The Prognostic Significance of p16^INK4a/p14^ARF and p15^INK4b Deletions in Adult Acute Lymphoblastic Leukemia

Stefan Faderl, Hagop M. Kantarjian, Taghi Manshouri, Chin-Yung Chan, Sherry Pierce, Kimberly J. Hays, Jorge Cortes, Deborah Thomas, Zeev Estrov, and Maher Albitar

Departments of Leukemia [S. F., H. M. K., S. P., J. C., D. T., Z. E.] and Laboratory Medicine [T. M., C.-Y. C., K. J. H., M. A.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Cytogenetic/molecular abnormalities significantly influence the prognosis of patients with acute leukemia. Recently, two genes, p16^INK4a and p15^INK4b, encoding two cyclin-dependent kinase inhibitor proteins of the INK4 family of M, 15,000 and 16,000, respectively, have been localized to 9p21. Remarkably, the p16^INK4a locus has been found to encode a second protein, p14^ARF, as p19^ARF in mice, with a distinct reading frame. Like p16^INK4a, p14^ARF is involved in cell cycle regulation, blocking cells at the G1 restriction point through the activity of MDM-2 and p53.

We studied bone marrow samples of 42 newly diagnosed and untreated patients with acute lymphoblastic leukemia for the incidence of deletions of p16^INK4a/p14^ARF and p15^INK4b using Southern blot analysis and determined the clinical outcome with regard to complete remission (CR) duration, event-free survival, and overall survival.

We found deletions of p16^INK4a/p14^ARF in 17 of 42 patients (40%), with homozygous deletions in 11 of 42 patients (26%) and hemizygous deletions in 6 of 42 patients (14%). The gene for p15^INK4b was codeleted in most, but not all, cases and was never deleted without deletion of p16^INK4a/p14^ARF. No correlation was observed between molecular studies and karyotype abnormalities as determined by conventional cytogenetics. Furthermore, no difference was found in the CR rate, CR duration, event-free survival, and overall survival in patients with homozygous gene deletions compared to patients with no deletions or loss of only one allele.

INTRODUCTION

ALLs result from clonal proliferation, accumulation, and tissue infiltration of neoplastic hematopoietic cells. Disruptions of the molecular mechanisms facilitating normal cell growth and differentiation frequently result from alterations of cell cycle control (1). Transitions of the eukaryotic cell cycle from G1 phase through DNA replication (S phase), G2, and cell division (M phase) are tightly regulated at multiple checkpoints known as restriction points. Progression through these stages is mediated by sequential accumulation of a family of serine-threonine protein kinases called CDKs and cyclins that activate the kinases (2–4). The CDKs are opposed by CDKIs that function like brakes in the cell cycle machinery, assuring the cells' functional integrity and readiness to progress across cell cycle restriction points, thus preventing uninhibited growth and proliferation (2, 4, 5).

Several CDKI proteins have been cloned and divided into families by homologies in their amino acid sequence (2). The INK4 family of CDKIs includes p16^INK4a (MTS1 and INK4b), p15^INK4b (MTS2 and CDKN2A), p15^INK4b (MTS2 and CDKN2B), p18^INK4a, and p19^ARF. They share a 90% homology in coding exon 2 and preferentially inhibit cyclin D-CDK-4/6 complexes (1, 5, 6). Kamb et al. (7) and Nobori et al. (8) localized the genes coding for p16^INK4a and p15^INK4b to chromosomal segment 9p21.

Deletions of p16^INK4a and p15^INK4b have been identified in up to 80% of human leukemia cell lines, with homozygous deletions as the most frequent mechanism of inactivation and p16^INK4a as the primary target for such deletions (7, 8, 9–11). However, analysis of primary leukemia samples revealed lower rates of detection than those in cell lines (9, 11).

Recently, the p16^INK4a locus was found to encode a second, distinct protein. The mRNA for p14^ARF is composed of exons 1β, 2, and 3, whereas the mRNA for p16^INK4a is derived from exons 1α, 2, and 3. Alternative splicing of exon 1 in p14^ARF results in a different reading frame for exons 2 and 3 (12). p14^ARF inhibits the expression of oncogene MDM-2, therefore preventing MDM-2-mediated inactivation of tumor suppressor gene p53 (13).

Most studies of 9p21 anomalies and molecular analyses for p16^INK4a/p14^ARF and p15^INK4b were performed in childhood ALL. We reported p16^INK4a/p14^ARF and p15^INK4b deletions in 178 cases of primary adult leukemias (14). Here we report an analysis of p16^INK4a/p14^ARF and p15^INK4b deletions in a series of 42 newly diagnosed adult ALL patients.

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2 The abbreviations used are: ALL, acute lymphoblastic leukemia; CR, complete remission; EFS, event-free survival; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; BM, bone marrow.
PATIENTS AND METHODS

Patient Samples

BM specimens were obtained from 42 newly diagnosed and untreated patients who presented to the Leukemia Department at The University of Texas M. D. Anderson Cancer Center between 1985 and 1997. Six of the patients had mature B-cell ALL, 30 patients displayed markers of pre-B-cell ALL (positive for CD10/calla in 25 patients), and 4 patients presented with T-lineage ALL. Results of immunophenotyping were not available for two patients (Table 1). The BM samples were obtained with informed consent, and the study was approved by the Human Experimentation Committee of our institution.

Treatment

All but four patients were treated with the “hyper-CVAD” regimen as described elsewhere (15). The remaining four patients (patient 12 from Table 3 and three patients without gene deletions) received VAD (vincristine, Adriamycin, and dexamethasone) chemotherapy (16).

Specimen Collection

All specimens were obtained during routine diagnostic procedures under approved protocols. Only BM specimens in which leukemic cells exceeded 80% of the population were used for analysis. Low-density cells were separated by Ficoll-Hypaque gradient centrifugation (Sigma, St. Louis, MO) and washed twice with PBS. Genomic DNA was extracted. Genomic DNA with 11q deletions or rearrangement of the MLL gene was not used. A few cases with deletion of chromosome 11 in less than 10% of metaphase cells and comparable intensities of 11q23 bands in Southern blot analysis were included. Cases with chromosomal translocations t(11;14)(q12;q32) and t(11;14)(q13;q32) were also included as long as the gene was not used. A few cases with deletion of chromosome 11 were considered as a homozygous deletion, and a ratio of <0.2 was considered as a homozygous deletion (Fig. 1).

Cytogenetic Analysis. BM cells were placed in 10 ml of Ham’s F-10 with 20% FCS to obtain a final concentration of 1–4 × 10^6 nucleated cells/ml. The cultures were incubated for 24 h at 37°C. Standard harvesting and fixation procedures were used, as described elsewhere (17). Up to three slides of each preparation were stained with Gurr’s Giemsa stain and placed in a 60°C oven overnight before Giemsa banding. A maximum of 25 metaphases were analyzed on the Giemsa-stained slides. Two abnormal metaphase cells with identical karyotype anomalies were required for establishing the diagnosis of a clonal abnormality (17).

Statistical Methods

The influence of clinical and cytogenetic parameters between groups of patients with homozygous, hemizygous, and no deletions was evaluated using the χ^2 test. Median values were compared using Kruskal-Wallis test statistics (18). The probability of surviving and remaining in CR was evaluated using Kaplan-Meier analysis (19).

RESULTS

Incidence of Deletions of p16INK4a/p14ARF and p15INK4b in Adult ALL. Table 1 summarizes the incidence of deletions in the p16INK4a/p14ARF and p15INK4b genes in our samples. Bi-allelic deletions of p16INK4a/p14ARF were observed in 11 of 42 (26%) patients. The gene for p15INK4b was codeleted inhomogeneously in nine patients (21%). In no patient could we demonstrate homozygous deletions of p15INK4b without deletions of p16INK4a/p14ARF. Hemizygous deletions of p16INK4a/p14ARF occurred in six cases (14%) and were associated with hemizygous deletions of p15INK4b in five patients (12%). As was the case in patients with homozygous deletions, no hemizygous deletions of p15INK4b were found without concomitant deletions of p16INK4a/p14ARF. Overall, deletions of p16INK4a/p14ARF
and p15INK4b were detected in 40% of our patients: homozygous deletions were detected in 26% of our patients (21% for p15INK4b); and hemizygous deletions were detected in 14% of our patients (12% for p15INK4b).

Deletions of p16INK4a/p14ARF/p15INK4b were highest among patients with precursor B-cell ALL (40 – 48% for homozygous gene deletions and 20 – 44% for hemizygous deletions) and lowest in patients with mature B-cell ALL (17% in either group). One of four patients with T-cell ALL showed homozygous loss of p16INK4a/p14ARF, whereas no deletions of p15INK4b could be associated with T-cell immunophenotype. No significant difference was found between T-cell immunophenotype and deletions of p16INK4a/p14ARF or p15INK4b (P > 0.05; Table 1).

Clinical and Cytogenetic Characteristics of Patients with Deletions of p16INK4a/p14ARF and p15INK4b. Clinical and cytogenetic characteristics were compared between patients with homozygous deletions, hemizygous deletions, and no deletions. Patients with homozygous deletions of p16INK4a/p14ARF or p15INK4b had a significantly higher WBC count when compared to the other groups (P = 0.0005). No significant difference existed between the groups for age, percentage of BM blasts at diagnosis, hepatomegaly and splenomegaly, and presence of the Philadelphia chromosome (Table 2).

Table 2  Clinical characteristics of patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Age (range; yrs)</th>
<th>WBC (range × 10^9/liter)</th>
<th>BM blast (range; %)</th>
<th>Splenomegaly (%)</th>
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<th>Ph+ (%)</th>
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<tbody>
<tr>
<td>Homozygous deletions of p16/p14 with or without p15</td>
<td>11</td>
<td>26 (16–79)</td>
<td>45.7 (13–298.5)</td>
<td>87 (76–98)</td>
<td>3 (27)</td>
<td>1 (9)</td>
<td>4 (36)</td>
</tr>
<tr>
<td>Hemizygous deletions of p16/p14 with or without p15</td>
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<td>57 (20–78)</td>
<td>5.1 (3.9–74.9)</td>
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<tr>
<td>No deletion detected</td>
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<td>42 (16–79)</td>
<td>8.3 (0.6–89)</td>
<td>81 (24–98)</td>
<td>4 (16)</td>
<td>2 (8)</td>
<td>6 (24)</td>
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<td>P</td>
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<td>b NS, not significant.</td>
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Clinical and Cytogenetic Characteristics of Patients with Deletions of p16INK4a/p14ARF and p15INK4b. Clinical and cytogenetic characteristics were compared between patients with homozygous deletions, hemizygous deletions, and no deletions. Patients with homozygous deletions of p16INK4a/p14ARF or p15INK4b had a significantly higher WBC count when compared to the other groups (P = 0.0005). No significant difference existed between the groups for age, percentage of BM blasts at diagnosis, hepatomegaly and splenomegaly, and presence of the Philadelphia chromosome (Table 2).

Association between Molecular Abnormalities and Cytogenetic Analysis of 9p21. Cytogenetic profiles of patients with deletions of p16INK4a/p14ARF and p15INK4b are shown in Table 3. In 6 of 17 patients (patients 4, 6, 10, 11, 12, and 16), determination of karyotype was not possible due to insufficient metaphases. The percentage of insufficient metaphases was not significantly different in the group of patients with deletions of p16INK4a/p14ARF/p15INK4b and patients without deletions in these two genes (35% versus 32%; P > 0.05). Two patients (patients 15 and 17) had a diploid karyotype. The remaining nine cases showed complex karyotypic abnormalities involving anomalies of the short arm of chromosome 9 in four patients. Three of these patients had homozygous deletions of p16INK4a/p14ARF/p15INK4b by Southern blot (Table 1). The 9p abnormalities consisted of del 9(p22) in patients 3 and 7 and 9p2 in patient 2. The fourth patient had no detectable deletion of p16INK4a/p14ARF/p15INK4b by molecular analysis but showed a 9p− abnormality by cytogenetics. No patient with hemizygous gene deletions had 9p− abnormalities. Overall, abnormalities of the short arm of chromosome 9 were detected in 27% of cases with biallelic deletions of p16INK4a/p14ARF/p15INK4b, in 4% of cases without deletions, and in no cases with hemizygous deletions. The Philadelphia translocation t(9;22)(q34;q22) was found in 4 of 11 patients (36%) with complete loss of p16INK4a/p14ARF/p15INK4b, in 1 of 6 patients (17%) with hemizygous deletions, and in 5 of 25 patients (20%) without deletions (P > 0.05; Tables 1 and 2).

Association of Gene Deletions with Clinical Outcome. Clinical outcome was assessed in all 42 patients. Median follow-up was 20 months (range, 6–157 months). For survival analysis, patients were classified as responders (cure, remission) or non-responders (secondary resistance, relapse).

Table 3  Clinical characteristics of patients

<table>
<thead>
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<th>n</th>
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</table>
analysis, patients were classified into two groups: (a) those with complete loss of \(p16^{INK4a}/p14^{ARF}\) and \(p15^{INK4b}\); and (b) those with either hemizygous deletions or no deletions. Patient numbers were too small for patients with hemizygous deletions to be analyzed separately. Ten of 11 patients (91%) with biallelic loss of \(p16^{INK4a}/p14^{ARF}\) achieved CR, whereas 30 of 31 patients (97%) with loss of one allele or no deletions achieved CR (Table 4). No significant differences between the two groups were found with respect to CR duration (Fig. 2), EFS (Fig. 3), and overall survival (Fig. 4), although a trend for worse outcome in homozygously deleted patients exists.

**DISCUSSION**

Abnormalities of the short arm of chromosome 9 including segment 9p21 occur in ALL with a frequency of 7–13% (9, 14, 20, 21). Some authors reported an association between abnormalities of 9p and characteristic clinical features such as high WBC and blast counts at presentation, prominent lymphadenopathy and splenomegaly, expression of predominantly T-cell surface markers, and significantly shorter survival than that of other patients with ALL (22). Other studies did not confirm this association (21, 23–25, 26). Inactivation of genes for \(p16^{INK4a}/p14^{ARF}\) and \(p15^{INK4b}\) by deletion and intragenic mutations has been described in a variety of cell lines and primary samples from patients with both hematological malignancies and solid tumors (10, 11, 27–29). The discovery of the \(p14^{ARF}\) gene arising by alternative splicing within the \(p16^{INK4a}\) locus raises questions regarding which of these genes represents the target gene of the 9p21 deletion. Loss of both genes, \(p16^{INK4a}\) and \(p14^{ARF}\), may be an important factor in the biological effects of deletions of 9p21. Most of the previous studies on 9p21 deletions do not discuss the loss of \(p14^{ARF}\), although it is most likely that deletions of \(p16^{INK4a}\) accompany deletions of \(p14^{ARF}\), because both genes share exons 2 and 3.

The incidence of homozygous and hemizygous deletions ranges from 20% to more than 70% in most series and is similar in children (26, 30–38) and adults (14, 21, 39, 40) with ALL. Our study showed deletions of \(p16^{INK4a}/p14^{ARF}\) and \(p15^{INK4b}\) in 40% of the patients. The most frequently deleted gene was \(p16^{INK4a}/p14^{ARF}\) with co deletions of \(p15^{INK4b}\) in most but not all cases. These data confirm \(p16^{INK4a}/p14^{ARF}\) as the primary target for inactivation by deletion of 9p21. Takeuchi et al. (38) undertook detailed deletional mapping of chromosome 9 by microsatellite analysis in 54 children with primary ALL. They found loss of heterozygosity on the short arm of chromosome 9 in 57% of the samples. Similar to data from our study, the smallest region of loss of heterozygosity included \(p16^{INK4a}/p14^{ARF}\) but not the locus for \(p15^{INK4b}\). Aquiar et al. (41) constructed a map of deletions at 9p21 using multiplex PCR. Although variable in size, the commonly deleted region included \(p16^{INK4a}/p14^{ARF}\) but did not include either \(p15^{INK4b}\) or the IFN-α gene cluster.

Importantly, our results demonstrated that most of deletions of \(p16^{INK4a}/p14^{ARF}\) and \(p15^{INK4b}\) are detected molecularly and not by conventional cytogenetics. Nine of the 17 patients (53%) who had either biallelic or hemizygous loss of \(p16^{INK4a}/p14^{ARF}/p15^{INK4b}\) showed karyotypic abnormalities. In the remaining cases, cytogenetics revealed diploid karyotypes in two (12%) patients or could not be performed due to insufficient

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### Table 3

<table>
<thead>
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<th>Patient no.</th>
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<th>(p16^{ho})</th>
<th>(p16^{he})</th>
<th>(p15^{ho})</th>
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<td>Calla</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>2</td>
<td>26/M</td>
<td>Calla</td>
<td>(5;46)x,t(9p:–)(1)t(1q:+4q–)(19;46),–5–7+der7t(7p:–?)(7q:–??)+M;15D</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>66/M</td>
<td>B</td>
<td>(3444)x,R(1)p1112,–8–9,del9(p22),–13,der14,t(8;14)q11q32,–16–21+3M;1 dip</td>
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<td>+</td>
<td>+</td>
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<td>4</td>
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<td>Calla</td>
<td>IM</td>
<td>+</td>
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<td>IM</td>
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<tr>
<td>14</td>
<td>70/M</td>
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<td>46xx</td>
<td>–</td>
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* IM, insufficient metaphases; dip, diploid karyotype.  
* ho, homozygous.  
* he, heterozygous.

### Table 4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CR rate (%)</th>
<th>CRD* (mo)</th>
<th>EFS (mo)</th>
<th>OS (mo)</th>
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<tr>
<td>Homozygous deletions of (p16^{INK4a}/p14^{ARF})</td>
<td>10/11 (91)</td>
<td>10</td>
<td>9</td>
<td>12</td>
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<tr>
<td>Normal and hemizygous deletions of (p16/p14/p15)</td>
<td>30/31 (97)</td>
<td>17</td>
<td>14</td>
<td>23</td>
</tr>
</tbody>
</table>

* CRD, complete remission duration; OS, overall survival; NS, nonsignificant.
metaphases in six patients (35%). Only three patients (18%) had gene deletions and 9p− abnormalities by cytogenetics. In all three of these patients, deletions of p16<sup>INK4a</sup>/p14<sup>ARF</sup> were homozygous. In one patient, 9p− was found without gene deletions. However, this karyotype was present in only a subpopulation of metaphases that were analyzed from that sample. In concordance with our results, Iolascon et al. (37) analyzed 21 children with T-cell ALL, scanning for deletions of p18<sup>INK4c</sup> and p16<sup>INK4a</sup>/p14<sup>ARF</sup> by multiplex PCR. They observed homozygous deletions of p16<sup>INK4a</sup>/p14<sup>ARF</sup> in 20 of 21 patients (95%). Of 13 cases for whom cytogenetic studies were available, 12 showed no detectable 9p alterations. Faienza et al. (32) found no correlation between karyotype and p16<sup>INK4a</sup>/p14<sup>ARF</sup> deletions in their series of childhood ALL. Our study confirmed these findings in adult ALL and emphasizes the need to perform molecular studies in patients with ALL at diagnosis to detect abnormalities that may have an impact on response to therapy and prognosis.

Patients with homozygous loss of p16<sup>INK4a</sup>/p14<sup>ARF</sup> and p15<sup>INK4b</sup> had no worse outcome than patients who had either no
deletions or loss of only one allele. CR duration, EFS, and overall survival were not significantly different. However, longer follow-up will be needed to substantiate these results in adults with ALL.

In an analysis of 79 children with ALL, Heyman et al. (36) found a correlation between inactivation of p16INK4a/p14ARF/p15INK4b and lower CR rates and shorter EFS. Deletion of genetic material from one allele but preservation of a normal coding sequence on the remaining allele conferred a similar prognosis to cases without deletions. The importance of this difference in survival between patients with homozygous and hemizygous deletions in this study is not understood. It is intriguing to think that patients with hemizygous deletions may carry a point mutation in the second or third allele that affects either p16INK4a or p14ARF but not both together. More knowledge of the molecular mechanisms of inactivation and of the role of each of the genes for p16INK4a, p14ARF, and p15INK4b in leukemogenesis will allow us to better understand the importance of genetic events and provide opportunities for new therapies in the future.

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The Prognostic Significance of $p16^{INK4a}/p14^{ARF}$ and $p15^{INK4b}$ Deletions in Adult Acute Lymphoblastic Leukemia

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