Clinical Cancer Research

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).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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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 reported 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 by univariate ($P = 0.0206$ 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 function through its interaction with MDM2 (8). *CDKN2B* lies adjacent to *INK4/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 cell growth regulator that controls cell-cycle G_1 progression.

Losses of *ARF* and *INK4*, eliminating the critical tumor surveillance mechanism, are hypothetically harmful because they facilitate proliferation and growth of incipient cancer cells with oncogenic mutations. Supporting this hypothesis, a genomic loss of *ARF* has been shown to enhance oncogenicity and limit imatinib response in mouse models of *BCR-ABL1*–induced acute lymphoblastic leukemia (ALL; ref. 9).

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. Seràgnoli", 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-

21,967,852)] probes, specific for the *MTAP-CDKN2A-CDKN2B* locus, as well as a BAC for *BCR* gene [RP11-164N13 (chr22:21,897,904–22,091,572)], were properly selected accordingly to the March 2006 release of the UCSC Human Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=169374627&clade=mammal&org=Human&db=hg18).

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/).

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=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 χ^2 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 $P \leq 0.05$ 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 1β 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 29 (28%) 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

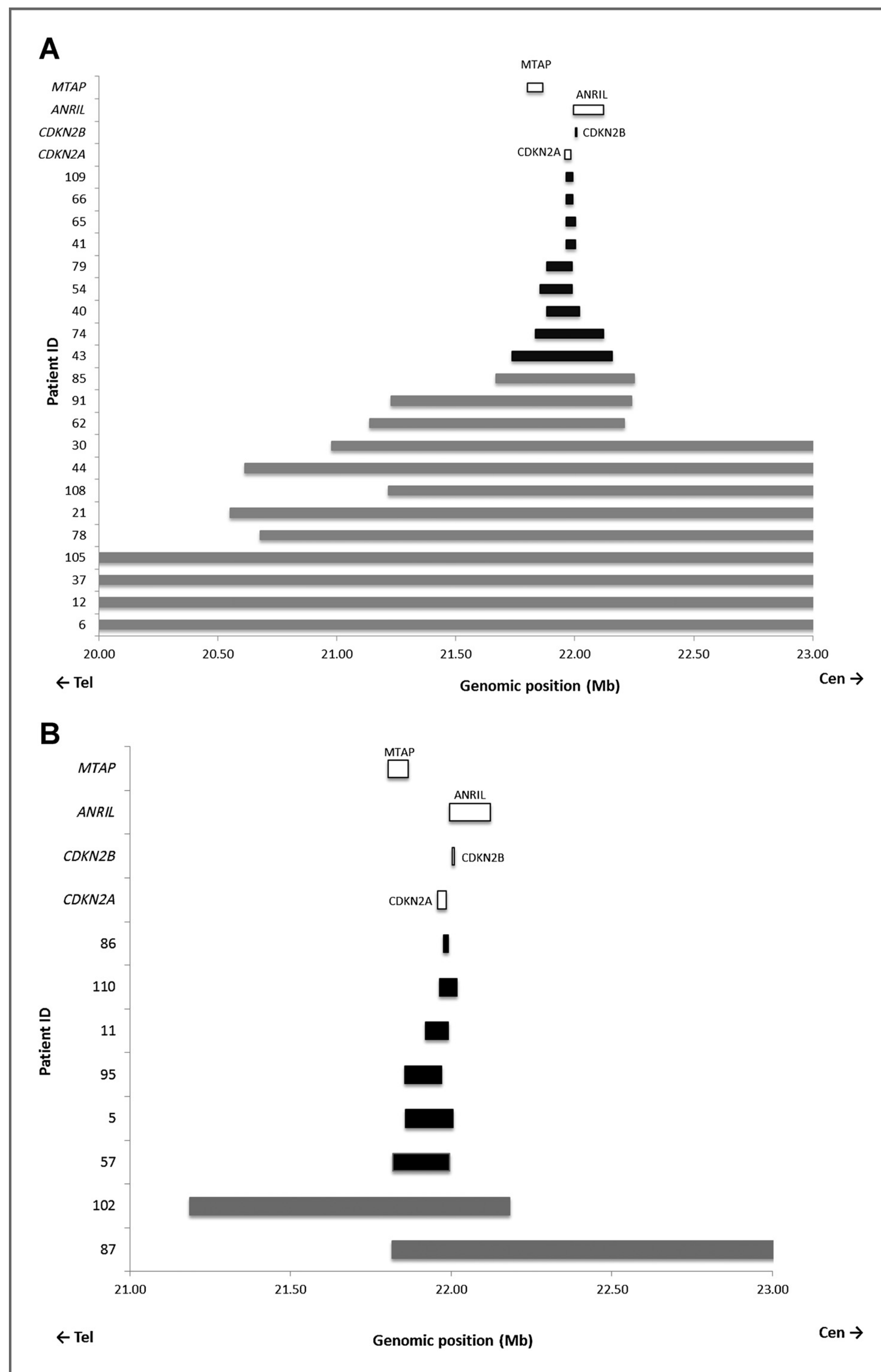

Figure 1. Schematic representation of heterozygous (A) and homozygous (B) deletions occurring on 9p21 genomic locus in adult *BCR-ABL1*–positive ALL patients. Gray bars indicate large deletions extending over the *CDKN2A/ANRIL/CDKN2B* genes; black bars indicate small deletions spanning only the *CDKN2A/ANRIL/CDKN2B* genes. A schematic position and size of *MTAP*, *CDKN2A*, *ANRIL*, and *CDKN2B* genes are also reported as white bars.

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

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

		Diagnosis (n = 101)	Relapse (n = 30)
CDKN2A Deletion	Heterozygous	21 (20.8)	10 (33.3)
	Homozygous	8 (7.9)	4 (13.3)
	Total	29 (28.7)	14 (46.6)
ANRIL Deletion	Heterozygous	23 (22.8)	10 (33.3)
	Homozygous	6 (5.9)	3 (10.0)
	Total	29 (28.7)	13 (43.3)
CDKN2B Deletion	Heterozygous	19 (18.8)	9 (30.0)
	Homozygous	6 (5.9)	3 (10.0)
	Total	25 (24.8)	12 (40.0)

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.

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 2exp(-$\Delta\Delta C_t$) 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

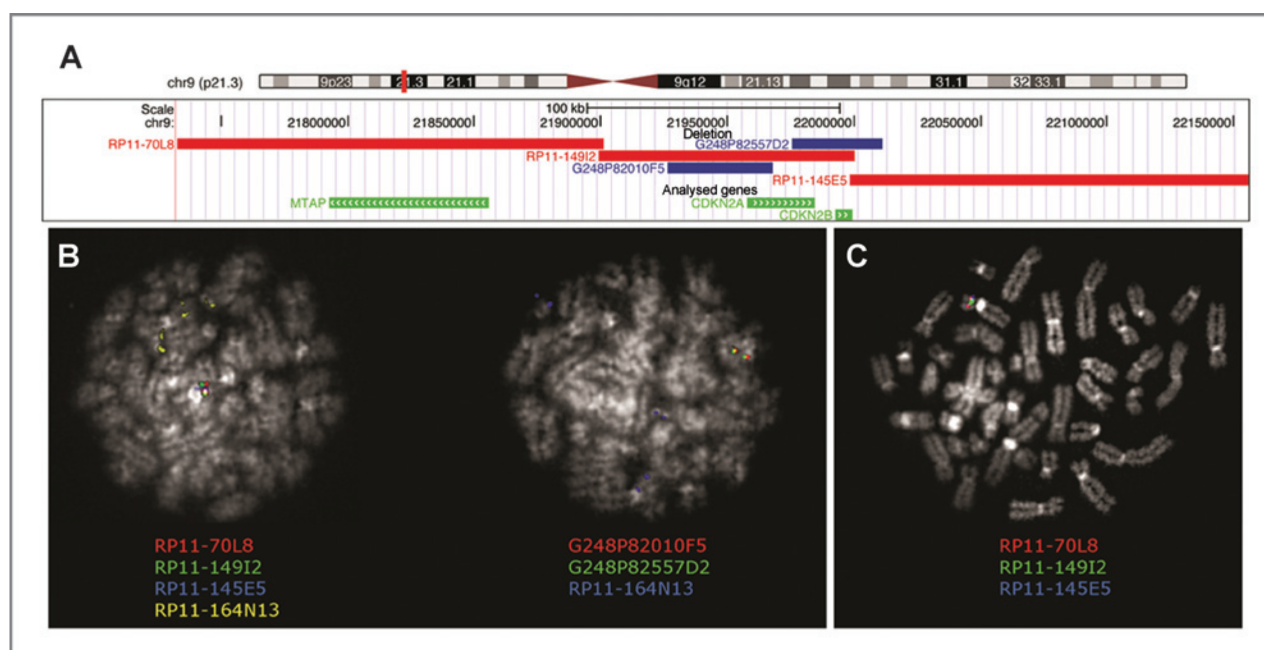

Figure 2. FISH results. A, map of the clones used in FISH experiments to detect *MTAP-CDKN2A-CDKN2B* deletions, showing BACs, fosmids, and genes, respectively, with red, blue, and green bars. B and C, FISH results obtained in patients #1 and #2, respectively, showing a *MTAP-CDKN2A-CDKN2B* heterozygous 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).

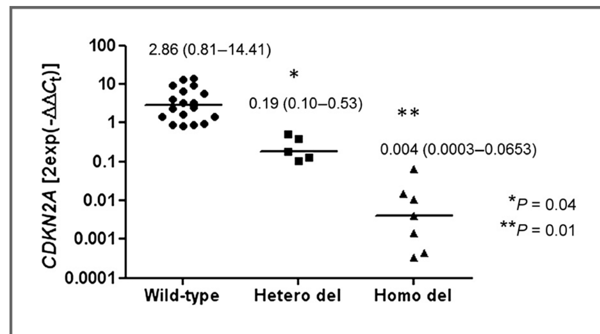

Figure 3. Expression levels of *CDKN2A* in *BCR-ABL1*–positive ALL patients with (i) wild-type *CDKN2A*; (ii) heterozygous *CDKN2A* deletion; (iii) homozygous *CDKN2A* deletion. Results are expressed as 2exp (-$\Delta\Delta C_t$). Nonparametric *t* test was used to compare group 1 versus group 2 ($P = 0.04$), group 1 versus group 3 ($P = 0.01$), and group 2 versus group 3 ($P = 0.004$).

(23–26), here we aimed to investigate the frequency of point mutations occurring in *INK4A/ARF* and *INK4B* genes. To address this issue, a mutation screening of all coding exons of *INK4A/ARF* (exons 1α, 1β, 2, and 3) and *INK4B* (exons 1 and 2) genes was successfully done on patients who were known to have retained at least 1 *CDKN2A/CDKN2B* allele or who were wild-type by SNP array (Supplementary Table S4). Samples tested were from the time of diagnosis ($n = 30$ for exon 1α of *CDKN2A*; $n = 35$ for exon 1β of *CDKN2A*; $n = 32$ for exon 2 of *CDKN2A*; $n = 36$ for exon 3 of *CDKN2A*; $n = 42$ for exon 1 and 2 of *CDKN2B*), and of relapse ($n = 10$ for exon 1α of *CDKN2A*; $n = 12$ for exon 1β of *CDKN2A* and exon 2 of *CDKN2A*; $n = 11$ for exon 3 of *CDKN2A*; $n = 14$ for exon 1 of *CDKN2B*; $n = 14$ for exon 2 of *CDKN2B*). Amplification and sequencing results showed that in the analyzed subset of patients, nonsynonymous point mutations in coding exons were rare with only 1 patient harboring a somatic nonsynonymous mutation. This was detected in a diagnosis sample and involved a base substitution of G→A in exon 2 of *CDKN2A* at codon 146 that resulted in a substitution of aspartic acid for asparagine (D146N). A base substitution of G→A in the same exon at codon 128 was identified in another case, but it resulted in a synonymous substitution of arginine (R128). Additional mutations have been identified in the 5′ untranslated region (UTR) of *CDKN2A* exon 1α at position 21965017, 191 bp before the start codon, (http://genome.ucsc.edu/cgi-bin/hgGateway?db=hg18; March 2006 release) with a heterozygous substitution of G→A in 46.7% of diagnosis patients and in 60.0% of relapsed patients. This substitution was homozygous in 36.6% of diagnosis patients and in 20.0% of relapse patients. In the same region, but at position 21964851, 25 bp before the start codon, a heterozygous substitution of C→T was found in only 1 patient at relapse (10%). Frequent nucleotide variations were identified in exons 2 and 3 of *CDKN2A*, but they resulted in known SNPs after comparison with the database dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). These variations included the following known SNPs: rs3731249 G/A, rs11515 C/G, and rs3088440 C/T. The rs3731249 G/A is located in exon 2 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* ($P < 0.0001$; χ^2 test) but not of *IKZF1* ($P = 0.5190$; χ^2 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*.

CDKN2A/B deletions and correlation with clinical outcome

Finally, to determine whether deletions of *CDKN2A/B* genes could impair response to treatment in *BCR-ABL1*–positive ALL patients, clinical data were collected from 81 patients. The median follow-up was 25.2 months (range: 2.1–148.1). Patient characteristics are reported in Supplementary Table S5. Briefly, the median age at diagnosis was 53.71 years (range: 18–76), the median white blood cell

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

		***CDKN2A/B loss* % (95% CI)**	***CDKN2A/B* wt % (95% CI)**	**Pr** > χ^2
CIR	**Patients**	**27**	**51**	0.0014
	At 24 mo	73.3 (71.6–75.1)	38.1 (37–39.2)	
	Median time	10.1 mo	56.1	
DFS	**Patients**	**27**	**51**	0.0010
	At 24 mo	22.2 (18.8–26.3)	57.6 (49.8–66.7)	
	Median time	10.1 mo	56.1 mo	
OS	**Patients**	**29**	**52**	0.0206
	At 24 mo	57.2 (46.5–70.4)	77.8 (68.7–88.1)	
	Median time	27.7 mo	38.2 mo	

Abbreviations: wt, wild type; mo, months.

(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% vs. 25.42%, P = 0.0014; 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; 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 know 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 1α 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)

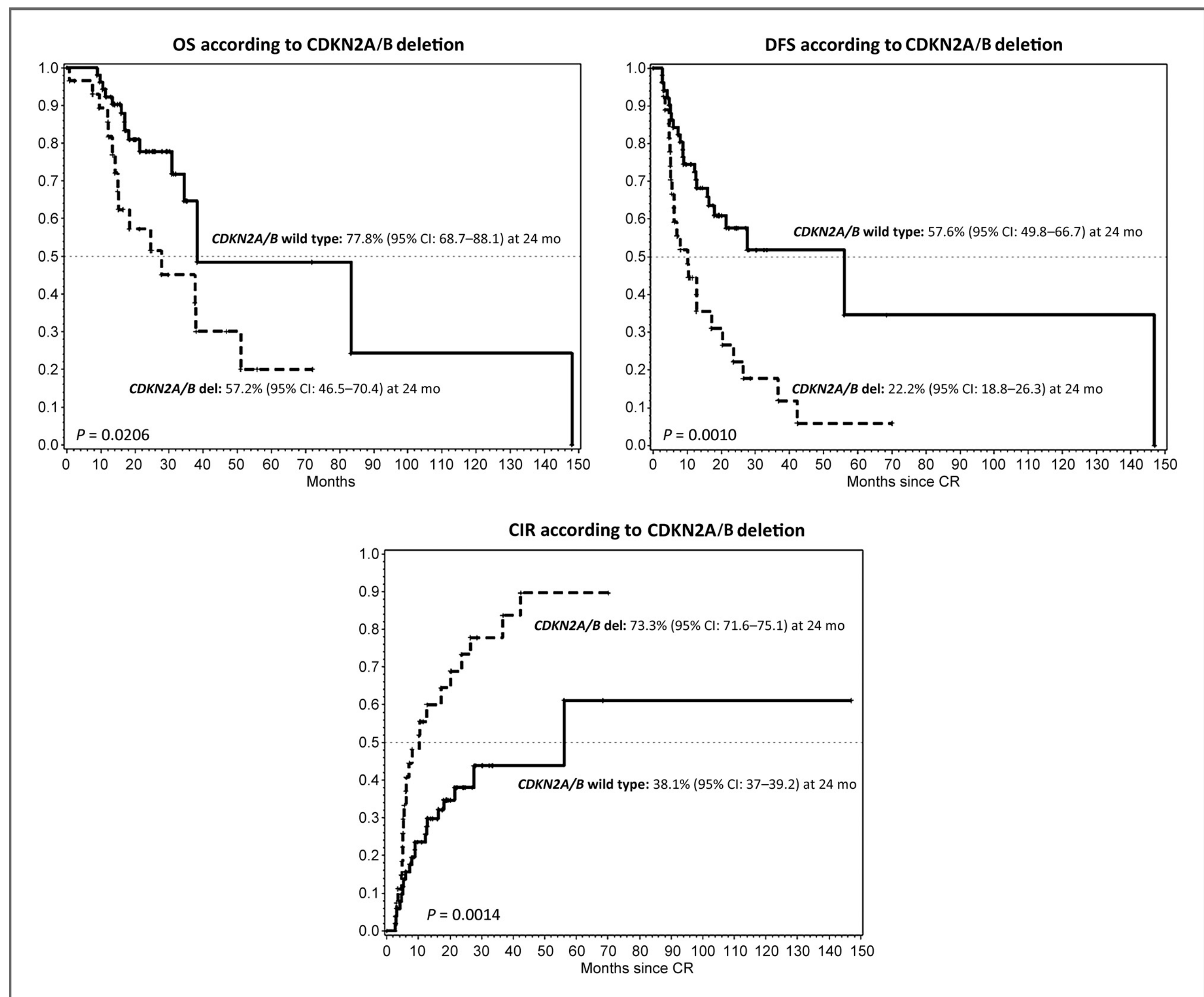

Figure 4. Correlation with clinical outcome. OS, DFS, and CIR of *de novo BCR-ABL1*–positive ALL patients enrolled in different clinical trials of GIMEMA according to *CDKN2A/B* deletions.

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 by univariate analysis 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 CDK4/6, attractive drugs could be represented by the inhibitors 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 RO5045337 [R7112], an MDM2 antagonist, is ongoing in patients with hematologic malignancies, including *BCR-ABL1*–positive ALL and CML in blast phase (ClinicalTrials.gov Identifier: NCT00623870).

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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References

1. Hamada K, Kohno T, Kawanishi M, Ohwada S, Yokota J. Association of CDKN2A(p16)/CDKN2B(p15) alterations and homozygous chromosome arm 9p deletions in human lung carcinoma. Genes Chromosomes Cancer 1998;22:232–40.
2. Illei PB, Rusch VW, Zakowski MF, Ladanyi M. Homozygous deletion of CDKN2A and codeletion of the methylthioadenosine phosphorylase gene in the majority of pleural mesotheliomas. Clin Cancer Res 2003;9:2108–13.
3. Raschke S, Balz V, Efferth T, Schulz WA, Florl AR. Homozygous deletions of CDKN2A caused by alternative mechanisms in various human cancer cell lines. Genes Chromosomes Cancer 2005;42: 58–67.
4. Krasinskas AM, Bartlett DL, Cieply K, Dacic S. CDKN2A and MTAP deletions in peritoneal mesotheliomas are correlated with loss of p16 protein expression and poor survival. Mod Pathol 2010;23:531–8.
5. Cobbers JM, Wolter M, Reifenberger J, Ring GU, Jessen F, An HX, et al. Frequent inactivation of CDKN2A and rare mutation of TP53 in PCNSL. Brain Pathol 1998;8:263–76.
6. Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell 1995;83:993–1000.
7. Pasmant E, Laurendeau I, Heron D, Vidaud M, Vidaud D, Bieche I. Characterization of a germ-line deletion, including the entire INK4/ARF locus, in a melanoma-neural system tumor family: identification of

ANRIL, an antisense noncoding RNA whose expression coclusters with ARF. Cancer Res 2007;67:3963–9.
8. Sherr CJ. The INK4a/ARF network in tumour suppression. Nat Rev Mol Cell Biol 2001;2:731–7.
9. Williams RT, Roussel MF, Sherr CJ. Arf gene loss enhances oncogenicity and limits imatinib response in mouse models of Bcr-Abl-induced acute lymphoblastic leukemia. Proc Natl Acad Sci U S A 2006;103:6688–93.
10. Usvasalo A, Savola S, Raty R, Vettenranta K, Harila-Saari A, Koistinen P, et al. CDKN2A deletions in acute lymphoblastic leukemia of adolescents and young adults: an array CGH study. Leuk Res 2008; 32:1228–35.
11. Mullighan CG, Williams RT, Downing JR, Sherr CJ. Failure of CDKN2A/B (INK4A/B-ARF)-mediated tumor suppression and resistance to targeted therapy in acute lymphoblastic leukemia induced by BCR-ABL. Genes Dev 2008;22:1411–5.
12. Niini T, Lopez-Guerrero JA, Ninomiya S, Guled M, Hattinger CM, Michelacci F, et al. Frequent deletion of CDKN2A and recurrent coamplification of KIT, PDGFRA, and KDR in fibrosarcoma of bone-An array comparative genomic hybridization study. Genes Chromosomes Cancer 2010;49:132–43.
13. Zhang H, Rosdahl I. Deletion in p16INK4a and loss of p16 expression in human skin primary and metastatic melanoma cells. Int J Oncol 2004;24:331–5.
14. Usvasalo A, Ninomiya S, Raty R, Hollmen J, Saarinen-Pihkala UM, Elonen E, et al. Focal 9p instability in hematologic neoplasias revealed by comparative genomic hybridization and single-nucleotide polymorphism microarray analyses. Genes Chromosomes Cancer 2010;49: 309–18.
15. Faderl S, Estrov Z, Kantarjian HM, Thomas D, Cortes J, Manshouri T, et al. The incidence of chromosome 9p21 abnormalities and deletions of tumor suppressor genes p15(INK4b)/p16(INK4a)/p14(ARF) in patients with acute lymphoblastic leukemia. Cytokines Cell Mol Ther 1999;5:159–63.
16. Kim M, Yim SH, Cho NS, Kang SH, Ko DH, Oh B, et al. Homozygous deletion of CDKN2A (p16, p14) and CDKN2B (p15) genes is a poor prognostic factor in adult but not in childhood B-lineage acute lymphoblastic leukemia: a comparative deletion and hypermethylation study. Cancer Genet Cytogenet 2009;195:59–65.
17. Iacobucci I, Storlazzi CT, Cilloni D, Lonetti A, Ottaviani E, Soverini S, et al. Identification and molecular characterization of recurrent genomic deletions on 7p12 in the IKZF1 gene in a large cohort of BCR-ABL1-positive acute lymphoblastic leukemia patients: on behalf of Gruppo Italiano Malattie Ematologiche dell'Adulto Acute Leukemia Working Party (GIMEMA AL WP). Blood 2009;114: 2159–67.
18. Storlazzi CT, Albano F, Dencic-Fekete M, Djordjevic V, Rocchi M. Late-appearing pseudocentric fission event during chronic myeloid leukemia progression. Cancer Genet Cytogenet 2007;174:61–7.
19. Kaplan E, Meier P. Non parametric estimation from incomplete observation. J Am Stat Assoc 1958;53:457–81.
20. Gooley TA, Leisenring W, Crowley J, Storer B. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. Stat Med 1999;18:695–706.
21. Simon R, Lee Y. Nonparametric confidence limits survival probabilities and median survival time. Cancer Treat Res 1982;66:37–42.
22. Cox D. Regression models and life-tables. J R Stat Soc 1972;34:187–220.
23. Drexler HG. Review of alterations of the cyclin-dependent kinase inhibitor INK4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells. Leukemia 1998;12:845–59.
24. Krug U, Ganser A, Koeffler HP. Tumor suppressor genes in normal and malignant hematopoiesis. Oncogene 2002;21:3475–95.
25. Wong IH, Ng MH, Huang DP, Lee JC. Aberrant p15 promoter methylation in adult and childhood acute leukemias of nearly all morphologic subtypes: potential prognostic implications. Blood 2000;95: 1942–9.
26. Chim CS, Tam CY, Liang R, Kwong YL. Methylation of p15 and p16 genes in adult acute leukemia: lack of prognostic significance. Cancer 2001;91:2222–9.
27. Iacobucci I, Lonetti A, Paoloni F, Papayannidis C, Ferrari A, Storlazzi CT, et al. The PAX5 gene is frequently rearranged in BCR-ABL1-positive acute lymphoblastic leukemia but is not associated with outcome. A report on behalf of the GIMEMA Acute Leukemia Working Party. Haematologica 2010;95:1683–90.
28. Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J, et al. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature 2008;453:110–4.
29. Martinelli G, Iacobucci I, Storlazzi CT, Vignetti M, Paoloni F, Cilloni D, et al. IKZF1 (Ikaros) deletions in BCR-ABL1-positive acute lymphoblastic leukemia are associated with short disease-free survival and high rate of cumulative incidence of relapse: a GIMEMA AL WP report. J Clin Oncol 2009;27:5202–7.
30. Bertin R, Acquaviva C, Mirebeau D, Guidal-Giroux C, Vilmer E, Cave H. CDKN2A, CDKN2B, and MTAP gene dosage permits precise characterization of mono- and bi-allelic 9p21 deletions in childhood acute lymphoblastic leukemia. Genes Chromosomes Cancer 2003;37: 44–57.
31. Lemos JA, Defavery R, Scrideli CA, Tone LG. Analysis of p16 gene mutations and deletions in childhood acute lymphoblastic leukemias. Sao Paulo Med J 2003;121:58–62.
32. Andrew S. Noncoding germline mutations in p16/CDKN2A in melanoma-prone families. Clin Genet 1999;56:188–90.
33. Wang SS, Cozen W, Severson RK, Hartge P, Cerhan JR, Davis S, et al. Cyclin D1 splice variant and risk for non-Hodgkin lymphoma. Hum Genet 2006;120:297–300.
34. Debniak T, Cybulski C, Gorski B, Huzarski T, Byrski T, Gronwald J, et al. CDKN2A-positive breast cancers in young women from Poland. Breast Cancer Res Treat 2007;103:355–9.
35. McCloud JM, Sivakumar R, Greenhough A, Elder J, Jones PW, Deakin M, et al. p16INK4a polymorphism: associations with tumour progression in patients with sporadic colorectal cancer. Int J Oncol 2004; 25:1447–52.
36. Zuchner S, Gilbert JR, Martin ER, Leon-Guerrero CR, Xu PT, Browning C, et al. Linkage and association study of late-onset Alzheimer disease families linked to 9p21.3. Ann Hum Genet 2008;72:725–31.
37. Kumar R, Smeds J, Berggren P, Straume O, Rozell BL, Akslen LA, et al. A single nucleotide polymorphism in the 3'untranslated region of the CDKN2A gene is common in sporadic primary melanomas but mutations in the CDKN2B, CDKN2C, CDK4 and p53 genes are rare. Int J Cancer 2001;95:388–93.
38. Williams RT, den Besten W, Sherr CJ. Cytokine-dependent imatinib resistance in mouse BCR-ABL+, Arf-null lymphoblastic leukemia. Genes Dev 2007;21:2283–7.
39. Notta F, Mullighan CG, Wang JC, Poeppl A, Doulatov S, Phillips LA, et al. Evolution of human BCR-ABL1 lymphoblastic leukaemia-initiating cells. Nature 2011;469:362–7.
40. Kees UR, Burton PR, Lu C, Baker DL. Homozygous deletion of the p16/MTS1 gene in pediatric acute lymphoblastic leukemia is associated with unfavorable clinical outcome. Blood 1997;89:4161–6.
41. Carter TL, Watt PM, Kumar R, Burton PR, Reaman GH, Sather HN, et al. Hemizygous p16(INK4A) deletion in pediatric acute lymphoblastic leukemia predicts independent risk of relapse. Blood 2001;97:572–4.
42. Fizzotti M, Cimino G, Pisegna S, Alimena G, Quartarone C, Mandelli F, et al. Detection of homozygous deletions of the cyclin-dependent kinase 4 inhibitor (p16) gene in acute lymphoblastic leukemia and association with adverse prognostic features. Blood 1995;85:2685–90.
43. Calero Moreno TM, Gustafsson G, Garwicz S, Grander D, Jonmundsson GK, Frost BM, et al. Deletion of the Ink4-locus (the p16ink4a, p14ARF and p15ink4b genes) predicts relapse in children with ALL treated according to the Nordic protocols NOPHO-86 and NOPHO-92. Leukemia 2002;16:2037–45.
44. Heyman M, Rasool O, Borgonovo Brandter L, Liu Y, Grander D, Soderhall S, et al. Prognostic importance of p15INK4B and p16INK4 gene inactivation in childhood acute lymphocytic leukemia. J Clin Oncol 1996;14:1512–20.
45. Heerema NA, Sather HN, Sensel MG, Liu-Mares W, Lange BJ, Bostrom BC, et al. Association of chromosome arm 9p

abnormalities with adverse risk in childhood acute lymphoblastic leukemia: A report from the Children's Cancer Group. Blood 1999;94:1537–44.
46. Mirebeau D, Acquaviva C, Suciu S, Bertin R, Dastugue N, Robert A, et al. The prognostic significance of CDKN2A, CDKN2B and MTAP inactivation in B-lineage acute lymphoblastic leukemia of childhood. Results of the EORTC studies 58881 and 58951. Haematologica 2006;91:881–5.
47. van Zutven LJ, van Drunen E, de Bont JM, Wattel MM, Den Boer ML, Pieters R, et al. CDKN2 deletions have no prognostic value in childhood precursor-B acute lymphoblastic leukaemia. Leukemia 2005; 19:1281–4.
48. Graf Einsiedel H, Taube T, Hartmann R, Wellmann S, Seifert G, Henze G, et al. Deletion analysis of p16(INKa) and p15(INKb) in relapsed childhood acute lymphoblastic leukemia. Blood 2002;99:4629–31.
49. Sulong S, Moorman AV, Irving JA, Strefford JC, Konn ZJ, Case MC, et al. A comprehensive analysis of the CDKN2A gene in childhood acute lymphoblastic leukemia reveals genomic deletion, copy number neutral loss of heterozygosity, and association with specific cytogenetic subgroups. Blood 2009;113:100–7.
50. Kurosu T, Wu N, Oshikawa G, Kagechika H, Miura O. Enhancement of imatinib-induced apoptosis of BCR/ABL-expressing cells by nutlin-3 through synergistic activation of the mitochondrial apoptotic pathway. Apoptosis 2010;15:608–20.