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Chronic myeloid leukemia: mechanisms of blastic transformation

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by excessive accumulation of apparently normal myeloid cells. It occurs with an annual incidence of 1.0–1.5 per 100,000 persons. CML occurs very rarely in children. In the Western world, the median age of onset is 50–60 years, which reflects the average age of the population. Although symptoms at presentation may include lethargy, weight loss, unusual bleeding, sweats, anemia, and splenomegaly, in more developed countries, 50% of patients are asymptomatic and are diagnosed as a consequence of blood tests performed for unrelated reasons. More than 90% of CML patients are diagnosed when their disease is in a relatively early phase known as the chronic phase (CP).

CML-CP is characterized by the presence of the Philadelphia (Ph) chromosome and the oncogene that it encodes in the vast majority of myeloid cells and some lymphocytes. The Ph chromosome results from a (9;22)(q34;q11) reciprocal translocation that juxtaposes the c-abl oncogene 1 (*ABL1*) gene on chromosome 9 with the breakpoint cluster region (*BCR*) gene on chromosome 22, generating the *BCR-ABL1* fusion oncogene with greatly enhanced ABL1 kinase activity. It is generally accepted that acquisition of the *BCR-ABL1* oncogene is the initiating event in the genesis of CML-CP, despite various lines of evidence suggesting that, at least in some cases, hematopoiesis may already be clonal before the acquisition of the Ph chromosome (1). It is believed that acquisition of the *BCR-ABL1* gene occurs initially in a single HSC that gains a proliferative advantage and/or aberrant differentiation capacity over its normal counterparts, giving rise to the expanded myeloid compartment (2).

Most CML-CP patients are currently treated with one of three drugs designed to block the enzymatic action of the BCR-ABL1 tyrosine kinase. The first of these to be developed was imatinib. Recent karyotype analyses show that 60%–70% of patients achieve complete disappearance of Ph-positive marrow cells and maintain exclusively Ph-negative bone marrow cells (a state designated as a complete cytogenetic response [CCyR]) 5 years after initiating imatinib treatment. The incidence of progression to a more advanced phase of leukemia in patients responding to imatinib is extremely low beyond the first two years (3). However, a small number of patients fail to respond to imatinib (primary resistance), while others respond initially and then lose their response (secondary resistance) (4). The reasons for imatinib resistance in CML-CP patients are poorly understood. Primary resistance may be related, at least in part, to the intrinsic heterogeneity of the disease (e.g., different BCR-ABL1 levels) in different patients and to the survival of variable numbers of quiescent cells from which the more mature leukemia cells are derived during CP (5). Secondary resistance may have a wide range of causes, of which the best characterized is the acquisition of mutations in the BCR-ABL1 kinase domain (such as the T315I mutation) (6).

In the last few years, two new tyrosine kinase inhibitors (TKIs), dasatinib and nilotinib, have become available, both of which are more potent in vitro inhibitors of the BCR-ABL1 kinase than imatinib. Both of these "second-generation" TKIs are effective at inducing or restoring CCyR in 40%–50% of patients who appear to have failed primary treatment with imatinib. However, approximately 20% of patients presenting with CML-CP fail to respond to both imatinib and a subsequent second-generation TKI; their prognosis is poor because of a higher risk of disease progression.

Before the advent of BCR-ABL1 TKIs, all patients with CML-CP progressed spontaneously to advanced phase CML after a median interval of approximately 5 years. The advanced phase is divided into an initial accelerated phase (AP), during which patients may still respond to treatment for some months or sometimes years, and a subsequent more aggressive blastic phase (BP). Patients with CML-BP have a median survival of approximately 6 months. Some patients progress directly to BP without an intermediate AP. The precise definitions of these three phases have been much debated in recent years (3, 7).

Conflict of interest: Catriona Jamieson has research grants from Pfizer, Coronado Biosciences, and Celgene and has also consulted for Bristol-Myers Squibb and Wintherix.

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The biological basis of BP is poorly understood. Although the majority of patients have a myeloblastic phenotype, approximately 25% of CML-BP patients show a pre–B lymphoblastic cell phenotype (8). Occasional cases of T lymphoblastic transformation have been identified (9). These findings lend support to the notion that the *BCR-ABL1* oncogene arises in a primitive cell, namely a leukemia stem cell (LSC), not yet committed to either myeloid or lymphoid differentiation. Conversely the blastic clone may originate either at the level of the multipotent LSC or at the level of a more committed leukemia progenitor cell (LPC). Here, we discuss genetic and epigenetic mechanisms leading to the transition of CML-CP into CML-BP and propose some novel therapeutic modalities that might prevent malignant progression.

CML-BP patients: a therapeutic challenge

In the past, CML-BP was often treated with drugs used for acute leukemias, but patients usually relapsed within a few months. The introduction of TKIs has improved prognosis to some degree. The majority of CML-BP patients not previously treated with TKIs do initially respond to treatment with these agents, either alone or in combination with conventional chemotherapeutic drugs, but most still relapse within a few months of achieving a seemingly complete hematologic or even cytogenetic response. Therefore, any CML-BP patient who does respond to modern therapy should proceed, if possible, to allogeneic stem cell transplant prior to relapse. In the 1990s, the results of allografting for CML-BP patients were not impressive — only 5%–10% of patients experienced long-term, leukemia-free survival (10) — but the use of a TKI after transplant may improve these results.

Extrapolating from the good clinical outcomes of treating CML-CP with TKIs and the dismal responses achieved in treating CML-BP, one might reasonably conclude that the best approach to CML-BP would be prevention. Indeed, it appears today that continued use of TKIs to treat CML-CP may prevent BP in a large proportion of patients, but 15%–20% of patients, most of whom will have been classified as nonresponders, may progress to BP (11). Indeed, the GIMEMA Working Party (Italian Group for Adult Hematologic Diseases) reported that the detection of TKI-resistant *BCR-ABL1* mutations in CML-CP is associated with a greater likelihood of disease progression (12). These patients may possess genetic/epigenetic abnormalities distinct from the patients with nonmutated *BCR-ABL1*, the appearance of which could be influenced by the duration of the BCR-ABL1–induced signals. Furthermore, the ability of TKIs to render residual CML cells "inactive" rather than to eradicate them entirely suggests that BP might still occur occasionally even in "responding" patients.

However, as a minority of patients will still progress to CML-BP, the routine use of TKIs may need to be supplemented with other agents, any of which might prevent BP. Possible examples are antioxidants (13), which protect against cancer-causing DNA mutations; farnesyl transferase inhibitors (14), which inhibit RAS signaling; hydroxychloroquine (15), which inhibits autophagy in some situations; sonic hedgehog pathway antagonists (16, 17), which impair self-renewal pathways only when used in combination with TKIs; and activators of protein phosphatase 2A (PP2A) (18), which targets BCR-ABL1 and other downstream oncogenic signaling cascades.

Biological complexity of CML-BP

At present, the molecular mechanisms underlying disease progression are still uncertain, but most likely involve activation of oncogenic factors and/or inactivation of tumor suppressors (19). A plausible assumption is that BP is a multistep, time-dependent process initiated by both BCR-ABL1–dependent and –independent DNA damage associated with inefficient and unfaithful DNA repair in CML-CP that, if facilitated by an increased level of BCR-ABL1 activity, leads to selection of one or more CML-BP clones.

The genetic lesions observed in CML-BP patients in the past and now since the introduction of TKIs mostly include the presence of additional chromosomes, gene deletions, gene insertions, and/or point mutations (including *BCR-ABL1* mutations) (20–22), but patterns differ in myeloblastic and lymphoblastic transformations (23). At the molecular level, the most common mutations detectable (other than those in the *BCR-ABL1* kinase domain) occur at the loci of the tumor suppressor genes *P53* (20%–30% of cases) and the runt-related transcription factor gene (*RUNX1*) (38% of cases) in myeloid BP and at the loci of cyclin-dependent kinase inhibitor 2A/2B (*CDKN2A/B*) (50% of cases) and Ikaros transcription factor (*IKZF1*) (55% of cases) in lymphoid BP (22, 24–28). As specific CML-BP–associated genetic alterations are relatively common, no one lesion occurs in the majority of CML-BP patients, and it is unlikely that any one specific secondary genetic aberration can be defined as the "culprit" causing disease progression. More likely, CML-BP results from the accumulation of a critical number or combination of different mutations.

Epigenetic changes are dependent mostly on the pleiotropic effect of constitutive BCR-ABL1 activity (19, 29), the levels of which start to increase in CML-AP (30). In support of this suggestion, expression studies revealed that BCR-ABL1 dramatically perturbs the CML transcriptome (31), resulting in altered expression of genes, some of which (e.g., *PRAME*, *MZF1*, *EVI-1*, *WT1*, and *JUN-B*) might play a role in BP (19, 32–34). Nonetheless, the posttranscriptional, translational, and posttranslational effects of high BCR-ABL1 levels result in the constitutive activation of factors with reported mitogenic, antiapoptotic, and antidifferentiation activity (e.g., MAPK$^{\mathrm{ERK1/2}}$, MYC, JAK2, YES-1, LYN, hnRNP-E2, MDM2, STAT5, BMI-1, and BCL-2) and inhibition of major key regulators of cellular processes, such as those regulated by the tumor suppressors p53, CCAAT/enhancer binding protein-α (C/EBPα), and PP2A (19, 29, 35). Interestingly, a signature based on six genes (*NOB1*, *DDX47*, *IGSF2*, *LTB4R*, *SCARB1*, and *SLC25A3*) was recently found to accurately discriminate early from late CP, CP from AP, and CP from BP (36); however, the biological role of these genes in disease progression is still unknown.

Thus, it is highly plausible that unrestrained and increasing BCR-ABL1 activity promotes and/or contributes to clonal evolution, thereby leading to CML-BP (37). This might occur at the level of LSCs, which display innate or acquired TKI resistance, and/or at the level of an LPC population that might have developed resistance and expanded during TKI therapy (38, 39).

Because there is a direct correlation between levels of BCR-ABL1, the frequency of clinically relevant *BCR-ABL1* mutations (40, 41), and the differentiation arrest of myeloid progenitors (42), it is likely that disease progression is triggered by the "right" combinations of genetic and epigenetic abnormalities (Figure 1). Thus, we can speculate that prevention or effective treatment of CML-BP will only be achieved if novel therapeutic strategies can be developed that are capable of interfering with the biological processes currently considered critical for the leukemic behavior of CML-BP progenitors.

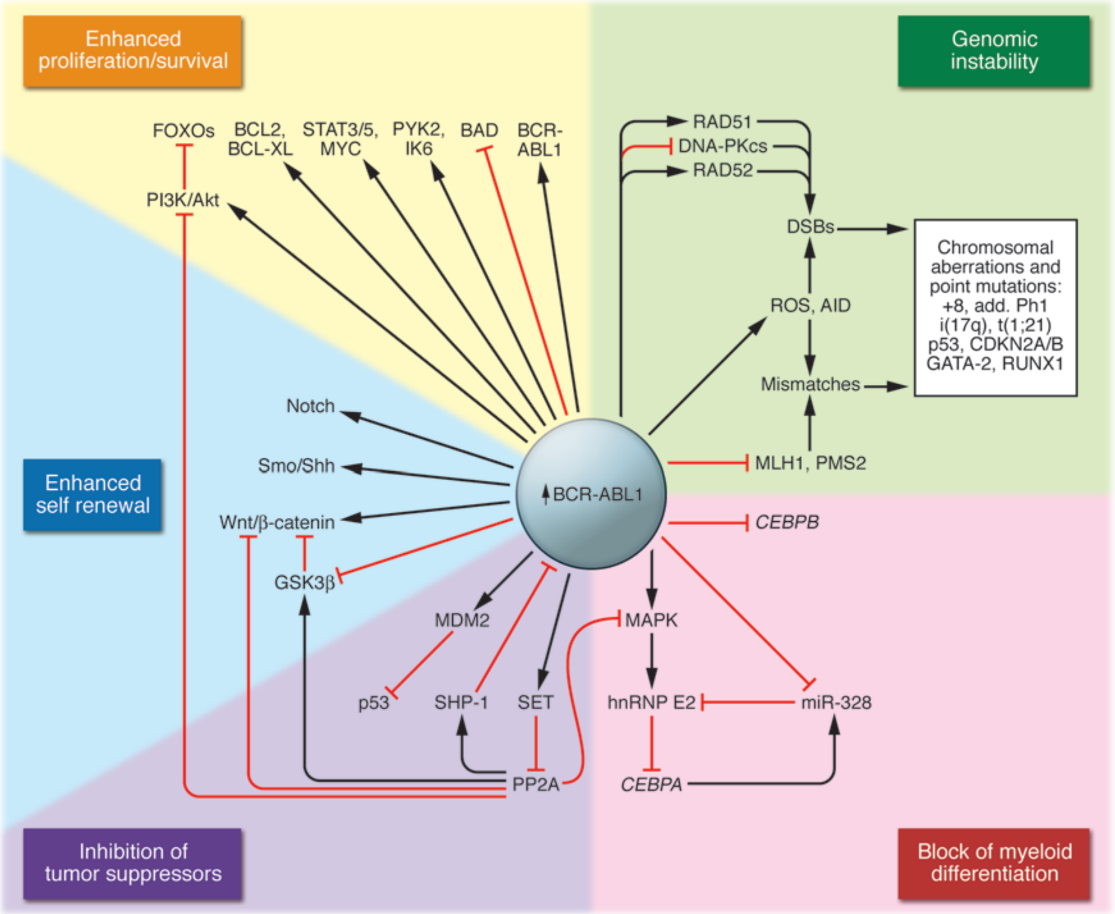

Figure 1
BCR-ABL1–dependent pathways to blastic transformation. Schematic representation of the potential BCR-ABL1–dependent molecular mechanisms leading to CML disease progression.The relatively high BCR-ABL1 expression/activity in CML-CP CD34$^+$CD38$^-$ stem cells and/or CD34$^+$ early progenitors compared with more committed progenitors, which is further markedly increased in CML-BP CD34$^+$ progenitors results in the following: enhancement of proliferation/survival pathways; increased genomic instability; and activation of pathways leading to a block in myeloid differentiation, acquisition of the ability to self renew, and inhibition of tumor suppressors with broad cell regulatory functions. BAD, BCL2 antagonist of cell death; DNA-PKcs, DNA-dependent protein kinase, catalytic subunit; FOXO, forkhead box O; IK6, Ikaros 6; miR-328, microRNA-328; MLH1, mutL homolog 1; PMS2, postmeiotic segregation increased 2; RAD51, RecA homolog in *Escherichia coli*; RAD52, RAD52 homolog (*Saccharomyces cerevisiae*); Shh, Sonic Hedgehog; wnt/β-catenin, wingless-int1/beta-catenin.

CML-BP LSCs: BCR-ABL1 overexpression, self renewal, and survival

According to the basic concept, LSCs should represent the most primitive cell able to initiate leukemia in animal xenograft limiting dilution experiments, to display self-renewal capacity, and to proliferate and differentiate (43, 44). In CML-CP, LSCs are located in the self-renewing Lin$^-$CD34$^+$CD38$^-$ population, but not in the non–self-renewing Lin$^-$CD34$^+$CD38$^+$ population (45), indicating that, in contrast to other oncogenes (e.g., *MOZ-TIF2* and *MLL-ENL*) *BCR-ABL1* cannot confer self-renewal properties (46, 47). While most human LSC research has focused on the Lin$^-$CD34$^+$ compartment, a recent study suggests that a Lin$^-$CD34$^-$ fraction of CML-CP cells also engrafts immunodeficient mouse strains, underscoring the complexity of the LSC compartment (48).

However, in CML-BP, Lin$^-$CD34$^+$CD38$^+$ granulocyte-macrophage progenitors (GMPs) that overexpress BCR-ABL1 behave like LSCs (49), suggesting that the acquisition of self renewal in GMPs may depend on epigenetic and/or genetic alterations caused by elevated expression of BCR-ABL1. Thus, LSCs in CML-BP patients may reside in at least 3 different subsets: Lin$^-$CD34$^+$CD38$^-$ and Lin$^-$CD34$^+$ cells remaining from CML-CP and the disease-driving Lin$^-$CD34$^+$CD38$^+$ GMPs.

Progression to BP is marked by overexpression of BCR-ABL1 in committed progenitors, leading to a multiplicity of genetic and epigenetic events. These cell type– and context-specific molecular events serve to enhance survival and self renewal, leading to impaired differentiation and generation of CML-BP LSCs. To date, the cell type– and context-specific effects of BCR-ABL1 overexpression have not been clearly elucidated in human stem cells, nor has the effect of the microenvironment on LSC maintenance. It seems, however, that increased BCR-ABL1 expression does play a critical role in promoting the genetic instability that drives progression to BP and the molecular evolution of LSCs in CML.

BCR-ABL1 overexpression and aberrant splicing. BCR-ABL1 induces alterations in pre-mRNA splicing in hematopoietic progenitor cells that result in aberrant adhesion, differentiation, survival, and self renewal as well as therapeutic resistance. Ectopic *BCR-ABL1* expression in human bone marrow and cord blood CD34$^+$ cells results in induction of factors involved in mRNA processing, export, and translation (50, 51). Interestingly, the metabolism of several mRNAs has been found to be altered in CML-BP (51). Among these, BCR-ABL1 induces alternative splicing of proline-rich tyrosine kinase 2 (*PYK2*) mRNA, thereby increasing expression of the β1-integrin–responsive *PYK2* kinase, which in turn may contribute to aberrant adhesion of CML-BP progenitors (50).

Likewise, BCR-ABL1–induced aberrant splicing might play an important role in those cases of CML-BP without deletion of the *IKZF1* gene (25). Indeed, a recent study suggests that BCR-ABL1 may inhibit differentiation and contribute to lymphoid CML-BP by promoting the production of a dominant negative splice isoform (IK6) of *IKZF1*, a transcription factor gene involved in pre–B cell differentiation (52). When this aberrant, non–DNA-binding splice isoform, IK6, was silenced in Ph-positive pre–B cells using siRNA or its production reduced by imatinib treatment, differentiation along the B cell lineage was partially restored (52). Notably, alternative splicing was also observed for *BCR-ABL1*. Aberrant *BCR-ABL1* mRNA splicing results in the generation of transcripts harboring a 35-kb insertion between *ABL1* domain exons 8 and 9, resulting in a frameshift with a truncation that, like IK6 expression, is associated with imatinib resistance (53, 54).

Finally, BCR-ABL1 overexpression is associated with mis-splicing of glycogen synthase kinase 3β (GSK3β) (55), a key component of the β-catenin destruction complex, leading to enhanced self renewal of GMPs that behave like LSCs (49). Lentiviral overexpression of wild-type GSK3β in CML-BP progenitors inhibits their capacity to engraft leukemia in immunocompromised mice (55).

Stem cell self renewal. Self renewal refers to division without differentiation and is a property normally ascribed to long-term HSCs. In mouse models, loss of junB/AP-1 enhances HSC proliferation and myeloid progenitor expansion, setting the stage for BP (56). In CML-BP, committed progenitors subvert this stem cell property of self renewal, lack the capacity to regulate it, and are able to propagate leukemia more readily. Various self-renewal pathways — including Wnt/β-catenin, sonic hedgehog, and Notch signaling — have been implicated in the generation and maintenance of CML-BP LSCs. Indeed, BCR-ABL1–independent (57) and –dependent (49, 58) mechanisms both seem to contribute to the acquisition of self renewal by CD34$^+$CD38$^+$ CD45RA$^+$CD123$^+$Lin$^-$ CML-BP GMPs. In fact, CML-BP is associated with accumulation of β-catenin, a key stem cell self-renewal mediator, in the nucleus of GMPs, thereby endowing them with self-renewal potential (49, 59). BCR-ABL1 stabilizes β-catenin through phosphorylation of tyrosines 86 and 654, which inhibits binding to axin/GSK3β, thereby enabling binding to T cell factor 4 (TCF4) and activation of transcription.

By inhibiting BCR-ABL1, imatinib prevents tyrosine phosphorylation of β-catenin and thus prevents nuclear translocation and transcriptional activation (58). Loss of β-catenin in a CML mouse model impairs self renewal of both normal HSCs and CML-BP LSCs, although the effects of decreased nuclear β-catenin on human normal HSC and CML-BP LSC maintenance remains to be established in xenograft models (60).

Decreased expression of functional GSK3β enhances CML progenitor self renewal by activating β-catenin and by elevating levels of sonic hedgehog pathway–mediators such as GLI family zinc finger 1 (GLI1) and GLI2 (32, 55). Recently, two independent studies demonstrated that overexpression of smoothened homolog (Drosophila) (Smo), an essential activator of sonic hedgehog signaling, enhanced LSC maintenance in mouse models of CML (16, 17). Conversely, Smo inhibition reduced LSC, but not normal HSC engraftment (16, 17), suggesting that the sonic hedgehog pathway is preferentially utilized by LSCs for self renewal.

Another recent study confirmed that sonic hedgehog signaling is dispensable for normal adult mouse HSC function, suggesting the possibility of targeting leukemic GMP without damaging residual HSCs (61). These findings provide the impetus for preclinical testing of a combination of Smo and BCR-ABL1 inhibitors to determine whether LSCs can be eradicated both in vitro and in xenogeneic transplantation models.

LSC survival. Resistance to apoptosis, an intrinsic property of normal HSCs, is also a hallmark of LSCs. In vivo inactivation of Dok-1 or Dok-2 decreases apoptosis, resulting in a myeloproliferative disorder (62). Moreover, the promyelocytic leukemia (*PML*) gene, a tumor suppressor that was first shown to be deregulated in acute promyelocytic leukemia, was recently found to play a pivotal role in LSC maintenance in a CML mouse model (63). Other investigators demonstrated that enhanced progenitor cell survival driven by B cell leukemia/lymphoma 2 (*BCL2*) and BCR-ABL1 overexpression promoted CML-BP development in a transgenic mouse model (64), underscoring the importance of resistance to apoptosis in BP evolution.

Although extensive SNP marker analyses demonstrated that a SNP (rs1801018) in the *BCL2* gene was associated with susceptibility to CML (65), the role of BCL2 in CML-BP progenitor survival remains to be elucidated. In CML-BP cell lines, expression levels of BCL2-interacting mediator of cell death (*BIM*), a proapoptotic *BCL2* family member, are low and can be induced by BCR-ABL1 inhibition (66). In recent studies, induction of apoptosis correlated with the magnitude and duration of BCR-ABL1 kinase inhibition (67). Transient, potent BCR-ABL1 inhibition was associated with *BIM* activation and induction of apoptosis, underscoring the importance of *BCR-ABL1* gene dosage in regulating apoptotic responses (67). In addition, JAK2-mediated activation of LYN kinase through the suppressor of variegation, enhancer of zeste, and Trithorax (SET)/PP2A/SHP1 pathway (68) may be important in promoting CML-BP LSC survival during imatinib therapy and disease progression. Pharmacologic inhibition of JAK2 induced apoptosis in imatinib-resistant CML-BP cells to a greater degree than in normal progenitors (68). Recently, targeted inhibition of arachidonate 5-lipoxygenase (ALOX5) with a 5-lipoxygenase inhibitor was shown to impair LSC survival in a CML mouse model, a finding that warrants further investigation into the role of *ALOX5* in CML-BP pathogenesis (69).

Interestingly, a recent study has shown that imatinib induces autophagy in CML-BP primitive progenitors through a mechanism that is independent of imatinib-induced, caspase-dependent apoptosis but is associated with ER stress and is suppressed by intracellular Ca^{2+} depletion (15). Suppression of autophagy genes enhanced imatinib-induced death of Ph-positive cells (15). Critically, the combination of TKIs with autophagy inhibitors resulted in killing of CML LSCs (15). Thus, autophagy inhibitors may enhance the therapeutic effects of TKIs in the treatment of CML-BP.

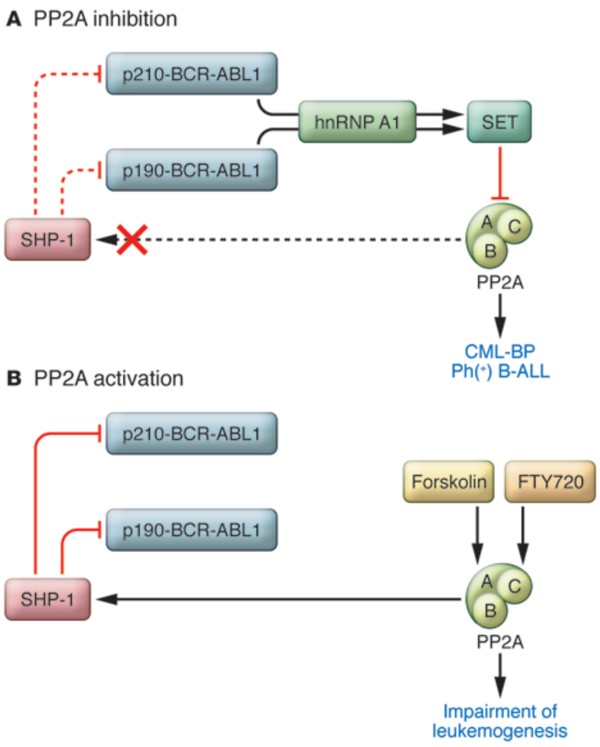

Figure 2
BCR-ABL1 and PP2A interplay. (**A**) In CML-BP and Ph-positive ALL $CD34^+$ progenitors, p210 and p190 BCR-ABL1 oncoproteins inhibit PP2A activity by inducing hnRNP-A1, which, in turn, enhances expression of SET. In BCR-ABL–positive myeloid progenitor cells, suppression of PP2A phosphatase activity is required for sustained activation of mitogenic and survival signals. (**B**) Restored PP2A activity, achieved by treatment with PP2A activators (e.g., Forskolin or FTY720), impairs in vitro and in vivo wild-type and T315I BCR-ABL1 leukemogenesis by antagonizing the effects of BCR-ABL1 on its downstream signal transducers (not shown) and promoting SHP-1–mediated BCR-ABL1 inactivation and proteasome-dependent degradation.

Global interference with BCR-ABL1–dependent and –independent signals

Since the late 1980s, when BCR-ABL1 was identified as a constitutively active tyrosine kinase, an impressive series of in vitro and in vivo studies have indicated a direct causal role of BCR-ABL1 activity in the acquisition of the molecular changes that characterize the phenotype of CML-BP progenitors (19).

In vivo resistance and in vitro sensitivity of CML-BP progenitors to TKI treatment: a biological paradox. Compelling research shows that CML-CP LSCs are resistant to imatinib as a result of various collaborating factors. These factors include quiescence, high BCR-ABL1 levels, lack of "oncogene addiction," increased activity of the drug efflux pumps ATP-binding cassette sub-family B member 1 (ABCB1) and transporter G2 (ABCG2), and decreasing organic cation transporter 1 (OCT1) expression (5, 70, 71).

In CML-BP, increased BCR-ABL1 expression (49, 72) accounts for activation of pathways transducing mitogenic, antiapoptotic signals and for differentiation arrest of the Ph-positive progenitors (42, 49, 73, 74). However, BCR-ABL1–independent mechanisms (e.g., LYN kinase–dependent mechanisms) also contribute to disease progression and imatinib resistance in some CML-BP cases with no *BCR-ABL1* amplification/overexpression (75–78). In this regard, the paradoxical in vitro and in vivo response of CML-BP progenitors to TKIs needs to be taken into consideration. While most CML-BP patients do not show long-term responses to TKIs and relapse within 12–24 months, CML-BP progenitors from these patients are still sensitive to the proapoptotic effects of imatinib when administered ex vivo. Thus, it is possible that the bone marrow environment elicits BCR-ABL1–independent signals conferring TKI resistance and sustaining in vivo survival of CML-BP blasts. In this scenario, BCR-ABL1–dependent and –independent signals likely synergize in inducing and maintaining the CML-BP phenotype. Furthermore, from this consideration, the concept emerges clearly that TKI treatment, especially at high dosage, might exert a selective pressure allowing clonal expansion of genetically unstable CML-BP progenitor cell clones that are more prone to acquire secondary chromosomal abnormalities and/or clinically relevant mutations in the *BCR-ABL1* oncogene itself and, likely, in other kinases targeted by TKIs. As CML-BP is also characterized by the loss of function of tumor suppressors, a rational and alternative therapeutic approach might envision the use of drugs capable of reactivating a tumor suppressor or suppressors.

Pharmacologic reactivation of the PP2A tumor suppressor gene. The notion that the serine-threonine phosphatase PP2A is inhibited in several types of cancer, through mechanisms that either involve the loss of expression/activity of one or more subunits or the enhanced expression of the endogenous PP2A inhibitors SET (79, 80) and cancerous inhibitor of PP2A (CIP2A) (81), led to the recognition of PP2A as a true tumor suppressor. In fact, loss of PP2A activity plays a central role in the pathophysiology of BCR-ABL1–driven leukemias. PP2A activity is slightly reduced in CML-CP $CD34^+$ progenitors but becomes markedly inhibited in CML-BP through the BCR-ABL1 dose- and kinase-dependent induction of SET (74, 82) (Figure 2). Remarkably, several targets that are shared by BCR-ABL1 and PP2A are either essential for BCR-ABL1 leukemogenesis or are altered in CML-BP (19).

Restoration of PP2A activity, either by chemical PP2A activators (e.g., forskolin and FTY720) (Figure 2) or by interfering with SET/PP2A interplay, promotes Src homology region 2–domain phosphatase 1 (SHP-1) tyrosine phosphatase–dependent BCR-ABL1 dephosphorylation (inactivation) which, in turn, triggers its degradation (74, 82, 83). Notably, SHP-1 expression is diminished in most leukemias and lymphomas (84, 85). Restoring normal PP2A activity induces marked apoptosis of $CD34^+$ CML (CP and BP) progenitors and suppresses in vivo leukemogenesis regardless of sensitivity to imatinib/dasatinib (74, 82) (Figure 2).

Loss of PP2A activity is also a feature of imatinib/dasatinib-insensitive $CD34^+CD38^-$*BCR-ABL1*$^+$ HSCs from CML (CP and BP) patients (57). Clonogenic, colony-forming cell (CFC)/replating, long-term culture–initiating cell (LTC-IC), and CFSE-mediated cell division–tracking assays revealed that FTY720 suppresses survival and self renewal and triggers apoptosis of *BCR-ABL1*$^+$ stem cells in a BCR-ABL1 kinase–independent and β-catenin–mediated manner (57). Notably, normal quiescent stem cells are not sensitive to FTY720 (57).

Because of the central role of PP2A in the regulation of survival, proliferation, self renewal, and differentiation of CML stem/progenitor cells, it is highly plausible that its loss of function contributes to BP. In this scenario, PP2A may have the role of a "gatekeeper," as its activation may control and restrain

BCR-ABL1 expression/activity, whereas its inhibition allows increased oncogene activity and induces a cascade of events that promotes disease development and progression. Thus, incorporating PP2A-activating drugs into current therapeutic protocols for CML-BP and imatinib/dasatinib-resistant (including T315I) patients has not only the potential to treat CML-BP but also to eradicate CML at the stem cell level.

Impaired myeloid differentiation in CML-BP: a true BCR-ABL1 dosage effect

Blastic transformation is phenotypically associated with the clonal expansion of the GMP pool (49), members of which have lost the ability to differentiate in response to cytokine stimuli. If we exclude the 20%–30% of CML-BP cases with *P53* mutations (28), the 11% of CML-BP cases with GATA-binding protein 2 (*GATA2*) mutations (86), and the 1%–2% of CML-BP cases with the t(3;21)(q26;q22) and t(7;11)(p15;p15) translocations associated with expression of AME (AML-1 [acute myeloid leukemia 1], MDS/EVI1 [Myelodysplastic syndrome–associated gene 1]) (87) and NUP98-HOXA9 (88) chimeric proteins, we can safely state that impaired myeloid maturation of Ph-positive GMPs is the consequence of increased *BCR-ABL1* dosage. Indeed, low BCR-ABL1 levels allow G-CSF–induced granulocytic maturation, while high oncogene expression impedes differentiation of Lin^- progenitors (89).

BCR-ABL1 levels and C/EBPα inhibition. Different genetic and epigenetic mechanisms may act alone or in cooperation to enhance *BCR-ABL1* expression and activity. Among them, *BCR-ABL1* gene amplification (90, 91), increased *BCR* promoter activity (92), decreased miR-203 expression (93), impaired PP2A activity (74), and genetic/epigenetic inhibition of SHP-1 phosphatase (74, 94) may all account for increased BCR-ABL1 expression/activity observed during disease progression (72). Interestingly, restoration of PP2A activity in myeloid precursors expressing high *BCR-ABL1* levels restores G-CSF–driven differentiation (74), suggesting that PP2A loss of function might play a central role in impairing maturation of Ph-positive GMPs.

The inhibitory effect of high BCR-ABL1 levels on differentiation depends on marked downregulation of C/EBPα (42), a transcription factor essential for granulocytic differentiation. The importance of the loss of C/EBPα activity as a central mechanism leading to differentiation arrest of myeloid CML blasts is supported by evidence that ectopic C/EBPα expression induces maturation of differentiation-arrested *BCR-ABL1*$^+$ myeloid precursors and CD34$^+$ CML-BP progenitors (42, 95, 96) and that a CML-BP–like process emerges in mice transplanted with BCR-ABL1–transduced *Cebpa*-null fetal liver cells (97). In CD34$^+$ CML-BP GMPs, loss of C/EBPα does not depend on *CEBPA* gene mutations (98), but results from the BCR-ABL1 dose–dependent induction of the RNA-binding protein heterogenous nuclear ribonucleoprotein E2 (hnRNP-E2) that, upon interaction with the *CEBPA* upstream open reading frame (uORF)/spacer element, inhibits *CEBPA* translation (42). hnRNP-E2 expression is high in CD34$^+$ CML-BP progenitors, where it suppresses C/EBPα and inhibits differentiation (42). Highlighting the importance of loss of C/EBPα expression in CML-BP, coexpression of BCR-ABL1 and AME also suppresses *CEBPA* translation and induces accumulation of blasts through activation of the *CEBPA* uORF-binding protein calreticulin (99, 100). Notably, C/EBPβ is also repressed in CML-BP (101), suggesting that loss of C/EBP activity contributes to differentiation arrest and aggressive behavior of CML-BP cells. In this regard, suppression of C/EBP proteins in CML-BP may also depend on BCR-ABL1–induced preferentially expressed antigen in melanoma (PRAME) expression, which impairs myeloid differentiation when ectopically expressed in CD34$^+$ progenitors (34).

The BCR-ABL1/hnRNP-E2/MAPK inhibitory pathway. The ability of hnRNP-E2 to suppress C/EBPα requires the constitutive activation of the MAPKs ERK1 and ERK2 (89, 102), which directly increases hnRNP-E2 stability (89). This is consistent with the observation that enhanced expression of various RNA-binding proteins is among the many imatinib-sensitive changes found in myeloid CML-BP (51). The involvement of ERK1/2 in the regulation of hnRNP-E2 is not surprising, as constitutive MAPK activation is readily detectable in CD34$^+$ CML-BP (102), while CML-CP progenitors show transient MAPK activation in response to mitogenic/survival signals induced by extracellular growth factors (103). Accordingly, levels of activated ERK1/2 in the absence of exogenous cytokines were similar in normal and CD34$^+$ CML-CP progenitors and were not affected by imatinib (103). Graded BCR-ABL1 expression correlates with a progressive increase in ERK1/2 activity (102), and ERK1/2 suppression rescues C/EBPα expression and allows G-CSF–driven maturation of differentiation-arrested progenitors expressing high BCR-ABL1 levels (89). Thus, constitutive ERK1/2 activation in CML-BP is not only essential for transduction of mitogenic/survival signals but also promotes the activation of antidifferentiation signals leading to translational (42) and, perhaps, posttranslational (104) inactivation of C/EBPα. Notably, a decrease in monophosphorylated ERK2 in imatinib-responsive but not -resistant patients suggests that ERK signaling may be important for transformation of TKI-resistant CML (105).

miR-328: a molecular relay in CML disease progression. A few miRNAs are aberrantly regulated in CML (93, 106, 107), but their involvement in disease progression is unclear. Interestingly, the correct functioning of the BCR-ABL1/MAPK/hnRNP-E2 inhibitory axis requires the inhibition of miR-328, which, otherwise, would bind hnRNP-E2 and prevent its interaction with *CEBPA* mRNA, thus restoring *CEBPA* mRNA translation. Loss of miR-328 occurs in CD34$^+$ CML-BP but not CML-CP myeloid progenitors, and forced miR-328 expression at levels resembling those observed in CML-CP rescues C/EBPα expression and reverses the CML-BP–like leukemia to a disease that resembles a myeloproliferative disorder in mice transplanted with *BCR-ABL1*–expressing myeloid precursors (108).

Genomic instability facilitates blastic transformation

Genomic instability usually results from an aberrant cellular response to enhanced DNA damage. In CML cells, these mechanisms can be modulated by BCR-ABL1 kinase (Figure 3) or may be kinase-independent.

Enhanced DNA damage. Much endogenous DNA damage arises from ROS such as superoxide radical anion ($\cdot O_2^-$), which may lead to the production of hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$). BCR-ABL1–transformed cell lines and CD34$^+$ CML cells contain, on average, 2–6 times more ROS than their normal counterparts (CML-BP > CML-CP > normal) (37, 109, 110); the mitochondrial respiratory chain, enhanced glucose uptake, and NADPH oxidase may play a role in this phenomenon (111). ROS can cause damage to all nucleobases and deoxyribose residues in DNA and free nucleotides, generating oxidized bases and DNA double-strand breaks (DSBs) (112). The number of oxidative "hits" to DNA per normal human cell per day is about 10^4, and normal cells contain approximately 50 DSBs per cell per cell cycle. CD34$^+$

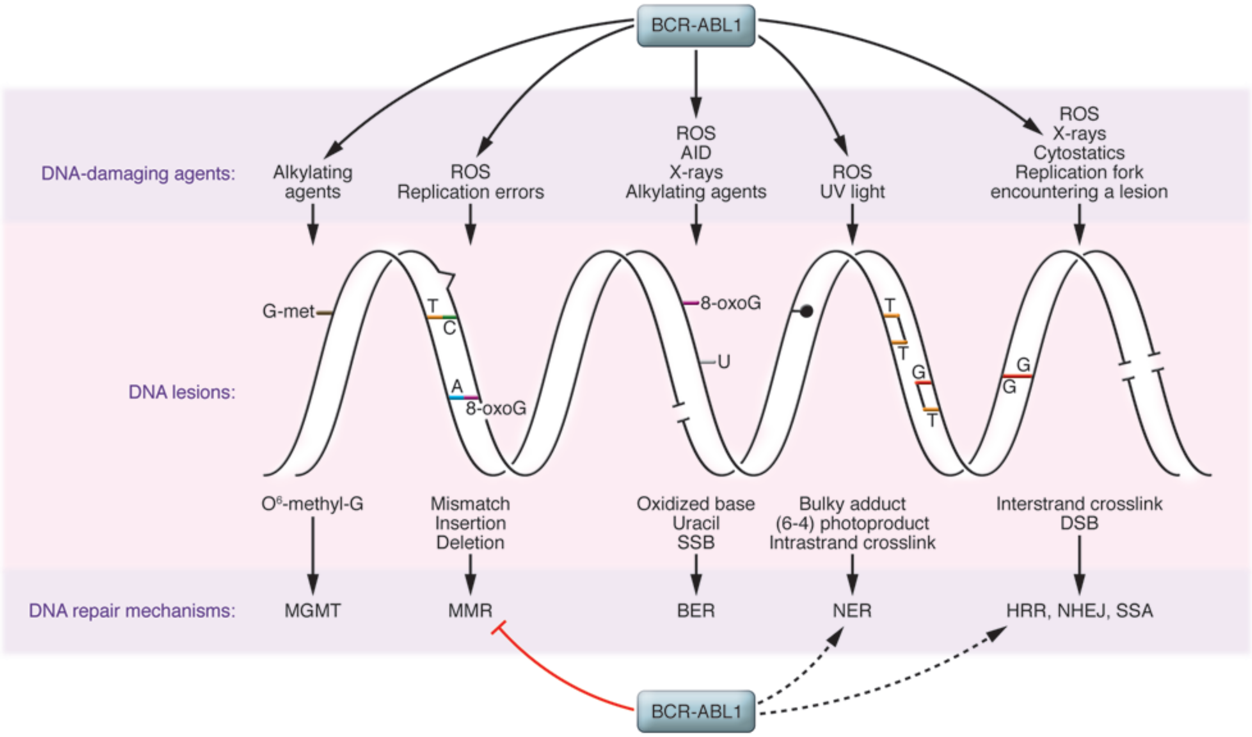

Figure 3

BCR-ABL1 regulates DNA damage and DNA repair, the 2 major components of genomic instability. BCR-ABL1–positive leukemia cells accumulate more DNA lesions, such as 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxoG), and DNA DSBs induced by ROS, AID, and genotoxic agents (e.g., γ-radiation, cisplatin, mitomycin C, hydroxyurea, and UV light) in comparison with normal cells. In addition, BCR-ABL1 inhibits MMR and stimulates mutagenic NER to generate point mutations including those causing TKI resistance. Moreover, BCR-ABL1 activates unfaithful DSB repair mechanisms, HRR, NHEJ, and SSA, which contribute to chromosomal aberrations. The effect of BCR-ABL1 on base excision repair (BER) and O(6)-methylguanine–DNA methyltransferase (MGMT) is not known. Altogether, elevated levels of DNA damage combined with inefficient/unfaithful DNA repair cause genomic instability in CML-CP and facilitate CML-BP.

CML cells display 3–8 times more oxidized nucleobases and 4–8 times more DSBs (37, 109, 110).

DNA damage could also be directly induced by ionizing radiation and genotoxic drugs, which are used as part of a conditioning regimen in hematopoietic transplantation for CML patients. BCR-ABL1–positive cells, in comparison with normal cells, accumulate more irradiation- and drug-induced DNA lesions, thus generating more chromosomal aberrations (113, 114).

Unfaithful and inefficient DNA repair. Unfaithful and/or inefficient repair of ROS-induced oxidized DNA bases and DSBs may lead to a variety of point mutations and chromosomal aberrations (115). CD34$^+$ CML cells display a malfunctioning mismatch repair (MMR) pathway, which can facilitate accumulation of point mutations (116) (Figure 3). BCR-ABL1 also promotes mutagenic nucleotide excision repair (NER) (117) and stimulates DSB repair, but the fidelity of the repair mechanisms (homologous recombination repair [HRR], nonhomologous end-joining [NHEJ], and single-strand annealing [SSA]) is compromised (37, 110, 118, 119). In BCR-ABL1–positive cells, point mutations were introduced during usually faithful HRR, extensive nucleobase loss was associated with NHEJ, and enhanced SSA generated large deletions. Overexpression and tyrosine phosphorylation of RAD51, a key element in HRR responsible for strand invasion and pairing, may result in aberrant HRR. Deregulation of DNA ligase IIIα, Werner helicase/exonuclease, and Artemis may contribute to excessive loss of DNA bases during NHEJ in BCR-ABL1–positive cells.

Consequences of genomic instability in CML. Genomic instability is probably responsible for two major problems in CML: TKI resistance and disease progression (13). Both phenomena could be induced by accumulation of point mutations and additional chromosomal aberrations in CML-CP cells irreversibly changing their phenotype toward that in CML-BP.

BCR-ABL1 point mutations have been detected in 50%–90% of patients displaying resistance to imatinib, including approximately 23% of imatinib-naive patients (120). Moreover, second-generation TKI treatment in imatinib-resistant cases led to selection of additional resistance mutations (121).

TKI-resistant BCR-ABL1 mutants exhibit altered kinase activity, substrate utilization, and transformation potency and are associated with clonal cytogenetic evolution, which may have an impact on disease progression (120, 122). Accordingly, BCR-ABL1 kinase mutations are associated with greater likelihood of disease progression, which suggests enhanced genomic instability in these cells (12).

Accumulation of various chromosomal aberrations and mutations is believed to be responsible for the transition of a relatively benign CP to aggressive BP (13). The frequency of additional chromosomal abnormalities is approximately 7% in CML-CP and increases to 40%–70% in the advanced phases of disease, as evaluated by standard cytogenetic analysis (23).

Numeric chromosomal changes are detected at a 50-fold higher frequency and structural changes at a 12-fold higher frequency in

CML-BP, in comparison with CML-CP. More sensitive comparative genomic hybridization (CGH) and SNP analyses detect multiple genetic aberrations already in CP, but CML-BP patients have much more complex karyotypes (123, 124). This observation suggests that genomic instability is an early event in CML. Patients from the pre-imatinib and imatinib era display similar types of genetic aberrations (125).

These aberrations involve acquisition of major alterations, such as the following: (a) the acquisition of additional chromosomes (e.g., +Ph, +8, +19); (b) the acquisition of isochromosome i(17q); (c) the acquisition of t(1;17), which is associated with loss of p53; (d) the acquisition of t(1;21), which affects RUNX1 (which is also known as AML1); (e) the acquisition of t(3;21), which generates the AML-1/EVI-1 fusion protein (a negative transcriptional regulator and cell signaling modulator); (f) the acquisition of t(7;11), which produces the NUP98-HOXA9 fusion protein that causes aberrant self renewal; and (g) the acquisition of translocations and inversions associated with AML/myelodysplasia (e.g., inv[3] and t[15;17]). In addition, minor genetic aberrations such as loss of heterozygosity (LOH) at 14q32, homozygous mutations/deletions of pRB, inactivating point mutations in *P53* and in interferon consensus sequence binding protein (*ICSBP*, which encodes an interferon regulatory transcription factor with leukemia-suppressor activity), gain-of-function mutations in *GATA-2* (which regulates myelomonocytic differentiation) and *RAS* (small GTP-binding signal transduction protein), and mutations in a zinc finger transcription factor PR domain containing 16 (*PRDM16*, mutated in myelodysplastic syndrome and AML) have been also detected. Numerous SNPs have been reported in additional genes regulating cell differentiation, such as *ICSBP*, *GATA-3*, and *AML1* in myeloid CML-BP (86); however, these results await confirmation.

In addition, mutations in *CDKN2A/B* and *IKZF1* facilitate CML-CP progression to CML-lymphoid BP (24, 25). Moreover, BCR-ABL1–mediated stimulation of activation-induced cytidine deaminase (*AID*) leads to a hypermutator phenotype, CML-lymphoid BP, and imatinib resistance (126).

Experimental findings support the conclusion that genetic aberrations contribute to malignant progression of CML. For example, loss of *p53* led to a CML-BP–like disorder in mice (127). *CDKN2A* gene loss enhanced oncogenicity in mouse models of BCR-ABL1–induced ALL (128). Coexpression of *BCR-ABL1* and *NUP98-HOXA9* caused CML-BP–like disease in mice (129). *GATA-2* gain-of-function mutations, partial deletions of *PMRD16* and *RUNX1*, and expression of *RUNX1-PMRD16* detected in CML-myeloid BP may disturb myelomonocytic differentiation, strongly suggesting their involvement in acute myeloid transformation (86, 130).

Moreover, genetic aberrations associated with CML-BP progression likely play a role in TKI resistance (131), causing a high risk of treatment failure (132). For example, additional chromosomal aberrations, loss of *P53*, and *CDKN2A* and *RUNX1* abnormalities may be responsible for disease persistence under imatinib treatment (128, 133–135).

BCR-ABL1 kinase–dependent and –independent genomic instability in CML-CP LSCs and/or LPCs. The (9;22) translocation that results in the formation of the Ph chromosome may be a random event or may result from preexisting conditions associated with genomic instability in HSCs. Therefore, additional genetic aberrations accumulated during the course of CML may be promoted by BCR-ABL1 kinase and also by a preexisting abnormality responsible for the formation of t(9;22)(q34;q11). The former statement is supported by reports that BCR-ABL1 kinase–positive cells acquire more oxidative DNA lesions than normal counterparts in response to endogenous ROS and genotoxic treatment (109, 110) and that BCR-ABL1 can inhibit some DNA repair mechanisms (MMR) and stimulate other mechanisms (NER, HRR, NHEJ, and SSA) at the cost of their fidelity (136) (Figure 3). However, the latter speculation about preexisting abnormality cannot be ruled out because chromosome abnormalities were detected in t(9;22)(q34;q11)-negative metaphases appearing during imatinib therapy in patients with newly diagnosed CML-CP (121).

Genomic instability in CML-CP most likely occurs in the LSC-enriched $CD34^+CD38^-$ population and/or the LPC-rich $CD34^+$ population because TKI-resistant *BCR-ABL1* mutants and chromosomal aberrations were detected in both subpopulations (38, 41, 114). As CML-CP can progress to either myeloid or lymphoid BP (sometimes a mixed myeloid/lymphoid phenotype) and chromosomal abnormalities are documented in both phenotypes (137), this suggests that genomic instability may occur at the LSC and/or LPC level. Mutations acquired by the LSCs are likely to be passed on to successive generations of LPCs. On the other hand, genetic aberrations acquired by CML-CP LPCs may "upgrade" them to the status of CML-BP LSCs (49).

Altogether, we postulate that elevated levels of DNA damage combined with unfaithful/inefficient DNA repair may generate mutations and chromosomal aberrations in CML-CP LSCs and/or LPCs, causing resistance to TKIs and progression toward CML-BP. These mechanisms at least partially depend on BCR-ABL1 kinase. Since LSCs, in contrast to LPCs, are not sensitive to TKIs, LSCs may be "ticking time bombs," eventually exploding to produce a TKI-resistant LPC clone that may evolve into a CML-BP clone.

Genomic instability in CML cells in the era of TKIs. BCR-ABL1 kinase induces genomic instability (13); therefore, imatinib and other TKIs should prevent accumulation of additional genetic changes in CML cells. In fact, imatinib reduced ROS, oxidative DNA damage, point mutations, and other genetic aberrations in BCR-ABL1–positive cells (109, 110, 138). Nevertheless, imatinib-treated CML patients continue to accumulate point mutations (including those causing resistance to other TKIs) and chromosomal aberrations (21, 121, 130, 139).

There are several possible explanations for persistent genomic instability during TKI treatment. First, although TKIs inhibit BCR-ABL1 kinase activity in CML-CP LPCs, their effectiveness in CML-CP LSCs is questionable. The effect of TKIs on BCR-ABL1 kinase–induced signaling may be obscured by growth factors, usually resulting in incomplete inhibition or even stimulation of signaling pathways, such as those involving STAT5, AKT, and MAPKs (140, 141). Therefore, TKIs cannot completely eliminate the effects of BCR-ABL1 kinase and may not effectively inhibit genomic instability. Second, imatinib may exert mutagenic activity to induce centrosome and chromosome aberrations (142). The appearance of cytogenetic aberrations in t(9;22)(q34;q11)-negative cells following imatinib therapy supports this hypothesis (143). Third, if CML-CP cells display an active preexisting genomic instability responsible for generation of t(9;22), this process should be BCR-ABL1 kinase independent and will continue generating errors despite treatment (121). This speculation implicates BCR-ABL1 kinase–dependent and –independent genomic instability in CML cells.

Prevention of genomic instability in CML-CP to improve therapeutic effects of TKIs and antagonize CML-BP. The majority of CML-CP patients at diagnosis do not have mutations or a "critical" combination of aberrations causing either TKI resistance or disease progression. However, a cohort of TKI-treated patients still develops mutations and chromosomal aberrations even though imatinib efficiently antagonizes genomic instability in experimental CML models. Given the fact that *BCR-ABL1*–negative patients, as assessed by reverse transcriptase PCR, may contain up to 10^6 CML cells in their body, that CML-CP patients can have approximately 5×10^7 CD34$^+$ cells displaying innate imatinib resistance (144), and that even imatinib-sensitive CD34$^+$ LPCs can still undergo up to 1–3 cell cycles in the presence of the drug and growth factors before eventually being eliminated (71), prevention of genomic instability may be critical for a better therapeutic effect or even eradication of CML.

ROS cause oxidative DNA damage resulting in both clinically relevant *BCR-ABL1* mutations and chromosomal aberrations often detected in CML-BP (i.e., aneuploidy, translocations, and truncations) (109, 113). Antioxidants diminished ROS-mediated oxidative DNA damage and reduced the appearance of TKI-resistant mutations and chromosomal aberrations (37, 109, 110). Because the combination of imatinib and an antioxidant exerted a synergistic/additive antimutagenic effect (109), it is possible that the combination of TKI and antioxidants may prevent CML-BP by reducing the appearance of TKI-resistant clones and accumulation of a "critical" combination of genetic aberrations.

Concluding remarks

To date, there is strong evidence supporting the idea that the level of BCR-ABL1 kinase activity plays a pivotal role in almost all CML patients undergoing progression and that BCR-ABL1–induced genetic/chromosomal abnormalities can predispose to transformation and/or markedly influence the aggressiveness of the blast crisis progenitor cell clone. However, there are several crucial and burning questions that remain to be answered. What controls BCR-ABL1 expression and activity during progression? Does malignant progression originate from CML-CP LSCs and/or LPCs? Is the acquisition of self renewal, impaired differentiation, and increased genomic instability of CML-BP stem and/or progenitor cells solely a BCR-ABL1–dependent effect? A possible scenario might envision a BCR-ABL1 autoregulatory loop that amplifies signals that positively influence *BCR-ABL1* gene transcription and enhance its protein stability. Likewise, it is highly plausible that, in CML-CP, BCR-ABL1–induced genomic aberrations and/or BCR-ABL1–independent preexisting genetic lesions function as "amplifiers" of a genetically unstable phenotype and thereby predispose CML to blastic transformation by affecting stemness, survival, proliferation, differentiation, and/or genome stability of the Ph-positive bone marrow stem and progenitor cells.

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