Brief report

p16INK4a immunocytochemical analysis is an independent prognostic factor in childhood acute lymphoblastic leukemia

Jean Hughes Dalle, Martine Fournier, Brigitte Nelken, Françoise Mazingue, Jean-Luc Laï, Francis Bauters, Pierre Fenaux, and Bruno Quesnel

We investigated the prognostic value of p16INK4a immunocytochemistry (ICC) analysis in 126 cases of newly diagnosed childhood acute lymphoblastic leukemia (ALL). The incidence of negative p16INK4a ICC was 38.1% and was more frequent in T-lineage ALL. The overall survival (OS) and event-free survival (EFS) of patients with positive p16INK4a ICC was 38.1% and was more frequent in T-lineage ALL. The median observation time for all patients was 4.8 years (range, 1.4-9.2 years). There were 31 other patients studied by ICC in limited cohort of adult ALL patients. The technique of p16INK4a ICC requires only bone marrow or blood smears; many samples can be processed in the same day, and this technique allows direct identification of leukemic cells, leading to an easier interpretation. We observed that negative p16INK4a ICC conferred an adverse outcome in adult ALL with standard-risk karyotype, but the small number of samples did not allow us to perform multivariate analysis. The low rate of high-risk karyotypic abnormalities in childhood ALL allowed us to expect a specific prognostic value of p16INK4a ICC in childhood ALL.

In a large, homogenously treated cohort of childhood ALL patients, we investigated the influence of negative p16INK4a ICC on overall survival (OS) and event-free survival (EFS) in univariate and multivariate analysis. Additionally, we sequentially investigated p16INK4a ICC in a cohort of those patients at relapse.

Study design
We analyzed p16INK4a gene expression by p16INK4a ICC in 126 childhood ALLs (89 B-precursor ALLs, 28 T-ALLs, and 9 acute undifferentiated leukemias). Patient bone marrow (n = 87) or blood (n = 39) smears were collected from May 1992 to December 2000. Median age was 4.9 years (range, 0.5-15). There were 73 males and 53 females. Of these patients, 80 were treated according to the European Organization for Research and Treatment of Cancer (EORTC) protocol 58881 between 1991 and December 1998, and 46 were treated by EORTC protocol 58951 between January 1998 and December 2000. The median observation time for all patients was 4.8 years (range, 1.4-9.2 years). There were 31 other patients studied by p16INK4a ICC in first relapse. Among them, 20 were also studied sequentially (14 at diagnosis and first relapse, 6 in first and second or third relapse).

Immunocytochemical detection of p16INK4a protein was performed with the immunoglobulin G1 mouse monoclonal antibody antihuman p16INK4a (clone DCS-50.1/H4) (Oncogene Research Products, Cambridge, United Kingdom), as previously described.12 The ICC reaction was performed with avidin-biotin-peroxidase technique by means of Vector reagents (Vector Laboratories, Burlingame, CA). Positive cells appeared with brownish granules. Samples were considered ICC-positive when more than 5% of cells showed p16INK4a protein, according to our previous study in adult ALL.13 The chi-square and Fisher exact test were used for comparison between initial parameters. OS and EFS were estimated according to the Kaplan-Meier method. Events were defined as induction death, relapse, and death in complete remission (CR). Multivariate analysis was based on the Cox proportional hazards regression model. Statistical analyses were performed on SPSS 9.1 analysis software (SPSS, Chicago, IL).

Results and discussion
As previously observed in adult ALL, a great variation in the percentage of p16INK4a ICC-positive cells was seen (median, 20%; range, 0-100).13 We found 51 samples (38.1%) to be p16INK4a ICC-negative. All patients achieved CR. No difference for sex, white blood cell count, presence of a bulky mass, central nervous system disease, hemoglobin level, chromosome 9p abnormalities,
or karyotype could be observed between p16\(^{INK4a}\) ICC–positive and p16\(^{INK4a}\) ICC–negative cases. However, positive p16\(^{INK4a}\) ICC was found significantly more frequently in B-precursor ALL (70.7%) than in T-ALL (43%) \((P = .006)\). Positive p16\(^{INK4a}\) ICC ALL patients were also more likely to be older than age 9 years than were negative p16\(^{INK4a}\) ICC ALL patients, but significance \((P = .046)\) disappeared after stratification by phenotype \((P = .205)\).

Increased sensitivity threshold for glucocorticoid-induced apoptosis induced by forced p16\(^{INK4a}\) expression in lymphoblastic leukemia cell line has been reported.\(^{15}\) However, we did not find significant association in our cohort of childhood ALL patients between p16\(^{INK4a}\) ICC status and prednisone response at day 8 \((P = .434)\). Hence, it remains unclear whether p16\(^{INK4a}\) expression influences in vivo glucocorticoid-induced apoptosis of ALL cells.

Univariate analysis showed significantly better OS and EFS in patients with positive p16\(^{INK4a}\) ICC at diagnosis (Figure 1). OS estimates at 6 years for patients with or without positive p16\(^{INK4a}\) ICC at diagnosis were 90% \((SE = 3.8\%)\) and 63% \((SE = 8.7\%)\), respectively \((P = .0014, \text{log-rank test})\). EFS estimates at 6 years were 78% \((SE = 5.8\%)\) and 54% \((SE = 8.4\%)\) for p16\(^{INK4a}\) ICC–positive and p16\(^{INK4a}\) ICC–negative subgroups, respectively \((P = .0033, \text{log-rank test})\). When cutoff values of 0% and 10%, rather than 5%, for negative versus positive p16\(^{INK4a}\) ICC were used, results were less significant. These findings indicate that, as in our previous analysis in adult ALL, 5% is a valid cutoff value for prognostic studies in ALL.\(^{13}\) Despite the association between negative ICC and T phenotype at diagnosis, p16\(^{INK4a}\) ICC remained a significant prognostic factor in the subgroup of B-precursor ALLs for both OS \((P = .0044)\) and EFS \((P = .011)\) (Figure 1). This was not the case for T-ALL \((OS, P = .23; EFS, P = .19)\).

Final models of multivariate analysis showed that p16\(^{INK4a}\) ICC remained an independent prognostic factor for both OS \((P = .02)\) and EFS \((P = .0188)\) in the whole cohort (Table 1). However, karyotype remained the main prognostic factor for OS \((relative \text{ risk} = 3.92 \text{ versus } 3.38)\). Incorporation of immunophenotype into the model did not

### Table 1. Multivariate Cox model analysis for overall and event-free survival of the 126 childhood acute lymphoblastic leukemia patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tr>
<td></td>
<td>(P)</td>
<td>(R) (\text{RR} )</td>
</tr>
<tr>
<td>OS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16(^{INK4a}) ICC(^*)</td>
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<td>.0204</td>
</tr>
<tr>
<td>Karyotype(^†)</td>
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<td>.0227</td>
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<td>Age(^‡)</td>
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<tr>
<td>WBC(^§)</td>
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<td>.0719</td>
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<td>Prednisone response day 8(^|)</td>
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<td>—</td>
</tr>
<tr>
<td>Hemoglobin(^¶)</td>
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<td>Bulky tumor mass(^#)</td>
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<td>—</td>
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<tr>
<td>Immunophenotype(^#)</td>
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<td>—</td>
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<tr>
<td>Sex(^+)</td>
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<tr>
<td>EFS</td>
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<tr>
<td>Sex(^+)</td>
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</table>

\(R\) indicates relative risk; CI, confidence interval; OS, event-free survival; ICC, immunocytochemistry; WBC, white blood count; EFS, event-free survival.

\(^*\)Greater than 5% acute lymphoblastic leukemia cells;

\(^†\)5% or fewer acute lymphoblastic leukemia cells.

\(^‡\)Younger than 1 and older than 9 years; 1 year or older and 9 years or younger.

\(^§\)Lower than 50 000/\(\mu\)L; 50 000/\(\mu\)L or higher.

\(^\|\)Circulating blasts at 1000/\(\mu\)L or more; fewer than 1000/\(\mu\)L circulating blasts.

\(^¶\)More than 11 g/dL; lower than 11 g/dL.

\(^#\)T-acute lymphoblastic leukemia; B-precursor acute lymphoblastic leukemia.

Figure 1. Survival of childhood ALL patients according to their p16\(^{INK4a}\) ICC status. OS (panel A) and EFS (panel B) of the 126 childhood ALL patients. OS (panel C) and EFS (panel D) of childhood B-precursor ALL patients according to their p16\(^{INK4a}\) ICC status.
modify the results. It has been suggested in previous studies that the significance of p16INK4a gene deletions would disappear within the subgroups of T-ALL and B-precursor ALL. Our data show that T phenotype does not account for the poorer outcome of p16INK4a ICC-negative patients. However, the results of gene deletion studies and protein expression analyses by ICC may differ. Indeed, several studies have shown that p16INK4a protein expression in leukemic cells is a complex phenomenon and can be altered not only by gene deletion but also by promoter methylation and other, unknown mechanisms. We also observed, both in adult ALL and in the pediatric study, that a few samples of leukemic cells with no detectable p16INK4a protein at diagnosis showed p16INK4a expression at relapse (Table 2, patient I120). These findings might explain why p16INK4a ICC provides prognostic information distinct from that derived through p16INK4a gene deletion analysis. The lower proportion of high-risk karyotypes such as t(9;22)(q34;q11) in children may explain why p16INK4a ICC has a specific prognostic value in childhood ALL as compared with adult ALL.

Of the children with ALL analyzed at first relapse, 11 (35.5%) showed negative p16INK4a ICC (Table 2). Previous studies had shown that p16INK4a gene deletion could be acquired during evolution of the disease, but these findings have not been observed by other groups. In our study, ICC status became negative between first and second relapse in two others (I102, I103). Substantial variations of the percentage of p16INK4a-positive cells were also observed in 5 patients, suggesting that p16INK4a expression varies widely among both patients and varieties of disease progression. Thus, p16INK4a ICC should be tested when drugs targeting p16INK4a protein are tested. These findings indicate that absence of p16INK4a expression is an early phenomenon in the evolution of ALL. Some patients may lose p16INK4a expression during further relapse, but the impact of p16INK4a inactivation in those leukemic cells, which probably have accumulated numerous other gene alterations, remains to be determined.

Thus, the simple and reproducible p16INK4a ICC method should provide important prognostic information in large-scale prospective therapeutic studies.

References


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