Gene Expression Profiling of Pediatric Acute Myelogenous Leukemia

Mary E. Ross1*, Rami Mahfouz2*, Mihaela Onciu2, Hsi-Che Liu2,5, Xiaodong Zhou2, Guangchun Song2, Sheila A. Shurtleff2, Stanley Pounds3, Cheng Cheng3, Jing Ma4, Raul C. Ribeiro1, Jeffrey E. Rubnitz1, Kevin Girtman2, W. Kent Williams2, Susana C. Raimondi2, Der-Cherng Liang5, Lee-Yung Shih6, Ching-Hon Pui1,2 & James R. Downing2,#

From the Departments of 1Hematology-Oncology, 2Pathology, 3Biostatistics, and the 4Hartwell Center for Bioinformatics and Biotechnology, St. Jude Children’s Research Hospital, Memphis, TN, 38105, USA, 5Division of Pediatric Hematology-Oncology, Mackay Memorial Hospital, Taipei, Taiwan, and the 6Division of Hematology-Oncology, Chang Gung Memorial Hospital, Taipei, Taiwan.

* These two authors contributed equally to this work and should both be considered as first authors.

This work was supported in part by National Cancer Institute grants P01 CA71907-06 (JRD), CA-21765 (Cancer Center CORE grant to SJCRH), T32-CA70089 and St. Jude Physician Scientist Training Program (MER), NHRI-EX92-9011SL (LYS) and by the American Lebanese and Syrian Associated Charities (ALSAC) of SJCRH. Dr. Pui is the recipient of the American Cancer Society F.M Kirby Clinical Professorship.

#Address reprint requests to: James R. Downing, M.D. at the Department of Pathology, St. Jude Children’s Research Hospital, 332 North Lauderdale, Memphis TN 38105, Tel: 901-495-3510, Fax: 901-495-3749, email: jim.downing@stjude.org

Running Title: Gene expression profiling in pediatric AML
Word Count: Abstract - 200  Paper - 5430
Scientific Heading: Neoplasia
Abstract

Contemporary treatment of pediatric acute myeloid leukemia (AML) requires the assignment of patients to specific risk groups. To explore whether expression profiling of leukemic blasts could accurately distinguish between the known risk groups of AML, we analyzed 130 pediatric and 20 adult AML diagnostic bone marrow or peripheral blood samples using the Affymetrix U133A microarray. Class discriminating genes were identified for each of the major prognostic subtypes of pediatric AML, including t(15;17)[PML-RARα], t(8;21)[AML1-ETO], inv(16)[CBFβ-MYH11], MLL chimeric fusion genes, and cases classified as FAB-M7. When subsets of these genes were used in supervised learning algorithms, an overall classification accuracy of > 93% was achieved. Moreover, we were able to use the expression signatures generated from the pediatric samples to accurately classify adult de novo AMLs with the same genetic lesions. The class discriminating genes also provided novel insights into the molecular pathobiology of these leukemias. Lastly, using a combined pediatric dataset of 130 AMLs and 137 acute lymphoblastic leukemias, we identified an expression signature for cases with MLL chimeric fusion genes irrespective of lineage. Surprisingly, AMLs containing partial tandem duplications of MLL failed to cluster with MLL chimeric fusion gene cases, suggesting a significant difference in their underlying mechanism of transformation.
Introduction

Acute myeloid leukemia is a relatively rare malignancy in the pediatric population, comprising only 15%-20% of the acute leukemias diagnosed in this age group. Nevertheless, it remains a challenging disease with an inferior treatment outcome compared to pediatric acute lymphoblastic leukemia (ALL). Despite the introduction of new drugs, the aggressive use of allogeneic and autologous bone marrow transplantation, and improvements in supportive care, overall cure rates of AML in most contemporary treatment protocols remain below 60%. Further improvements in cure rates are likely to come from a better understanding of both the molecular abnormalities responsible for the formation and growth of the leukemic cells, and the mechanisms underlying drug resistance.

Increasingly, contemporary treatment protocols are incorporating methods for both accurate diagnosis and subsequent risk-stratification. To achieve this requires not only distinguishing myeloblasts from lymphoblasts, but also assessing the extent of lineage commitment and differentiation, as well as the presence of specific molecular lesions or chromosomal abnormalities. Efforts over the last several decades have revealed AML to be a heterogeneous disease, with marked differences in cure rates between various genetic subtypes. Acute promyelocytic leukemia was the first clear example of a clinically distinct AML subtype, being characterized by FAB-M3 morphology and expression of the t(15;17)-encoded PML-RARα fusion protein. Treatment with all-trans retinoic acid, which targets the PML-RARα fusion protein, induces differentiation and significantly improves cure rates when combined with chemotherapy. More recent work has resulted in the classification of AMLs into one of three prognostic or risk groups: favorable, including t(15;17)[PML-RARα], t(8;21)[AML1-ETO], inv(16)[CBFβ-MYH11] and in the pediatric population t(9;11); intermediate, including other MLL chimeric fusion genes or normal karyotypes; and unfavorable, including -5/del(5q), -7/del(7q), or t(8;21)[AML1-ETO].
7/del(7q), inv(3)/t(3;3), +8, and complex karyotypes. An additional poor risk subtype of AML that is seen primarily in the pediatric population is acute megakaryocytic leukemia (FAB-M7).

High throughput parallel expression analysis using DNA-based microarrays has recently been applied to the diagnosis of acute leukemias and to the exploration of their underlying molecular pathology. Work from a number of different laboratories have identified unique expression signatures for the three major subtypes of favorable risk adult AML - t(15;17), t(8;21), and inv(16), and for several rare genetic subtypes of AML. Whether these expression profiles will allow the accurate diagnosis of these specific subtypes of AML in the pediatric population remains to be determined. Similarly, it remains to be determined whether distinct expression signatures exist for some of the standard and high risk forms of pediatric AML.

To address these issues, we utilized oligonucleotide microarrays to analyze the expression of over 22,000 genes in diagnostic leukemic blasts from 130 pediatric AML patient samples using the Affymetrix U133A oligonucleotide microarray. Our data demonstrate that expression profiling is not only a robust approach for the accurate identification of known lineage and molecular subtypes of pediatric AML, but also provides new insights into their underlying biology. In addition, only minimal differences were identified between the expression profiles of pediatric and adult AML cases that contained the same genetic lesions, suggesting that these de novo leukemia subtypes are the same diseases in different age groups. Lastly, a common expression signature was identified for acute leukemias that contain MLL chimeric fusion genes, irrespective of their lineage. This signature provides novel insights into the altered transcriptional program induced by the presence of an MLL fusion gene. Importantly, cases of AML that contain a partial tandem duplication of the MLL gene failed to express this
transcriptional program, suggesting that these leukemias have a mechanism of transformation that differs from that of cases with \textit{MLL} chimeric fusion genes.

**Materials and Methods**

\textit{Samples}

Bone marrow (BM) aspirates or peripheral blood (PB) samples were obtained at the time of diagnosis from pediatric (130) or adult (20) patients with \textit{de novo} AML. Informed consent for the use of the leukemic cells for research was obtained from parents, guardians or patients (as age-appropriate) in accordance with local Institutional Review Board (IRB) guidelines.

Mononuclear cells were purified from the diagnostic BM or PB samples by density gradient centrifugation and cryopreserved in liquid nitrogen. Samples included BM aspirates (n = 139), PB (n = 10), or therapeutic apheresis sample (n = 1). The majority (122/130) of the pediatric samples were from patients that were subsequently treated on SJCRH AML protocols: AML83, AML87, AML91, or AML97. Results of these protocols have been published elsewhere.\textsuperscript{32-35} After 1992, children with t(15;17) positive acute promyelocytic leukemia were not treated on AML studies but instead were treated either on POG9710 or by best clinical management. To increase the number of cases with \textit{PML-RAR}\textalpha{} and \textit{AML1-ETO}, diagnostic samples (3 \textit{PML-RAR}\textalpha{} and 5 \textit{AML1-ETO}) were obtained from pediatric patients in El Salvador that were treated through the International Outreach Program of St. Jude Children’s Research Hospital.

All pediatric AML samples used in this study with the exception of one had a blast percentage $\geq$ 65\% post ficoll purification (Table S1). The average blast percentage for the samples included in this study was 86.9\%. Only 10 samples had blast counts < 75\% and only two
samples had a blast count < 70%. All adult AML samples evaluated in this study had a blast count > 75% post ficoll purification.

The diagnosis and classification of AML was based on morphologic, cytochemical and immunophenotypic criteria according to the revised French-American-British (FAB) classification. The diagnostic samples were also characterized by conventional cytogenetics, RT-PCR assays for PML-RARα, AML1-ETO, CBFβ-MYH11, and for the presence of MLL chimeric fusion genes by at least two of the following methods: cytogenetics, 11q23 fluorescence in situ hybridization (FISH), or RT-PCR for t(9;11)[MLL-AF9], t(11;19)[MLL-ENL], t(11;19)[MLL-ELL], t(10;11)[MLL-AF10], or t(4;11)[MLL-AF4]. Samples that lacked evidence of any of these chromosomal rearrangements were evaluated for the presence of internal tandem duplications of the MLL gene using a previously described RT-PCR-based assay (Table S6).

Gene Expression Profiling

Detailed protocols for RNA extraction, assessment of integrity, and generation of labeled cRNA has been previously described, and can be obtained at http://www.stjude.org/data/ALL1 and http://www.stjude.org/data/ALL3. Arrays were scanned using a laser confocal scanner (Agilent, Palo Alto, CA) and then analyzed with Affymetrix Microarray Suite 5.0 (MAS 5.0). Detection values (present, marginal or absent) were determined by default parameters, and signal values were scaled by global methods to a target value of 500. Minimal quality control parameters for inclusion in the study included greater than 10% present calls and a GAPDH 3’/5’ ratio of ≤ 3. Microarrays included in the study had an average % present call of 38.75% (range 20.2-50.4). Primary data is available at our website http://www.stjude.org/data/AML1.
**Statistical Analysis**

Analysis was performed using two-dimensional hierarchical clustering, principal component analysis (PCA), and discriminant analysis with variance (DAV) (GeneMaths software version 2.01, Applied Maths, Belgium). For class prediction the pediatric dataset was split into training (100 samples) and test (30 samples) sets, which were stratified with regard to AML1-ETO, CBFβ-MYH11, MLL chimeric fusion genes, PML-RARα, and FAB-M7 (Table 1). Prior to analysis a variation filter was applied that removed any probe sets absent in all samples, had a maximum signal value within the dataset less than or equal to 100, or had a maximum – minimum signal value of less than or equal to 100 (Table S5). Significance Analysis of Microarrays (SAM) was performed exclusively using cases in the training set to select class discriminating genes. The discriminating genes were then used in an artificial neural network (ANN) supervised learning algorithms and class assignment accuracies were initially assessed by three-fold cross validation on the randomly selected stratified training set. The true accuracy was then determined on a blinded test group consisting of the remaining one-fourth of the samples. Details of the supervised learning algorithms and their use have been previously described.

**Results**

**Expression profile of genetic risk groups of pediatric AML**

Expression profiles were obtained from diagnostic samples of leukemic blasts from 130 pediatric AML patients using the Affymetrix® HG-U133A microarrays. The leukemia samples had an average blast percentage close to 90%. Cases were selected to provide a representation of the known morphologic, genetic and prognostic subtypes of pediatric AML, and included cases with t(15;17)[PML-RARα], t(8;21)[AML1-ETO], inv(16)[CBFβ-MYH11], MLL chimeric fusion
genes, acute megakaryocytic morphology (FAB-M7), or lacking any of these features (Table 1, Table S1, Table S2). The primary data is available at http://www.stjuderesearch.org/data/AML1.

Table 1. Pediatric AML subgroup distribution.*

<table>
<thead>
<tr>
<th>Genetic subgroup</th>
<th>Training</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PML-RARα</em></td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td><em>AML1-ETO</em></td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>CBFβ-MYH11</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>MLL chimeric fusion genes</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>FAB-M7</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Other</td>
<td>38</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>30</td>
</tr>
</tbody>
</table>

*Cases were randomly distributed into training and test sets, stratifying for genetic subgroups and FAB-M7.

To reduce the complexity of the data a variation filter was applied to remove probe sets that showed minimal variation across the data set (see methods). In an initial analysis of the filtered data using an unsupervised two-dimensional hierarchical clustering algorithm, relatively good clusters were observed for several of the genetic and morphologic AML subtypes, although the tightness of clustering was less than that seen in pediatric ALL (Figure 1, Figure S1, and 26,41). As shown in Figure 1, relatively tight grouping was observed for the genetic subgroups *AML1-ETO, PML-RARα*, and *MLL* chimeric fusion genes, and for the morphologic subgroups FAB-M3, M7, and M4/M5. Unexpectedly, however, AMLs that expressed the inv(16)-encoded *CBFβ-MYH11* failed to cluster using a variety of different unsupervised clustering algorithms. The failure of this subgroup to tightly cluster indicates significant heterogeneity within the gene expression profile of these cases (see below).
Figure 1. **Unsupervised cluster analysis of pediatric AMLs.** Expression profiles of the diagnostic leukemic blasts from 130 cases of pediatric AML were obtained using the U133A Affymetrix microarray. The expression data was then filtered to remove any probe sets that failed to show significant variation in expression across the dataset. The remaining 17,051 probe sets were then used in an unsupervised two-dimensional hierarchical clustering algorithm and the resultant dendogram is shown. Indicated below the dendogram are the genetic subtype and FAB morphology for each case according to the indicated color codes.

We next set out to identify expression signatures for each of the known prognostically important AML subtypes including *AML1-ETO*, *PML-RARα*, *CBFβ-MYH11*, *MLL* chimeric fusion genes, and AML M7. Discriminating genes were selected using SAM on a training set of pediatric AML cases. The numbers of discriminating probe sets per leukemia subtype at a 5% false discovery rate (FDR) were: *AML1-ETO*, 764; *PML-RARα*, 2521; *CBFβ-MYH11*, 63; *MLL* chimeric fusion genes, 2218; and AML M7, 1242. Consistent with the observed heterogeneity noted above in the expression profile of *CBFβ-MYH11* leukemias, this subtype also had the smallest number of class discriminating genes.

The expression profiles obtained using the top 50 ranked genes for the five prognostically important subgroups are illustrated in Figure 2A using a two-dimensional hierarchical clustering algorithm (see also Figure S2 and Table S7-S11). As shown, using the unique class specific expression signatures, we were able to obtain relatively tight clustering of cases within each of the five leukemia subtypes, including those expressing *CBFβ-MYH11*. Thus, distinct expression signatures can be identified for each of the known prognostically important AML leukemia subtypes.
Despite the ability to identify class specific expression signatures, significant heterogeneity in the signatures continued to be observed among cases in both the \( CBF\beta-MYH11 \) and \( MLL \) chimeric fusion gene subgroups (Figure 2A). To further assess the relationship both within and between leukemia subtypes, we next analyzed the expression data in pair-wise comparisons to assess the degree of relatedness between cases. The data is displayed using a two-dimensional plot in which similarities are plotted using a scale that is based on Pearson correlation coefficients calculated for pair-wise comparisons using the expression data for the 250 class discriminating genes. As shown in Figure 2B, the similarities of cases within a leukemia subtype were very high for \( AML1\)-ETO, \( PML\)-\( RAR\alpha \), and FAB-M7. By contrast, more heterogeneity was observed among cases within the \( CBF\beta-MYH11 \) and \( MLL \) chimeric fusion gene subgroups.

Close examination of the expression profiles and similarity plots for cases with either \( MLL \) chimeric fusion genes or \( CBF\beta-MYH11 \) suggests the existence of distinct subgroups. However, the observed variation could not be completely explained by differences in the structure of chromosomal rearrangements, extent of differentiation, or presence of specific secondary mutations (see Figure S2 and associated description). Thus, the underlying reason for the observed heterogeneity remains unknown.
Figure 2. Expression profiles of pediatric AMLs. (A) Hierarchical clustering of 130 diagnostic pediatric AML samples (columns) versus 250 class discriminating genes (rows). The genes used in this analysis are the top 50 ranked genes per group as selected by SAM. For genes that had more than one probe set selected as a class discriminator, the highest ranked probe set was used for this figure. Probe set signal values were normalized to the mean across the entire dataset and the relative value for each case is represented by a color, with red representing high expression and green representing low expression (scale shown in the lower right). The genetic subtype of each case is indicated by colored bars across the top and bottom of the panel. (B) Similarity plot of 130 pediatric AML diagnostic samples using the top 50 ranked genes (one probe sets per gene) for each subgroup as selected by SAM.
Similarities are plotted using a scale that is based on Pearson correlation coefficients calculated for pair wise comparisons using the expression data. The degree of similarity between cases is displayed using the blue color-scale at the bottom of the figure. Genetic groups are indicated by the color bars along the top and side of the similarity plot and are arranged identically to that shown in panel A.

**Biologic insights from the class defining genes**

The identified class discriminating genes should provide unique insights into the underlying biology of the different leukemia subtypes and have the potential to serve as unique class specific diagnostic or therapeutic targets. The patterns of expression of a select subset of class discriminating genes are shown in Figure 3. For each leukemia subtype the class specific genes shown include examples of previously defined class specific markers, as well as subtype specific genes defined exclusively by our analysis.

The aberrant co-expression of the hematopoietic progenitor marker CD34 and the B-cell antigen CD19 are a hallmark of AML1-ETO expressing leukemic blasts. Similarly, the unique high-level expression of ETO (CBFA2T1) is expected. More surprising is the number of AML1-ETO class discriminating genes whose homologs in Drosophila have

![Figure 3](image_url)

**Figure 3. AML subtype specific class discriminating genes.** Shown are representative genes that are highly correlated with the individual genetic subtypes of AML. Probe set signal values are normalized to the mean for the dataset and the expression for each case is then represented by color, with red representing deviation above the mean and green representing below the mean. The leukemia subtype is indicated at the top of the figure, and the Affymetrix probe set number and gene symbol are listed on the right side of the panel.
been shown to be involved in developmental processes. These include *roundabout* (*ROBO1*), *twisted* (*TWSG1*), and *pellino homolog 2* (*PEGI2*). Although no direct functional relationship has been established between the Drosophila AML1 homolog (*runt* a pair-rule gene) and these other genes, their specific high expression in this leukemia subtype warrants further exploration. Lastly, the *POU4F1* transcription factor, a proposed modulator of p53 transcription, is overexpressed >30 fold, and is the second highest-ranking *AML1-ETO* class discriminating gene (Table S7).43

The class discriminating genes for *PML-RARα*, *CBFβ-MYH11*, and FAB-M7 each include genes that encode proteins characteristic of the specific myeloid differentiation stage or lineage. For example, *PML-RARα* includes *hepatocyte growth factor* (*HGF*), *myeloperoxidase* (*MPO*) and *carboxypeptidase A3* (*CPA3*); *CBFβ-MYH11* includes *CDW52*, and *chitinase 3-like* (*CHI3L1*); and FAB-M7 includes the megakaryocytic lineage markers *glycoprotein Ib and IIb* (*GPIBB & ITGA2B*). Also prominent among the list of class discriminating genes are growth factors, growth factor receptors, and somewhat surprising, putative tumor suppressors. The latter includes *meningioma (disrupted in balanced translocation) 1* (*MN1*), and *suppressor of tumorigenicity 18* (*ST18*) in *CBFβ-MYH11*, and *deleted in liver cancer 1* (*DLC1*) in FAB-M7. Direct sequence analysis of these genes will be required to determine if they encode wild type or mutant proteins. The genes included in the *MLL* chimeric fusion gene signature are described in more detail below.

*CBFβ-MYH11* and *AML1-ETO* target the genes that encode the AML1/CBFβ transcription factor complex and constitute two of the major subtypes of “so-called” core-binding factor leukemias. To better assess the relationship between these leukemia subtypes, we determined the number of genes whose expression significantly differed between subtypes. In this analysis the smaller the number of differentially expressed genes between two leukemia
subtypes the closer their relationship. As shown in Table 2, *CBFβ-MYH11* leukemias were most closely related to cases having either *AML1-ETO* or *MLL* gene rearrangements. By contrast, *AML1-ETO* leukemias showed significantly more similarity to *CBFβ-MYH11* than to *MLL* chimeric fusion gene cases. Thus, as expected, a high degree of similarity is identified between the two major subtypes of core-binding factor leukemias; however, a surprising degree of similarity is also seen between *CBFβ-MYH11* and *MLL* chimeric fusion gene cases, and may be reflective of similarities in their extent of monocytic differentiation.

Table 2. Pair-wise comparisons showing the number of probe sets that differ between groups by SAM (90 percentile and a 1% FDR*).

<table>
<thead>
<tr>
<th>Group 2</th>
<th>Group 1</th>
<th>CBFβ-MYH11</th>
<th>AML1-ETO</th>
<th>MLL</th>
<th>PML-RARα</th>
<th>M7</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBFβ-MYH11</td>
<td>-</td>
<td>637</td>
<td>547</td>
<td>2081</td>
<td>1519</td>
<td></td>
</tr>
<tr>
<td>AML1-ETO</td>
<td>637</td>
<td>-</td>
<td>2140</td>
<td>1533</td>
<td>1599</td>
<td></td>
</tr>
<tr>
<td>MLL</td>
<td>547</td>
<td>2140</td>
<td>-</td>
<td>2742</td>
<td>1610</td>
<td></td>
</tr>
<tr>
<td>PML-RARα</td>
<td>2081</td>
<td>1533</td>
<td>2742</td>
<td>-</td>
<td>1992</td>
<td></td>
</tr>
<tr>
<td>FAB-M7</td>
<td>1599</td>
<td>1599</td>
<td>1610</td>
<td>1992</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

- indicates identity
* FDR – false discovery rate

Based on the known relationship between *CBFβ-MYH11* and *AML1-ETO* leukemias, and the observed similarities outlined above, we also selected genes that could discriminate these two subtypes of core-binding factor leukemias from all other leukemia subtypes. The top 50 discriminating genes are shown in Figure 4 and listed in Table S12. As illustrated, a subset of the discriminating genes are either over- or under-expressed in both *CBFβ-MYH11* and *AML1-ETO* subgroups, whereas the expression of other discriminating genes appears to be primarily expressed in *AML1-ETO* cases. Most of the latter genes were also selected as *AML1-ETO* only class discriminating genes as shown in Figure 2A.
Expression profiling as a diagnostic tool

A major goal of this study was to assess the ability of gene expression profiling to accurately diagnose the prognostically important AML subtypes. To examine this, class discriminating genes identified using SAM were used in an ANN-based supervised learning algorithm to classify cases into *PML-RARα, AML1-ETO, CBFβ-MYH11, MLL* chimeric fusion gene, or FAB-M7. The assignment to one of these leukemia subtypes required that the ANN generated node value for classification exceeded a 95% confidence level (see Supplemental Information). Any case that was not classified with high confidence into one of these five subgroups was labeled as “other”. Using the top 20-50 ranked discriminating genes for each subgroup, very high prediction accuracies were achieved on a randomly selected training set that consisted of three-fourths of the total cases. When this classification model was then applied to a blinded test set consisting of the remaining 30 samples, 100% diagnostic accuracies were achieved.

Figure 4. Expression signature of core-binding factor AMLs. Two-dimensional hierarchical clustering of the 130 AML cases using the top 50 ranked discriminating probe sets for the core-binding factor (CBF) leukemias (*AML1-ETO* and *CBFβ-MYH11* cases). The genetic subtype of each case is presented by a color coded bar at the bottom of the figure, using the same color scheme noted in the preceding figures. The probe set number and gene symbol for the discriminating genes are listed on the right. The normalized expression level for each gene is represented by a color using the scale shown in the lower left hand corner. Cases were clustered using a cosine function.
obtained for \textit{PML-RAR}, \textit{AML1-ETO}, \textit{CBF}/\textit{MYH11}, and FAB-M7, and 93% accuracy for cases with \textit{MLL} chimeric fusion gene, for an overall accuracy of 93% (95% CI, 79%-99%, Table 3). Optimal class assignment was achieved with as few as five genes (the smallest number tested) for \textit{PML-RAR}, \textit{AML1-ETO}, \textit{CBF}/\textit{MYH11}, and FAB-M7, and 35 genes for cases with \textit{MLL} gene rearrangements (data not shown).

\begin{table}[h]
\centering
\begin{tabular}{lcc}
\hline
 & Training (n=100) & Test (n=30) \\
\hline
\textit{PML-RAR} & 100 & 100 \\
\textit{AML1-ETO} & 100 & 100 \\
\textit{CBF}/\textit{MYH11} & 100 & 100 \\
\textit{MLL} & 98 & 93 \\
FAB-M7 & 100 & 100 \\
\hline
\end{tabular}
\caption{Diagnostic accuracies for pediatric AML*.}
\end{table}

* using 25 genes selected by SAM at 90th percentile with 5% FDR rate for \textit{PML-RAR}, \textit{AML1-ETO}, \textit{CBF}/\textit{MYH11} and FAB-M7 and 50 genes for \textit{MLL}.

Since the incidence of AML is significantly higher in adults than pediatric patients, we next assessed whether the expression profiles identified using pediatric patients could be used to accurately classify these specific subtypes of \textit{de novo} AML in adults. For this analysis, a selected set of 20 adult \textit{de novo} AML diagnostic samples were used (Table S3). This sample dataset contained examples of the specific leukemia subtypes under study, with the exception of FAB-M7, which is exceedingly rare in adult patients. We first used SAM to calculate the number of genes at a 1% FDR that differed between like subgroups of pediatric and adult \textit{de novo} AML. For this analysis, a sufficient number of adult cases were available only for \textit{PML-RAR}, \textit{AML1-ETO}, and \textit{MLL} chimeric fusion gene subgroups. As shown in Table 4, minimal or no significant differences existed between adult and pediatric cases within these specific subtypes of AML. Next we used the adult cases as a second blinded test set and applied the discriminating genes and supervised learning algorithms developed using pediatric AML cases to see if we could
accurately classify the adult cases. As shown in Table 5, using ANN we were able to obtain very high diagnostic accuracies for each of the genetic and morphologic subtypes, with an overall accuracy of 90% (95% CI, 68%-98%). Although no FAB-M7 cases were included in this dataset, no samples were misclassified as FAB-M7. These data demonstrate that there are minimal differences between pediatric and adult cases of these specific subtypes of de novo AML. Moreover, the class discriminating genes selected using the pediatric cases can be used to accurately diagnose adult cases.

### Table 4. Number of probe sets selected to differentiate between pediatric and adult AML subtypes.*

<table>
<thead>
<tr>
<th></th>
<th>1% FDR</th>
<th>5% FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PML-RARα</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AML1-ETO</td>
<td>0</td>
<td>172</td>
</tr>
<tr>
<td>MLL</td>
<td>0</td>
<td>17</td>
</tr>
</tbody>
</table>

*genes selected with SAM at 90th percentile with the indicated false discovery rate (FDR).

### Table 5. Adult AML case distribution and classification accuracy*

<table>
<thead>
<tr>
<th></th>
<th>No. cases</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PML-RARα</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>AML1-ETO</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>CBFβ-MYH11</td>
<td>2</td>
<td>95†</td>
</tr>
<tr>
<td>MLL</td>
<td>6</td>
<td>95</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>Overall</td>
<td>20</td>
<td>90</td>
</tr>
</tbody>
</table>

* There were no FAB-M7 cases in the adult dataset. No samples were mistakenly predicted as FAB-M7.
† This dataset contains one case that expresses both CBFβ-MYH11 and BCR-ABL, a combination described once before. The ANN supervised learning algorithm failed to classify this case as CBFβ-MYH11.

Although knowing the specific subtypes of AML can influence our therapeutic decisions, additional prognostic markers are needed to more accurately predict whether a patient can be cured by a specific therapeutic approach. Identifying a gene expression-based outcome predictor that could provide additional prognostic information, either independent of, or within a genetic subtype would be a significant advance. We examined the association of expression with
outcome in pediatric AML. For this analysis, we used a cohort of 98 patients treated on the AML 87, AML 91, and AML 97 protocols, excluding those patients with t(15;17). Genes were selected using a generalized Mantel statistic that examined the association of expression with time to relapse (see Supplemental Information, Section VI). This procedure selected three probe sets whose significance at the $\alpha = 0.001$ level was robust against the exclusion of any one patient from analysis. Two of the probe sets were also significant in a multivariable Cox proportional hazards regression analysis applied to the training cohort. The training cohort data was then used to develop a prognostic score function based on the expression of the two probe sets. In the validation cohort, time to relapse or progression became significantly shorter ($p = 0.0442$) as the value of this score increased. To further explore the prognostic significance of these genes, we applied the score function for the two identified genes to the adult cases included in our study (see Supplemental Information, Section VI). Although the same trend was observed in this small adult cohort, the association was not statistically significant at the traditional $\alpha = 0.05$ level ($p = 0.0898$). Thus, the value of these genes as predictors of prognosis independent of the genetic subtypes of de novo AML will require assessing their performance in larger cohorts of pediatric and adult patients.

A recently published paper reported the identification of 35 genes that could serve together as an outcome predictor in pediatric AML. When tested on our data set, the expression level of these genes did not show correlation with outcome (see Supplemental Information, Section V).

The significance of MLL gene rearrangements in pediatric ALL and AML

Translocations targeting the MLL gene are seen across the spectrum of acute leukemias including both B- and T-lineage ALLs and AMLs. Cases of B-precursor ALL with MLL chimeric fusion
genes, like several other genetic subtypes of ALL, can be easily identified as a unique biological subtype by their expression profile. As noted above, we can also identify an expression signature that can accurately identify cases of AML with MLL chimeric fusion genes. Defining expression signatures associated with MLL chimeric fusion genes irrespective of the lineage of the acute leukemia should provide valuable insights into common downstream pathways that are required for MLL-mediated transformation. To explore this possibility, we combined the pediatric AML dataset from this study with a dataset of 132 pediatric ALL cases, and five additional pediatric cases of T-ALL with MLL translocations (Table S4). These 267 pediatric acute leukemias include 48 cases with MLL chimeric fusion genes (20 B-lineage ALLs, 5 T-lineage ALLs and 23 AMLs), and 219 acute leukemias that lack this genetic lesion (98 B-lineage ALLs, 14 T-lineage ALLs, and 107 AMLs). No examples of therapy-induced AMLs are included in this dataset.

We initially analyzed this dataset using the unsupervised clustering algorithm PCA to assess the major grouping of the cases based solely on their gene expression profiles. Using all genes that passed a variation filter, three major subgroups were identified and shown to correspond to B-lineage ALL (B-ALL), T-lineage ALL (T-ALL), and AML (Figure 5A). Importantly, the cases with MLL chimeric fusion genes segregated according to their lineage (Figure 5B). Thus in this analysis, cases with MLL chimeric fusion gene did not cluster as a unique subgroup, but instead clustered according to their lineage of origin.
Figure 5. Gene expression profiles of pediatric acute leukemia with MLL chimeric fusion genes. (A) Multidimensional scaling plot generated using unsupervised principle components analysis with a combined dataset containing 130 AML cases, 132 ALL cases, and 5 additional T-lineage ALL (T-ALL) cases that contain MLL chimeric fusion genes. A variation filter was applied to remove any probe sets that showed minimal variation in expression across this dataset, and the analysis was performed with the remaining 17,944 probe sets. Each case is represented by a colored sphere, with AML cases indicated by blue, B-progenitor lineage ALLs (B-ALL) by yellow, and T-ALL by green. Acute leukemia cases cluster based on lineage. (B) The same PCA analysis as shown in panel A, except cases that contain an MLL chimeric fusion gene are indicated in red. The MLL chimeric fusion gene containing cases continue to cluster according to lineage. (C) Multidimensional scaling plot generated using the supervised learning algorithm, discriminants analysis with variance (DAV) with the expression data from the 267 acute leukemia samples generated using the 17,944 probe sets that passed the variation filter. Cases are color coded as described for panel B. Cases with an MLL chimeric fusion gene (in red) can be separated in gene space from the leukemias that lack this genetic lesion. (D) Expression profile of the top 50 ranked MLL discriminating genes. The probe set number and gene symbol for the discriminating genes are listed on the right. The normalized expression level for each gene is represented by color using the scale shown.

To see if a unique expression signature could be defined for the MLL chimeric fusion gene containing leukemias irrespective of their lineage, we next analyzed the data using the supervised learning algorithm discriminate analysis with variance (DAV). As shown in Figure 5C, although this algorithm continued to demonstrate a strong correlation between lineage and gene expression profile, we could now also appreciate a separation in gene space of MLL versus non-MLL chimeric fusion gene cases. Thus, this analysis suggested the existence of a shared gene expression signature among cases with MLL chimeric fusion genes.

To identify the genes that contributed to this signature, SAM was used to identify non-lineage restricted MLL class discriminating genes. This analysis identified 1059 genes whose expression patterns were statistically associated with the presence of an MLL chimeric fusion gene at a 1% FDR (Top 100 genes listed in Table S13). The expression patterns of the top 50 ranked non-lineage restricted MLL class discriminating genes are illustrated in the two-dimensional dendogram shown in Figure 5D. In this analysis all cases with MLL chimeric fusion genes were grouped on the left by lineage. As shown, the majority of the MLL class discriminating genes are overexpressed in this leukemia subtype. Among these are genes that are
expressed in the majority of MLL chimeric fusion gene containing cases including MBNL1, MEIS1, HOXA4, HOXA5, HOXA9 HOXA10, and MYH9 (Table S14), as well as genes that show a more lineage restricted pattern of expression, being preferentially expressed in either AMLs or T- and B-lineage ALLs. Use of the MLL specific discriminating genes in an ANN supervised learning algorithm yielded an overall diagnostic accuracy of 96% (95% CI; 90%-99%) when tested on a blinded test set of 100 cases.

In addition to chromosomal translocation, the MLL gene can also be altered by an internal partial tandem duplication (PTD). MLL-PTD are typically found in AML cases that have either normal cytogenetics or a trisomy of chromosome 11. The 47 cases of pediatric AML that lacked evidence of PML-RARα, AML-ETO, CBFβ-MYH11, MLL chimeric fusion genes, or FAB-M7 morphology were analyzed for evidence of MLL-PTD using a RT-PCR-based assay. Thirteen cases (28% of the analyzed samples) contained MLL-PTD. Quite surprisingly, these cases failed to cluster with the other MLL chimeric fusion gene AML cases when all 130 AML cases were analyzed using a two-dimensional hierarchical clustering algorithm (Figure S1). Moreover, many fewer discriminating genes were identified for the combined group of MLL-PTD and MLL chimeric fusion gene cases, than for a group consisting exclusively of cases with MLL chimeric fusion genes. In addition, use of these discriminating genes in an ANN supervised learning algorithm yielded a very low accuracy of class prediction (data not shown). Lastly, when MLL-PTD was considered as a single leukemia subgroup, no class discriminating genes could be identified at a 5% FDR. Thus taken together, these data suggest that MLL-PTD are heterogeneous at a molecular level and are distinct from AMLs that contain chromosomal translocation of MLL that result in the formation of chimeric fusion genes.
Discussion

Gene expression profiling using microarray-based methodologies has provided new insights into the biology of a variety of hematopoietic malignancies, and has shown promise as a tool to aid in the accurate diagnosis and risk-stratification of patients. Recent applications to the acute leukemias have revealed distinct expression signatures for the individual lineages of the leukemic blasts, as well as for many of the known prognostic subtypes of pediatric ALL and adult AML. These gene signatures have proven to be robust discriminators of the specific subtypes of leukemia, showing diagnostic accuracies that, in many cases, exceed that achieved using routine diagnostic approaches. We now extend these studies by reporting the results from the expression analysis of diagnostic leukemic blasts from 130 pediatric and 20 adult patients with de novo AML. Our results demonstrate distinct expression signatures for each of the known prognostic subtypes of pediatric AML, including t(8;21)[AML1-ETO], inv(16)[CBFβ-MYH11], t(15;17)[PML-RARα], MLL chimeric fusion genes, and FAB-M7. Moreover, using the identified expression signatures in an ANN-based supervised learning algorithm, we achieved an overall diagnostic accuracy of 93%. More importantly, the pediatric AML subtype specific expression signatures were present essentially unchanged in adult AML cases containing the identical genetic lesions. Thus, the identified class discriminating expression signatures should prove valuable in the development of custom AML diagnostic microarrays for use in the clinical setting. In addition, we identified a limited set of genes whose high expression correlated with a poor outcome. However, because of the relative small size of our dataset, the true prognostic significance of these genes will require validation in larger cohorts of pediatric and adult patients.

Lastly, by combining the described AML dataset with a previously published pediatric ALL dataset, we identified an expression signature that was specific for the presence of an MLL chimeric fusion gene, irrespective of the lineage of the leukemic blasts. This signature provides
novel insights into the downstream transcriptional cascade resulting from the expression of an MLL chimeric fusion gene.

The diagnostic expression signatures identified in this paper, as well as those presented in several other recently published studies, represent only a first step in moving this methodology in the clinical setting. Importantly, the signatures developed to date allow the identification of a limited subset of the known prognostically important AML subtypes. It remains to be determined whether expression signatures can be identified for some of the other known AML subtypes, including -5/del(5q), -7/del(7q), and inv(3)/t(3;3). Moreover, between 25%-45% of adult and pediatric cases of AML are reported to lack evidence of a clonal chromosomal abnormality. A variety of genetic lesions including mutations of AML1, N-RAS, K-RAS, C/EBPα and the FLT-3 receptor have been identified in varying proportions of these cases. Whether unique expression signatures can be defined for some of these lesions remains unknown. Although only a minority of the cases in our study were analyzed for these lesions, we nevertheless were able to detect a suggestion of clustering for a small subset of the cases containing FLT-3 activating mutations (data not shown). The examination of a large number of well-characterized cases will be required to determine if unique expression signatures can be defined for these genetic lesions, and if so whether they are robust enough to accurately diagnose the presence of the lesions in blinded clinical samples. Two recent studies have demonstrated that for some genetic lesions this may be possible.

The identified expression signature for each of the different leukemia subtypes provides a unique insight into their underlying pathobiology. Although many testable hypotheses can be generated from the list of class discriminating genes, they remain at best speculative. Direct experimentation will be required to determine which of the identified genes play a mechanistic role in the growth of the leukemic cells. Moreover, defining the genes that are aberrantly
expressed within the leukemic cells as compared to normal bone marrow derived hematopoietic stem cells and lineage committed progenitors will provide important insights into the altered biology of the leukemic cells. The generated dataset provides an invaluable resource for the latter kinds of analyses.

The presented FAB-M7 expression signature represents the first detailed analysis of this specific subtype of AML. FAB-M7 is known to be a heterogeneous leukemia subtype with a minority of patients having Down syndrome, and an independent subset having leukemic blasts that contains the t(1;22). In the examined cohort, only two patients had Down syndrome and no examples of the t(1;22) were included. Thus, the data provides a view of only a limited subset of FAB-M7 leukemias. In this cohort, most of the identified genes have previously been shown to be expressed in cells of the megakaryocyte lineage. These include glycoproteins IIb/IIIa (GP1BB and ITGA2B), GATA1, and MRPS12. In addition, we identified a number of novel genes not previously associated with this leukemia subtype. Included in this latter list are the BMP-2 inducible nuclear serine/threonine kinase (BMP2K) and the putative tumor suppressor deleted in liver cancer 1 (DLC1). Exploring the functional role of the identified genes in the pathogenesis of this leukemia subtype should provide insights that could lead to improvements in our ability to treat this poor risk AML subtype.

The identified expression signature of *MLL* chimeric fusion gene cases provides an important view into the downstream targets of these mutant transcriptional regulatory proteins. It is likely that within the identified set of genes are targets whose altered expression is essential for the development and growth of the leukemic clone. Among the *MLL* class discriminating genes we identified a subset of 21 genes that showed a relatively uniform level of expression in all *MLL* chimeric fusion gene cases, irrespective of lineage (Table S14). Many of the genes in this list have previously been implicated in *MLL* chimeric fusion protein-mediated transformation,
including MEIS1 and the HOX genes (HOXA4, HOXA5, HOXA7, HOXA9, and HOXA10).64-68 Others, however, are unique to this analysis. A comparison of the 21 relative expression levels of the identified genes to their level of expression in normal BM-derived CD34+ hematopoietic progenitors, revealed that approximately half were expressed at levels nearly identical to that seen in normal hematopoietic progenitors (Table S14). By contrast, other genes in this list were significantly overexpressed, including the HOXA9, HOXA10, CAPG, NICAL, ABCA7, MYH9, VLDLR, ARPC2, PTPRC, and RAC2. Several of these genes including CAPG, ABCA7 and MYH9 are normally highly expressed on cells of the monocyte/macrophage lineage, and thus likely reflect a degree of myeloid/monocytic gene expression, or more correctly, inappropriate expression in the B- and T-lineage cases. Others genes encode products that suggest that their inappropriate expression may be functionally important. Rac2 in particular has recently been shown to play an important role in intergrin-mediated stem cell signaling, and to inhibit cell death under certain growth conditions.69 Deciphering which genes are simply reflective of the stage of differentiation, and which are mechanistically important will require direct experiments to assess the functional effects that result from the loss of expression.

One of the most surprising results from our analysis was the lack of a distinct expression signature for AMLs with MLL-PTD and the inability to define a clear relationship of these cases to other AMLs with MLL chimeric fusion genes. These data suggest that MLL-PTD induces the altered growth of hematopoietic progenitors through mechanism that shows minimal, if any, relationship to the altered transcriptional pathway that result from the expression of MLL chimeric fusion genes. Comparing and contrasting the mechanisms of transformation induced by these two distinct classes of MLL mutations should provide valuable insights that will eventually lead to better ways to treat these AML subtypes.
In summary, the data presented suggest that expression profiling could provide a robust platform for the accurate diagnosis and classification of the major prognostic subtypes of AML in both pediatric and adult patients. Moreover, the database generated through this study coupled with those generated through our past efforts should provide valuable resources for the investigation of the altered biology that underlies the various genetic subtypes of acute leukemia.

Acknowledgements

The authors thank the staff of the Molecular Pathology laboratory and the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital (SJCRH) for technical support. We also thank the SJCRH Tumor Bank and Michael Jaynes for assistance in obtaining cryopreserved samples. This work was supported in part by National Cancer Institute grants P01 CA71907-06 (JRD), CA-21765 (Cancer Center CORE grant to SJCRH), T32-CA70089, and by the American Lebanese and Syrian Associated Charities (ALSAC) of SJCRH. Dr. Pui is the recipient of the American Cancer Society F.M Kirby Clinical Professorship.

References


Gene Expression Profiling of Pediatric Acute Myelogenous Leukemia

Mary E Ross, Rami Mahfouz, Mihaela Onciu, Hsi-Che Liu, Xiaodong Zhou, Guangchun Song, Sheila A Shurtleff, Stanley Pounds, Cheng Cheng, Jing Ma, Raul C Ribeiro, Jeffrey E Rubnitz, Kevin Girtman, W K Williams, Susana C Raimondi, Der-Cherng Liang, Lee-Yung Shih, Ching-Hon Pui and James R Downing

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include digital object identifier (DOIs) and date of initial publication.