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

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Background and Objectives. Deletion and methylation of the 9p21 chromosomal region are frequent in childhood acute lymphoblastic leukemia (ALL) but the prognostic significance is controversial. They inactivate *CDKN2A*, a gene encoding both $p16^{INK4a}$ and $p14^{ARF}$ and, in some cases, contiguous genes that may influence chemosensitivity, such as *CDKN2B* encoding p15INK4b or *MTAP* encoding methylthioadenosine phosphorylase.

Design and Methods. *CDKN2A* inactivation by deletion or methylation was studied using gene dosage and methyl-specific polymerase chain reaction.

Results. Bi-allelic and mono-allelic inactivation were found in, respectively, 38 (17%) and 31 (14%) of 227 children with B-lineage ALL enrolled in EORTC trials. Although *CDKN2A* inactivation was more often associated with poor prognostic features in B-lineage ALL, it failed to influence the outcome of the patients significantly. Bi-allelic *CDKN2B* and *MTAP* co-inactivation were found in 36 (16%) and 24 (11%) of patients, respectively, and did not influence the 6-year event-free survival rate either, even when the analysis was restricted to *CDKN2A* inactivated ALL.

Interpretion and Conclusions. In this study of 227 cases of childhood B-lineage ALL, inactivation of *CDKN2A, CDKN2B* and *MTAP* did not influences the patients' outcome.

Key words: acute lymphoblastic leukemia, 9p21, *CDKN2A*, *CDKN2B*, *MTAP*.

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Deletions of the 9p21 chromosomal region are frequent in childhood acute lymphoblastic leukemia (ALL) and encompass *CDKN2A* (*MTS1*), a gene encoding both $p16^{INK4a}$ and $p14^{ARF}$. $p16^{INK4a}$ and $p14^{ARF}$ transcripts have different promoters and first exons (exons 1α and exon 1β respectively) but share exons 2 and 3 in alternative reading frames.[1] They encode two proteins which are potent regulators of the cell cycle. $p16^{INK4a}$, an inhibitor of cyclin-dependent kinase, inhibits Rb phosphorylation, whereas $p14^{ARF}$ activates TP53 via interaction with the MDM2 protein.[1] Hypermethylation of promoter has been shown to be an alternate way of inactivation for these proteins in a variety of malignancies, including ALL.[2,3] The incidence of 9p21 alterations suggests that inactivation of $p16^{INK4a}$ and $p14^{ARF}$ is relevant not only in T-ALL leukemogenesis but also in B-precursor ALL.[4,5] Despite their high frequency, the prognostic importance of 9p21 alterations is still controversial in ALL and has been reported to be either unfavorable or similar to that of other patients.[5-18] However, most of the series published so far are small, retrospective, and use techniques that did not enable small and/or mono-allelic deletions to be detected. Moreover, T and B-lineage ALL were usually mixed for analysis. This latter point may be of particular importance since 9p21 deletions occur at a very high frequency in T-ALL, which account for approximately 15% of childhood ALL but are often associated with unfavorable features and a shorter event-free survival.

An important level of complexity for prognostic assessment comes from the heterogeneity of 9p21 alterations. Mono- and/or bi-allelic deletions of various size are found, which can be associated in some patients with gene inactivation by hypermethylation of gene promoters. Using a gene dosage assay, we previously demonstrated that while *CDKN2A* is always involved in 9p21 deletions, the extent of the deletions is heterogeneous, leading to variable co-inactivation of contiguous genes such as *CDKN2B* (*MTS2*) encoding $p16^{INK4b}$ and *MTAP* encoding methyl-thioadenosine phosphorylase.[4] The co-deletion of these genes might influence chemosensitivity. In particular, MTAP is involved in purine and methionine salvage metabolism. *In vitro* experiments have shown that its loss makes cancer cells more sensitive to drugs that interfere with purine metabolism such as methotrexate, which is used in all treatment protocols for childhood ALL.[19-22]

We tried to refine the prognosis evaluation of childhood B-lineage ALL with 9p21 alterations and to determine whether co-inactivation of contiguous genes influence this prognosis. We were particularly interested in investigating the possible positive influence of *MTAP* co-inactivation on the prognosis of ALL with *CDKN2* inactivation. For this purpose, we performed loss of heterozygosity screen-

Table 1. Characteristics of patients according to *CDKN2A* status.

	Total population studied	*CDKN2A subgroup* Bi-allelic deletion	Mono-allelic deletion	Normal
Variable/Total (%)	227 (100%)	38 (100%)	31 (100%)	158 (100%)
Sex				
Male	129 (57%)	20 (53%)	17 (55%)	92 (58%)
Female	98 (43%)	18 (47%)	14 (45%)	66 (42%)
Age at diagnosis (yrs.)				
<1	7 (3%)	0 (0%)	1 (3%)	6 (4%)
1-9	190 (84%)	32 (84%)	24 (77%)	134 (85%)
≥10	30 (13%)	6 (16%)	6 (19%)	18 (11%)
Median	4	4	4	3
Range	0-16	1-15	0-16	0-16
WBC ($\times 10^9$/L)				
< 50	178 (78%)	26 (68%)	20 (65%)	132 (84%)
≥50	49 (22%)	12 (32%)	11 (35%)	26 (16%)
Median	13.4	32.7	31.6	11.5
Range	0.9–582	1.1-582	2.2-369	0.9–495
NCI risk group				
Standard	153 (67%)	22 (58%)	16 (52%)	115 (73%)
High	74 (32%)	16 (42%)	15 (48%)	43 (27%)
Immunophenotype¶				
B-lineage	63 (28%)	9 (24%)	8 (26%)	46 (29%)
Pre-preB	14 [8%]	1 [3%]	2 [9%]	11 [10%]
cALL (CD10$^+$)	106 [65%]	21 [72%]	16 [70%]	69 [62%]
PreB (ICμ$^+$)	41 [25%]	7 [24%]	5 [22%]	29 [26%]
Mature B (sIG$^+$)	3 [2%]	0 [0%]	0 [0%]	3 [3%]
Cytogenetics				
ND-failure	35 (15%)	8 (21%)	9 (29%)	18 (11%)
Hyperdiploidy >50 chr.	62 [32%]	2 [7%]	6 [27%]	54 [38%]
t(9;22)	4 [2%]	1 [3%]	2 [9%]	1 [<1%]
MLL/11q23$^+$	3 [2%]	0 [0%]	2 [9%]	1 [<1%]
t(1;19)	10 [5%]	1 [3%]	1 [5%]	8 [6%]
Normal or other abnormalities	113 [59%]	26 [87%]	11 [50%]	74 [53%]
t(12;21)				
ND-failure	45 (20%)	4 (11%)	7 (23%)	34 (22%)
No	139 [76%]	24 [70%]	19 [79%]	96 [77%]
Yes	43 [24%]	10 [30%]	5 [21%]	28 [23%]
CDKN2B				
Bi-allelic deletion	27 (12%)	25 (66%)	2 (6%)	0 (0%)
Mono-allelic deletion and promoter methylation	9 (4%)	3 (8%)	6 (19%)	0 (0%)
Mono-allelic deletion	15 (7%)	3 (8%)	12 (39%)	0 (0%)
Normal	176 (78%)	7 (18%)	11 (36%)	158 (100%)
MTAP				
Bi-allelic deletion	24 (11%)	24 (63%)	0 (0%)	0 (0%)
Mono-allelic deletion	28 (12%)	10 (26%)	18 (58%)	0 (0%)
Normal	175 (77%)	4 (11%)	13 (42%)	158 (100%)

¶*Percentages between [ ] were calculated taking into account only documented cases. cALL: common ALL; ICμ: intra-cytoplasmic μ chains; sIG: surface immunoglobulins; ND: not done.*

ing and gene dosage in order to reliably discriminate between mono-and bi-allelic deletions and to delineate precisely the extent of the deletion. We also determined DNA methylation patterns in the CpG island of *CDKN2A* and *CDKN2B* by methylation-specific polymerase chain reaction (PCR).

Design and Methods

Patients

We studied 227 children (aged 2 months to 17 years) consecutively diagnosed as having a B-lineage ALL. All patients were treated according to the protocols of the EORTC 58881 (n=200) or 58951 (n=27) between August 1989 and June 2001.[23] In the EORTC 58881 study, patients were randomized to receive either *E. coli* asparaginase or erwiniase and erwiniase was associated with a poorer outcome. At the end of the 58881 trial and in the 58951 trial, only *E. coli* asparaginase was administered. The majority of patients included in our studies (82%) received *E. coli* asparaginase. Informed consent was provided according to the Declaration of Helsinki. The main characteristics of the patients studied are reported in Table 1. The median follow-up was 6 years. The overall 6-year event-free survival rate was 74% and the 6-year overall survival rate was 85%.

Bone marrow was collected for molecular analyses before induction therapy and at the time of complete remission. The diagnosis of B-lineage ALL was based on the expression of B-cell-associated antigens (CD19$^+$, CD22$^\pm$, CD10$^\pm$, ICμ$^\pm$, sIG$^\pm$). Karyotypes, determined systematically at diagnosis, were centrally reviewed. The presence of a *TEL-AML1* fusion was systematically screened for by reverse transcriptase PCR or fluorescence *in situ* hybridization analysis.

9p21 deletions: detection and delineation

9p21 deletions were studied using both loss of heterozygosity screening, and a gene dosage assay based on real-time PCR. Loss of heterozygosity screening was performed as previously described by PCR amplification of a panel of microsatellites spanning the 9p21 chromosomal region (from centromere to telomere: *D9S265*; D9S171, D9S958, D9S1604, D9S1748, D9S942, INFA).[4,24] For each patient, allelic patterns obtained for the tumor sample were compared with those obtained during complete remission. Allelic losses were scored as previously described.[4] The gene dosage assay, its validation and comparison with loss of heterozygosity data have been described elsewhere.[4] Five targets were amplified on 9p21: *CDKN2B*-exon 1 (e1), *CDKN2A*-e1β, e1α, and e3, and *MTAP*-e8. Two sequences were used as reference: *59KB* (8q11), and *AK1* (9q23). Real-time PCR was performed using the SYBR Green I dye and an ABI PRISM 7700 Sequence detector system (Applied Biosystems, Foster City, CA, USA). The ratio of the value obtained for each target to the reference sequence value was calculated and adjusted to the percentage of blasts as described before.[4] This ratio was close to 1 if no deletion was present, and to 0.5 or 0 in the case of mono- or bi-allelic deletion, respectively.

Methylation-specific PCR

Methylation-specific PCR was performed in patients displaying mono-allelic deletions. After bisulphite modification of the DNA according to Herman *et al.*,[25] DNA (1 μg) was treated with bisulphite, purified using Wizard DNA clean-up purification resin (Promega, Madison, WI, USA) and eluted into 50 μL of water. DNA methylation patterns of *CDKN2A*-e1α, *CDKN2A*-e1β, and *CDKN2B*

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promoters were then determined using two sets of primers for each gene, one amplifying methylated DNA and one amplifying unmethylated DNA.[3,25] Blood from a healthy donor was used as an unmethylated control, the Raji cell line as a methylated control for *CDKN2B* and *CDKN2A*-e1α, and the KG-1 cell line as methylated control for *CDKN2A*-eβ.

Statistical analysis

Event-free survival was calculated from the date of complete remission to the date of first relapse or death. For patients who failed to reach complete remission by the end of induction-consolidation, the failure was considered as an event at time 0. All patients alive and in first complete remission were censored at their last follow-up. Actuarial event-free survival curves were calculated according to the Kaplan-Meier technique.[26] The standard errors (SE) of the estimates were computed using the Greenwood formula.[26] The two-tailed log-rank test was used to compare the differences between event-free survival curves.[26] All analyses followed the intent-to-treat principle. The relationship between two categorical variables was statistically tested using the χ^2 test.

Results

Leukemia cells from 227 children with a B-lineage ALL were studied. *CDKN2A* bi-allelic and mono-allelic deletion was found in 38 (17%) and 31 (14%) patients respectively. These data are in keeping with a recent report on B-lineage ALL5. Promoter methylation of either *CDKN2A*-e1α/$p16^{INK4A}$ or e1β/$p14^{ARF}$ was observed in only 4/31 (6%) of ALL with *CDKN2A* mono-allelic deletion. Methylation of both $p16^{INK4A}$ and $p14^{ARF}$ promoters was not observed. Since point mutations are very unfrequent in ALL,[24] we can assume that virtually all patients with *CDKN2A* inactivation were detected using our approach. Patients with a *CDKN2A* inactivation (bi- or mono-allelic) did not differ from other (*normal*) patients regarding age, sex, immunophenotype and response to prephase treatment, but they had a higher incidence of WBC count $>50\times10^9$/L at diagnosis (p=0.02) and, therefore, belonged more often (p=0.03) to the NCI high-risk group (Table 1). The incidence of hyperdiploidy with more than 50 chromosomes was significantly (p=0.002) lower in patients with *CDKN2A* inactivation, bi-allelic (7%) or mono-allelic (27%), than in *normal* patients (38%) (Table 1). The presence of a *TEL-AML1* fusion was quite well balanced in the three *CDKN2A* inactivation groups. The lack of exclusion between both abnormalities suggests that *TEL-AML1* and *CDKN2A* inactivation can functionally co-operate. This is consistent with a study showing that, compared to *TEL-AML1* mice, *TEL-AML1/CDKN2A* inactivated mice develop leukemia more rapidly and at a higher incidence.[27] It has been shown that $p14^{ARF}$ is a transcriptional target of AML1 and that TEL-AML1 is a dominant inhibitor of AML1.[28,29] In this respect, $p14^{ARF}$ expression should be repressed in TEL-AML1-positive ALL. The simultaneous presence of 9p21 deletion and t(12;21) in a subset of ALL suggests that $p14^{ARF}$ inhibition is not sufficient for leukemogenesis and that $p16^{INK4A}$ inhibition also plays an important role. Another possibility is that that *TEL-AML1* is less potent than AML1-ETO in repressing AML1-mediated transcription of $p14^{ARF}$.

Although some studies reported an absence of 9p21 deletion in ALL with a t(1;19) and/or expression of the *E2A-PBX1* associated fusion transcript, *CDKN2A* deletion was present in two of our ten patients with a t(1;19) (Table 1). This apparent discrepancy may be due to the small number of patients in each series and to the fact that only homozygous deletions were investigated in previous studies.[30,31] Since $p14^{ARF}$ is down-regulated by E2A-PBX1,[32] the finding of *CDKN2A* deletions in these patients suggests here again that the additional loss of $p16^{INK4}$ provides a selective advantage to leukemia cells. These observations reinforce the assumption that $p14^{ARF}$ and $p16^{INK4A}$ are non redundant tumor suppressor genes in ALL. This may be one of the reasons why deletional events, which result in the concomitant inactivation of both genes, are preferentially found in this disease.

At the time of analysis, the median follow-up was 6 years (range 2-12.5 years), a total of 55 events had been reported, and the overall 6-year event-free survival rate was 74%. The 6-year event-free survival rates (SE%) of patients with bi-allelic, mono-allelic and no *CDKN2A* inactivation were 68% (6%), 80% (5%), and 75% (3%) respectively, and did not differ significantly (Figure 1). Moreover *CDKN2A* status had no impact on the type of relapse or on the overall survival (Table 2).

When present, *CDKN2B* and *MTAP* deletions were always associated with a *CDKN2A* deletion (Table 1). A significant proportion (9/24, 38%) of cases with *CDKN2B* mono-allelic deletion showed *CDKN2B* promoter methylation, leading to a total inactivation of the gene in 36 (16%) patients. The 6-year event-free survival rate (SE%) of these patients was similar to that of normal patients: 71% (6%) versus 75% (3%) (Figure 1). *MTAP* bi-allelic inactivation was found in 24 (11%) of patients. A role as a tumor suppressor gene has been proposed for *MTAP* in some types of cancer.[33,34] The absence of isolated *MTAP* deletion argues against such a role in ALL. It has also been suggested that *MTAP* inactivation in tumor cells could be important for treatment sensitivity because the action of drugs inhibiting *de novo* purine synthesis, such as methotrexate, could be enhanced in cells lacking the salvage pathways. Indeed, *MTAP*-deficient cell lines are more sensitive to methionine deprivation[19] and to methotrexate[20,21] than their *MTAP*-positive counterparts. Although MTAP-deficient cells are very sensitive *in vitro* to methotrexate, neither the response to a 8-day prephase treatment including methotrexate nor the 6-year event-free survival rate (70% versus 75%) differed significantly (p=0.50) from that of MTAP-positive patients. Similar results were obtained when analyses were restricted to the more homogeneous group of patients with *CDKN2A* inactivated ALL (Figure 1). Furthermore, the occurrence of relapses, central nervous system relapses in particular, which are known to be efficiently prevented by high doses of methotrexate, was not clearly decreased in patients with *MTAP* inactivation as compared to in those without *MTAP* inactivation (*data not shown*). Although we did not find any positive effect of *MTAP* inactivation on the outcome of patients, we cannot exclude that such an effect exists in

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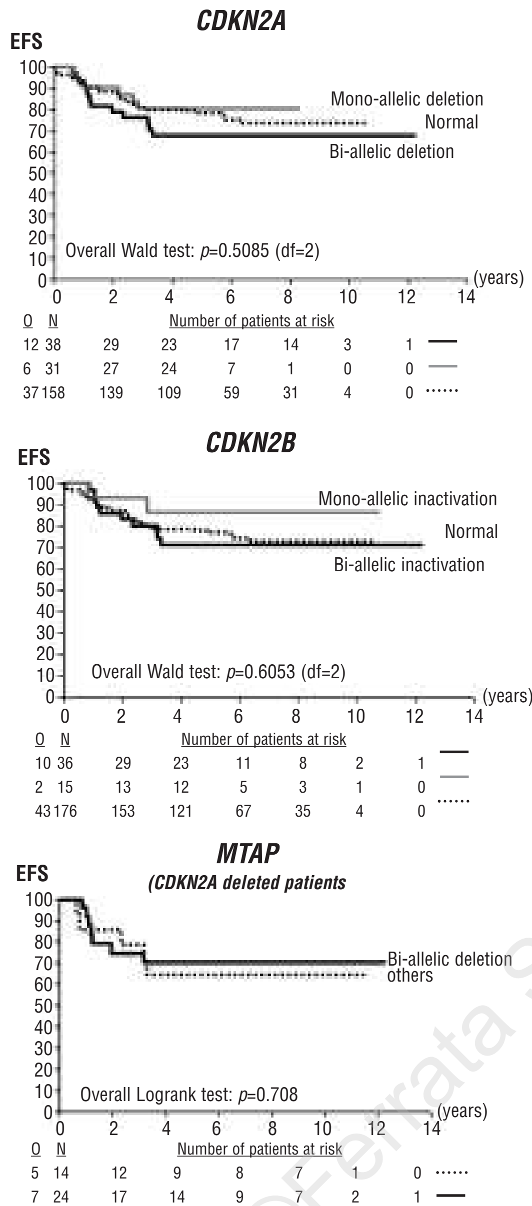

Figure 1. Kaplan-Meier estimates of event-free survival according to the status of *CDKN2A*, *CDKN2B*, and *MTAP* (restricted to the group of patients with a *CDKN2A* alteration). N=number of patients; O=observed number of events; the *p* value was determined from the overall logrank test.

patients treated with other treatment protocols with different dosages or modalities of methotrexate administration. However, the metabolic influence of MTAP on leukemia may be much more complex *in vivo* than it is *in vitro*.[35] In any case, *MTAP* deletion may provide an interesting new target for therapy in the near future.[36] L-alanosine is one of these selective therapies and its use may be proposed in MTAP-deficient B-lineage ALL.[37]

Discussion

In conclusion, although bi-allelic *CDKN2A* inactivation was more often associated with bad prognostic features, it failed to significantly influence the outcome of the patients, in agreement with the only other study focused on B-lineage ALL.[5] 9p21 alterations have been associated with a poor prognosis in some studies,[8,10-12,14,18] including one with EORTC-treated patients,[18] but not in others.[6,7,9,17,19] The importance of a prognostic factor is closely linked to the treatment received. When treatment results are better, it is normal that a prognostic factor loses its importance. Even within a group, treatment of patients may differ with time or according to the randomization performed. In the EORTC 58881 study, patients were randomized to receive *E. coli* asparaginase or erwiniase and erwiniase was associated with a poorer outcome.[38] However, patients analyzed in the previous EORTC single center study[18] and in our series were included in the same two trials (58881 and 58951), and there is a large overlap between recruitment periods. This makes it very unlikely that the difference in asparaginase accounted for the difference in prognostic relevance of 9p21 alterations between the two studies. Although differences in treatment regimen could account for some discrepant results in the literature, small cohorts of retrospectively selected patients, technical differences, mixed analysis of B-ALL with T-ALL in which the frequency of *CDKN2A* alterations is at least 3 times higher, and, perhaps preferential publication of positive results, may have induced a bias in prognostic evaluation. The large size of our study, and its agreement with the results of the largest series of B-lineage ALL reported before,[5] suggest that the poor prognosis associated with 9p21 alteration, if any, would be weak in B-lineage ALL and mainly due to a preferential association with classical unfavorable parameters. In contrast to *in vitro* studies, we did not observe any influence of the co-inactivation of *CDKN2B* and, more surprisingly, of *MTAP* on the outcome.

Table 2. Clinical outcome according to *CDKN2A* status.

	Total population studied	*CDKN2A subgroup*		
		Bi-allelic deletion	*Mono-allelic deletion*	*Normal*
Response to prephase*				
<1000 blasts/μL	198 (87%)	35 (92%)	23 (74%)	140 (89%)
≥1000 blasts/μL	29 (13%)	3 (8%)	8 (26%)	18 (11%)
Type of event				
No event (i.e. in CCR)	173 (76%)	26 (68%)	25 (81%)	122 (77%)
No CR	4 (2%)	0 (0%)	0 (0%)	4 (3%)
BM relapse only	27 (12%)	4 (10%)	2 (6%)	21 (13%)
CNS relapse only	9 (4%)	3 (8%)	1 (3%)	5 (3%)
Other isolated relapse	1 (<1%)	0 (0%)	0 (0%)	1 (<1%)
Combined relapse	11 (5%)	4 (10%)	2 (6%)	5 (3%)
Death in CR	3 (1%)	1 (3%)	1 (3%)	1 (<1%)
Survival status				
Alive	196 (86%)	31 (82%)	28 (90%)	137 (87%)
Dead	31 (14%)	7 (18%)	3 (10%)	21 (13%)

**Response to prephase treatment was evaluated by the number of peripheral leukemia cells persisting after an 8-day treatment course of corticoids and methotrexate. CR: complete remission; CCR: continuous complete remission; BM: bone marrow; CNS: central nervous system.*

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DM, CA, RB are students who set up the biological assays and performed the analyses (gene dosage and MS-PCR); DM also contributed to the analysis of data and the writing of the manuscript; SS is the statistician who analyzed the data and contributed

to writing the manuscript; ND is in charge of the cytogenetic review of all patients enrolled in EORTC protocols; AR, PB, FM, EP and EV were physicians at the clinical centers participating in the study; JO is the coordinator of the EORTC 58881 and 58951 trials and contributed to writing the manuscript; HC is the coordinator of the present study (supervision of students' work, analysis of data and writing of the manuscript).

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