Molecular Analysis of Infant Acute Lymphoblastic Leukemia: MLL Gene Rearrangement and Reverse Transcriptase-Polymerase Chain Reaction for t(4;11)(q21;q23)

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Molecular techniques to detect MLL (11q23) and AF-4 (4q21) gene rearrangements are being evaluated for use in stratification of patients into prognostic groups. We studied 15 cases of infant acute lymphoblastic leukemia (ALL) with Southern blotting for MLL gene rearrangement and reverse transcriptase-polymerase chain reaction (RT-PCR) for t(4;11) fusion transcripts and compared the results to cytogenetic and clinical data. Our results indicate that classic t(4;11)(q21;q23) translocations are detected by RT-PCR; however, unusual 4;11 translocations still require additional investigation. We also extended and updated our original study of MLL gene rearrangement in infant ALL to 40 patients with longer follow-up and show that the group with germline configuration of the MLL gene continues to have an excellent outcome. The results of salvage therapy (bone marrow transplantation or chemotherapy) suggest that transplant may show an advantage. Preliminary results of the use of RT-PCR to assess minimal disease are also reported.

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INFANT ACUTE lymphoblastic leukemia (ALL), because of its poor response to therapy, has been extensively studied to define prognostic indicators. Disease characteristics associated with poor outcome are age less than 6 months, white blood cell (WBC) count greater than 50,000/µL, and CD10− phenotype.1–4 Infant ALL has a high incidence of cytogenetic 11q23 rearrangements (43% to 60%),5–8 with the t(4;11)(q21;q23) being the most common translocation among many different 11q23 translocation partners.9–12 Studies using Southern analysis of the MLL (mixed-lineage leukemia) gene at chromosome band 11q23 consistently show that infant ALL with rearrangement of this gene has a dismal prognosis.1,13–14 However, the survival of infants without this rearrangement has been less consistent, from 50%11 to 67%12 to the 80% reported by Chen et al.14 Current debate centers around which patients will need intensified therapy. For this reason we have added to and updated the group of infants originally reported by Chen et al14; the infants with germline MLL configuration continue to do very well.

Among MLL-rearranged patients, cytogenetic data suggest that it is the t(4;11) that confers a poor prognosis7,10,15,16 and that infants with 11q23 translocation partners other than the AF-4 gene at chromosome band 4q21 do somewhat better, but the number of such cases is small.8 The prognostic significance of specific 11q23 translocations may become more clear with the use of molecular techniques to detect these translocations in cases with inadequate cytogenetic data. Reverse transcriptase-polymerase chain reaction (RT-PCR) has been used to detect fusion transcripts resulting from specific translocations; however, this technique relies on known sequence data to design PCR primers around classic translocation breakpoints. We and others have reported an RT-PCR assay for t(4;11) fusion transcripts.15–20 We report here 15 cases of infant ALL examined with both Southern analysis of the MLL gene and RT-PCR for MLL/AF-4 fusion transcripts. Those with nonclassic cytogenetic t(4;11) breakpoints show that MLL rearrangement (reliably detected by Southern blot) does not always lead to standard fusion transcripts detectable by PCR. This finding has implications as to which molecular tests are used to determine prognostic groups.

Intensification of therapy involves chemotherapy or bone marrow transplantation (BMT); there are few data available as to their efficacy in salvage therapy. The patients in our study whose disease relapsed were salvaged either with BMT or chemotherapy; the outcome for these infants is reported. In addition, we report preliminary results of a study assessing minimal residual disease (MRD) in t(4;11) acute leukemia using RT-PCR.

MATERIALS AND METHODS

Patients and specimens. Bone marrow (BM) or peripheral blood (PB) samples were obtained from 15 infants (≤12 months of age) with newly diagnosed ALL who were enrolled and treated on Childrens Cancer Group (CCG) 1883, the most recently completed CCG infant ALL protocol. Treatment includes induction with vincristine, prednisone, L-Asparaginase, and daunomycin (VPLD) and intrathecal methotrexate and postinduction therapy with rotating combinations of high-dose Ara-C, very high dose methotrexate with citrovorum factor rescue, VPLD, 6MP, and cytoxan. Cases were selected only on the basis of availability of cryopreserved blasts. These 15 cases were studied both by RT-PCR for MLL/AF-4 fusion transcripts and by Southern analysis for MLL gene rearrangement. The results of Southern analysis have been previously reported for 6 of these infants in a study of the impact of MLL gene rearrangement on the outcome of 30 infants treated for ALL on 3 CCG protocols.14 In total, 40 (CCG 191,21 2 patients; CCG 107,3 14 patients; and CCG 1883, 24 patients) infants have now been studied for MLL gene rearrangement, and their outcome has been updated for this report, including the results of salvage therapy.

In addition, 4 other t(4;11) ALL patients, who were 4 months to
20 years of age at diagnosis, were studied at various timepoints on therapy for the presence of MLL/AF-4 fusion transcripts by RT-PCR. PB or BM or postmortem spleen or liver specimens (homo- geneized with a Dounce homogenizer) were studied. RNA and DNA were prepared as described.\(^\text{3}\) MLL/AF-4–positive (RS411\(\text{2}\) and B1\(\text{4}\)) and -negative (Nalm-6\(\text{3}\)) cell lines have been previously described.

**Molecular Analysis of Infant ALL**

**Immunophenotyping.** The immunophenotyping was performed in CCG reference laboratories at Children’s National Medical Center (Washington, DC) or the University of Minnesota (Minneapolis, MN). Cells from patients were reviewed; to include a normal case as adequate, analysis of at least 20 metaphases was required. All cases were banded, with a minimum band level of 400.

**Statistical analysis.** Life table comparisons were made for the groups with and without MLL gene rearrangement using the Mantel-Peto-Cox summary \(\chi^2\). The endpoint used in the life table analysis was event-free survival (EFS); an event was defined as occurrence of any relapse or death after first remission.

**RT-PCR for the MLL/AF-4 fusion transcript.** Polyadenylated RNA was extracted and cDNA synthesized as previously described.\(^\text{7}\) The PCR primers (M1 in MLL exon 6, and A1 and A2 in AF-4) that amplify the derivative 11 fusion transcript resulting from the t(4;11) translocation have been previously reported.\(^\text{17}\) In addition, we have used two nested primers, M5b (AAGTTGCTCAGCCCAAGGTAT) in MLL exon 5 and A43b (TGGGGTTACAGAATGACATG) in AF-4. The location of the AF-4 primers is such that classical AF-4 breakpoints as well as breakpoints up to 400 bp downstream from the cluster region would be detected. Amplification was performed with Taq polymerase as previously reported.\(^\text{17}\)

Any initially negative RT-PCR reactions with the first set of primers were repeated using the nested primers with 5% of the original PCR product. Amplification of \(\beta\)-actin RNA was performed to demonstrate integrity of isolated RNA. Strict precautions were used to prevent contamination of samples, and positive and negative controls were included at all steps. PCR products were separated by electrophoresis on a 1% agarose gel. The DNA was transferred to a nylon membrane and probed with \(\gamma^3\)-P-labeled oligonucleotides specific for MLL (GAATTCGTCAGACAGCAAACTGACATG) and AF-4 (TGACCCATTCCATGGCTCCTC), PCR products were cloned and sequenced as previously reported.\(^\text{17}\) The AF-4 exon terminology is as described in Downing et al.\(^\text{18}\)

**Detection of MLL gene rearrangement by Southern analysis.** Genomic DNA was extracted, digested with restriction endonucleases (EcoRI, HindIII, and BamHI), and analyzed by Southern blotting as described.\(^\text{14}\) The probes used to detect rearrangement of the MLL gene at chromosome band 11q23 have been described.\(^\text{14,26}\)

**RESULTS**

**RT-PCR for detection of the t(4;11) (MLL/AF-4) fusion transcript.** We tested 15 infants with ALL for MLL gene rearrangements and MLL/AF-4 fusion transcripts; the results are shown in Table 1. Three patients had a germline MLL gene configuration (patients no. 1 through 3) without evidence of fusion transcripts by RT-PCR. Two of these patients had insufficient metaphases for cytogenetic testing, and 1 had a normal male karyotype. Five patients (no. 4 through 8) with MLL gene rearrangement by Southern analysis were also negative for MLL/AF-4 fusion transcripts. Of interest, 2 of these (patients no. 6 and 7) had unusual 4;11 translocal-
were taken off protocol for BMT in first remission (Table 3). One of these patients (no. 32), who was treated with an unrelated donor transplant 7 months after achieving remission, remains disease-free 38 months after BMT. The other patient (no. 11), who was treated with an allogeneic BMT 2.5 months after achieving remission, has relapsed and died.

The results of salvage therapy in those infants with MLL gene rearrangement who have relapsed are shown for those that received BMT (Table 3) and chemotherapy (Table 4). The median survival after relapse for babies salvaged with chemotherapy was 140 days; 1 of the 12 children treated remains alive without disease. For those receiving transplants in second or later remission, the median survival after relapse was 310 days; 2 of 8 are alive and disease-free.

DISCUSSION

MRD analysis. RT-PCR for detection of MLL/AF-4 fusion transcripts was undertaken in 4 patients at different timepoints on therapy (Fig 2). Dilutions of RNA from the RS(4;11) cell line in RNA from the non-*(4;11) cell line Nalm-6 were tested. This method detects message from 1 cell in 10^5, which is comparable to other PCR assays for minimal disease.\cite{19,20,25,26} Note that more than one size transcript may be detected, resulting from alternative splicing, as previously reported.\cite{19}

Patient A, an infant, was studied in first remission at 1.5 years into maintenance therapy. No t(4;11) transcripts were detected in testing this single marrow specimen in duplicate. Patient B is 14 years old and was treated with unrelated donor BMT after consolidation (preparative regimen Cytoxan and total body irradiation [TBI]). We detected transcripts in the diagnostic blasts (PB1), in the PB and BM after consolidation (PB2 and BM2), and in the postmortem specimens from spleen (Sp13) and BM (BM3), all in morphologic remission. Blood (PB3) on the last day of life (the patient died 6 months post-BMT from infection) was negative.

Patient C, an infant, was treated with unrelated donor BMT (prepared with Cytoxan/TBI) during maintenance; he died 4 months after transplantation of interstitial pneumonia. Transcripts were detected pre-BMT (BMI) and in (morphologic remission) marrows at day 100 (BM2), day 125 (BM3), and day 157 (BM4) post-BMT. Blood on the last day of life (PB4) was negative. Patient D, who was 20 years of age, received an unrelated donor BMT after consolidation (prepared with VP-16, cytoxan, TBI, and B43-PAP [anti-CD19] immunotoxin).\cite{21} He died from infection 6 months after transplantation. Transcripts were detected at diagnosis (BM1) and after consolidation (BM2, BM3, and BM4, all remission marrows), but were not detected 21 (BM5) or 52 (BM6) days after transplantation or in the postmortem spleen, liver, or marrow (BM7). All specimens were positive when tested for β-actin, indicating the isolation of intact RNA.

Table 1. Results of Cytogenetics, Southern Analysis of the MLL Gene, and RT-PCR for the MLL/AF4 Fusion Transcript in Infant ALL

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Karyotype [no. of cells]</th>
<th>MLL</th>
<th>PCR</th>
<th>der11 Fusion Transcript: MLL Exon/AF4 Exon</th>
<th>Duration CR1 (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Inadequate study</td>
<td>G</td>
<td>–</td>
<td>ND</td>
<td>50+</td>
</tr>
<tr>
<td>2</td>
<td>Inadequate study</td>
<td>G</td>
<td>–</td>
<td>ND</td>
<td>18+</td>
</tr>
<tr>
<td>3</td>
<td>46,XY(20)</td>
<td>G</td>
<td>–</td>
<td>ND</td>
<td>35+</td>
</tr>
<tr>
<td>4</td>
<td>46,XX(20)</td>
<td>R</td>
<td>–</td>
<td>ND</td>
<td>29+</td>
</tr>
<tr>
<td>5</td>
<td>not available</td>
<td>R</td>
<td>–</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>46,XX,t(4;11)(q21;q23)</td>
<td>R</td>
<td>–</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>46,XX,+X,+t(4;11)(q35)</td>
<td>R</td>
<td>–</td>
<td>ND</td>
<td>13</td>
</tr>
<tr>
<td>8</td>
<td>Inadequate study</td>
<td>R</td>
<td>–</td>
<td>ND</td>
<td>37+</td>
</tr>
<tr>
<td>9</td>
<td>Inadequate study</td>
<td>R</td>
<td>+</td>
<td>MLL8/AF4a</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>46,XX,der(4)(71;4)(q21; q21),del(7)(p13),der(11)(4;11)(q21;q23)</td>
<td>R</td>
<td>+</td>
<td>MLL6/AF4b</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>46,XX,t(4;11)(q23;14)/46, XX(6)</td>
<td>R</td>
<td>+</td>
<td>MLL8/AF4a*</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td>46,XY,t(4;11)(q23;7)/46,XY,t(4;11)(q21;q23;14;18)iq32;q11.2) (7)/46,XY(6)</td>
<td>R</td>
<td>+</td>
<td>MLL7/AF4a</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>46,XX,t(4;11)(q23;12)</td>
<td>R</td>
<td>+</td>
<td>MLL8/AF4a</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>46,XX,t(4;11)(q23;10)/46,XX(10)</td>
<td>R</td>
<td>+</td>
<td>MLL8/AF4a*</td>
<td>4</td>
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<tr>
<td>15</td>
<td>46,XX,t(4;11)(q23;3)/46,XX(10)</td>
<td>R</td>
<td>+</td>
<td>MLL6/AF4a</td>
<td>3</td>
</tr>
</tbody>
</table>

Abbreviations: G, germline; R, rearranged; ND, not detected.

* Sequence previously reported.

Table 2. Details of Infants Studied for MLL Gene Rearrangement

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>MLL Rearranged</th>
<th>MLL Germline</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>11</td>
</tr>
<tr>
<td>CD10</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td>CD19</td>
<td>25 (3 ND)</td>
<td>8 (2 ND)</td>
</tr>
<tr>
<td>Median WBC</td>
<td>225 (5-2,810)</td>
<td>24 (2-50)</td>
</tr>
<tr>
<td>Median age</td>
<td>5 (1-11)</td>
<td>10 (4-11)</td>
</tr>
<tr>
<td>No. achieving remission by day 28</td>
<td>27</td>
<td>11</td>
</tr>
<tr>
<td>No. relapsing</td>
<td>22</td>
<td>1</td>
</tr>
</tbody>
</table>

Cytogenetics

11q23 abnormality: 19 (0)
Other abnormalities: 1 (3)
Insufficient or NA: 7 (2)
Normal: 2 (6)
molecular markers in the assignment of treatment groups must be performed with concurrent comparison to established parameters such as cytogenetics and immunophenotyping. In infant ALL, cytogenetic data show that the t(4;11)(q21;q23) translocation portends a poor prognosis. Several groups have shown that MLL (11q23) gene rearrangement, as detected by Southern blot, carries a poor prognosis in infant ALL. The purpose of this study was to simultaneously evaluate cytogenetics, Southern analysis of MLL gene rearrangement, and RT-PCR for detection of the MLL/AF-4 fusion transcript in infant ALL in the context of outcome. With other investigators reporting intermediate survival (from 50% reported by the Pediatric Oncology Group to the 67% reported by Cimino et al) in infants with germline MLL configuration, in contrast to the 80% survival seen in infants treated on CCG protocols, we also sought to add to and update the survival curve for the CCG infants studied for MLL status.

The results show, as expected, that molecular techniques detect rearrangement of the MLL and AF-4 genes not found by cytogenetics and that PCR at this point cannot replace Southern blotting for MLL gene status. They also underscore the continued need for cytogenetic testing. Patients no. 6, 7, and 9 illustrate the questions clinicians will consider to make therapeutic decisions based on these tests. In patients no. 6 and 7 (rearranged at MLL), cytogenetics show t(4;11) with nonclassic breakpoints, and, as anticipated, PCR does not detect a classic MLL/AF-4 fusion transcript. In patient no. 9, there were insufficient metaphases for analysis, but RT-PCR identified a t(4;11) fusion transcript. We must decide how a recommendation for BMT for t(4;11) leukemia in first remission would apply to these patients.

RT-PCR for t(4;11) is difficult because the AF-4 gene remains largely uncharacterized, and a particular set of primers may miss uncommon AF-4 translocation breakpoints. Several groups have cloned and sequenced most of the AF-

### Table 3. BMT in Infant MLL-Rearranged ALL

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Donor Type</th>
<th>Timing</th>
<th>Prep</th>
<th>Duration CR1 (mo)</th>
<th>From BMT</th>
<th>From Relapse</th>
<th>Cause of Death</th>
<th>11q23 Cytogenetic Abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>URD</td>
<td>CR1</td>
<td>Cy/TBI</td>
<td>NA</td>
<td>38+</td>
<td>NA</td>
<td>NA</td>
<td>t(4;11)(q21;q23)</td>
</tr>
<tr>
<td>11</td>
<td>MSD</td>
<td>CR1</td>
<td>Cy/TBI</td>
<td>NA</td>
<td>2</td>
<td>NA</td>
<td>PD</td>
<td>t(4;11)(q21;q23)</td>
</tr>
<tr>
<td>7</td>
<td>Haplo</td>
<td>CR2</td>
<td>Bu/Cy/Ara-C/TBI</td>
<td>13</td>
<td>3</td>
<td>3</td>
<td>LPD</td>
<td>t(4;11)(q35;q14-22)</td>
</tr>
<tr>
<td>23</td>
<td>Haplo</td>
<td>CR2</td>
<td>Cy/TBI</td>
<td>4</td>
<td>5</td>
<td>7</td>
<td>PD</td>
<td>t(4;11)(q21;q23)</td>
</tr>
<tr>
<td>15</td>
<td>URD</td>
<td>CR2</td>
<td>Bu/Cy/Ara-C/TBI</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>PD</td>
<td>t(4;11)(q21;q23)</td>
</tr>
<tr>
<td>13</td>
<td>MSD</td>
<td>CR2</td>
<td>Bu/Cy/VP-16</td>
<td>4</td>
<td>12+</td>
<td>13+</td>
<td>NA</td>
<td>t(4;11)(q21;q23)</td>
</tr>
<tr>
<td>12</td>
<td>Auto</td>
<td>CR2</td>
<td>Ara-C/TBI</td>
<td>4</td>
<td>9+</td>
<td>14+</td>
<td>NA</td>
<td>Not available</td>
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<tr>
<td>14</td>
<td>URD</td>
<td>CR2</td>
<td>Ara-C/TBI</td>
<td>4</td>
<td>9</td>
<td>11</td>
<td>PD</td>
<td>t(4;11)(q21;q23)</td>
</tr>
<tr>
<td>21</td>
<td>Auto</td>
<td>CR3</td>
<td>Cy/TBI</td>
<td>9</td>
<td>3</td>
<td>5</td>
<td>PD</td>
<td>Inadequate study</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not applicable; LPD, lymphoproliferative disease; PD, progressive disease; Haplo, haplidentical family donor; MSD, matched sibling donor; URD, unrelated donor; Auto, autologous donor; CR, complete remission.
4/FEL gene. Limited work has been performed at the genomic level; however, breakpoints in AF-4 are distributed over 38 kb, which is a large genomic area compared with the breakpoint clustering seen in the MLL gene. 55 MLL/AF-4 fusion transcripts have been reported so far by several investigators; all have shown the fusion of MLL to one of three AF-4 exons. The RT-PCR primers used in this study encompass this area and an area 400 bp downstream as well. Nonetheless, RT-PCR primers designed based on these data may miss a rare breakpoint elsewhere in the AF-4 gene or one involving a different gene on chromosome 4. Thus, treatment decisions based on this test alone must be carefully considered, and Southern blot for MLL rearrangement remains the important test detecting poor-prognosis patients.

MLL gene rearrangement is clearly associated with age less than 6 months and hyperleukocytosis, and it portends a poor prognosis in infants. However, it remains unclear whether infants with germline MLL are a group with a good prognosis. Chen et al reported an 80% EFS at 4 years for infants with germline MLL treated on CCG protocols; others report an EFS as low as 50% for infants with germline MLL. Because of this discrepancy, we have updated and extended the results for the CCG infants and report that our germline group continues to do extremely well, with only 1 relapse among 11 infants. Different groups may be seeing other high-risk translocations among their infants without MLL rearrangement; there were no such translocations in our germline infants. Because therapy is intensified for infants with ALL, those without MLL gene rearrangement should be excluded from more toxic therapy.

Our group of infants with MLL gene rearrangement has done poorly even with more aggressive management; 16 of the 29 infants were treated on the recently completed protocol 1883 consisting of aggressive cytoreductive therapy administered immediately after induction and again with a reinstitution. They continue to relapse early. In collecting follow-up data on these infants, we ascertained the salvage therapy used for relapsed patients. Table 3 shows the outcome for those infants salvaged with BMT (and also shows 2 treated in first remission). Table 4 shows outcomes for patients treated with chemotherapy. Two patients receiving transplants in CR2 remain disease-free 13 and 14 months, respectively, after relapse; the others died of subsequent relapsed disease (5/6) or of lymphoproliferative disease (1/6). The median postrelapse survival was 310 days for those receiving transplants. Of those treated with chemotherapy, 1 patient still survives 59 months from relapse. The median postrelapse survival was 140 days, and all but 1 died of progressive disease.

![Fig 2](https://example.com/fig2.png)

**Fig 2.** Detection of MLL/AF-4 fusion transcripts in patients on therapy. (A) t(4;11) RT-PCR products after Southern blotting. (B) β-actin PCR products, ethidium bromide staining. Spl, spleen; Liv, liver. The timing of each specimen relative to therapy is described in the text.
Resistant disease, and not toxicity of therapy, remains the problem for both forms of salvage therapy. This raises the question of timing of BMT. Of 2 patients receiving transplants in first remission (Table 3), 1 patient had a relapse within 2 months (BMT 2.5 months after achieving CR) and 1 patient who received an unrelated donor BMT (6 months after CR) remains free of disease 38 months after BMT and 47 months from diagnosis.

The preliminary results we present here of the assessment of minimal disease in t(4;11) leukemia are from a small but intriguing group of patients. Residual disease was detected in some patients in morphologic remission even after intensive therapy including BMT. These findings will be pursued in further studies of minimal disease using quantitative techniques in infants on therapy.

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We thank the clinicians who submitted specimens from their patients to the CCG ALL reference laboratory. The following clinicians referred the patients new to this study: Dr. Jerry Finklestein, Dr. Jeffrey Geyer, Dr. Stuart Gold, Dr. Martha Greenwood, Dr. Neil Grossman, Dr. Christopher Moertel, Dr. Joseph Neglia, Dr. Mark Nesbit, Dr. Greg Reaman, Dr. Margaret Robinson, Dr. Frederick Ruymann, Dr. Leticia Valdez, and Dr. Michael Willoughby. Thanks also to the CCG Clinical Data Managers who were very instrumental in collecting follow-up data.

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