Pre-Pre-B Acute Lymphoblastic Leukemia: High Frequency of Alternatively Spliced ALL1-AF4 Transcripts and Absence of Minimal Residual Disease During Complete Remission


We used the polymerase chain reaction (PCR) to detect ALL1-AF4 rearrangements, the molecular hallmark of t(4;11), in a series of 48 pre-pre-B (CD19<sup>+</sup>, CD24<sup>+</sup>, CD10<sup>-</sup>/CD20<sup>-</sup>/cyt<sup>M</sup>/slg<sup>M</sup>) acute lymphoblastic leukaemias (ALL). Eighteen patients (39%) exhibited fusion transcripts including 4 ALL1-AF4 rearrangements, the molecular hallmark of t(4;11). In contrast, only two patients from a group of 67 common (CD19<sup>+</sup>/CD10<sup>-</sup>, cyt<sup>M</sup>/slg<sup>M</sup>) and pre-B ALLs (CD19/cyt<sup>M</sup><sup>+</sup>, CD10<sup>+</sup>, slg<sup>M</sup>) showed ALL1-AF4 mRNA. All PCR-positive cases showed multiple amplification products representing alternative splicing events. Moreover, reciprocal (4) -derived AF4-ALL1 transcripts were observed in 65% of the cases analyzed. Eight of the 18 pre-pre-B ALL patients with an ALL1-AF4 recombination are currently in complete continuous remission for up to 54 months (median, 26 months). Twelve remission samples were available from seven cases, and all of them lacked evidence of minimal residual disease. Overall this study documents a similarly high incidence of ALL1-AF4 recombinations in children (infants excluded) and adults with pre-pre-B ALL and demonstrates the decline of the leukemic cell clone below the detection level of PCR in a remarkable proportion of patients under intensive treatment protocols.

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MATERIALS AND METHODS

Patients. The 113 B-cell precursor ALL patients (41 children, 62 adults) included in the present study were treated according to the German prospective multicenter ALL trials for children (ALL/NHL Berlin-Frankfurt-Münster (BFM)) and adults (ALL/AUL Bundesministerium für Forschung und Technologie (BMFT)).<sup>5,6</sup> We investigated all 46 pre-pre-B ALL (12 children, including four infants and 34 adults) diagnosed between April 1989 and July 1993, of whom adequate cryopreserved cell material had been stored at the central immunologic laboratory of the trials and compared the incidence of ALL1-AF4-positive cases between this group of patients and 59 common ALLs (38 children, including one infant and 21 adults) as well as eight pre-B ALL (one child, seven adults) diagnosed over the same period.

Immunophenotyping. Fresh bone marrow or peripheral blood samples were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and cell-surface antigens were detected by a standard indirect immunofluorescence (IF) assay, as previously described.<sup>5</sup> Non-specific binding was avoided by adding heat-inactivated 10% rabbit serum in both the first and second incubations. Leukemic cells were evaluated for IF by flow cytometry (FACScan, Becton Dickinson Immunocytometry Systems, Mountain View, CA), and data acquisition was performed with FACScan Research or Lysis II software (Becton Dickinson). Isotype identical nonreactive monoclonal antibodies (MoAbs) were used as negative controls.

The panel of MoAbs included antilymphoid (CD3/Leu-4, CD7/
Leu-9, CD19/HD37, CD20/B1, CD24/OKB2), antimyeloid (CD13/Myl7, CD33/Myl9, CD65/VIM-2), and nonlineage-specific reagents (CD10/JS, CD34/Myl0, HLA-DWOKal), all available from commercial sources.

For cytoplasmic (cy) and nuclear staining, cytospin preparations of leukemic blasts were fixed in acetone (cyCD3, cyIgM) or methanol (terminal deoxynucleotidyl transferase, TdT), subsequently applied by using VIM-2 (CDw65) and HD37 (CD19) MoAbs conjugated with fluorescein isothiocyanate and phycoerythrin, as described elsewhere.23

All cases were B-lineage in origin. On the basis of their pattern of reactivity, leukemic blasts were classified as pre-pre-B (CD19+, CD10-, CD20-, cyIgM-), common (CD19+, CD10+, cyIgM+), and pre-B ALL (CD19+, CD10+, cyIgM+, surface IgM+).24

PCR amplification and sequencing. RNA was isolated from cryopreserved leukemia cells by standard techniques. Ten micrograms of genomic DNA was digested with BamHI or HindIII, separated in a 0.6% agarose gel, denatured, and blotted onto nylon filters (Nytran, Schleicher and Schuell, Dassel, Germany). Filters were hybridized to the 5' and 3' ALL1 probes pEX5/GEB13 and pL from a total of 20 pL cDNA, 30 pmol of each 5' and 3' oligonucleotide primer, 0.2 mmol/L of each deoxynucleotide triphosphate, 10 mmol/L TRIS-HCl pH 8.3, 50 mmol/L KCl, 2.5 mmol/L MgCl2, 0.001% gelatin (w/vol), and 1 unit Taq polymerase (Amplitaq, Perkin-Elmer, Norwalk, CT). Denaturing, annealing, and extension steps were performed at 93°C for 60 seconds, 60°C for 90 seconds, and 72°C for 90 seconds, respectively, in an automatic PCR processor (BioMed, Theres, Germany) including an initial 3-minute denaturation step at 93°C and a final extension step at 72°C for 10 minutes. Two microliters of the first round were used in a second round. For the analysis of ALL1-AF4 products in leukemia cell samples, both PCR rounds comprised 25 and 20 cycles, respectively. Dilution experiments determined the detection level of leukemia cells under these conditions as 1 in 10^5 cells. To increase the sensitivity of the PCR approach for the detection of residual leukemia cells in remission samples to 10^-3 to 10^-6, we applied 35 and 30 cycles, respectively. The quality of RNA was controlled by amplification of AF4 sequences in a single round of PCR with 35 cycles. For the analysis of ALL1-ENL fusion transcripts, we used 35 cycles (first round) and 25 cycles (second round), respectively. PCR products (30 μL) were visualized by electrophoresis in 2.5% agarose gels stained with ethidium bromide. Scrupulous precautions were taken to avoid contamination.25

Oligomers were prepared using the solid-phase phosphate triester method according to ALL1,26 AF4,10,11,16 and ENL119 sequences. One ALL1 primer was biotinylated at the 5' end to allow single strand isolation. Primer positions are indicated in Fig 1. We used the follow-
ALL1-AFI TRANSCRIPTS IN PRE-PRE-B ALL

**Table 2. Immunophenotype of Patients With ALL1-AF4 Fusion Transcripts**

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Results are expressed as percent positive cells. Abbreviation: ND, not done.

**RESULTS**

Frequency of ALL1-AF4 recombinations. To estimate the frequency of ALL1-AF4 rearrangements in pre-B ALL, we performed RT-PCR analysis in a relatively large series of 46 cases comprising 34 adults and 12 children (including four infants). Four children (32%) and 14 adults (41%) exhibited ALL1-AF4 transcripts. Clinical and immunophenotypic features of these patients (cases 1 through 18) are shown in Tables 1 and 2. Our data document a significant association of the ALL1-AF4 recombination with coexpression of myeloid associated antigens, in particular CDw65 (P < .001), compared with pre-B ALL patients without this genetic anomaly (Table 3). Moreover, a highly signifi-

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For sequence analysis major PCR fragments were cut out from the gel and purified with Qiaex gel extraction kit (Qiagen Inc, Chatsworth, CA). A portion of the purified product was used for 45 minutes at room temperature in 30 μL STE (100 mmol/L NaCl, 10 mmol/L TRIS-HCl pH 8.0, 1 mmol/L EDTA pH 8.0) washed Dynabeads M280 streptavidin (Dyana, Hamburg, Germany). Dynabeads were collected via a DynaM MPC concentrator, washed in 200 μL 1 X STE, incubated with 100 μL 0.15 N NaOH for 15 minutes at room temperature, and washed in 100 μL 0.15 N NaOH. Finally, single-stranded templates bound to the beads were washed twice in 200 μL H2O and resuspended in 10 μL H2O. The supernatant of the desiccation step was directly precipitated with 0.2 mol/L Na-acetate pH 4.2, glycogen and 2.5 volume of ethanol, washed in 70% ethanol, and finally resuspended in 10 μL H2O. Minor amplification products were cloned into the pMOS blue T-vector according to methods described by the manufacturers (Amersham, Buckinghamshire, UK). We used the sequencing kit (Pharmacia, Freiburg, Germany) following the instructions of the manufacturer together with the AF4/2 and ALL1/6 sequencing primers for analysis of the beads fraction and the ALL1/5 and AF4/3 primers for sequence analysis of the supernatant fraction. Statistical analysis. Significance of the differences in leukocyte count and coexpression of myeloid antigens at the time of diagnosis between patients with and without ALL1-AF4 recombination was examined by the Wilcoxon-Mann-Whitney test and chi-square tests, respectively.
cant difference (P value .0055) was observed between the leukocyte count of ALL1-AF4-positive cases (mean 199,100/μL ± 36,586, median 176,000/95% CI: 87,000 to 231,000) versus ALL1-AF4-negative pre-pre-B patients (mean 96,381/μL ± 34,883, median 11,550/95% CI: 7,300 to 24,500). To compare the incidence of ALL1-AF4 rearrangements with pre-pre-B ALL and other B-cell precursor subtypes, we also analyzed 59 common ALL and eight pre-B ALL patients. Only one ALL patient of each group, however, showed this genetic anomaly (cases 19 and 20, Tables 1 and 2). Karyotypic data were available from eight of the 20 ALL1-AF4-positive patients. Only one ALL1-AF4-negative pre-pre-B ALL, of whom cytogenetic results were not available from these leukemias, but PCR analysis (not shown) disclosed in one patient an ALL1-ENL rearrangement corresponding to a t(11;19).

Alternatively spliced fusion transcripts. We next characterized the PCR fragments of the 20 ALL1-AF4-positive cases by sequence analysis. In virtually all patients, we observed one major amplification product together with one, two, or occasionally three additional fragments (Table 4, Fig 3). Overall, eight ALL1-AF4 versions were detectable. This large number of differently sized fusion transcripts results from alternative splicing events. Thus, case 1 (Fig 3) exhibits 669-bp, 555-bp, and 423-bp fragments representing ALL1-AF4 exons 8-b, 7-b, and 6-b junctions, respectively. Alternatively heterogeneous pattern is observed in the other patients (Fig 3), eg, case 13 (423-bp and 349-bp fragments corresponding to 6-b and 5-b junctions), case 8 (513-bp, 423-bp, and 381-bp fragments representing 7-c, 6-b, and 6-c junctions), case 15 (555-bp, 513-bp, and 423-bp fragments corresponding to 7-b, 7-c, and 6-b junctions), and case 16 (381-bp and 307-bp fragments representing 6-c and 5-c junctions). Moreover, ALL1-AF4 junctions containing AF4 exon b exist in two versions because of the alternate usage of two adjacent CAG trinucleotides at the splice acceptor site of this exon (not shown).16,14,16,13

We also investigated the expression of reciprocal recombination products from the der (4) chromosome. Sufficient RNA was available from 17 of the 20 ALL1-AF4-positive patients and showed AF4-ALL1 transcripts in 11 cases (65%). Similar to the situation on the der (11) chromosome, multiple amplification products emerged per patient from the der (4) fusion because of alternative splicing. Sequence analysis of the major products showed AF4-ALL1 RNA species, which generally correspond precisely to the reciprocal transcripts from the der (11) chromosome, although skipping of one or two ALL1 exons was observed in patients No. 4 through 6 and 19 (Table 4).

Detection of minimal residual disease. Nine of the 20 ALL1-AF4-positive leukemia patients are presently in con-
Fig 2. Detection of ALL1 rearrangements by Southern blot analysis. DNA samples (10 μg) were obtained from patient 9 (a), a healthy proband (b) and three ALL patients (c through e) who were ALL1-AF4-negative according to PCR analysis. HindIII (A) and BamH1 digests (B) were hybridized to an ALL1-specific probe, pEX5/GEB13, showing 12-kb and 8.4-kb germline fragments (dashes), respectively. Rearranged ALL1 fragments are indicated by dots.

Table 5. Minimal Residual Disease in Seven Patients With ALL1-AF4 Transcripts

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<th>PCR Result</th>
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</tr>
<tr>
<td>10</td>
<td>52 (46)</td>
<td>BM</td>
<td>--</td>
</tr>
<tr>
<td>11</td>
<td>29 (22)</td>
<td>PB/BM</td>
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</tr>
<tr>
<td>13</td>
<td>9 (4)</td>
<td>PB/BM</td>
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DISCUSSION

In this study, we show a significantly higher frequency of ALL1-AF4 recombinations in ALL patients with a pre-pre-B phenotype as compared with other B-cell precursor leukemias. Taking into account overall incidences of 5% and 11% of the pre-pre-B subtype in the ongoing German multicenter trials for children and adults, respectively, our results match well with cytogenetic analyses that have identified a t(4;11) in 2% to 4% of ALL patients.1,2 ALL1 at 11q23 is notorious for its promiscuous involvement in more than 20 different chromosomal abnormalities.3 Along this line, 10.6% (3 of 28) of the ALL1-AF4-negative pre-pre-B leukemias in the continuous complete remission (Table 1). Four of them received an autologous or allogeneic bone marrow transplant (BMT) in the course of treatment. We were able to obtain 12 BM and PB samples of seven pre-pre-B ALL patients for the analysis of residual leukemia (Table 5). A series of dilution experiments using cell lines RS 4;11 and MV 4;11 established that our PCR conditions reliably allow the detection of one ALL1-AF4-positive cell among $10^5$ to $10^6$ cells (Fig 4). Interestingly, all 12 remission samples lacked evidence of ALL1-AF4 fusion transcripts in repeated experiments including Southern transfer from respective gels and consecutive hybridization with ALL1 probes. Control analyses of the same samples readily detected AF4 amplification products even under standard PCR conditions (Fig 4).
present study showed an ALL1 recombination. In one patient, the fusion partner could be identified as the ENL gene on 19p13. Thus, it is possible that about 45% of pre-pre-B ALL may exhibit ALL1-recombinations. Our data also show a significant association of ALL1-AF4-positive ALL with distinct clinical and phenotypic characteristics, namely hyperleukocytosis and coexpression of myeloid markers, thereby confirming previous reports based on cytogenetic or molecular investigations.

The ALL1-AF4 fusion generates a large set of alternatively spliced transcripts. In our series, sequence analysis identified eight different versions representing combinations of ALL1 exons 5, 6, 7, or 8 with AF4 exons b or c. Additional heterogeneity stems from two adjacent CAG trinucleotides at the intron-exon-boundary of AF4 exon b resulting in minor codon variation in respective splice junctions. In every patient, we identified two or three RNA species with predominant expression of one transcript. The biologic significance, if any, of this heterogeneity among ALL1-AF4 RNA species remains enigmatic. Moreover, the leukemogenic potential of ALL1-AF4 products still awaits formal proof by in vitro or in vivo studies. Transcription from the reciprocal der(4) chromosome represents a less consistent event and has been found in 11 of 17 cases (65%) in our study and in 16 of 19 patients (84%) in a recent report. At the present time, the oncogenic potential of reciprocal AF4-ALL1 or RARα-PML products has not been defined by in vitro or in vivo assays. Therefore, their role in leukemogenesis is far from being clear.

The t(4;11) in ALL has been associated with a very poor prognosis. However, recently various high-risk leukemia subgroups have improved substantially following the introduction of intensified treatment strategies. This issue cannot sufficiently be addressed in the present study and awaits prospective evaluation in the ongoing multicenter trials. Yet, it appears noteworthy that nine of the 19 ALL1-AF4-positive leukemias with adequate follow-up remain in complete remission for up to 54 months (median, 26 months). This outcome may be partly attributed to the fact that four patients received marrow transplants.

The most interesting result of this report emerges from PCR analysis of BM and PB samples obtained from seven patients in complete remission. In fact, none of the 12 samples showed evidence for minimal residual disease. This observation in conjunction with a recent report on serial PCR analysis in two ALL1-AF4-positive ALL patients demonstrates the possibility to reduce the leukemia clone below the detection level of PCR. These data may encourage current efforts to induce and maintain remissions in respective patients by intense protocols. Along the same line PCR-negativity has recently been established as a therapeutic goal in acute promyelocytic leukemia. The clinical significance of PCR monitoring in other leukemia entities is less clear. Thus, virtually all acute myeloid leukemia patients characterized by the t(8;21) show persistence of AML1-ETO-positive cells even in long-term remission. Also, in chronic myeloid leukemia (CML) patients treated by interferon (IFNα), BCR-ABL-positive cells generally remain detectable during complete cytogenetic and clinical remission. Yet another situation exists in CML patients after allogeneic BMT. Here, minimal amounts of neoplastic cells may persist for years depending on immunologic factors, but eventually become eradicated in long-term survivors. Overall, these data emphasize the view that it is important
to evaluate the significance of minimal residual disease independently for each hematopoietic neoplasm, taking into account biologic and genetic discrepancies, as well as different treatment modalities.

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This work is dedicated to Professor Bernhard Kubanek on the occasion of his 60th birthday.

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