Priority Report

Cancer Research

Imatinib Sensitivity in BCR-ABL1–Positive Chronic Myeloid Leukemia Cells Is Regulated by the Remaining Normal *ABL1* Allele

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Abstract

Chronic myeloid leukemia in chronic phase (CML-CP) cells that harbor oncogenic *BCR-ABL1* and normal *ABL1* allele often become resistant to the ABL1 kinase inhibitor imatinib. Here, we report that loss of the remaining normal *ABL1* allele in these tumors, which results from cryptic interstitial deletion in 9q34 in patients who did not achieve a complete cytogenetic remission (CCyR) during treatment, engenders a novel unexpected mechanism of imatinib resistance. BCR-ABL1–positive $Abl1^{-/-}$ leukemia cells were refractory to imatinib as indicated by persistent BCR-ABL1–mediated tyrosine phosphorylation, lack of BCR-ABL1 protein degradation, increased cell survival, and clonogenic activity. Expression of ABL1 kinase, but not a kinase-dead mutant, restored the antileukemic effects of imatinib in ABL1-negative chronic myelogenous leukemia (CML) cells and in BCR-ABL1–positive $Abl1^{-/-}$ murine leukemia cells. The intracellular concentration of imatinib and expression of its transporters were not affected, although proteins involved in BCR-ABL1 degradation were downregulated in $Abl1^{-/-}$ cells. Furthermore, 12 genes associated with imatinib resistance were favorably deregulated in $Abl1^{-/-}$ leukemia. Taken together, our results indicate that loss of the normal ABL1 kinase may serve as a key prognostic factor that exerts major impact on CML treatment outcomes. *Cancer Res; 71(16); 5381–6. ©2011 AACR.*

Introduction

BCR-ABL1 results from t(9;22)(q34;q11) reciprocal translocation or variants generating the Philadelphia chromosome (Ph), which initiates chronic myeloid leukemia in chronic phase (CML-CP) cells. The second (wild-type) *ABL1* allele remains intact on the nonrearranged homologue of chromosome 9, and CML-CP cells at early stages express both forms of the ABL1 kinase, oncogenic BCR-ABL1 and normal ABL1 (1).

ABL1 and BCR-ABL1 can exert opposite effects on a variety of cellular functions (2). For example, BCR-ABL1 can act upstream and downstream of cytochrome *c* to inhibit apoptosis. In contrast, ABL1 kinase may facilitate apoptosis by stimulation of p73, p53, and caspase 9.

Tyrosine kinase inhibitor (TKI) imatinib revolutionized the treatment of BCR-ABL1–positive leukemias (3). The incidence of a continuous complete cytogenetic remission (CCyR) in CML-CP patients treated for 12 months with the drug was 66% (4). Mutations within the kinase domain of *BCR-ABL1*, overexpression of LYN kinase, and loss of *p53* and *BCR-ABL1* amplification were implicated in the lack of achieving CCyR (5).

Here we show that loss of expression of normal ABL1 kinase due to cryptic deletion in remaining normal chromosome 9 [del(9q34)] reduced the sensitivity of BCR-ABL1 leukemia cells to imatinib and may contribute to drug resistance in chronic myeloid leukemia (CML) patients.

Materials and Methods

Chromosome and whole genome analysis of CML-CP samples

Bone marrow cells (BMC) of CML-CP patients who failed to achieve CCyR within 12 months of TKI treatment were obtained after informed consent and analyzed at presentation and at 3 monthly intervals by G-banding and dual color/dual fusion probe FISH (D-FISH) with a range of bacterial artificial chromosome (BAC) probes to detect the loss of normal 9q34

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(6). DNA from BMCs of patients 1 and 3 was also analyzed by array comparative genomic hybridization (aCGH) using DNA Analytics (105K Agilent) and Formatter software (7). All genome addresses are derived from NCBI36/hg18 (March 2006) of the Human Genome. Additional information about the patients and cytogenetic and molecular results are described in Supplementary Materials and Supplementary Table S1.

Cells

Abl1$^{+/-}$ mice were kindly obtained from Dr. A.J. Koleske (Department of Molecular Biophysics and Biochemistry, Yale University) and bred to obtain −/− and +/+ littermates. Animal studies were approved by the Institutional Animal Care and Use Committee at Temple University. p210BCR-ABL1–positive growth factor–independent leukemia cells were generated by retroviral infection of *Abl1*$^{+/+}$ and *Abl1*$^{-/-}$ BMCs with pMIG-*BCR-ABL1*-IRES-*GFP* retroviral construct as previously described (8). CML-BP LAMA84R and KCL22 cell lines were described before (9, 10). LAMA84R and KCL22 cells, and green fluorescent protein (GFP+) BCR-ABL1–positive *Abl1*$^{-/-}$ leukemia cells were infected with pKI retroviral construct encoding YFP-ABL1 fusion protein or kinase-dead YFP-ABL1 (K290R) mutant (kindly obtained from Dr. Koleske). GFP, yellow fluorescent protein (YFP), and YFP/GFP-positive cells were sorted and expanded in growth factor–free conditions.

Sensitivity to imatinib

Cells were treated with imatinib (Novartis Pharma) and evaluated by clonogenic assay as described before (8). TKI-resistant BCR-ABL1 kinase mutations were not detected in cells used for these experiments.

Imatinib retention

Radiolabeled drug uptake was carried out using ^{14}C-labeled imatinib (Novartis) as previously described with modifications (11). Briefly, 2×10^6 cells were incubated with 1.6 μmol/L ^{14}C-labeled imatinib (3,052 MBq/mg) at 37°C for 2 hours. After incubation, the cells were washed twice with ice-cold PBS and incubated in culture medium at 37°C for another 15 minutes. Cell pellet was then solubilized in 50 μL of distilled water and radioactivity was counted using β-counter (Perkin Elmer).

Protein expression

Total cell lysates were analyzed by Western blotting using primary antibodies recognizing ABL1, Abcb1, CHIP, and tubulin (Calbiochem); phosphotyrosine (Upstate), Bag1, Cbl, and GFP (Santa Cruz Biotechnology); Oct-1 (Novus Biologicals); Abcg2, HSP90, and cathepsin B (Abcam Inc.); and Hsc70 (Enzo Life Sciences International, Inc.) as described previously (8).

Genome-wide expression array

Affymetrix Mouse gene 1.0ST array containing 28,815 probe sets (Affymetrix) was used to measure mRNA expression levels. Affymetrix arrays were processed by Partek Genomic Suite at the Penn Molecular Profiling Facility–Bioinformatics Group (University of Pennsylvania) to determine whether a given transcript was present and if there were consistently significant differences between BCR-ABL1–positive *Abl*$^{-/-}$ and BCR-ABL1–positive *Abl*$^{+/+}$ BMC based on 3 separate experiments. Genes were considered to have a significant differential expression between the 2 groups when displayed a False Discovery Rate (FDR) not exceeding 5% and a cut-off value more than 1-fold (upregulated or downregulated).

Results and Discussion

Using G-banding, D-FISH, and aCGH, we detected that 3 CML patients who initially failed to achieve CCyR within 12 months of TKI treatment acquired a cryptic deletion in 9q34 region in the normal chromosome 9 [del(9q34)] resulting in the loss of normal *ABL1* allele (Figs. 1 and 2, Supplementary Table S1 and Fig. S1). Two of these are among 21 CML-CP patients without CCyR on first line TKI, we analyzed in the lab. Importantly, in addition to the cryptic loss at 9q34.1, all 3 patients showed karyotype evolution—from the presence of a second Ph (Fig. 2A) to multiple numerical and structural aberrations (Supplementary Table S1).

In patient 1, FISH analysis revealed an aberrant signal pattern due to a missing *ABL1* signal, which was mapped to the morphologically normal chromosome 9 indicating a cryptic deletion [Fig. 1A (iii) and 1B]. In patient 2, the deletion was detectable by G-banding and assessed as del(9q31;q34) (Supplementary Table S1). In patient 3, the loss of ABL1 was revealed by aCGH and confirmed by FISH mapping (Fig. 1B, Supplementary Fig. S1). aCGH results for the 9q34 region in patient 1 (blue) and patient 3 (red) show the extent of the cryptic deletions [Fig. 1B (i)]. The common loss is defined proximally by the *ABL1* breakpoint in patient 3 and distally by the telomeric breakpoint in patient 1 [arrow in Figs. 1B (i), and 2C and D, and Supplementary Fig. S1]. The estimated size of the common genome loss is 567 kb, which includes *ABL1* exons a2 to a11 together with downstream sequences encompassing the *LAMC3* and *NUP214* genes. These deletions differ significantly from the deletions at der(9), where the genome loss involves only *ABL1* exons 1a and 1b and spans toward the centromere [Fig. 1A (ii) and B (ii)].

The observed loss of the wild allele of *ABL1* is an evolutionary event as evidenced by the presence of Ph(+) cells with and without *ABL1* deletion in patient 1 (Fig. 2B). Furthermore, it is the *ABL1*-deficient cell clone that sustains the disease progression by acquiring a second copy of Ph (Fig. 2A, B, and D). Our observation combined with other report that inhibition of Abl1 kinase compromises genomic stability suggests that loss of ABL1 not only decreases imatinib sensitivity but also promotes accumulation of chromosomal aberrations (12).

Cryptic deletions in 9q34 causing the loss of normal *ABL1* allele may be underreported in CML-CP patients probably because they would be missed unless either D-FISH or aCGH have been used for monitoring therapy response. In concordance, similar del(9q34) was found in several CML-BP cell lines (Supplementary Table S2). To detect loss of the *ABL1* signal from the normal 9 homologue, FISH using a *BCR-ABL1*-ASS

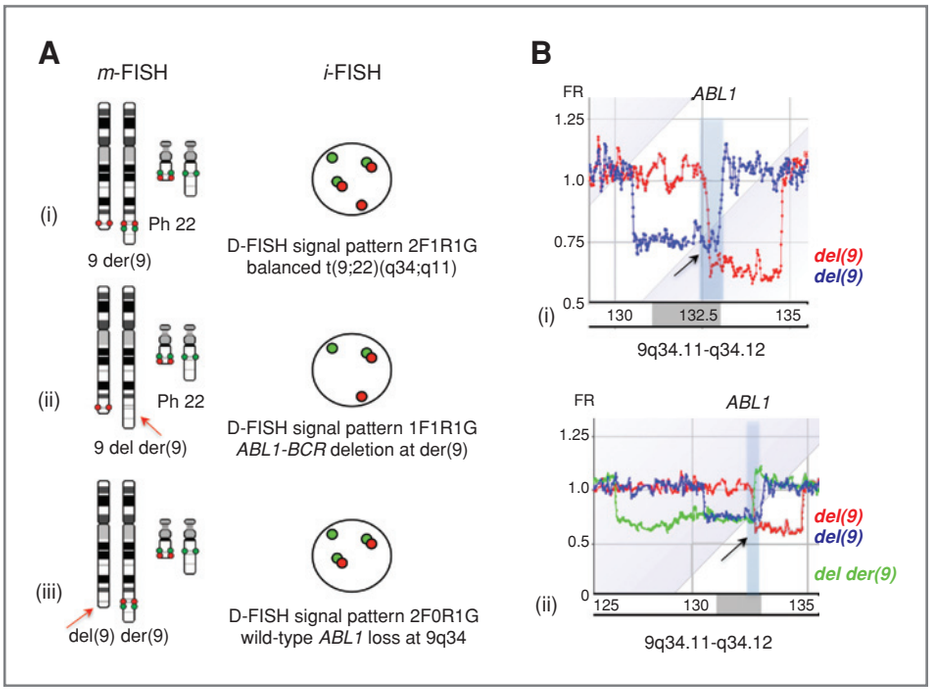

Figure 1. Deletions of the normal *ABL1* allele in CML-CP patients. A, the classical and variants of t(9;22)(q34;q11) are usually balanced and reciprocal translocations; however, the translocation may be unbalanced. (i) balanced t(9;22)(q34;q11) with 2 fusion signals on Ph and der(9), 1 red signal and 1 green signal on the normal 9q34 and 22q11, respectively (2F1R1G), (ii) t(9;22)(q34;q11) accompanied by the loss of *ABL1-BCR* signal at der(9) (1F1R1G), and (iii) t(9;22)(q34;q11) accompanied by the loss of wild-type *ABL1* at del(9) (2F0R1G). B, aCGH results: (i) the 9q34-qter region of patient 1 showing a 2.6 Mb genome loss, including the entire *ABL1* together with proximally flanking sequences leading to 2F0R1G aberrant FISH signal pattern (blue), and a 1.8 Mb loss in patient 3 encompassing sequences downstream of the *ABL1* breakpoint (red), and (ii) added for comparison the "classic" der(9) deletion (graph in green) involves the 5′ *ABL1* and sequences proximal to the *ABL1* breakpoint thus resulting in aberrant 1F1R1G pattern.

tricolor dual fusion translocation probe could be recommended (Supplementary Fig. S2), which produces unique signals for the translocation products and the normal nonrearranged loci at 9q34 and 22q11. Importantly, identification of del(9q34) using any of the 2 double fusion *BCR-ABL1* probe sets is as reliable on interphase cells as on chromosome preparations. In contrast, the popular ES-FISH probe creates in *BCR-ABL1*–positive cell with *ABL1* loss a signal pattern (1R1G1F) that cannot reliably differentiate the "wild" *ABL1* allele from the *ABL1-BCR* fusion (Supplementary Fig. S2) thus misreporting del(9q34) as deletions at der(9).

To prove that loss of ABL1 directly contributes to imatinib resistance, BCR-ABL1 was expressed in $Abl1^{-/-}$ and $^{+/+}$ BMCs. The absence of Abl1 reduced the sensitivity of BCR-ABL1 leukemia cells to imatinib whereas expression of YFP-ABL1 fusion kinase, but not the kinase-dead YFP-ABL1 (K290R) mutant, in BCR-ABL1–positive $Abl1^{-/-}$ leukemia cells restored antileukemia effect of the drug (Fig. 3A). YFP-ABL1, but not YFP-ABL1 (K290R), also increased imatinib sensitivity in drug-resistant CML-BP cell line LAMA84R (*ABL1*-negative, Supplementary Table S2; Fig. 3B). Because *BCR-ABL1* gene amplification and overexpression of the multidrug resistance P-glycoprotein was observed in LAMA84R cells, loss of *ABL1* may collaborate with other genetic/epigenetic abnormalities to promote drug resistance in CML-BP (9). Moreover, expression of YFP-ABL1 kinase in KCL22 CML-BP cells [relatively low ABL1 expression (10)] increased their sensitivity to imatinib suggesting that increased BCR-ABL1:ABL1 ratio observed during the course of disease can also limit the effect of imatinib (Fig. 3C; ref. 13).

The fact that ABL1 kinase may regulate the sensitivity of CML cells to imatinib is rather unexpected because BCR-ABL1 and ABL1 kinases are equally sensitive to imatinib *in vitro* (3). However, inhibition of intracellular ABL1 kinase usually requires higher concentration of the drug in comparison to BCR-ABL1 kinase; in addition ABL1 may work in a kinase-independent manner (14, 15). Moreover, imatinib-induced inhibition of BCR-ABL1 kinase is associated with release of ABL1 from the complex with 14-3-3 sigma, which promotes ABL1 relocation to the nucleus (triggers p73-dependent apoptosis), to the mitochondrial membranes (causes the loss of mitochondrial membrane potential), and to the complex with caspase 9 (activates caspase cascade) (2, 16). In summary, the presence of ABL1 kinase may exert a significant impact on anti-CML effect of imatinib. This speculation is supported by the observation that expression of YFP-ABL1 fusion kinase, but not its kinase-dead K290M mutant restored sensitivity to imatinib in BCR-ABL1–positive $Abl1^{-/-}$ leukemia cells and LAMA84R (*ABL1*-negative) CML-CP cells (Fig. 3A and B).

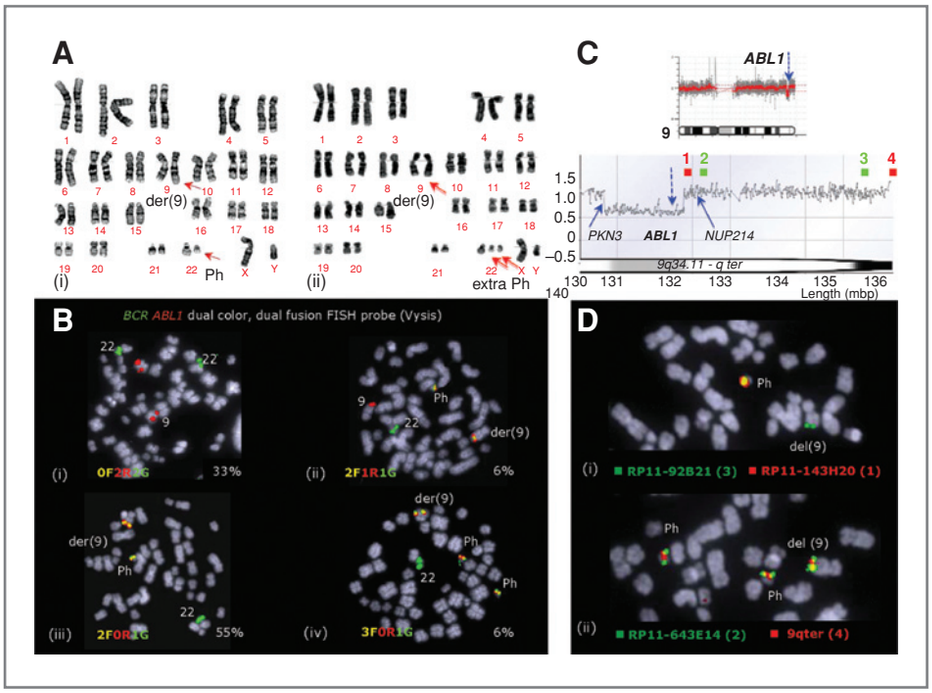

Figure 2. Analysis of del(9) causing a loss of the wild-type *ABL1* allele during imatinib treatment in patient #1. A, G-banding analysis identified (i) a t(9;22)(q34;q11) and (ii) an additional Ph in 20% of cells. B, FISH analysis showed that (i) 33% of the cells were *BCR-ABL1* negative (0F2R2G), (ii) 6% were *BCR-ABL1* positive with fusion products on both the Ph and der(9) chromosome and signals from the normal 9q34 and 22q11 regions (2F1R1G) as expected, (iii) 55% had an abnormal 2F0R1G signal pattern indicating cryptic loss of the *ABL1* signal at 9q34 [del(9)], and (iv) 6% had in addition to del(9) also an extra Ph. C, chromosome 9 aCGH profile indicates the loss at 9q34.1 (top, blue arrow) with a close-up of 9q34.1-qter region (bottom), showing that the breakpoints fall within the *PKN3* gene and downstream of *ABL1*. The locations of BAC probes (1–4) are shown in green and red. D, FISH mapped the missing *ABL1* sequences to del(9) affecting the wild-type allele of *ABL1* and confirmed the location of the distal breakpoint between (i) BACs RP11-143H20 [note the missing red signal from del(9)] and (ii) RP11-643E14 within a 128 Kb region containing *NUP214* gene.

In $Abl1^{-/-}$ leukemia cells, imatinib displayed reduced capability to inhibit BCR-ABL1 kinase-mediated tyrosine phosphorylation and to induce BCR-ABL1 protein degradation in comparison with $Abl1^{+/+}$ counterparts and $Abl1^{-/-}$ leukemia reconstituted with YFP-ABL1 (Fig. 4A). Moreover, genome-wide array confirmed imatinib-resistant signature of BCR-ABL1–positive $Abl1^{-/-}$ cells by detecting deregulated expression of 12 genes previously reported in imatinib-resistant CML patients (Fig. 4B; refs. 17, 18). Intracellular retention of imatinib, and expression of drug importer Oct-1 and exporters Abcb1 and Abcg2 seem unaffected by Abl1 (Fig. 4C and D), but the impact of Abl1 on metabolism of imatinib cannot be excluded. On the other hand, more than 10-fold downregulation of Cbl E3 ligase, which induce

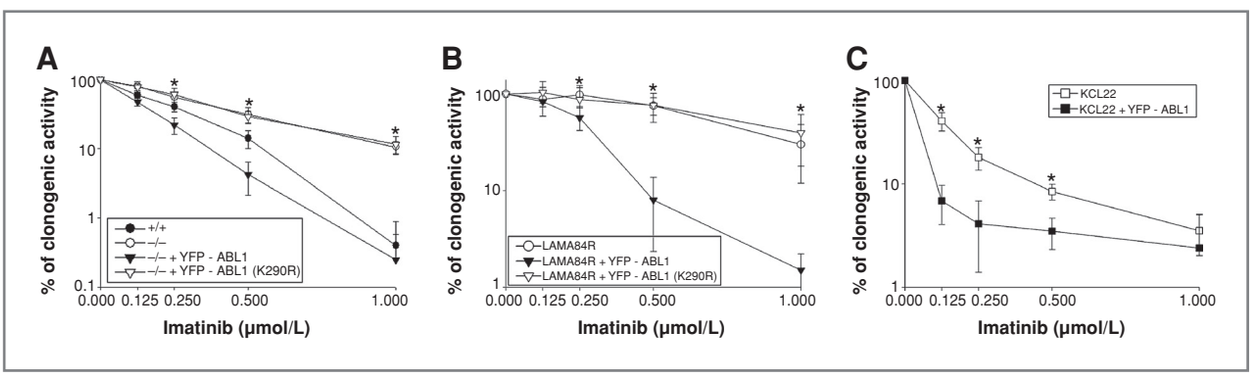

Figure 3. ABL1 kinase regulates imatinib sensitivity of BCR-ABL1 leukemias. A, BCR-ABL1–positive $Abl1^{-/-}$ leukemia cells (−/−), BCR-ABL1–positive $Abl1^{+/+}$ leukemia cells (+/+), and BCR-ABL1–positive $Abl1^{-/-}$ leukemia cells reconstituted with YFP-ABL1 and YFP-ABL1(K290R), B, LAMA84R cells and these transfected with YFP-ABL1 and YFP-ABL1(K290R), and C, KCL22 cells and these transfected with YFP-ABL1 were incubated with imatinib and clonogenic cells were counted. Results represent mean percentages ± SD of control untreated cells; *, $P < 0.05$ in comparison with other group(s) as determined by 2-tailed Student's *t* test.

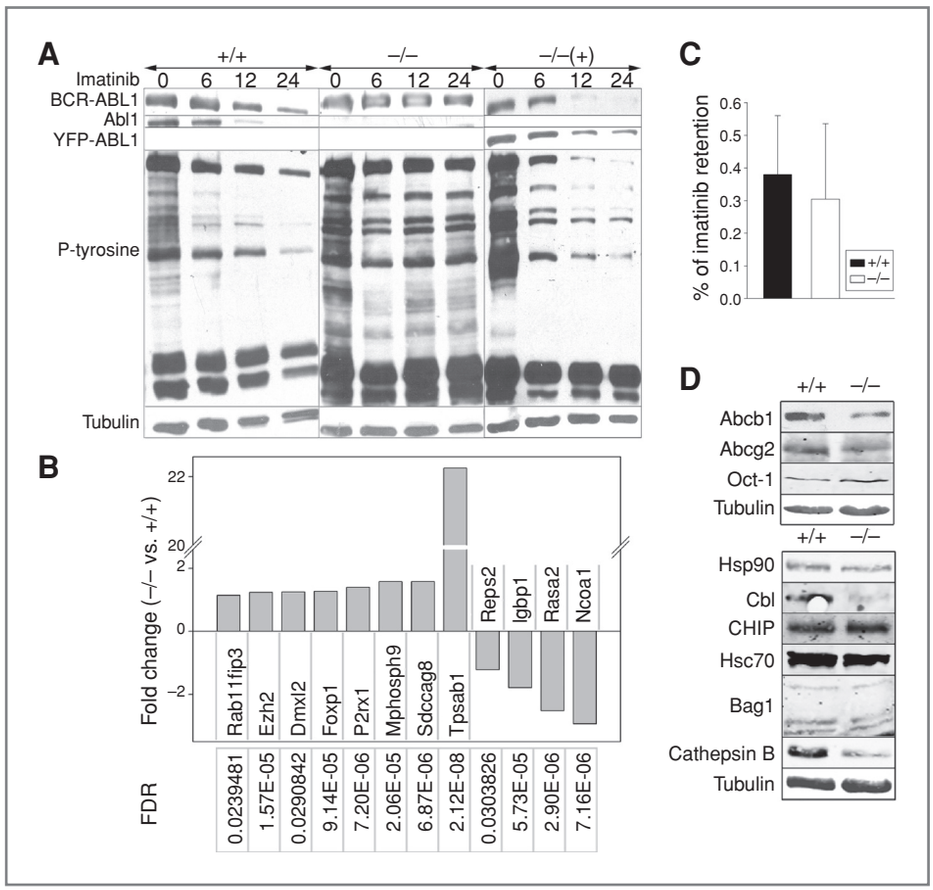

Figure 4. Imatinib-resistant phenotype of BCR-ABL1–positive *Abl1*$^{-/-}$ leukemia cells. BCR-ABL1–positive *Abl1*$^{-/-}$ leukemia cells (−/−), BCR-ABL1–positive *Abl1*$^{+/+}$ leukemia cells (+/+), and BCR-ABL1–positive *Abl1*$^{-/-}$ leukemia cells reconstituted with YFP-ABL1 [−/− (+)] were used. A, Western analysis of the total cell lysates from cells incubated with 1 μmol/L imatinib for 0, 6, 12, and 24 hours. B, statistically significant (FDR < 0.05) fold-changes (>1) of the expression of indicated genes in BCR-ABL1–positive *Abl1*$^{-/-}$ versus BCR-ABL1–positive *Abl1*$^{+/+}$ samples. C, intracellular retention of imatinib; results represent mean percentages ± SD of total ^{14}C-imatinib. D, Western blots of total cell lysates to detect imatinib transporters (top) and proteins involved in BCR-ABL1 degradation (bottom).

ubiquitin-dependent degradation of "mature" BCR-ABL1 protein, and/or more than 3-fold downregulation of cathepsin B, which cleaves BCR-ABL1 may be responsible for lack of degradation of BCR-ABL1 protein in imatinib-treated *Abl1*$^{-/-}$ cells (Fig. 4D; refs. 19, 20). Hsc70, Bag1, and E3 ligase CHIP responsible for degradation of "immature" BCR-ABL1 protein, and chaperone protein Hsp90 protecting BCR-ABL1 from proteasomal degradation, are not affected by Abl1 (Fig. 4D; ref. 19). Downregulation of BCR-ABL1 in imatinib-treated CD34+ CML-CP cells was implicated in regulating their sensitivity to the drug (20).

Altogether, it can be postulated that loss of expression of ABL1 kinase may contribute to imatinib resistance in CML-CP patients which do not achieve CCyR during 12 months on imatinib and eventually progress to CML-BP. ABL1 loss in CML-CP can be achieved by interstitial deletion in chromosome 9 [del(9q34)] causing a loss of normal *ABL1* allele (this report), which may be combined with epigenetic silencing of the alternative *ABL1* promoter retained in t(9;22) (13). Therefore, detection of del(9q34) may serve as an important prognostic factor and have a significant impact on CML treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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