Brief report

# Deletion analysis of p16$^{INKa}$ and p15$^{INKb}$ in relapsed childhood acute lymphoblastic leukemia

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**This study aimed at determining the prevalence of *INK4* deletions and their impact on outcome in 125 children with acute lymphoblastic leukemia (ALL) at first relapse using real-time quantitative polymerase chain reaction. Patients were enrolled into relapse trials ALL-REZ BFM (ALL-Relapse Berlin–Frankfurt–Münster) 90 and 96. The prevalence of p16$^{INK4a}$ and p15$^{INK4b}$ homozygous deletions was 35% (44 of 125) and 30% (38 of 125), respectively. A highly significant association of both gene deletions was found with the 2 major adverse prognostic factors known for relapsed childhood ALL: T-cell immunophenotype and first remission duration. There was no correlation between *INK4* deletions and probability of event-free survival. These findings argue against an independent prognostic role of *INK4* deletions in relapsed childhood ALL. (Blood. 2002;99:4629-4631)**



## Introduction

Homozygous deletions of the tumor suppressor genes p16$^{INK4a}$ and p15$^{INK4b}$ are found in approximately 25% of childhood acute lymphoblastic leukemia (ALL) at first presentation (5% to 20% in B-cell precursor and 60% to 80% in T-cell ALL). The prognostic importance of these alterations is controversial. Whereas an adverse prognostic effect of p16$^{INK4a}$ and p15$^{INK4b}$ deletions has been found in some studies on pediatric[1,2] and adult ALL,[3] others studying a larger number of patients were not able to confirm these findings.[4,5] However, the coincidence of these deletions with T-cell immunophenotype and high white blood cell (WBC) count is unequivocal.[6,7] The prevalence of *INK4* deletions in relapsed ALL and their prognostic importance have not been determined yet.

We investigated bone marrow (BM) samples from 125 children with first relapse of ALL enrolled into relapse trials ALL-REZ BFM (ALL-Relapse Berlin–Frankfurt–Münster) 90/96 for *INK4* deletions to compare the prevalence with that in initial disease and to elucidate their possible prognostic importance in ALL relapse.

## Study design

### Patients and treatment

BM samples (containing more than 75% BM blasts) collected prior to chemotherapy at first relapse of ALL from 125 children enrolled into trials ALL-REZ BFM 90 and 96[8] were analyzed retrospectively. The studied patients are representative of the overall population enrolled in these trials with regard to sex, time of relapse, blast count, and immunophenotype. Treatment consisted of chemotherapy and central nervous system irradiation; bone marrow transplantation was performed when indicated. BM samples were obtained after informed and written consent. Median follow-up was 894 days (range, 176-2058 days).

### DNA extraction and real-time quantitative polymerase chain reaction

Preparation of cell samples and DNA extraction were performed as described previously.[9]

We developed a gene-dosage assay using TaqMan real-time quantitative (rq-) polymerase chain reaction (PCR), with p16$^{INK4a}$ and p15$^{INK4b}$ as test sequences (TS) and *β-globin* as a reference gene (RG). The normalized ratio of TS/RG yields a value of approximately 1 in samples without the deletion, whereas ratios with values close to 0 indicate homozygous deletion.[10,11] Rq-PCR reactions were carried out in separate tubes with specific primers and dually labeled TaqMan probes for each sequence. Serial dilutions of genomic DNA with known concentrations were amplified simultaneously to generate a calibration curve. Samples were analyzed in duplicate (TS and RG), and diagnosis was made only when concordant results were achieved in 3 independent runs. The cutoff value between homozygous deletion and absence of homozygous deletion was set at 0.3 (contaminating normal cells up to 25% + 5% variation). Because of considerations discussed in detail previously,[12] values greater than 0.3 were interpreted as absence of homozygous deletions, and hemizygous deletions were not assessed. The assay was validated using serial dilutions of cell line Jurkat DNA (*INK4*$^{-/-}$) in the background of normal DNA, as described by Carter et al.[13] The correlation coefficient for the linear function obtained in these experiments was 0.97, demonstrating the capability of the assay to measure normal cell contamination. Oligonucleotides and TaqMan probes used were as follows: p16$^{INK4a}$: p16se 5′-GGGGCACCAGAGGCAGTA, p16as 5′-GGGCTGAACTTTCTGTGCTG, E2 TaqMan: 5′-FAM-ATGCC-CGCATAGATGCCGCGGAAG-TAMRA; p15$^{INK4b}$: 15F 5′-AGCCGC-CCACAACGACTT, 15R 5′-CGTTGGCAGCCTTCATCGAA, 15TM 5′-FAM-TACCCAATTTCCCACCCCCACCCACC-TAMRA; *β-globin:* PCO3: 5′-CTGACACAACTGTGTTCACTAGC, KM38: 5′-TATTG-GTCTCCTTAAACCTGTCTTG, TMglobin03: 5′-FAM-TCCTGAG-GAGAAGTCTGCCGTTAC-TAMRA (TIB Molbiol, Berlin, Germany).

Reactions were performed in a final volume of 50 μL with 50 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 200 μM each deoxyribonucleotide triphosphate (GIBCO BRL, Life Technologies, Karlsruhe, Germany), 0.5 U Platinum-Taq (GIBCO BRL), 0.2 μM

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each oligonucleotide, and 0.1 μM each TaqMan probe (both TIB Molbiol). PCR was performed as follows: An initial denaturation step of 10 minutes at 97°C was followed by 5 cycles at 97°C for 60 seconds and 65°C for 60 seconds; then 35 cycles at 95°C for 20 seconds and 65°C for 45 seconds. Fluorescence was detected at the end of the 65°C step.

## Results and discussion

A homozygous deletion of p16$^{INK4a}$ and p15$^{INK4b}$ was found in 44 (35%) and 38 (30%) of the 125 samples, respectively (Table 1). Six samples showed an isolated deletion of p16$^{INK4a}$, and one sample had only a p15$^{INK4b}$ deletion. The homozygous deletion frequencies of p16$^{INK4a}$ and p15$^{INK4b}$ were 30% and 25% in 109 B-cell precursor (BCP) ALL samples, respectively, and 73% for both in 15 T-cell ALL samples ($P = .001$). The median first-remission duration was significantly shorter ($P < .01$) in the groups with p16$^{INK4a}$ and p15$^{INK4b}$ deletions (773 and 739 days, respectively) than in those without deletions (both cohorts 1108 days) (Table 1). Furthermore, we observed a significant association between homozygous p16$^{INK4a}$ deletion and higher peripheral blast cell and WBC counts (median 2410 blasts/μL for patients with p16$^{INK4a}$ deletion and 440 blasts/μL for those without this alteration; $P = .004$). Patients with p15$^{INK4b}$ deletion had median peripheral blast counts of 1700 blasts/μL, compared with 500 blasts/μL in patients without the deletion (not significant; Table 1).

The probability of event-free survival (pEFS) at 5 years for children with p16$^{INK4a}$ or p15$^{INK4b}$ deleted leukemic blasts was not significantly different from that in children without these alterations (.46 ± .08 and .39 ± .08 versus .43 ± .07 and .46 ± .07, respectively; Figure 1A,B).

The deletion prevalences in relapsed childhood ALL were 35% for p16$^{INK4a}$ and 30% for p15$^{INK4b}$, slightly higher than those reported for initial ALL. In this disease, the reports on deletion prevalences vary considerably, especially with regard to BCP ALL. The *INK4* deletion prevalences in initial BCP ALL reported in 9 different studies ranged from 10% to 20%, and the respective values for T-cell ALL ranged from 60% to 80%.[1,4,5,7,14-18] The higher *INK4* deletion prevalence in relapsed ALL could be interpreted as an indication of an adverse prognostic role for this alteration in initial ALL; alternatively, some deletions could have been acquired in the interval between initial and relapse diagnosis.

T-cell immunophenotype and high WBC count are known to correlate with *INK4* deletion in initial childhood ALL.[1,2,4,5] We also observed this correlation in our cohort of patients; additionally, we found a significant association of *INK4* deletion and the major adverse prognostic parameter known for ALL relapse, a short first-remission duration.[19] Interestingly, despite this correlation, no significant difference in pEFS was found between the group with *INK4* deletion and those without, a fact that renders a prognostic role of *INK4* deletions in relapsed childhood ALL unlikely.

**Table 1. Clinical characteristics of children with first ALL relapse presenting with or without *INK4* deletion**

| Parameter | Patients | Number of patients with deletion (%) | | | |
|---|---|---|---|---|---|
| | | p15$^{INK4b}$ | *P* | p16$^{INK4a}$ | *P* |
| Total number | 125 | 38 (30) | | 44 (35) | |
| Sex | | | | | |
| Female | 40 | 15 (38) | | 17 (43) | |
| Male | 85 | 23 (27) | n.s. | 27 (32) | n.s. |
| Immunophenotype | | | | | |
| T cell | 15 | 11 (73) | | 11 (73) | |
| BCP | 109 | 27 (25) | .000 | 33 (30) | .001 |
| First-remission duration, d* | | 739/1108 | .000 | 773/1108 | .003 |
| Peripheral blast count, 1000/μL* | | 1.7/0.5 | n.s. | 2.41/0.44 | .004 |

n.s. indicates not significant; BCP, B-cell precursor.
*Data are presented as median with deletion/median without deletion.

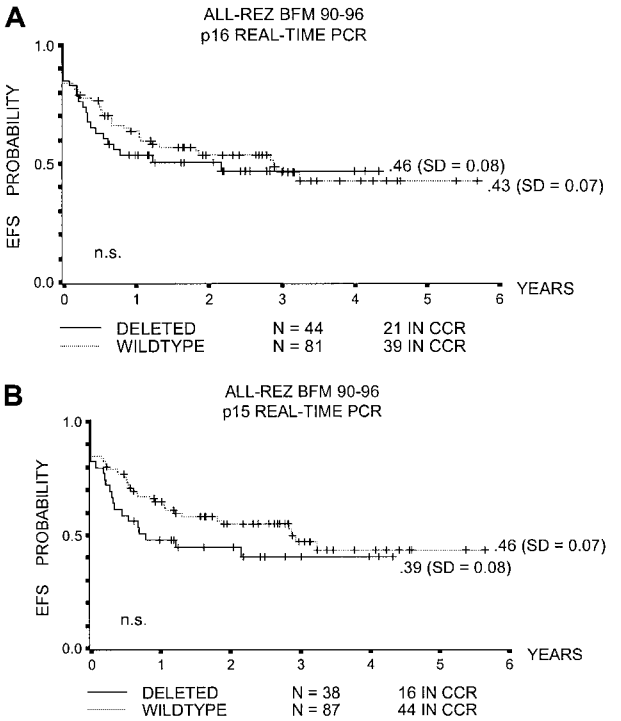


**Figure 1. Event-free survival with p16$^{INK4a}$ and p15$^{INK4b}$ deletion.** (A) pEFS and p16$^{INK4a}$ deletion. (B) pEFS and p15$^{INK4b}$ deletion. CCR indicates complete continuous remission; n.s., not significant.

Our data on relapsed patients provide a new possible explanation for the discordant results seen in initial ALL: Patients with *INK4* deletion tend to relapse approximately 1 year earlier (median first-remission duration approximately 2.1 years versus approximately 3 years). Accordingly, the follow-up time in some studies could be too short, leading to a bias toward more adverse events in the group of patients with *INK4* deletion. The median follow-up time in 2 studies reporting a correlation between *INK4* deletion and adverse outcome was 3.25 years in one (Heyman et al[2]) and was not provided in the other.[1] In contrast, the study of Rubnitz et al[5] examining the largest number of patients to date (n = 155) had a follow-up of more than 5 years in both subgroups and found no prognostic effect.

Carter et al[13] reported that hemizygous p16$^{INK4a}$ deletions correlated with adverse outcome in childhood ALL. Because of methodologic and theoretical considerations discussed in detail elsewhere,[12] we did not assess hemizygous deletions of the *INK4* genes in our study. Theoretically, this could veil an adverse prognostic impact of *INK4* deletions because the potentially present hemizygous deletions would appear in the group with absence of homozygous deletions. However, in contrast to Carter et al,[13] Heyman et al[2] found no difference in pEFS between patients with hemizygous deletion and those with wild-type *INK4* genes. Nevertheless, a validation of our and other's data with an appropriate method (ie, fluorescence in situ hybridization) would be worthwhile.

It remains the task of further studies to examine alternative mechanisms of *INK4* tumor suppressor gene inactivation by appropriate methods (ie, immunohistochemistry or methylation-specific PCR) to clarify their prognostic significance.

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