

## Demonstration of Philadelphia chromosome negative abnormal clones in patients with chronic myelogenous leukemia during major cytogenetic responses induced by imatinib mesylate

ME O'Dwyer<sup>1</sup>, KM Gatter<sup>2</sup>, M Loriaux<sup>2</sup>, BJ Druker<sup>1</sup>, SB Olson<sup>3</sup>, RE Magenis<sup>3</sup>, H Lawce<sup>3</sup>, MJ Mauro<sup>2</sup>, RT Maziarsz<sup>2</sup> and RM Brazier<sup>2</sup>

<sup>1</sup>Division of Hematology and Oncology, Department of Medicine, Oregon Health & Science University, Portland, OR, USA;

<sup>2</sup>Department of Pathology, Oregon Health & Science University, Portland, OR, USA; and <sup>3</sup>Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR, USA

**Imatinib mesylate, an Abl-specific kinase inhibitor, produces sustained complete hematologic responses (CHR) and major cytogenetic responses (MCR) in chronic myeloid leukemia (CML) patients, but long-term outcomes in these patients are not yet known. This article reports the identification of clonal abnormalities in cells lacking detectable Philadelphia (Ph) chromosome/BCR-ABL rearrangements from seven patients with chronic- or accelerated-phase CML, who were treated with imatinib. All seven patients were refractory or intolerant to interferon therapy. Six of seven patients demonstrated MCR and one patient, who had a cryptic translocation, achieved low-level positivity (2.5%) for BCR-ABL by fluorescence *in situ* hybridization. The median duration of imatinib treatment before the identification of cytogenetic abnormalities in BCR-ABL-negative cells was 13 months. The most common cytogenetic abnormality was trisomy 8, documented in three patients. All patients had varying degrees of dysplastic morphologic abnormalities. One patient exhibited increased numbers of marrow blasts, yet consistently demonstrated no Ph-positive metaphases and the absence of morphologic features of CML. The presence of clonal abnormalities in Ph-negative cells of imatinib-treated CML patients with MCR and CHR highlights the importance of routine metaphase cytogenetic testing and long-term follow-up of all imatinib-treated patients.**

*Leukemia* (2003) 17, 481–487. doi:10.1038/sj.leu.2402848

**Keywords:** CML; clonal abnormalities; MDS; imatinib mesylate; ST1571

### Introduction

Chronic myelogenous leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by a specific chromosomal translocation, t(9;22)(q34;q11), resulting in a shortened chromosome 22, commonly referred to as the Philadelphia (Ph) chromosome.<sup>1,2</sup> The molecular consequence of this translocation is the generation of a *BCR-ABL* fusion oncogene, resulting in the production of a constitutively activated *BCR-ABL* tyrosine kinase.<sup>3</sup> This kinase is capable of inducing leukemias in mice.<sup>4</sup> Importantly, the tyrosine kinase activity of the *BCR-ABL* protein is essential to its transforming ability, making this an ideal therapeutic target.<sup>5</sup>

Imatinib mesylate (trade name Gleevec, formerly known as ST1571), a specific Abl kinase inhibitor, blocks the ATP-binding site of the Abl kinase and has been shown to arrest growth and induce apoptosis in *BCR-ABL*-expressing hematopoietic cells.<sup>6</sup> In phase II clinical trials, imatinib has shown activity in all phases of CML (chronic phase, accelerated phase, and blast

crisis), inducing major cytogenetic responses (MCR; defined as 1–35% Ph+ bone marrow metaphases) in 41%, and complete cytogenetic responses (CCR; defined as 0% Ph+ bone marrow metaphases) in 19% of the late chronic phase patients.<sup>7</sup> Preliminary results of a phase III randomized study in newly diagnosed CML patients show even more impressive results with CCR in up to 40% of patients within 6 months of therapy and superior progression-free survival compared to interferon.<sup>8</sup> While imatinib was well tolerated to date in clinical trials and appears as a safe agent for chronic administration, little is known about its potential for long-term efficacy and toxicity because of the relatively short follow-up time since its introduction into clinical use.

While clonal evolution within the context of the Ph+ clone is documented in patients treated with imatinib,<sup>9,10</sup> the identification of clonal abnormalities in cells lacking the Ph chromosome during treatment is rare and has not been previously studied following imatinib. In this report, we present seven CML patients treated with imatinib at our institution in whom we identified clonal abnormalities in *BCR-ABL*-negative cells. All seven patients had achieved an MCR on imatinib when these clonal abnormalities were identified. This article documents the morphologic and cytogenetic findings in these patients. Some of these patients are from the initial cohort of phase I chronic-phase CML patients treated with imatinib, with follow-up intervals of up to 33 months.

### Materials and methods

#### Patients

Review of Leukemia Center records at Oregon Health & Science University (OHSU) identified seven CML patients (chronic and accelerated phases), who had achieved low-level Ph+ disease following treatment with imatinib (on Novartis studies 01, 109, 110, and 114) and who had evidence of clonal cytogenetic abnormalities in Ph- cells. The seven patients were from a total of 231 patients who received imatinib therapy for CML on study at our institution. Seven matched imatinib-treated CML patients with complete hematologic responses (CHR) and MCR, but without clonal abnormalities in Ph- cells were selected for comparison. All 14 patients were treated with interferon before being treated with imatinib. The Institutional Review Board at OHSU approved this retrospective study. In all patients, the diagnosis of CML was confirmed prior to imatinib treatment by morphologic review of peripheral blood (PB) and bone marrow, and by documentation of the presence of the *BCR-ABL* translocation by conventional metaphase cytogenetic analysis

Correspondence: RM Brazier, Department of Pathology, L471, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97201, USA; Fax: 1-503-494-6787  
Received 4 July 2002; accepted 7 November 2002

or molecular studies. The patients were initially treated with imatinib at oral doses of 250–1000 mg per day (with subsequent escalations and reductions as per protocol), and were followed with complete blood counts (CBCs) and bone marrow aspiration and biopsy every 2–6 months. Bone marrow specimens were subjected to morphologic evaluation, routine metaphase karyotypic analysis, and fluorescence *in situ* hybridization (FISH) cytogenetic studies. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for the *BCR-ABL* translocation was not performed regularly as part of this study, but limited PCR results were available for some patients at later times.

At the time of entry into the study, the seven patients with documented clonal chromosomal abnormalities had a mean age of 50 years (range 39–60 years). The mean approximate duration of documented disease was 2.6 years (range 0.6–10 years). There were three male patients and four female patients. Four patients had chronic-phase CML, three patients were interferon refractory and one patient was interferon intolerant. Three patients were in the accelerated phase of the disease. Six of the seven patients had a classic t(9;22)(q34;q11) Ph translocation on routine karyotyping at the beginning of imatinib therapy. One patient of the seven demonstrated a cryptic Ph translocation, with *BCR-ABL* positivity detected by FISH on metaphase preparations but not by routine G-banded karyotype analysis. One patient showed evidence of clonal evolution before imatinib therapy, with del(7)(q22) in two of 20 Ph+ cells. Pretreatment patient characteristics including age, sex, disease duration, and results of their karyotypic analysis (preimatinib therapy) are shown in Table 1.

The seven comparison patients used as a control cohort, lacking evidence of clonal chromosomal abnormalities in Ph– cells, had a mean age of 56 years (range 21–72 years) at the time of entry into the study. The mean approximate duration of documented disease was 4.3 years (range 2.3–9.6 years). All seven patients were male and all seven patients demonstrated the classic Ph chromosomal translocation on routine karyotyping at the beginning of imatinib therapy. Four of the seven comparison patients entered study in the chronic phase of their disease and three of the patients had progressed to accelerated phase of CML.

### Morphologic review

PB smears were evaluated for red cell morphology, platelet abnormalities, and the presence of circulating myeloid precursors, basophilia, and blasts. Wright-stained bone marrow aspirate smears and hematoxylin- and eosin-stained paraffin sections of bone marrow biopsy and aspirate clot specimens were examined for overall marrow cellularity, cell maturation and proportion of blasts, basophilia, the presence or absence of

focal lesions, and myelodysplastic morphology. Bone marrow aspirate differential counts were used to derive a myeloid:erythroid cellular ratio (M:E ratio), which was correlated with overall cellularity.

### Cytogenetic and molecular analyses

Complete karyotyping on metaphase spreads was performed in all patients at baseline and thereafter at 2–6 month intervals, depending on the individual study requirements. *BCR-ABL* interphase FISH studies were performed in the majority of patients at the time of metaphase analysis, and quantitative RT-PCR analysis for *BCR-ABL* was performed in selected patients at the discretion of the investigator, but usually following a CCR. The results of bone marrow metaphase karyotyping, *BCR-ABL* FISH results, and when available, *BCR-ABL* RT-PCR analyses were correlated with the hematologic and marrow findings. Results were viewed specifically to determine whether there was any correlation between secondary cytogenetic findings, residual morphologic evidence of CML, and morphologic evidence of dysplasia.

### Chromosome preparations

Bone marrow aspirate was introduced into culture medium and three different culture methods were used: One culture was harvested at 24 h, one synchronized with  $10^{-7}$  M methotrexate and harvested at 24 h, and one supplemented with giant cell tumor-conditioned medium and harvested at 48 h. The harvest and slide preparations were made according to standard methods. Chromosomes were Giemsa–trypsin–Wright (GTW)-banded. Cytogenetic responses, based on metaphase analysis of at least 20 cells, were defined as complete (no Ph+ cells), major (1–35% Ph+ cells), partial (36–65% Ph+ cells), or absent (> 65% Ph+ cells). A yield of fewer than 20 cells suitable for analysis was encountered in certain patients, more often early in therapy.

### FISH

Cells fixed in 3:1 methanol:acetic acid were dropped onto slides and treated to optimize spreading, similar to routine metaphase chromosome preparation. Slides were baked at 95°C for 5–6 min, incubated in  $2 \times$  SSC at 37°C for 30 min, dehydrated through an alcohol series (70, 80, and 95% for 2 min each), and dried. Direct labeled probes for the t(9;22) translocation breakpoints (Ventana Medical Systems, Tucson, AZ, USA [Oncor] double fusion *BCR-ABL* D-FISH set, catalog #P5161-DC) were codenatured with the target DNA at 72°C for 2 min

**Table 1** Pretreatment patient characteristics of CML patients with clonal abnormalities in Ph– cells

Patient no.	Age (years)	Sex	Disease duration (years)	Phase	IFN status	Karyotype (number of metaphases)
1	54	F	3	Chronic	Resistant	46,XX,t(9;22)(q34;q11) (20)
2	54	F	10	Chronic	Resistant	46,XX,t(9;22)(q34;q11) (20)
3	41	M	2.5	Accelerated	Intolerant	46,XY,t(9;22)(q34;q11) (20)
4	60	F	1.8	Accelerated	Resistant	46,XX,t(9;22)(q34;q11) (20)
5	51	M	1	Chronic	Resistant	46,XY,t(9;22)(q34;q11)(10)/46,XY (10)
6	39	F	0.6	Chronic	Resistant	46,XY (20) (Cryptic translocation)
7	53	M	1.8	Accelerated	Resistant	46,XY,t(9;22)(q34;q11) (18)/46,idem,del(7)(q22) (2)

and allowed to renature overnight at 37°C. Slides were then rinsed in  $0.5 \times$  SSC at 72°C for 5 min and transferred to phosphate-nonadet buffer for 3 min. Preparations were counterstained with DAPI and viewed with appropriate filters for visualization of red, green, and yellow probe signals through a Zeiss Axiophot. Images were captured using a CytoVision system (Applied Imaging, Santa Clara, CA, USA).

At least 200 interphase cells were scored for signal patterns. FISH results for at least one metaphase cell were analyzed, when possible, to aid in interpretation of complex signal patterns. The normal signal pattern in this system is two red and two green. The common abnormal pattern for the double fusion system (D-FISH) is one red, one green, and two yellow signals (representing both derivatives 9 and derivative 22). Observation of a single interphase cell with one red, one green, and two yellow signals with the D-FISH kit is considered to be positive for the Ph rearrangement. Variations in the signal pattern may reflect underlying complex karyotypes.

A subset of patients was characterized for the t(9;22) and trisomy 8 by interphase FISH. *BCR-ABL* and chromosome 8 aqua direct labeled alpha satellite (gift of Vysis, Inc., Downer Grove, IL, USA) probes were mixed and hybridized according to the above protocol. A total of 100 interphase cells were scored

using green and red filters followed by an aqua filter to view all probe signals within each cell (Figure 1).

### RT-PCR analysis for *BCR-ABL*

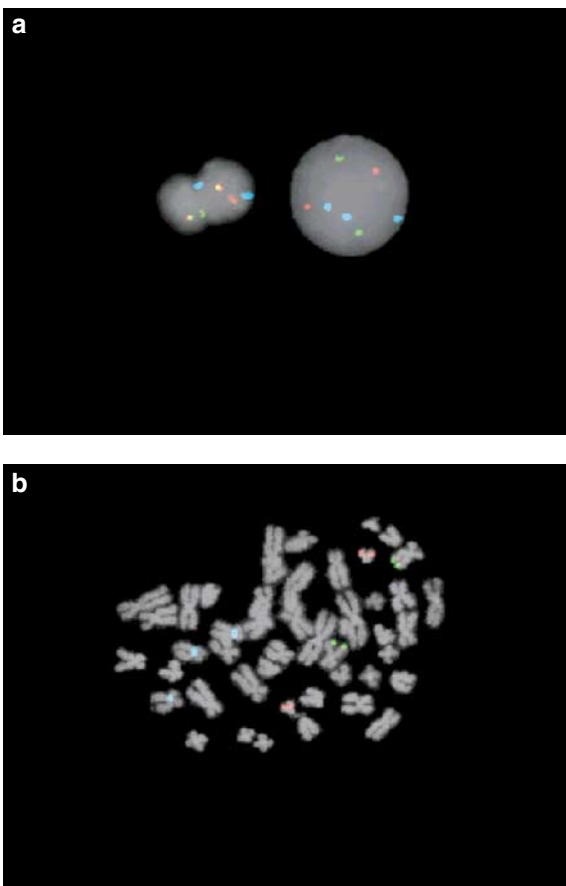
The reported RT-PCR analyses were performed on peripheral blood or bone marrow aspirate specimens using a real-time PCR technique. Briefly, mRNA was isolated on a MagNA Pure LC instrument (Roche Molecular Biochemicals) using recommended protocols. Approximately 3 million white cells from PB or bone marrow were added to red cell lysis buffer, centrifuged, and resuspended in 0.3 ml lysis buffer. The poly-A-tailed mRNA was purified by oligo(dT) affinity binding with typical yields of 10–50 µg mRNA in 50 µl buffer. Reverse transcription, cDNA amplification, and product quantitation were performed with slight modification using the LightCycler-t(9;22) Quantification Kit and a LightCycler Instrument (Roche Molecular Biochemicals). Briefly, 10 µl of poly-A mRNA (2–10 µg) was mixed with AMV RT enzyme and random hexamer primers in a 20 µl final volume. cDNA synthesis was performed in a normal thermal cycler using the recommended reaction conditions. In all, 5 µl of the resulting cDNA was added to reaction buffer, extension primers, detection probes, and thermostable polymerase per kit protocol. Real-time RT-PCR was performed using the recommended reaction conditions. Primers and probes that produce and quantitate both *BCR-ABL* and constitutively expressed housekeeping gene glucose-6-phosphate-dehydrogenase (*G6PDH*) transcripts were used. Results were reported as the ratio (%) of *BCR-ABL* mRNA to *G6PDH* mRNA.

### Results

#### *PB and bone marrow findings*

All seven patients with clonal abnormalities had documented CHR for CML while treated with imatinib. CHR is defined as sustained normalization (greater than 4 weeks) of the platelet count and the white blood cell (WBC) count, with no immature forms or basophilia, as well as resolution of splenomegaly. None of the patients on imatinib developed leukocytosis, basophilia, circulating blasts, or thrombocytosis subsequent to CHR. Means and ranges of the PB findings in the study patients were also determined after confirmation of the clonal abnormalities in Ph<sup>-</sup> cells (Table 2). Six of the seven study patients had mild anemia (mean hemoglobin range 11.0–12.0 g/dl). Five of seven patients had mean corpuscular volume (MCV) values above 100 (range 101–108 fl) and two had mean values of 95 and 97. Four patients were mildly neutropenic (range  $1.0\text{--}1.5 \times 10^9/l$ ). None of the patients had circulating blasts. Three of the seven were mildly thrombocytopenic (range  $81\text{--}86 \times 10^9/l$ ) and none had thrombocytosis.

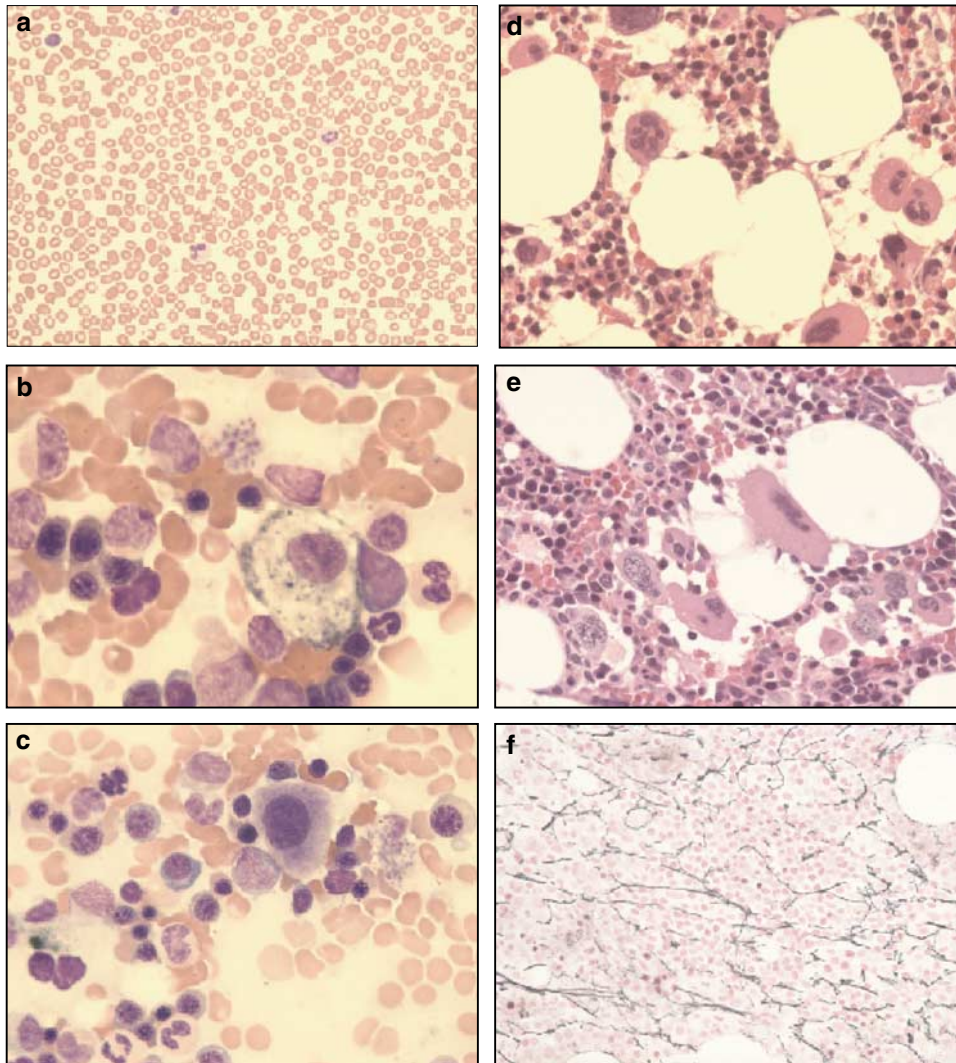
Bone marrows obtained after identification of clonal cytogenetic abnormalities in Ph<sup>-</sup> cells were interpreted as morphologically consistent with remission. The marrows from the seven study patients showed an estimated cellularity ranging from 30 to 60%. One of the seven bone marrows (patient #5; chronic phase at time of entry) had increased numbers of blasts (7%), with multilineage dysplasia, including atypical megakaryocytic hyperplasia, but no hypercellularity, granulocytic hyperplasia or marrow basophilia. All seven marrows had megaloblastic changes with varying degrees of dysplasia in the erythroid precursors. Four of the seven patients had mild relative increases



**Figure 1** FISH study using probes for Bcr, Abl and chromosome 8. Panel a: The interphase cell on the left shows one red, one green, two yellow fusion, and two aqua signals, consistent with the presence of the *BCR-ABL* fusion and two copies of chromosome 8. The interphase cell on right shows two red, two green, and three aqua signals consistent with trisomy 8 in the absence of the *BCR-ABL* fusion. Panel b: The metaphase cell shows trisomy 8 (three aqua signals) in the absence of *BCR-ABL* translocation (no yellow fusion signals).

**Table 2** Comparison of PB and bone marrow values in imatinib-treated CML patients with and without secondary clonal abnormalities in Ph-cells

	<i>CML patients (7) with clonal abnormalities in Ph- cells</i>	<i>CML patients (7) without clonal abnormalities in Ph- cells</i>
Mean WBC count $\times 10^9/l$ (range)	4.4 (2.6–5.4)	3.7 (2.5–5.2)
Mean ANC $\times 10^9/l$ (range)	2.2 (0.9–3.6)	2.1 (1.4–3.0)
Mean hemoglobin (g/dl) (range)	12.2 (10.5–15.5)	12.0 (8.9–13.2)
Mean MCV (fl) (range)	104.5 (95.7–111.2)	97.0 (93.7–101.8)
Mean platelet count $\times 10^9/l$ (range)	169 (74–365)	148 (70–279)
Mean PB basophil count $\times 10^9/l$	<0.1	<0.1
Mean % marrow cellularity (range)	50 (40–60)	30 (10–60)
Mean M:E ratio	1.5:1	2:1
Mean % marrow blasts (range)	1–2 (0–7)	1–2 (0–3)



**Figure 2** PB and bone marrow findings in CML patients on imatinib who have developed additional chromosomal abnormalities. (a) Typical PB smear shows adequate counts with low normal WBC and no basophilia, circulating granulocytic precursors or blasts. (b) Typical marrow findings include increased numbers of histiocytes (Wright stain). (c) Bone marrow with megaloblastic erythropoiesis, mild dysplastic erythropoiesis, and atypical megakaryocytes (Wright stain). (d) and (e) Occasional marrow findings included megakaryocytic hyperplasia with focal atypical clustering (hematoxylin and eosin stain). (f) Typical marrow findings included mild to moderate reticulin fibrosis (reticulin stain).

in numbers of erythroid precursors and four of the seven patients had atypical megakaryocytes (Figure 2c, d, e). Consistent findings also included increased numbers of hemosiderin-laden

histiocytes on the aspirate smears and core biopsies (Figure 2b). Two of the seven patients had persistent lymphoid aggregates. Marrow fibrosis, as determined by a reticulin stain on sections of

the core biopsies, was mild to moderate in all the marrows (Figure 2f).

The PB findings in the control cohort of seven matched imatinib-treated CML patients with documented CCR and lacking clonal chromosomal abnormalities in Ph<sup>-</sup> cells were not significantly different from the patients with the clonal cytogenetic abnormalities in Ph<sup>-</sup> cells. Mean CBC values, derived from the most recent results from these seven comparison patients, revealed a WBC count of  $3.7 \times 10^9/l$ , MCV of 97.0, hemoglobin of 12.0, and a platelet count of 148 000 (Table 2). Bone marrow findings in the seven matched control CML patients were similar to the seven study patients. All of the most recent marrows from these comparison patients had varying degrees of megaloblastic changes and erythroid hyperplasia, three had hypolobated megakaryocytes, and one showed atypical clustering of megakaryocytes. All seven marrows had mild to moderate reticulin fibrosis. None of these marrows had increased numbers of blasts.

### Cytogenetic findings

Six of seven patients with cytogenetic abnormalities in Ph<sup>-</sup> cells achieved an MCR, within 3 months in three cases and 14 months in the other three cases. One patient with a cryptic *BCR-ABL* translocation achieved low-level positivity (2.5%) for *BCR-ABL* by FISH after 18 months of treatment. CCRs were seen in four patients. All seven patients had less than 5%

*BCR-ABL* positivity by FISH on at least one occasion (Table 3). Clonal cytogenetic abnormalities were detected in Ph<sup>-</sup> cells after a median time of 13 months of imatinib therapy (range 3–24 months) (Table 4). A variety of cytogenetic abnormalities were documented to be present on repeated examinations. In four patients, the majority of metaphases were involved on repeated occasions, including: (1) trisomy 8 in 19/20 metaphases, (2) *inv(1)(p32.3p31.2)* and *del(10)(q22q22)* involving all 20 metaphases, (3) monosomy 7 in 19/20 metaphases, and (4) complex karyotype in many metaphases. The most common clonal abnormality seen in Ph<sup>-</sup> cells was trisomy 8, which was present in three of seven patients. Patients with trisomy 8 were further characterized for the *t(9;22)* and trisomy 8 using both *BCR-ABL* and chromosome 8 probes in the same interphase FISH analysis (Table 4). Trisomy 8 was clearly seen in cells with normal *BCR* and *ABL* signals (Figure 1). In the three patients who developed trisomy 8, the respective values for *BCR-ABL* and trisomy 8 FISH were 0 and 7.5%, 2 and 34%, and 33.5 and 13.5%. Control samples showed 0% FISH positivity for *BCR-ABL* and 0–2% FISH positivity for trisomy 8, which is within normal limits.

### *BCR-ABL* RT-PCR findings

Although not performed regularly as part of this study, at least two quantitative RT-PCR analyses for the *BCR-ABL*

**Table 3** Summary of hematologic, bone marrow morphologic, cytogenetic, and molecular responses to imatinib in seven CML patients with Ph<sup>-</sup> clonal abnormalities

Patient no.	Dose imatinib (mg/day)	Hematologic status at 18 months	Best cytogenetic response	% Ph+ metaphases at best response (time observed)	% Ph+ metaphases at last follow-up	Best <i>BCR-ABL</i> RT-PCR response <sup>a</sup>	<i>BCR-ABL</i> RT-PCR at last follow-up <sup>a</sup>
1	250 ≥ 600	CHR	Complete	0% Ph+ (20 mo)	0% Ph+ (24 mo)	Not Detected (29 mo)	0.055% (34 mo)
2	300	CHR	Partial	5% Ph+ (14 mo)	15% Ph+ (33 mo)	0.41% (28 mo)	0.41% (28 mo)
3	1000 ≥ 600	CHR	Partial	5% Ph+ (24 mo)	5% Ph+ (24 mo)	0.33% (24 mo)	0.33% (24 mo)
4	600	CHR	Complete	0% Ph+ (3 mo)	0% Ph+ (26 mo)	Not Detected (15 mo)	0.028% (26 mo)
5	400	CHR	Complete	0% Ph+ (6 mo)	0% Ph+ (24 mo)	0.27% (14 mo)	0.27% (14 mo)
6	400	CHR	Cryptic	FISH 2% <sup>b</sup> (18 mo)	FISH 2% <sup>b</sup> (18 mo)	1.6% (16 mo)	1.6% (16 mo)
7	600	CHR	Complete	0% Ph+ (9 mo)	0% Ph+ (15 mo)	0.0039% (12 mo)	0.15% (19 mo)

<sup>a</sup>Quantitative RT-PCR results are reported as the ratio (%) of *BCR-ABL* mRNA to G6PDH mRNA. <sup>b</sup>Cryptic translocation not visualized by metaphase karyotyping. CHR, complete hematologic response; Ph+, Philadelphia chromosome-positive metaphases. Mo, months

**Table 4** Changes in karyotype observed during the course of treatment with STI571. No changes in karyotype were observed in the seven comparison CML patients without clonal changes in Ph<sup>-</sup> cells

Patient	Pretreatment karyotype	Karyotypic change during imatinib treatment (after 12–24 months)	Corresponding FISH	
			<i>Bcr-Abl</i> (%)	+8 (%)
1	46,XX,t(9;22)(q34;q11) (20)	46,XX,t(9;22)(q34;q11) (1)/47,XX,+8 (6)/46,XX (23)	0	7.5
2	46,XX,t(9;22)(q34;q11) (20)	46,XX,t(9;22)(q34;q11),-14,-15 (1)/47,XX,+8 (19)	2	34
3	46,XY,(9;22)(q34;q11) (20)	46,XY,t(9;22)(q34;q11) (1)/47,XY,+8 (3)/46,XY (16)	33.5	13.5
4	46,XX,t(9;22)(q34;q11) (20)	46,XX,t(3;10)(q26;q22) (3),46,XX (19)	0	—
5	46,XY,t(9;22)(q34;q11) (10)/46,XY (10)	46,XY, <i>inv(1)(p32.3p31.2)</i> , <i>del(10)(q22q22)</i> (20)	0.5	—
6	46,XY (20) (Cryptic translocation)	45,XX,-7 (19)/46,XX (1)	2.5	—
7	46,XY,t(9;22)(q34;q11) (18)/46,idem, <i>del(7)(q22)</i> (2)	46,XY,t(1;12)(q21;q22) (7)/45,XY,t(4;22)(p16;q11.2), <sup>a</sup> t(5;6)(6;8)(q13;p23q23;q22),-21 (8)/46,XY, t(1;2)(q21;q3?5),t(4;7) (q21;q32) (2)/46,XY (6)	5.5	—

<sup>a</sup>The translocation between chromosomes 4 and 22 may represent further rearrangement of Philadelphia *t(9;22)*.

translocation were available for each of the seven patients. For all patients, quantitative RT-PCR analyses were performed a minimum of 8 months after initiation of imatinib therapy. *BCR-ABL* transcripts were undetectable by RT-PCR in patients #1 and #4 at 28 and 15 months, respectively; however, both patients had subsequent intermittent positive results as well as positive results at last follow-up. The remaining patients showed consistently positive *BCR-ABL* RT-PCR results.

## Discussion

Imatinib mesylate, the first molecularly targeted therapy for CML, has produced dramatic response rates in patients with both chronic- and accelerated-phase CML. Phase II studies documented MCR and CCR rates of 41 and 19%, respectively, in late chronic-phase patients. Similar responses were seen in accelerated-phase patients, with an MCR in 24% and a CCR in 17% of patients.<sup>7,11</sup> These response rates are associated with durable hematologic remissions and the possibility of improved long-term survival for these patients. However, prolonged treatment with imatinib could introduce new or unmask previously unrecognized long-term complications that could adversely impact prognosis.

This report documents seven CML patients, of approximately 230 total, whose Ph<sup>-</sup> bone marrow cells developed clonal cytogenetic abnormalities after achievement of MCR on therapy with imatinib. The patients who developed these clonal cytogenetic abnormalities could not be predicted by PB or bone marrow morphologic findings; the PB and bone marrow findings at baseline (postinterferon, preimatinib therapy) and at a median follow-up interval of 13 months after initiation of imatinib therapy were not significantly different between CML patients with and without clonal cytogenetic abnormalities in Ph<sup>-</sup> cells. Several of the clonal cytogenetic abnormalities seen in the Ph<sup>-</sup> cells in these patients have a clear association with myelodysplasia (MDS), in particular trisomy 8 (three patients) and monosomy 7 (one patient).<sup>12</sup> In fact, all of the marrows showed varying degrees of dysplastic morphologic changes, particularly in the erythroid and megakaryocytic cell lines. However, comparison of these patients to seven imatinib-treated CML patients with MCR and CHR but without clonal chromosomal abnormalities in Ph<sup>-</sup> cells showed similar morphologic findings, with at least mild myelodysplastic morphologic abnormalities in all cases.

The etiology and the clinical significance of the mild myelodysplastic abnormalities seen in imatinib-treated CML patients with and without additional documented clonal cytogenetic changes in Ph<sup>-</sup> cells is not yet clear. The morphologic changes are not specific and some changes may be related to a reversible direct drug effect of imatinib, but these findings raise the concern that a proportion of patients with favorable responses to imatinib may ultimately develop myelodysplasia. The presence of cytogenetically abnormal subclones has been documented in CML patients treated with interferon and hydroxyurea, and some of these patients have been reported to have subsequent development of myelodysplasia. Fayad and colleagues reported three CML patients with presumed new detectable clonal abnormalities while in complete cytogenetic remission for CML (demonstrated by 0% Ph<sup>+</sup> cells by FISH), between 23 and 90 months after the start of interferon therapy. One patient with 5q abnormalities and monosomy 7 developed myelodysplasia within 2 years. A second patient with 18p11 abnormalities subsequently developed a new myeloproliferative syndrome. Both patients

remained in complete cytogenetic remission for their Ph<sup>+</sup> CML.<sup>11</sup> It is possible that the MDS-like changes seen in our patients are secondary to their previous interferon- $\alpha$  therapy, rather than imatinib. The resolution of this question will require comparison of the outcomes of this cohort of interferon-treated CML patients to a cohort of *de novo* CML patients treated with imatinib without any prior chemotherapy. These data are not yet available.

Although a variety of abnormal karyotypes were documented in Ph<sup>-</sup> cells of these CML patients, the most common secondary cytogenetic abnormality was trisomy 8, present in three of our patients. The development of trisomy 8 is not uncommon in CML patients after autografting or interferon therapy. However, the trisomy 8 typically develops as an additional abnormality in Ph<sup>+</sup> cells. The finding of metaphases containing trisomy 8 as the sole abnormality is distinctly unusual in CML patients.<sup>12,13</sup> Using dual probes for *BCR-ABL* and an additional probe for chromosome 8 in the same FISH analysis, we have shown that the trisomy 8 in these patients is present in cells with two normal *ABL* and *BCR* signals. The presence of two normal *ABL* and *BCR* signals shows that the trisomy 8+ cells are not clonally evolved CML cells that have lost *BCR-ABL* through deletion of the Ph chromosome (Figure 2). The additional chromosomal abnormality is clearly present in a different cell line from that containing the Ph chromosome.

The mechanism of the emergence of these abnormal clones is unclear. Through its antileukemic effect, imatinib may be unmasking abnormal hematopoiesis initially present at only a low level in these patients. Since these patients all had late chronic- or accelerated-phase CML, it is possible that clonal abnormalities may have arisen as a result of the previous duration of the disease process or prior therapy. Prior therapy or the leukemic clone may also exert an inhibitory effect on normal stem cells, providing the necessary selective pressure for outgrowth of an abnormal resistant Ph<sup>-</sup> clone. Alternatively, trisomy 8 may be derived from a constitutional mosaicism.<sup>14-16</sup> Some reports suggest that the frequency of such mosaicism in hematologic dysplastic and neoplastic disorders might be as high as 20%. However, constitutional mosaicism for trisomy 8 cannot explain the other abnormalities observed, two of which (monosomy 7 and t(3;10)) are associated with development of myelodysplasia and acute myeloid leukemia.<sup>12,17</sup>

It is also possible that the cytogenetic abnormalities in Ph<sup>-</sup> cells are a direct consequence of imatinib toxicity. Imatinib inhibits the c-kit receptor tyrosine kinase at equimolar concentrations to *BCR-ABL*.<sup>18</sup> c-Kit is the receptor for stem cell factor (SCF or a c-kit ligand), an important early hematopoietic growth factor, and is expressed on stem cells in the (long-term reconstituting) stem cell compartment. Most of the hematopoietic activities of SCF appear to require a synergistic interaction with other early acting or lineage-selective cytokines. However, c-kit might be critical for maintenance and self-renewal of long-term reconstituting stem cells, particularly in adult hematopoiesis and may also be essential for optimal production of mature hematopoietic cells from stem cells. Stem cells deficient in c-kit are defective in their ability to reconstitute hematopoiesis in myelo-ablated animals, and mice with mutant forms of c-kit develop a severe macrocytic anemia.<sup>19</sup> Conceivably, long-term administration of imatinib could have a suppressive effect on normal stem cells, leading to the development of hypoplasia and/or dysplasia.

Whereas any conclusions about underlying mechanisms are premature and require additional investigation, the clinical implications are more concrete. The advent of quantitative RT-PCR monitoring of *BCR-ABL* -positive cells has led to

suggestions that periodic monitoring of bone marrow or PB PCR results may obviate the need for marrow examinations with metaphase karyotyping. The results from the seven patients reported herein indicate that quantitative *BCR-ABL* RT-PCR and FISH analysis for *BCR-ABL* are not sufficient for disease monitoring, as these techniques will not uncover other chromosomal abnormalities. Follow-up, at least in CML patients with previous chemotherapies, requires periodic metaphase cytogenetic analysis. The long-term clinical outcome in imatinib-treated CML patients with newly detected clonal cytogenetic abnormalities in Ph<sup>-</sup> cells is not yet known.

In conclusion, this report raises the question of potential adverse cytogenetic events in CML patients with good responses to imatinib, and introduces issues regarding how these patients are best monitored following therapy. It also raises questions about potential mechanisms underlying the development of secondary clonal cytogenetic changes in Ph<sup>-</sup> cells of imatinib-treated patients. The answers to these questions will require further study,<sup>20</sup> including longer-term follow-up to determine the frequency of progression to MDS or acute leukemia. Comparison of the patient cohort treated with other chemotherapy prior to imatinib to a cohort of CML patients treated from the outset of their diagnosis with imatinib alone is underway, and will likely provide answers to some of the questions raised by this study.

#### Acknowledgements

We thank Susan Oliver for administrative assistance.

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