Identification of Genes with Abnormal Expression Changes in Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) is one of the most common and deadly forms of hematopoietic malignancies. We hypothesized that microarray studies could identify previously unrecognized expression changes that occur only in AML blasts. We were particularly interested in those genes with increased expression in AML, believing that these genes may be potential therapeutic targets. To test this hypothesis, we compared gene expression profiles between normal hematopoietic cells from 38 healthy donors and leukemic blasts from 26 AML patients. Normal hematopoietic samples included CD34+ selected cells ($N = 18$), unselected bone marrows ($N = 10$), and unselected peripheral bloods ($N = 10$). Twenty genes displayed AML-specific expression changes that were not found in the normal hematopoietic cells. Subsequent analyses using microarray data from 285 additional AML patients confirmed expression changes for 13 of the 20 genes. Seven genes (*BIK*, *CCNA1*, *FUT4*, *IL3RA*, *HOMER3*, *JAG1*, *WT1*) displayed increased expression in AML, while 6 genes (*ALDHA1A*, *PELO*, *PLXNC1*, *PRUNE*, *SERPINB9*, *TRIB2*) displayed decreased expression. Quantitative RT/PCR studies for the 7 over-expressed genes were performed in an independent set of 9 normal and 21 pediatric AML samples. All 7 over-expressed genes displayed an increased expression in the AML samples compared to normals. Three of the 7 over-expressed genes (*WT1*, *CCNA1*, and *IL3RA*) have already been linked to leukemogenesis and/or AML prognosis, while little is known about the role of the other 4 over-expressed genes in AML. Future studies will determine their potential role in leukemogenesis and their clinical significance. This article contains Supplementary Material available at http://www.interscience.wiley.com/jpages/1045-2257/suppmat.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease with variable response to therapy that affects thousands of patients each year. Over the last 10 years, research has identified several novel prognostic factors such as *FLT3* and nucleophosmin mutations that have provided insight into the biology of AML (Kottaridis et al., 2001; Kelly et al., 2002; Schnittger et al., 2002; Thiede et al., 2002; Alcalay et al., 2005; Boissel et al., 2005; Cazzaniga et al., 2005; Dohner et al., 2005; Lee et al., 2005; Verhaak et al., 2005; Wilson et al., 2006; Falini et al., 2007). However, the overall prognosis for AML patients remains dismal, with the majority of patients dying from relapse of their disease (National Cancer Institute, 2004). A better understanding of the biology causing AML may lead to the development of more specific and less toxic therapies for the disease. To investigate the biology of this complex disease, investigators have turned to high-throughput technologies such as microarrays that have the capacity to examine the expression of thousands of genes in a single sample (Golub et al., 1999; Clark et al., 2000; Bullinger et al., 2004; Lacayo et al., 2004; Valk et al., 2004; Haferlach et al., 2005; Wilson et al., 2006). Although AML microarray studies have identified novel prognostic factors, studies have not extensively compared expression profiles between normal hematopoietic cells and AML blasts (Bullinger

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et al., 2004; Lacayo et al., 2004; Valk et al., 2004; Haferlach et al., 2005; Heuser et al., 2005; Marcucci et al., 2005; Steinbach et al., 2006). Microarray studies comparing AML blasts and normal hematopoietic cells may uncover critical genes or pathways that promote leukemogenesis. In addition, this research may discover novel prognostic factors, minimal residual disease markers, and therapeutic targets that will improve clinical outcomes for AML patients.

AML blasts vary in their differentiation stages between patients and, sometimes, within a single patient. In addition, these malignant cells often aberrantly express differentiation markers, making it impossible to determine their "normal" hematopoietic counterpart. These two factors pose a challenge for studies comparing AML blasts to normal hematopoietic cells. We were interested in identifying genes with unique expression changes in AML blasts that were not found in normal hematopoietic cells (i.e. AML-specific expression changes). Also, we wanted to determine if these AML-specific expression changes were generalizable across different populations of AML patients. Therefore, microarray studies compared expression profiles between AML blasts and normal hematopoietic cells. For these studies, we examined normal hematopoietic cells at variety of different stages of maturation (immature CD34+ cells, unselected BM, and unselected peripheral blood) to compensate for the challenge of identifying the optimal normal hematopoietic counterpart for AML comparisons. The microarray results were also confirmed across different populations of AML patients to ensure that the findings were not restricted to a specific demographic population. The results indicate that there are several genes with AML-specific expression changes. Future studies are planned to investigate the biology and possible clinical significance of these genes with AML-specific expression changes.

MATERIALS AND METHODS

Acquisition of Samples from Healthy Donors and AML Patients

The CD34+ cells from bone marrows (BM CD34+, $N = 8$), CD34+ cells from peripheral blood stem cell products (PBSC CD34+, $N = 10$), unselected bone marrows (BM, $N = 10$), and unselected peripheral blood samples (PB, $N = 10$) were either obtained from volunteer donors at the Fred Hutchinson Cancer Research Center (FHCRC) or purchased from commercially available vendors (Cambrex, Rutherford, NJ or AllCells, Emeryville, CA). BM ($N = 7$) and PB ($N = 19$) samples from 26 adult AML patients were obtained from the FHCRC's Leukemia Repository. The diagnosis of AML for all FHCRC patients was confirmed by the director of hematopathology using WHO definition for AML as previously described (Harris et al., 1999). All AML samples had a blast count $\geq$ 65% (Supplement 1). Samples for validation studies (Supplement 1) were obtained from 21 pediatric AML patients enrolled onto Children's Cancer Group clinical protocol (CCG) 2961 and nine healthy donors (3 PBSC CD34+, 3 BM, and 3 PB). All samples were obtained under Institutional Review Board (IRB) approved protocols, and consent was provided according to the Declaration of Helsinki. CD34+ cells were selected using anti-CD34 immunomagnetic beads (Miltenyi Biotec, Auburn, CA) as previously described (Colter et al., 1996; Yu et al., 2000). Based on quality control experiments, the purity of CD34+ cells was $\geq$95%. Student's *t*-test with two-tail distribution and two-sample unequal variance was used to determine statistical significance between different populations of subjects.

RNA Extraction and Microarrays

RNA was extracted from cells using TRIzol® reagent as per the standard protocol (Invitrogen, Carlsbad, CA) and analyzed on HP 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) to assess the integrity of total RNA (Stirewalt et al., 2004). The Eukaryotic Target Labeling protocol was performed as per the recommended guidelines in GeneChip Expression Analyses Technical Manual (Affymetrix Inc., 2003a; Stirewalt et al., 2004). Five micrograms of total RNA was used from each sample. Biotin-labeling was performed using the Enzo BioArray™ High Yield™ RNA Transcript Labeling Kit (Enzo Life Technologies, Farmingdale, NY). Fragmentation and hybridization were performed as per Affymetrix protocol (Affymetrix Inc., 2003a; Stirewalt et al., 2004). Fifteen micrograms of fragmented antisense cRNA was hybridized to the HG-U133A arrays (Affymetrix, Santa Clara, CA).

Microarray Analyses Identify AML-Specific Expression Changes

DAT files were generated using GCOS 1.2.1 software (Affymetrix). CEL and CHP files were generated using MAS 5.0 software (Affymetrix) with target signals for probe sets scaled to 500. Individual arrays were screened for quality, such that any array with a 3′/5′ *GAPDH* or *ACTB* ratio >

1.5 or background > 100 was eliminated from further analyses (Affymetrix, Inc., 2003a; Affymetrix, Inc., 2003b). In addition, the scaling factors of all arrays were within three-fold of each other and displayed similar average intensities (Brazma et al., 2001). Log_2 expression values for individual probe sets were generated from CEL files using robust multi-array average (gcRMA) as previously described (Bolstad et al., 2003; Irizarry et al., 2003). These expression values were imported into GenePlus™ software (Enodar Biologic, Seattle, WA) for downstream statistical analyses. Expression changes were deemed to be statistically significant if the probe set displayed a Number of False Discovery (NFD) $\leq$ 1.0 (Xu et al., 2002; Storey and Tibshirani 2003;). Detailed clinical, cytogenetic, and molecular cytogenetic information along with the CEL files for each of the normal and AML samples is available at the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo).

Two analyses were performed to identify genes with AML-specific expression changes. The first analysis (Analysis 1) compared expression profiles between AML samples and the entire group of 38 normal hematopoietic samples. The second analysis (Analysis 2) compared expression profiles between AML cells and individual subgroups of normal hematopoietic cells (e.g. AML vs. BM CD34+; AML vs. PBSC CD34+, AML vs. BM, and AML vs. PB). Probe sets that displayed significant expression differences in analysis 1 and all four comparisons in Analysis 2 were determined to be candidate genes with AML-specific expression changes.

Microarray Analyses Validate AML-Specific Expression Changes in Another AML Dataset

Analyses were also performed using the data from our 38 normal samples and 285 AML samples from a Dutch-Belgian study (GEO, GEO accession: GSE 1159) (Valk et al., 2004; NCBI:GEO, 2006). Since Dutch-Belgian study also used HG-U133A microarrays, a direct comparison between the normal hematopoietic samples and Dutch-Belgian AML samples was possible. MAS 5.0 signals were used for the comparisons as previously described (Valk et al., 2004). Because of the poor discriminative power of MAS 5.0 at low signal intensity (Valk et al., 2004), MAS 5.0 signals < 10 were set to 10. All signals were then log_2 transformed for future analyses. Log_2 transformed signals were imported into GenePlus™ software (Zhao et al., 2001; Xu et al., 2002), and the same analyses as described above were performed to identify potential AML-specific genes. We then determined the overlap of significant genes between FHCRC and Dutch-Belgian AML studies.

Network Generation and Pathway Analyses

Data sets with unique Affymetrix identifiers and corresponding *Z*-scores were uploaded and analyzed through the use of Ingenuity® Pathways Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com). Only genes identified as being statistically significant as defined as having an NFD $\leq$ 1.0 were loaded into the IPA software. The significance of canonical pathways was determined using Fischer's exact test (Ingenuity Systems, 2006a). All relationships are supported by at least one literature reference and/or information from the Ingenuity Pathways Knowledge Base (Ingenuity Systems, 2006b). Canonical pathways with a *P*-value $\leq$ 0.05 were considered to be statistically significant.

Expression of AML-Specific Genes Are Associated with Molecular Markers and Other Characteristics

Associations of expression levels of selected genes with molecular markers and other characteristics were evaluated using data from the 285 AML samples in the Dutch-Belgian study. Differences in mean expression levels between two groups (defined, for example, by the presence of a specific mutation) or among 3+ groups (FAB classes, cytogenetic categories, etc.) were tested using regression models fit by the method of maximum likelihood to account for the truncation of expression levels at a lower level of 10 in the Dutch-Belgian data set. These analyses used log-transformed expression levels, which were found to have roughly symmetric distributions. Calculations were performed using the SAS LIFEREG procedure (SAS version 9, SAS Institute, Inc. Cary NC).

Quantitative RT/PCR Assays Validate AML-Specific Expression Changes for Selected Genes

To validate over-expressed genes in an independent population of normal donors and AML patients, quantitative reverse transcription/polymerase chain reactions (Q-RT/PCR) were performed using standard TaqMan® conditions and the ABI PRIZM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described (Tse et al., 2004). RT was performed using 0.6 μg of total RNA, AMVRT (Invitrogen, Carlsbad, CA), and Oligo dT primer (Invitrogen) as per manufacturer's protocol. The cDNA equivalent of 0.05 μg of starting total RNA was

used as template for Q-PCR. Taqman® Gene expression assays for *CCNA1*, *FUT4*, *IL3RA*, *JAG1*, and *GUSB* (endogenous control) were purchased from Applied Biosystems. Primers and dual-labeled fluorogenic probes for *WT1* have previously been described (Cilloni et al., 2002). Primer and probe sequences for *BIK* and *HOMER3* are provided in Supplement 2. The fold expression difference for each gene was calculated relative to normal bone marrow (the calibrator) using the $2^{-\triangle\triangle C_T}$ method (Livak and Schmittgen, 2001). Student's *t*-test with one-tail distribution and two-sample unequal variance was used to determine statistical significance.

RESULTS

Microarray Analyses Identify AML-Specific Expression Changes

Demographics were available for 24 of the 38 normal donors (Supplement 1). Leukemic samples were obtained from 26 AML patients from FHCRC Leukemia Repository (Supplement 1). Normal donors were younger than the AML patients (median age 30 vs. 56, $P < 0.01$), but there was no significant difference with respect to gender between the two populations ($P = 0.20$). Unfavorable cytogenetics were common in the FHCRC AML patients (favorable 5%, intermediate 52%, unfavorable 43%), and 35% of FHCRC AML patients had preceding myelodysplasia or treatment-related AML. In addition, 43% of FHCRC AML patients had a FLT3 internal tandem duplication (ITD), which is slightly higher than previous reports (Kiyoi et al., 1999; Stirewalt et al., 2001; Kottaridis et al., 2002; Schnittger et al., 2002; Thiede et al., 2002). Only samples with high blast counts (median 83, range 65–97%) were examined.

The goal was to identify genes with expression changes that were unique to AML blasts and outside of the normal range of expression occurring in the normal hematopoietic cells. To achieve this goal, two separate analyses were performed. The first analysis compared the expression profile from 26 AML samples to the entire group of 38 normal hematopoietic samples. This analysis identified a total of 475 genes (555 probe sets) with significant expression differences (NFD $\leq$ 1) between the AML and normal samples. However, a subset of the 475 genes had expression in AML blasts that were similar to one or more subpopulations of normal hematopoietic cells (e.g. *PBX1* and *LEF1*, Figs. 1A and 1B).

Therefore, a second analysis was performed that compared the expression profiles of the AML cells to individual subpopulations of normal hematopoietic cells (e.g. AML vs. BM CD34+, AML vs. PBSC CD34+, AML vs. BM, and AML vs. PB). In this multiple group analysis, 1750, 1988, 1420, and 3567 genes displayed significant expression differences between AML and BM CD34+, PBSC CD34+, BM, and PB samples, respectively. By determining the overlap of genes with significant expression differences in *all* four comparisons, genes like *PBX1* and *LEF1* (Figs. 1A and 1B) were filtered out. Seventy genes had significant expression changes between AML and *all* four normal hematopoietic subpopulations. However, 50 of the 70 genes were not significant in Analysis 1 because their average expression in AML was not significantly different from their average expression in the hematopoietic samples as a group. Further review of these 50 genes found that their average expression in AML samples fell within the upper and lower expression ranges for normal hematopoietic subpopulations (e.g. *CCL5*, Fig. 1C). By selecting only genes with significant expression differences in *all* comparisons in both Analyses 1 and 2, we identified a set of 20 "candidate" genes with AML-specific expression changes (Supplement 3) that were not expressed at similar levels in any of the subpopulations of normal hematopoietic cells and whose average expression fell outside the normal ranges for normal hematopoietic cells (e.g. *WT1*, Fig. 1D). Eleven genes had an increased expression in AML, while nine genes had a decreased expression in AML.

Microarray Analyses Validate AML-Specific Expression Changes in Another AML Dataset

Since we were interested in identifying genes with the most robust AML-specific expression changes, we examined the expression profiles of another AML population with different demographics and clinical characteristics. Microarray data was obtained from AML patients enrolled on protocols from the Dutch-Belgian Hematology-Oncology Cooperative group (Supplement 1) (Valk et al., 2004). The cytogenetic profiles of Dutch-Belgian AML patients (favorable 25%, intermediate 57%, and adverse 18%) were consistent with other large AML studies (Grimwade et al., 1998) and had higher percentage of favorable cytogenetics than the FHCRC AML group ($P < 0.01$). In addition, the Dutch-Belgian AML patients had fewer FLT3 ITDs than the FHCRC AML patients (27% vs. 43%, $P = 0.15$). The frequency of FLT3

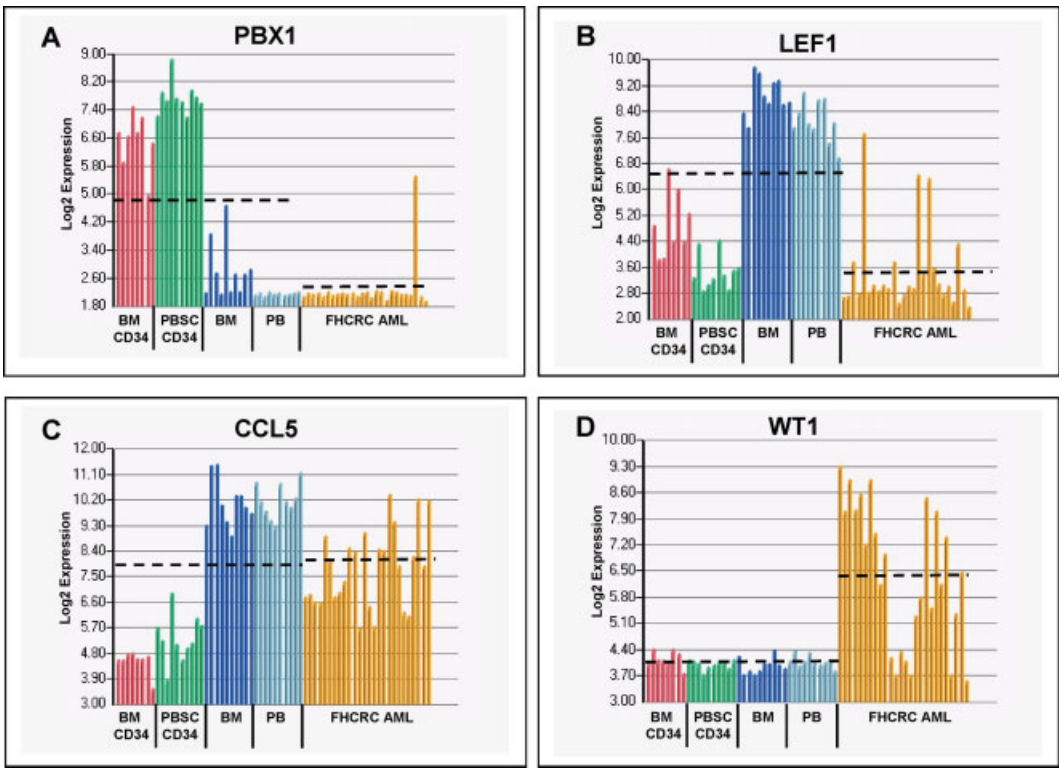

Figure 1. Expression differences between normal hematopoietic cells and AML blasts. Log_2 transformed expressions (*y*-axis) were derived from microarray data from normal and FHCRC AML samples. Normal samples included 8 BM CD34+ 10 PBSC CD34+ 10 BM and 10 PB. The average signals for the all 38 normal hematopoietic and 26 AML samples are represented by black dotted lines. A: The average expression of *PBX1* is significantly different between all 38 normal hematopoietic samples (left dotted line) and AML samples (right dotted line), but average expression of *PBX1* in the FHCRC samples is not significantly different than the its average expression in either BM or PB samples. B: The average expression of *LEF1* is significantly different between all 38 normal hematopoietic samples (left dotted line) and AML samples (right dotted line), but average expression of *LEF1* in FHCRC AML samples is not significantly different than its average expression in either BM CD34+ or PBSC CD34+ samples. C: The average expression of *CCL5* is significantly different between the AML samples and each individual subpopulation of the normal hematopoietic samples (AML vs. BM CD34+; AML vs. PBSC CD34+; AML vs. BM; and AML vs. PB), but the average expression of *CCL5* is not significantly different between the 38 normal hematopoietic samples as a group (right dotted line) and the FHCRC AML samples (left dotted line). As demonstrated in the figure, the average expression of CCL5 falls within the upper and lower expression ranges for the gene in the normal hematopoietic samples. D: The average expression of *WT1* is significantly different between the AML samples and each individual subpopulation, and its average expression is also significantly different between the 38 normal hematopoietic samples as a group (right dotted line) and the FHCRC AML samples (left dotted line). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tyrosine kinase domain mutations (TKD) was similar in both the Dutch-Belgian (12%) and FHCRC (14%) AML patients ($P = 0.79$). Age, gender, and ethnicity data were not available for individual patients from the Dutch-Belgian cohort, but the majority of the Dutch-Belgian AML patients (89%) were $\leq$ 60 years old, suggesting that the Dutch-Belgian AML patients were slightly younger than the FHCRC AML patients.

The same two analyses (Analysis 1 and 2) were performed using the 285 Dutch-Belgian AML patients and the 38 normal hematopoietic samples. We then determined which genes displayed significant expression differences using both the FHCRC and Dutch-Belgian AML patients. For Analysis 1, 288 genes (61%, 288 of 475) displayed significant expression differences in both the FHCRC and Dutch-Belgian AML patients. A list of these 288 genes is provided in Supplement 4. Approximately 75% of significant genes from the FHCRC Analysis 2 were also significant in the Dutch-Belgian AML patients. Specifically, we found the following numbers of genes with significantly expression differences in the comparisons for Analysis 2 using both populations of AML patients: BM CD34+ vs. AML = 1375; PBSC CD34+ vs. AML = 1465; BM vs. AML = 1059; and PB vs. AML = 2829. Lists of these genes are provided in Supplements 5–8.

The results from individual analyses were imported separately into Ingenuity® Pathways Analysis software to identify canonical pathways with significant expression changes for each list of significant genes (i.e. Supplement 4–8). A few canonical pathways (e.g. cell cycle regulation) displayed significant expression changes in more than

TABLE 1. List of 13 Genes with AML-Specific Expression Changes

			FHCRC AML study		Dutch-Belgian AML study	
Affymetrix ID	Gene symbol	Gene name	Z	NFD	Z	NFD
206067_s_at	*WT1*	Wilms tumor 1	6.35	0.00	29.35	0.00
205780_at	*BIK*	BCL2 interacting killer	4.80	0.23	17.66	0.00
209893_s_at	*FUT4*	fucosyltransferase 4	5.01	0.11	16.86	0.00
205899_at	*CCNA1*	cyclin A1	5.45	0.02	15.24	0.00
206148_at	*IL3RA*	interleukin 3 receptor, alpha	7.40	0.00	14.36	0.00
215489_x_at	*HOMER3*	homer homolog 3	7.08	0.00	13.78	0.00
216268_s_at	*JAG1*	jagged 1	6.50	0.00	11.86	0.00
212224_at	*ALDH1A1*	aldehyde dehydrogenase 1 family, member A1	−9.20	0.00	−23.38	0.00
202478_at	*TRIB2*	tribbles homolog 2	−8.85	0.00	−15.59	0.00
209723_at	*SERPINB9*	serpin peptidase inhibitor, clade B, member 9	−7.05	0.00	−15.26	0.00
210988_s_at	*PRUNE*	prune homolog	−8.04	0.00	−14.65	0.00
218472_s_at	*PELO*	pelota homolog	−7.48	0.00	−11.45	0.00
213241_at	*PLXNC1*	plexin C1	−8.15	0.00	−8.40	0.00

The Affymetrix probe set ID, gene symbol, and gene name are provided in the first three columns. Statistics from the analyses using the FHCRC AML (Columns 4 and 5) and the Dutch-Belgian AML samples (Column 6 and 7) are provided. The *Z*-scores (*Z*) between normal and AML samples are shown in Columns 4 and 6. A negative *Z*-score indicates decreased expression in AML samples compared to normal samples, while a positive *Z*-score indicates increased expression in the AML samples. The numbers of false discoveries (NFD) are shown in Columns 5 and 7.

one of the analyses. However, the majority of significant canonical pathways were unique to an individual comparison (e.g. BM CD34+ vs. AML). For example, Notch signaling was one of the most significant pathways identified in the comparison between all normal hematopoietic cells and AML cells (Supplement 4), but this pathway was not significant in the other comparisons. All significant canonical pathways are provided with each gene set in Supplements 4–8.

We then determined the overlap of significant AML-specific expression changes that were found in all comparisons for Analyses 1 and 2 using both FHCRC and Dutch-Belgian AML patients. Thirteen of the 20 "candidate" genes (65%) originally identified in the FHCRC AML patients also displayed significant AML-specific expression changes in all analyses with the Dutch-Belgian AML patients (Table 1). AML expression was increased for seven genes and decreased for the other six genes (Fig. 2). Most of these AML-specific candidate genes displayed relatively uniform expression in the subpopulations of normal cells, with more variable expression within the AML population (Fig. 2).

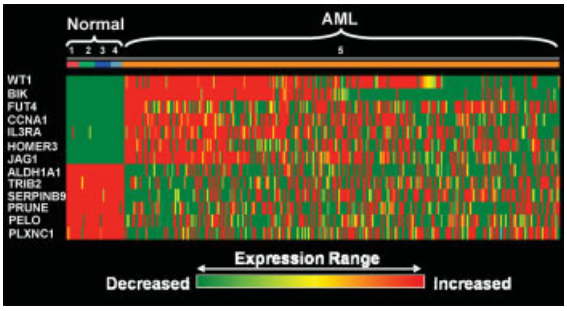

Figure 2. Heatmap of expression for 13 AML-specific genes from microarray studies**.** Normalized expression values for 13 AML-specific genes in the five populations of cells are shown using a heatmap. Red cells indicate increased levels of expression, while green cells indicate decreased levels of expression. Each row represents the expression for one gene, and each column represents a single sample. The 38 samples from normal donors (Normal) and 285 Dutch-Belgian AML samples (AML) are shown. The normal samples have been grouped by CD34 status and donor source (1, red = BM CD34+; 2, green = PBSC CD34+; 3, dark blue = BM; and 4, light blue = PB). Orange bar (5) indicates the columns with the 285 AML samples.

Expression of AML-Specific Genes Are Associated with Other Molecular Markers

To investigate further the significance of the 13 genes, we examined the associations between their expression levels and previously identified genetic abnormalities, using the Dutch-Belgian data set. The genetic abnormalities examined included *FLT3* internal tandem duplications (ITD), *FLT3* tyrosine kinase domain (TKD) mutations, *NRAS* and *KRAS* mutations, *EVI1* expression, and *CEBPA* mutations. FAB classification and cytogenetics were also investigated. Detailed results of these analyses are provided in Supplement 9. All 13 genes were significantly associated ($P \leq 0.001$) with at least one of the molecular variables, FAB or cytogenetics (Table 1 in Supplement 9). Expression levels of *WT1*, *FUT4*, *CCNA1*, *HOMER3*, *JAG1*, *TRIB2*, and *SERPINB9* were strongly associated with FAB classification ($P < 0.001$). The

mean expressions of *WT1*, *CCNA1*, *HOMER3*, and *JAG1* were highest in FAB M3 samples, while *FUT4* expression was highest in FAB M5 samples. *TRIB2* and *SERPINB9*, both of which displayed a decreased expression in AML, were expressed lowest in FAB M5 and FAB M3 samples, respectively (Table 2 in Supplement 9).

With respect to cytogenetics, *WT1*, *JAG1*, *ALDH1A1*, *TRIB2*, and *PLXNC1* were expressed at different levels in AML patients with normal cytogenetics as compared to those with cytogenetic abnormalities ($P < 0.01$). All the genes except *TRIB2* were expressed at higher levels in samples with normal cytogenetics (Table 3 in Supplement 9). Further analyses found a significant association between gene expression and cytogenetic risk groups (i.e. favorable, intermediate, or unfavorable) for most genes. Specifically, *FUT4*, *CCNA1*, and *HOMER3* were over-expressed in AML samples with favorable cytogenetics, while *ALDH1A1* and *PLXNC1* displayed increasing expression in patients with unfavorable cytogenetics (Table 4 in Supplement 9). The expression of all 13 genes except *IL3RA* and *PRUNE* were associated with individual cytogenetic abnormalities (Table 5 in Supplement 9). Of the genes with increased expression in AML, *WT1* expression was over-expressed in patients harboring either inv(16) or t(15;17), and *CCNA1* expression was highest in patients harboring t(15;17). *JAG1* displayed increased expression primarily in patients with inv(16), t(15;17), and normal cytogenetics, and was low in patients with t(8;21). *BIK* had one of the most significant associations between expression and individual cytogenetic abnormalities. Although *BIK* displayed an increased expression in AML compared to normal hematopoietic cells, *BIK* expression was absent or extremely low in AML patients with t(8;21) as compared all other types of cytogenetic abnormalities ($P < 0.00001$). For those genes with a decreased expression in AML, *ALDH1A1* displayed one of the more unique expression patterns. *ALDH1A1* was expressed at very low levels in the favorable and intermediate cytogenetic subgroups, with its expression increasing primarily in patients with either trisomy 8, 5q-, or -7. *SERPINB9* was expressed at low levels in patients with t(15;17), and PLXNC1 was expressed at low levels in patients with inv(16) or t(8;21).

We also examined whether expressions of the 13 genes were associated with other AML molecular markers. *WT1*, *BIK*, *CCNA1*, *IL3RA*, and *JAG1* were expressed at higher levels in AML patients with FLT3 ITDs than those without the mutation, while *TRIB2* and *SERPINB9* were expressed at lower levels in *FLT3* ITD+ patients ($P < 0.01$, Table 6 in Supplement 9). *BIK* ($P = 0.001$) and *JAG1* ($P = 0.013$) also displayed increased expression in AML patients with *FLT3* TKD mutations (Table 7 in Supplement 9). The expression levels of only two genes (*CCNA1* and *TRIB2*) were possibly associated with *NRAS* mutations (Table 8 in Supplement 9), and expression of none of the 13 genes was significantly associated with *KRAS* mutations (Table 9 in Supplement 9). However, the power to detect significant associations between expression and *RAS* mutations may be limited by the small numbers of AML patients harboring these mutations. *EVI1* expression, which has been linked to a poor outcome in AML, was associated with decreased expression for *FUT4*, *HOMER3*, *JAG1*, and *PELO*, with only *ALDH1A1* displaying increased expression in *EVI1* positive samples (Table 10 in Supplement 9) (Valk et al., 2004). *BIK*, *IL3RA*, *HOMER3*, and *JAG1* displayed a decreased expression in AML patients harboring *CEBPA* mutations, while *PELO* and *PLXNC1* had an increased expression in patients with *CEBPA* mutations (Table 11 in Supplement 9).

Quantitative RT/PCR Assays Validate AML-Specific Expression Changes

To validate our microarray results and to determine the generalizability of the findings, we used Q-RT/PCR to examine the expression of AML-specific genes in a cohort of pediatric AML patients. We focused on the seven over-expressed candidate genes, believing that the over-expressed genes were more likely to be useful as future minimal residual biomarkers and therapeutic targets. Subjects for the Q-RT/PCR studies included nine normal donors (3 PBSC CD34+, 3 BM, and 3 PB) and 21 pediatric AML patients (Supplement 1). None of the subjects were previously examined in microarray analyses. The median age of the normal donors was 40 years (range 25–59), and the majority (78%) were male. The median age of the pediatric AML population was 9.6 years (range <1–18), with the slight majority (61%) being male. All of the pediatric AML samples were obtained from BM. The median blast count for the pediatric AML patients was 85% (range 30–99%). Cytogenetics were available for 16 pediatric AML patients (31% favorable, 38% intermediate, and 31% unfavorable).

All seven genes displayed increased expression in the pediatric AML samples as compared to the

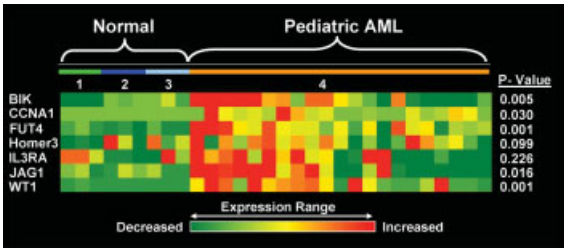

Figure 3. Heatmap of fold difference in expression for seven genes with increased expression. Q-RT/PCR studies determined the fold difference in expression of each normal hematopoietic and AML sample relative to the median expression of BM. The fold change expression for each sample was imported into GenePlus to create a heatmap displaying the relative amount of expression for each gene (rows) in each sample (column). Red cells indicate increased levels of expression, while green cells indicate decreased levels of expression. The nine normal samples have been grouped by CD34 status and donor source (1, green = PBSC CD34+; 2, dark blue = BM; and 3, light blue = PB). Orange bar (4) indicates the columns with the 21 pediatric AML samples. *P* values are provided (far right) for comparison between expression of all normal ($N = 9$) samples and the pediatric AML samples ($N = 21$). *P* values were computed using standard Student's *t*-test (one-tail distribution, unequal variance).

normal hematopoietic samples (Fig. 3). *BIK*, *FUT4*, and *WT1* displayed the most significant increase in expression ($P = 0.005$, 0.001, and 0.001, respectively); followed by *CCNA1* and *JAG1* ($P = 0.016$ and 0.030, respectively). Both *HOMER3* and *IL3RA* also displayed an overall increase in expression in AML samples compared to normal hematopoietic cells, but their expression differences were not statistically significant ($P = 0.099$ and 0.226, respectively). Together, the Q-RT/PCR results confirm the robustness of the microarray findings, determining that the over-expressed genes identified using microarray data in adult AML patients are also over-expressed in pediatric AML patients.

DISCUSSION

The study of AML-specific expression changes may provide critical insight into the biology governing leukemogenesis. In addition, genes with AML-specific expression may serve as potential prognostic factors, minimal residual disease biomarkers, and targets for the future therapies. Therefore, we used microarrays to identify genes with AML-specific expression changes. Four different types of normal hematopoietic cells were examined, ranging from immature CD34-expressing cells to unselected peripheral blood. Initial analyses with 26 AML samples identified 20 genes with significant expression differences between normal and AML cells (Supplement 3). Additional analyses examining 285 AML samples from another microarray data set (Valk et al., 2004) validated AML-specific expression for 13 of the 20 genes (Table 1, Fig. 2). Seven genes displayed an increased expression in AML, while 6 genes had a decreased expression. Since we were primarily interested in over-expressed genes, Q-RT/PCR assays examined the seven over-expressed genes in another population of AML patients. Five of the seven genes (*BIK*, *CCNA1*, *FUT4*, *JAG1*, and *WT1*) displayed a significant increase in expression in these AML patients as compared to normal hematopoietic cell donors (Fig. 3). *HOMER3* and *IL3RA* also had an increased expression in the AML patients, but this increase in expression was not statistically significant (Fig. 3).

Other studies have found an increased expression of *CCNA1*, *IL3RA*, and *WT1* in AML patients (Budel et al., 1989; Schwarzinger et al., 1990; Marosi et al., 1992; Miwa et al., 1992; Miyagi et al., 1993; Inoue et al., 1994; Bergmann et al., 1997; Inoue et al., 1997; Schmid et al., 1997; Munoz et al., 2001; Testa et al., 2002; Nakamaki et al., 2003; Holm et al., 2006; Lapillonne et al., 2006; Steinbach et al., 2006). In these studies, high levels of *WT1* and *ILR3A* expression were associated with unfavorable clinical outcomes, while increased expression of *CCNA1* was associated with a favorable prognosis (Budel et al., 1989; Munoz et al., 2001; Testa et al., 2002; Nakamaki et al., 2003; Lapillonne et al., 2006). *WT1* and *CCNA1* have also been directly linked to leukemogenesis, with overexpression promoting leukemia in transgenic mice (Liao et al., 2001; Nishida et al., 2006). In addition, recent work suggests that *WT1* and other overexpressed genes may be valid targets for the development of immunologic therapies and molecular inhibitors (Gaiger et al., 2000; Elisseeva et al., 2002; Scheibenbogen et al., 2002; Anuchapreeda et al., 2006; Glienke et al., 2006). Less is known about the functional and clinical significance of the other four over-expressed genes in AML. *FUT4* has been found to be over-expressed in a few myeloid cell lines (Taniguchi et al., 2000; Watkins and Clarke 2001), but its biological significance in AML is unknown. There has been a recent flurry of studies examining the JAG1/Notch pathway in normal hematopoiesis and stem cell renewal (Tohda and Nara 2001; Alcalay et al., 2003; Chiaramonte et al., 2005; Zweidler-McKay et al., 2005). However, it is unclear what role *JAG1* may or may not be playing in leukemogenesis. Although the significance of *HOMER3* expression changes in leukemic blasts is unknown, *HOMER3* probably regulates transcription and may play a role in the differentiation for some tissues (e.g. muscle) (Ishiguro and Xavier, 2004; Bortoloso et al., 2006). *BIK* displayed one of the largest and most significant

expression differences between normal and AML cells. *BIK* is a proapoptotic BH3-only gene within the BCL2 family; thus, a high level of expression seems counterintuitive for a malignant cell (Boyd et al., 1995). However, the increased expression of *BIK* may be an appropriate cellular response due to either a disruption in the downstream effectors of BCL2 pathway or the over-expression of anti-apoptotic BCL2 family members (Manion and Hockenbery, 2003; Certo et al., 2006). Together, all seven over-expressed genes have some link to hematopoiesis, and for three of the genes (*WT1*, *CCNA1*, and *IL3RA*), there is data directly linking their increased expression to leukemogenesis or clinical responses. Therefore, studies are underway to determine how *JAG1*, *FUT4*, *BIK*, and *HOMER3* may contribute to the biology of AML.

ALDH1A1 displayed the largest and most significant decrease in expression in AML (Fig. 2 and Table 1). *ALDHA1A* is one of several aldehyde dehydrogenase (ALDH) genes, catalyzing the oxidation of retinal to retinoic acid (Hsu et al., 1989; Duester 2000). Normal primitive hematopoietic progenitor cells have increased ALDH activity as compared to their mature counterparts, and ALDH activity may be required for the maintenance and repopulating potential of normal hematopoietic stem cells (Storms et al., 1999). However, the role of ALDH activity in leukemogenesis and the malignant stem cell is uncertain. Pearce at al. found that most AML blasts lacked ALDH activity (Pearce et al., 2005), which seems to correlate well with our microarray findings. In addition, these investigators found no correlation between ALDH activity in leukemic blasts and repopulating potential in irradiated NOD/SCID mice, suggesting that ALDH activity may not be required for leukemic stem cell renewal (Pearce et al., 2005). There is less known about the potential relationship of the other five genes and hematopoiesis. However, increased protein expression of TRIB2 was recently found to reduce CEBPA protein levels, and forced expression of TRIB2 promoted leukemia in mice (Keeshan et al., 2006). The investigator also found increased *TRIB2* expression at the RNA level in AML patients with *CEBPA* defects, which represented a relatively small subset of the total AML patients examined (Keeshan et al., 2006). We found that *TRIB2* expression was significantly decreased in most AML samples. However, our microarray studies were designed to identify the most frequent AML-specific expression changes, and as demonstrated in Figure 2, there is considerable variability in the expression of the 13 candidate genes in the AML samples, such that some genes with an overall decreased expression in AML samples (e.g. *TRIB2*) were expressed at high levels in small subsets of AML patients. The other genes help to regulate cell signaling (e.g. *PLXNC1*), cell division/cycle control (e.g. *PELO*), and cell adhesion (e.g. *PRUNE*) (Eberhart and Wasserman, 1995; Aurandt et al., 2002; Perrot et al., 2002; Walzer et al., 2005; Zollo et al., 2005; Aurandt et al., 2006; Kobayashi et al., 2006). Their role in hematopoiesis and leukemia has not been examined, but the functions of these genes make them attractive candidates for future biological and clinical studies.

To our knowledge, this is the first study to compare the global expression patterns of AML cells to a large number of different types of normal hematopoietic cells. Other microarray studies have primarily examined expression changes in transfected leukemic cell lines or focused on the discovery of novel prognostic factors in AML (Golub et al., 1999; Alcalay et al., 2003; Mizuki et al., 2003; Ross et al., 2003; Bullinger et al., 2004; Lacayo et al., 2004; Valk et al., 2004; Haferlach et al., 2005; Heuser et al., 2005; Kim et al., 2005; Marcucci et al., 2005; Neben et al., 2005; Verhaak et al., 2005; Camos et al., 2006; Ritter et al., 2006; Steinbach et al., 2006). In a study by Valk et al., the investigators identified 16 unique AML subgroups that had prognostic significance. In their studies, the investigators examined the hematopoietic expression profiles from eight healthy donors (five bone marrows and 3 CD34-selected products), determining that these normal samples clustered within two of the AML subgroups. However, the investigators did not specifically examine expression changes between normal hematopoietic and AML cells. In another study, Gal et al. identified 409 genes with expression changes between CD34+/CD38− and CD34+/CD38+ leukemic blasts ($N = 5$ AML patients) (Gal et al., 2006), and then, compared their list of 409 genes to a list of 4206 genes with expression changes during normal hematopoietic differentiation (Ivanova et al., 2002; Georgantas et al., 2004; Toren et al., 2005; Gal et al., 2006). Approximately 34% of the genes overlapped between the two gene lists, suggesting that a significant number of genes governing differentiation in normal cells may be promoting differentiation in leukemic blasts (Gal et al., 2006), but this later study also did not specifically examine expression differences between AML and normal hematopoietic subpopulations.

The 13 AML-specific genes identified in the microarray studies represent a highly selective

population of candidates for future studies. Because of the strict statistical criteria and the requirement of genes to be significant in all analyses, genes critical to the development of AML have certainly been excluded from the final list of 13 genes with AML-specific expression changes. Therefore, we have provided lists of all genes identified from each of the analyses (Supplement 4–8), so that investigators may examine how their gene of interest is expressed in AML cells relative to normal hematopoietic cells. Also, the expression profiles for these AML samples may not represent the expression profile of the "leukemic stem cell." Currently, there is considerable debate about what constitutes the leukemic stem cell and how to identify these cells. Investigators often functionally define leukemic stem cells by their ability to repopulate the hematopoietic compartment in transplant models (Lapidot et al., 1994), and the classic markers for normal hematopoietic stem cells (e.g. ALDH activity) may not be found in leukemic stem cells (Pearce et al., 2005). In addition, certain genetic abnormalities have been found to convert committed normal hematopoietic cells into repopulating transformed blasts that behave like malignant stem cells (Huntly et al., 2004; Krivtsov et al., 2006). These issues make it difficult to determine the optimal normal hematopoietic cells for comparison studies and impractical to isolate leukemic stem cells for microarray studies. Therefore, we examined a broad spectrum of normal hematopoietic cell types, identifying genes with expression differences in AML cells that were not present in a variety of different types of normal cells. Additional studies found that the expressions of most of these genes are significantly associated with other molecular markers (e.g. cytogenetic abnormalities, FLT3 ITDs, etc.) and with FAB classification. This may be expected, given that molecular biology and genetic expression are probably driven by and dependent on these underlying genetic events. Future studies will be required to determine the functional and clinical significance of these AML-specific genes in normal hematopoietic cells and their transformed counterparts. In addition, it will be important to correlate the expression of these 13 genes with other molecular markers such as nucleoporin mutations (NPM), which were not available in the data sets. However, as described above, several of these 13 genes have already been linked to leukemogenesis or AML prognosis, suggesting that the microarray analyses have identified functionally relevant genes.

In conclusion, our studies identified 13 genes with significant expression changes between normal hematopoietic cells and AML blasts. Dysregulation of three of the 13 genes (*CCNA1*, *TRIB2*, *WT1*) has already been directly linked to leukemogenesis (Liao et al., 2001; Keeshan et al., 2006; Nishida et al., 2006), and three other genes (*ALDH1A1*, *IL3RA*, *JAG1*) are known to play critical roles in the regulation of normal hematopoiesis. In addition, expression changes in *CCNA1*, *IL3RA*, and *WT1* may be associated with AML prognosis (Budel et al., 1989; Munoz et al., 2001; Testa et al., 2002; Nakamaki et al., 2003; Lapillonne et al., 2006). Most of the remaining 13 genes (*BIK*, *FUT4*, *HOMER3*, *PLXNC1*, *SERPINB9*, *PELO*, *PRUNE*), however, have not been studied in the context of hematopoiesis or leukemogenesis. Many of these later genes regulate cellular division, apoptosis, adhesion, or intracellular signaling, making them attractive candidates for future functional studies. We believe that this work provides novel insights into the expression differences between normal hematopoietic cells and AML blasts, identifying genes with AML-specific expression changes that may play a role in the biology of this disease.

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