

# Gene expression profiling of adult acute myeloid leukemia identifies novel biologic clusters for risk classification and outcome prediction

Carla S. Wilson, George S. Davidson, Shawn B. Martin, Erik Andries, Jeffrey Potter, Richard Harvey, Kerem Ar, Yuexian Xu, Kenneth J. Kopecky, Donna P. Ankerst, Holly Gundacker, Marilyn L. Slovak, Monica Mosquera-Caro, I-Ming Chen, Derek L. Stirewalt, Maurice Murphy, Frederick A. Schultz, Huining Kang, Xuefei Wang, Jerald P. Radich, Frederick R. Appelbaum, Susan R. Atlas, John Godwin, and Cheryl L. Willman

To determine whether gene expression profiling could improve risk classification and outcome prediction in older acute myeloid leukemia (AML) patients, expression profiles were obtained in pretreatment leukemic samples from 170 patients whose median age was 65 years. Unsupervised clustering methods were used to classify patients into 6 cluster groups (designated A to F) that varied significantly in rates of resistant disease (RD;  $P < .001$ ), complete response (CR;  $P = .023$ ), and disease-free survival (DFS;

$P = .023$ ). Cluster A ( $n = 24$ ), dominated by *NPM1* mutations (78%), normal karyotypes (75%), and genes associated with signaling and apoptosis, had the best DFS (27%) and overall survival (OS; 25% at 5 years). Patients in clusters B ( $n = 22$ ) and C ( $n = 31$ ) had the worst OS (5% and 6%, respectively); cluster B was distinguished by the highest rate of RD (77%) and multidrug resistant gene expression (*ABCG2*, *MDR1*). Cluster D was characterized by a "proliferative" gene signature with the highest proportion of detectable

cytogenetic abnormalities (76%; including 83% of all favorable and 34% of unfavorable karyotypes). Cluster F ( $n = 33$ ) was dominated by monocytic leukemias (97% of cases), also showing increased *NPM1* mutations (61%). These gene expression signatures provide insights into novel groups of AML not predicted by traditional studies that impact prognosis and potential therapy. (Blood. 2006;108:685-696)

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## Introduction

In most patients, particularly those over 55 years of age, acute myeloid leukemia (AML) is a highly resistant disease (RD) and overall outcomes remain extremely poor.<sup>1-5</sup> While improved survival has been achieved in younger AML patients or in selected cytogenetic subsets, older patients are either unable to receive intensive chemotherapy or such therapy results in remission rates of only 25% to 55% and overall survival (OS) rates of 10% or less.<sup>1,6-10</sup> In addition to age and white blood cell (WBC) count, the presence of recurring cytogenetic abnormalities provides the most important prognostic information in AML. Unfortunately, cytogenetic abnormalities associated with favorable outcomes account for only 5% to 12% (*t(8;21)*), 5% to 8% (*inv(16)*), and 10% to 12% (*t(15;17)*) of all AML cases and are disproportionately seen in younger patients.<sup>11,12</sup> In contrast, approximately 50% to 70% of all AMLs have normal or risk-indeterminate karyotypes.<sup>11,13,14</sup>

Gene mutations confer additional prognostic information that may be useful in refining cytogenetic risk classification.<sup>15-19</sup> The most frequently acquired mutation in AML is a mutation at exon 12 of the nucleophosmin (*NPM1*) gene. This multifunctional, nucleocytoplasmic shuttling protein primarily resides in the nucleolus,

playing a role in maintenance of genomic integrity, *ARF-p53* pathway regulation, and centrosome duplication.<sup>20,21</sup> Mutated *NPM1* relocates to the cytoplasm and disrupts normal *NPM1* function. Approximately 25% to 35% of AML patients have *NPM1* mutations,<sup>22-24</sup> with a higher percentage (47%-60%) seen among those with a normal karyotype.<sup>22,25-26</sup> The impact on survival is variable, but likely favorable, with secondary influences such as concurrent *FLT3* mutations having potentially significant roles.<sup>23,24,26,27</sup> The *FLT3* mutations occur as internal tandem duplications (ITDs), observed in 15% to 35% of AML, or point mutations of the intracellular tyrosine-kinase domain (TKD), seen in an additional 5% to 10% of patients.<sup>19</sup> The prognostic impact of *FLT3* mutations trends toward decreased survivals or increased relapse rates primarily for patients with *FLT3*-ITDs.<sup>28-30</sup>

In contrast to traditional cytogenetic analysis or the detection of mutations in individual genes, global gene expression profiling provides a powerful method to probe the marked biologic heterogeneity of AML. Comprehensive expression profiles have the power to provide new insights into mechanisms of leukemogenesis and to enhance risk classification and therapeutic targeting in AML. A

From the Department of Pathology and the Cancer Research and Treatment Center, University of New Mexico (UNM), Albuquerque; Sandia National Laboratories, Albuquerque, NM; Departments of Computer Science, Mathematics and Statistics, and Physics and Astronomy, and the UNM Center for High Performance Computing, University of New Mexico, Albuquerque; Southwest Oncology Group Statistical Center, Seattle, WA; City of Hope National Medical Center, Duarte, CA; Fred Hutchinson Cancer Research Center, Seattle, WA; and Loyola University, Chicago, IL.

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Reprints: Cheryl L. Willman, UNM Cancer Research and Treatment Center, MSC08 4630, 1 University of New Mexico, Albuquerque, NM 87131; e-mail: cwillman@salud.unm.edu.

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number of laboratories using supervised learning algorithms have identified unique gene expression signatures associated with karyotypic abnormalities, normal karyotypes, and *NPM1* mutation status.<sup>31-39</sup> In contrast, we wished to determine whether gene expression profiling using an entirely unsupervised approach could reveal intrinsic biologic groups of AML among a set of well-characterized older AML patients, with a high frequency of normal and unfavorable cytogenetic abnormalities. We further wished to determine whether the gene expression signatures we derived were useful in risk classification and therapeutic targeting in this poor-risk disease.

## Patients, materials, and methods

### Patients

This study used pretreatment samples from patients with previously untreated de novo or secondary AML by French-American-British (FAB) criteria who were registered to Southwest Oncology Group (SWOG) clinical trials for patients over the age of 55 years (studies S9031, S9333), patients aged 15 to 55 years (S9034, S9500), and patients with secondary AML (S9126). Trial details have been previously reported.<sup>2,9,40-42</sup> All trials except S9031 excluded patients with acute promyelocytic leukemia (FAB-M3); S9031 evaluation was limited to non-M3 AML patients who received induction chemotherapy with Ara-C and an anthracycline. Case selection was restricted to patients with cryopreserved blood or bone marrow containing more than 80% leukemic blasts, stored in the SWOG Myeloid Leukemia Repository (University of New Mexico) after appropriate informed consent. Microarrays were performed for 185 eligible patients between February 2003 and September 2003, and 170 had high-quality gene expression data that fulfilled technical criteria for study inclusion (outlined in "Gene expression profiling"). Clinical, morphologic, cytogenetic, and outcome data on the 170 patients, along with all gene expression profiles, are provided at the National Cancer Institute Gene Expression Data Portal website. Conventional cytogenetic banding was performed in SWOG-approved laboratories with review and risk classification assessment performed by members of the SWOG Cytogenetic Committee per published criteria.<sup>11</sup> For studies S9031, S9126, S9333, and S9500, response to induction chemotherapy was assessed according to SWOG criteria.<sup>43</sup> Study S9034, an intergroup trial coordinated by the Eastern Cooperative Oncology Group (E3489), used slightly different response criteria.

### Gene expression profiling

RNA was prepared from thawed cryopreserved samples with the Qiagen RNeasy mini kit (Qiagen, Valencia, CA). All specimens had more than 80% blasts as confirmed by microscopic review of Wright-stained cytopsin preparations of the thawed cell suspensions. Total RNA concentration was quantified with the RiboGreen assay (Molecular Probes, Eugene, OR); RNA integrity and DNA contamination were evaluated as described at the University of New Mexico Cancer Research and Treatment Center website.<sup>44</sup> The isolated RNA was reverse transcribed into cDNA and retranscribed into cRNA after double amplification using a modification reported by Ivanova et al to enhance detection of low-abundance genes.<sup>44,45</sup> Biotinylated cRNA was fragmented and hybridized to HG\_U95Av2 oligonucleotide microarrays (Affymetrix, Santa Clara, CA).<sup>44</sup> After analysis with Affymetrix Microarray Suite (MAS 5.0), the data were scaled to minimize experimental variation.<sup>44</sup> Technical criteria for case inclusion of the 185 initial specimens evaluated included adequate total RNA more than 2.5  $\mu$ g, good quality cRNA, good quality scanned images, and good experimental quality. Experimental quality was assessed by *GAPDH* at least 1800, at least 10% expressed genes, and *GAPDH* 3'/5' amplification ratios of 4 or below. High-quality expression data were obtained on 170 of the 185 specimens, 133 from marrow and 37 from peripheral blood. Of the original 12 625 probe sets in the Affymetrix HG\_U95Av2 probe sets, 9463 genes were "present" in at least 1 case; these genes were further analyzed after transformation to Savage rank scores (VxInsight).<sup>44</sup>

### *NPM1* and *FLT3* mutational status

Samples were evaluated for *NPM1* mutations using cDNA amplified to generate a 249 bp fragment spanning portions of exons 11 and 12 (Document S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).<sup>44</sup> The polymerase chain reaction (PCR) products were subjected to dissociation analysis (65°C to 80°C) with appropriate controls. Samples with characteristic melting profiles underwent agarose gel electrophoresis and hybridization with *NPM1* variant A probe or a pool of 13 probes for variants B to Q.<sup>26</sup> Cases were also evaluated for *FLT3*-ITDs in exons 14 and 15 as previously described and screened for *FLT3*-TKD in exon 20 using 2 methods (see Document S1).<sup>44,46</sup> Suspected *FLT3*-ITDs and TKD mutations were confirmed by sequencing.<sup>46</sup>

### Statistical analysis

VxInsight,<sup>47</sup> developed at Sandia National Laboratories for extremely large datasets, was the primary unsupervised data mining tool used in this study.<sup>48-51</sup> Using a force-directed placement algorithm, clusters were formed 100 times using different starting conditions for the random number generator. The most representative single ordination (the most central member of the whole set) was then determined by measurement of the total overlap of local neighborhoods around the individual genes. Analysis of variance (ANOVA) was used to identify rank-ordered gene lists characterizing each cluster; bootstrap resampling was applied to estimate the stability of these lists.<sup>51</sup> Receiver operator characteristic (ROC) curves and genetic algorithm K-nearest neighbor method (GA/KNN) were additionally employed to identify top characterizing genes for the VxInsight-derived clusters, as further explained in Tables S1-S14.<sup>44</sup> The full rank-ordered gene lists derived from ANOVA with bootstrapping, ROC, and GA/KNN are provided in Tables S1-S14.<sup>44</sup> Principal component analysis (PCA) and hierarchical clustering were performed using MATLAB (MathWorks, Natick, MA).<sup>52,53</sup> Concordance between VxInsight and hierarchical clusters was measured by the adjusted Rand index, with Monte Carlo estimation of statistical significance ( $n = 10\,000$  replications).<sup>54</sup>

Comparisons between clusters were based on the Kruskal-Wallis test for continuous variables (age, laboratory values) and on the  $\chi^2$  approximation of the Fisher exact test and Pearson  $\chi^2$  test for independence for dichotomous and categorical variables (complete response [CR], resistant disease [RD], FAB classification, cytogenetic characteristics, *FLT3* mutations). Overall survival (OS) was measured from registration on treatment study until death from any cause, with observation censored for patients last known alive. Disease-free survival (DFS) was measured from the date the CR was established until the relapse of leukemia or death from any cause, with observation censored for patients last known to be alive without report of relapse. Distributions of OS and DFS were estimated by the method of Kaplan and Meier<sup>55</sup> and compared between clusters using the log-rank test.<sup>56</sup> Multivariate analyses of cluster differences and prognostic factors were based on logistic regression models for CR and RD and on proportional hazards regression models for OS and DFS.<sup>57</sup> In logistic regression models, differences in proportions between clusters are represented as odds ratios relative to a defined cluster. This permits the cluster differences to be compared on a consistent scale regardless of other terms in the model. The hazard ratio plays an analogous role for proportional hazards regression models. All *P* values were 2-tailed and, in view of the exploratory nature of these analyses, were calculated without adjustment for multiple testing.

## Results

### AML cohort

Gene expression profiles were obtained from a retrospective cohort of 170 patients with previously untreated AML. Clinical, morphologic, cytogenetic, and mutation status of the cohort, outlined in Table 1, showed no sex predominance and most patients (80%)

**Table 1. Clinical, morphologic, cytogenetic, and mutation characteristics of adult AML cohort (N = 170 patients)**

Characteristic	Data
<b>Age, no. patients (%)</b>	
Younger than 56 y	34 (20)
56 y or older	136 (80)
<b>Sex, no. patients (%)</b>	
Female	85 (50)
Male	85 (50)
<b>FAB classification, no. patients (%)</b>	
M1	40 (24)
M2	60 (35)
M4	42 (25)
M5	13 (8)
M6	1 (1)
M7	2 (1)
M0	10 (6)
Other	2 (1)
<b>Evaluable cytogenetics, no. patients (%)</b>	
No	29 (17)
Yes	141 (83)
<b>Cytogenetic risk category, no. patients (%)*</b>	
Favorable	12 (9)
Intermediate	83 (59)
Unfavorable	44 (31)
Not assigned	2 (1)
<b>Specific cytogenetic features, no. patients (%)†</b>	
Normal	65 (46)
t(8;21)	8 (6)
inv(16)	4 (3)
<b>NPM1 mutation status, no. patients (%)‡</b>	
Type A	45 (27)
Non-type A§	5 (3)
Type A or non-A	50 (30)
<b>FLT3 mutation status, no. patients (%)  </b>	
ITD	46 (27)
TKD	13 (12)
Median age, y (range)	65 (20-84)
Median WBC count, × 10 <sup>9</sup> /L (range)	22.9 (0.7-272.5)
Median peripheral blasts, % (range)	43 (0-99)
Median marrow blasts, % (range)	70 (5-99)
Median platelet count, × 10 <sup>9</sup> /L (range)	53 (2-1052)
Median hemoglobin level, g/dL (range)	9.1 (4.3-14.4)

ITD indicates internal tandem duplication; TKD, point mutations of intracellular tyrosine-kinase domain.

To convert hemoglobin level from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

\*n = 141. Cytogenetic risk categories are defined by the following cytogenetic abnormalities (abn): favorable: inv(16)/t(16;16)/del(16q), t(8;21), or t(15;17) with any additional abn; intermediate: +8, -Y, +6, del(12p), or normal karyotype; unfavorable: -5/del(5q), -7/del(7q), inv(3q), abn 11q, t(6;9), t(9;22), abn 17p, or complex karyotype defined as more than 3 abn. Other findings are listed as not assigned or nonevaluable.<sup>12</sup>

†n = 141.

‡n = 165.

§Non-type A includes 4 cases that hybridized to probes for 13 known variants (B to Q)<sup>26,44</sup> and 1 case that was sequenced.

||n = 105.

over the age of 55 years with a median age of 65 years (range, 20-84 years). Thirty-two cases (19%) were judged by clinical history to have secondary AML, while 104 (61%) had clinically de novo AML (clinical onset was not recorded in the 2 trials for patients of age 15 to 55, and in none of the other trials was secondary AML further classified as myelodysplastic syndrome [MDS]- versus treatment-related). All FAB subtypes were included except AML-M3, with a preponderance of acute myeloblastic leukemia with maturation (FAB-M2, 35%). Adequate cytoge-

netic analyses were obtained on 141 (83%) of the patients, and 139 of these could be assigned to cytogenetic risk categories. Most cytogenetically evaluable cases fell into the intermediate cytogenetic risk group (59%) due to the high percentage of patients with normal karyotypes (46%).

**Unsupervised clustering algorithms**

VxInsight analysis partitioned the AML patients into 6 distinct and stable groups based on strong similarities in gene expression among the 9463 genes, visualized in Figure 1.

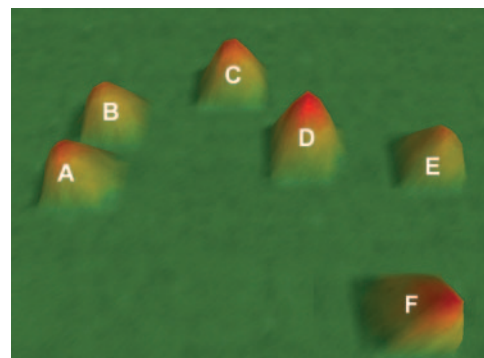
Membership among clusters ranged from a low of 18 patients (cluster E, 11%) to a high of 42 patients (cluster D, 25%). Clusters derived from PCA and unsupervised hierarchical clustering showed significant levels of concordance with the VxInsight-derived clusters (P < .001) (Figure 2).

**VxInsight cluster membership, treatment outcomes, and clinical correlates**

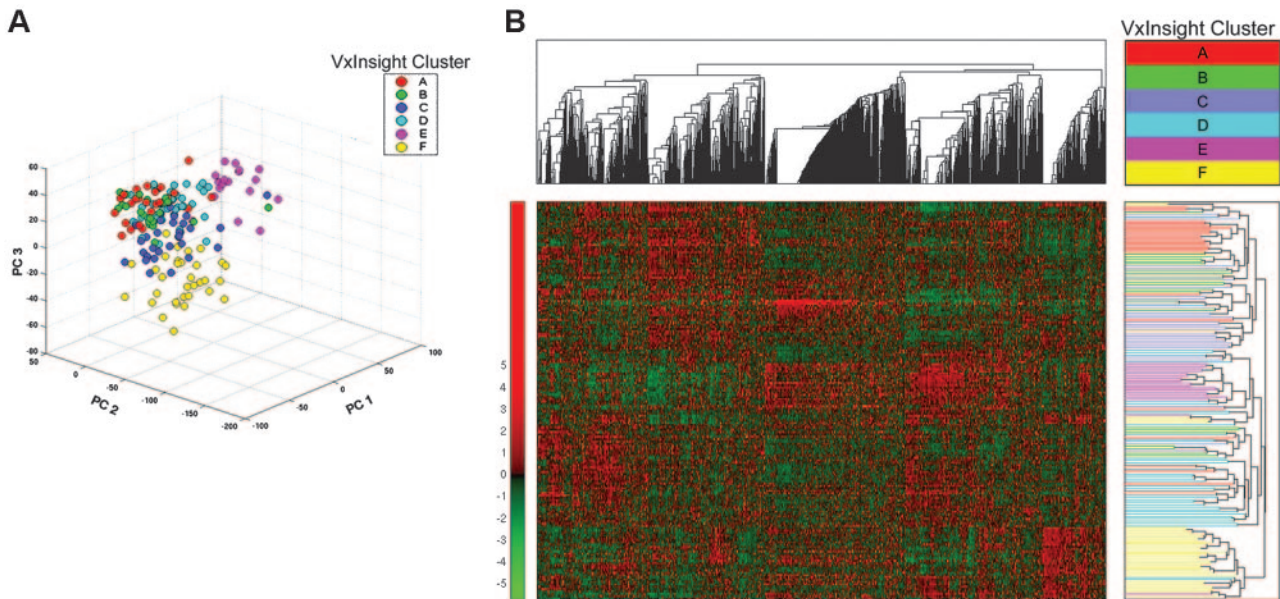
DFS varied significantly between VxInsight clusters (Figure 3; P = .023). Clusters A and C had the lowest and highest hazard ratios, respectively, for relapse or death in remission (Table 2), and all 3 remitting patients in cluster B relapsed within 16 months. Of the 170 patients, 145 have died and the remaining 25 were last known to be alive at 13 months to 10.9 years after starting treatment (median, 6.1 years). OS did not vary significantly among clusters (P = .40) but generally paralleled the DFS results, with cluster A having the best OS and clusters B and C generally the worst (Table 2; Figure 3).

Response to induction chemotherapy varied significantly among the 6 clusters (Table 2). Sixty (35%) of the 170 patients were resistant to their protocol induction chemotherapy, with a significantly different RD rate seen between clusters (P < .001). This was largely due to an exceptionally high RD rate in cluster B (77%) compared with all other clusters combined (43 of 148; 29%), although heterogeneity among the remaining 5 clusters was also significant (P = .021). Roughly complementary results were observed for CR. Seventy-three patients (43%) achieved CR, and the CR rate varied significantly among clusters (P = .023), being lowest in cluster B (14%). Forty-seven of the remitting patients have relapsed, and 11 others have died without report of relapse.

VxInsight cluster membership was not significantly correlated with patient age or de novo versus secondary onset of disease (Figure 4; Table 3). Despite the absence of a significant association



**Figure 1. VxInsight clusters in adult AML.** Six distinct clusters of AML patients are identified based on gene expression profiles and designated A to F. The data are visualized as a 3-dimensional terrain map with 2-dimensional distances reflecting gene expression profile correlates and the third dimension representing cluster membership density. Additional information on VxInsight is available at the University of New Mexico Health Sciences Center website and Sandia National Laboratories website.<sup>44,47</sup>



**Figure 2. Alternative clustering algorithms of adult AML cohort.** (A) A multidimensional scatterplot generated using principal component analysis (PCA) reduces the dimensionality of the data by projecting the expression data matrix into 3 dimensions. The largest sources of gene expression signal variance are represented as principal components (labeled PC1, PC2, PC3).<sup>52</sup> (B) Two-dimensional unsupervised clustering dendrograms and “heat map” of gene expression data from the 170 AML cases using 9463 genes. Pearson correlation coefficient was used to compute gene and patient similarity. The cluster-to-cluster distance was computed using the average linkage. The relative gene expression scale is depicted on the left with the normalized scores ranging from  $-5$  to more than  $5$ . Gene cluster and patient cluster dendrograms are plotted to the top and right sides of the heat map, respectively. After PCA and hierarchical clustering was performed, the individual patients were color coded for comparison with their VxInsight cluster membership. These methods showed a significant degree of concordance with VxInsight cluster membership (adjusted Rand index = 0.3457,  $P < .001$ ) (Table S15).<sup>44</sup>

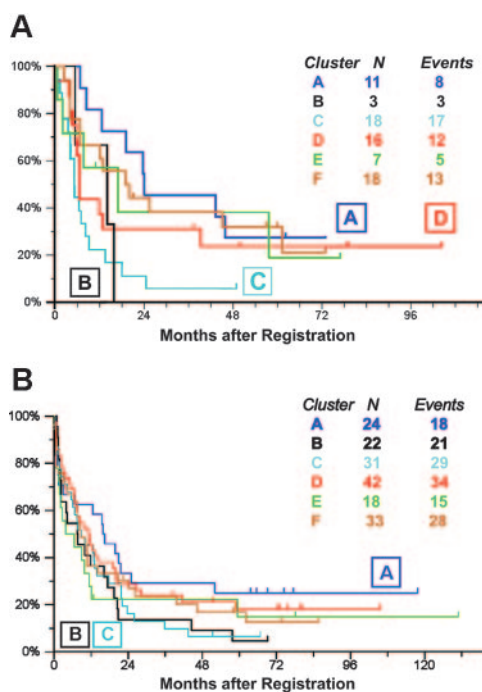
with age, it was noteworthy that only 4% of patients in the 2 clusters with worse outcomes (B, C) were under age 56, compared with 27% (32 of 117) of patients in the remaining clusters.

Clinical and laboratory parameters that showed significant correlation with VxInsight cluster membership were pretreatment

WBC counts, blast percentages, platelet counts, FAB classification, normal or t(8;21) karyotypes, and *NPM1* mutation status (Tables 1 and 3). The lower WBC and blast counts in the poor-risk clusters (B, C) suggest underlying marrow damage. Clusters were segregated by their degree of blast maturation and more specifically by myeloid versus monocytic derivation (FAB classification) (Figure 4). Cluster F consisted almost entirely of monocytic leukemias, with 97% of members having FAB-M4 or FAB-M5, although monocytic leukemias were present in lower proportions in the 5 other clusters. Cytogenetic risk groups varied with cluster membership (Table 3; Figure 4). Cluster A, with the best OS, had the highest percentage of normal karyotypes (75%). In contrast, cluster D had the highest percentage of karyotypic abnormalities (76%), including those associated with both favorable (8 of 8 patients with t(8;21) and 2 of 4 with inv(16)) and unfavorable risk.

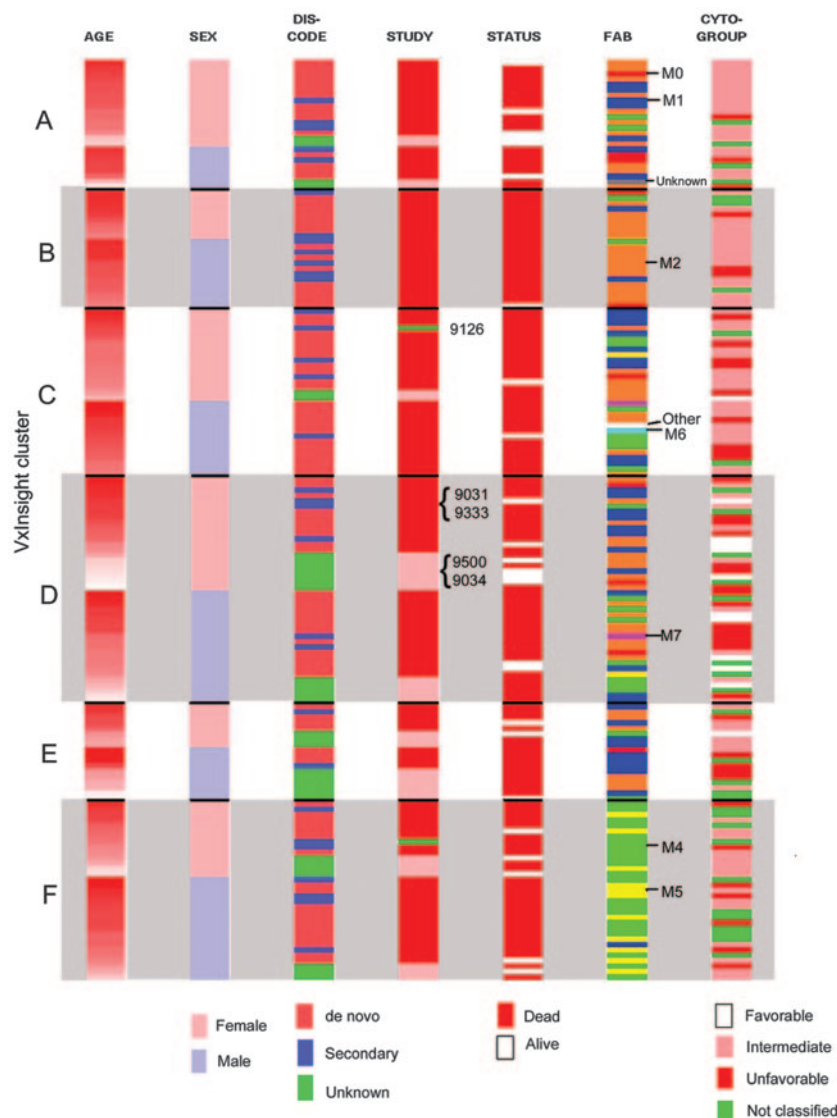
*NPM1* mutations were present in 30% (50 of 165) of cases with significant differences observed between VxInsight clusters (Tables 1 and 3; Figure 5). The highest prevalences were seen in cluster A (78%), which also had the highest percentage of females and normal karyotypes, and in cluster F (61%) with the predominance of monocytic leukemias. *FLT3*-ITD mutations were identified in 27% of cases (Table 1) with no significant differences among VxInsight groups (Table 3). A significant number of patients with *FLT3*-ITDs also had *NPM1* mutations (Table 3). *FLT3*-TKDs were found in 12% of the AMLs investigated; cluster A had the highest percentage of point mutations (*FLT3*-TKDs).

Further analyses were performed to investigate whether comparisons of outcomes between the clusters might be biased by confounding effects of the other factors considered. In multivariate logistic regression analysis, increasing age ( $P = .024$ ), secondary AML onset ( $P = .010$ ), and unfavorable cytogenetic risk category ( $P = .030$ ) had independent detrimental prognostic effects on RD. AML onset and/or cytogenetic risk group were unknown for 57 of



**Figure 3. Estimated distributions for disease-free survival and overall survival of AML patients by VxInsight cluster membership.** (A) DFS varied significantly among the 6 clusters (log-rank,  $P = .023$ ). (B) OS showed a trend that paralleled the DFS findings among the 6 clusters (log-rank,  $P = .40$ ). Tick marks indicate censored observations for patients last known to be alive without report of relapse.





**Figure 4. Clinical characteristics of 170 AML patients separated by VxInsight cluster membership.** Each horizontal row represents an individual AML patient, and each column is the clinical variable for that individual. Age is presented as a continuum with the lightest color (white) representing the youngest patients and the darkest color (dark red) representing the oldest patients. Discode relates to AML onset; it is color coded and categorized similar to the remaining clinical variables, as described below the associated columns. Distribution of FAB classification varied significantly among clusters ( $P < .001$ ).

cluster F has the most distinguishing genetic profile due to the significant number of genes associated with monocyte differentiation and function (*LILRB1*, *AOAH*, *TIL3*, *CASP1*, *LGALS3*). The multidrug resistant gene for vault-transporter lung resistance protein (*LRP*, ranked 54) is also found in this group.

Alternative gene lists using different statistical and normalization methods are provided in Tables S7-S14.<sup>44</sup> These show extensive overlap with the ANOVA-derived gene lists.

## Discussion

We used a novel unsupervised clustering algorithm (VxInsight) to analyze gene expression profiles from older AML patients with a high proportion of intermediate- and poor-risk outcome factors. This type of analysis, without knowledge of prior class definitions, allows for identification of fundamental subsets of patients sharing similar expression signatures. Unanticipated similarities between cytogenetically diverse patient groups, as discovered in this study and reported by others,<sup>35</sup> would have been harder to detect with a more restrictive supervised approach. The result is an interesting separation of the AML cases into 6 distinct clusters with outcome differences.

In contrast to previous studies using unsupervised computational methods alone,<sup>32,34,35</sup> we found significant outcome differences between the clusters defined by gene expression for RD after induction therapy ( $P < .001$ ), CR rate after induction therapy ( $P = .023$ ), and DFS ( $P = .023$ ). The heterogeneity of RD and CR rates among clusters was not explained by confounding effects of age, AML onset, and unfavorable cytogenetics, indicating that the clustering conveyed prognostic information independent of the other factors. For some patients, data were absent regarding prognostic factors, particularly de novo versus secondary onset of AML and cytogenetics. However, in the multivariate regression analyses of treatment outcomes, it was evident that excluding the patients with incomplete data did not markedly influence the magnitude or statistical significance of differences between clusters. This was most clearly evident for RD, for which both the statistical significance of cluster differences and the ORs representing the magnitudes of those differences were essentially unchanged by the adjustment for covariates. Evaluation of DFS was limited by the small number of remitting patients with complete data. For CR, adjusting for the covariates decreased the statistical significance from  $P = .023$  to  $.051$ , which is not a profound change, especially given the necessity of excluding patients with incomplete data from the multivariate analysis.

**Table 3. Clinical and laboratory correlates of VxInsight clusters derived from 170 adult AMLs**

	A	B	C	D	E	F	P*
No. patients	24	22	31	42	18	33	—
Median age, y (range)	67 (22-76)	68 (58-76)	65 (44-84)	62 (20-83)	60 (21-81)	64 (34-83)	.27
No. with secondary AML (%)	5 (25)	7 (32)	5 (17)	6 (20)	2 (22)	7 (27)	.87
<b>Median pretreatment laboratory values</b>							
WBC count, × 10 <sup>9</sup> /L	29	6	14	20	33	57	< .001
% PB blasts	76	28	38	48	85	11	< .001
% BM blasts	82	59	55	71	80	70	.004
Platelet count, × 10 <sup>9</sup> /L	36	91	62	42	52	62	.002
Hemoglobin level, g/dL	9.8	8.7	9.4	8.7	9.5	9.3	.20
<b>Cytogenetic risk group, no. (%)*</b>							
Favorable	0 (0)	0 (0)	1 (3)	10 (29)	1 (8)	0 (0)	< .001
t(8;21)	0 (0)	0 (0)	0 (0)	8 (24)	0 (0)	0 (0)	< .001
Intermediate	16 (80)	15 (79)	19 (66)	9 (27)	7 (54)	17 (71)	< .001
Abnormal	1 (5)	7 (37)	3 (10)	1 (3)	1 (8)	5 (21)	—
Normal	15 (75)	8 (42)	6 (55)	8 (24)	6 (46)	12 (50)	.011
Unfavorable, no. (%)†	4 (20)	4 (21)	9 (31)	15 (44)	5 (38)	7 (29)	.41
<b><i>NPM1</i> mutation status, no. (%)‡</b>							
<i>NPM1</i> + (%)	18/23 (78)	1/22 (5)	4/30 (13)	2/41 (5)	6/18 (33)	19/31 (61)	< .001
<b><i>FLT3</i> mutation status, no. (%)§</b>							
ITD+	8/24 (33)	3/22 (14)	6/31 (19)	13/42 (31)	5/18 (28)	11/32 (34)	.467
TKD+	5/19 (26)	0/9 (0)	2/18 (11)	3/27 (11)	1/8 (13)	2/24 (8)	.404
Both <i>NPM1</i> + and <i>FLT3</i> -ITD	7/23 (30)	1/22 (5)	2/30 (7)	2/41 (5)	3/18 (17)	10/31 (32)	.003

P values were determined using  $\chi^2$  test.

To convert hemoglobin level from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

PB indicates peripheral blood; BM, bone marrow.

\*n = 139.

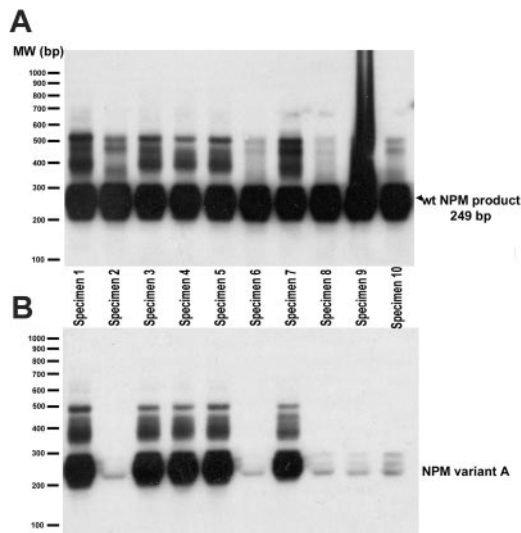
†11q23 abnormalities were seen in 9 cases and distributed in clusters A to F as 0, 1, 2, 1, 1, 3, respectively.

‡n = 165; values given indicate number of patients with the mutation out of number of patients tested.

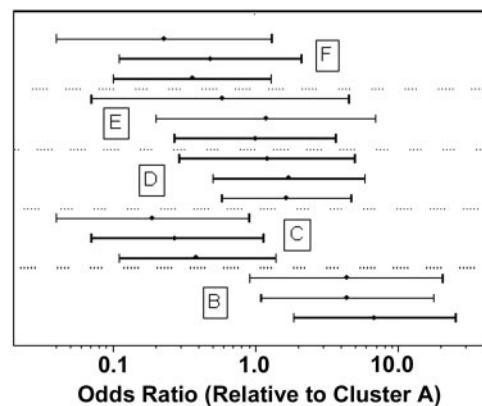
§For ITD, n = 169; for TKD, n = 105. Values given indicate number of patients with the mutation out of number of patients tested.

Members of cluster A had the best DFS and OS: 27% and 25%, respectively, at 5 years. The striking finding for this group was the high percentage of *NPM1* mutations (78%). This group has many of the characteristics emerging for cases of AML with *NPM1* mutations, including the disproportionate number of women (67%), increase in normal karyotypes (75%), older age (but not significantly different than other cluster groups), and higher WBC counts.<sup>22,24-27</sup> Genes responsible for the better outcome were not

clearly identified, but significant overlap was discovered between top genes predicting for cluster A and for those previously reported for *NPM1* mutations based on the data by Alcalay et al (Tables S18-S19).<sup>39,44</sup> For example, 8 of the top 15 ranked genes (53%) for cluster A were also genes found to be predictive of *NPM1* mutations.<sup>39</sup> This finding is particularly striking given the use of different Affymetrix platforms with different probe sets (see Supplement). Genes predictive of cluster A were also examined in the AML dataset of Valk et al<sup>34</sup>; their cluster group 6 showed a



**Figure 5. Nucleophosmin (*NPM1*) gel analysis/hybridization.** (A) Hybridization to wild-type *NPM1* probe confirms the presence of wild-type *NPM1* in 7 patient specimens and 3 cell lines (specimens 8 to 10). All 165 AML samples contained wild-type *NPM1*. (B) Patient specimen (1, 3-5, 7) hybridized to *NPM1* variant A probe consistent with an *NPM1* variant type A mutation (see Supplement).<sup>44</sup>



**Figure 6. Estimated odds ratios for resistant disease in each of clusters B through F, relative to cluster A.** For each cluster 3 estimates are shown: based on all 170 patients without adjustment for other factors (bottom); based on 113 patients with known AML onset (de novo versus secondary) and cytogenetic risk category without adjustment for other factors (middle); and based on the same 113 patients but with adjustment for age, AML onset, and cytogenetic risk category (unfavorable versus favorable/intermediate) (top). Bars indicate 95% confidence intervals. Results of the 3 analyses are generally consistent and, in particular, the heterogeneity of RD rates among the 6 clusters remained statistically significant after adjusting for possible confounding ( $P < .001$ ).

**Table 4. Rank-ordered gene lists characterizing each cluster; genes are up-regulated or down-regulated in relation to the other clusters**

Cluster and order	P	Gene	Probe set	Description
<b>Up-regulated genes</b>				
<b>A</b>				
1	.002	<i>LTBP1</i>	1495_at	Latent transforming growth factor beta binding protein 1
2	.003	<i>CASP3</i>	36143_at	Caspase-3, apoptosis-related cysteine protease
4	.011	<i>FTO</i>	37242_at	Fatso
5	.015	<i>FOXC1</i>	41027_at	Forkhead box C1
6	.003	<i>COL4A5</i>	32667_at	Collagen, type IV, alpha 5 (Alport syndrome)
11	.015	<i>RASGRP3</i>	34748_at	RAS guanyl-releasing protein 3 (calcium and DAG-regulated)
19	.025	<i>MYCN</i>	35158_at	v-myc myelocytomatosis viral-related oncogene, neuroblastoma derived
<b>B</b>				
1	.005	<i>BIA2</i>	36713_at	BIA2
2	.003	<i>CXorf6</i>	38916_at	Chromosome X open reading frame 6
3	.014	<i>PLOD2</i>	34795_at	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2
4	.011	<i>OPTN</i>	41744_at	Optineurin
5	.016	<i>CLIC2</i>	40013_at	Chloride intracellular channel 2
6	.017	<i>RHD</i>	37164_at	Rhesus blood group, D antigen
7	.021	<i>CDC42BPA</i>	39962_at	CDC42 binding protein kinase alpha (DMPK-like)
8	.038	<i>ANK3</i>	36965_at	Ankyrin 3, node of Ranvier (ankyrin G)
<b>C</b>				
1	.003	<i>SDR1</i>	40782_at	Short-chain dehydrogenase/reductase 1
2	.01	<i>SDS</i>	40390_at	Serine dehydratase
6	.058	<i>SERPINF1</i>	40856_at	Serine (or cysteine) proteinase inhibitor, clade F
8	.092	<i>MALT1</i>	38575_at	Mucosa-associated lymphoid tissue lymphoma translocation gene 1
9	.051	<i>HERPUD1</i>	39733_at	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
10	.056	<i>IRF4</i>	37625_at	Interferon regulatory factor 4
11	.034	<i>IL10RA</i>	1062_g_at	Interleukin-10 receptor, alpha
12	.035	<i>BCS1L</i>	31842_at	BCS1-like (yeast)
13	.054	<i>RAB9A</i>	39628_at	RAB9A, member RAS oncogene family
<b>D</b>				
1	.001	<i>RNASEP1</i>	37471_at	Ribonuclease P1
2	.006	<i>PGDS</i>	35523_at	Prostaglandin D2 synthase, hematopoietic
3	.006	<i>NHP2L1</i>	41746_at	NHP2 nonhistone chromosome protein 2-like 1 ( <i>S cerevisiae</i> )
4	.010	<i>UNG</i>	37686_s_at	Uracil-DNA glycosylase
5	.011	<i>POP1</i>	38513_at	Processing of precursors 1
6	.008	<i>HSU79274</i>	31838_at	Protein predicted by clone 23733
7	.005	<i>CGI-51</i>	34845_at	CGI-51 protein
8	.010	<i>NASP</i>	33255_at	Nuclear autoantigenic sperm protein (histone-binding)
9	.010	<i>CDK4</i>	1942_s_at	Cyclin-dependent kinase 4
<b>E</b>				
1	.002	<i>CAPN1</i>	33908_at	Calpain 1, (mu/l) large subunit
2	.010	<i>HSF1</i>	40200_at	Heat shock transcription factor 1
3	.005	<i>ACTN4</i>	41753_at	Actinin, alpha 4
5	.007	<i>TNRC11</i>	40998_at	Trinucleotide repeat containing 11
6	.007	<i>G2AN</i>	37040_at	Alpha glucosidase II alpha subunit
7	.005	<i>NFIC</i>	440_at	Nuclear factor I/C (CCAAT-binding transcription factor)
<b>F</b>				
1	.001	<i>EPB41L3</i>	41385_at	Erythrocyte membrane protein band 4.1-like 3
2	.001	<i>FCGR2A</i>	37688_f_at	Fc fragment of IgG, low affinity IIa, receptor for (CD32)
3	.001	<i>HK3</i>	36372_at	Hexokinase 3 (white cell)
4	.002	<i>CSPG2</i>	31682_s_at	Chondroitin sulfate proteoglycan 2 (versican)
5	.005	<i>PGAM1</i>	41221_at	Phosphoglycerate mutase 1 (brain)
6	.003	<i>LILRB1</i>	32475_at	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1
7	.006	<i>CYBB</i>	37975_at	Cytochrome b-245, beta polypeptide (chronic granulomatous disease)
9	.004	<i>CD86</i>	36270_at	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)
<b>Down-regulated genes</b>				
<b>A</b>				
3	.004	<i>HLA-DPB1</i>	38095_i_at	Major histocompatibility complex, class II, DP beta 1
7	.010	<i>HLA-DMA</i>	37344_at	Major histocompatibility complex, class II, DM alpha
8	.012	<i>HLA-DPB1</i>	38096_f_at	Major histocompatibility complex, class II, DP beta 1
9	.007	<i>CD74</i>	35016_at	CD74 antigen (invariant polypeptide of MHC, class II antigen-associated)
12	.001	<i>HLA-DRB3</i>	41723_s_at	Major histocompatibility complex, class II, DR beta 3
16	.009	<i>HLA-DRA</i>	37039_at	Major histocompatibility complex, class II, DR alpha
20	.007	<i>RAB31</i>	33371_s_at	RAB31, member RAS oncogene family



Table 4. Continued

Cluster and order	P	Gene	Probe set	Description
<b>B</b>				
12	.01	<i>LGALS1</i>	33412_at	Lectin, galactoside-binding, soluble, 1 (galectin 1)
34	.046	<i>STX4A</i>	37911_at	Syntaxin 4A (placental)
37	.032	<i>PTPRC</i>	40520_g_at	Protein tyrosine phosphatase, receptor type, C
40	.036	<i>EMP3</i>	39182_at	Epithelial membrane protein 3
44	.026	<i>CORO1A</i>	38976_at	Coronin, actin binding protein, 1A
51	.048	<i>INPPL1</i>	36598_s_at	Inositol polyphosphate phosphatase-like 1
<b>C</b>				
3	.015	<i>DNCL1</i>	34891_at	Dynein, cytoplasmic, light polypeptide 1
4	.022	<i>RAB9P40</i>	109_at	Rab9 effector p40
5	.035	<i>BDH</i>	37211_at	3-hydroxybutyrate dehydrogenase (heart, mitochondrial)
7	.018	<i>CGI-87</i>	41590_at	CGI-87 protein
12	.035	<i>BCS1L</i>	31842_at	BCS1-like (yeast)
14	.027	<i>POP5</i>	39516_at	RNase MRP/RNase P protein-like
<b>D</b>				
24	.047	<i>HOXA10</i>	41448_at	Homeobox A10
34	.028	—	32021_at	<i>H sapiens</i> transcribed sequence with weak similarity to protein ref: NP_060265.1 ( <i>H sapiens</i> ) hypothetical protein FLJ20378
40	.046	<i>KIAA0669</i>	41788_i_at	KIAA0669 gene product
42	.056	<i>HOXA9</i>	37809_at	Homeobox A9
<b>E</b>				
4	.002	<i>DPM1</i>	34879_at	Dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit
16	.010	<i>MCP</i>	38441_s_at	Membrane cofactor protein (CD46, trophoblast-lymphocyte cross-reactive antigen)
19	.019	<i>STXBP3</i>	37962_r_at	Syntaxin binding protein 3
25	.007	<i>PSMC6</i>	949_s_at	Proteasome (prosome, macropain) 26S subunit, ATPase, 6
35	.033	<i>CHUK</i>	33770_at	Conserved helix-loop-helix ubiquitous kinase
36	.019	<i>COPB</i>	34326_at	Coatomer protein complex, subunit beta
<b>F</b>				
50	.019	<i>CCND2</i>	36650_at	Cyclin D2
57	.020	<i>ERG</i>	914_g_at	v-ets erythroblastosis virus E26 oncogene like (avian)
80	.035	<i>IMPDH2</i>	36624_at	IMP (inosine monophosphate) dehydrogenase 2
82	.022	<i>6-SEP</i>	38826_at	Septin 6
84	.025	<i>RPL17</i>	32440_at	Ribosomal protein L17

ANOVA was used to identify rank-ordered gene lists with bootstrap resampling to estimate the stability of these lists. *P* represents the estimated fraction of time that a gene was ranked at or above its observed position after tabulation of rankings from 1000 bootstrap resamplings (Document S1).<sup>44</sup> — indicates gene symbol not available.

similar gene expression pattern to cluster A as well as a high incidence of *NPM1* mutations (100%) (Table S17 and Figure S2).<sup>33,44</sup>

Gene expression profiles associated with *NPM1* mutations are dominated by a stem-cell molecular signature.<sup>39</sup> Activation of HOX

genes and TALE partner genes (ie, *MEIS*) is found in *NPM1* gene signatures.<sup>39</sup> The reportedly favorable impact of *NPM1* mutations on survival has included higher CR rates<sup>23,26</sup> and a trend to longer OS and EFS.<sup>26</sup> However, other studies have observed either no significant effect<sup>22,25</sup> or an impact only when *NPM1*-mutated cases are also *FLT3*-ITD negative.<sup>24,26,27</sup> While AML with *NPM1* mutations is associated with increased *FLT3* mutations,<sup>22-24</sup> this relationship was not observed for cluster A members. Cluster A had a disproportionate number of *FLT3* mutations involving TKDs rather than ITDs, but the overall *FLT3* mutation incidence was similar to the other VxInsight groups. *FLT3*-TKDs have been linked to increased release of IL-12 by leukemic blasts; IL-12A was overexpressed by members of cluster A.<sup>64</sup> IL-12 has antiangiogenic and antitumor effects and, unless offset by an increased level of proangiogenic regulators, may have a role in improving outcomes.<sup>64,65</sup>

Cluster A members had overexpression of Wilms tumor (*WT1*) gene; this gene is overexpressed at variable levels in 75% to 100% of AMLs at diagnosis.<sup>18,66</sup> A lower level of expression of *WT1* has been seen among the more differentiated AMLs in most but not all series.<sup>18,67</sup> Because of the increased *WT1* expression, patients in cluster A may be more likely to benefit from *WT1*-specific immunotherapy than other AML patients, either in the form of a T-cell approach or a vaccine.<sup>14,67</sup> One potential problem is that a number of the MHC class II genes were down-regulated in cluster

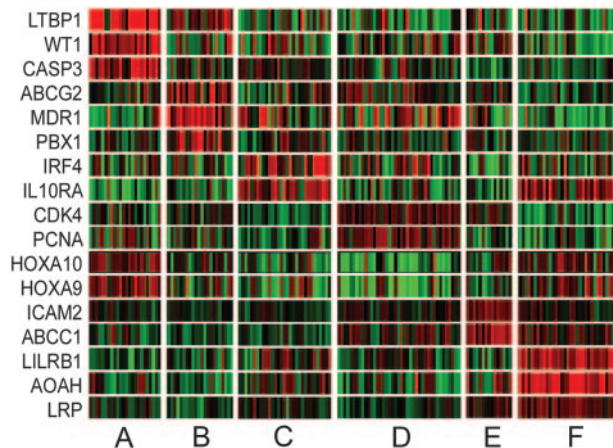


Figure 7. Differential expression of select genes among VxInsight clusters. The columns represent the 170 AML samples ordered by VxInsight membership. Rows represent select genes that are differentially expressed among the VxInsight clusters. Red indicates high expression relative to the mean; green, low expression relative to the mean.

A. Because tumor cells are poorly immunogenic when deficient in MHC class II molecule expression, the leukemic cells may escape host immunity. Cluster A also had overexpression of genes that promote apoptosis (*LTBP1*, *CASP3*), with *LTBP1* being a particularly important gene for predicting cluster A membership (ranked 1) and for predicting *NPM1* mutations.<sup>24,39</sup>

Patients in clusters B and C had the worst DFS, with estimated probabilities of 0% and 6%, respectively, at 5 years. They also had the poorest OS, although OS did not vary significantly among clusters. Cluster B, in particular, is an interesting group of 22 patients: 77% were unresponsive to induction chemotherapy, and its 3 remitting patients all relapsed within 16 months. This group of patients might be considered prone to disease resistance, because the patients had the highest median age (68 years) and highest incidence of secondary disease (32%), yet these factors did not vary significantly in the 6 clusters. Despite 42% of cases having a normal karyotype, only one individual (5%) had an *NPM1* mutation. One gene overexpressed by these patients was *ABCG2*. *ABCG2*, also termed breast cancer resistance protein (BCRP) and mitoxantrone resistance protein (MRX), is a member of the ATP-binding cassette (ABC) superfamily of membrane transporters that function as drug efflux pumps to remove chemotherapeutic agents from cells.<sup>68</sup> *ABCG2* is expressed by approximately one third of adult AMLs when measured using semiquantitative reverse transcriptase (RT)-PCR or flow cytometric analysis.<sup>69-72</sup> Of relevance to our study is the report by Steinbach and colleagues, who found significantly higher median *ABCG2* gene expression levels in 24 pediatric AML patients who failed to achieve remission after initial induction chemotherapy compared with the 21 patients who achieved remission.<sup>73</sup> Similar and discrepant results have been reported by others using varying and sometimes discordant analytic methods and study designs.<sup>72,74,75</sup>

The significant *ABCG2* overexpression among members of our high induction failure cluster, B, supports a role for *ABCG2* in chemoresistance, possibly in combination with *MDR1*.<sup>76,77</sup> Permeability glycoprotein (*MDR1*, P-gp, or *ABCB1*) was concurrently overexpressed among patients in cluster B, but *MDR1* alone did not significantly differentiate this group from the other AML patients. Drug-sensitive cells transfected with *ABCG2* become resistant to mitoxantrone, doxorubicin, daunorubicin, and topotecan,<sup>70</sup> while *ABCG2*-expressing cells from AML patients are resistant to daunorubicin in vitro.<sup>78</sup> Therefore, treatment methods circumventing *ABCG2*-mediated multidrug resistance should be considered for evaluation in future patients with gene profiles similar to cluster B members. These include the use of *ABCG2* inhibitors and antineoplastic agents that show poor *ABCG2*-mediated efflux (ie, idarubicin or newer agents in development).<sup>79</sup>

The other poor-outcome cluster, C, had the highest rate of CR to induction therapy (58%), with only 16% of the 31 patients showing initially resistant disease. However these CRs were comparatively short lived: In the analysis of DFS, cluster C had the largest hazard ratio of the 6 clusters. Many high-ranking genes defining this cluster were down-regulated (54% of the top 50 genes). Among the significantly overexpressed genes were genes involved in immunoregulation, including interferon regulatory factor-4 (*IRF4*), a gene regulated by NF- $\kappa$ B member *c-rel*<sup>80</sup>; *IL-10RA*, a member of the interferon receptor family; and *MALT1*, a factor required for NF- $\kappa$ B activation. Immune-mediated antitumor effects may have played a role in the initial therapeutic responses in this ultimately poor-outcome group.

Cluster D had the largest membership (n = 42), the highest prevalence of karyotypic abnormalities (76% of members), and a

low prevalence of *NPM1* mutations (5% of members). This cytogenetically diverse group contained most of the patients in the favorable cytogenetic risk group (10 of 12; 83%; including all t(8;21)) as well as the largest percentage of patients with unfavorable karyotypes (15 of 44, 34%; 44% of members). A study of 116 adult AML patients initially using an unsupervised clustering approach also found karyotypic diversity within cluster groups.<sup>35</sup> Analogous to their findings, primary translocating events may be less important in the transformation to leukemia than the overall dysregulation of signaling pathways or other genetic events better reflected in gene expression profiles. In cluster D, the “high proliferative activity” gene signature dominated and may have obscured gene signatures more specific to the divergent karyotypes. Most of the top-ranked genes in cluster D were associated with DNA proliferation and repair. It is unclear whether the “high proliferative” signature led to an increase in detectable cytogenetic abnormalities, because in vitro proliferation is required to detect such karyotypic abnormalities, or whether the cytogenetic abnormalities led to the increase in proliferative genes. Notable in this cluster was the low expression of class I homeobox A genes (*HOXA9*, *HOXA10*). *HOXA* gene expression has been shown to be lower among AMLs with favorable karyotypes compared with unfavorable<sup>33,81,82</sup> and higher among patients with *NPM1* mutations,<sup>39</sup> normal karyotypes, or subsets of patients with intermediate-risk karyotypes.<sup>33,83</sup> Down-regulation of *HOXA9* and *HOXA10* in cluster D may reflect the lower proportion of patients with *NPM1* mutations or decreased normal karyotypes (24%) relative to the other clusters (42%-75%).

Cluster E represented a small group of patients (n = 18), many of whom were registered to SWOG protocol S9500 for the treatment of younger adults (younger than 56 years). Cluster F (n = 33) was defined by AML with monocytic differentiation (97% of members), the highest pretreatment WBC counts, and a high percentage of *NPM1* mutations (61%). *NPM1* mutations have been shown to be increased among monocytic leukemias, and the gene expression profiles contained many genes pertinent to monocyte function.<sup>25-27</sup> When our gene lists were compared with those in the study by Valk et al,<sup>34</sup> 23% of the top 40 genes defining cluster F were similar to those seen in the cluster of Valk et al<sup>34</sup> containing monocytic leukemias (see Table S16).<sup>44</sup> Similarly, when significant cluster-defining genes in our study were analyzed using datasets of Valk et al<sup>34</sup> and Bullinger et al,<sup>35</sup> the strongest gene expression relationships were found among the monocytic groups in all the studies (see Figures S2-S8).<sup>44</sup> This confirms the importance of monocyte morphology on AML gene expression signatures and raises the question of whether the strong “monocyte signature” masks other genes of potential biologic significance in these groups.<sup>35</sup> We are currently evaluating whether the outcome of monocytic leukemias with *NPM1* mutations and a “monocytic gene signature” differs significantly from AMLs with a “stem-cell molecular signature” seen in cluster A and reported by Alcalay et al.<sup>39</sup>

This gene expression profiling study highlights the divergent mechanisms and pathways of leukemic transformation that are not appreciated by current methods of AML diagnosis, classification, and risk assignment. No bias was induced during cluster selection in this analysis, and therefore these subsets represent true reflections of the intrinsic biology in this cohort of patients. For example, the significance of *NPM1* mutations in AML was unknown at the initiation of this study, yet the gene expression profiles clustered groups of patients together with this unique genetic abnormality. Additional studies will be important to determine whether the

improved survivals in cluster A with increased *NPM1* mutations relate to the gene expression signatures displayed by these cases, regardless of the *FLT3* mutational status. We are now evaluating the relative significance of these genes in predictive models of outcome using supervised learning methods in this same cohort. It is hoped that the gene signatures identified in this study will provide clues to new therapeutic interventions for older AML patients who have historically done poorly with current treatment regimens. Confirmatory studies and prospective validation of our results are required to continue to understand the significance of

our clusters of patients, such as cluster B with increased RD. These analyses are important to enhance risk classification and the identification of individual genes and pathways that can be exploited for improved therapeutic interventions.

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