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ORIGINAL ARTICLE: RESEARCH

Leukemic cells with increased telomerase activity exhibit resistance to imatinib

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Abstract

Imatinib mesylate (imatinib), previously known as STI571 (Gleevec), is currently utilized in the treatment of chronic myeloid leukemia (CML). However, its effect on telomerase activity and the correlation of this to its observed antitumor effect has yet to be defined. We investigated the effects of this agent on human telomerase reverse transcriptase (*hTERT*) expression and telomerase activity and found that it significantly down-regulated telomerase activity in both K562 cells and primary leukemic cells. The telomerase activity of primary leukemic cells from CML patients in blastic crisis showed less suppression than that of cells from patients in chronic phase. Additionally, data also demonstrate that inhibition of telomerase was due to the direct action of imatinib on *hTERT* transcription, rather than an increase in cell death. These results suggest a novel mechanism in the antitumor activity of imatinib and may provide a basis for future development of anti-telomerase therapies, as well as leading to better understanding of the regulation of telomerase in leukemic cells.

Keywords: *Telomerase, imatinib, CML, tyrosine kinase*

Introduction

Chronic myeloid leukemia (CML) is a hematological stem cell disorder characterized by excessive myeloid cell proliferation. The Philadelphia chromosome is the hallmark of CML and is characterized on a molecular basis by the *bcr-abl* fusion gene that produces the P190, P210, and P230 chimeric proteins [1]. These proteins exhibit increased tyrosine phosphorylation activity compared with normal P145 tyrosine kinase [2,3]. The *bcr-abl* gene has been proven to induce either acute or chronic leukemia in mice, indicating that the abnormal tyrosine kinase activity is the basis of Ph-positive leukemia [4,5]. Therefore, the targeting of bcr-abl protein tyrosine kinase (PTK) activity is an attractive therapeutic strategy for CML or *bcr-abl*-positive acute lymphoblastic leukemia (ALL). Recent studies have shown that administration of a relatively specific bcr-abl PTK inhibitor (imatinib) can eradicate *bcr-abl*-positive leukemic cells [6]. However, the mechanisms responsible for antitumor activity in relation to an influence on human telomerase reverse transcriptase (hTERT) expression and telomerase activity are poorly defined.

Telomerase is a ribonucleoprotein polymerase that adds specific hexanucleotides (TTAGGG) to the 3′ end of DNA strands in the telomere regions of chromosomes [7]. Increased telomerase activity is associated with cellular immortality and a malignant phenotype in most cancers [8]. However, the mechanism by which telomerase activity is regulated in cancer cells is presently unknown. In this study, the effects of tyrosine kinase inhibitors on telomerase activity were examined in K562 cells and primary leukemic cells from patients with CML in the chronic phase or during blastic crisis.

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Materials and methods

Cells and culture

K562 cells(derived from a patient with CML in blastic crisis) and Reh (pre-B ALL) [9,10] were obtained from the American Type Culture Collection (Manassas, VA). SUSM-1 cells (γ−radiation immortalized fibroblastic cells) were kindly provided by Dr. M. Namba [11]. Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 μ/mL), streptomycin (100 μg/mL) and 2 mM L-glutamine (Gibco BRL, Gaithersburg, MD, USA) at 37°C in a humidified atmosphere of 5% CO2 in air. Primary leukemic cells were obtained from 10 patients with CML, six of whom had been newly diagnosed (untreated) in the chronic phase, two of whom had been newly diagnosed (untreated) in blastic crisis and two of whom had progressed from the chronic phase to blastic crisis during treatment with interferon-α. Leukemic cells were isolated from peripheral blood samples with Ficoll-Conray density gradient, washed twice, suspended in a cell banker (Juji Kagaku, Tokyo, Japan) and stored in liquid nitrogen. Cells were thawed prior to use, and their viability determined using the trypan blue dye exclusion method. Cells showing >80% viability were used in experiments; six out of 10 samples were found to be suitable at the time of thawing. Because minute changes of telomerase activity are hard to detect when the initial level of telomerase activity is very low, such cell samples were also omitted to exclude unreliable data. Therefore, of the initial 10 patients recruited, only cells from two patients in the chronic phase and two patients in blastic crisis (one had undergone transformation from the chronic phase) were studied. All samples were obtained after informed consent was given, and the study protocol was approved by the Human Investigation Committee of our institution.

Subcloning of telomerase promoter region and generation of reporter gene constructs

DNA was isolated from Reh cells using standard methods, and the promoter region of telomerase was amplified by polymerase chain reaction (PCR) [12]. The primers used to amplify the promoter region, which we named ProL-PGL3, were: ProL1125Kpn (5′-TGGGTACCGTCTGGATTCCTGGGAAGT CCTCA) and ProL43Bgl (5′-AGAGATCTGAC GAGCGCTGCCTGAAACTGG). The 5′-termini of the primers were modified to create KpnI/BglII restriction sites on the ends of the amplified product to facilitate subcloning into the PGL3-Basic vector (Promega, Madison, WI).

For generation of the green fluorescent protein (GFP) reporter construct, the following primers were designed to amplify ProL-GFP: ProL1125Xho (CTGCTCGAGGTCTGGATTCCTGGGAAGT CCTCA) and ProL43Kpn (AGGGTACCGACG CAGCGCTGCCTGAAACTGG). The PCR conditions were the same as those used to amplify ProL-PGL3. The amplified product was digested with XhoI/KpnI and subcloned into the pEGFP1 vector (Clontech, Palo Alto, CA). Sequence analysis revealed that the inserted DNA sequence was identical to the published sequence [13].

Luciferase assay

The 1.2 kb *hTERT* promoter sequence upstream of the initiating ATG was cloned into the plasmid PGL3-Basic (Promega, Madison, WI) in a sense orientation and examined using the dual luciferase reporter assay system. For better comparisons among cell lines with different transfection efficiencies, the PGL3-control plasmid (Promega), which has the firefly luciferase gene under the transcriptional control of the SV40 enhancer or promoter, and PGL3-basic were cotransfected with PRL-SV40 into K562 and SUSM-1 cells. In all experiments, cells were cultured for 48 h following transfection, and cell lysates were examined using an MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA). All experiments were performed at least three times and the mean luciferase activity was determined.

Establishment of stable transformants expressing hTERT and GFP

The plasmid containing the full-length cDNA of *hTERT* (PCI-neo-*hTERT*) was a generous gift from R. Weinberg (White Head Institute, MIT, MA). TelProL-pEGFP1, a GFP reporter construct that is driven under the control of the telomerase promoter, was also used. The expression vector and a mock vector were introduced into cells by electroporation using a Gene Pulser apparatus (BioRad, Hercules, CA) at 280 V and 960 μF at room temperature. After electroporation, transfected cells were cultured in selection medium containing 800 μg/mL of G418 (Gibco) and finally a single colony was isolated from methyl cellulose semisolid medium [14]. Stable transfectants were confirmed by reverse transcriptase PCR (RT-PCR).

Detection of apoptosis

Apoptosis was analyzed by labeling the cells with annexin V-FITC and propidium iodide using an

apoptosis kit (Immunotech, Beckman Coulter, Marseille, France). Briefly, cells were treated with imatinib for 48 h, washed twice with cold phosphate buffer solution (PBS), and suspended again in binding buffer provided by the manufacturer. After fluorescein-labeled annexin V and propidium iodide were added, the cells were immediately analyzed by flow cytometry using a Coulter EPICS cytometer equipped with a single laser (excitation at 488 nm).

Nonradioisotopic telomerase assay and quantitation of enzyme activity

Telomerase activity was assayed by a modified TRAP method in a total assay volume of 50 μL. In brief, aliquots of untreated cell extracts or heat-treated extracts (10 min at 85°C) were incubated with 0.1 μg of 6-carboxyfluorescein (FAM)-labeled telomerase substrate (TS) oligonucleotide (5′-AATCCGTC-GAGCAGAGTT-3′) for 20 min at 22°C in a reaction mixture as reported previously [12,15]. Following elongation of the [F]-TS primer by telomerase, the products were amplified by PCR in the presence of 0.1 μg of the CX primer (5′-CCCTTACCCT TACCCTTACCCTAA-3′) and Taq polymerase. PCR involved 27 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s. Fluorescent amplification products were detected by electrophoresis on 4.5% polyacrylamide or 6% urea sequencing gels using a 377 automatic sequencer (Perkin-Elmer Cetus, Norwalk, CT). Telomerase products displayed a shark-tooth pattern with a periodicity of six nucleotides in the fluorescent amplification products. Each peak was automatically assessed for size, height, and area using the GeneScan software package (Perkin-Elmer). Telomerase activity was expressed in arbitrary units as reported elsewhere [12,15].

Real-time quantitative PCR

The expression of *bcr-abl* transcripts in leukemic cells was determined by real-time quantitative PCR (RQ-PCR) using the ABI Prism 7700 Sequence Detector System (Perkin-Elmer). Primer sequences for *bcr-abl* were as follows: 5′-GCATTCCGCTGACCATCA ATA-3′ (sense) and 5′-TCCAACGAGCGGCTT CAC-3′ (antisense). The TaqMan probe for *bcr-abl* (5′-CAGCGGCCAGTAGCATCTGACTTTGA-3′) was labeled at the 5′-end with carboxy-fluorescein phosphoramidite as a reporter dye and at the 3′-end with carboxy-tetramethyl-rhodamine for quenching (Takara Shuzou, Kyoto, Japan). To correct for differences in RNA quality, *β-actin* mRNA expression was measured in parallel and the ratio between the expression of *bcr-abl* and *β-actin* mRNA transcripts was calculated in each sample. Amplification and data analysis were carried out using the Sequence Detector V 1.6.3 program (Perkin-Elmer) as previously reported [12,16].

Detection of point mutations

To amplify the kinase domain of *bcr-abl*, PCR was performed with the following primers: 5′-CGCAA

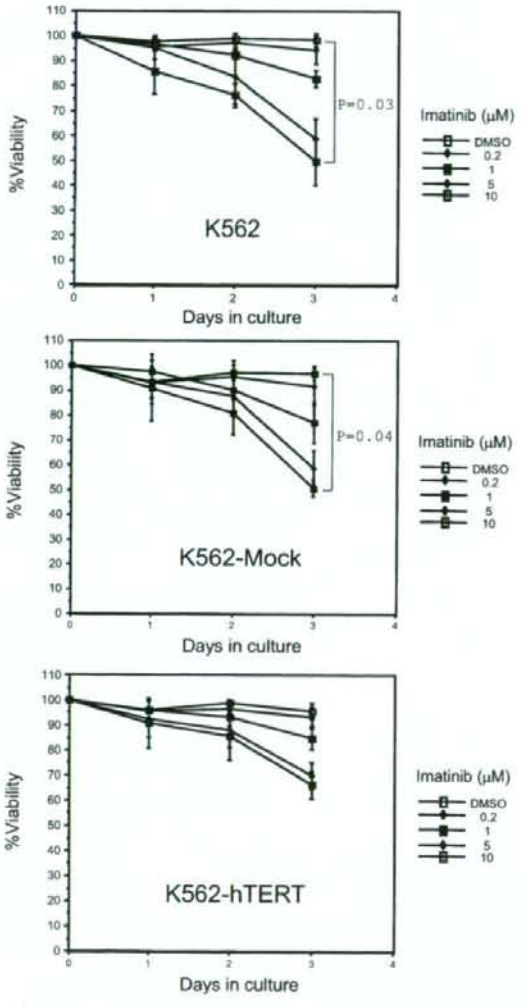

Figure 1. Effect of imatinib on the proliferation of K562-*hTERT*, K562-Mock and parental K562 cells. Cells at a density of 2×10^5/mL were cultured for 72 h with various concentrations of imatinib. Control cultures were treated with the highest DMSO concentration (0.02%). The inhibitory effect of imatinib at 10 μM was more prominent in K562 ($p = 0.03$) and K562-Mock ($p = 0.04$) cells than K562-hTERT cells. Data is expressed as mean ± standard deviation ($n \geq 3$).

CAAGCCCACTGTCT-3′ (sense) and 5′-CTCCA TGGCTGACGAGATCT-3′ (antisense). The 359-bp product encoding the *bcr-abl* adenosine triphosphate (ATP) binding site and the kinase activation loop were then cloned into the PGEM-T vector (Promega) and introduced into JM109 cells. Randomly selected white colonies were screened for the appropriate insert and sequenced with the forward and reverse universal primers using the ABI377 system.

Statistical analysis

Results are expressed as the mean ± standard deviation of at least three separate experiments. Data were analyzed by Student's *t*-test and a *p*-value of <0.05 was accepted as statistically significant.

Results

Correlation between upregulation of telomerase activity and sensitivity to imatinib-induced cell death

Since telomerase is implicated in the anti-apoptotic phenotype [14], we attempted to determine whether a correlation exists between telomerase activity and imatinib-induced apoptosis. As can be seen in Figure 1, during 48 h of culture, K562-*hTERT* cells and K562-Mock control cells together with their parental K562 cells, did not demonstrate any significant decrease in sensitivity to treatment with either 0.2 μM or 1 μM imatinib when compared with dimethyl sulfoxide (DMSO)-treated cells as suggested by the overlapping error bars at each time interval examined. After 72 h of culture, when cells were treated with imatinib,

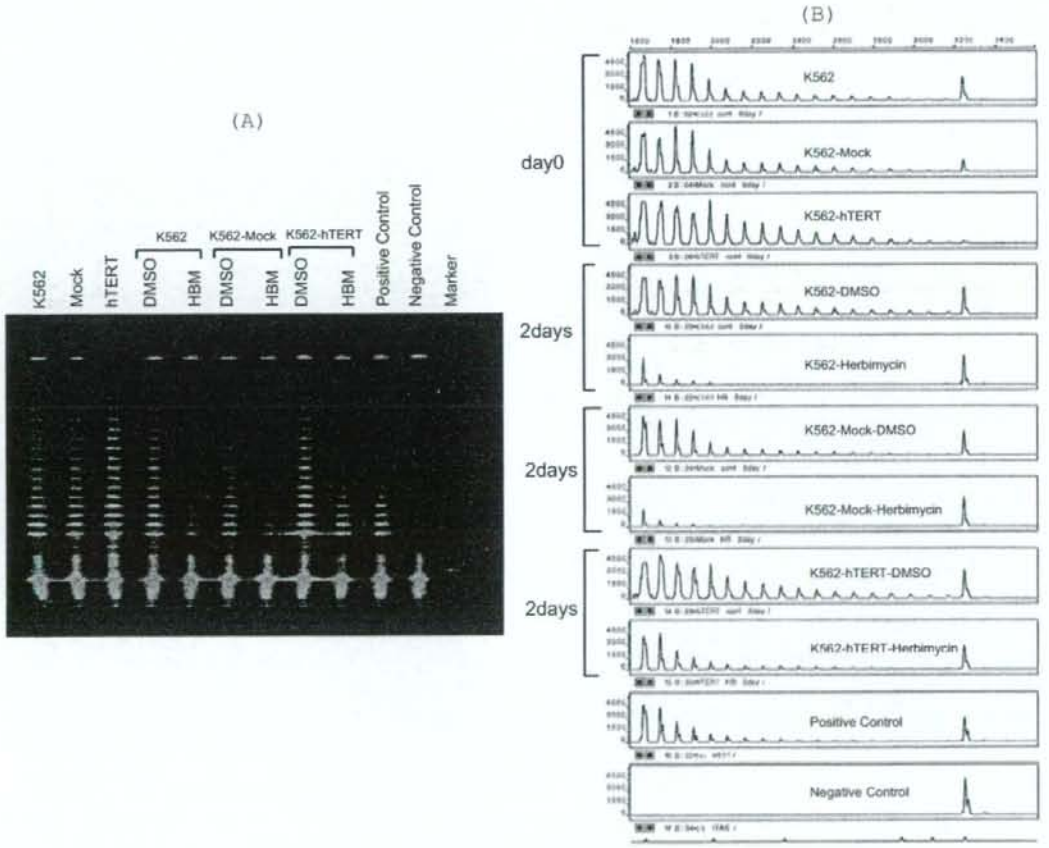

Figure 2. Effect of herbimycin A on telomerase activity. Cell extracts obtained from K562-*hTERT* cells, K562-Mock control cells and parental K562 cells were analyzed by [F]-TRAP assay following incubation with 0.8 μM herbimycin A. (A) The characteristic 6-bp DNA ladder of telomerase products is represented by fluorescence curves. (B) The peak height, peak area, and size of fluorescence curves starting with the 50-bp band were calculated automatically using GeneScan software. Samples were evaluated using 0.1 and 0.01 μg of total protein to confirm telomerase status and only the result for 0.1 μg of protein is shown. The peak at 160 bp represents the internal standard amplified with the same two primers used for the telomerase assay.

over a higher concentration range, 5–10 μM, a large fraction of dead cells was observed among both K562-Mock control and parental K562 cells. These results suggest that K562-*hTERT* cells may exhibit resistance to imatinib partially through the activation of telomerase. To assess the effects of imatinib in cell lines which do not express the *bcr-abl* gene, HL60 cells (a cell line derived from acute myelocytic leukemia) were cultured under the same conditions as the K562 cells. The growth and viability of treated HL60 cells showed no differences from those of control cells (data not shown).

Modulation of telomerase activity in K562 cells by imatinib

To determine the effect of PTK inhibition on telomerase activity, first we investigated the effects of herbimycin A, a widely used PTK inhibitor, in *hTERT*-transformed K562 cells, K562-Mock cells and parental K562 cells. After two days of incubation with 0.8 μM herbimycin A [Figure 2 (A) and (B)], telomerase activity in *hTERT*-transformed, mock and parental K562 cells declined to 31%, 10% and 9.7% of control values, respectively. The proliferation of all three cell lines was suppressed by herbimycin A compared to solvent (DMSO)-treated cells, but total cell numbers and the number of viable cells showed little difference between these cell lines (data not shown).

Next we examined the effects of imatinib, a more specific inhibitor of bcr-abl fusion kinase, on these three cell lines. Taking into consideration the half life of the telomerase protein, we used a non-cytotoxic concentration of imatinib (1 μM) and compared the telomerase activity of these cell lines after 48 h in culture [Figure 3 (A) and (B)]. Treatment of all three cell lines with imatinib downregulated endogenous telomerase activity.

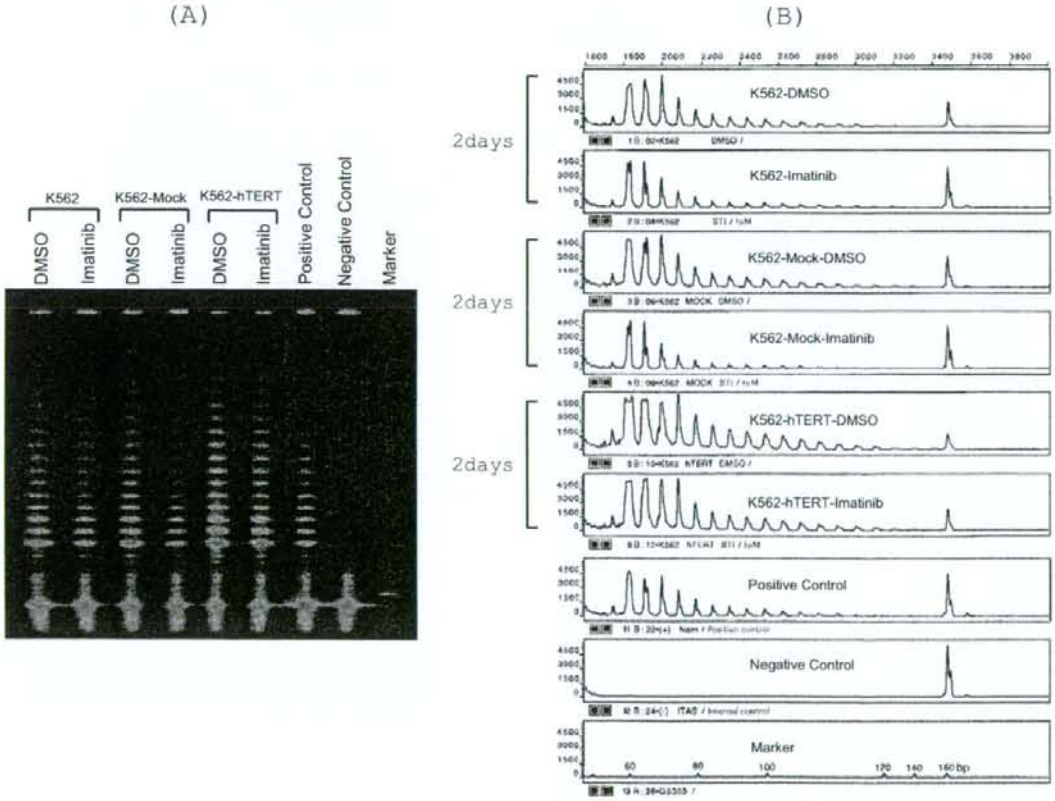

Figure 3. The effects of imatinib on telomerase activity. Cell extracts obtained from K562-*hTERT* cells, K562-Mock control cells and parental K562 cells were analyzed by F-TRAP assay following incubation with 1 μM imatinib. (A) Downregulation of endogenous telomerase activity in all three cell lines. (B) The characteristic 6-bp DNA ladder of telomerase products was calculated automatically using GeneScan software. The enzyme activity was determined as described in Figure 2.

However, the suppressive effect was less prominent (55%, 32% and 31% of control values, respectively) than in herbimycin A-treated cells. The inhibition of telomerase activity was not due to the direct action of imatinib against the telomerase enzyme, since extracts from cells when mixed directly with 0.2 – 1 μM imatinib did not abrogate the activity (data not shown) and was independent of imatinib-induced apoptosis as was shown using flow cytometric analysis (Figure 4).

Effects of imatinib on telomerase activity in primary leukemic blasts from patients with CML

The sensitivity of telomerase to imatinib in fresh leukemic cells obtained from CML patients in different stages of the disease was also evaluated (Table I). Primary leukemic blasts were incubated in suspension cultures in the presence of 0.2 μM and 1 μM of imatinib for 48 h and the telomerase activity was determined (Figure 5). Primary leukemic cells obtained in chronic phase showed a dose-dependent

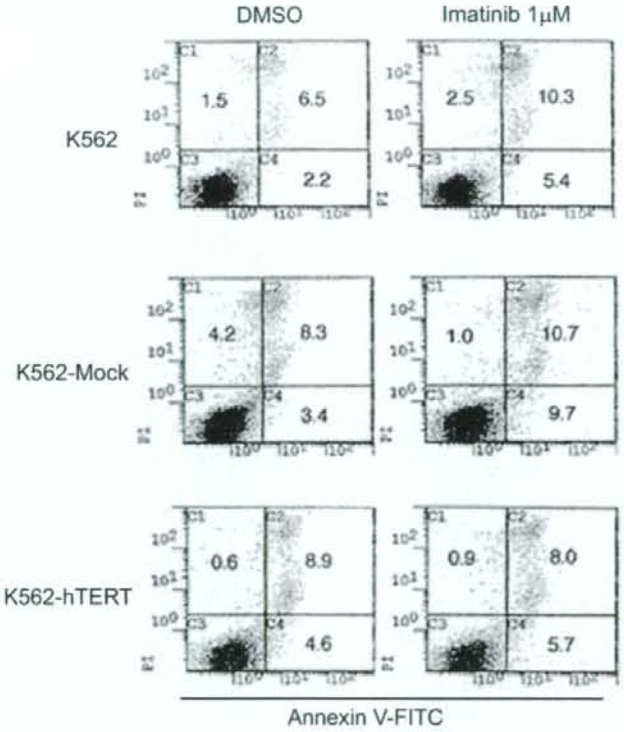

Figure 4. Detection of apoptosis in imatinib-treated cells by the annexin V/PI assay. K562-*hTERT*, K562-Mock and parental K562 cells were incubated with 1 μM of imatinib for 48 h and analyzed by flow cytometry. Live cells are distinguished as unstained with fluorochrome, apoptotic cells are stained only with annexin V, and necrotic cells are stained with both annexin V and PI. The percentage of cells located in each quadrant of dot plots was recorded. The experiment was repeated twice with similar results.

Table I. Patient characteristics.

Patient	Age (years)	Sex	CP/BC	BM/PB	WBC (10^9/L)	HB (g/L)	Platalet (10^9/L)	Chromosome	Outcome
1	51	F	CP	PB	151	88	480	46,XX t(9;22)	MMR
2	61	F	CP-BC	PB	110	106	255	46,XX t(9;22),ins(15;8)	Dead
3	57	M	BC	BM	230	69	22	46,XY t(8;15,9;22)	Dead
4	56	M	CP	PB	300	120	290	46,XY t(9;22)	MMR

CP, chnonic phase; BC, blast crisis; CP-BC, progression from CP to BC; BM, bone marrow; PB, peripheral blood; MMR, major molecular response.

inhibition of telomerase activity when treated with 0.2 μM or 1 μM imatinib (12/8% and 40/7% of each control value, respectively). On the other hand, the changes in the telomerase activity of leukemic cells in blastic crisis were totally different from those in chronic phase. Cells still expressed telomerase activity after 48 h (83/91% and 88/77% of control values, respectively) (Table II).

Correlation between upregulation of telomerase activity and sensitivity to imatinib

To determine whether the differences in the suppressive effect of imatinib on primary leukemic cells are derived from genomic changes in these cells, we examined the correlation between telomerase activity and sensitivity to imatinib. Leukemic cells obtained from the two patients in blastic crisis expressed higher *bcr-abl* mRNA levels compared to cells from the patients in the chronic phase, as determined by RQ-PCR. In all patients, no single amino acid substitution, including the commonly reported ATP binding site and kinase activation loop between codon 227 and 345, was observed (Table II).

Transcriptional activity of subcloned telomerase promoter region

The degree of luciferase activity under the control of telomerase promoter was examined in telomerase-positive K562 cells and in telomerase-negative SUSM-1 cells. After 48 h of transfection, a luciferase assay was performed using the Dual luciferase reporter assay system. As shown in Figure 6, the

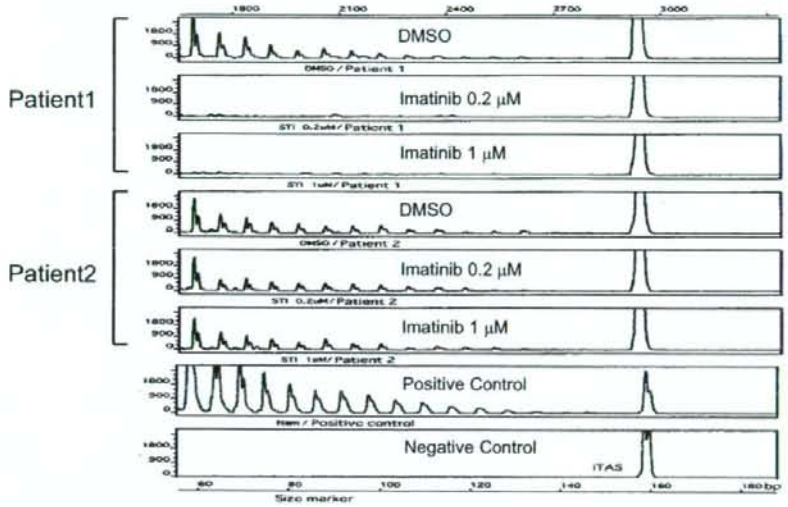

Figure 5. Effects of imatinib on telomerase activity in primary leukemic cells from patients with CML. Leukemic cells were isolated from six patients in the chronic phase and four patients in blastic crisis. Primary leukemic cells were incubated in suspension culture in the presence of 0.2 or 1 μM imatinib for 48 h, after which telomerase activity was examined. Representative data analyzed by GeneScan software from patients 1 and 2 are shown.

Table II. Response to imatinib and the results of gene analysis.

Patient	CP/BC	Relative telomerase activity (% control) ST1571(0.2 μM/1 μM)	Fold changes in mRNA relative to that of K562 cells Major bcr	Minor bcr	Point mutation Binding site/activation loop
1	CP	12/8	0.5	0.125	E255K(−);T315I(−)
2	CP-BC	83/91	2	8	E255K(−);T315I(−)
3	BC	88/77	4	16	E255K(−);T315I(−)
4	CP	40/7	1	4	E255K(−);T315I(−)
K562			1	1	

CP, chronic phase; BC, blastic crisis; CP-BC, progression from CP to BC.

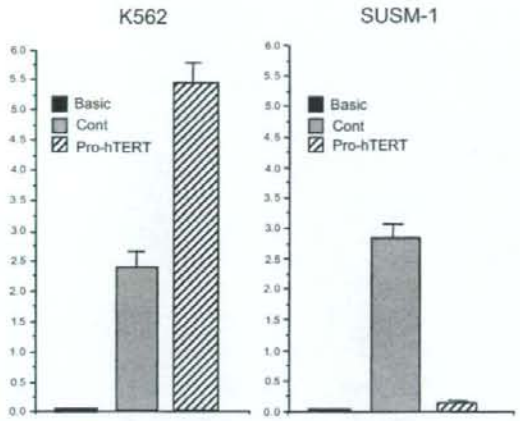

Figure 6. *hTERT* promoter activity in K562 and SUSM-1 Cells. The 1.2 Kb *hTERT* promoter sequence upstream of the initiating ATG was cloned into the plasmid PGL3-Basic (Promega) in a sense orientation and examined using the Dual luciferase reporter assay system. After 48 h of transfection, luciferase activity was assayed and values normalized for transfection efficiency via control plasmid activity. Data are expressed as mean ± standard deviation, $n \geq 3$.

transcriptional activity of K562 cells was significantly higher than that of SUSM-1 cells ($p \leq 0.05$).

Effect of imatinib on the transcriptional activity of hTERT in K562 cells

The transcriptional activity of the 1.2 kb *hTERT* promoter sequence cloned upstream of the pEGFP1 plasmid (Clontech) was examined in the absence or presence of imatinib. Fluorescence-activated cell sorting (FACS) analysis of K562 cells after 2 days of incubation with imatinib showed a significant, dose-dependent decrease in GFP expression, suggesting that *hTERT* expression is decreased in the presence of imatinib (Figure 7).

Discussion

The aim of this study was to investigate the effect of imatinib on telomerase activity and to clarify the relationship between telomerase dynamics and PTK. Although some differences in cell proliferation were observed during the initial two days of culture with imatinib (0.2 – 1 μM), the viability of the *hTERT*-transfected cells, empty vector-transfected control cells, and parental cells was not statistically significant. Alternatively, when cells were treated with imatinib over a higher concentration range (5 – 10 μM) for 72 h, the viable cell count in cultures of *hTERT*-transformed K562 cells significantly exceeded that in cultures of empty vector-transfected control and parental K562 cells. It has been reported that hTERT expression is associated with resistance to apoptosis *in vitro* in addition to the prevention of telomere shortening [17 – 19]. Our results suggest that K562-*hTERT* cells may in part escape imatinib-induced apoptosis via increased telomerase activation.

Herbimycin A, a widely used PTK inhibitor and is also reported to bind Hsp90 and alter its function, reduced the telomerase activity of *hTERT*-transformed K562 cells, K562-Mock cells and parental K562 cells to 31%, 10% and 9.7% of control values.

Treatment of these cell lines with imatinib also downregulated endogenous telomerase activity. However, the suppression was less marked than in herbimycin A-treated cells. The difference in sensitivity of leukemia cells to the suppressive activity of these two PTK inhibitors may reflect the role of multiple signal transduction pathways in the control of *hTERT* gene transcription and the inhibition of HSP90 by herbamycin [14,20]. In the current study, the telomerase activity of primary leukemic cells from CML patients in blastic crisis was less sensitive to therapeutic concentrations of imatinib than that of cells from patients in the chronic phase. We used only cells with good viability and excluded cases in which constitutive telomerase activity levels were low to remove unreliable data [21]. As a result, only four out of 10 patients were eligible for the study. The patients in blastic crisis have already died while those in chronic phase are still receiving imatinib.

In patients who do not respond to imatinib therapy, both *bcr-abl*-dependent and independent mechanisms have been reported [22,23]. Mutations in the kinase domain of *bcr-abl* are the most common mechanism of acquired imatinib resistance, and more than 30 different point mutations have been identified *in vivo* [24,25]. In the current study, we examined a limited section of the *abl* oncogene, which included the multi-inhibitor-resistant T315I, the ATP-binding site, and the kinase activation loop. None of the four CML patients had point mutations in any of the seven different amino acids tested in the abl kinase domain. However, a high level of *bcr-abl* transcript expression was observed in the two patients with blastic crisis.

CML invariably progresses to acute leukemia. However, the mechanisms involved in this transformation are poorly understood. The acquisition of other molecular and cytogenetic abnormalities is likely to be responsible for the progression of this disease [26]. Primary leukemic cells isolated from patients in chronic phase retained sensitivity to imatinib, while cells from patients in blastic crisis

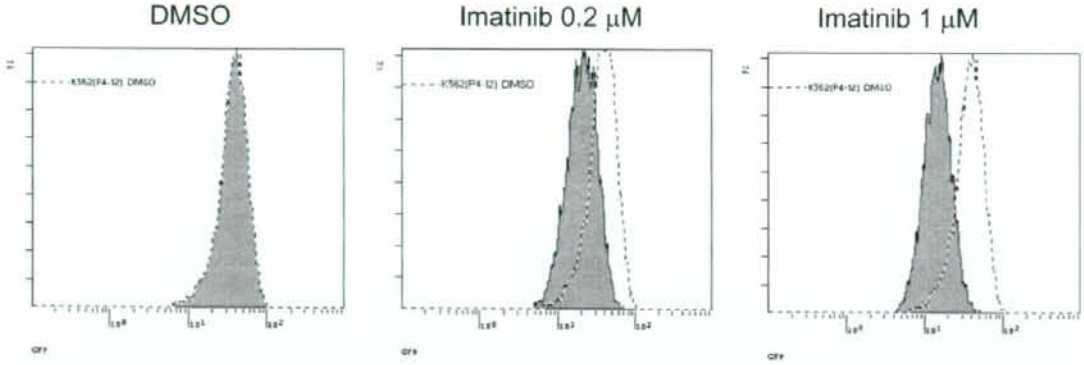

Figure 7. Imatinib-induced suppression of *hTERT* transcriptional activity. The 1.2-kb *hTERT* promoter sequence was cloned into the plasmid pEGFP1 (Clontech) and K562 cells with stable GFP expression were obtained. Promoter activity was evaluated by flow cytometric analysis of GFP expression in K562 cells using an EPICS-XL flow cytometer (Coulter Electronics, Hialeah, FL). Expression levels in DMSO (control cells) treated cells are plotted as dotted lines, STI571-treated cells are shown as shaded peaks. FACS analysis of K562 cells after 2 days of incubation with imatinib showed a dose-dependent decrease in GFP expression.

showed strong telomerase activity and resistance to imatinib. Increased expression of bcr-abl is associated with enhanced expression/activation of several effector proteins, such as Ras, Rac, Raf-1, phosphatidylinositol 3-kinase, Akt (which is a known signal transducer of telomerase), and signal transducers and activators of transcription [20,27,28]. It is possible that in these patients, additional chromosomal aberrations may have resulted in reactivation of the bcr-abl tyrosine kinase and/or stimulation of bcr-abl-independent signal transduction pathways, thus increasing the transcription of telomerase [20,29]. Further study would have to be performed to test this theory. The results of the promoter assay indicate that imatinib suppresses telomerase gene transcription through inhibition of *bcr-abl*-dependent transcriptional activation of the telomerase promoter in K562 cells. It is difficult from our experiment to say whether the effect of imatinib for telomerase inhibition is direct or indirect. However, the finding that imatinib induces the repression of *hTERT* expression and a decrease in telomerase activity suggests a novel mechanism for antitumor activity of imatinib.

Currently, second-generation bcr-abl tyrosine kinase inhibitors have been developed and are in use in clinical practice [30,31]. Due to resistance to these drugs in a subset of patients, additional treatment strategies are clearly needed. It has been reported that *hTERT* expression is associated with resistance to apoptosis in addition to prevention of telomere shortening [32–34]. Our results suggest that resistance to imatinib-induced apoptosis in K562-*hTERT* cells (telomerase over-expressing cells) may be due to telomerase activation. Treatment strategies that combine a bcr-abl PTK inhibitor with an agent that lowers telomerase activity may prove to be an effective treatment strategy in patients exhibiting resistance to imatinib [35–37].

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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, $\times 10^9$/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

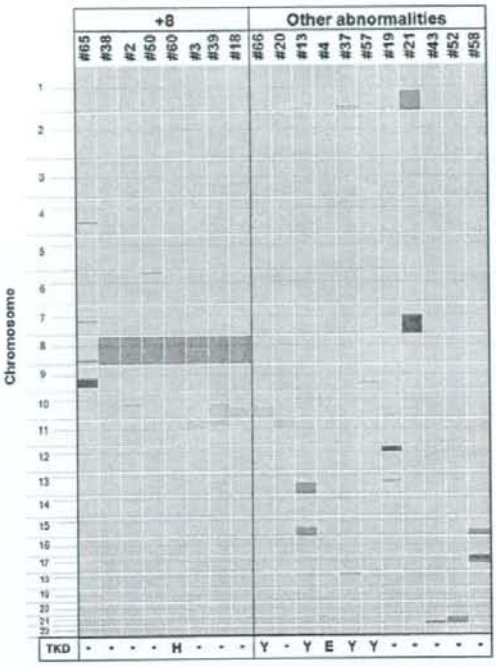

Figure 1. Summary of genomic abnormalities in t(15;17) APL samples. Genomic DNA of 47 t(15;17) APL samples were subjected to SNP-chip analysis, and genomic abnormalities are summarized. Color boxes are used to denote the type and size of abnormalities: pink (copy-number-neutral loss of heterozygosity; CNN-LOH); green (deletion); and red (duplication including trisomy). A total of 28 patients (60%) showed no detectable genomic abnormalities (data not shown). In contrast, 19 patients (40%) had one or more genomic abnormalities: trisomy 8 or duplication of the *MYC* gene region either with or without genomic abnormalities was found in 8 patients (17%, referred as "+8") and 11 patients (23%, referred as "other abnormalities") had genomic abnormalities without trisomy 8. Six patients (13%) had CNN-LOH; and 1 sample in +8 group and 5 samples in other abnormalities group had *FLT3* point mutations that are shown by their amino acid change at codon 835 from D (aspartic acid) to either Y (tyrosine), E (glutamic acid), or H (histidine).

no. 21 (1q21–q-terminal, 102.63 Mb), case no. 43 (21q22.12–q-terminal, 10.69 Mb), and case no. 58 (15q24.1–q-terminal, 27.97 Mb; and 17p11.2–q21.1, 14.05 Mb). The duplicated region at 21q of case no. 43 and at 17p of case no. 58 included the *ERG* and *ERBB2* genes, respectively. Importantly, the duplicated region at 8q of case no. 65 contained the *MYC* gene (Figure 2A). Seven cases had trisomy 8, and one of the candidate genes on chromosome 8 is the *MYC* gene; therefore, we classified case no. 65 within the +8 group.

Of note, the NB4 APL cell line had amplification of the *MYC* gene region (8q24.21), whereas the PL-21 APL cell line showed duplication of the region (Figure S2A). We compared levels of c-Myc mRNA in these cell lines, and found that NB4 cells had approximately 6-fold higher expression of c-Myc than did PL-21 cells (Figure S2B). This result indicated that copy-number change was associated with mRNA levels of the target gene.

CNN-LOH in t(15;17) samples

Several cases had the same chromosomal region involved in CNN-LOH. Chromosome 10q CNN-LOH was found in 3 cases (6%) including case no. 18 (10q22.2–q-terminal), case no. 39 (10q21.1–q-terminal), and case no. 66 (10q22.2–q-terminal; Figure 1; Figure S3A). This region included the tyrosine kinase receptor gene *FGFR2* and the tumor suppressor gene *PTEN* (Table 2). Cases no. 3, no. 20, and no. 39 had CNN-LOH on 11p-terminal–p11.12, 11p-terminal–p11.12, and 11p-terminal–p14.1, respectively; and the common region was 28.7 Mb (Figure 1; Figure S3B) containing the tumor suppressor genes *WT1* and *CDKN1C*, and the oncogene *HRAS* (Table 2). CNN-LOH of 19q13.2–q-terminal (17.3 Mb) occurred in one case (no. 4, Table 2).

Comparison of chromosomal changes between diagnosis and complete remission samples

To assess whether chromosomal alterations detected by SNP-chip analysis were acquired abnormalities, germ-line mutations, or copy-number variants, we compared chromosomal changes between diagnosis and complete remission (CR) samples in the same patients. CR samples of cases no. 2, no. 3, no. 18, no. 19, no. 38, no. 39, and no. 50 were available, and these CR samples were subjected to SNP-chip analysis. As shown in Figure 4, trisomy 8 of case no. 2 and CNN-LOH of case no. 18 at diagnosis were not present in the samples obtained at CR. Other alterations including trisomy 8 (cases no. 3, no. 18, no. 38, no. 39, and no. 50), CNN-LOH at chromosomes 10 (case no. 39) and 11 (cases no. 3 and no. 39), and deletions (cases no. 2, no. 19, and no. 50) also were not present at CR (Figure S4). Taken together, these results showed that chromosomal alterations detected by SNP-chip analysis were acquired somatic changes.

Relationship between genomic abnormalities and FLT3 mutations

Finally, we compared genomic abnormalities and *FLT3* status. Twenty-four samples (51%) had wild-type *FLT3;* whereas 12 samples (26%) had *FLT3*-TKD mutation (aspartic acid at codon 835, D835) and another 11 samples (23%) had *FLT3*-ITD form. Interestingly, all 11 samples with *FLT3*-ITD were found only in the normal-copy-number (NC) group (Tables 1 and 3). One sample in the trisomy 8 group had a *FLT3*-TKD mutation. Samples in the "other abnormalities" group did not have *FLT3*-ITD; and 6 samples in NC group and 5 samples in the "other abnormalities" group had a *FLT3*-TKD mutation. These results suggested that the pathway of development of APL differs in each group; in a mutually exclusive fashion, *FLT3*-ITD, trisomy 8, and unknown factor(s) were involved in each group.

Discussion

Our genome-wide SNP-chip analysis showed that 40% of t(15;17) APL samples had one or more genomic abnormalities including deletions, duplications, and/or CNN-LOH. Since the PML-RARα fusion protein is probably not sufficient to cause APL in murine model systems, the additional genetic changes that we found may be necessary to cause the leukemia.

Our analysis revealed that 6 samples (13%) of t(15;17) APL samples had CNN-LOH. Previously, we analyzed AML with normal karyotype samples and found that 32% of cases had CNN-LOH (Akagi et al[28]); and other investigators also demonstrated CNN-LOH in AML samples at a frequency of 15% to 20%.[22-25,27] Of interest, approximately 40% of relapse AML had CNN-LOH.[26] CNN-LOH in t(15;17) APL is about half as frequent as the other AMLs. Two CNN-LOH regions occurred in multiple samples: chromosomes 10q (58.2 Mb, 3 cases) and 11p (28.7 Mb, 3 cases). Of note, case no. 39 had both 10q and 11p CNN-LOHs, suggesting that 10q and 11p might contain novel APL-related gene(s). CNN-LOH is a genomic abnormality that

Table 2. Chromosomal alterations in t(15;17) APL samples

Group/case no.	Status	Location	Physical localization		Size, Mb	Gene(s) in the region
			Proximal	Distal		
+8						
65	Del	4q28.1	125 190 507	126 521 903	1.33	*KIAA1223*
	Del	7q21.11-q21.12	85 414 972	86 445 002	1.03	*GRM3, KIAA1324L,* and *DMTF1*
	Dup	8q24.13-q24.22	122 607 785	132 092 760	9.48	> 10 genes including *MYC*
	Del	9q12-q31.3	64 207 745	111 479 523	47.27	> 10 genes
38	Tri	Trisomy 8				
2	Tri	Trisomy 8				
	Del	10q21.2-q21.3	62 496 958	68 046 104	5.55	>10 genes
50	Del	6p25.1-p24.3	5 545 437	8 054 930	2.51	> 10 genes
	Tri	Trisomy 8				
60	Tri	Trisomy 8				
3	Tri	Trisomy 8				
	CNN-LOH	11p.ter-p11.12	1 938 894	49 879 899	47.9	> 10 genes including *WT1, CDKN1C* and *HRAS*
39	Tri	Trisomy 8				
	CNN-LOH	10q21.1-q-ter	59 576 047	135 228 726	75.7	> 10 genes including *PTEN* and *FGFR2*
	CNN-LOH	11p.ter-p14.1	1 938 894	30 627 880	28.7	> 10 genes including *CDKN1C* and *HRAS*
18	Tri	Trisomy 8				
	CNN-LOH	10q22.2-q-ter	76 995 152	135 228 726	58.2	> 10 genes including *PTEN* and *FGFR2*
Other						
66	CNN-LOH	10q22.2-q-ter	76 289 513	135 295 604	59.0	> 10 genes including *PTEN* and *FGFR2*
20	CNN-LOH	11p.ter-p11.12	1 938 894	49 330 228	47.4	> 10 genes including *WT1, CDKN1C* and *HRAS*
13	Dup	13q21.1-q-ter	56 784 440	114 051 465	57.27	> 10 genes
	Dup	15q22.2-q-ter	57 244 668	100 182 183	42.94	> 10 genes including *PML*
4	CNN-LOH	19q13.2-q-ter	46 160 099	63 437 743	17.3	> 10 genes
37	Del	1q42.2	227 843 862	227 867 765	0.02	*EGLN1*
	Dup	18p11.31-p11.23	7 192 739	7 657 575	0.46	*PTPRM*
57	Dup	9q22.32	94 435 025	94 710 006	0.27	*FBP2, FBP1,* and *C9orf3*
19	Del	12p13.31-p11.22	6 755 671	29 248 257	22.49	> 10 genes including *ETV6* and *CDKN1B*
	Del	13q14.2-q14.3	49 630 676	50 510 777	0.88	*FAM10A4, DLEU7, FLJ11712,* and *GUCY1B2*
21	Dup	1q21-q-ter	142 487 224	245 120 412	102.63	> 10 genes
	Del	7q11.21-q-ter	61 522 282	158 554 645	97.03	> 10 genes
43	Dup	21q22.12-q-ter	36 234 195	46 924 583	10.69	>10 genes including *ERG*
52	Tri	Trisomy 21				
58	Dup	15q24.1-q-ter	72 224 840	100 192 115	27.97	> 10 genes
	Del	17p-ter-p11.2	18 901	21 459 693	21.44	> 10 genes including *TP53*
	Dup	17p11.2-q21.1	21 491 135	35 542 587	14.05	> 10 genes including *NF1* and *ERBB2*

Physical localization, size (Mb), and gene(s) at the chromosomal regions were obtained from UCSC Genome Browser. If known gene(s) in the chromosomal regions are less than 10, all gene names are displayed.

Copy number changes as previously described as copy number variant at Database of Genomic Variants (http://projects.tcag.ca/variation/)[29] and UCSC Genome Browser (http://genome.ucsc.edu/)[32] were excluded.

Del indicates deletion; Dup, duplication; Tri, trisomy; ter, terminal; and CNN-LOH, copy-number-neutral loss of heterozygosity.

normally cannot be detected by conventional cytogenetic analysis. These regions usually contain a mutation of a key gene. For example, a constitutively active form of either *JAK2* V617F mutant, *FLT3*-ITD, *AML1/RUNX1* frameshift, and/or mutations of *WT1* and *NPM1* were found in CNN-LOH regions in AML.[22-25] CNN-LOH regions identified in this study contain genes coding for several tyrosine kinase and/or tumor suppressors. Further studies are required to identify the key dysregulated gene(s) in these regions. In addition to CNN-LOH, we also found several copy-number changes that may be sites containing novel disease-related genomic regions in t(15;17) APL. Although we cannot rule-out copy-number variants (CNVs) at several sites, we think it is unlikely. We had 7 genomic DNA at complete remission samples and confirmed for each that the chromosomal changes were only in the leukemia cells. Furthermore, for each of these sites, we interrogated a collated library of CNVs (Database of Genomic Variants and UCSC Genome Browser) to assure that these regions were not known CNVs.

FLT3 is a tyrosine kinase receptor involved in normal hematopoiesis, and mutations of the gene often occur in AML. Incidence of *FLT3*-ITD and *FLT3*-TKD was 23% and 26% in our samples, respectively. Experiments have shown that *FLT3*-ITD and *FLT3*-TKD have differences in their downstream signaling.[33-35] Interestingly, bone marrow transplantation in mice showed that *FLT3*-ITD induced an oligoclonal myeloproliferative disease,[33] whereas *FLT3*-TKD produced an oligoclonal lymphoid disorder with a long latency.[34] Furthermore, only *FLT3*-ITD caused activation of STAT5 and repression of C/EBPα and PU.1.[34,35]

Here, t(15;17) APL samples were divided into 3 groups based on genomic status detected by SNP-chip analysis: normal-copy-number group (NC group, 28 samples); trisomy 8 group (+8 group, 8 samples); and other abnormalities group (11 samples). Notably, our subclassifications did reveal an interesting relationship between genomic status and *FLT3* mutation. Eleven samples of NC group (39% of the NC group samples) had *FLT3*-ITD, whereas no *FLT3*-ITD occurred in samples from the other 2 groups. In contrast, one good candidate gene in the +8 cohort is the oncogene *MYC*. In fact, case no. 65 had duplication localized to 8q24.13-q24.22 that included the

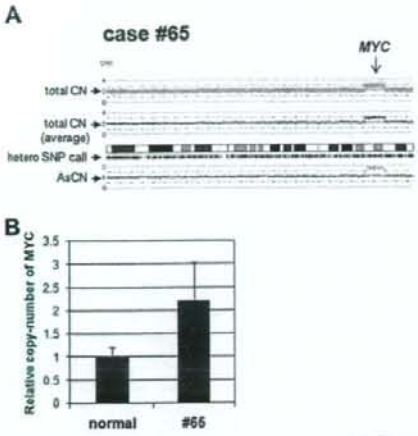

Figure 2. Validation of copy-number change in case no. 65. (A) SNP-chip data of chromosome 8 in case no. 65. Top dots are SNP sites as probes and indicate total copy number (CN). Middle line is an average of the copy number and shows gene dosage. Bars are heterozygous (hetero) SNP calls. Bottom 2 lines show allele-specific copy number (AsCN). (B) Duplication of the *MYC* gene region in case no. 65. Copy number of the *MYC* gene in case no. 65 was compared with normal genomic DNA with quantitative genomic real-time PCR. Level of the copy number was determined as a ratio between the *MYC* gene and the reference genomic region 2p21. Results represent mean of 3 experiments plus or minus SD.

MYC gene. Previous karyotype analysis showed that the PL-21 cell line, which was established from an APL patient, had a polyploid male karyotype with 13q+ chromosome, but a translocation between chromosome 15 and 17 was not identified.[36] NB4 cells are cytogenetically very complex, with a hypotetraploid karyotype and multiple chromosomal alterations.[37,38] Our SNP-chip analysis of NB4 cells also showed ploidy = 3.67, indicating that the karyotype is hypotetraploid. Of interest, NB4 cells have amplification of the *MYC* gene. Importantly, expression of c-Myc mRNA is stimulated by *FLT3*-ITD[39,40]; and AML samples with *FLT3*-ITD have increased expression of c-Myc mRNA compared with normal bone marrow.[41] These data indicate that the *MYC* gene may be dysregulated by either copy-number change or *FLT3*-ITD as a secondary abnormality to enhance the development of APL. Of course, our sample population is small, therefore, additional studies are needed to confirm these findings.

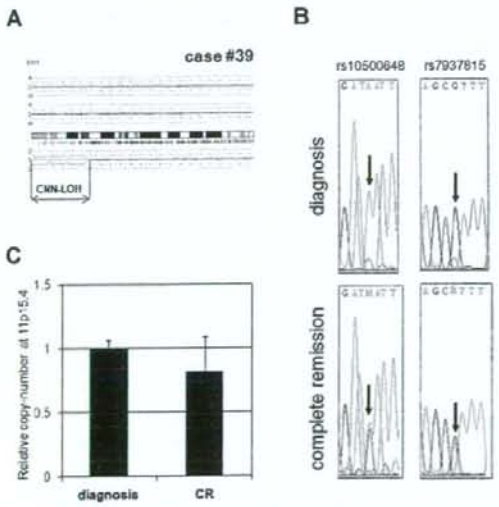

Figure 3. Validation of CNN-LOH in case no. 39. (A) SNP-chip data of chromosome 11 in case no. 39. The samples had CNN-LOH on chromosome 11 (11p-terminal–p14.1, 28.7 Mb). (B) Determination of SNP sequence in 11p CNN-LOH region in case no. 39. 2 SNP sites (rs10500648 and rs7937815) were sequenced. Both SNP sites showed heterozygosity in the complete remission sample, whereas they showed homozygosity in the diagnosis sample. (C) Determination of copy number in the 11p15.4 region. Copy number of 11p15.4 (CNN-LOH region) in case no. 39 at diagnosis was compared with complete remission (CR) sample with quantitative genomic real-time PCR. Levels of the copy number were determined as a ratio between 11p15.4 and the reference genomic DNA, 2p21. Results represent the mean of 3 experiments plus or minus SD.

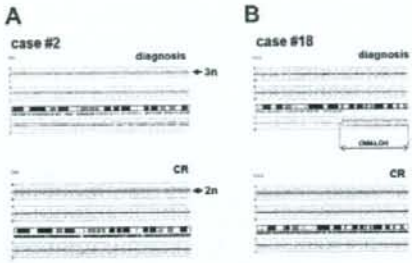

Figure 4. Comparison of chromosomal changes between diagnosis and complete remission samples. (A) Trisomy 8 in case no. 2. Case no. 2 had trisomy 8 at diagnosis, whereas chromosome 8 was 2n at complete remission (CR). (B) CNN-LOH in case no. 18. Case no. 18 had CNN-LOH in chromosome 10 (10q22.2–q-terminal, 58.2 Mb), and the alteration was not present in the matched CR sample.

Our APL cohort "other abnormalities" group has neither *FLT3*-ITD nor trisomy 8, but has several other genomic changes including deletion of *ETV6/TEL* (case no. 19) and duplication of *ERG* (case no. 43). ETV6/TEL is a transcriptional repressor, and approximately 30% of AML patients have loss of expression of ETV6/TEL protein.[42,43] In addition, mutation of the *ETV6/TEL* gene occurs in approximately 2% of AML samples, and these mutants behaved in a dominant-negative fashion.[43] ERG is a member of the ETS family of transcription factors and is a proto-oncogene. Overexpression of ERG predicts a worse outcome in AML with normal karyotype.[44] Taken together, our observed copy-number changes in these regions may be involved in development of APL.

Our findings extend those of Le Beau et al[13] who recently reported elegant models of APL using transgenic mice coexpressing PML-RARα and either BCL2, IL3, activated IL3R, or activated murine FLT3 ($FLT3^{W51}$). *PML-RARα/BCL2* mice developed leukemia, and these cells had a complex karyotype including trisomy 15 (100% of these mice), where the oncogene *MYC* is located. In contrast, *PML-RARα/*$FLT3^{W51}$ mice develop

Table 3. Relationship between chromosomal abnormality and *FLT3* mutations

	Group			Total (%), 47 (100%)
	NC, 28 (60%)	+8, 8 (17%)	Other, 11 (23%)	
*FLT3*WT	11	7	6	24 (51)
*FLT3*TKD	6	1	5	12 (26)
*FLT3*ITD	11	0	0	11 (23)

Mutational status of the *FLT3* gene is shown.

NC indicates normal-copy-number; +8, trisomy 8 or duplication of the *MYC* gene region; other, other abnormalities; and WT, wild-type.

leukemia, and these cells had normal karyotype except for trisomy of either chromosomes 8 (29%), 10 (43%), or 15 (43%), and monosomy X (86%). These models suggest that different cooperating events are involved in the development of murine APL. Taken together, these findings strongly suggest that the pathway of development of APL differs in each of our cohorts; *FLT3*-ITD, *MYC*, and unknown factor(s) are involved in the development of APL; and these finding should facilitate the screening for novel therapeutic targets in each case.

Further studies in a larger cohort of patients will begin to stratify prognostically the APL patients in relation to the genomic changes of their leukemic cells; and new therapeutic targets, which are involved in the development of APL, should be discovered.

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Authorship

Contribution: T.A. performed research, analyzed the data, and wrote the paper; M.K., S.O., G.Y., and M.S. performed SNP-chip analysis and developed CNAG; N.K., R.O., and C.W.M. assisted in data analysis; L.-Y.S. and D.-C.L. provided APL samples and clinicohematologic data for all APL patients, and performed *FLT3* mutation analysis; and H.P.K. directed the overall study.

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Frequent genomic abnormalities in acute myeloid leukemia/myelodysplastic syndrome with normal karyotype

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ABSTRACT

Background

Acute myeloid leukemia is a clonal hematopoietic malignant disease; about 45-50% of cases do not have detectable chromosomal abnormalities. Here, we identified hidden genomic alterations and novel disease-related regions in normal karyotype acute myeloid leukemia/myelodysplastic syndrome samples.

Design and Methods

Thirty-eight normal karyotype acute myeloid leukemia/myelodysplastic syndrome samples were analyzed with high-density single-nucleotide polymorphism microarray using a new algorithm: allele-specific copy-number analysis using anonymous references (AsCNAR). Expression of mRNA in these samples was determined by mRNA microarray analysis.

Results

Eighteen samples (49%) showed either one or more genomic abnormalities including duplication, deletion and copy-number neutral loss of heterozygosity. Importantly, 12 patients (32%) had copy-number neutral loss of heterozygosity, causing duplication of either mutant *FLT3* (2 cases), *JAK2* (1 case) or *AML1/RUNX1* (1 case); and each had loss of the normal allele. Nine patients (24%) had small copy-number changes (< 10 Mb) including deletions of *NF1*, *ETV6/TEL*, *CDKN2A* and *CDKN2B*. Interestingly, mRNA microarray analysis showed a relationship between chromosomal changes and mRNA expression levels: loss or gain of chromosomes led, respectively, to either a decrease or increase of mRNA expression of genes in the region.

Conclusions

This study suggests that at least one half of cases of normal karyotype acute myeloid leukemia/myelodysplastic syndrome have readily identifiable genomic abnormalities, as found by our analysis; the high frequency of copy-number neutral loss of heterozygosity is especially notable.

Key words: normal karyotype acute myeloid leukemia/myelodysplastic syndrome, SNP-chip, CNN-LOH.

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