To investigate the relationship of bcr-ab\textsubscript{1} fusion mRNAs with childhood acute lymphoblastic leukemias (ALL), we examined 27 pediatric Philadelphia chromosome (Ph\textsuperscript{1})-positive acute leukemias using a reverse polymerase chain reaction (PCR) procedure. In cells from 24 leukemias, single bcr-ab\textsubscript{1} PCR products were detected that corresponded to breakpoints in the minor breakpoint cluster region (mbcr in intron 1 of the bcr gene) associated with production of the P190 fusion protein. Cells from the three remaining leukemias contained breakpoints in the major breakpoint cluster region (Mbcr) as shown by PCR and Southern blot analyses. These three leukemias also contained low levels of the mbcr PCR product that may have resulted from alternative splicing of the bcr-ab\textsubscript{1} precursor RNA. A screen of 35 additional leukemias from patients who failed therapy before day 180 (induction failures or early relapses) found one case with unsuccessful cytogenetics to express Mbcr-ab\textsubscript{1} RNA. All four children with Mbcr breakpoints had white blood cell levels in excess of 250,000 at presentation (compared with 2 of 24 with mbcr breakpoints) and two had hematologic and clinical features suggestive of chronic myelogenous leukemias (CML) in lymphoid blast crisis. Our results indicate that in Ph\textsuperscript{1}-positive pediatric leukemias, all 9;22 breakpoints occur in one of the two known breakpoint cluster regions in the bcr gene on chromosome 22. The reverse PCR reliably detected all patients with cytogenetic t(9;22) and is capable of detecting additional Ph\textsuperscript{1}-positive leukemias that are missed by standard cytogenetics. Furthermore, the Mbcr-type breakpoint, associated with production of p210, can be seen in childhood leukemias presenting either as clinical ALL or as apparent lymphoid blast crisis of CML, suggesting that t(9;22) breakpoint locations do not exclusively determine the biologic and clinical features of pediatric Ph\textsuperscript{1}-positive ALL.

THE PHILADELPHIA chromosome (Ph\textsuperscript{1}) is observed in over 95% of adult chronic myelogenous leukemias (CML), 15% to 20% of adult acute lymphoblastic leukemias (ALL), 3% to 5% of pediatric ALL, and rarely in adult acute myelogenous leukemias (AML).\textsuperscript{1,2} The Ph\textsuperscript{1} chromosome results from a reciprocal exchange of genetic material between the long arms of chromosomes 9 and 22 with breakpoints in the abl and bcr genes, respectively.\textsuperscript{3,4} This exchange creates a fusion gene containing the 5\textsuperscript{'} portion of bcr and most of the abl gene. Although cytogenetically all Ph\textsuperscript{1}’s appear the same, at the molecular level there appear to be two widely separated areas in bcr where translocation breakpoints occur, one primarily (but not exclusively) associated with CML and the other with ALL. The former, referred to as the major breakpoint cluster region (Mbcr), lies within one of two introns between bcr exons 11-13; the other occurs within the first intron of bcr, a region referred to as the minor breakpoint cluster region (mbcr).\textsuperscript{5,6} This difference accounts for the variability in the number of bcr exons in the fusion transcript although abl exon 2 always serves as the acceptor exon that is fused to bcr sequences for each type of breakpoint.

Two different mRNAs may be transcribed from the fused bcr-ab\textsubscript{1} genes, one of 8.0 kb resulting from Mbcr breakpoints and the other 7.0 kb from mbcr breakpoints.\textsuperscript{7,8} Variability in splicing of transcripts from the Mbcr-type translocation gives rise to microheterogeneity of transcripts, either containing or lacking exon 12.\textsuperscript{9,10} The fusion Mbcr and mbcr transcripts code for proteins with altered tyrosine kinase activities designated p210 and p190, respectively.\textsuperscript{11,12} Gene transfer studies showed that the p190 induces a more aggressive phenotype consistent with its role in ALL.\textsuperscript{13} However, a controversial and unsettled issue is whether there are biologic and clinical differences associated with expression of p210 versus p190 in Ph\textsuperscript{1}-carrying leukemias as determined by the Mbcr and mbcr breakpoint locations.

Although only 3% to 5% of pediatric ALLs carry a Ph\textsuperscript{1} detected by cytogenetic analysis, its presence is associated with a poor prognosis as patients with Ph\textsuperscript{1}-positive leukemias have a much poorer treatment outcome that patients who are Ph\textsuperscript{1} negative.\textsuperscript{1} Given the prognostic significance of Ph\textsuperscript{1} in pediatric ALL, it appears important to reliably detect this cytogenetic abnormality because alternative treatment strategies (such as early bone marrow transplantation) may improve outcome.\textsuperscript{14} However, cytogenetic studies, the conventional means of Ph\textsuperscript{1} identification, are not always successful. Alternatively, Southern blot analyses for bcr gene rearrangements are useful for detecting Mbcr-type breakpoints but are limited in their ability to detect mbcr-type breakpoints as they presumably occur over a large region within the first intron of the bcr gene.\textsuperscript{5,15,16} Pulsed-field gel analysis may be a more accurate means of molecular diagnosis but it is quite cumbersome and labor intensive.\textsuperscript{17,18} More recently, the polymerase chain reaction (PCR) has

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been shown to be a useful means of identifying the bcr-abl fusion mRNA in Ph1-carrying cell lines and patient materials.23 Because mRNA does not contain introns, the technique is oblivious to the large first intron of bcr. However, it is not clear from previous investigations whether all Ph1 and variant breakpoints occur in the two known chromosome 22 breakpoint cluster regions and, thus, whether PCR as currently performed can detect most or all bcr-abl fusion products.24

The current study was undertaken to determine the distribution of t(9;22) breakpoints on chromosome 22 in pediatric Ph1-positive acute leukemias to establish the frequency of bcr gene involvement and to assess the potential biologic and clinical differences associated with the various breakpoint locations.

MATERIALS AND METHODS

Patients and leukemia samples. All but one (see no. 26 below) patient in this study met the standard criteria for ALL. Frozen bone marrow and peripheral blood specimens from pediatric ALL patients were obtained from the Pediatric Oncology Group (POG), St Louis, MO. Fresh specimens from patients 16 and 17 were obtained from the pediatric oncology service of The Children's Hospital at Stanford, CA. A follow-up remission sample from patients was obtained from the Pediatric Oncology Group, St Louis, MO. Fresh specimens from patients 16 and 17 were obtained from the Pediatric Oncology Group and the oncology service of The Children's Hospital at Stanford. Additional clinical information (see below) on selected patients was provided by their physicians.

Case reports. Patient 25 was a 14-month-old white male who presented with a 2-week history of pallor, bruising, and fatigue. Physical examination showed hepatosplenomegaly and cervical/axillary adenopathy. A complete blood cell (CBC) count disclosed: hemoglobin (Hb) 6.9 g/dL, hematocrit (Hct) 18%, platelets 21 × 10^10/L, and white blood cells (WBC) 647 × 10^9/L (97% blasts, 2% neutrophils, and 1% bands). Lumbar puncture showed evidence of central nervous system (CNS) leukemia. Bone marrow aspirate showed ALL (non-B/non-T). The patient was enrolled in a POG pilot treatment study (ALInF-C) and karyotyping showed the Ph1 chromosome (see Table 1). The patient entered a complete remission; marrow karyotype in remission on several occasions was 46,XY with no evidence of Ph1. The patient experienced two CNS relapses but currently continues in first marrow remission and third CNS remission, 34 months after initial diagnosis.

Patient 26 was a 12-year-old white female who presented with a several week history of headache, abdominal pain, fatigue, and weight loss. A CBC count showed: Hb 11.0 g/dL, Hct 29.5%, platelets 121 × 10^9/L, and WBC 396 × 10^9/L (34% lymphocytes, 12% neutrophils, 17% bands, 3% monocytes, 19% metamyelocytes, 12% promyelocytes, and 3% blasts). Bone marrow aspirate showed 12% blasts; cytogenetic study showed 46,XX,t(9;22) (q34;

### Table 1. Clinical Hematologic, and PCR Data Obtained From Pediatric Acute Leukemias

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*+ denotes presence of specific PCR product as detected by hybridization to internal oligonucleotide probe; (+), specific PCR product present at very low levels; -, indicates absence of specific PCR product as detected by hybridization analysis with an internal oligonucleotide.*

Clinical data were provided by the statistical office of the Pediatric Oncology Group and the oncology service of Children's Hospital at Stanford. Additional clinical information (see below) on selected patients was provided by their physicians.
The patient was felt to have CML and treatment with hydroxyurea was initiated. Four weeks after initial presentation a CBC count showed: Hb 6.5 g/dL, platelets 5 x 10^11/L, and WBC 46.9 x 10^9/L (2% neutrophils, 1% bands, 5% lymphs, 1% monos, 40% blasts, and 51% immature granulocytes). A bone marrow aspirate showed 70% lymphoblasts, and karyotyping confirmed that the Ph' chromosome was still present (see Table 1). The patient was excluded from the treatment study because of the preceding “chronic” phase and the prior administration of chemotherapy. The patient then received ALL induction chemotherapy and achieved a complete remission. Cytogenetic studies were not performed at this time. Seven months after initial presentation, a routine bone marrow aspirate showed 13% lymphoblasts, and 20 of 20 cells were Ph'-positive. A second remission was induced but the patient relapsed again and died of progressive leukemia.

The patient underwent transplantation, but died approximately 8 weeks post-BMT from pulmonary aspergillosis.

Patient 28 was an 11-1/2-year-old white male who presented with a 4-week history of headache and upper respiratory infection followed by progressive fever, weakness, and pallor. Physical examination disclosed no adenopathy or hepatosplenomegaly. A CBC count showed: Hb 9.0 g/dL, HCT 26.3%, platelets 110 x 10^11/L, and WBC 256 x 10^9/L (86% blasts, 1% lymphocytes, 1% monocytes, and 1% bands). Lumbar puncture showed no evidence of CNS leukemia. A bone marrow aspirate disclosed CALLA positive ALL. Karyotyping was performed and showed the t(9;22) (see Table 1). The patient entered a complete remission and remained in hematologic remission. Because of high-risk features, she was referred for allogeneic bone marrow transplant (BMT) from her HLA-identical sibling. Immediately before BMT a bone marrow aspirate showed her to be in complete remission, but cytogenetics showed 19 of 20 metaphases to be Ph'-positive. The patient underwent transplantation, but died approximately 8 weeks post-BMT from pulmonary aspergillosis.

Cytogenetics. Cytogenetic analyses were performed using standard techniques at the POG reference laboratory at Birmingham, AL. Cell samples (usually bone marrow) were placed in sterile tubes containing RPMI 1640 supplemented with 15% fetal calf serum (FCS) and shipped overnight to the reference cytogenetics laboratory. On arrival, cells were placed in fresh medium and subjected to short-term (24-hour) culture. Routine methods were used for culture harvest, slide preparation, and Giemsa-Trypsin-Wright (GTW)-banding. Chromosomes were identified and assigned according to an International System for Human Cytogenetic Nomenclature. Initial studies on patient 17 were performed at an outside hospital and confirmed on relapse samples at Stanford.

RNA isolation. WBC were isolated from fresh blood or bone marrow specimens by lysing red blood cells in a 10 to 1 solution of 0.14 mol/L NH₄Cl and 0.01 mol/L NH₄HCO₃ and washing with phosphate-buffered saline (PBS). RNA was isolated using the guanidinium-acid phenol method. Reverse transcription and PCR. Part of the total cellular RNA, 2.25 μg, was converted to single-stranded cDNA, which was then subjected to 36 cycles of PCR using primers and conditions described elsewhere to detect the Mbcr-type transcripts. An oligonucleotide primer (AL22) corresponding to nucleotides 205-225 of the bcr first exon was used in conjunction with the ablX3 oligonucleotide to amplify a 506-bp mbcr fusion product. Reaction products were analyzed by hybridization using an oligonucleotide probe homologous to abl exon 2, which was end-labeled with 32P by polynucleotide kinase. To control for possible contamination, 10 Ph'-negative and 10 Ph'-positive samples were analyzed blind. None of the Ph'-negative samples showed evidence of either type of fusion transcript while all showed amplification of the normal abl transcript. Also, each time PCR was performed, control samples of RNA from two leukemic cell lines were run in parallel. One leukemic cell line was K562, a CML cell line producing a Mbcrr-type transcript, and the other was SUP-B15, an ALL cell line producing a mbcrr-type transcript. False positive results were never observed in these controls or in a blank containing no input RNA. To verify the results on patients 25 and 27, separately frozen aliquots of WBCs were obtained from the POG cell bank and analyzed with fresh reagents in two different laboratories.

Southern blot analysis. DNA was isolated from peripheral blood or bone marrow, digested with restriction enzymes BamHI or BglII, and subjected to Southern blot analysis according to previously described procedures. The probe consisted of a 1.2-kb BamHI-BglII restriction fragment between bcr exons 12 and 13 (Oncogene Sciences, Inc, Mineola, NY) radiolabeled by primer extension with the Klenow fragment of DNA polymerase I.

RESULTS

Twenty-seven cases of pediatric ALL known to carry the Ph' chromosome were analyzed by PCR for the presence of bcr-ab1 fusion transcripts. The patients' ages, WBC counts, and karyotypes at presentation are shown in Table 1. The results tabulated in Table 1 indicate that all samples exhibited at least one of the three forms of bcr-ab1 fusion transcripts. Twenty-four cases showed a single predominant band corresponding to the mbcrr-type transcript, whereas three cases contained a predominant PCR product corresponding to one or both of the Mbcrr-type transcripts.

An autoradiogram from Southern analysis of PCR products from cases 2, 3, 25, 26, and 27 is shown in Fig 1. The A lanes contain the 290-bp fragment generated by amplification of a region within abl exons 2 and 3 that served as an internal positive control. The B lanes contain the 324- or 399-bp product resulting from amplification across an Mbcrr-ab1 fusion transcript, and the C lanes contain the 506-bp fragment resulting from amplification across a mbcrr-ab1 fusion transcript. Cases 2 and 3 illustrate the results seen in 24 of 27 of the Ph'-positive leukemias where bands are seen in lanes A and C. The positive control band in lane A is more intense than the mbcrr-type transcript in lane C, presumably due to amplification of transcripts from both the translocated and nontranslocated abl genes. There was no evidence of Mbcrr-type transcripts (lane B) in these cases.

In cases 25, 26, and 27 the predominant fusion product...
Fig 1. Reverse PCR analysis of bcr-abl fusion transcripts in pediatric ALL. Patient numbers correspond to those listed in Table 1. (A lanes) Reaction products (290 bp) from amplification of exons 2 and 3 of abl transcript (positive control); (B lanes) reaction products (324 and 399 bp) from amplification of bcr-abl fusion due to Mbcr-type breakpoints; and (C lanes) reaction products (506 bp) from amplification of bcr-abl fusion due to mbcr-type breakpoints. Autoradiograph exposures for patients 2 and 3 and for patients 25, 26, and 27 were 1 hour and 3 hours, respectively. Long exposure on patients 25, 26, and 27 demonstrates faint bands in (C lanes). Sizes of reaction products are shown in base pairs.

Further support for Mbcr-type breakpoints in cases 25 through 27 was obtained by Southern blot analyses performed on DNA from the leukemia cells (Fig 2). The 2.8-kb BamHI and 4.75-kb BgIII fragments seen in each sample represent the germline bcr gene from the nontranslocated chromosome 22. At least one nongermline band was seen in all of these cases on both enzyme digests. These bands corresponded to translocated bcr genes whose sizes varied due to variability of breakpoint loci within Mbcr. Southern blot analyses for mbcr-type breakpoints were not performed.

To test the utility of the bcr-abl PCR technique we examined a small series of pediatric leukemias from patients who had failed treatment by day 180 (induction failures or early relapses), as this group of patients would likely be enriched for cases with t(9;22). One leukemia carrying an mbcr-type breakpoint was included as a positive control. The samples were coded and PCR was performed without knowledge as to their identities. Of 35 cases examined, one (no. 28 in Table 1) was found to express bcr-abl fusion transcripts of the Mbcr type with no detectable expression of the mbcr-abl transcript. The internal positive control with bcr-abl transcript expression was also correctly identified. All other samples were negative. Many of these other leukemias (12 of 35) had other high-risk corresponded to an Mbcr-type transcript as judged by the intensity of hybridization bands seen in lanes B versus C in these cases (Fig 1). Although these three cases each contained detectable mbcr-type transcripts (C lanes), the hybridization intensities suggested they were much less abundant than the Mbcr transcripts. Patient 25 showed a single band of 399 bp in lane B corresponding to a fusion transcript containing both bcr exons 11 and 12. Patient 26 contained a single band of 324 bp corresponding to a transcript containing only bcr exon 11. Patient 27 contained both sizes of Mbcr-type transcripts. PCR analysis of remission bone marrow from patient 25 showed no evidence of either type of fusion transcript. Remission samples from patients 26 and 27 were not available.

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cytogenetic features such as t(4;11) (q21;q23) or t(8;14) (q24;q32) that would explain their early treatment failure (data not shown).

DISCUSSION

Ph'-positive ALL is an aggressive disease in which patients have a vastly inferior treatment outcome.20 Because conventional treatment is not effective in Ph'-positive disease, the use of alternative therapeutic modalities such as BMT may provide a higher rate of disease-free survival.20 Thus, accurate diagnosis of Ph'-positive leukemias is essential to providing appropriate treatment. In consideration of these factors, the current study was undertaken to assess the diagnostic and prognostic usefulness of bcr-ab1 fusion transcript expression in Ph'-positive pediatric leukemias.

Our data demonstrate that in all pediatric patients with a cytogenetically documented Ph1 chromosome, reverse PCR analysis reliably detected expression of bcr-ab1 fusion transcripts. Our study, the largest series of pediatric ALLs reported to date, helps establish that chromosome 22 breakpoints in Ph'-carrying childhood leukemias consistently involve the bcr gene. Furthermore, our preliminary screening also showed that some leukemias lacking adequate cytogenetic studies have molecular evidence of bcr-ab1 fusion, confirming the utility of the PCR procedure as an adjunct to conventional cytogenetic studies for diagnosing Ph'-positive ALL.23 PCR is also ideally suited for detection of minimal residual disease in ALL, as our laboratory and others have shown in CML.28 In CML, many patients have been shown to have detectable Mbcr-type transcripts post-BMT but the clinical significance of this is not clear. Our results indicate it should be possible to perform similar studies with all Ph1-positive pediatric ALL patients during the course of their treatment.

The observed consistent involvement of the bcr gene by Ph1 breakpoints in ALL contrasts with the results of one previous study using PCR. Hooberman et al showed that two out of nine cytogenetically Ph1-positive adult ALL patients lacked bcr-ab1 fusion transcripts by PCR even though genomic analysis would have predicted a fusion transcript.22 It is not clear whether the negative PCR results were due to a low ratio of leukemic to normal cells or an idiosyncratic failure of the oligonucleotides to amplify bcr-ab1 fusion products in a subset of patients. By Southern blot techniques, Heisterkamp et al showed only five of nine Ph1-chromosome positive ALLs to contain bcr gene rearrangements using several bcr probes.34 In both studies, the cases showing lack of bcr gene involvement were leukemias arising in adults, whereas we studied pediatric ALLs exclusively. Our results are consistent with previous studies on smaller numbers of pediatric Ph1-positive ALLs showing bcr gene involvement in most or all cases.21

An unexpected finding in this study was the simultaneous presence of both Mbcr- and Mbcr-type transcripts in 3 of 28 patients. Expression of additional bcr-ab1 fusion species may be involved in leukemia progression and has been reported previously in a case of adult Ph1-positive ALL.24 The presence of an additional Ph1 chromosome could theoretically account for this phenomenon, but of the three patients exhibiting both Mbcr- and Mbcr-type transcripts, only no. 27 was mosaic for +Ph1. There is only one report where gain of Ph1 resulted from a second rearrangement and this case was not studied for sites of RNA fusion.33 Our studies cannot rule out the possibility that other cases expressing dual fusion transcripts were mosaic and contained low numbers of cells with +Ph1 that were not detected cytogenetically. PCR contamination is unlikely based on the precautions used (see Materials and Methods for description).

Alternative splicing of a bcr-ab1 fusion precursor RNA would appear to be a more likely explanation of dual transcript expression in the leukemias studied here. In a previous study, this mechanism was suggested to give rise to a novel fusion transcript 411 bp shorter than what was predicted by the configuration of the fusion gene in a patient with CML.28 If alternative splicing gives rise to coexistent Mbcr- and mbcr-type transcripts, they would only be observed if the 9;22 crossover occurred in the Mbcr, as was the case in our three patients. Splicing out of sequences for exons 2 through 12 from an Mbcr-ab1 precursor RNA would produce an mbcr-ab1 mature mRNA possibly reflecting the unusual splice-acceptor properties of abl exon 2.27 Precursor RNA from an mbcr-type translocation would not contain sequences for exons 2 through 12 and could therefore not generate an Mbcr-ab1 transcript by alternative splicing. This fact and the observation that none of 24 mbcr breakpoint patients expressed dual transcripts is consistent with their origin from alternative splicing as opposed to a second t(9;22) event. Alternative splicing could also explain the dual expression of both species of Mbcr transcripts as seen in patient 27.28-30

Our observations that 27 of 28 ALL patients expressed mbcr-type transcripts, even if in very small quantities, appear to be supportive of previous suggestions that the P190 product is necessary for induction of acute leukemia,41 especially in light of its enhanced tyrosine kinase activity compared with the p210 product.19 However, one of our cases (no. 28) with clinical features typical of ALL contained an Mbcr-type breakpoint and did not express detectable P190 transcripts. Mbcr-breakpoint patients have been reported previously in pediatric ALL, but expression of abl transcripts and proteins were not studied to rule out low level P190 products as observed in three of our cases.21 In lymphoid blast crisis of CML, expression of Mbcr-type transcripts with no evidence of mbcr-type transcripts has been reported in two patients and two blast crisis-derived cell lines.42 In another report, two Ph1-positive patients in chronic phase showed only mbcr-type transcripts even though one of the two patients had a DNA breakpoint very near Mbcr.35 These data and our case no. 28 are more consistent with conclusions drawn from recent gene transfer studies comparing the v-abl, P190 bcr-abl, and P210 bcr-ab1 products which showed that because each may induce an acute or chronic leukemia a more important determinant for disease phenotype may be the type of cell
targeted for infection as opposed to the specific amino-terminal ABL truncation.

The presence of Mbcr-type transcripts in 4 of 28 patients raises the issue of whether these patients presented clinically in lymphoid blast crisis of CML following a clinically silent chronic phase. All four patients presented with WBC counts in excess of 250,000, whereas only 2 of 24 patients with mbcr-type breakpoints presented with counts this high. Patient 26 was, in fact, initially diagnosed in chronic phase and rapidly developed lymphoid blast crisis (see Materials and Methods for detailed history). Patient 27 also had features suggestive of CML in lymphoid blast crisis with a high presenting WBC count (416,000) and a differential that showed all stages of myeloid differentiation. Both patients had almost exclusively Ph<sup>+</sup>-positive metaphases at the time they were in complete (no. 27) or very good partial (no. 26) remission, suggesting that their diseases originated in pleuripotent stem cells as opposed to lymphoid progenitors. The atypical clinical findings in conjunction with the molecular data suggest that these two patients presented in lymphoid blast crisis of CML. It would appear from these data that a significant proportion of pediatric ALLs with an Mbcr-type breakpoint (7% of pediatric Ph<sup>+</sup>-positive ALL) may represent blast crisis of CML, a proportion consistent with the overall incidence of Ph<sup>+</sup>-positive CML in childhood.

Our findings indicate that not all ALLs with an Mbcr breakpoint represent blast crisis CML. For example, patient 28 had a clinical history that appeared to be typical of ALL. Another Mbcr patient (no. 25) had no evidence of the Ph<sup>+</sup> on remission cytogenetics nor did he show either type of fusion transcript on PCR analysis of a remission bone marrow sample (data not shown). Thus, it appears from these data that pediatric ALL can be associated with an Mbcr-type breakpoint and Mbcr-abl transcript expression consistent with previous suggestions based on observations in adult ALL and lymphoid blast crisis of CML that breakpoint locations apparently do not determine leukemia phenotype or biology. The molecular determinants of the latter may relate more to the cellular stage at which 9;22 translocation occurs or secondary genetic alterations acquired during leukemogenesis.

In conclusion, molecular analysis for bcr-abl fusion transcripts was feasible in this setting when performed under controlled conditions to minimize known technical limitations. Tests for bcr-abl transcript expression were informative in all cases of pediatric ALL studied and, therefore, should aid in patient management to allow for more timely and appropriate treatment of this very aggressive subset of pediatric leukemias.

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