MYELOID NEOPLASIA

Genomic instability may originate from imatinib-refractory chronic myeloid leukemia stem cells

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Key Points

- Imatinib does not prevent accumulation of genomic instability in CML-CP.
- Imatinib-refractory leukemia stem cells may be a source of genomic instability in CML-CP.

Genomic instability is a hallmark of chronic myeloid leukemia in chronic phase (CML-CP) resulting in BCR-ABL1 mutations encoding resistance to tyrosine kinase inhibitors (TKIs) and/or additional chromosomal aberrations leading to disease relapse and/or malignant progression. TKI-naive and TKI-treated leukemia stem cells (LSCs) and leukemia progenitor cells (LPCs) accumulate high levels of reactive oxygen species (ROS) and oxidative DNA damage. To determine the role of TKI-refractory LSCs in genomic instability, we used a murine model of CML-CP where ROS-induced oxidative DNA damage was elevated in LSCs, including quiescent LSCs, but not in LPCs. ROS-induced oxidative DNA damage in LSCs caused clinically relevant genomic instability in CML-CP–like mice, such as TKI-resistant BCR-ABL1 mutations (E255K, T315I, H396P), deletions in ikzf1 and Trap53, and additions in Zfp423 and Idh1. Despite inhibition of BCR-ABL1 kinase, imatinib did not downregulate ROS and oxidative DNA damage in TKI-refractory LSCs to the levels detected in normal cells, and CML-CP–like mice treated with imatinib continued to accumulate clinically relevant genetic aberrations. Inhibition of class I p21-activated protein kinases by IPA3 downregulated ROS in TKI-naive and TKI-treated LSCs. Altogether, we postulate that genomic instability may originate in the most primitive TKI-refractory LSCs in TKI-naive and TKI-treated patients. (Blood. 2013;121(20):4175-4183)

Introduction

Chronic myeloid leukemia in chronic phase (CML-CP) is initiated by t(9;22), which encodes p210BCR-ABL1 tyrosine kinase that transforms hematopoietic stem cells (HSCs).1 CML-CP is leukemia stem cell (LSC)–induced, but a leukemia progenitor cell (LPC)–manifested, disease. In CML-CP patients, ABL1 tyrosine kinase inhibitors (TKIs)–such as imatinib, dasatinib and nilotinib—may induce complete cytogenetic response (CCR), major molecular response (MMR), or even complete molecular response (CMR), or even complete molecular response (CMR), but it is unlikely that any TKI will eradicate CML.2 While most LPCs are eventually eliminated by TKIs, LSCs are intrinsically insensitive and thus usually survive treatment.3 CML-CP cells display genomic instability and may acquire additional genetic changes that cause disease relapse due to TKI resistance and/or induce more advanced CML accelerated phase (CML-AP) or blast phase (CML-BP).4

Genomic instability results from an aberrant cellular response to enhanced DNA damage. The primary candidates that cause DNA damage are reactive oxygen species (ROS) such as superoxide anion (·O2−), hydrogen peroxide (H2O2), and hydroxyl group (·OH).5 ROS can damage DNA bases to produce 7,8-dihydro-8-oxo-2-deoxyguanosine (8-oxoG) and other oxo-base derivatives, which may lead to a variety of point mutations. ROS also induce DNA double-strand breaks (DSBs), of which unfaithful repair may produce chromosomal aberrations.6

We postulated that genomic instability in CML cells results from a combination of high levels of ROS-induced oxidative DNA damage and unfaithful/inefficient DNA repair mechanisms. ROS appears to be a major driver of genomic instability because the antioxidant N-acetyl-cysteine reduced oxidative DNA damage in Lin−CD34− LSCs/LPCs to the levels detected in normal counterparts.7 Moreover, specific targeting of mitochondrial ROS by quinone, and genetic and chemical targeting of the mitochondrial respiratory chain (MRC) complex III MRC-cIII significantly diminished ROS and oxidative DNA damage in Lin−CD34−...
LPCs/LSCs, Lin−CD34−CD38− LSCs, and/or quiescent LSCs. Antioxidants also prevented accumulation of TKI-resistant BCR-ABL1 kinase mutants and additional chromosomal aberrations in vitro and in vivo.7-9

Other findings revealed that, in addition to the t(9;22) translocation, CML-CP at diagnosis may already harbor a variety of sporadic genetic aberrations affecting prognosis and treatment.1 The frequency of additional chromosomal abnormalities in CML-CP at diagnosis is ~7% as evaluated by standard cytogenetic analysis, but more sensitive comparative genomic hybridization and single nucleotide polymorphism (SNP) analyses detected multiple genetic aberrations in all CML-CP samples.10,11 Moreover, ~23% of freshly diagnosed patients carry TKI-resistant BCR-ABL1 kinase mutations and some patients may also harbor other genetic aberrations that likely play a role in TKI resistance, for example, loss of p53 and CDKN2A, and RUNX1 abnormalities.4 These mutations may increase the risk of treatment failure and/or clonal evolution to CML-BP.12,13 Numeric chromosomal changes were detected at a 50-fold higher frequency and structural changes at a 12-fold higher frequency in CML-BP, in comparison with CML-CP, reflecting more complex karyotypes.14,15

In addition, imatinib-treated CML-CP cells and patients may continue to accumulate TKI-resistant BCR-ABL1 mutants and additional chromosomal aberrations.16-19 Altogether, genomic instability is an early event in CML-CP, and it persists during the course of disease/treatment generating TKI-resistant clones and additional chromosomal aberrations causing disease relapse and/or malignant progression to CML-BP.

Genomic instability in TKI-sensitive LPCs has the potential to upgrade their status to TKI-refractory/resistant LSCs and to prevent mutations from disappearing during proliferation, maturation, and/or TKI treatment. On the other hand, genetic aberrations in TKI-refractory LSCs may not cause problems if acquired in quiescent LSCs, but if the aberrations induce proliferation or are present in cycling LSCs, they may generate TKI-resistant and/or more malignant LSC and/or LPC clones. Therefore, identification of the cellular compartment which displays genomic instability may have a significant impact on future therapeutic modalities.

We report here that genomic instability may originate from the most primitive TKI-naive and TKI-treated LSCs, and it may depend on persistent activation of class I p21-activated protein kinases (PAKs). Because LSCs are usually not sensitive to TKIs, genomic instability in this subpopulation is a major concern.3 Thus, our work supports the necessity of developing strategies to eliminate the “last CML stem cell,” especially in patients presenting higher risk of relapse.20

**Methods**

**Human cells**

Bone marrow (BM) samples from CML-CP patients at diagnosis (90%–100% Philadelphia chromosome–positive by fluorescence in situ hybridization) were obtained from the Stem Cell and Leukemia Core Facility (University of Pennsylvania, Philadelphia, PA), the Institute of Hematology and Blood Transfusion (Warsaw, Poland), and the UK SPIRIT 2 Clinical Trial. Healthy donor samples were purchased from Cambrex BioScience and Blood Transfusion (Warsaw, Poland), and the UK SPIRIT 2 Clinical Trial. Healthy donor samples were purchased from Cambrex BioScience.

**Immunostaining of murine hematopoietic stem and progenitor cells**

Mononuclear BM cells (BMCs) were stained with rat anti-mouse APC- or PerCP-Cy5.5-conjugated lineage antibody cocktail (Lin), PE- or PerCP-Cy5.5-conjugated CD117 (c-Kit), and PE-Cy7-conjugated Ly-6a/E (Sca-1) (BD Pharmingen) and sorted for Lin−c-Kit−Sca-1− population was further stained with PE-conjugated anti-CD34 and APC-conjugated anti-Flt3 antibodies (BD Pharmingen) to distinguish long-term and short-term stem cells (CD34+Flt3+ LT-SCs and CD34+Flt3+ ST-SCs, respectively) and multipotent progenitors (CD34+Flt3+ MPPs). To identify quiescent stem cells, BMCs were stained with rat anti-mouse APC- or PerCP-Cy5.5–conjugated Lin antibody cocktail, sorted for Lin− cells, stained with 2 μM CFSE or cell proliferation dye (eFluor670; eBioscience), and cultured in Iscove modified Dulbecco medium containing 10% fetal bovine serum supplemented with pretested minimal concentrations of IL-3 and SCF (WEHI- and BHK–conditioned media) for 4 days. Then, cells were restained with anti-Lin−, c-Kit−, and Sca-1 antibodies. BCR-ABL1 kinase activity was assessed by fluorescence-activated cell sorting (FACS) using FITC-conjugated anti-phosphotyrosine (P-Tyr) antibody (4G10; Millipore).

**Small-molecule inhibitor treatment**

For in vivo treatment, syngeneic mice transplanted with 1 × 10^6 BMCs from leukemic SCLITa/p210BCR-ABL1 donors were kept tet-off directly after transplantation. Imatinib (100 mg/kg body weight) was administered by oral gavage twice daily for 4 weeks as described previously.21 Lin− BMCs were harvested 3 weeks after completion of imatinib treatment when mice developed CML-CP–like disease. For in vitro treatment, Lin−CD34+ cells from CML-CP patients and healthy donors, and/or BMCs from leukemic (induced) and healthy (noninduced) SCLITa/p210BCR-ABL1 mice were incubated with 1 mM imatinib, 90 mM dasatinib, and 2 μM nilotinib (LC Laboratories), 30 μM IPA3 (Sigma-Aldrich), or a combination of a TKI and IPA3 for 48 hours as previously described.3 BCR-ABL1 kinase activity was assessed by FACS analysis using FITC-conjugated anti-P-Tyr or anti-ABL1 phospho-Y245 (ABL1-pY245) (Cell Signaling) followed by Alexa Fluor 488– or 647–conjugated goat anti-rabbit immunoglobulin G (Invitrogen). STAT5-pY694 (Cell Signaling), and PKA-pT423 (Santa Cruz Biotechnology) antibodies, followed by Alexa Fluor 488– or 647–conjugated goat anti-rabbit immunoglobulin G, were applied.
ROS assays
Cells were incubated with redox-sensitive fluorochromes 2',7'-dichlorodihydrofluorescein-diacetate (DCFDA), Redox Sensor Red CC-1, and dihydroethidium (DHE) (Invitrogen) and analyzed using the FACSCan or LSR II flow cytometry system (BD Biosciences) as described previously. DCFDA detects H$_2$O$_2$ and OH, DHE measures O$_2$ · and CC-1 detects O$_2$ · and H$_2$O$_2$.

Oxidative DNA damage
Cells were stained with the Biotin-OxyDNA test (Biotrin) following the manufacturer’s protocol to detect 8-oxoguanine (8-oxoG) or with HTC-conjugated anti-phospho-S139 histone H2AX (γH2AX) antibody (Millipore) to assay for DSBs by FACS. In addition, 8-oxoG and γH2AX (DSBs) were assessed on cytospins as described previously (see supplemental Methods).

Detection of BCR-ABL1 kinase mutations
Total RNA was extracted from Lin$^-$ BMCs harvested from mice with CML-CP–like disease using TRizol Reagent (Invitrogen) and subjected to denaturing high-performance liquid chromatography (D-HPLC) and allele-specific polymerase chain reaction (ASO-PCR) to detect imatinib-resistant BCR-ABL1 kinase mutants. BCR-ABL1–positive imatinib-resistant clones were detected as described previously. Briefly, Lin$^-$ BM and spleen cells from CML-CP–like SCLtTA/p210BCR-ABL1 mice were incubated with 1μM imatinib for 7 days in the absence of growth factors followed by plating in methylcellulose. Colonies were harvested and the BCR-ABL1 kinase domain was amplified followed by sequencing. Negative controls included cells from noninduced mice, and those which were induced only during the in vitro incubation with imatinib.

SNP array
High-molecular-weight DNA was extracted from total or FACS-sorted Lin$^-$ BMCs and spleen cells harvested from CML-CP–like mice and healthy noninduced littermates using the DNeasy Tissue kit (Qiagen) according to the manufacturer’s protocol, and resuspended in 2 mM Tris-Cl, 0.1 mM EDTA buffer. In addition, high-molecular-weight DNA samples were extracted from tail tissue of CML-CP–like mice. Three to five micrograms of each sample was subjected to the JAX high-density genotyping Affymetrix array (The Jackson Laboratory), which can simultaneously assay over 600,000 SNPs. Results from CML-CP–like animals were first compared with those obtained from their own tail DNA to subtract germline mutations followed by comparison with the data from BMCs harvested from healthy littermates. Results from imatinib-treated CML-CP–like mice were compared with those from healthy littermates.

Statistical analysis
Data are expressed as mean ± SD and were compared using the unpaired Student t test, 1-sample t test, Mann-Whitney rank sum test, and 1-way ANOVA; P < .05 was considered statistically significant.

Results
TKI-naive and TKI-treated LSCs and LPCs from CML-CP patients accumulate high levels of ROS and oxidative DNA damage
To determine whether genomic instability could originate not only in TKI-naive, but also in TKI-treated LSCs and/or LPCs, human Lin$^-$, CD34$^+$ CML-CP cells were treated or not with 1 μM imatinib for 48 hours in the presence of growth factors at concentrations similar to that found in stroma-conditioned medium from long-term BM cultures. In concordance with a previous report, we detected a twofold to fourfold increase of ROS levels in the most primitive Lin$^-$, CD34$^+$, CD38$^-$ cells, Lin$^-$, CD34$^+$, CD38$^-$ quiescent LSCs, Lin$^-$, CD34$^+$, CD38$^-$, and Lin$^-$, CD34$^+$, CD38$^+$ LPCs when compared with normal counterparts (Figure 1A–C, respectively; ROS panels). Elevated levels of ROS were accompanied by 2 to 4 times more DNA lesions, such as 8-oxoG and DSBs (determined by γH2AX) in LSCs and LPCs (Figure 1A–C oxidative DNA damage panels). Unexpectedly, despite inhibition of BCR-ABL1 kinase by imatinib (Figure 1 ABL1–pY245 panels) ROS and oxidative DNA damage were only modestly inhibited (20%-30%) in living, annexin V–negative quiescent LSCs (Figure 1A) and not significantly affected in proliferating LSCs and early LPCs (Figure 1B–C).

We also compared ROS and oxidative DNA damage at diagnosis in TKI “good responders” who achieved imatinib- or dasatinib-induced CR by 12 months and MMR/CMR by 24 to 42 months, and in “poor responders” who did not achieve CR by 12 months (supplemental Table 1). Interestingly, imatinib-naive and/or imatinib-treated Lin$^-$, CD34$^+$, CD38$^-$, CFSE$^{max}$/CTV$^{max}$ quiescent LSCs (Figure 1D), Lin$^-$, CD34$^+$, CD38$^-$, LSKs (Figure 1E), and Lin$^-$, CD34$^+$, CD38$^+$, LPCs (Figure 1F) from poor responders accumulated more ROS, 8-oxoG, and γH2AX than these from good responders.

To determine and compare the impact of first- and second-generation TKIs on ROS, we applied them in concentrations reflecting patient’s plasma levels at steady state: imatinib (1–5 μM), dasatinib (90nM), and nilotinib (2–4 μM). All TKIs abrogated BCR-ABL1 kinase activity, but did not reduce ROS in annexin V–negative Lin$^-$, CD34$^+$ CML-CP cells (supplemental Figure 1). Altogether, it appears that in the presence of growth factor cocktails mimicking physiologic conditions, TKIs inhibited BCR-ABL1 kinase activity, but were incapable of reducing ROS and oxidative DNA damage in the most primitive TKI-refractory LSCs and also in TKI-sensitive LPCs to the levels detected in normal counterparts.

Primitive LSCs from CML-CP–like SCLtTA/p210BCR-ABL1 transgenic mice accumulate more ROS than normal counterparts and LPCs
To determine whether TKI-refractory LSCs contribute to genomic instability in imatinib-naive and imatinib-treated CML-CP patients, we used previously generated SCLtTA/p210BCR-ABL1 transgenic mice which develop CML-CP–like disease or B-lymphoblastic malignancies. Mice displaying CML-CP–like disease were identified by detection of p210BCR-ABL1 transcript in BMCs and spleen (SPL) (supplemental Figure 2A), expansion of mature (Gr1$^+$CD11b$^+$) and immature (Gr1$^{low}$CD11b$^+$) granulocytes/macrophages and also megakaryocytes (CD41$^+$), but reduction of B (B220$^+$) and T (CD3$^+$) cells in BM, SPL, and/or peripheral blood (supplemental Figure 2B), and splenomegaly (supplemental Figure 2C).

Using DCFDA to detect DNA-damaging ROS such as H$_2$O$_2$ and ·OH, we found that Lin$^-$ c-Kit$^+$ Sca-1$^+$ LSCs from CML-CP–like mice accumulated more ROS in comparison with healthy counterparts and Lin$^-$ c-Kit$^+$ Sca-1$^+$ LPCs (Figure 2A). In addition, there was no difference in ROS levels between Lin$^-$ c-Kit$^+$ Sca-1$^+$ LPCs and hematopoietic progenitor cells (HPCs). Accordingly, it appears that Lin$^-$ c-Kit$^+$ Sca-1$^+$ LSCs, but not Lin$^-$ c-Kit$^+$ Sca-1$^+$ LPCs, from CML-CP–like mice are exclusively prone to accumulate high levels of ROS.

The Lin$^-$ c-Kit$^+$ Sca-1$^+$ population consists of the long-term and short-term HSCs/LSCs (CD34$^+$ Flt3$^+$ LT-HSCs/LSCs and CD34$^+$ Flt3$^+$ ST-HSCs/LSCs, respectively) and multipotent progenitors (CD34$^+$ Flt3$^+$ MPPs). LT-LSCs and ST-LSCs accumulated ~2.5 and 2.1 times more H$_2$O$_2$ and ·OH than their normal counterparts, respectively, while leukemic MPPs displayed an only 1.3-fold increase of ROS in comparison with normal MPPs (Figure 2B).

Quiescent LSCs represent a reservoir of TKI-refractory “dormant” leukemia cells. Lin$^-$ c-Kit$^+$ Sca-1$^+$ CFSE$^{max}$ quiescent LSCs contained...
1.7 to 2.1 times more $\cdot$O$_2$ and H$_2$O$_2$ than normal counterparts (Figure 2C).

Primitive LSCs from CML-CP–like mice accumulate more oxidative DNA damage than normal counterparts

ROS-induced oxidative DNA damage can be measured by detecting 8-oxoG. Elevation of ROS in Lin$^-$c-Kit$^+$Sca-1$^+$ quiescent HSCs/LSCs and Lin$^-$c-Kit$^+$Sca-1$^+$Fluor670$^{\max}$ quiescent LSC subpopulation was accompanied by 1.3- and 2.4-fold accumulation of 8-oxoG, respectively (Figure 3A-B, respectively; 8-oxoG panels). Because oxidized bases may trigger DSBs, we evaluated these DNA lesions by detecting $\gamma$H2AX. Similarly to 8-oxoG, Lin$^-$c-Kit$^+$Sca-1$^+$LSCs and Lin$^-$c-Kit$^+$Sca-1$^+$Fluor670$^{\max}$ quiescent LSCs displayed 1.3- and 2.6-fold increase, respectively, of DSB number in comparison with healthy counterparts (Figure 3A-B, respectively).

~1.7 to 2.1 times more $\cdot$O$_2$ and H$_2$O$_2$ than normal counterparts (Figure 2C).

**Figure 2. Elevated levels of ROS in the most primitive LSCs from CML-CP–like mice.** BMCs were obtained from healthy (black bars) and CML-CP–like (gray bars) mice. (A) ROS were detected with DCFDA in HSCs/LSCs (Lin$^-$c-Kit$^+$Sca-1$^+$) and HPCs/LPCs (Lin$^-$c-Kit$^+$Sca-1$^+$) from 7 healthy (black bars) and 4 CML-CP–like (gray bars) animals. *P < .001 in comparison with HSCs; and **P < .05 in comparison with LPCs. (B) ROS were detected with DCFDA in BM-derived subpopulations of Lin$^-$c-Kit$^+$Sca-1$^+$ cells from 2 healthy and 2 CML-CP–like mice: CD34$^+$Flt3$^+$LT, CD34$^+$Flt3$^+$ST, and CD34$^+$Flt3$^+$MPPs. *P < .05 in comparison with healthy counterparts. (C) ROS were measured with DHE (left panel) and CC-1 (right panel) in Lin$^-$c-Kit$^+$Sca-1$^+$CFSE$^{\max}$ quiescent cells from 7 and 4 normal, and 3 and 3 CML-CP–like mice, respectively. *P < .05 and **P < .005 in comparison with healthy counterparts.
Figure 3. The most primitive LSCs from CML-CP–like mice accumulate oxidative DNA damage. (A) 8-oxoG (163 and 110 cells from 5 and 4 normal and CML-CP–like mice, respectively) and γ-H2AX (162 and 279 cells each from 4 normal and CML-CP–like mice) were detected by immunofluorescence in DAPI-counterstained nuclei of BM-derived Lin c-Kit Sca-1+ HSCs/LSCs from healthy (black bars) and CML-CP–like (gray bars) mice. *P < .05 in comparison with healthy counterparts. DAPI, 4,6 diamidino-2-phenylindole.

LSCs-restricted oxidative DNA damage in CML-CP–like mice is associated with genomic instability

To determine whether exclusive enhancement of ROS-mediated oxidative DNA damage in LSCs is associated with genomic instability, we examined whether CML-CP–like mice acquired imatinib-resistant BCR-ABL1 kinase mutations and chromosomal aberrations after 6 to 25 weeks of disease induction. Although D-HPLC did not detect clinically relevant imatinib-resistant BCR-ABL1 kinase mutations, low frequencies of E255K (0.065%) and T315I (0.17%) were detected in 1 of 7 CML-CP–like mice using more sensitive ASO-PCR (Table 1). However, the most sensitive method, clonogenic assay followed by sequencing of BCR-ABL1 kinase domain identified leukemic colonies expressing E255K, T315I, and H396P in 5 of 7 mice (Table 1).

SNP array was performed in Lin BM and/or SPL cells harvested from 8 CML-CP–like mice (6-30 weeks of the disease induction), 4 normal littersmates, and tail genomic DNA (gDNA) from 4 CML-CP–like mice to detect chromosomal abnormalities. Various counts of copy number gains (CNGs) and copy number losses (CNLs) were detected in CML-CP–like mice; these aberrations displayed a wide-range of base pair gains and losses (Table 2). Averaging 113 CNGs and 84 CNLs per mouse, which displayed a broad range of base pair additions and deletions (Table 2). Averaging 113 CNGs and 84 CNLs per mouse, which displayed a broad range of base pair additions and deletions (Table 2).

Table 1. Imatinib-naive and imatinib-treated CML-CP–like mice harbor leukemia clones expressing imatinib-resistant BCR-ABL1 kinase mutations

<table>
<thead>
<tr>
<th>Mouse*</th>
<th>Assay</th>
<th>Sensitivity</th>
<th>Detection†</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>D-HPLC</td>
<td>10⁻² to 10⁻³</td>
<td>0/7</td>
<td>—</td>
</tr>
<tr>
<td>Imatinib</td>
<td>(wt.mut mRNA)</td>
<td>0/5</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>ASO-PCR</td>
<td>10⁻³</td>
<td>1/7</td>
<td>E255K, T315I</td>
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<tr>
<td>Imatinib</td>
<td>(wt.mut mRNA)</td>
<td>0/5</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>Resistant colonies</td>
<td>10⁻⁶ cells</td>
<td>5/7</td>
<td>E255K, T315I, H396P</td>
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</tbody>
</table>

*Leukemic mice were untreated or treated with imatinib as described in “Methods.”
†Number of mice with mutations/total number of mice analyzed.

Imatinib neither abrogates ROS and oxidative DNA damage in quiescent LSCs nor prevents genomic instability in CML-CP–like mice

Because quiescent TKI-refractory LSCs exhibit elevated ROS-induced oxidative DNA damage, we determined the impact of imatinib treatment on ROS and oxidative DNA damage in these cells. Incubation with 1 μM imatinib caused inhibition of cellular phosphotyrosine in Lin c-Kit Sca-1+ Fluor670max quiescent LSCs to the levels similar to normal counterparts (Figure 4A). Although imatinib partially reduced ROS in quiescent LSCs (P = .033), the levels of ROS in these cells remained almost twofold higher than in normal counterparts (Figure 4B). At the same time, 8-oxoG lesions were not significantly affected by imatinib (Figure 4C).

Because the length of induction of CML-CP–like disease in tet-off SCLrTA/p210BCR-ABL1 transgenic mice may vary, we used a transplant model to obtain more uniform disease latency. These “secondary” CML-CP–like mice were then treated with imatinib for 4 weeks and 3 weeks later (total 7 weeks after the disease induction) and gDNA was extracted from Lin BMCs and splenocytes and compared with that from healthy animals to examine genomic instability. Imatinib-resistant BCR-ABL1 kinase mutation (T315I) was detected with low frequency (0.135%) in 1 of 5 CML-CP–like mice treated with imatinib (Table 1). Because of the small amounts of gDNA obtained from BM and splenic samples from individual mice treated with imatinib, samples were pooled together for SNP analysis. Imatinib-treated CML-CP mice continued to accumulate various chromosomal aberrations averaging 113 CNGs and 84 CNLs per mouse, which displayed a broad range of base pair additions and deletions (Table 2). Again, several genes (supplemental Table B) including these known for their potential role/presence in patients which developed TKI resistance (Ddx11, Recq1, Mcm9) and/or progressed to CML-AP/BP (Aaf6, Ptpn14, Wdr26, Pax6, Nup85, Rars2, Map3k7, Pdss2, Rbl1, mir-103) were found mutated in imatinib-treated CML-CP–like mice. Because gene copy number and range of CNGs/CNLs detected in imatinib-treated and untreated animals are similar, it is unlikely that the aberrations in former animals were accumulated exclusively before and/or after treatment.
ROS are downregulated in TKI-naive and TKI-treated LSCs by the PAK serine/threonine kinase inhibitor IPA3

We have reported before that persistent activation of a small GTP-binding protein Rac2 may be responsible for elevation of mitochondrial ROS resulting in oxidative DNA damage in LSCs.7 Using flow cytometry, we now demonstrated that inhibition of BCR-ABL1 kinase by imatinib resulted in the expected decrease of phospho-Y964 STAT5 in most primitive Lin2CD341CD38-CFSEmax/CTVmax quiescent human LSCs, and in Lin2CD341CD382LSCs (Figure 5A-B). However, high levels of activation-associated PAK phospho-T423, a Rac downstream signaling effector, were detected in both imatinib-naive and imatinib-treated LSCs (Figure 5A-B) and also in dasatinib- and nilotinib-treated LSCs (Figure 5C).34 Moreover, coflin, a member of the PAK-induced signaling pathway, was also highly phosphorylated in LSCs before and after imatinib treatment (Figure 5A-B). These results identified the PAK serine/threonine kinase signaling pathway as a potential target for antioxidant treatment.

To determine whether inhibition of PAK reduces ROS in LSCs, we have used IPA3, a highly selective non-ATP-competitive small-molecule inhibitor of class I PAK kinases.35 IPA3 inhibited activation-associated phospho-T423 PAK (Figure 5D inset) and downregulated ROS levels by 2-fold in LSCs (Figure 5D).

Table 2. Imatinib-naive and imatinib-treated CML-CP–like mice accumulate genetic aberrations in leukemia cells

<table>
<thead>
<tr>
<th>Mice*</th>
<th>CNs†</th>
<th>CNGs‡, counts</th>
<th>CNGs, bp range</th>
<th>CNLs, counts</th>
<th>CNLs, bp range</th>
<th>CNG/L§, median</th>
</tr>
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<tr>
<td>Untreated</td>
<td>2.6 ± 1.8</td>
<td>1-410</td>
<td>3-448908</td>
<td>7-343</td>
<td>3-271869</td>
<td>659 (104-2377)</td>
</tr>
<tr>
<td>Imatinib</td>
<td>2.7 ± 1.6</td>
<td>113</td>
<td>3-63658</td>
<td>84</td>
<td>3-288609</td>
<td>527 (64-2688)</td>
</tr>
</tbody>
</table>

**CN, copy number.**

*Leukemic mice were untreated (n = 8) or treated (n = 3) with imatinib as described in “Methods” and compared with healthy untreated mice (n = 4 and n = 3, respectively).

†Median ± SD of gene CNs calculated by the Student t test (P = .383).

‡The lowest and highest counts of CNGs and CNLs per imatinib-treated CML-CP–like mouse; imatinib-treated mice are represented by average number of CNGs and CNLs per mouse. The shortest and longest base pair loss/gain (bp range) is shown. Segments displaying CN < 0.5 and CN > -0.5 and a minimal segment size of 5 probes were selected for investigation. CN = log2 (sample/reference).

§Results are expressed as median 25 to 75 percentile (range) calculated from CNG and CNL bp range values with the Mann-Whitney rank sum test (P = .074).

Discussion

Preexisting and acquired genomic instability in TKI-naive and TKI-treated CML-CP patients, respectively, is a major obstacle to achieve a cure.7 We show here that LSCs, including the TKI-refractory quiescent subpopulation, and also LPCs from CML-CP, accumulate ROS-induced oxidative DNA damage. Unexpectedly, inhibition of BCR-ABL1 kinase by TKIs (imatinib, dasatinib, nilotinib) exerted only a very modest inhibitory effect on ROS and oxidative DNA damage in TKI-refractory quiescent LSCs, and did not affect proliferating LSCs and early LPCs in the presence of

![Figure 4](https://www.bloodjournal.org/16MAY13/VOLUME121/NUMBER20/Figure4.png)

**Figure 4. Elevated levels of ROS and oxidative DNA damage in quiescent LSCs treated with imatinib.** Lin-c-Kit+Sca-1+Fluor670™ quiescent HSCs/LSCs from healthy (black bars) and CML-CP–like (gray bars) mice were untreated or treated with 1 μM imatinib (–IM and +IM, respectively) followed by FACS analysis of phosphotyrosines (P-Tyr), ROS, and 8-oxoG. (A) P-Tyr was measured in 7 LSC and 4 HSC samples; *P = .039 in comparison with untreated HSCs and **P = .028 in comparison with untreated LSCs. (B) Fold change of ROS detected in 3 LSCs vs 2 HSCs samples; *P = .019 and **P = .017 in comparison with corresponding HSCs. (C) 8-oxoG was examined in 4 LSCs and 5 HSCs samples; *P = .002 and **P = .031 in comparison with corresponding HSCs.
physiologic concentrations of hematopoietic growth factors. In addition, TKI-naive and TKI-treated LSCs and LPCs obtained at diagnosis from patients who never achieved CCR contained more ROS and oxidative DNA damage than these from patients achieving CCR followed by MMR/CMR. In concordance, gene expression signature of primary imatinib-resistant patients includes genes regulating oxidative stress and DNA repair.36,37 These observations in conjunction with unfaithful/inefficient repair of DNA lesions explain why these patients are more prone to progress to CML-AP or CML-BP.4,38

High levels of ROS-induced oxidative DNA damage may be especially dangerous in LSCs because they may display some intrinsic disadvantages in DNA repair in comparison with LPCs.29

To determine whether LSCs can accumulate genomic instability, an inducible murine model of CML-CP-like disease was used.22 In this model, LSC capacity is restricted to Lin Kit Sca-1 + cells, in particular to a population of cells with LT-LSC phenotype.23,40 We show here that TKI-naive Lin Kit Sca-1 + LSCs (equivalent of human Lin CD34 CD38 ), their LT, ST, and MPP subpopulations, and especially the most primitive Lin Kit Sca-1 CFSEmax/ eFluor670max quiescent LSCs, but not Lin Kit Sca-1 LPCs accumulate ROS and oxidative DNA damage. As in CML-CP primary cells, TKI-refractory Lin Kit Sca-1 + eFluor670max quiescent LSCs from CML-CP-like mice displayed high levels of ROS and oxidative DNA damage despite inhibition of BCR-ABL1 kinase by imatinib.

Elevated levels of ROS and oxidative DNA damage in Lin Kit Sca-1 + LSCs, but not in Lin Kit Sca-1 LPCs, may depend on enhanced BCR-ABL1 expression in the former cells (supplemental Figure 2A). This speculation is in agreement with our observation that ROS and oxidative DNA damage in CD34 + cells transfected with BCR-ABL1 were proportional to the “kinase dosage” (supplemental Figure 3). The fact that cells expressing higher levels of BCR-ABL1 display accelerated frequency of acquiring TKI resistant mutants supports the overall hypothesis that the rate of genomic instability is proportional to BCR-ABL1 expression levels.45 Because CML-CP LSCs appear to express higher levels of BCR-ABL1 than LPCs,42,43 it could be postulated that the former cells may be more prone to accumulate genomic instability. However, we cannot exclude the possibility that differences in ROS and oxidative DNA damage between LSCs and LPCs may also depend on intracellular regulatory mechanisms specific for the cell compartment.34

Accumulation of ROS-induced oxidative DNA damage in TKI-refractory LSCs, including the quiescent population, resulted in the accumulation of clinically relevant genetic aberrations in TKI-naive and TKI-treated CML-CP-like mice. TKI-resistant BCR-ABL1 kinase mutations and a wide range of scattered genetic aberrations have been detected in concordance with reports from CML-CP patients.10 TKI-resistant BCR-ABL1 kinase mutations E255K and T315I results from G:C→A:T substitutions, which are common for ROS-induced oxidative DNA damage. Chromosomal abnormalities found in CML-CP-like mice include major and minor deletions/ additions, which are common in patients with CML-AP and CML-BP4 and have also been detected in TKI-resistant patients.17,33 Some of these aberrations, for example, deletions of Trp53 and Rb1 (encoding tumor suppressors p53 and Rb1, respectively), Ikaros family zinc finger protein 1, and mir-103, and gains of Zfp423 (encoding zinc finger protein 423) and Idh1 (encoding isocitrate dehydrogenase 1) affected genes already identified for their role in the induction of CML-BP and/or contribution to TKI resistance.17,29,33,45 The fact that leukemic mice displayed CML-CP but not CML-BP-like disease while harboring these genomic aberrations supports the hypothesis that CML-BP progression is usually triggered by accumulation of a specific set of mutations.4

The presence of cytokines presumably plays a pivotal role in enhancement of ROS-induced oxidative DNA damage, especially in TKI-treated LSCs.40,46,47 This speculation is supported by our data showing that BCR-ABL1(K1172R) kinase-dead mutant does not significantly elevate ROS and oxidative DNA damage (supplemental Figure 4). However, preexisting conditions responsible for generation of t(9;22)(q34;q11) encoding BCR-ABL1 oncogene may not be ruled out in LSCs from CML-CP patients.

BCR-ABL1 kinase-independent mechanisms most probably do not involve SRC kinases because dasatinib, a dual inhibitor of ABL1 and SRC, did not downregulate ROS. Our recent report indicated that activation of small GTP-binding protein Rac2, which in turn modulates electron flow through MRC, results in generation of high levels of ROS in imatinib-naive and imatinib-treated CML cells.7 Here we report that the PAK kinase–phospho-cofilin pathway, which is downstream of Rac, remains activated in TKI-
naive and TKI-treated LSCs and may lead to F-actin polymerization, which in turn causes reduction of mitochondrial membrane potential and enhances ROS production (supplemental Figure 5). 

Moreover, genome-wide microarray analysis (GSE18446) detected elevated expression of genes involved in the generation of ROS (eg, Ant, Cox6a2, Paox, CoxD-like) and diminished expression of genes responsible for downregulation of ROS (eg, Gstm2, Pklr, Achr7d3) (supplemental Table 2) in Lin−c-Kit+Sca1− LSCs. Interestingly, 4 of 7 aberrantly expressed genes regulate oxidative phosphorylation (Ant), mitochondrial electron transport (Cox6a2, CoxD-like), and mitochondrial fatty acid oxidation (Echd3-3), further supporting the hypothesis that dysregulation of mitochondrial metabolism in LSCs may lead to enhanced production of ROS.

Altogether, we postulate that genomic instability may originate from LSCs, including the most primitive quiescent LSCs, and contribute to the emergence of mutations and/or chromosomal aberrations in CML-CP patients at diagnosis and also during TKI treatment. This conclusion is supported by the report that TKI-resistant BCR-ABL1 kinase mutations have been detected in Lin−CD34−CD38− long-term culture initiating cell LSCs from CML-CP patients at diagnosis and after imatinib treatment. In addition, CML-CP can progress to either myeloid or lymphoid blast phase, or even a mixed myeloid/lymphoid phenotype, and chromosomal abnormalities are documented in all phenotypes, suggesting that genomic instability originates, at least in some cases, in LSCs.

It is plausible that before achieving CCR or MMR, TKI-resistant mutants and additional chromosomal aberrations may originate not only from numerous LPCs, but also from LSCs. However, LSCs are the most likely source of these abnormalities in patients relapsing after a CCR and MMR, when the majority of leukemia cells consist of imatinib-refractory LSCs. The frequency of these events diminishes during years after achieving CCR/MMR, highlighting the heterogeneity among LSCs and complexity of genetic aberrations required to stimulate expansion of TKI-refractory/resistant LSCs, especially the quiescent subpopulation.

Genomic instability in LSCs is of particular concern because these cells are not eliminated by TKIs and can even expand in the presence of inhibitors to acquire TKI-resistant BCR-ABL1 kinase mutations and additional chromosomal aberrations. This could be especially deleterious because CML-CP patients at diagnosis have estimated to have ~5 × 10^7 CD34^+ cells displaying innate imatinib resistance. Imatinib-treated patients in CCR may carry 10^7 to 10^9 leukemia cells and even BCR-ABL1-negative patients (by PCR) in CMR may have up to 10^6 leukemia cells in the Lin−CD34^+CD38^- compartment, most of which belong to the pool of quiescent and/or TKI-refractory LSCs. Therefore, LSCs, especially in the subpopulation of patients poorly responding to TKIs and displaying the highest levels of ROS, may act as “ticking time bombs,” which at some stage acquire additional genetic changes that cause disease relapse and induce more advanced CML-AP or CML-BP. Thus, novel strategies to eradicate LSCs and/or to prevent genomic instability (eg, targeting PAK class I kinases suggested here) are needed.

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Authorship

Contribution: E.B.-G. performed the majority of the experiments with tet-off SCLtTA/p210BCR-ABL1 transgenic mice with the assistance of M.S., M.K. and L.K., and analyzed cells expressing various levels of BCR-ABL1 kinase and kinase-dead BCR-ABL mutant; G.H., S.F., and M.N.-S. generated data from CML-CP primary cells; H.-U.K. analyzed SNPs; T.L. and M.C.M. detected BCR-ABL1 kinase mutations; J.M. optimized and performed ligation-PCR; H.M. provided CD34^+ human hematopoietic cells transfused with BCR-ABL1 kinase and kinase-dead BCR-ABL mutant; T. Stoklosa, I.S., and T.L.H. supplied CML cells and collected patient’s data; S.K. and R.B. helped to design experiments and interpret the data, and provided valuable input for the final version of the manuscript; and T. Skorski conceived the hypothesis, designed the experiments, supervised the project, and wrote the manuscript.

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References

Genomic instability may originate from imatinib-refractory chronic myeloid leukemia stem cells

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