unknown whether patients who progressed from IE to PV had the *JAK2* V617F mutation.

More recently, Scott et al. and Williams et al. [15, 21] reported that the novel *JAK2* mutations in exon 12 and *MPL* gene mutations were detected in *JAK2* V617F-negative PV and IE. Therefore, we performed direct sequence analysis on exon 12 of the *JAK2* gene, and there was no mutation detected in any of the 11 IE patients. The previous papers as well as our findings suggest that IE is a heterogeneous disorder.

In conclusion, *JAK2* V617F mutation is a rare event in IE in contrast to the incidence of PV. The majority of IE patients may have a genetic lesion different from PV, and the molecular mechanism responsible for IE remains unclear. With respect to the V617F mutation, IE may not be the first stage of PV. However, in our study, a small proportion of V617F mutation-positive cells were detected in one patient with IE. A further longitudinal study monitoring V617F positive-cells will clarify the process of progression from IE to PV in such a patient.

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Short Communication

Infrequent V617F mutation of the *JAK2* gene in myeloid leukemia and its absence in lymphoid malignancies in Japan

Naoki Mori[1], Kentaro Yoshinaga[1], Makiko Tada[1,2], Yanhua Wang[1], Masayuki Shiseki[1] and Toshiko Motoji[1]

[1]*Department of Hematology, Tokyo Women's Medical University, Tokyo, Japan.*
[2]*Meiji Pharmaceutical University, Tokyo, Japan.*

Abstract

A unique mutation of the *JAK2* gene, V617F, has recently been identified in polycythemia vera, essential thrombocythemia and myeloid metaplasia with myelofibrosis. To determine the relevance of this mutation in other types of hematological neoplasms in Japan, we performed allele-specific polymerase chain reaction analysis on the *JAK2* gene. The V617F mutation was detected in one out of 130 myeloid neoplasms, but in none of 114 lymphoid malignancies and four biphenotypic acute leukemias. Although a favorable chromosomal alteration t(8;21)(q22;q22) was observed in one acute myeloid leukemia (AML) patient with the mutation, two courses of chemotherapy resulted in induction failure and short survival. Sequencing of *JAK2* cDNA revealed expression of the mutant allele in the patient. The V617F mutation might play a role in the pathogenesis of certain AML cases.

Key words: JAK2 gene, V617F mutation, signal transduction, acute myeloid leukemia, lymphoid malignancies.

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Myeloproliferative disorders (MPD) comprise a heterogeneous group of diseases including chronic myelocytic leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and myeloid metaplasia with myelofibrosis (MMM). With the exception of CML, characterized by the Philadelphia chromosome and the *BCR-ABL* fusion gene, genetic events causing these disorders have remained unidentified for a long time. The *Janus kinase 2 (JAK2)* gene encodes a tyrosine kinase involved in cytokine signal transduction. JAK phosphorylates cytoplasmic targets including signal transducers and activators of transcription (STAT). In hematological neoplasms, several chromosomal translocations involving the *JAK2* gene locus have been identified (Khwaja, 2006). Recently, a unique mutation of the *JAK2* gene, G to T transversion at nucleotide 1849 resulting in valine to phenylalanine substitution at amino acid position 617 (V617F), has been identified in MPD (Baxter *et al.*, 2005; James *et al.*, 2005; Kralovics *et al.*, 2005; Levine *et al.*, 2005a). This mutation was present in most patients with PV and in half of the patients with ET and MMM. Since the V617F mutation is located in the JH2 negative regulatory domain of the *JAK2* gene, it disrupts the auto-inhibitory activity of JAK2. In consequence, the mutation leads to constitutive tyrosine phosphorylation activity, promoting cytokine hypersensitivity and inducing erythrocytosis in a mouse model (James *et al.*, 2005).

Send correspondence to Naoki Mori. Department of Hematology, Tokyo Women's Medical University, 8-1 Kawada-cho, 162-8666 Shinjuku-ku, Tokyo, Japan. E-mail: mori@dh.twmu.ac.jp.

Recent studies have shown the V617F mutation to be present in some cases of acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and chronic myelomonocytic leukemia (CMMoL) (Jelinek *et al.*, 2005; Levine *et al.*, 2005b). However, limited information is available to determine whether the mutation is specifically associated with myeloid neoplasms, and most of the studies are from Europe and North America. In order to assess the range and frequency of the mutation in Japanese patients with hematological neoplasms, we performed allele-specific polymerase chain reaction (PCR) analysis on the *JAK2* gene.

Bone marrow, peripheral blood or lymph node samples from 248 hematological neoplasms were analyzed after obtaining written informed consent (Table 1). Of the 248 samples, 130 were myeloid neoplasms, 114 were lymphoid neoplasms, and four were biphenotypic acute leukemias (BAL). The current study was conducted within the guidelines and with the approval of the institutional review board. The primer sequences for allele-specific PCR were previously published (Baxter *et al.*, 2005):

ALLF-S, 5'-AGCATTTGGTTTTAAATTATGGAGTATATT-3';

ALLF-IC, 5'-ATCTATAGTCATGCTGAAAGTAGGAGAAAG-3'; and

Table 1 - *JAK2* V617F mutations in hematological neoplasms.

	Number of samples	Number of V617F myeloid malignancies
Myeloid malignancies		
AML	38	1
MDS	38	0
RAEB-t[1]	2	0
MDS/AML	16	0
CML CP[2]	18	0
CML AP[3]	1	0
CML BC[4]	11	0
CMMoL	5	0
aCML[5]	1	0
Lymphoid malignancies		
MM	48	0
MGUS[6]	2	0
ALL	40	0
CLL	7	0
ML	8	0
Macroglobulinemia	2	0
ATL	7	0
Biphenotypic acute leukemia	4	0
Total	248	1

[1]RAEB-t, refractory anemia with excess of blasts in transformation; [2]CP, chronic phase; [3]AP, accelerated phase; [4]BC, blast crisis; [5]aCML, atypical chronic myeloid leukemia; [6]MGUS, monoclonal gammopathy with undetermined significance.

ALLR-S, 5'-CTGAATAGTCCTACAGTGTTTTC AGTTTCA-3'.

ALLF-S is specific for the mutant allele and contains an intentional mismatch at the third nucleotide from the 3' end, to improve specificity. After 5 min at 94 °C, 36 amplification cycles of 60 s at 94 °C, 60 s at 58 °C and 60 s at 72 °C were performed, with a subsequent 7 min extension at 72 °C. Electrophoresis was repeated two or three times in each sample, using independent PCR products. Primers ALLF-S and ALLR-S amplify 203-bp (base pair) products, while the size of products using primers ALLF-IC and ALLR-S is 364 bp. The 203-bp products indicated the V617F mutation, while the 364-bp products indicated the internal control. PV samples with the V617F mutation were used as positive control. Aberrant bands were detected in one of the 248 samples (AML02, Figure 1 lane 5).

To confirm the aberrant bands detected by allele-specific PCR, DNA from AML02 was subsequently sequenced in both directions on a MegaBase sequence system (Amersham, Buckingham, UK). PCR products were purified and ligated into pGEM-T vector (Promega, Madison, WI, USA). Sequencing revealed G to T transversion at nucleotide 1849, resulting in the V617F mutation (Figure 2). Several samples without aberrant bands by allele-specific

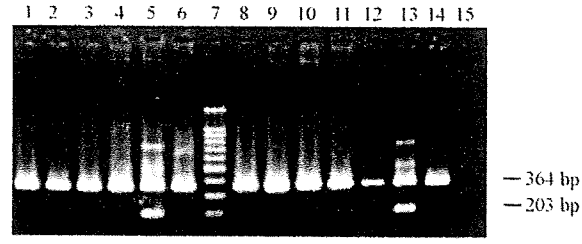

Figure 1 - Allele-specific PCR analysis of the *JAK2* gene in hematological neoplasms. Ten of 20 µL of PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide and visualized by ultraviolet illumination. The size of the products is indicated on the right. Aberrant bands were detected in one of the AML samples (lane 5). Lane 1, AML01; lane 2, MDS01; lane 3, ALL01; lane 4, BAL01; lane 5, AML02; lane 6, AML03; lane 7, 100 bp ladder; Lane 8, ALL02; lane 9, AML04; lane 10, AML05; lane 11, AML06; lane 12, CLL01, lane 13, PV01; lane 14, CML01, lane 15, water. Aberrant bands of PV01 are shown as positive control (lane 13).

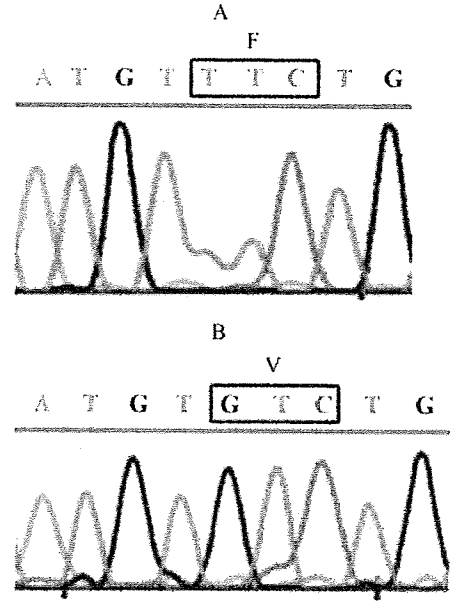

Figure 2 - Sequence analysis of the *JAK2* gene in AML. Sequencing identified G to T transversion at nucleotide 1849, resulting in the V617F mutation in the AML sample (A, AML02). Wild-type sequences are shown as control (B, MM01).

PCR were also sequenced, but only wild-type sequences were obtained.

To determine the expression of the JAK2 V617F mutation, we performed reverse transcriptase-PCR (RT-PCR) analysis in AML02 (Mori *et al.*, 1990). The following sequences were used for the primers: RT-11F1, 5'-AAAGC CTTGGCCAAGGCACTT-3'; and RT-13R2, 5'-ATGCA TGGCCCATGCCAACTG-3'. After 5 min at 94 °C, 30 amplification cycles of 60 s at 94 °C, 60 s at 55 °C and 60 s at 72 °C were performed, with a subsequent 10 min extension at 72 °C. Primers RT-11F1 and RT-13R2 amplify

340-bp products. Sequencing of *JAK2* cDNA revealed the expression of the mutant allele in 20 out of 41 AML02 clones.

The V617F mutation of the *JAK2* gene was found in one of the 130 myeloid neoplasms, but not in the 114 lymphoid malignancies or in the four biphenotypic acute leukemias. The mutation was detected in the majority of Japanese patients with PV and ET (K Yoshinaga, N Mori, Y Wang, M Shiseki, T Motoji, unpublished data). In contrast to classic MPD, the V617F mutation was infrequent in myeloid leukemia and absent in lymphoid malignancies. Recent reports show that the JAK2 V617F mutation was not identified in either acute lymphoblastic leukemia (ALL) or chronic lymphocytic leukemia (CLL) (Levine *et al.*, 2005b). Our study showed that also in Japan the V617F mutation is absent in ALL and CLL. Furthermore, it was also undetectable in adult T-cell leukemia (ATL), macroglobulinemia, multiple myeloma (MM), and malignant lymphoma (ML).

AML02 was obtained from a patient with AML showing M2 morphology according to the French-American-British (FAB) classification at diagnosis. This patient was a 70-year-old woman with no history of preceding MPD or MDS. Peripheral blood tests revealed 50.1 x 10^9/L leukocytes with 76.5% blast cells, 7.8 g/dL hemoglobin, and 55 x10^9/L platelets. Although the favorable chromosomal alteration t(8;21)(q22;q22) accompanied by *AML1/ETO* transcripts was the only chromosomal abnormality observed in the patient, two courses of chemotherapy resulted in induction failure and short survival. The mutation was found in nine out of 14 AML02 clones. Since this sample was expected to contain more than 90% blast cells after mononuclear cell isolation, this case may have been heterozygous for the V617F mutation. Another possibility is that a substantial proportion of the leukemic cells from AML02 did not harbor the mutation.

The V617F mutation was detected in one of the 38 patients with AML (3%). Other recent studies found a similar incidence of the V617F mutation: 0/17 (Jones *et al.*, 2005), 5/90 (6%) (Scott *et al.*, 2005), 2/39 (5%) (Jelinek *et al.*, 2005), 4/222 (2%, three had preceding MPD) (Levine *et al.*, 2005b), 1/152 (0.7%) (Frohling *et al.*, 2006), and 2/112 (2%) (Lee *et al.*, 2006). The V617F mutation in AML was initially found in FAB M6 (1/53) (Frohling *et al.*, 2006) and FAB M7 (2/11) (Jelinek *et al.*, 2005), while the FAB subtype of AML was not described in other reports. In the current study, this mutation was also found to be present in one out of 12 patients with AML M2 (8%): one of the four patients with t(8;21)(q22;q22). After submission of our paper, expression of the JAK2 V617F mutation was reported in two out of 18 MDS/AML but not in 198 *de novo* AML cases (Nishii *et al.*, 2007). In contrast to their result, in the present study expression of the mutant allele was detected in AML02, as described above.

Our study revealed that the V617F mutation is infrequent in myeloid leukemia and absent in lymphoid malignancies in Japan. Nevertheless, it was detected in AML02, along with the favorable cytogenetic alteration t(8;21)(q22;q22). Previous studies have demonstrated that additional event(s) are required for the development of AML in the presence of t(8;21)(q22;q22) (Yuan *et al.*, 2001; Kuchenbauer *et al.*, 2006). Tyrosine kinases have been involved in several disorders including BCR-ABL in CML, PDGFRβ in CMMoL, and PDGFRα in chronic eosinophilic leukemia. Furthermore, activating mutations in the *FLT3* receptor tyrosine kinase gene are the most common genetic events in AML. Patients with FLT3 mutations have a poor prognosis, suggesting the important role of tyrosine kinase gene mutations in leukemogenesis. The relation between the V617F mutation and short survival in AML02 is unclear. The V617F mutation might confer a proliferative advantage to blast cells and play a role in the pathogenesis of certain AML cases. As suggested for FLT3 mutations, constitutive signaling in the absence of ligand may result in reduced apoptosis of the leukemic cells or may grant increased repair capacity following cell damage; either of these mechanisms could be considered as inducing chemoresistance (Kottaridis *et al.*, 2001). Further studies will clarify the incidence and significance of the V617F mutation in AML with t(8;21)(q22;q22).

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ORIGINAL ARTICLE: RESEARCH

Leukemic cells with increased telomerase activity exhibit resistance to imatinib

OSAMU YAMADA[1,2], KIYOTAKA KAWAUCHI[3], MASAHARU AKIYAMA[4], KOHJI OZAKI[5], TOSHIKO MOTOJI[1], TOMOKO ADACHI[6], & EIZO AIKAWA[7]

[1]*Department of Hematology,* [2]*Medical Research Institute, and* [3]*Department of Medicine, Tokyo Women's Medical University, Tokyo, Japan,* [4]*Department of Pediatrics, Jikei University School of Medicine, Japan,* [5]*IREIIMS,* [6]*Department of Gynecology, and* [7]*Department of Anatomy and Developmental Biology, Tokyo Women's Medical University, Tokyo, Japan*

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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.

Correspondence: Osamu Yamada, Medical Research Institute, Hematology, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 162-8666 Japan.
E-mail: yamadao@lab.twmu.ac.jp

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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

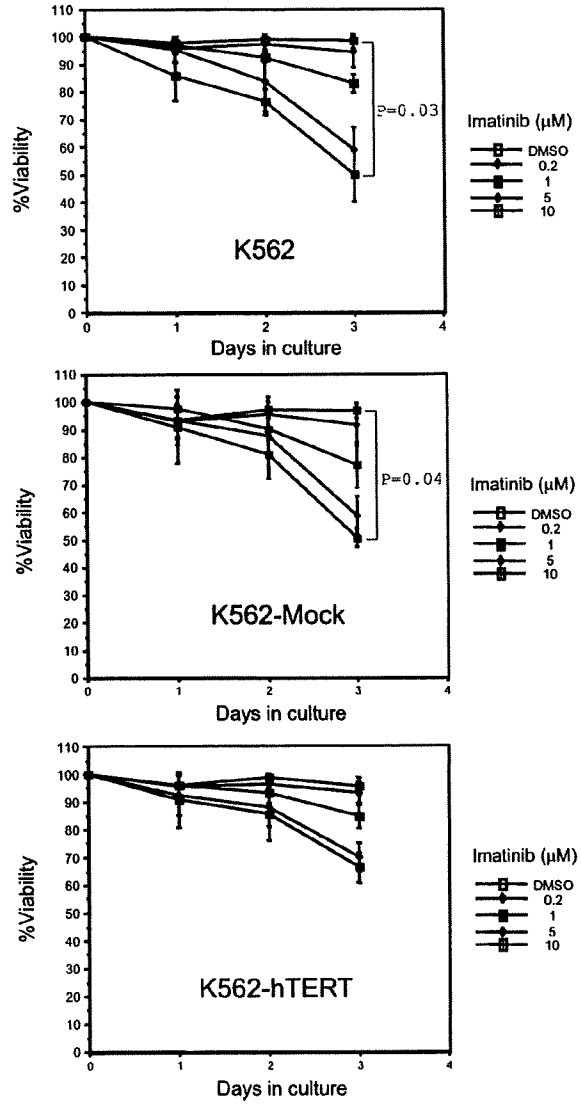

Figure 1. Effect of imatinib on the proliferation of K562-*hTERT*, K562-Mock and parental K562 cells. Cells at a density of 2×10^5/mL were cultured for 72 h with various concentrations of imatinib. Control cultures were treated with the highest DMSO concentration (0.02%). The inhibitory effect of imatinib 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,

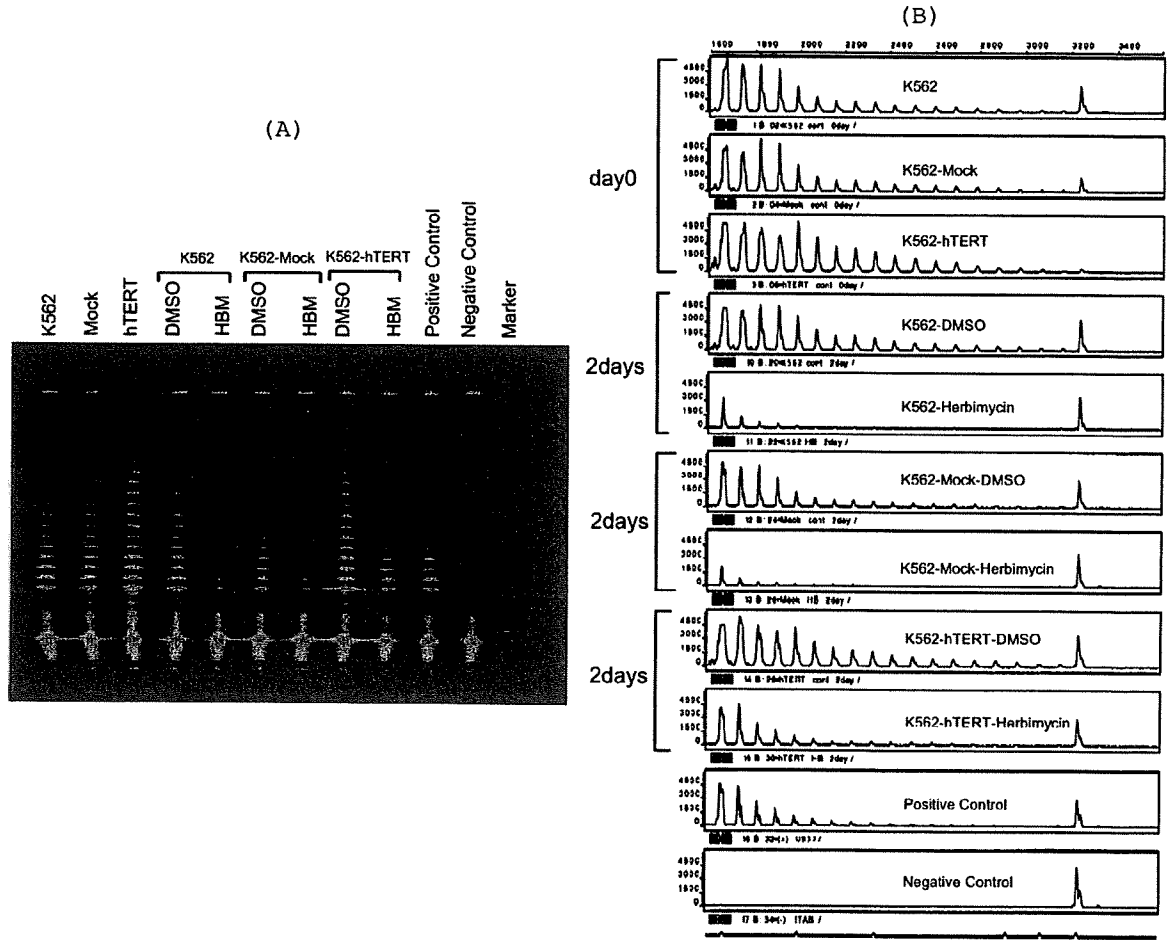

Figure 2. Effect of herbimycin A on telomerase activity. Cell extracts obtained from K562-*hTERT* cells, K562-Mock control cells and parental K562 cells were analyzed by [F]-TRAP assay following incubation with 0.8 μM herbimycin A. (A) The characteristic 6-bp DNA ladder of telomerase products is represented 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.

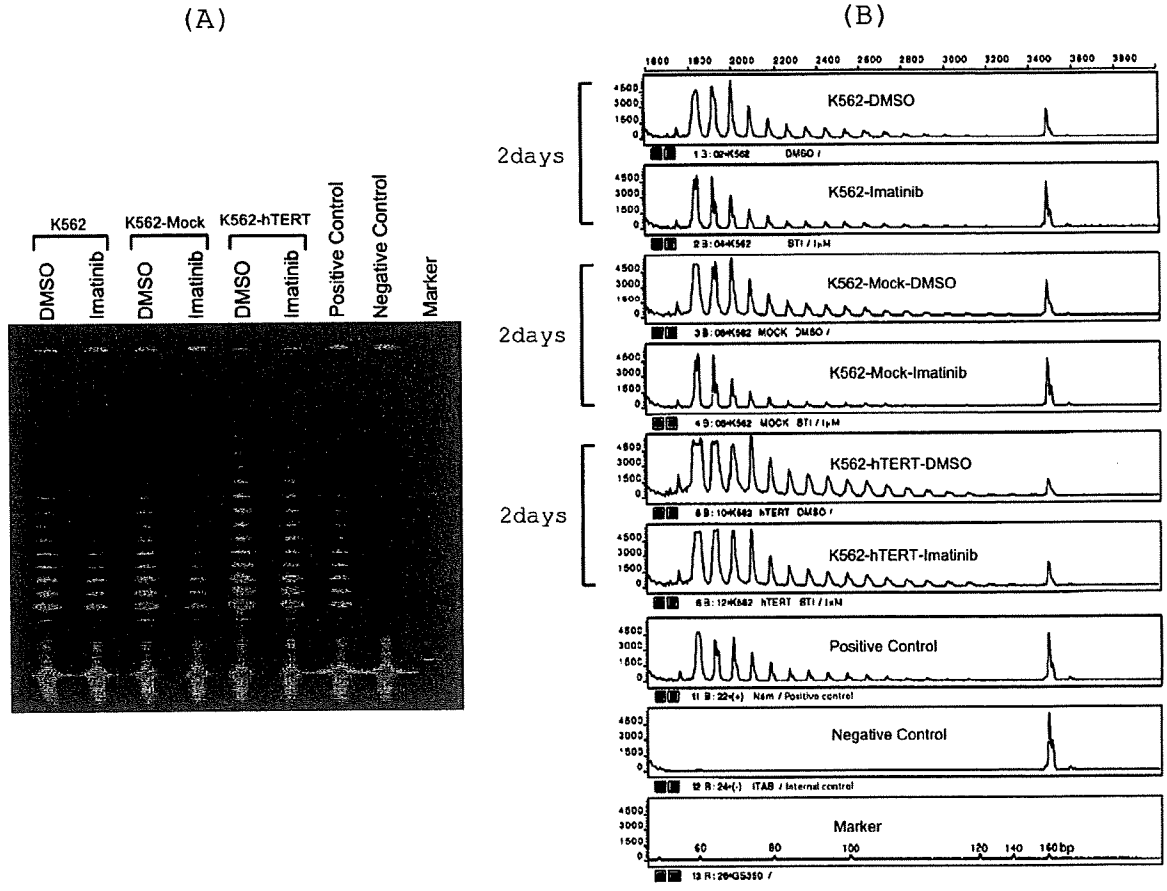

Figure 3. The effects of imatinib on telomerase activity. Cell extracts obtained from K562-*hTERT* cells, K562-Mock control cells and parental K562 cells were analyzed by F-TRAP assay following incubation with 1 μM imatinib. (A) Downregulation of endogenous telomerase activity in all three cell lines. (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

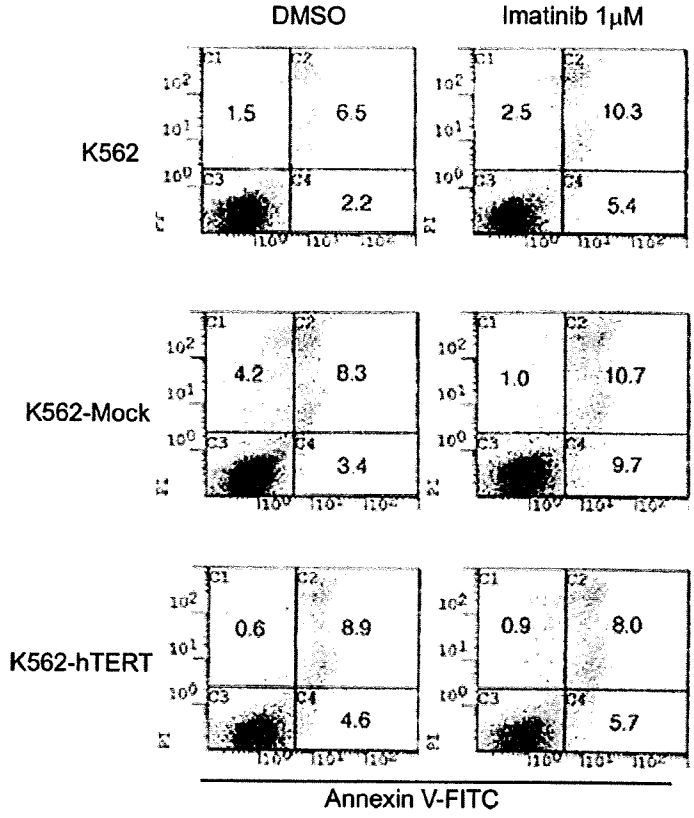

Figure 4. Detection of apoptosis in imatinib-treated cells by the annexin V/PI assay. K562-*hTERT*, K562-Mock and parental K562 cells were incubated with 1 μM of imatinib for 48 h and analyzed by flow cytometry. Live cells are distinguished as unstained with fluorochrome, apoptotic cells are stained 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

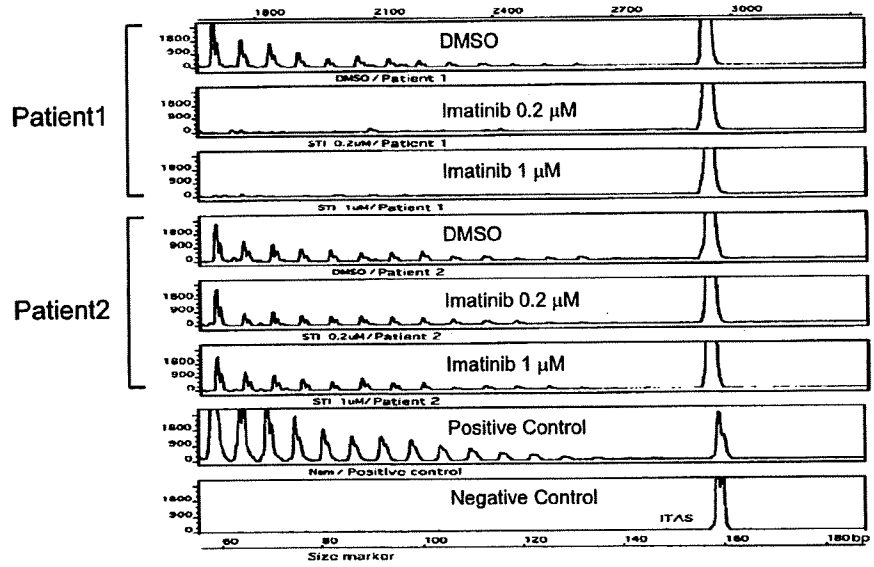

Figure 5. Effects of imatinib on telomerase activity in primary leukemic cells from patients with CML. Leukemic cells were isolated from six patients in the chronic phase and four patients in blastic crisis. Primary leukemic cells were incubated in suspension culture in the presence of 0.2 or 1 μM imatinib 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.

	Relative telomerase activity (% control)		Fold changes in mRNA relative to that of K562 cells		
Patient	CP/BC	ST1571(0.2 μM/1 μM)	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.

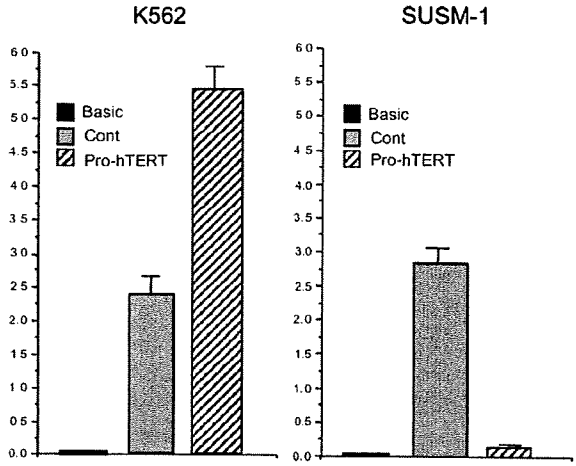

Figure 6. *hTERT* promoter activity in K562 and SUSM-1 Cells. The 1.2 Kb *hTERT* promoter sequence upstream of the initiating ATG was cloned into the plasmid PGL3-Basic (Promega) in a sense orientation and examined using the Dual luciferase reporter assay system. After 48 h of transfection, luciferase 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

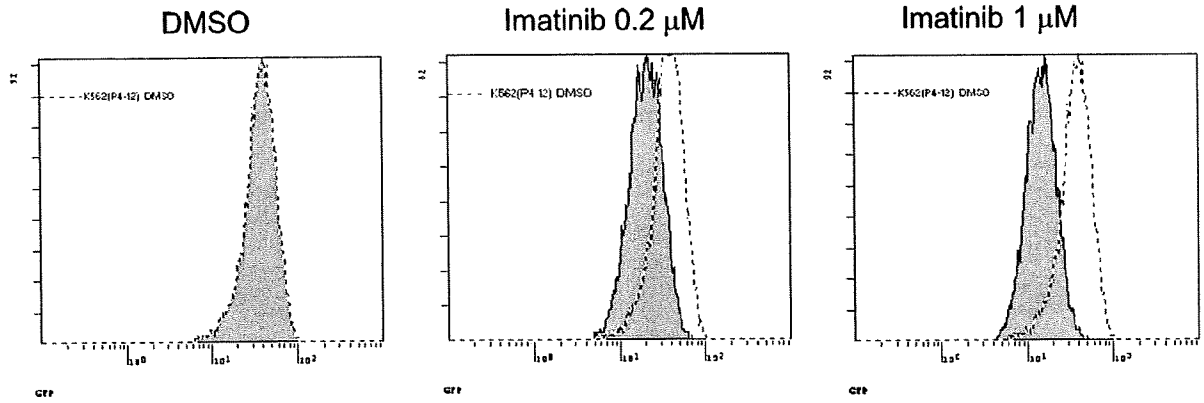

Figure 7. Imatinib-induced suppression of *hTERT* transcriptional activity. The 1.2-kb *hTERT* promoter sequence was cloned into the plasmid pEGFP1 (Clontech) and K562 cells with stable GFP expression were obtained. Promoter activity was evaluated by flow cytometric analysis of GFP expression in K562 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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Genetic profiling of myeloproliferative disorders by single-nucleotide polymorphism oligonucleotide microarray

Norihiko Kawamata[a,*], Seishi Ogawa[b,*], Go Yamamoto[b], Soren Lehmann[a], Ross L. Levine[c], Yana Pikman[c], Yasuhito Nannya[b], Masashi Sanada[b], Carl W. Miller[a], D. Gary Gilliland[c], and H. Phillip Koeffler[a]

[a]*Hematology/Oncology, Cedars-Sinai Medical Center/UCLA School of Medicine, Los Angeles, Calif., USA;* [b]*Regeneration Medicine of Hematopoiesis, University of Tokyo, School of Medicine, Tokyo, Japan;* [c]*Hematology, Brigham and Women's Hospital, Harvard University, School of Medicine, Boston, Mass., USA*

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Objective. **Myeloproliferative disorders (MPD) are clonal hematopoietic diseases that include polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF). Mutations in *JAK2* are present in many MPD patients. Additional genomic abnormalities are not fully examined in MPD.**

Materials and Methods. **We used single-nucleotide polymorphism DNA microarray (SNP-chip) to analyze 43 patients with MPD (10 PV, 17 ET, and 16 PMF) for genomic aberrations.**

Results. **Genomic abnormalities were rare in ET. The region containing either *RB* (13q14) or *NF1* (17q11) was deleted in 4 of the 16 PMF, especially PMF with no *JAK2* mutations. All five cases of PV having homozygous *JAK2*V617F had loss of heterozygosity with normal copy number [uniparental disomy] involving the gene. A subpopulation with 9p uniparental disomy was detected in 11 MPD (3 PV, 1 ET, 7 PMF). Uniparental disomy at 1p was found in one PV and three PMF. A novel mutation of *MPL* (Y591D), which was involved in this uniparental disomy, was found in 1 PV with *JAK2* mutation. The other three cases of PMF with 1p uniparental disomy had point mutations of the *MPL* gene, either a novel mutation (S204F) or the previously described W515L.**

Conclusion. **Genomic abnormalities, including 9p uniparental disomy/*JAK2* point mutations, 1p uniparental disomy/*MPL* point mutations, deletions of *RB1* and *NF1* are common alterations in MPD, especially in PMF.** © **2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Myeloproliferative disorders (MPD) are clonal hematopoietic diseases that are classified based on their clinical features into subtypes that include polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF) [1–3]. Recently, activating point mutations of *JAK2* kinase in exon 14 (V617F) [4–8] and exon 12 [9] have been detected in MPDs. These alterations transform JAK2 into a constitutively active kinase, leading to dysregulated proliferation of hematopoietic cells. *JAK2*V617F is detected in >90% of cases of PV and ~50% of cases of ET and PMF [4–8]. Further, a point mutation of the *MPL* gene (W515L) has been detected in PMF [10,11]. This point mutation also activates Jak2/Stat signal transduction and induces proliferation of hematopoietic cells and an MPD phenotype in murine models of disease [10].

Nevertheless, clonality analysis suggests that, at least in some cases, the *JAK2*V617F allele is acquired as a secondary event [12]. Thus, further genetic analysis is required to understand the pathophysiology of *JAK2*V617F-negative cases of ET and PMF, and to identify other acquired somatic mutations that contribute to disease pathogenesis in collaboration with *JAK2*V617F.

Recently, genomic microarray has been developed in which oligonucleotide probes corresponding to 50,000 to 1,000,000 chromosomal locations over the whole genome are placed on small glass plates. DNA extracted from cancer cells and normal cells are fragmented and labeled with fluorescence. The fluorescence-labeled DNA fragments are hybridized on the microarray, and the amount of DNA

*Drs. Kawamata and Ogawa contributed equally contributed to this work as the first authors.

Offprint requests to: Norihiko Kawamata, M.D., Hematology/Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, 8700 Beverly Boulevard, Los Angeles, CA 90048; E-mail: kawamatan@cshs.org

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hybridized to each probe is measure on the scanner, leading to quantification of alleleic dosage at each genomic site. This technique allows detection of subcytogenetic deletions and amplification over the genome. Further, new screening platforms using arrayed oligonucleotide probes that contain single-nucleotide polymorphisms (SNP), so-called "SNP-chip," have been developed [13–15]. All probes on the SNP-chip are oligonucleotides containing SNPs and two types of probes (alleles A and B) are placed at each genomic locus. The probes are designed so that one nucleotide difference in two probes (alleles A and B) leads to different hybridization affinity of the probes. Therefore, SNP-chips are able to distinguish each parental allele (allele A or B) at heterozygous sites and to detect sensitively any alleleic imbalance at these sites [13–15]. Because probes on SNP-chips are designed based on reported SNP sequences, this technique cannot detect somatic mutations acquired in cancer cells. This technique allows detection of loss of heterozygosity with neutral allelic dosage [uniparental disomy] that cannot be identified by conventional methods, as well as, gain/loss of genomic materials at very high resolution [13–15]. We employed this technique for analysis of a set of *JAK2*V617F-positive (24 cases) or negative (19 cases) MPD samples to identify novel genomic abnormalities.

Materials and methods

Clinical samples and DNA preparation

Forty-three patients with MPD, including 10 PV (all *JAK2*V617F-positive); 17 ET, including 7 *JAK2*V617F–positive; and 10 *JAK2*V617F-negative cases and 16 PMF, including 10 *JAK2*V617F-negative and 6 *JAK2*V617F-positive cases were enrolled in this study after informed consents were obtained. DNA was extracted from neutrophils in the peripheral blood, using the standard proteinase K-phenol–chloroform extraction method. Bone marrow or peripheral blood cells were cultured in RPMI-1640 medium with 10% fetal bovine serum for 48 hours at 37°C. Chromosomes were G-banded using trypsin and Giemsa; karyotypes were described according to the International System for Human Cytogenetic Nomenclature.

SNP-chip analysis

SNP-chip of GeneChip Human mapping 50 k array XbaI 240 was used for this study (Affymetrix, Tokyo, Japan). Fragmentation and labeling of DNAs were performed using GeneChip resequencing kit (Affymetrix) according to manufacturer's protocols. Hybridization, washing, and signal detection were performed on a GeneChip Fluidics Station 400 and GeneChip scanner 3000 according to manufacturer's protocols (Affymetrix).

To reduce noise, we used "local mean analysis" as described previously [16]. In brief, the sequence of the mean log 2 ratios,

$$ {}^{c}\overline{A}_{i,k}^{test,\,REF} = 1/k \sum_{i}^{i+k-1} {}^{c}A_{i}^{test,\,REF} $$

is calculated where k is the number of terms to be averaged and arbitrarily set from 3 to 10, according to the SD values after compensations of log 2 ratios and optimization of nonreferences for diploid SNPs. In the alternative analysis using a hidden Markov model, the inference of copy number is more efficient and automated, in which a real state of the copy number sequence (a hidden state) along a chromosome is inferred from the observed sequence ${}^{c}\Lambda_{i}^{test,\,REF}$ as the state of maximum likelihood that is calculated from the state transition load and the probability of the hidden state to "emit" the observed sequence of log 2 ratios, using the Viterbi algorithm [16]. We assumed that copy number change is the result of a genetic recombination event between the two adjacent SNP loci, and Kosambi's map function $(1/2)\tanh(2\theta)$ is used to transform the genomic distance, or recombination fraction between the two SNPs (θ) to state "transition probability," where θ is expressed in cM units; for simplicity, 1 cM should be 1 Mbp. The observed log 2 ratio is assumed to follow the normal distribution according to real copy number states, which gives the "emission probability." The variables of normal distribution were empirically determined from the experimental data [16].

Allele-specific PCR for JAK2 mutation

Allele-specific polymerase chain reaction (PCR) was performed according to the method previously reported to detect *JAK2* V617F mutations [4]. All 43 MPD cases were examined using mutation-specific primers and wild-type–specific primers for the *JAK2* gene as reported previously [4]. All PCR products were electrophoresed in 2% agarose gels containing ethidium bromide. PCR bands were visualized under ultraviolet light and photographed.

Quantitative genomic PCR

Quantitative genomic PCR was performed using real-time PCR technique as described previously. Gene dosage of the *NF1* and *RB* was measured [14]. All primers used for the quantitative genomic PCR are listed in Supplementary Table 1.

PCR and sequencing

All coding exons of *FGR1*, *TIE1*, and *MPL* genes were amplified from selected samples using the PCR technique. The primers used for amplification of the *MPL* gene are listed in the Supplementary Table 1. The PCR conditions were as follows: 40 cycles of 94°C, 30 seconds for denaturing, 55°C, 30 seconds for annealing, and 73°C, 30 seconds for extension. All PCR products were electrophoresed in a 2% agarose gel; PCR bands were excised, and purified using Qiagen Gel extraction kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocols. Purified PCR bands were directly sequenced using the Big-dye sequence reaction (Applied Biosystems, Foster City, CA, USA) and analyzed on an Autosequencer 3100 (Applied Biosystems). PCR products which had mutations of S204F-*MPL* (*MPL* exon 4), W515L (*MPL* exon 10), Y591D (*MPL* exon 12) as detected by direct sequencing were cloned into pGEM-T vectors (Promega) and transformed into *Escherichia coli*–competent cells. Plasmids were extracted from eight independent clones of each PCR product and their nucleotide sequences were determined.

Screening of normal DNA

DNA from 100 normal volunteers and 42 MPD patients were screened for S204F and Y591D mutations of the *MPL* gene.

PCR products of exon 4 of *MPL* were digested with AlwNI for the S204F mutation (wild-type is digested and mutant is not digested); PCR products of exon 12 of *MPL* were digested with BsiEI for the Y591D mutation (wild-type is not digested and mutant is digested). The treated PCR products were run in 2% agarose gel.

Expression of MPL in BaF/3 cells

Human full-length *MPL* cDNA was cloned into pMSCV vector (Clontech, Mountain View, CA, USA) from normal human bone marrow cells using PCR. EGFP cDNA (Clontech) was ligated into the pMSCV vector as a marker protein driven by the pGK promoter. S204F, W515L, and Y591D mutations were introduced into pMSCV-EGFP-hMPL vector by the PCR mutagenesis method. Expression of wild-type and mutant MPL was driven by LTR. The constructs were electroporated into the murine interleukin-3 (IL-3)–dependent B-cell line, BaF/3, and cultured in media containing IL-3 for 2 days. Green fluorescent protein–positive cells were selected by fluorescein-activated cell sorting and cultured in media without IL-3. Cell numbers were counted on days 2, 4, and 6.

Results

Chromosomal duplications and deletions in MPDs

Genetic abnormalities, including deletions and duplications, are shown in Figures 1A to C and summarized in Table 1. Gain of genomic materials was detected in 5 of the 43 MPD samples; loss of genomic materials was detected in 9 of 43 MPD cases. Trisomy 9 was detected in one PV and one ET; duplication of 9 p was identified in one PV; duplication of 9q21.11-qter was detected in one PMF.

Seven of 16 samples of PMF had loss and/or gain of genomic material. Notably, five of the nine cases of PMF with no *JAK2* mutations had genomic alterations, including deletion involving *RB1* (13q14) (PMF case nos. 29, 84, and 287); deletion involving *NF1* (17q11) (PMF case no. 485), duplication of 8q21.3, duplication of 9q21.11-qter, duplication of 4q28.1-qter, deletion of 12p13 and deletion of 4q24. Deletion of 4q24 (PMF case no. 109) was also detected by conventional cytogenetics confirming the SNP-chip results (data not shown). Deletions of *NF1* and *RB1* were also confirmed by quantitative genomic PCR (Suppl. Fig. 1).

Only 2 of 17 ET cases had genomic alterations; those with wild-type *JAK2* (10 cases) had a normal genomic pattern (Table 1). One case of ET had deletion at 5q23.1 involving a single gene, LOC51334. We analyzed nucleotide sequences of all exons of the remaining allele, as well as, methylation status of the promoter region of this gene in this case; and found neither methylation nor mutation of the gene (data not shown).

9p uniparental disomy and JAK2 point mutations in MPD

One of the advantages of SNP-chip analysis is the ability to detect uniparental disomy [17]. This novel analysis can detect uniparental disomy even when clones with uniparental disomy are not dominant [17]. We found uniparental disomy in the 9 p region that involved *JAK2* in five PV samples, in which the clones with the uniparental disomy were dominant (Fig. 2A and Table 1) because large regions of loss of heterozygosity without loss of DNA copy number were detected. Each of these cases showed homozygous *JAK2*V617F mutations by allele specific PCR (Table 1).

Further, we identified 11 cases with 9 p uniparental disomy (3 PV, 1 ET, and 7 PMF), in which these clones were not dominant (Table 1). Although regions with loss of heterozygosity of 9 p in these cases were quite obvious, allele-specific gene-dosage analysis clearly indicated that dosage level of one of the parental alleles was lower than normal level, and the other parental allele was higher (Fig. 2B).

One PV and one ET had both wild-type *JAK2* and *JAK2*V617F with trisomy 9. Another PV had both wild-type *JAK2* and *JAK2*V617F with duplication of 9 p, but we were unable to determine if either the mutant or wild-type allele was duplicated in these cases.

Point mutations of the MPL gene in MPD with 1p uniparental disomy

SNP-chip analysis showed 1 p uniparental disomy in two cases of PMF (case nos. 29 and 84) (Fig. 3A), suggesting the possibility of a transforming allele in this region. Analysis of the common region of uniparental disomy on 1 p of these two cases of PMF showed two tyrosine kinase (*FGR* and *TIE1*) genes and the thrombopoietin receptor (*MPL*) gene; each is known to be expressed in normal hematopoietic cells [18–20]. We sequenced all coding exons of the *FGR*, *TIE1*, and *MPL* genes in these two PMF cases. No mutations in the *FGR* and *TIE1* genes were detected (data not shown). However, we found a point mutation in the *MPL* gene that changed codon 204 from TCT to TTT (S204F) in case no. 29 (Fig. 3B) and changed codon 515 from TGG to TTG (W515L) in case no. 84 (Fig. 3C). Each sample had no wild-type allele as shown by direct sequencing analysis (Figs. 3B and C).

In another sample of PMF (case no. 325) and PV (case no. 298), 1 p uniparental disomy was detected in a subpopulation of the tumor cells (Fig. 4). We sequenced all exons of the *MPL* gene in these two cases and found point mutations of the *MPL* gene (W515L and Y591D) (Fig. 4). In case no. 325 of PMF, TGG changed to TTG at amino-acid 515, leading to substitution of leucine for tryptophan (Fig. 4A); in case no. 298 of PV, TAC changed to GAC at amino-acid position 591, leading to substitution of aspartic acid for tyrosine (Fig. 4B). In these cases, wild-type sequences were detected as well, suggesting that clones with these mutations were not predominant. All point mutations were also confirmed after subcloning of the PCR products into plasmids (data not shown). Positions of the *MPL* mutations are shown in Figure 5; S204F mutation is in the extracellular region; W515L and Y591D are in the cytoplasmic domain.

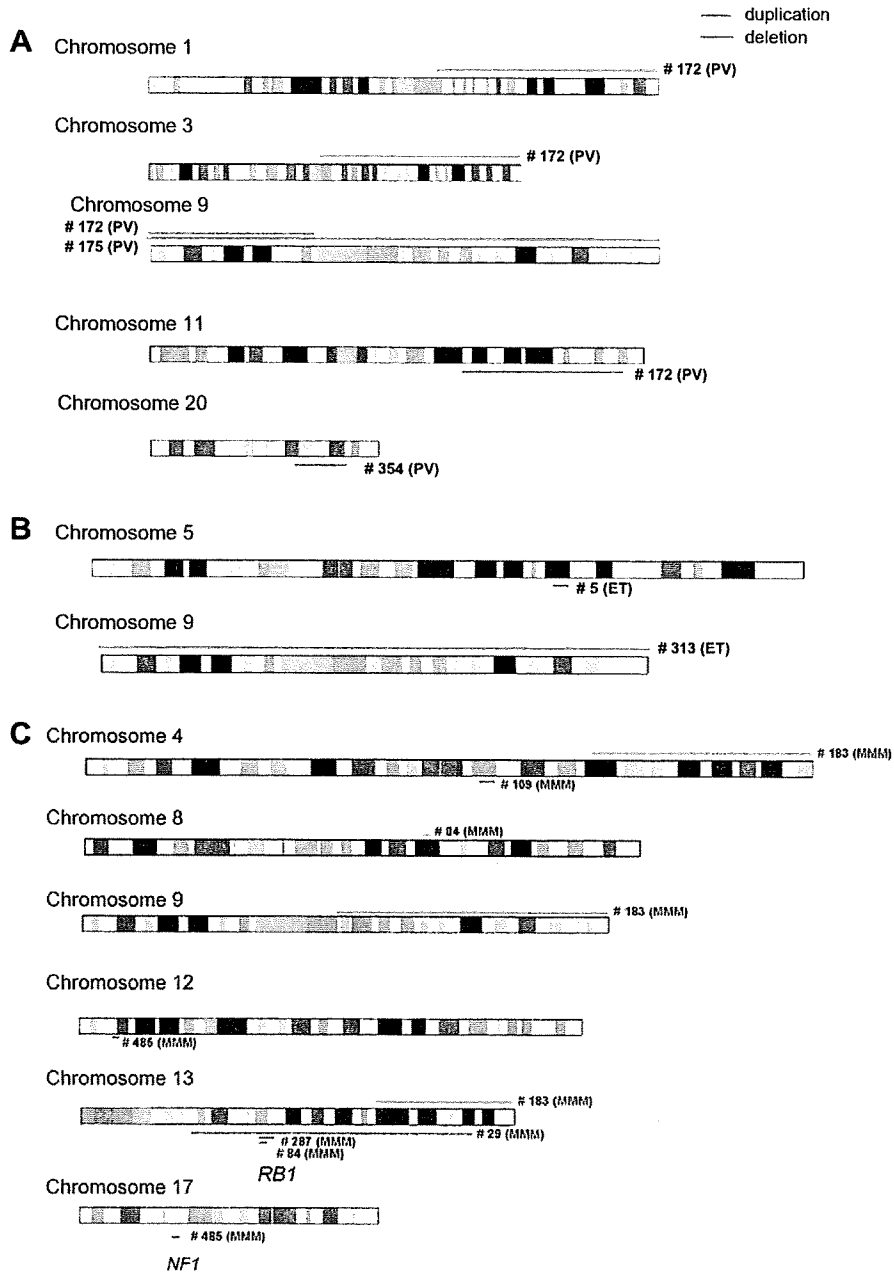

Figure 1. Chromosomal duplications and deletions in myeloproliferative disorder samples as detected by single-nucleotide polymorphism DNA microarray (SNP-chip). Duplications are indicated as red lines above the chromosomes; deletions are displayed as blue lines under the chromosomes. Case numbers are shown to the right of the chromosomes. Results of polycythemia vera (PV) (**A**), essential thrombocytosis (ET) (**B**), and myelofibrosis with myeloid metaplasia (**C**) are shown. PMF = primary myelofibrosis.

Discussion

Recently, we developed a new algorithm for SNP-chip analysis that reduced noise (false-positive and false-negative results), making the data more reliable [16,17]. We have previously validated the sensitivity and specificity of the SNP-chip analysis using genomic real time PCR, fluorescence in situ hybridization, and nucleotide sequencing [14–17]. This platform and our novel algorithm allow detection of very small genomic abnormalities at a very high sensitivity [14–17].

Comparison between tumor samples and matched control samples is the best way to detect genomic loss/gain in cancer samples. However, it is not always possible to obtain matched control samples. As reported by a number of

Table 1. Genomic abnormalities detected by SNP-Chip in MPDs

Disease	case #	JAK2	UPD	UPD*	Recurrent	Other abnormalities	MPL**
PV	78	1		9p		dup 9p, dup 3q, Trisomy 4, del 11q	
	172	1				Trisomy 9	
	175	1					
	298	1		1p/9p			Y591D
	375	1		9p			
	86	2	9p				
	249	2	9p				
	307	2	9p				
	327	2	9p				
	354	2	9p			Del 20q	
ET	114	0					
	115	0					
	146	0					
	201	0					
	240	0					
	281	0					
	285	0					
	314	0					
	446	0					
	461	0					
	5	1				Del 5q23.1(LOC51334)	
	186	1					
	271	1					
	272	1					
	313	1				Trisomy 9	
	330	1		9p			
	374	1					
MMM	29	0	1p		RB del		S204F
	56	0					
	84	0	1p		RB del	Dup 8q21.3	W515L
	138	0					
	183	0				Dup 9q21.11-qter, Dup 4q28.1-qter	
	196	0					
	325	0		1p			W515L
	459	0					
	485	0			NF1 del	Del 12p13	
	109	1		9p		Del 4q24	
	120	1		9p			
	122	1		9p			
	191	1		9p			
	287	1		9p	RB del		
	442	1		9p			
	457	1		9p			

UPD: uniparental disomy, Del: deletion, Dup: duplication.
JAK2: Mutational status of JAK2 gene; 0, only wild-type JAK2 allele was detected;
1, both wild-type and mutated alleles of JAK2 were detected;
2, only mutated allele of JAK2 was detected.
*: Subpopulation had UPD which was detected by allele-specific gene dosage analysis.
**: Mutational status of MPL gene (see Result Section)

investigators, the human genome has copy number variants (CNV), and many genomic sites of CNV have been reported [21–24]. When allelic copy numbers of tumor samples are compared with nonmatched control samples, differences of copy numbers at CNV sites could be interpreted as either a deletion or amplification/duplication. However, these are not pathological abnormalities, but caused by variants. In our study, we used non–self-reference data for comparison. We excluded the known regions of CNV in our study, therefore, the abnormalities found here are unlikely to be areas of CNV.

SNP-chip is a powerful tool for genome-wide analysis of genetic abnormalities [14,15]. It allows for detection of small deletions and amplification that cannot be detected by standard karyotyping techniques [14,15]. Using this approach, commonly deleted and/or amplified regions of the genome can be defined to facilitate the identification of target tumor suppressor genes and/or oncogenes in these