, imatinib is the only tyrosine kinase inhibitor indicated in newly diagnosed CML and Ph+ ALL [2–4]. However, resistance to imatinib gradually develops in many patients with CML and Ph+ ALL, particularly those with advanced disease. Among CML patients treated with imatinib, 31% discontinue the drug within 5 years because of insufficient responses or unacceptable toxicity [5]. As a major factor responsible for development of resistance to imatinib, numerous point mutations in BCR-ABL have been reported [6–8]. Additional factors including *BCR-ABL* gene amplification [6, 9], excretion of the drug through a P-glycoprotein efflux pump [10, 11], and activation of the signal transduction pathway for SRC family kinase and other signals [12, 13] have also been implicated. Therefore the development of new treatments is desirable for patients with insufficient response to imatinib and in whom imatinib cannot be continued at effective doses due to toxicity.

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Dasatinib (BMS-354825) is a novel oral tyrosine kinase inhibitor that exerts inhibitory activity against BCR-ABL and SRC family kinase. In vitro, dasatinib binds to both active and inactive BCR-ABL and is 325 times more potent than imatinib and 16 times more potent than nilotinib against wild-type BCR-ABL-expressing cells [14]. Dasatinib has demonstrated activity against all reported types of imatinib-resistant mutant BCR-ABL, except for T315I [14–18]. Five phase 2 studies collectively known as START (SRC/ABL Tyrosine kinase inhibition Activity Research Trials of dasatinib) studies demonstrated that dasatinib is safe and elicits hematologic and cytogenetic response at all stages of CML and Ph+ ALL resistant or intolerant to imatinib [18–22]. Against chronic phase (CP)-CML, dasatinib was highly effective with 91% of patients showing complete hematologic responses (CHR) and 62% major cytogenetic responses (MCyR). Efficacy for CP-CML was durable and duration of MCyR was 88%, progression-free survival was 80% and overall survival was 94% at 2-year follow-up [23]. Dasatinib (Sprycel®) was initially approved in the United States in June 2006 and has received marketing approvals in numerous other countries world-wide.

We conducted an open-label phase 1/2 study of dasatinib in Japanese patients with CP-CML, accelerated phase (AP)/blast crisis (BC)-CML or Ph+ ALL resistant or intolerant to imatinib. This study comprised two parts. Phase 1 evaluated the safety of dasatinib at escalating doses in patients with CP-CML. Phase 2 evaluated the efficacy and safety of dasatinib in patients with all-stage CML or Ph+ ALL.

2 Methods

2.1 Patients

Adult CML or Ph+ ALL patients aged 20–75 years who were resistant or intolerant to imatinib were conducted from 2005 to 2007. Because imatinib had no registered indication for Ph+ ALL in Japan at the start of this study, patients with Ph+ ALL resistant to or intolerant of prior therapies were eligible. Treatment and analysis were conducted in three cohorts with CP-CML, AP/BC-CML and Ph+ ALL (Table 1).

CP-CML was considered to be resistant to imatinib when given at a dose level $\geq$ 400 mg/day if the following occurred: (1) white blood cell count (WBC) showed a $\geq$2-fold increase from nadir to >20000/mm^3 or rose from nadir to $\geq$50000/mm^3; (2) CHR was not achieved despite $\geq$3-month treatment with imatinib; (3) cytogenetic response was not achieved despite $\geq$6-month treatment with imatinib; (4) MCyR was not achieved despite $\geq$12-month

Table 1 Definition of CML phases

Phase	Description
	Patients satisfying all the following requirements:
CP	• Percentage of blasts in peripheral blood and bone marrow <15%
	• Percentage of basophils in peripheral blood or bone marrow <20%
	• Total percentage blasts and promyelocytes in peripheral blood and bone marrow <30%
	• Platelet count ≥100,000/mm^3 (rated at chronic stage if thrombocytopenia due to prior therapy is present)
	• Extramedullary leukemia absent
AP	• Nonacute patients satisfying ≥1 of the following requirements:
	• Percentage blasts in peripheral blood or bone marrow ≥15 and <30%
	• Percentage basophils in peripheral blood or bone marrow ≥20%
	• Total percentage blasts and promyelocytes in peripheral blood or bone marrow ≥30% and percentage blasts <30%
	• Platelet count <100,000/mm^3 (not associated with treatment)
BC	• Patients satisfying ≥1 of the following requirements:
	• Percentage blasts in peripheral blood or bone marrow ≥30%
	• Extramedullary leukemia, excluding that affecting liver or spleen

treatment with imatinib; (5) relapse after MCyR or CHR; or (6) mutation in *ABL* gene suggestive of resistance to imatinib (L248V, G250E, Q252H/R, Y253H/F, E255K/V, T315I/D, F317L or H369P/R) was noted in patients of chronic CML. AP-CML was considered as resistant to imatinib if the following occurred in patients treated with imatinib at a dose level ≥600 mg/day, or ≥400 mg/day if the initial diagnosis was CP-CML intolerant to imatinib: (1) progressed to BC; (2) hematologic response was not achieved in ≤4 weeks; or (3) progressed to AP after hematologic response. BC-CML was considered as resistant to imatinib if the following patients occurred: (1) the condition progressed into BC after hematologic response; or (2) the condition remained BC-CML despite ≥4-week treatment. Ph$^+$ ALL was considered as resistant to prior therapies if the following occurred: (1) CHR was not achieved at least 2 weeks after the start of treatment; or (2) progressed from CHR.

Patients with CP-CML were assessed as intolerant to imatinib if grade ≥3 nonhematologic toxicity was observed or grade 4 hematologic toxicity persisted ≥7 days. Patients with AP/BC-CML were considered intolerant to imatinib if treatment had to be discontinued or the dosage kept <400 mg/day for reasons of toxicity. Ph$^+$ ALL patients were considered intolerant to prior therapy if grade ≥3 nonhematologic toxicity was noted, grade 4 hematologic toxicity persisted ≥7 days, or existing therapy could not be given for other reasons. This study was carried out in accordance with the principles of the Declaration of Helsinki, ICH-GCP, and requirements set forth by Japanese Good Clinical Practice. Prior to the study, written informed consent was obtained from each subject. The study was approved by the Institutional Review Board at each participating institution. The study was designed by academic investigators in conjunction with representatives from the sponsor, Bristol-Myers K.K. Both parties contributed to the collection and analysis of the data. This study was registered at http://www.clinicaltrials.gov as NCT00227454.

2.2 Two-part study design: phases 1 and 2

Phase 1 was designed as a dose-escalation study in patients with CP-CML, evaluating the safety of dasatinib. Phase 2 was designed as a fixed-dose study in patients with CP or AP/BC-CML resistant or intolerant to imatinib and Ph$^+$ ALL resistant or intolerant to prior therapies, evaluating the efficacy and safety of dasatinib. In this phase, the primary endpoint was cytogenetic response in patients with CP-CML and hematologic response in those with AP/BC-CML and Ph$^+$ ALL.

2.3 Dasatinib treatment

During phase 1, dasatinib was orally administered twice daily at 50, 70, or 90 mg/dose for 24 weeks. Dose-limiting toxicity (DLT) defined as grade ≥3 nonhematologic toxicity, grade 3–4 QTc interval prolongation, grade 4 neutropenia lasting ≥7 days, grade 4 thrombocytopenia, bleeding requiring platelet transfusion, and other toxicity requiring discontinuation of the drug was evaluated during the first 4 weeks of treatment.

Phase 2 was started after the safety of 70 mg twice daily was confirmed. During phase 2, dasatinib was orally administered at 70 mg twice daily for 24 weeks in the CP-CML group and for 12 weeks in the AP/BC-CML and Ph$^+$ ALL groups. Upon completion of the observation period, an extension study involving continued treatment was planned.

The dose level of dasatinib was reduced if the following occurred: (1) grade ≥2 nonhematologic toxicity (grade ≥3 nonhematologic toxicity in patients of CP-CML); or (2) grade 4 neutropenia in patients of AP/BC-CML and Ph$^+$ ALL when bone marrow cell density and percentage of blasts were checked ≥15 days after the start of treatment. The dose level of dasatinib for CP-CML patients was increased if: (1) progression of disease (PD) was noted; (2)

Table 2 Criteria for efficacy evaluation

Hematologic response[a]	
(1) CP-CML	
CHR	
• WBC count less than or equal to institutional upper limit of normal	
• Platelet count <450,000/mm^3	
• Absence of blasts or promyelocytes in peripheral blood	
• Total percentage myelocytes and metamyelocytes in peripheral blood <5%	
• Percentage basophils in peripheral blood <20%	
• Absence of extramedullary leukemia (including hepatomegaly and splenomegaly)	
(2) AP/BC-CML and Ph+ ALL	
Major HR	
(a) CHR	
• WBC count less than or equal to institutional upper limit of normal	
• Neutrophil count ≥1000/mm^3	
• Platelet count ≥100,000/mm^3	
• Absence of blasts/promyelocytes in peripheral blood	
• Percentage of blasts in bone marrow <5%	
• Total percentage myelocytes and metamyelocytes in peripheral blood <5%	
• Percentage basophils in peripheral blood <20%	
• Absence of extramedullary leukemia (including hepatomegaly and splenomegaly)	
(b) NEL	
• WBC count less than or equal to institutional upper limit of normal	
• Absence of blasts or promyelocytes in peripheral blood	
• Percentage blasts in bone marrow <5%	
• Total percentage myelocytes and metamyelocytes in peripheral blood <5%	
• Percentage basophils in peripheral blood <20%	
• Absence of extramedullary leukemia (including hepatomegaly and splenomegaly)	
• Platelet count ≥20,000/mm^3 and <100,000/mm^3 and/or neutrophil count ≥500/mm^3 and <1000/mm^3	
Minor HR	
• Percentage blasts in bone marrow/peripheral blood <15%	
• Total percentage blasts/promyelocytes in peripheral blood <30%	
• Percentage basophils in peripheral blood <20%	
• Absence of extramedullary leukemia other than in spleen and liver	
Cytogenetic response	
Percentage Ph+ cells in bone marrow	
MCyR	
(a) CCyR	0%
(b) PCyR	>0 and ≤ 35%
Minor CyR	>35 and ≤65%
Minimal CyR	>65 and ≤95%
No response	>95 and ≤100%

CHR Complete hematologic response, *NEL* no evidence of leukemia, *MCyR* major cytogenetic response, *CCyR* complete cytogenetic response, *PCyR* partial cytogenetic response

[a] Hematologic response is confirmed if the remitted state lasts ≥4 weeks

CHR was not achieved despite ≥8 weeks of treatment; and (3) MCyR was not achieved despite ≥12 weeks of treatment. For AP/BC-CML and Ph+ ALL patients, the dose level of dasatinib was increased if: (1) PD was noted; (2) the percentage of blasts in peripheral blood showed an increase from that recorded ≥1 week previously; and (3) CHR was not achieved despite ≥4-week treatment. During the study period, concomitant use of anticancer drugs other

than dasatinib was prohibited in both CML and Ph^+ ALL patients, except for short term (≤14 days) use of hydroxycarbamide in patients in whom WBC was >50000/mm^3.

2.4 Patient evaluation

Evaluation of peripheral blood findings was performed every week during the first 4 weeks in phase 1, every other week during the first 4 weeks in phase 2, and every 4 weeks thereafter. Evaluation of bone marrow findings was made at the end of the study. Table 2 shows the criteria for efficacy evaluation. Cytogenetic response was evaluated in bone marrow by G-band test and in bone marrow and peripheral blood samples by fluorescence in situ hybridization (FISH) for *BCR-ABL* at baseline and at week 12 in AP/BC-CML and Ph^+ ALL patients and at week 24 in those with CP-CML. *BCR-ABL* point mutation was assessed by direct sequencing of PCR products of peripheral blood cells before the start of treatment. Adverse events were graded according to NCI Common Terminology Criteria for Adverse Events (CTCAE) Version 3.0.

3 Results

3.1 Patient demographics and dasatinib treatment

A total of 55 patients were registered for this trial, of whom dasatinib was administered to 54 (18 and 36 patients during phases 1 and 2, respectively). Median age was 43 (range 27–66) and 60 (29–73) years in patients entered in phases 1 and 2, respectively. Of the 54 patients, 35 were males and 19 females. Thirty-five patients were resistant to imatinib at daily dose of 400 mg or more, and 19 patients were intolerant to imatinib. Table 3 shows patient characteristics. Phase 1 involved 18 patients of CP-CML (12 resistant/6 intolerant); phase 2 involved 12 patients of CP-CML (6 resistant/6 intolerant), 11 AP/BC-CML (8 resistant/3 intolerant), and 13 Ph^+ ALL (9 resistant/4 intolerant). Major causes for intolerance to imatinib were rash ($n = 6$), myalgia and vomiting ($n = 3$ each), and hepatic dysfunction ($n = 2$). Although prior treatment with imatinib was not a requirement for enrollment in the Ph^+ ALL group, all patients enrolled had a history of imatinib therapy and were either resistant or intolerant to imatinib.

The duration of prior imatinib therapy was 1–3 years in 19 patients (35%), and >3 years in 19 patients (35%). The dosage of imatinib during prior therapy was ≥400 mg/day in all patients. Forty-three patients (80%) had previously received therapy other than imatinib, seven patients (13%) had undergone hematopoietic stem cell transplantation.

In phase 1, dose reduction was performed for 3 of 7 patients in the 70 mg group and 3 of 4 patients from the 90 mg group because of hematologic toxicity in 5 patients and nonhematologic toxicity in one patient. In phase 2, dose reduction was performed for 10 of 12 patients in the CP-CML group, 3 of 11 patients in the AP/BC-CML group, and 5 of 13 patients in the Ph^+ ALL group because of hematologic toxicity in 10 patients and nonhematologic toxicity in 8 patients. Dose increase was performed in one

Table 3 Patients' baseline characteristics

	CP-CML, phase 1 ($n = 18$)	CP-CML, phase 2 ($n = 12$)	AP/BC-CML ($n = 11$)	Ph^+ ALL ($n = 13$)
Median age, range (years)	43 (27–66)	60 (30–68)	57 (31–73)	64 (29–70)
Median time after diagnosis, range (years)	6.9 (0.3–19)	3.6 (0.7–15)	1.6 (0.0–14)	1.1 (0.2–6.3)
Imatinib resistant, *n* (%)	12 (67)	6 (50)	8 (73)	9 (69)
Imatinib intolerant, *n* (%)	6 (33)	6 (50)	3 (27)	4 (31)
Length of prior imatinib therapy, *n* (%)				
<1 years	3 (17)	4 (33)	2 (18)	7 (54)
1–3 years	4 (22)	3 (25)	6 (55)	6 (46)
>3 years	11 (61)	5 (42)	3 (27)	0
Prior imatinib dosage, *n* (%)				
400–600 mg/day	16 (89)	11 (92)	5 (45)	13 (100)
>600 mg/day	2 (11)	1 (8)	6 (55)	0
Prior chemotherapy, *n* (%)	12 (67)	9 (75)	9 (82)	13 (100)
Prior IFN therapy, *n* (%)	9 (50)	6 (50)	3 (27)	0
Prior HSCT, *n* (%)	0	1 (8)	3 (27)	3 (27)
BCR-ABL mutation, *n* (%)	4 (22)	1 (8)	2 (18)	4 (31)

IFN Interferon, *HSCT* hematopoietic stem cell transplantation

patient with Ph^+ ALL because of insufficient response. The median treatment period was 24 weeks in phase 1 and 24, 12, and 11 weeks in the CP-CML, AP/BC-CML, and Ph^+ ALL groups, respectively, in phase 2. Median dose was 96.20 (range 46.5–179.5) mg/day in phase 1 and 99.05 (44.7–141.8) mg/day in phase 2.

Forty-four patients completed the trial (17 in phase 1 and 27 in phase 2). One patient in phase 1 and 9 patients (2 patients of AP/BC-CML and 7 of Ph^+ ALL) in phase 2 discontinued study treatment prematurely, because of insufficient response in 6 patients and adverse events in 4 patients.

3.2 DLT evaluation: phase 1

In phase 1, DLT was evaluated in 15 patients (6 each in the 50 and 70 mg groups and 3 in the 90 mg group). One patient in the 50 mg group was not evaluated who was diagnosed as AP-CML after registration, one in the 70 mg group had violated the protocol, and one in the 90 mg group reduced dosage. One patient in each of the 50 and 70 mg groups developed grade 4 thrombocytopenia as DLT, whereas no patient in the 90 mg group developed DLT. Two patients in the 50 mg group exhibited grade 3 elevation of ALT, but this change was not deemed DLT since it was transient and subsided without requiring treatment. There was no dose level at which DLT appeared in ≥2 patients; thus dasatinib was well tolerated at dose levels ≤90 mg twice daily.

Following this finding, dasatinib 70 mg twice daily, which was previously demonstrated safe and effective in an overseas phase 1 and 2 studies, was adopted as the regimen for the second phase of this study.

3.3 Efficacy: phases 1 and 2

3.3.1 CP-CML

Table 4 shows the efficacy results for 30 patients with CP-CML in phase 1 ($n = 18$) and 2 ($n = 12$). A high response rate was achieved, with 90% of CP-CML patients achieving a CHR (83% in imatinib-resistant and 100% -intolerant). CHR was achieved rapidly and median time to CHR was 10 days. Fifty-three percent of CP-CML patients exhibited a MCyR following dasatinib therapy. The rate of CCyR was 43%. MCyR was achieved in 33% of imatinib-resistant and 83% of -intolerant patients. In phase 1, CHR, MCyR and CCyR were 89, 50 and 44%, respectively. In phase 2, CHR, MCyR, and CCyR were 92, 58 and 42% respectively. Dasatinib therapy was not discontinued in any CP-CML patient due to insufficient response.

3.3.2 AP/BC-CML

MaHR was achieved in a high percentage (64%) of AP/BC-CML patients (63% imatinib-resistant, 67% -intolerant). Median time to MaHR was 34 days. MCyR was achieved in 27% of AP/BC-CML patients, whereas CCyR was observed in 9%. MCyR was achieved in 38% of imatinib-resistant and 0% -intolerant patients. Dasatinib therapy was not discontinued in any AP/BC-CML patient due to insufficient response.

3.3.3 Ph^+ ALL

MaHR was achieved in 38% of Ph^+ ALL patients (33% imatinib-resistant, 50% -intolerant). Median time to

Table 4 Treatment response

	CP-CML			AP/BC-CML			Ph^+ ALL		
	Imatinib resistant $n = 18$	Imatinib intolerant $n = 12$	Total $n = 30$	Imatinib resistant $n = 8$	Imatinib intolerant $n = 3$	Total $n = 11$	Imatinib resistant $n = 9$	Imatinib intolerant $n = 4$	Total $n = 13$
Hematologic response, *n* (%)									
Major	–	–	–	5 (63)	2 (67)	7 (64)	3 (33)	2 (50)	5 (38)
Complete	15 (83)	12 (100)	27 (90)	2 (25)	0	2 (18)	0	1 (25)	1 (8)
NEL	–	–	–	3 (38)	2 (67)	5 (45)	3 (33)	1 (25)	4 (31)
Minor	–	–	–	1 (13)	0	1 (9)	2 (22)	2 (50)	4 (31)
Cytogenetic response, *n* (%)									
Major	6 (33)	10 (83)	16 (53)	3 (38)	0	3 (27)	3 (33)	4 (100)	7 (54)
Complete	5 (28)	8 (67)	13 (43)	1 (13)	0	1 (9)	2 (22)	4 (100)	6 (46)
Partial	1 (6)	2 (17)	3 (10)	2 (25)	0	2 (18)	1 (11)	0	1 (8)
Minor	3 (17)	1 (8)	4 (13)	2 (25)	0	2 (18)	0	0	0
Minimal	3 (17)	1 (8)	4 (13)	1 (13)	1 (33)	2 (18)	0	0	0

CHR + NEL = Major hematologic response, CCyR + PCyR = major cytogenetic response, NEL = no evidence of leukemia

MaHR was 57 days. CCyR was achieved in 46% of Ph+ ALL patients. MCyR was seen in 33% of imatinib-resistant and 100% -intolerant patients. Dasatinib treatment was discontinued because of insufficient response in 6 patients.

3.3.4 *Efficacy by baseline BCR-ABL mutation status*

Of the 54 subjects, 11 (20%; 5 CP-CML; 2 AP/BC-CML; 4 Ph+ ALL) showed 8 different *BCR-ABL* point mutations (L248V, G250E, Y253H, E255K, F311I, T315I, E355A, and H396R) at baseline. All these 11 patients were resistant to imatinib (Table 3). Seven patients (64%) had mutation of kinase domain P-loop (amino acids 244–255) and one that of T315I, which are highly resistant mutations to imatinib. Nonetheless, even in patients with various BCR-ABL point mutations, dasatinib conferred a MaHR in 5 (45%; 3 CP-CML; 1 AP/BC-CML; 1 Ph+ ALL) of 11 patients and MCyR in 4 patients (36%; 2 CP-CML; 1 AP/BC-CML; 1 Ph+ ALL), comparable to the MaHR and MCyR rates for patients without *BCR-ABL* mutation. Six patients had no hematologic or cytogenetic response; 2 patients early discontinued dasatinib due to adverse events, 1 patient had T315I mutation at baseline and 2 patients had additionally emerging T315I mutation during dasatinib treatment period.

3.4 Safety

Overall, dasatinib was well tolerated. Most of the nonhematologic adverse events were mild or moderate and required no intervention or disappeared following dose interruption or reduction of dasatinib. Frequently observed adverse events possibly related to dasatinib were headache (41%), fever (33%), diarrhea (33%), rash (31%), edema (31%), and malaise (30%) (Table 5). Pleural effusion was seen in 14 patients (26%), but was mostly mild or moderate except for one patient with grade ≥3. In all patients, the adverse events recovered to a level that allowed resumption of study treatment upon administration of diuretics or dose interruption/reduction of dasatinib. Hematologic toxicity was observed in a high percentage of patients, as expected, but was often reversible and subsided following dose interruption or reduction. Grade ≥3 thrombocytopenia was seen in 50% of CP-CML, 64% of AP/BC-CML, and 62% of Ph+ ALL patients. Neutropenia was observed in 47, 73, and 77%, respectively (Table 6). The incidence of grade >3 anemia was highest in Ph+ ALL patients.

Treatment was discontinued in 4 (7%) of the 54 patients because of adverse events; pneumonia in 2 patients, neutropenia in 1 patient and arrhythmia and heart failure in 1 patient.

Table 5 Cumulative possibly dasatinib related adverse events in the total treated population ($n = 54$) at 24 weeks (CP-CML) or 12 weeks (AP/BC-CML, Ph+ ALL) of follow-up

Adverse event	Cumulative incidence rate, *n* (%)	
	All grade	Grades 3–4
Headache	22 (41)	0
Fever	18 (33)	0
Diarrhea	18 (33)	1 (2)
Rash	17 (31)	1 (2)
Edema	17 (31)	0
Malaise	16 (30)	0
Pleural effusion	14 (26)	1 (2)
Weight gain	14 (26)	0
Nausea	11 (20)	0
Constipation	11 (20)	0
Anorexia	10 (19)	0
Cough	10 (19)	0
Stomatitis	7 (13)	0
Weight loss	7 (13)	0
Pain in extremity	6 (11)	1 (2)
Vomiting	6 (11)	0
Arthralgia	6 (11)	0

4 Discussion

This two-part study was designed to evaluate the safety of escalating doses of dasatinib in Japanese patients with CP-CML (phase 1) and its safety and efficacy in patients with CP-CML, AP/BC-CML, and Ph+ ALL (phase 2).

Although the results shown in this paper cover relatively short treatment periods of 6 and 3 months in CP-CML and AP/BC-CML or Ph+ ALL, respectively, dasatinib demonstrated clinical efficacy in Japanese patients in all stages of CML and Ph+ ALL resistant or intolerant to imatinib. Among patients with CP-CML, more than half achieved MCyR and most retained their cytogenetic response throughout the study period. These observations are clinically significant in view of reports that long-term prognosis may be improved in patients with CP-CML achieving MCyR [24, 25]. Also, in patients with AP/BC-CML and Ph+ ALL, dasatinib monotherapy resulted in rapid achievement of a high rate of MaHR (64 and 38%, respectively) and the percentage of patients showing hematologic response among imatinib-resistant patients was comparable to that of imatinib-intolerant patients. The rate of cytogenetic response seemed to be higher in imatinib-intolerant patients than in imatinib-resistant patients in this study. Most patients enrolled in this study had a history of long-term imatinib therapy and of many other therapies such as interferon and chemotherapy, and were therefore expected to have a poor prognosis.

Table 6 Hematologic adverse events grade 3–4

	Cumulative incidence rate, *n* (%)		
	CP-CML ($n = 30$)	AP/BC-CML ($n = 11$)	Ph$^+$ ALL ($n = 13$)
Leukopenia	8 (27)	5 (45)	10 (77)
Neutropenia	14 (47)	8 (73)	10 (77)
Thrombocytopenia	15 (50)	7 (64)	8 (62)
Anemia	5 (17)	2 (18)	4 (31)

However, these patients without effective treatment options showed favorable responses to dasatinib. The observation period was short in this study to be able to fully assess the efficacy of dasatinib in CML and Ph$^+$ ALL patients and it would be expected that the response rate would be higher than the result in the present study.

At baseline, 20% of the subjects had *BCR-ABL* point mutations reported associated with resistance to imatinib [26]. Moreover, 64% of mutations observed were P-loop mutations, which are associated with high resistance to imatinib. Even these highly resistant patients achieved hematologic and cytogenetic responses. It is known that mutations associated with imatinib resistance reduce the potential of imatinib to bind to the ATP-binding site of BCR-ABL. Since the mode of binding by dasatinib differs from that by imatinib, dasatinib retains its activity even in the presence of mutation associated with imatinib resistance.

Although 35 (65%) of the 54 subjects in the present study were resistant to imatinib, mutation associated with imatinib resistance was seen in only 31% of the 35 imatinib-resistant subjects. This finding suggests that resistance to imatinib involves not only BCR-ABL point mutation but also other mechanisms. Since dasatinib exerted clinical efficacy even in patients without *BCR-ABL* point mutation, treatment with dasatinib is expected to overcome resistance to imatinib attributable not only to BCR-ABL mutation but also to other mechanisms.

In phase 1 of this study, dasatinib was shown to be safe in patients with chronic CML with dose escalations up to 90 mg twice daily. The only DLT observed in this study was grade 4 thrombocytopenia in 2 patients. Cytopenia is common adverse events in leukemia patients who have long-term and intensive prior therapy. Although cytopenia following dasatinib treatment could be controlled by dose interruption or reduction, close monitoring of blood cell counts is advisable during use of this drug.

Treatment had to be discontinued in 4 (7%) of the 54 patients because of adverse events. These results indicate that dasatinib is safe in patients with all phases of CML and Ph$^+$ ALL resistant or intolerant to imatinib. Pleural effusion was noted in 14 (26%) patients, but the incidence of edema (a frequent toxicity of imatinib) was low in the present study. Grade $\geq$3 pleural effusion was seen in only one patient, and treatment did not have to be discontinued. The mechanism by which dasatinib induces pleural effusion is likely related to off-target kinase inhibition, platelet-derived growth factor receptor beta (PDGFRβ) in particular [27]. Pleural effusion was successfully treated by interruption of dasatinib and was reversible. There was low incidence of rash, muscle cramp, and nausea, which are frequent toxicities associated with imatinib. There was no apparent difference in the safety profile of dasatinib among Japanese and non-Japanese CML and Ph$^+$ ALL patients [18–22]. It was rare that patients who had been intolerant to imatinib experienced the same severe nonhematologic toxicity following treatment with dasatinib. Therefore it is possible to treat imatinib-intolerant patients safely with dasatinib.

It has been reported that most Japanese CML patients are treated with lower dosages of imatinib than the standard recommended dosage, because of toxicities [28–31]. Imatinib treatment at low dosage is related with low rate of cytogenetic response [30]. Dasatinib is a meaningful option for those patients intolerant to the standard dosage of imatinib.

In the overseas phase 3 study designed to determine the optimal dose level and dosing method of dasatinib in patients with CP-CML [32], the efficacy of dasatinib 100 mg once daily in terms of hematologic response and cytogenetic response was comparable to that of 70 mg twice daily while the incidence of adverse events was lower. Dasatinib 100 mg once daily is currently being evaluated in Japanese patients with CP-CML. A multinational study (including Japan) is underway to access the efficacy and safety of dasatinib in newly diagnosed CML patients. In the past, only limited options were available for the treatment of imatinib-resistant or -intolerant CML and Ph$^+$ ALL and patients often had a poor prognosis. The results of the present study indicate that dasatinib is promising as a new treatment for Japanese CML and Ph$^+$ ALL patients resistant or intolerant to imatinib.

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Hidden abnormalities and novel classification of t(15;17) acute promyelocytic leukemia (APL) based on genomic alterations

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Acute promyelocytic leukemia (APL) is a hematopoietic malignant disease characterized by the chromosomal translocation t(15;17), resulting in the formation of the *PML-RARA* gene. Here, 47 t(15;17) APL samples were analyzed with high-density single-nucleotide polymorphism microarray (50-K and 250-K SNP-chips) using the new algorithm AsCNAR (allele-specific copy-number analysis using anonymous references). Copy-number-neutral loss of heterozygosity (CNN-LOH) was identified at chromosomes 10q (3 cases), 11p (3 cases), and 19q (1 case). Twenty-eight samples (60%) did not have an obvious alteration (normal-copy-number [NC] group). Nineteen samples (40%) showed either one or more genomic abnormalities: 8 samples (17%) had trisomy 8 either with or without an additional duplication, deletion, or CNN-LOH (+8 group); and 11 samples (23%) had genomic abnormalities without trisomy 8 (other abnormalities group). These chromosomal abnormalities were acquired somatic mutations. Interestingly, *FLT3*-ITD mutations (11/47 cases) occurred only in the group with no genomic alteration (NC group). Taken together, these results suggest that the pathway of development of APL differs in each group: *FLT3*-ITD, trisomy 8, and other genomic changes. Here, we showed for the first time hidden abnormalities and novel disease-related genomic changes in t(15;17) APL. (Blood. 2009;113:1741-1748)

Introduction

Acute promyelocytic leukemia (APL) is a hematopoietic malignant disease characterized by the chromosomal translocation t(15;17), resulting in the fusion of the promyelocytic leukemia (*PML*) gene and retinoic acid receptor α (*RARA*) gene (*PML-RARA*).[1,2] The fusion product PML-RARα homodimerizes, binds to DNA, and works as a transcriptional repressor together with corepressors including histone deacetylase.[3] Therefore, reactivation of RARα-dependent transcription is one of the major strategies to treat APL patients. In fact, all-*trans* retinoic acid (ATRA), which binds to RARα and leads to the activation of the transcription factor, is a highly effective compound for the induction of remission of APL patients.[4,5]

Transgenic mice revealed that PML-RARα is necessary but not sufficient for the development of APL.[6,7] APL occurred in these mice only after a long latency (8.5 to 12 months) and penetrance was 15% to 30%.[6,7] These findings suggest that additional genetic mutations are also required for the development of APL. Candidate genes include the tyrosine kinase receptor gene, *FLT3*, and the oncogene, *RAS*. Activating *FLT3* mutations occur in approximately 30% to 35% APL samples[8,9]; and *NRAS* and *KRAS* mutations are found in 4% to 5% and 5% to 10% of APL samples, respectively.[9,10] Interestingly, transgenic mice coexpressing PML-RARα and either FLT3[W51] (constitutively activated form of murine FLT3), FLT3-ITD, or K-Ras (K12D) develop APL with a short latency and a high penetrance.[11-14]

Comparative genomic hybridization (CGH) is one of the genome-wide screening methods to identify chromosomal abnormalities. However, CGH analysis cannot detect copy-number-neutral loss of heterozygosity (CNN-LOH). Single-nucleotide polymorphism microarray (SNP-chip) is a powerful method to examine genomic alterations including small copy-number changes and/or CNN-LOH in several cancers.[15-17] SNP-chip analysis has been used for chronic lymphocytic leukemia (CLL),[18,19] childhood acute lymphoblastic leukemia (ALL),[20,21] acute myeloid leukemia (AML),[22-26] and AML with normal karyotype (Gorletta et al[27]; Akagi et al[28]).

In the present study, we focused on t(15;17) APL and examined whether additional genomic alterations could be found to subcategorize this disease on the basis of genomic status. The use of CNAG (copy-number analysis for Affymetrix GeneChips; Santa Clara, CA) program[15] and a new algorithm AsCNAR (allele-specific copy-number analysis using anonymous references)[17] provides a highly sensitive technique to detect CNN-LOH as well as copy-number changes in APL.

Methods

Patient samples

DNA from the bone marrow of 47 anonymized cases of t(15;17) APL at diagnosis as well as 7 complete remission bone marrow samples were

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examined. Sample information including the form of PML-RARα (long, short, or variant), sex, age, white blood cell counts (WBCs), blast percentage in the bone marrow, mutational status of the *FLT3* gene, *FLT3*-ITD level, and karyotype are shown in Table 1. This study received IRB approval from the Cedars-Sinai Medical Center and informed consent was obtained in accordance with the Declaration of Helsinki.

High-density SNP-chip analysis

Genomic DNA was isolated from bone marrow samples from t(15;17) APL patients at diagnosis and complete remission, as well as APL cell lines NB4 and PL-21. The DNA was subjected to GeneChip Human mapping 50-K or 250-K microarray (SNP-chip; Affymetrix) as described previously.[15,17] Hybridization, washing, and signal detection were performed on GeneChip Fluidics Station 400 and GeneChip scanner 3000 according to the manufacturer's protocols (Affymetrix). Microarray data were analyzed for determination of both total and allelic-specific copy number (AsCN) using the CNAG program as previously described[15,17] with minor modifications, where the status of copy numbers as well as CNN-LOH at each SNP was inferred using the algorithms based on hidden Markov models.[15,17] For clustering of AML samples with regard to the status of copy-number changes, as well as CNN-LOH, GNAGraph software (Tokyo University, Tokyo, Japan) was used.[21] Size, position, and location of genes were identified with UCSC Genome Browser (http://genome.ucsc.edu/). Germline copy-number changes previously described as copy-number variant at Database of Genomic Variants (http://projects.tcag.ca/variation/) and UCSC Genome Browser were excluded. This microarray data are available for public viewing in the Gene Expression Omnibus (GEO) database[29] under accession number GSE14016.

Determination of SNP sequences in cases of CNN-LOH and *FLT3* mutations

To validate CNN-LOH, 2 SNP sequences (rs10500648 and rs7937815) in chromosome 11p of case no. 39 at diagnosis and complete remission, and 6 SNP sequences (rs10491032, rs363221, rs2099803, rs2104543, rs7075893, and rs7918018) in chromosome 10q of case no. 18 at diagnosis were determined. The genomic region of each SNP site was amplified by genomic polymerase chain reaction (PCR) using specific primers (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), and PCR products were purified and sequenced. For determinations of *FLT3*-TKD and *FLT3*-ITD mutations, genomic PCR was performed as described previously.[30]

Cell culture, mRNA isolation, and quantitative real-time PCR

APL cell lines, NB4 and PL-21, were cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA). Total RNA was isolated from these cells and case no. 48 bone marrow sample at diagnosis using RNeasy kit (QIAGEN, Valencia, CA), and 1 μg total RNA was converted into cDNA by reverse transcription with Superscript III (Invitrogen). Gene expression of c-Myc mRNA was quantified with real-time quantitative PCR (iCycler; Bio-Rad, Hercules, CA) using Sybr Green. β-Actin was used as control.

Copy number of chromosome 11p15.4 in case no. 39, 10q24.31 in case no. 18, the *MYC* gene in cases no. 2, no. 18, and no. 65, and the *ERG* gene in case no. 43 were determined by quantitative real-time PCR (Bio-Rad) using Sybr Green. The region on chromosome 2p21 was used as control.[21] Copy number of the 2p21 region was normal as determined by SNP-chip analysis in these samples. The delta threshold cycle value (ΔCt) was calculated from the given Ct value by the formula $\Delta Ct = (Ct\ sample - Ct\ control)$. The fold change was calculated as $2^{-\Delta Ct}$. Primer sequences are shown in Table S2.

Results

SNP-chip analysis of t(15;17) APL samples

We examined the genomic changes in 47 samples of t(15;17) APL using 50-K and 250-K SNP-chip analyses. A total of 28 patients (60%) showed no detectable genomic abnormalities (normal-copy-number [NC] group). In contrast, 19 patients (40%) had one or more genomic abnormalities: 8 patients (17%) had trisomy 8 or duplication on chromosome 8 in the region of the *MYC* gene either with or without other genomic abnormalities (+8 group), and 11 patients (23%) had genomic abnormalities without trisomy 8 (other abnormalities group; Figure 1; Table 2).

One case (case no. 65, 2%) had 4 chromosomally altered regions; 2 cases (4%; no. 39 and no. 58) had 3 chromosomally altered regions; 8 cases (17%; no. 2, no. 50, no. 3, no. 18, no. 13, no. 37, no. 19, and no. 21) had 2 chromosomally altered regions; and 8 cases (17%; no. 38, no. 60, no. 66, no. 20, no. 4, no. 57, no. 43, and no. 52) had 1 chromosomally altered region. Importantly, 6 patients (13%) had CNN-LOH.

Validation of SNP-chip analysis

As proof of principal, we validated SNP-chip results using quantitative genomic real-time PCR (QG RT-PCR) and nucleotide sequencing of SNP sites. Case no. 65 had a duplicated region at chromosome 8, and this region contained the *MYC* gene (Figure 2A). QG RT-PCR showed that levels of the *MYC* copy number were approximately 2-fold higher than normal genomic DNA (Figure 2B). Other copy-number changes including duplication of the *MYC* gene in cases no. 2 and no. 18, and duplication of the *ERG* gene in case no. 43 were also confirmed by QG RT-PCR (data not shown).

Next, we validated CNN-LOH detected by SNP-chip analysis (Figure 3). If a chromosome has LOH, SNP sequences in this region should have homozygosity at diagnosis but heterozygosity at complete remission. Therefore, we examined 2 independent SNP sequences in case no. 39 on chromosome 11p in the CNN-LOH region using diagnosis and complete remission samples. Two SNP sites (rs10500648 and rs7937815) clearly showed a single signal at diagnosis (homozygosity), whereas, the sites showed a double signal at complete remission (heterozygosity; Figure 3B). These results demonstrated that this region had LOH. Next, we determined copy number of the region to exclude the possibility of a hemizygous deletion. As shown in Figure 3C, level of DNA at the 11p15.4 region of case no. 39 at diagnosis was almost the same as level of the complete remission sample, indicating that this region had a normal copy number and the region represented CNN-LOH. CNN-LOH region of case no. 18 was also validated by SNP sequencing and QG RT-PCR (Figure S1). Taken together, these results indicated that SNP-chip analysis clearly reflected real chromosomal abnormalities.

Copy-number changes in t(15;17) APL samples

As shown in Table 2, several copy-number changes were detected by SNP-chip analysis. Deletions were found in 7 cases (15%) including case no. 65 (4q28.1, 1.33 Mb; 7q21.11-q21.12, 1.03 Mb; and 9q12-q31.3, 47.27 Mb), case no. 2 (10q21.2-q21.3, 5.55 Mb), case no. 50 (6p25.1-p24.3, 2.51 Mb), case no. 37 (1q42.2, 0.02 Mb), case no. 19 (12p13.31-p11.22, 22.49 Mb; and 13q14.2-q14.3,

Table 1. Baseline clinical characteristic of 47 t(15;17) APL patients

	PML-RARα					*FLT3*		*FLT3*-ITD	
Group/case no.	Isoform	Sex	Age, y	WBC, ×10⁹/L	Blast, %	D835	ITD	Level, %	Chromosome
NC									
5	S	F	43	1.9	88	-	-		46,XX,t(15;17)(q22;q21)
48	S	M	45	1.3	76	-	-		46,XY,t(15;17)(q22;q21)
23	L	F	38	0.4	84	-	-		ND, RT-PCR(+)
28	L	F	36	9.9	87	-	-		ND, RT-PCR(+)
35	L	F	60	1.0	94	-	-		46,XX,t(15;17)(q22;q21)
40	L	M	32	0.9	75	-	-		46,XY,t(15;17)(q22;q21)
12	L	M	54	1.3	97	-	-		46,XY,t(15;17)(q22;q21)
55	L	M	36	2.0	76	-	-		46,XY,t(15;17)(q22;q21)
56	L	M	17	1.9	85	-	-		ND, RT-PCR(+)
24	V	M	32	0.9	77	-	-		46,XY,t(15;17)(q22;q12)
46	V	M	33	2.8	62	-	-		ND, RT-PCR(+)
33	S	M	42	31.4	88	+H	-		46,XY,t(15;17)(q22;q21)
1	L	F	68	1.3	96	+Y	-		46,XX,t(15;17)(q22;q21)
9	L	F	30	1.4	87	+Y	-		46,XX,t(15;17)(q22;q21)
11	L	M	30	4.3	78	+D	-		ND, RT-PCR(+)
53	L	M	36	36.2	96	+E	-		46,XY,t(15;17)(q22;q21)
61	L	M	32	0.3	93	+E	-		ND, RT-PCR(+)
7	S	F	31	120.0	95	-	+	42	46,XX,t(15;17)(q22;q12)
8	S	M	49	4.5	96	-	+	37	46,XY,t(15;17)(q22;q21)
14	S	M	9	28.0	80	-	+	40	46,XY,t(15;17)(q22;q21)
17	S	F	47	51.1	98	-	+	24	46,XX,t(15;17)(q22;q21)
29	S	M	75	1.5	88	-	+	35	ND, RT-PCR(+)
63	S	F	57	9.7	96	-	+	42	46,XX,t(15;17)(q22;q21)
64	S	M	28	24.8	90	-	+	57	46,XY,t(15;17)(q22;q21) [25/26] 46,XY[1/26]
6	L	F	37	29.5	93	-	+	32	ND, RT-PCR(+)
42	L	F	24	1.2	87	-	+	4	46,XX,t(15;17)(q22;q21)
51	L	M	51	45.4	78	-	+	47	46,XY,t(15;17)(q21;q12)
62	L	F	46	45.0	89	-	+	42	46,XX,t(15;17) (q22;q21)
+8									
65	S	F	58	1.5	87	-	-		46,XX,RT-PCR(+)
38	L	F	22	7.0	78	-	-		47,XX,+8,t(15;17)(q22;q12)
2	S	M	42	2.4	79	-	-		47,XY,+8,t(15;17)(q22;q21)
50	S	F	58	19.8	84	-	-		47,XX,+8,t(15;17)(q22;q21)
60	L	M	22	9.9	90	+H	-		46,XY,t(15;17)(q22;q21) [16/21] 47XY,+8,t(15;17) (q22;q21) [4/21] 46,XY [1/21]
3	V	M	33	3.2	67	-	-		47,XY,+8,t(15;17)(p22;q12)
39	L	M	38	1.0	51	-	-		47,XY,+8,t(15;17)(q22;q21)
18	L	M	23	18.1	94	-	-		ND, RT-PCR(+)
Other									
66	S	F	41	15.6	89	+Y	-		46,XX,t(15;17)(q22;q11.2)
20	L	F	51	77.9	88	-	-		46,XX,t(15;17)(q22;q21)
13	L	F	7	1.6	99	+Y	-		ND, RT-PCR(+)
4	S	M	68	0.7	91	+E	-		46,XY,t(15;17)(q22;q21)
37	S	F	26	2.0	75	+Y	-		46,XX,t(15;17)(q22;q12)
57	L	F	30	1.4	87	+Y	-		46,XX,t(15;17)(q22;q21)
19	L	M	51	1.1	73	-	-		46,XY,t(15;17)(q22;q21)
21	L	M	45	4.7	87	-	-		ND, RT-PCR(+)
43	L	M	7	8.6	75	-	-		46,XY,t(15;17)(q22;q21)
52	L	M	54	8.6	82	-	-		46,XY,t(15;17)(q21;q12) [19/20] 47,XY, +21, t(15;17)(q21;q12) [1/20]
58	L	F	27	6.9	90	-	-		46,XX,t(15;17)(q22;q21)ider(17) (q10)t(15;17) [23/26] 46,XX [3/26]

Chromosomal translocation of t(15;17) was determined by karyotype studies and/or RT-PCR analysis specific for PML-RARα fusion products. Three types of PML-RARα (long, short, and variant) are shown as L, S, and V, respectively. The recorded number of white blood cells (WBCs) and bone marrow blast percentages were obtained at diagnosis. Mutations of *FLT3* were either tyrosine kinase domain (TKD) at codon 835 or internal tandem repeat (ITD). The APL samples are divided into 3 groups based on SNP-chip analysis: normal-copy-number (NC), trisomy 8 including duplication of the *MYC* gene region (+8), and other abnormalities (other).

0.88 Mb), case no. 21 (7q11.21–q-terminal, 97.03 Mb), and case no. 58 (17p-terminal–p11.2, 21.44 Mb). Of note, deleted region at 12p of case no. 19 and 17p of case no. 58 contained the *ETV6/TEL* and *TP53* genes, respectively.

Duplications were found in 7 cases (15%) including case no. 65 (8q24.13-q24.22, 9.48 Mb), case no. 13 (13q21.1–q-terminal, 57.27 Mb; and 15q22.2–q-terminal, 42.94 Mb), case no. 57 (9q22.32, 0.27 Mb), case no. 37 (18p11.31–p11.23, 0.46 Mb), case