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Chronic myeloid leukemia: mechanisms of blastic transformation

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The BCR-ABL1 oncoprotein transforms pluripotent HSCs and initiates chronic myeloid leukemia (CML). Patients with early phase (also known as chronic phase [CP]) disease usually respond to treatment with ABL tyrosine kinase inhibitors (TKIs), although some patients who respond initially later become resistant. In most patients, TKIs reduce the leukemia cell load substantially, but the cells from which the leukemia cells are derived during CP (so-called leukemia stem cells [LSCs]) are intrinsically insensitive to TKIs and survive long term. LSCs or their progeny can acquire additional genetic and/or epigenetic changes that cause the leukemia to transform from CP to a more advanced phase, which has been subclassified as either accelerated phase or blastic phase disease. The latter responds poorly to treatment and is usually fatal. Here, we discuss what is known about the molecular mechanisms leading to blastic transformation of CML and propose some novel therapeutic approaches.

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).

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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 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 disappointing and expanded during TKI therapy (38, 39). 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., PRAE, 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 antidiifferentiation activity (e.g., MAPK[^ERK1/2], MYC, JAK2, YES-1, LYN, hnRNPE2, 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 (NOBI1, DDX47, IGFS2, 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.
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.

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; MPM2, postmeiotic segregation increased 2; RAD51, RecA homolog in Escherichia coli; RAD52, RAD52 homolog (Saccharomyces cerevisiae); Shh, Sonic Hedgehog; wnt/l-catenin, wingless-int1/beta-catenin.
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 and LSCs 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 xenogenic 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 Ca2+ depletion (15). Suppression of autophagy genes enhanced imatinib-induced death of Ph-positive cells (15). Critical- ly, 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.
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–dependent 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 phosphatases.

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-ABL–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 or FTY720) (Figure 2) or by interfering with SET/PP2A interplay, promotes Src homology region 2–domain phosphatase 1 (SHP-1) tyrosine phosphatase–dependent repression of the proapoptotic serine/threonine phosphatase PP2A as a true tumor suppressor. In fact, loss of PP2A expression or activity in CML-BP is associated with both 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 phosphatases. The loss of PP2A activity, either by chemical PP2A activators (e.g., forskolin or FTY720) (Figure 2) or by interfering with SET/PP2A interplay, promotes Src homology region 2–domain phosphatase 1 (SHP-1) tyrosine phosphatase–dependent repression of the proapoptotic serine/threonine phosphatase PP2A as a true tumor suppressor. In fact, loss of PP2A expression or activity in CML-BP is associated with both 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 phosphatases.
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 PS3 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 ([AME-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/EβPα 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 progenitors 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/EβPα (42), a transcription factor essential for granulocytic differentiation. The importance of the loss of C/EβPα activity as a central mechanism leading to differentiation arrest of myeloid CML blasts is supported by evidence that ectopic C/EβPα expression induces maturation of differentiation-arrested BCR-ABL1+ myeloid progenitors and CD34− CML-BP progenitors (42, 95, 96) and that a CML-BP-like process emerges in mice transplanted with BCR-ABL1–transduced Cebpα-null fetal liver cells (97). In CD34+ CML-BP GMPs, loss of C/EβPα 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 (hnRNPE2) that, upon interaction with the CEBPA upstream open reading frame (uORF)/spacer element, inhibits CEBPA translation (42). hnRNPE2 expression is high in CD34+ CML-BP progenitors, where it suppresses C/EβPα and inhibits differentiation (42).

Highlighting the importance of loss of C/EβPα 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/EβPα is also repressed in CML-BP (101), suggesting that loss of C/EβPα activity contributes to differentiation arrest and aggressive behavior of CML-BP cells. In this regard, suppression of C/EβPα 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/hnRNPE2/MAPK inhibitory pathway.** The ability of hnRNPE2 to suppress C/EβPα requires the constitutive activation of the MAPKs ERK1 and ERK2 (89, 102), which directly increases hnRNPE2 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 hnRNPE2 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/EβPα 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 antidiifferentiation signals leading to translational (42) and, perhaps, posttranslational (104) inactivation of C/EβPα. 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/hnRNPE2 inhibitory axis requires the inhibition of miR-328, which, otherwise, would bind hnRNPE2 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/EβPα 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 (O2−), which may lead to the production of hydrogen peroxide (H2O2) and hydroxyl radical (•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 deoxyribosyl 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 10⁴, and normal cells contain approximately 50 DSBs per cell per cell cycle. CD34+...
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. Derepression of DNA ligase IIIɛ, 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 cytokine 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 RUNXI-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 mutational profile in CML-CP was also documented 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 centosome 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 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 10^7 CML cells in their body, that CML-CP patients can have approximately 5 × 10^14 CD34+ cells displaying innate imatinib resistance (144), and that even imatinib-resistant 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 clones and accumulation of a “critical” combination of genetic aberrations. This 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 blast 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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