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Chronic myeloid leukemia cells refractory/resistant to tyrosine kinase inhibitors are genetically unstable and may cause relapse and malignant progression to the terminal disease state

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Abstract

BCR-ABL1 kinase-induced chronic myeloid leukemia in chronic phase (CML-CP) usually responds to treatment with ABL tyrosine kinase inhibitors (TKIs) such as imatinib, dasatinib and nilotinib. In most patients TKIs reduce the leukemia cell load substantially, but some leukemia cells, for example leukemia stem cells (LSCs) are intrinsically refractory to TKIs. In addition, some patients who respond initially may later become resistant to TKIs due to accumulation of point mutations in BCR-ABL1 kinase. LSCs or their progeny, leukemia progenitor cells (LPCs), at some stage may acquire additional genetic changes that cause the leukemia to transform further to a more advanced blast phase (CML-BP), which responds poorly to treatment and is usually fatal. We postulate that LSCs and/or LPCs refractory or resistant to TKIs may be “ticking time-bombs” accumulating additional genetic aberrations and eventually “exploding” to generate additional TKI-resistant clones and CML-BP clones with complex karyotypes.

Keywords

CML; leukemia stem cells; leukemia progenitor cells; genomic instability; relapse

Biological significance of genomic instability in CML

BCR-ABL1 fusion tyrosine kinase encoded by t(9;22) transforms hematopoietic stem cells (HSCs) and cause chronic myeloid leukemia in chronic phase (CML-CP) [1]. LSCs are capable of generating large numbers of leukemia progenitor cells (LPCs) such as leukemia common myeloid progenitors (LCMPs) and leukemia granulocyte-macrophage progenitors (LGMPs), which cannot self-renew and eventually differentiate into mature elements. Thus, CML-CP is a stem cell-derived but progenitor-driven disease [1]. In more aggressive CML-BP, leukemia progenitors (LCMP, LGMP) gain self-renewal capability [2] and are therefore LSC candidates.

Small molecule ABL1 tyrosine kinase inhibitors (TKIs), such as imatinib, dasatinib and nilotinib, have revolutionized the treatment of CML-CP. After 12-months of treatment with imatinib, dasatinib or nilotinib a complete cytogenetic response (CCyR) was achieved in 65–66%, 77% and 78–80% CML-CP patients, respectively [3,4]; however, the rate of major molecular response was only 22–28%, 46% and 43–44%, respectively.

Although TKIs eventually eliminate the majority of LPCs in patients responding to the treatment, their effect on LSCs is questionable. CML-CP patients can initially have $\sim 5 \times 10^7$ leukemia cells displaying innate resistance to imatinib and even PCR-negative patients may harbor up to 10^6 CML cells [5].

Although several mechanisms of acquired TKI resistance have been proposed, the appearance of mutated BCR-ABL1 isoforms plays a major role; overall, point mutations in the kinase domain of *BCR-ABL1* have been detected in 23% of the imatinib-naïve patients and in approximately 50% of patients with acquired resistance to imatinib [6,7]. TKI-resistant BCR-ABL1 kinase mutants exhibit altered kinase activity and transformation potency, and are associated with clonal cytogenetic evolution, which may facilitate disease progression [7–9]. In concordance, the presence of mutations in BCR-ABL1 kinase were associated with greater likelihood of progression to blast phase, which suggests enhanced genomic instability in these cells [10,11]. In addition to TKI-resistant BCR-ABL1 mutants additional chromosomal aberrations, loss of *P53* and *CDKN2A* and *RUNX1* abnormalities are likely to play a role in TKI resistance [12–16], increasing the risk of treatment failure [17].

Transition of a relatively benign CML-CP to the aggressive CML-BP is believed to be due to accumulation of additional chromosomal aberrations and mutations [18]. The frequency of additional chromosomal abnormalities is around 7% in CML-CP and increases to 40–70% in the advanced phases of disease, as evaluated by standard cytogenetic analysis [19]. More sensitive comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) analyses detected multiple genetic aberrations already in CML-CP, but CML-BP patients carried much more complex karyotypes [20,21]. Thus genomic instability is an early event in CML-CP, which accumulates in CML-BP.

Point mutations in BCR-ABL1 kinase and chromosomal aberrations have been detected in the CD34+ leukemic sub-population (LSCs and LPCs) including CD34+CD38– LSCs [22–24]. In addition, the fact that CML-CP can progress to either myeloid or lymphoid blast phase (sometimes even a mix myeloid/lymphoid phenotype is observed) and that chromosomal abnormalities are documented in both phenotypes [25] suggests that genomic instability occurs at the level of LSC and/or LCMP/LGMP. Furthermore, mutations detected in LSCs are likely to be passed onto successive generations of LPCs [23,24,26].

Since BCR-ABL1 kinase induces genomic instability [27], TKIs should prevent accumulation of additional genetic changes in CML cells. In fact, imatinib diminished ROS and oxidative DNA damage, and reduced point mutations and other genetic aberrations in BCR-ABL1-positive cell lines [28,29]. However, TKI-treated CML patients continue to accumulate point mutations and chromosomal aberrations eventually leading to the disease relapse and/or malignant progression (Figure 1) [30–33].

There are several possible explanations for persistent genomic instability during TKI treatment. *First*, LSCs (CD34+CD38– cells and the quiescent sub-population) are resistant to TKIs [34–36]. *Second*, the inhibitory effect of TKIs on BCR-ABL1 kinase-induced signaling is obscured in CD34+CD38– LSCs and CD34+CD38+ LPCs especially in the

presence of growth factors, usually resulting in incomplete inhibition or even stimulation of signaling pathways, such as those involving STAT5, AKT, and MAPK [37,38]. In addition, CD34+ LPCs can still undergo up to 1–3 cell cycles and persist in the presence of TKI and growth factor before eventually being eliminated [26,39]. Thus, LSCs, which undergo cell cycle to replenish their population, should survive during TKI treatment. *Third*, we cannot exclude the possibility that genomic instability in LSCs and/or LPCs, at least to some extent, is driven by the presence of BCR-ABL1 protein, but not by its kinase activity, which is inhibited by TKI [40]. *Fourth*, we should not ignore the potential mutagenic effect of TKIs used for a prolonged period of time [41]. Therefore, TKIs do not abrogate genomic instability.

In addition, if CML cells display an active pre-existing genomic instability responsible for generation of t(9;22), this process is BCR-ABL1 kinase-independent and will continue generating errors despite treatment [32]. This speculation implicates BCR-ABL1 kinase-dependent and -independent genomic instability in CML cells.

Second generation TKIs (dasatinib, nilotinib) active against some imatinib-resistant mutants led to development of novel clones resistant to these TKIs [32,42] probably due to elevated genomic instability, which produced imatinib-resistant clones and/or to ineffectiveness of TKIs in stem/quiescent cells. Thus, it is likely that CML cells will respond to next generation TKIs by developing resistant clones. In addition, patients harboring TKI-resistant clones are more prone to accumulate additional genomic aberrations leading to progression to blast phase [10]. These clinical observations indicate that (1) TKIs do not prevent accumulation of TKI-resistant mutants and other chromosomal aberrations, and (2) a novel strategy is needed to prevent genomic instability in order to achieve better therapeutic effect or even a cure in CML.

It is expected that due to prolonged survival on TKI therapy the number of CML patients will grow continuously. Most of these patients will be PCR-positive. Moreover, increasing numbers of these patients will accumulate TKI-resistant mutations enhancing their risk of disease relapse and CML-BP progression.

Sources of genomic instability in CML: Reactive oxygen species (ROS) and oxidative DNA damage

Mutations usually result from enhanced DNA damage and/or deregulated mechanisms of DNA repair [43–48]. Much endogenous DNA damage arises from ROS [49]. ROS are generally small, short-lived and highly reactive molecules, formed by incomplete one-electron reduction of oxygen. The most important primary ROS is superoxide radical anion ($\bullet\text{O}_2^-$), which may lead to the production of hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet\text{OH}$) [50] (Figure 2). While $\bullet\text{O}_2^-$ exerts a moderate effect on DNA, H_2O_2 and $\bullet\text{OH}$ have strong DNA damaging capability.

BCR-ABL1–transformed cell lines and CD34+ CML cells contain, on average, 2–6 -times more ROS (H_2O_2 and $\bullet\text{OH}$) than normal counterparts (CML-BP>CML-CP>normal) [28,29,51]. Although mitochondrial respiratory chain and NADPH oxidase 4 were suggested

as BCR-ABL1 - PI-3k - Akt downstream effectors responsible for overproduction of ROS [52,53], the mechanisms generating an excess of ROS in CML cells remain largely unknown.

ROS can cause damage to all nucleobases and deoxyribose residues in DNA, and free nucleotides generating oxidized bases and DNA double-strand breaks (DSBs) [54]. The number of oxidative “hits” to DNA (for example 7,8-dihydro-8-oxo-2'-deoxyguanosine, 8-oxoG) per normal human cell per day is about 10^4 , and normal cells contain ~50 DSBs per cell per cell cycle. CD34+ CML cells display about 3–8 -times more oxidized nucleobases and 4–8 -times more DSBs [28,29,51].

Elevation of ROS in CML cells appears to be a “driving force” of genomic instability in BCR-ABL1-positive leukemia cells. ROS-induced oxidative DNA damage resulted in mutations in *BCR-ABL1* kinase encoding resistance to TKIs and in accumulation of chromosomal aberrations often detected in CML-BP [28,55].

Sources of genomic instability in CML: unfaithful and inefficient repair of the oxidative DNA lesions

Cellular DNA repair systems act to remove DNA damage and ultimately preserve the informational integrity of the genome; if too much damage is inflicted, the apoptotic pathways are activated to eliminate cells with irreparable and potentially mutagenic DNA lesions [56].

Oxidized bases often cause misincorporation of a nucleotide during DNA synthesis, for example 8-oxoG:A, forming a mismatch [57]. Several lines of evidence indicate that mismatch repair (MMR), in addition to removing post-replicative errors from DNA is also involved in protection from accumulation and repair of lesions resulting from ROS such as 8-oxoG:A [58]. The role of MMR in genomic instability in CML was initially investigated by measuring microsatellite instability, which did not provide conclusive results [59–62]. However, recent studies with the use of a reporter plasmid containing mismatched GFP gene revealed that BCR-ABL1 kinase suppressed MMR [63]. Therefore an elevated level of oxidative DNA damage (e.g., 8-oxoG) combined with inefficient MMR activity may contribute to accumulation of point mutations in CML cells, including these in BCR-ABL1 kinase encoding TKI-resistant mutants.

Oxidative DNA damage can also generate DSBs that represent a “clear and present danger” to survival and genomic integrity. Human cells may use three different repair mechanisms: homologous recombination repair (HRR), non-homologous end-joining (NHEJ), and single-strand annealing (SSA) to repair DSBs [64]. HRR is considered as faithful mechanism, while NHEJ and SSA usually generate small or large deletions.

HRR depends on DNA strand exchange and homology-dependent repair of DSBs, which is usually an accurate process resulting from the use of an undamaged copy of broken DNA as a template for the repair process [65,66]. NHEJ normally has an important function in maintaining genomic integrity, but not necessarily the fidelity. It joins sequences at the

broken ends, which have little or no homology, in a non-conservative manner, and usually some genetic information is lost [67,68]. SSA arises from the annealing of complementary single strands formed after resection at a DSB [69,70]. When sequence repeats are present near a DSB, they can undergo SSA, resulting in a deletion of sequences between the repeats. BCR-ABL1 kinase stimulates all three mechanisms of DSB repair to enhance genomic instability. HRR products incorporated point mutations, NHEJ resulted in more extensive deletions in some products and SSA generated large deletions [29,51,71–74].

Concluding remarks

In conclusion, TKI-refractory cells appear to display elevated levels of ROS-induced oxidative DNA damage and inefficient/unfaithful DNA damage repair mechanisms. This process may occur at the level of LSCs and/or LPCs (Figure 3). The combination of enhanced DNA damage and unfaithful DNA repair may turn these cells into “ticking time-bombs”, eventually “exploding” to produce TKI-resistant LPC clone and/or CML-BP clone upon accumulation of additional aberrations.

Reports from our laboratory demonstrated that anti-oxidants such as vitamin E and N-acetylcysteine prevented accumulation of genetic aberrations in the experimental models of CML [28,29,55]. Since TKIs cannot completely eliminate the effects of BCR-ABL1 kinase and thus may not effectively inhibit genomic instability, antioxidants were applied in combination with imatinib. This treatment resulted in a synergistic effect abrogating the accumulation of TKI-resistant clones carrying point-mutations in BCR-ABL1 kinase [28]. Accordingly, we postulate that antioxidants may prevent accumulation of TKI-resistant mutants and chromosomal aberrations in CML-CP LSCs and/or LPCs and improve the therapeutic effect of TKIs (Figure 4).

In addition, various hematologic malignancies (myeloproliferative disorders, acute myeloid leukemia, acute lymphoblastic leukemia, anaplastic large-cell lymphoma) expressing other fusion tyrosine kinases (FTKs) such as BCR-FGFR1, TEL-ABL1, TEL-JAK2, TEL-PDGFR, TEL-TRKC(L), NPM-ALK, ZNF198-FGFR1, and PCM1-JAK2 may also display elevation of ROS and enhanced spontaneous DNA damage [18,51,75]. Therefore therapeutic modalities abrogating genomic instability in CML may have broader application in other tumors induced by these FTKs [18].

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Figure 1. Model of CML disease relapse and progression in the TKI era

At diagnosis CML-CP cells in addition to Philadelphia chromosome may harbor additional sporadic genetic aberrations; some patients also have TKI-resistant mutants. TKIs eliminate most leukemia cells, but cannot inhibit genomic instability in TKI-refractory LPCs, in pre-existing TKI-resistant LPCs and also in TKI-resistant LPCs emerging during treatment. Thus, these cells eventually accumulate multiple chromosomal aberrations. CML-BP clones appear when these cells acquire a critical number and/or combination of genetic aberrations.

Figure 2. General schema of ROS generation

At neutral pH, inside the cellular system, superoxide anion ($\cdot\text{O}_2^-$) is a moderately stable free radical, which can be generated by various mechanisms, e.g., mitochondrial respiratory chain and NADPH oxidase. Its toxicity is principally based on the generation of further reactive species, which are then able to attack DNA. The dismutation of $\cdot\text{O}_2^-$ by superoxide dismutase (SOD) results in H_2O_2 production, which can easily penetrate through membranes, due to its intercharged nature and poor reactivity. On the other hand, H_2O_2 may be converted by iron (Fe^{2+})-driven cleavage of H_2O_2 in a Fenton reaction to generate the highly reactive $\cdot\text{OH}$. DNA damage capability of different ROS is indicated.

Figure 3. A schema illustrating genomic instability making CML cells ticking time-bombs
 Normal HSC or HSC displaying pre-existing genomic instability (?) acquires t(9;22) to become LSC and initiate CML-CP. Time-dependent BCR-ABL1-driven (and perhaps also pre-existing) genomic instability in CML-CP LSCs, LCMPs and/or LGMPs (brown nuclei) is responsible for the acquisition of genetic aberrations encoding resistance to imatinib, including point mutations in BCR-ABL1 kinase (cells with blue nuclei and “small explosion mark”). These cells display higher level of genomic instability leading to accumulation of additional mutations causing resistance to other TKIs. TKI-refractory cells continue to accumulate genetic aberrations until the critical combination is achieved, which promotes CML-BP progression (cells with red nuclei and “large explosion mark”). These processes may originate in CML-CP LSCs and be passed on to descendant cells, and/or in LPCs (LCMPs, LGMPs). In summary, LSCs and/or LPCs may be “ticking time-bombs” eventually exploding to generate TKI-resistant and/or CML-BP clones.

Figure 4. Role of antioxidants in CML-CP treatment

BCR-ABL1 kinase stimulates ROS, which cause enhanced oxidative DNA damage. Unfaithful and inefficient repair of numerous oxidative DNA lesions generates point mutations in *BCR-ABL1* kinase encoding resistance to TKIs and produces chromosomal aberrations contributing to CML-BP. In addition, mechanisms responsible for the appearance of t(9;22) (?) and growth factor-mediated signaling (GFs) may be able to co-stimulate ROS and genomic instability. Since TKIs are not effective or are only partially effective in TKI-refractory LSCs and TKI-resistant LPCs, scavenging of ROS should inhibit/delay mutagenesis. Therefore, antioxidants (AO) may disarm LSCs/LPCs “ticking time-bombs” by reducing ROS and preventing genomic instability. In such case LSCs will not gain additional aberrations and will continue to generate TKI-sensitive LPCs, which will be eliminated by continuous TKI treatment. Since BCR-ABL1-positive LSCs have limited self-renewal capability they will eventually exhaust during long-term therapy.