Hemizygous $p16^{INK4A}$ deletion in pediatric acute lymphoblastic leukemia predicts independent risk of relapse


The genes at the $INK4A/ARF$ locus at 9p21 are frequently involved in human cancer. Virtually all $p16^{INK4A}$ exon 2 (henceforth called $p16$) inactivation in pediatric acute lymphoblastic leukemia (ALL) occurs by gene deletion. The results of this study illustrate that real-time quantitative polymerase chain reaction is capable of detecting gene deletion in primary patient specimens with a precision not previously achieved by conventional methods. Importantly, this assay includes the detection of hemizygous deletions. The study revealed, strikingly, that the risk ratio for relapse for hemizygous deletion compared with no deletion was 6.558 ($P = .00687$) and for homozygous deletion was 11.558 ($P = .000539$). These results confirm and extend the authors’ previous findings that homozygous deletion of $p16$ in pediatric ALL patients is an independent prognostic indicator of outcome from therapy. (Blood. 2001;97:572-574)

Study design

Patients

Diagnosis bone marrow specimens were studied from 45 ALL patients, on the basis of the availability of cryopreserved specimens (Table 1). The Princess Margaret Hospital, Perth, Australia, provided 30 patients, and the Children’s National Medical Center, Washington, DC, provided 15. There was no selection on the basis of either $p16$ genotype or time-to-relapse. Informed consent was obtained from all patients or their guardians to use specimens for research. Specimens were collected between 1981 and 1997. The leukemia immunophenotype (B-lineage or T-cell ALL) was determined with the use of a panel of monoclonal antibodies. Cytogenetic results revealed that none of the patients were known to have either t(4;11) or t(9;22). For all but 2 patients studied, therapy was administered according to risk-adjusted protocols of the Children’s Cancer Group, most of which were based upon modifications of the Berlin-Frankfurt-Munster trials; the exception consisted of 2 patients treated on a previously reported intensive therapy protocol for high-risk patients.

Real-time PCR analysis in multiplex format

Genomic DNA was isolated from cryopreserved specimens and control cell lines by standard methodology. All primers and probes were designed by means of Perkin-Elmer Primer Express software (Perkin-Elmer, Foster City, CA), and primers were supplied by Geneworks (Adelaide, Australia). The sequences were as follows: $p16$ exon 2 forward (F): gcgtcactaaagaccttctccttc; $p16$ exon 2 reverse (R): tcacgctcgagacacagaga; $\beta$-actin F: aggcggctagcatgtc; and $\beta$-actin R: gtacgatcgctctcttcagg. The probe for $p16$ had the sequence cccccacctggcttggaac and was labeled with FAM, whereas the probe for $\beta$-actin, attctccgctgctgctttagt, was labeled with VIC (both probes manufactured by Perkin-Elmer). The reactions were optimized first individually and then for multiplexing. The reaction was performed in a...
final volume of 50 μL. The final concentrations of primers and probes were as follows: p16 F 50 ng/μL, p16 R 50 ng/μL, p16 probe 200 nM, β-actin F 50 ng/μL, β-actin R 200 ng/μL, and β-actin probe 200 nM. Each reaction contained 50 ng DNA as template, and the Taqman Universal Master Mix (Perkin-Elmer) was used. The standard thermal cycling conditions of the ABI PRISM 7700 Sequence Detection instrument were applied. A standard calibration curve was included with each experiment with the use of a range of concentrations of DNA extracted from Raji B cells (range, 1.56-100 ng DNA).

P16 gene deletion analysis in specimens containing normal cells

We simulated normal cell contamination by using mixtures of DNA from Raji B cells, which are wild type for p16 (G/G), and K562 cells, which show homozygous deletion of p16 (D/D). The experimentally determined ratio for p16/β-actin was expressed as a function of the input ratio of Raji B/K562 cells. The test yielded a linear graph with a correlation coefficient of 0.9687, indicating that normal cell contamination (here simulated by Raji B cells) in a p16 D/D sample can be accurately measured by this technique. On the basis of this result, the bone marrow specimens were interpreted as follows. Ratio for p16/β-actin less than 0.4: p16 deletion (D/D); ratio 0.4 to 0.8: hemizygous p16 (G/D); ratio exceeding 0.8: germline p16 (G/G). The method was compared with Southern blot analysis, and the 2 methods agreed in all 11 cases tested, including G/D specimens. All but one of the 45 specimens contained fewer than 25% normal cells, according to an independent review by a hematologist, and the experimentally determined ratio for p16/β-actin was used to determine the genotype directly. The remaining specimen contained more than 50% normal cells, a factor that was taken into account.

Statistical analysis

The main analysis was based on methods appropriate for censored failure times. The primary time scale was calendar time from diagnosis; the primary response was relapse. Univariate analysis was based upon Kaplan-Meier survival functions and the Mantel-Cox (log-rank) test statistic. Multivariate analysis was based on the Cox proportional hazards regression model and the likelihood-ratio test. Covariates known to modulate the risk of relapse were included in the primary model whether they were statistically significant or not. Secondary modeling demonstrated that removal of the nonsignificant covariates did not modify substantive conclusions. Final models were subjected to (and passed) standard tests of goodness of fit. Analysis was undertaken in SAS version 6.12 (Cary, NC) for Unix.

Results and discussion

Deletion analysis of p16 was performed on 45 pediatric ALL patients at diagnosis, and the results are summarized in Table 1. Of the 45 patients, 11 (25%) demonstrated a homozygous deletion; 6 (13%) were hemizygous; and 28 (62%) were wild type for the p16 gene. In a previous study using Southern blot analysis performed in this laboratory, the incidence of homozygous p16 deletions at diagnosis was 18.3% (9/48).5 These findings for homozygous deletions are in agreement with the frequency of homozygous deletion reported for pediatric ALL patients: 23% for B-lineage and 64% for T-lineage ALL (T-ALL) (reviewed by Drexler6). When the distribution of T-ALL versus B-lineage ALL cases in our study is taken into account, the observed frequency is higher than expected, most likely owing to the higher sensitivity of the PCR technique.

Hemizygous and homozygous p16 deletions are independent prognostic indicators for poor outcome

Figure 1 illustrates the Kaplan-Meier curves for relapse-free survival stratified by p16 status. Among those patients who were
Table 2. Hemizygous and homozygous p16 deletions are independent prognostic indicators for poor outcome

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Risk ratio*</th>
<th>Confidence interval</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/D versus G/G</td>
<td>6.5</td>
<td>1.858-23,880</td>
<td>.00687</td>
</tr>
<tr>
<td>D/D versus G/G</td>
<td>11.5</td>
<td>2.625-47,284</td>
<td>.000539</td>
</tr>
</tbody>
</table>

See Table 1 footnote for explanation of abbreviations. *Multivariate analysis was performed to determine risk ratios.

Hemizygous status as determined in this study could be due to a mixture of leukemia cells (D/D, G/D, and G/G) or true hemizygosity in all leukemia cells. Owing to lack of material, it was not possible to study the G/D specimens by means of the fluorescence in situ hybridization technique. However, the clonality could be assessed by analyzing the rearrangement of the T-cell receptor and immunoglobulin heavy chain genes. Examination of the 5 G/D specimens from patients who relapsed revealed that in 3 cases there was clear evidence for clonal disease as only one rearranged band was detected whereas the 2 remaining cases showed 2 bands suggesting bclonal disease (data not shown). This test does not exclude the possibility that leukemia cells in these specimens were also hemizygous for p16. Clearly, 3 patients were diagnosed with a monoclonal disease showing hemizygous deletion of p16, excluding the potential for false hemizygous readout due to a mixture of G/G with D/D leukemic cells in the specimens.

In the current study, we identify hemizygous and homozygous loss of p16 as a major independent negative prognostic indicator in pediatric ALL. These results are consistent with the reported general sensitivity of pediatric ALL to current chemotherapy. Unlike the majority of good-prognosis pediatric ALL patients, who appear to have an intact apoptosis pathway, the subpopulation that is refractory to treatment with apoptosis-inducing drugs may have bypassed normal regulation by mutation of key regulators such as p16. Independent evidence that INK4A/ARF mutations promote resistance to chemotherapeutic drugs has recently been reported in a transgenic lymphoma model. The findings from this animal model provide direct evidence that mutations at the INK4A/ARF locus have a negative impact on the outcome of cancer therapy. The quantitative PCR method used in our study is suitable for high-throughput screening of patient specimens and has many clinical applications as it can be adapted to the deletion analysis of other tumor suppressor genes and other cancers.

References

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