CDKN2A/B Alterations Impair Prognosis in Adult BCR-ABL1–Positive Acute Lymphoblastic Leukemia Patients

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Abstract

**Purpose:** The 9p21 locus, encoding three important tumor suppressors (p16/CDKN2A, p14/ARF, and p15/CDKN2B), is a major target of inactivation in the pathogenesis of many human tumors.

**Patients and Methods:** To explore, at high resolution, the frequency and size of alterations affecting this locus in adult BCR-ABL1–positive acute lymphoblastic leukemia (ALL) and to investigate their prognostic value, 112 patients (101 de novo and 11 relapsed cases) were analyzed by genome-wide single-nucleotide polymorphism arrays and gene candidate deep exon sequencing. Paired diagnosis–relapse samples were further available and analyzed for 19 (19%) cases.

**Results:** CDKN2A/ARF and CDKN2B genomic alterations were identified in 29% and 25% of newly diagnosed patients, respectively. Deletions were monoallelic in 72% of cases, and in 43% of them, the minimal overlapping region of the lost area spanned only the CDKN2A/B gene locus. An analysis conducted at relapse showed an increase in the detection rate of CDKN2A/ARF loss (47%) compared with the time of diagnosis ($P = 0.06$). Point mutations within the 9p21 locus were found at very low levels, with only a nonsynonymous substitution in the exon 2 of CDKN2A. Of note, deletions of CDKN2A/B were significantly associated with poor outcomes in terms of overall survival ($P = 0.0206$), disease free-survival ($P = 0.0010$), and cumulative incidence of relapse ($P = 0.0014$).

**Conclusions:** Inactivation of the 9p21 locus by genomic deletion is a frequent event in BCR-ABL1–positive ALL. Deletions are frequently acquired during leukemia progression and are a poor prognostic marker of long-term outcomes. Clin Cancer Res; 17(23); 7413–23. ©2011 AACR.

Introduction

Disruption of tumor suppressor genes and/or activation of oncogenic pathways result in constitutive mitogenic signaling and defective responses to antimitogenic stimuli that contribute to unscheduled proliferation and genomic instability in tumor cells. Taken together, these alterations result not only in proliferative advantages but also in increased susceptibility to the accumulation of additional genetic alterations that contribute to tumor progression and acquisition of more aggressive phenotypes. In almost all tumors (1–5), these cell-cycle defects are mediated by the inactivation of a region located in humans at chromosome arm 9p21.

This region has a complex and unique genomic organization containing 2 candidate tumor suppressor genes, CDKN2A [cyclin-dependent kinase (CDK) inhibitor 2A] and CDKN2B, which encode 3 critical factors for the regulation of cell cycle and/or apoptosis (6): p16/INK4A (inhibitor of CDK4) and p14/ARF (alternative reading frame) encoded by CDKN2A and p15/INK4B encoded by CDKN2B. Moreover, this region includes a recently discovered noncoding RNA, designed ANRIL (antisense noncoding RNA in the INK4 locus) or CDKN2BAS, with a first exon located in the promoter of the ARF gene and overlapping the 2 exons of CDKN2B. Its transcription occurs in the opposite strand to the CDKN2A/B locus and coclusters mainly with p14/ARF in both physiologic and pathologic conditions (7).
**Translational Relevance**

CDKN2A/ARF and CDKN2B encode 3 tumor suppressors involved in the regulation of cell cycle and/or apoptosis and often lost in several cancers. Here, we report a genetic loss in 29% (CDKN2A/ARF) and 25% (CDKN2B) of newly diagnosed Philadelphia-positive (Ph+) ALL (acute lymphoblastic leukemia) patients and in 47% (CDKN2A/ARF) and 40% (CDKN2B) of relapsed cases. The novelty of this article is represented by the correlation, for the first time, found in adult Ph+ ALL patients, among the genomic status of CDKN2A/B and clinical outcome. Our results showed that deletions of CDKN2A/B are significantly associated with clinical outcome (P = 0.0026 for overall survival, P = 0.0010 for disease free-survival, and P = 0.0014 for cumulative incidence of relapse) and multivariate analysis (P = 0.0051) with poor outcome. Our findings strongly suggest that there are genetically distinct Ph+ ALL patients with a different risk of leukemia relapse and that testing for CDKN2A/B alterations at diagnosis may aid risk stratification.

INK4A and ARF share common second and third exons, but a different first exon (exon 1α for INK4 and exon 1β for ARF) and therefore are translated in alternate reading frames, exhibiting no protein sequence similarity. Functionally, INK4A is a CDK inhibitor, whereas ARF (p19 Arf in mice) regulates p53 tumor suppressor pathway. CDKN2B lies adjacent to INK4A/ARF and it encodes p15/INK4B, a CDK inhibitor, which forms a complex with CDK4 or CDK6 and prevents the activation of the CDK kinases; thus, the encoded protein functions as a suppressor involved in the regulation of cell cycle and/or apoptosis and often lost in several cancers. Here, we report a genetic loss in 29% (CDKN2A/ARF) and 25% (CDKN2B) of newly diagnosed Philadelphia-positive (Ph+) ALL (acute lymphoblastic leukemia) patients and in 47% (CDKN2A/ARF) and 40% (CDKN2B) of relapsed cases. The novelty of this article is represented by the correlation, for the first time, found in adult Ph+ ALL patients, among the genomic status of CDKN2A/B and clinical outcome. Our results showed that deletions of CDKN2A/B are significantly associated with clinical outcome (P = 0.0026 for overall survival, P = 0.0010 for disease free-survival, and P = 0.0014 for cumulative incidence of relapse) and multivariate analysis (P = 0.0051) with poor outcome. Our findings strongly suggest that there are genetically distinct Ph+ ALL patients with a different risk of leukemia relapse and that testing for CDKN2A/B alterations at diagnosis may aid risk stratification.

Deletions of p16/INK4A, p14/ARF, and p15/INK4B have been shown to frequently occur in all lymphoid malignancies (10–14), with homozygous deletions as the most frequent mechanism of inactivation (15). However, despite their high frequency, the prognostic importance of 9p21 alterations is still controversial in ALL (10, 16). Moreover, most of the series published so far are small, and they used techniques that did not enable small and/or monoallelic deletions to be detected.

To overcome these limits, in this study, we aimed at exploring, at a high resolution, the frequency and the type of deletions occurring at the 9p21 locus in adult BCR-ABL1–positive ALL, at determining the main mechanism of inactivation and at investigating the influence of 9p21 inactivation on prognosis.

**Materials and Methods**

**Patients**

All patients gave informed consent to blood collection and biological analyses, in agreement with the Declaration of Helsinki. The study was approved by the Department of Hematology and Oncological Sciences “L. and A. Seragno”, University of Bologna, Bologna, Italy. Overall 112 adult BCR-ABL1–positive ALL patients, enrolled from 1996 to 2008 in different clinical trials of the GIMEMA (Gruppo Italiano Malattie Ematologiche dell’Adulto) Acute Leukemia Working Party or in Institutional protocols, were analyzed. Clinical trials included: LAL0201-B enrolling elderly (>60 years) Philadelphia-positive (Ph+) ALL patients who received imatinib, 800 mg daily, associated to steroids as frontline treatment; LAL2000 enrolling adult (>18 years) ALL patients, including Ph+ cases, who received induction and consolidation chemotherapy followed by maintenance therapy with imatinib; LAL1205 enrolling adult Ph+ ALL patients who received dasatinib 70 mg twice a day for 84 consecutive days, as induction therapy, initially associated to steroids without further chemotherapy as frontline treatment.

A total of 101 patients (90%) were de novo ALL cases analyzed at the time of diagnosis, whereas 11 (10%) were relapse cases analyzed only at the time of treatment failure. In 19 of 101 (19%) cases, both diagnosis and relapse samples were collected and, thereafter, analyzed. Demographics and main clinical characteristics of the patients are summarized in Supplementary Table S1.

**Single-nucleotide polymorphism microarray analysis**

Genomic DNA was extracted using the DNA Blood Mini Kit (Qiagen) from mononuclear cells isolated from peripheral blood or bone marrow aspirate samples by Ficoll gradient centrifugation. DNA was quantified using the Nanodrop Spectrophotometer and quality was assessed using the Nanodrop and by agarose gel electrophoresis. A total of 83 samples (63 diagnosis, 20 relapse) were genotyped with GeneChip Human Mapping 250K NspI and 48 samples (38 diagnosis, 10 relapse) with Genome-Wide Human SNP 6.0 array microarrays (Affymetrix Inc.) following the manufacturer’s instructions and as previously described (17).

**FISH and probes**

FISH analysis was done as previously described (18). Bacterial artificial chromosome [BAC; RP11-70L8; accession number AL359922; chr9:21,732,609–21,901,258, RP11-149I2 accession number AL449423; chr9:21,899,259–22,000,413, and RP11-145E5 accession number AL354709; chr9:21,998,414–22,155,946] and fosmid [G248P82557D2 (accession number WIBR2-1053H3; chr9:21,975,653–22,011,179, and G248P82010F5 (accession number WIBR2-1034K10; chr9:21,926,491-
Fluidigm Dynamic Array 48 using Hs00924091_m1 assay (Applied Biosystems). Quantitative PCR analysis was done transcribed using the High Capacity cDNA Archive Kit RNA isolation kit (Qiagen) and 1 microgram was reverse CDKN2A gene expression levels.

Total cellular RNA was extracted using the RNeasy total RNA isolation kit (Qiagen) and 1 microgram was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems). Quantitative PCR analysis was done using Hs00924091_m1 assay (Applied Biosystems) and the Fluidigm Dynamic Array 48 × 48 system (Fluidigm; http://www.fluidigm.com/).

CDKN2A gene expression levels

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INK4A/ARF and INK4B point mutation screening

Genomic resequencing of all coding exons of INK4A/ARF and INK4B was done in search of mutations using primers listed in Supplementary Table S2. All sequence variations were detected by comparison using the BLAST software tool (www.ncbi.nlm.nih.gov/BLAST/) to reference genome sequence data (GenBank accession number NM_000077.4, NM_058195.3 and NM_004936, for CDKN2A, ARF, CDKN2B, respectively) obtained from the UCSC browser (http://genome.ucsc.edu/cgi-bin/hgGateway?db=human&db=hg18; March 2006 release).

Collection of DNA from saliva samples

Almost 2 mL of saliva samples were collected from 5 patients and mixed with 2 mL DNA preserving solution contained in the Oragene DNA Self-Collection Kit Oragene (OG-500 Tube Format; DNA Genotek Inc.), according to the manufacturer’s instructions.

Statistical analysis

Differences in the distributions of prognostic factors in subgroups were analyzed by χ² or Fisher exact test and by Kruskal–Wallis test. Median follow-up time was estimated by reversing the codes for the censoring indicator in a Kaplan–Meier analysis (19). The probabilities of overall survival (OS) and disease-free survival (DFS) were estimated using the Kaplan–Meier method (19) and the probability of cumulative incidence (20) of relapse (CIR) was estimated using the appropriate nonparametric method, considering death in complete remission as competing risk. The log-rank test was used to compare treatment effect and risk factor categories for the Kaplan–Meier curves, and the Gray test for the incidence curves. CIs (95% CIs) were estimated using the Simon and Lee method (21). Cox proportional hazard regression model (22) was done to examine and check for treatment results and the risk factors affecting DFS. All tests were 2-sided, accepting P ≤ 0.05 as indicating a statistically significant difference.

A full, detailed description of the Methods is provided in the Supplementary Material.

Results

Single-nucleotide polymorphism microarray analysis detects frequent and recurrent deletions in CDKN2A, ANRIL, and CDKN2B genes at diagnosis and during leukemia progression

To detect the frequency and size of deletions occurring at the 9p21 locus in BCR-ABL1–positive ALL, data generated by high-resolution single-nucleotide polymorphism (SNP) arrays were analyzed in adult patients at diagnosis (n = 82), relapse (n = 11), or at both time points (n = 19). At diagnosis, CDKN2A and ANRIL genomic alterations were identified in 29/101 (29%) patients. ANRIL has a first exon located about 300 bp upstream of the transcription start site of exon 1B of CDKN2A and overlapping at its 5’ end with the two exons of CDKN2B. Genomic deletions also included the two exons of CDKN2B in 25/101 patients (25%). Deletions were monoallelic in the majority of cases (72%) with a median of 1,012 kb in size (range: 2.8–31,319 kb). In 9 of 29 (43%) patients with CDKN2A/ANRIL/CDKN2B losses, the minimal overlapping region of the lost area spanned only the 2 genes, but more often (12/29, 57%) the loss was considerably larger and extended sometimes (3/29, 10%) over the entire short chromosome arm, eliminating a large number of genes (Fig. 1A). In contrast, cases with biallelic inactivation occurred in 8 of 28 (29%) patients, with the majority of deletions (6/8, 75%) limited to the CDKN2A/ANRIL/CDKN2B genes (Fig. 1B).

Next, to investigate whether the deletions of CDKN2A/ANRIL/CDKN2B genes could be involved in disease progression, the genomic status of the 9p21 locus was assessed at the time of relapse in 30 patients (11 unpaired and 19 paired relapsed cases). In an unpaired analysis, an almost significant increase in the detection rate of CDKN2A loss (47%) compared with diagnosis (29%; P = 0.06) was found by a nonparametric t test. When we analyzed the type of deletion, we found that deletions were heterozygous in the majority of cases, both at diagnosis and at relapse (72% vs. 28%; Table 1). Thereafter, we assessed the genomic status of CDKN2A/ANRIL/CDKN2B in the 19 paired relapse cases (Supplementary Table S3 and Fig. S2): CDKN2A deletion was acquired at the relapse in 6 cases (31.6%: 4 heterozygous and 2 homozygous cases), it was maintained with the same pattern of diagnosis in 4 cases (21.1%), and it was lost at the relapse in 3 cases (15.8%).

FISH analysis confirmed large deletions

FISH experiments with BACs and fosmids, encompassing the whole MTAP–CDKN2A–CDKN2B locus (Fig. 2A), were done in 6 BCR-ABL1–positive ALL patients, to confirm the deletion disclosed by SNP array analysis. Heterozygous deletion was detected (at FISH resolution) in 2 cases, as shown by the use of MTAP–CDKN2A–CDKN2B probes on chromosome der(9) in Ph– metaphases identified by the splitting signal of RP11-164N13, observed, respectively, on der(9) and Ph chromosomes (Fig. 2B and C and data not shown). The same probes failed to identify the deletion in the other 4 patients on study (data not shown) because of the
limited size of the deletion spanning only the 2 genes and below the limits of FISH resolution, as verified by SNP array.

Deletions lead to a downregulation of CDKN2A levels

To investigate the functional consequences of genomic deletions affecting the 9p21 locus, CDKN2A transcript levels were assessed by quantitative reverse transcriptase PCR in 3 different groups of BCR-ABL1–positive ALL patients: (i) cases lacking CDKN2A deletions (n = 18); (ii) cases with heterozygous deletion of CDKN2A (n = 5); (iii) cases with homozygous deletion of CDKN2A (n = 7). The Hs00924091_m1 assay (Applied Biosystems) amplifying
the 1–2 exon boundary of CDKN2A (reference sequence NM_058195.3) was used. The results showed a significant decrease in the expression of CDKN2A in heterozygous deleted cases ($P = 0.04$) and in homozygous deleted cases ($P = 0.01$) compared with cases without the deletion. The median CDKN2A expression level expressed as $2^{-\Delta \Delta C_t}$ in diploid cases was 2.86 (range: 0.81–14.41) versus 0.19 (range: 0.10–0.53) and 0.004 (range: 0.0003–0.0653) of cases with monoallelic and biallelic losses, respectively (Fig. 3). A significant difference in the expression of CDKN2A was also observed among cases with heterozygous and homozygous deletions ($P = 0.004$). Overall, these results suggest that CDKN2A deletions lead to a gene dosage effect.

**INK4A/ARF and INK4B mutation screening**

The 9p21 locus can be inactivated in many tumors due to several mechanisms in addition to deletions, such as hypermethylation of promoter regions, and inactivating mutations. Because hypermethylation is a rare event in ALL.

### Table 1. Deletion rates of CDKN2A/ANRIL/CDKN2B at diagnosis and relapse

<table>
<thead>
<tr>
<th></th>
<th>Diagnosis ($n = 101$)</th>
<th>Relapse ($n = 30$)</th>
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<tbody>
<tr>
<td><strong>CDKN2A Deletion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>21 (20.8)</td>
<td>10 (33.3)</td>
</tr>
<tr>
<td>Homozygous</td>
<td>8 (7.9)</td>
<td>4 (13.3)</td>
</tr>
<tr>
<td>Total</td>
<td>29 (28.7)</td>
<td>14 (46.6)</td>
</tr>
<tr>
<td><strong>ANRIL Deletion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>23 (22.8)</td>
<td>10 (33.3)</td>
</tr>
<tr>
<td>Homozygous</td>
<td>6 (5.9)</td>
<td>3 (10.0)</td>
</tr>
<tr>
<td>Total</td>
<td>29 (28.7)</td>
<td>13 (43.3)</td>
</tr>
<tr>
<td><strong>CDKN2B Deletion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>19 (18.8)</td>
<td>9 (30.0)</td>
</tr>
<tr>
<td>Homozygous</td>
<td>6 (5.9)</td>
<td>3 (10.0)</td>
</tr>
<tr>
<td>Total</td>
<td>25 (24.8)</td>
<td>12 (40.0)</td>
</tr>
</tbody>
</table>

**NOTE:** Hundred and one patients were analyzed at diagnosis, whereas 30 patients (including 19 paired and 11 unpaired cases) were analyzed at the time of relapse. The table shows the numbers and percentages of BCR-ABL1–positive ALL patients with heterozygous and homozygous deletions at diagnosis and at relapse. The values in parenthesis are taken in percent.

Figure 2. FISH results. A, map of the clones used in FISH experiments to detect MTAP-CDKN2A-CDKN2B deletions, showing BACs, fosmids, and genes, respectively, with red, blue, and green bars. B and C, FISH results obtained in patients #1 and #2, respectively, showing a MTAP-CDKN2A-CDKN2B heterozygous deletion. B, only one fluorescent signal of both BACs (on the left) and fosmids (on the right) observed on normal chromosome 9 in Ph-positive metaphases (as shown by the 3 signals of RP11-164N13); C, colocalization of all the MTAP-CDKN2A-CDKN2B BAC probes only on normal chromosome 9. No signal on der(9).
exons of CDKN2A and it is responsible for a nonsynonymous substitution of alanine with threonine at codon 148. The rs11515 C/G and rs3088440 C/T polymorphisms are located in the 3’-UTR. The first and second exons of CDKN2B were wild type, except for the silent mutation at codon 83 (P83) identified in one case at diagnosis (Supplementary Table S4 and Fig. S3).

Comparison between leukemia and germline DNA samples
To assess whether the nucleotide substitutions identified in CDKN2A/B genes were acquired at the time of leukemia (somatic mutations), we compared the leukemia DNA samples with those obtained from collection of saliva after written informed consent. For this analysis, 5 cases were available. PCR was done on the promoter region and exon 1α of CDKN2A to assess the mutational status of the substitution at position 21965017 because the remaining exons resulted wild-type or containing SNPs. Results showed that the mutation was inherited for cases #1, #3, and #5; it was acquired by the leukemia blast cells in case #4. Interestingly, case #2 showed an unusual pattern with the mutation in the germline/saliva sample and with the wild-type allele in the leukemia sample (Supplementary Fig. S4).

To exclude a potential contamination of saliva samples with blood leukemia cells, we assessed the genomic status of IKZF1 gene in the 3 cases (#1, #3, and #5), in whom the mutation was inherited. Patients #3 and #5 showed a deletion of IKZF1 in the leukemia samples but not in the saliva (data not shown), excluding a contamination with leukemia cells. Sample #1 lacked IKZF1 deletion in both leukemia and saliva samples, avoiding us to exclude a potential contamination.

CDKN2A/B deletions and correlation with known molecular alterations
After having shown that deletions are the main mechanism of inactivation, we investigated their association with other known molecular alterations reported by our group and others (17, 27, 28) to occur frequently in BCR-ABL1–positive ALL, such as IKZF1 and PAX5 losses. In our study cohort, CDKN2A/B deletions were strongly associated with deletions of PAX5 (P < 0.0001; χ² test) but not of IKZF1 (P = 0.5190; χ² test). In details, 72 of 101 (71.29%) newly diagnosed cases were IKZF1 deleted, whose 22 (30.56%) harbored also CDKN2A/B deletions; 31 of 101 (30.69%) patients had a loss of PAX5, whose 19 (61.29%) showed a simultaneously loss of CDKN2A/B.
(WBC) count was 21.95 \( (\times 10^9)/L; \) range: 0.40–302.00) and CDKN2A/B was lost in 29 (35.80%) cases. A total of 72 patients (89%) were enrolled in the GIMEMA clinical trials (12 patients in GIMEMA LAL0201-B protocol, 13 in LAL2000, and 47 in the LAL1205 protocols), whereas 9 patients (11%) were enrolled into institutional protocols. Details of the treatment schemes have been previously reported (29). First of all, to investigate whether clinical patterns correlate with CDKN2A/B status, we evaluated, by univariate analysis, a potential association between the genomic status of CDKN2A/B and variables, such as age, WBC, and treatment regimens. We found a strong association between deletions of CDKN2 and higher WBC \( (P = 0.0291) \) and between deletions and protocol. In particular, deletions have been found to be more frequent in the group of patients treated without TKIs \( (63.64\% \text{ vs. } 25.42\% , P = 0.0014; \text{Supplementary Table S6}) \). Thereafter, a univariate analysis of the CDKN2A/B genomic status and its association with outcome was done. A shorter OS and DFS were found in patients with CDKN2A/B deletion compared with wild-type patients (OS: 27.7 vs. 38.2 months, respectively, \( P = 0.0206 \); DFS: 10.1 vs. 56.1 months, respectively, \( P = 0.0010 \)). Moreover, a higher CIR for patients with CDKN2A/B deletion versus wild-type patients (73.3 vs. 38.1; \( P = 0.0014 \)) was also recognized (Table 2 and Fig. 4). To investigate whether there may be any confounding factors that could be influencing the relapse, we conducted a multivariate analysis which confirmed the negative prognostic impact of CDKN2A/B deletion on DFS \( (P = 0.0051; \text{Supplementary Table S7}) \).

### Discussion

Several groups have investigated how the 9p21 chromosome band is inactivated in ALL, but most of them referred to small cohorts of patients, mainly pediatric and using low resolution methodologies. For example, traditional techniques that have a limited number of probes are not able to detect small deletions that often occur in this locus and may underestimate the real incidence of deletions. Therefore, in this study, we carried out high resolution Affymetrix SNP arrays in 112 Ph+ ALL adult patients with the aim of exploring the frequency and size of deletions affecting the INK4A/ARF/INK4B genes in adult BCR-ABL1–positive ALL patients, of determining the main mechanism of inactivation, and of correlating deletions with clinical outcome.

In ALL patient samples, the size and position of 9p21.3 deletions seem to vary substantially, but in most cases, CDKN2A is codeleted with CDKN2B and the frequency of genomic deletions is 21% in B-cell precursor ALL and 50% in T-ALL patients (30). Here, we identified CDKN2A/ARF and ANRIL genomic alterations in 29% of BCR-ABL1–positive ALL patients at diagnosis. In 25% of cases, genomic deletions also included the 2 exons of CDKN2B. Deletions were predominantly monoallelic and in more than half of leukemia cases (57%), the minimal overlapping region of the lost area was considerably large, eliminating a large number of genes.

At relapse, a strong trend in the detection rate of CDKN2A/ARF loss (47%) compared with diagnosis \( (P = 0.06) \) was found, suggesting that loss of this genomic region may be involved in disease progression. But are the deletions the only mechanism of 9p21 inactivation? It is well known that in addition to deletions, the CDKN2A/B locus can also be inactivated by epigenetic silencing through DNA methylation or by point mutations. Methylation of CDKN2A and CDKN2B seems to lack prognostic significance in ALL (26), and the rate of point mutations has been extremely low in ALL (31). In line with these findings, ARF, CDKN2A, and CDKN2B point mutations were found at very low levels, with only a nonsynonymous substitution in the exon 2 of CDKN2A (D146N). Additional mutations, previously identified in melanoma cases (32), have been identified in the 5′-UTR/promoter of CDKN2A exon 1c at position 21965017, 191 bp before the start codon, and at position 21964851, 25 bp before the start codon.

Moreover, frequent nucleotide variations, known as SNPs, were identified in exons 2 and 3 of CDKN2A: rs3731249 G/A, rs11515 C/G, and rs3088440 C/T. These SNPs have been phenotypically associated with solid tumors such as non-Hodgkin lymphoma (33), breast cancer (34), colorectal cancer (35), and other diseases, such as Alzheimer’s (36).

### Table 2. Clinical outcome related to CDKN2A/B loss in univariate analysis

<table>
<thead>
<tr>
<th>CIR</th>
<th>Patients</th>
<th>CDKN2A/B loss % (95% CI)</th>
<th>CDKN2A/B wt % (95% CI)</th>
<th>Pr &gt; χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFS</td>
<td>At 24 mo</td>
<td>73.3 (71.6–75.1)</td>
<td>38.1 (37.9–39.2)</td>
<td>0.0014</td>
</tr>
<tr>
<td></td>
<td>Median time</td>
<td>10.1 mo</td>
<td>56.1</td>
<td></td>
</tr>
<tr>
<td>OS</td>
<td>At 24 mo</td>
<td>22.2 (18.8–26.3)</td>
<td>57.6 (49.8–66.7)</td>
<td>0.0010</td>
</tr>
<tr>
<td></td>
<td>Median time</td>
<td>10.1 mo</td>
<td>56.1 mo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>At 24 mo</td>
<td>57.2 (46.5–70.4)</td>
<td>77.8 (68.7–88.1)</td>
<td>0.0206</td>
</tr>
<tr>
<td></td>
<td>Median time</td>
<td>27.7 mo</td>
<td>38.2 mo</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: wt, wild type; mo, months.
and melanoma (37). However, their role in leukemia has not yet been well established and a larger number of patients is required to show any potential association.

After having shown that deletions are the main mechanism of inactivation, we investigated their implications in leukemia. Preliminary results have shown that in mice, the combination of BCR-ABL1 and ARF loss are sufficient to induce aggressive B-cell ALL, increased self-renewal capacity, inhibition of apoptosis, and independence on cytokines, contributing to resistance to targeted therapy with TKIs (9, 38). Moreover, recently Notta and colleagues (39) showed that Ph+ ALL patient samples with a loss of CDKN2A/B had a tendency to poorer survival, correlated with aggressive dissemination in xenografts and higher leukemia-initiating cell frequency, compared with patients with normal CDKN2A/B, showing that the loss of CDKN2A/B contributes to clonal predominance at diagnosis and competitive xenograft growth.

How could this be translated *in vivo* in Ph+ ALL patients? To address this issue, we investigated the prognostic relevance of CDKN2A/B deletions in our study cohort. This matter is still controversial in the literature, with some studies suggesting that CDKN2A/B deletion is a poor prognostic factor in ALL (40–45), whereas others show no correlation (46–48). Recently, Usvasalo and colleagues did not observe any difference in the incidence of deletion between diagnosis and relapse in childhood ALL (49). Furthermore, reports focused only on the BCR-ABL1–positive subgroup and investigating a correlation between CDKN2A/B deletions and outcome are still lacking.

Our results showed that deletions of CDKN2A/B are significantly associated with higher WBC (\(P = 0.0291\)) and with poor outcome in terms of OS (\(P = 0.0206\)), DFS (\(P = 0.0010\)), and CIR (\(P = 0.0014\)). The negative prognostic impact of CDKN2A/B deletion on DFS was thereafter also confirmed by a multivariate analysis.
(\(P = 0.0051\)). It is likely that the impact of CDKN2A/B deletion might also be dependent on the coexisting aberrations, such as the BCR-ABL1 rearrangement, and this may, in part, explain the controversial reports existing on the prognostic value of 9p21 deletions in ALL. Anyway, our results show that there are genetically distinct Ph+ ALL patients with a different risk of leukemia relapse and that testing for CDKN2A/B alterations at diagnosis may help in risk stratification. Furthermore, the awareness that genetically distinct patients experience different responses to treatment points to the need to develop more effective therapies able to eradicate all genetic leukemia cells and to prevent disease recurrence. Because the loss of CDKN2A/B eliminates the critical tumor surveillance mechanism and allows proliferation, cell growth and tumor formation by the action of MDM2 and CDK4/CDK6, in line with these therapeutic suggestions, recently nutlin-3, a small-molecule antagonist of MDM2, has been shown to inhibit in vitro proliferation and to induce apoptosis more effectively in BCR-ABL1–driven Ton B210 cells than in those driven by IL-3 (50). Moreover, nutlin-3 drastically enhanced imatinib-induced apoptosis in a p53-dependent manner in primary leukemic cells from patients with chronic myeloid leukemia (CML) blast crisis and Ph+ ALL, including cells expressing the imatinib-resistant E255K BCR-ABL1 mutant, providing a molecular rationale for concomitant activation of p53 and inhibition of BCR-ABL1 in effective killing of BCR-ABL1–expressing leukemic cells (50). In vivo a multicenter, open-label study to investigate the maximum tolerated dose of ROS045337 [R7112], an MDM2 antagonist, is ongoing in patients with hematologic malignancies, including BCR-ABL1–positive ALL and CML in blast phase (ClinicalTrials.gov Identifier: NCT010623870).

In conclusion, our findings indicate that the inactivation of the CDKN2A/B locus is a frequent event in Ph+ ALL. Deletions are frequently acquired at leukemia progression and are a poor prognostic marker, impairing OS, DFS, and CIR. Novel treatment strategies targeting the ARF-MDM2-p53 and the CDKN2A/B-CDK4/6–retinoblastoma pathways may be effective in this subset of patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Clin Cancer Res 2011;17:7413-7423.

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