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Gene Expression Profiling of Adult Acute Myeloid Leukemia Identifies Novel Biologic Clusters for Risk Classification and Outcome Prediction

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Abstract

To determine if gene expression profiling could improve risk classification and outcome prediction in older AML patients, expression profiles were obtained in pre-treatment leukemic samples from 170 patients whose median age was 65 years. Unsupervised clustering methods were used to classify patients into six cluster groups (designated A-F) that varied significantly in rates of resistant disease (RD, *P*<.0001), complete remission (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.

Introduction

In the majority of patients, particularly those over 55 years of age, acute myeloid leukemia (AML) is a highly resistant disease and overall outcomes remain extremely poor. [1-5] 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 25-55% and overall survival rates of 10% or less.[1,6-10] In addition to age and white blood cell 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 5-12% [t(8;21)], 5-8% [inv(16)] and 10-12% [t(15;17)] of all AML cases, and are disproportionately seen in younger patients. [11,12] In contrast, approximately 50-70% of all AMLs have normal or risk-indeterminate karyotypes. [11,13,14]

Gene mutations confer additional prognostic information that may be useful in refining cytogenetic risk classification.[15-19] 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.[20,21] Mutated NPM1 relocates to the cytoplasm and disrupts normal NPM1 function. Approximately 25-35% of AML patients have NPM1 mutations, [22-24] with a higher percentage (47-60%) seen among those with a normal karyotype.[22,25-26] The impact on survival is variable, but likely favorable, with secondary influences, such as concurrent FLT3 mutations having potentially significant roles.[23,24,26,27] The *FLT3* mutations occur as internal tandem duplications (ITD), observed in 15-35% of AML, or point mutations of the intracellular tyrosine-kinase domain (TKD), seen in an additional 5-10% of patients.[19] The prognostic impact of *FLT3* mutations trends towards decreased survivals or increased relapse rates primarily for patients with FLT3 ITDs. [28-30]

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 number of laboratories using supervised learning algorithms have identified unique gene expression signatures associated with karyotypic abnormalities, normal karyotypes, and NPM1 mutation status.[31-39] 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 utilized pre-treatment samples from patients with previously untreated *de novo* or secondary AML by FAB criteria, who were registered to SWOG clinical trials for patients over the age of 55 years (studies S9031, S9333), patients aged 15-55 years (S9034, S9500) and patients with secondary AML (S9126). Trial details have been previously reported.[2,9,40-42] 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 >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 below). 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.[11] For studies S9031, S9126, S9333 and S9500, response to induction chemotherapy was assessed according to SWOG criteria.[43] 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 >80% blasts as confirmed by microscopic review of Wright stained cytospin 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 http//hsc.unm.edu/crtc/willmanresearch.[44] The isolated RNA was reverse transcribed into cDNA and re-transcribed into cRNA after double amplification using a modification reported by Ivanova *et al* to enhance detection of low abundance genes.[44,45] Biotinylated cRNA was fragmented and hybridized to HG_U95Av2 oligonucleotide microarrays (Affymetrix).[44] After analysis with Affymetrix Microarray Suite (MAS 5.0); the data was scaled to minimize experimental variation.[44] Technical criteria for case inclusion of the 185 initial specimens evaluated included: adequate total RNA >2.5 ug, good quality cRNA, good quality scanned images, and good experimental quality. Experimental quality was assessed by GAPDH $\geq$ 1800, $\geq$ 10% expressed genes, and GAPDH 3'/5' amplification ratios of $\leq$ 4. High quality expression data were obtained on 170 of the 198 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*).[44]

NPM1 and FLT3 mutational status

Samples were evaluated for NPM1 mutations utilizing cDNA amplified to generate a 249 bp fragment spanning portions of exons 11 and 12 (see Supplement for details).[44] The PCR products were subjected to dissociation analysis (65ºC to 80ºC) 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-Q[26]. Cases were also evaluated for *FLT3* ITDs in exon 14 and 15, as previously described and screened for *FLT3* TKD in exon 20 using two methods (see Supplement).[44,46] Suspected *FLT3* ITDs and TKD mutations were confirmed by sequencing.[46]

Statistical analysis

VxInsight™, developed at Sandia National Laboratories for extremely large datasets, was the primary unsupervised data mining tool utilized in this study (http://www.cs.sandia.gov/projects/VxInsight.html).[47-50] Using a force-directed placement algorithm, clusters were formed one hundred 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.[50] 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 the Supplement.[44] The full rank ordered gene lists derived from ANOVA with bootstrapping, ROC, and GA/KNN are provided in the Supplement.[44] Principal component analysis (PCA) and hierarchical clustering were performed using MATLAB (MathWorks, Inc, Natick, MA).[51,52] Concordance between VxInsight and hierarchical clusters was measured by the adjusted Rand index, with Monte Carlo estimation of statistical significance (N=10000 replications).[53]

Comparisons between clusters were based on the Kruskal-Wallis test for continuous variables (age, lab values), and on the χ^2 approximation of the Fisher exact test and Pearson's χ^2 test for independence for dichotomous and categorical variables [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 complete response 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 [54] and compared between clusters using the log rank test.[55] 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.[56] 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 two-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 gender predominance and a majority of patients (80%) over the age of 55 years with a median age of 65 years (range 20-84). 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 two trials for patients of age 15-55, and in none of the other trials was secondary AML further classified as 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 cytogenetic analyses were obtained on 141 (83%) of the patients, and 139 of these could be assigned to cytogenetic risk categories. The majority of 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 six distinct and stable groups based on strong similarities in gene expression among the 9,463 genes, visualized in Figure 1.

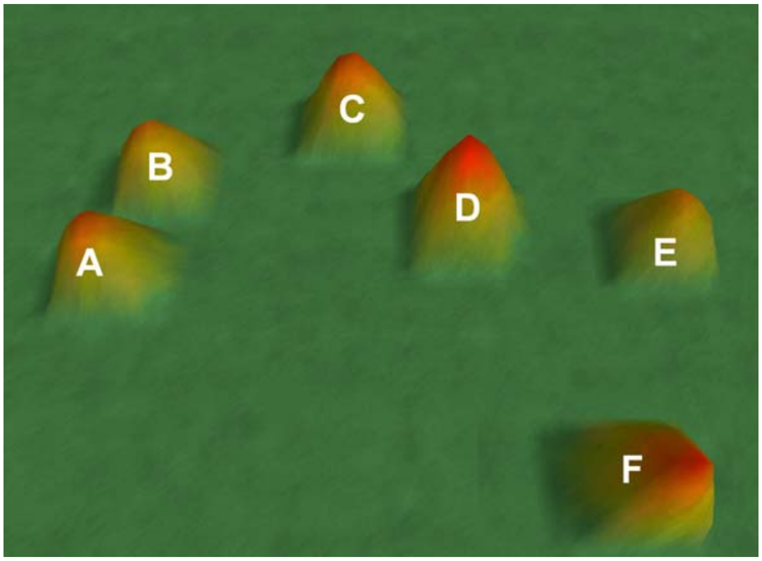

Figure 1

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*<.0001*)* (Figure 2).

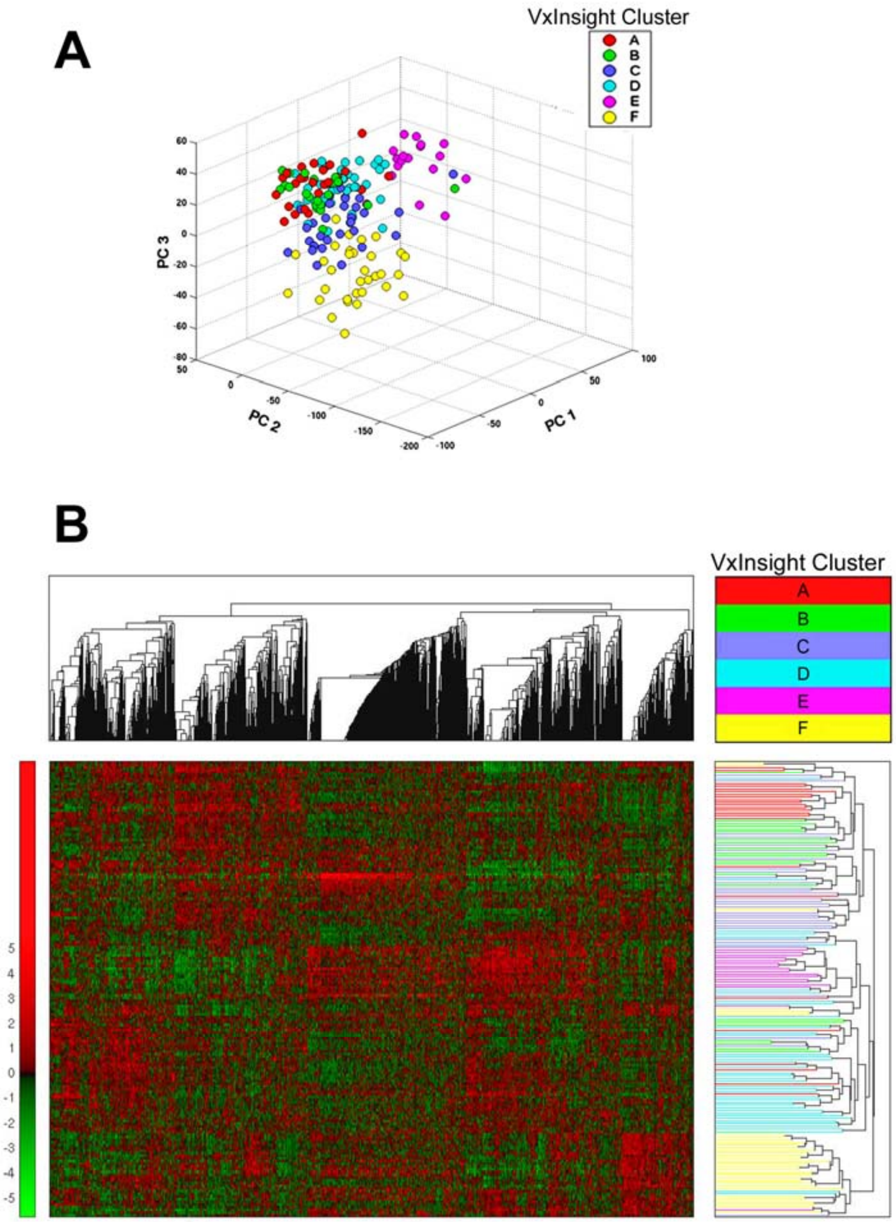

Figure 2

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 three 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). Overall survival 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).

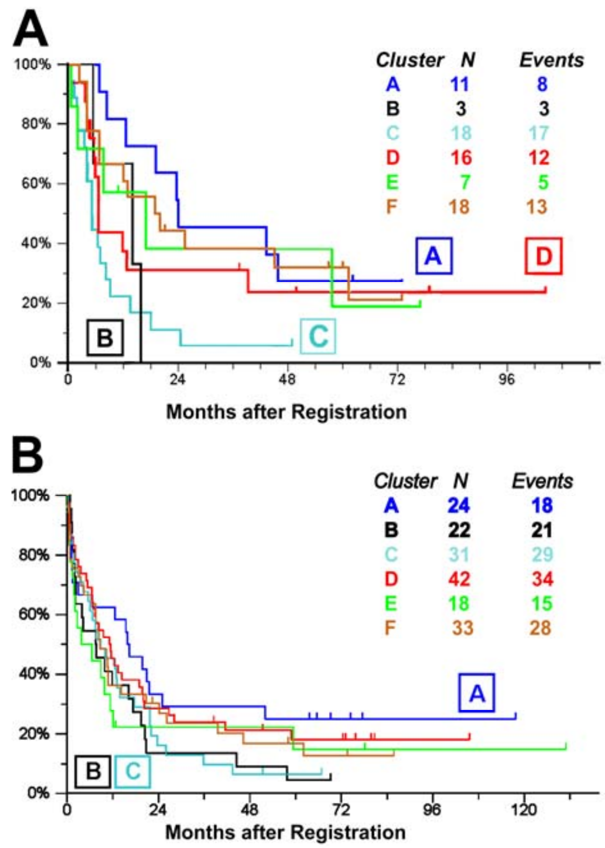

Figure 3

Response to induction chemotherapy varied significantly among the six 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* < .0001). This was largely due to an exceptionally high RD rate in cluster B (77%) compared to all other clusters combined (43/148, 29%), although heterogeneity among the remaining five 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 with age, it was noteworthy that only 4% of patients in the two clusters with worse outcomes (B, C) were under age 56, compared to 27% (32/117) of patients in the remaining clusters.

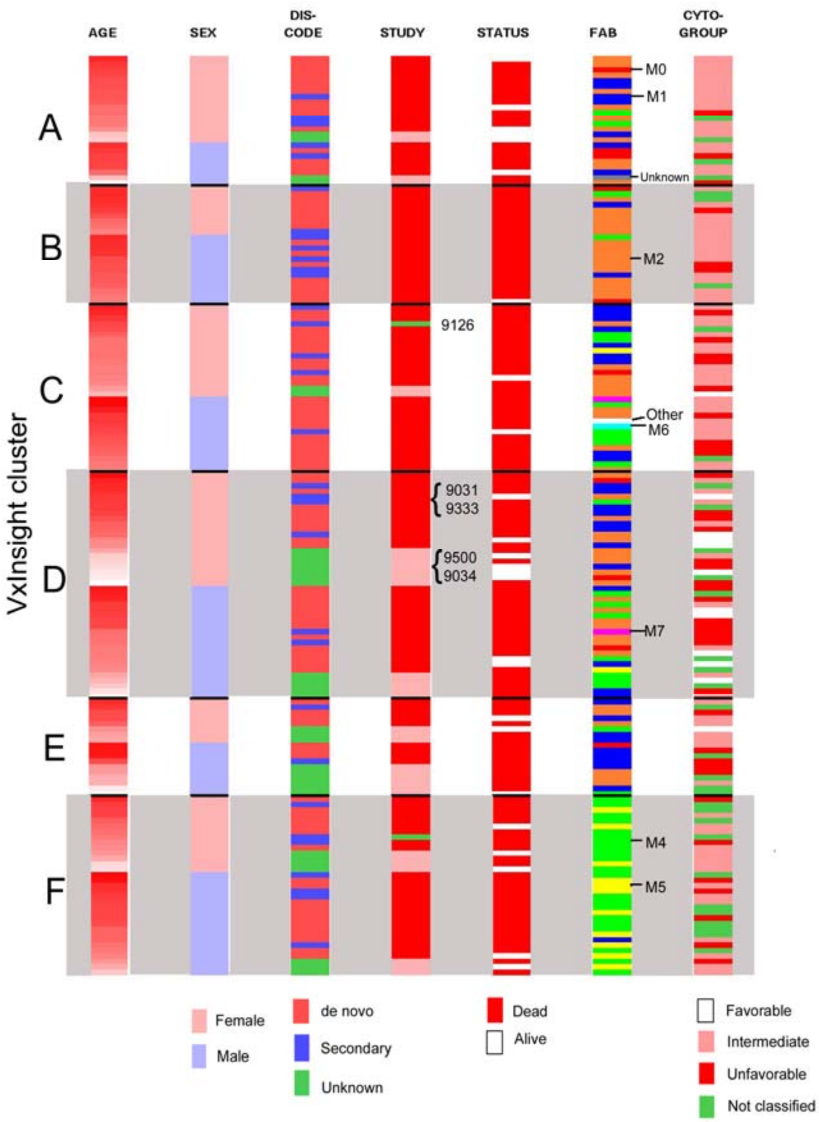

Figure 4

Clinical and laboratory parameters that showed significant correlation with VxInsight cluster membership were: pretreatment white blood cell counts, blast percentages, platelet counts; FAB classification; normal or t(8;21) karyotypes, and NPM1 mutation status (Table 1, 3). The lower white blood cell 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 five other clusters. Cytogenetic risk groups varied with cluster membership (Table 3, Figure 4). Cluster A, with the best overall survival, 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 [8/8 with t(8;21) and 2/4 patients with inv(16)] and unfavorable risk.

NPM1 mutations were present in 30% (50/165) of cases with significant differences observed between VxInsight clusters (Table 1, 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).

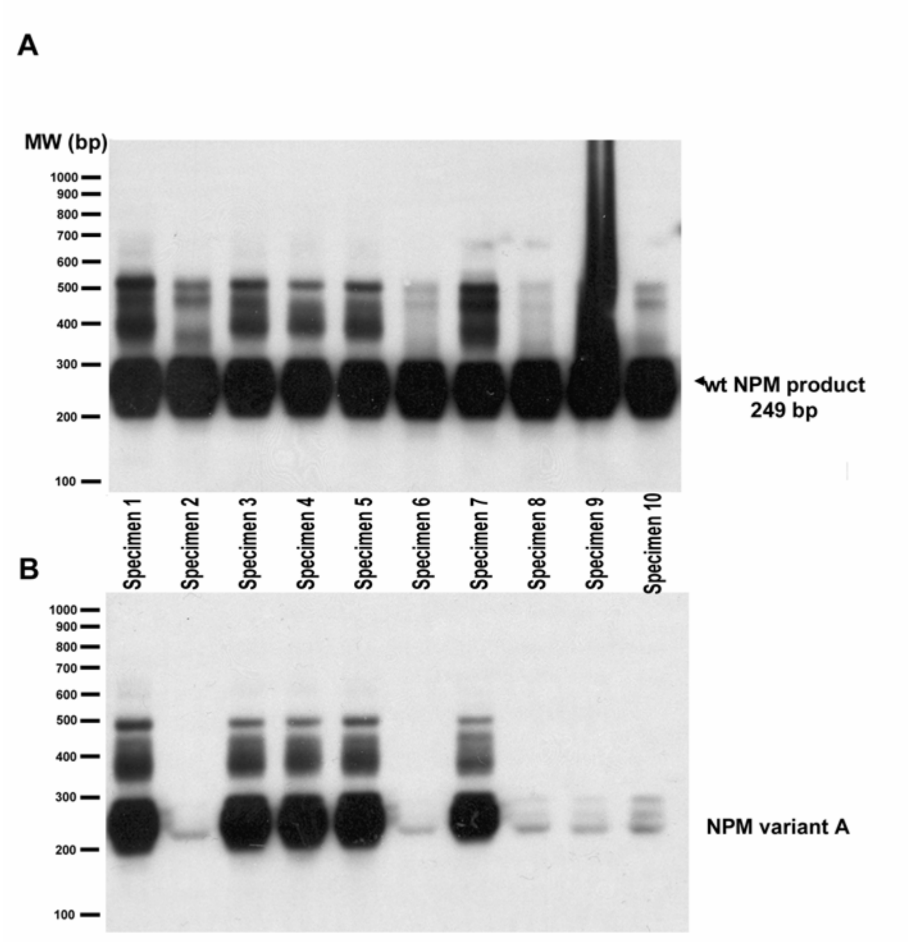

Figure 5

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 the 170 patients. Therefore to allow for the possibility that results might be biased by the exclusion of these patients, the association between RD rate and cluster was estimated with and without adjustment for age, AML onset and cytogenetic category, for the 113 patients with complete data. The results, shown in Figure 6, confirm that heterogeneity of RD rates among the six clusters remained statistically significant (*P*<.0001) after adjusting for possible confounding. The variation of CR rates among the six clusters remained marginally significant after adjusting for age, AML onset and unfavorable cytogenetics (P=.051). In proportional hazards regression analyses adjusting for age, AML onset and cytogenetic risk category, the variation of OS among clusters remained nonsignificant (P=.56). DFS also did not vary significantly among clusters after accounting for similar effects (P=.22), however this analysis was inconclusive since only 49 remitting patients had both AML onset and cytogenetic risk group data.

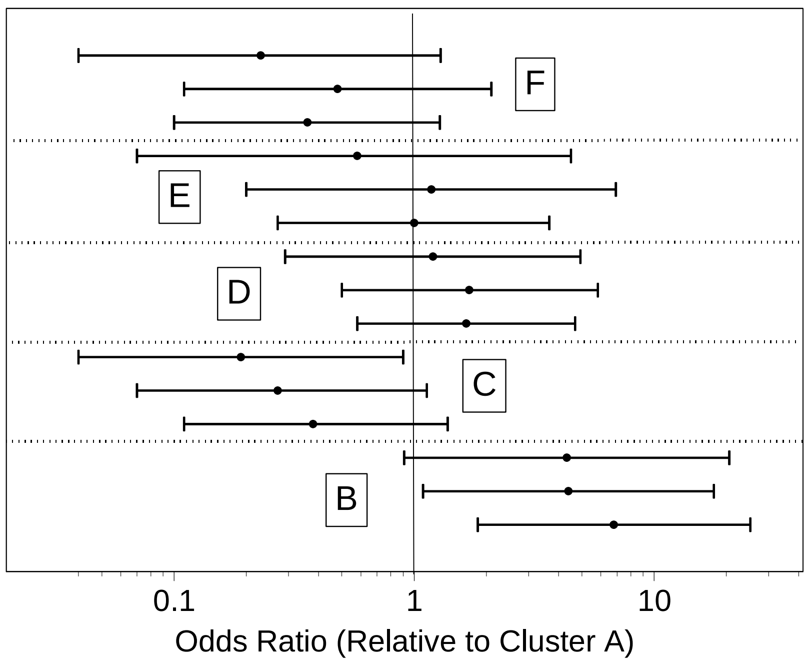

Figure 6

Genes distinguishing VxInsight clusters

Using ANOVA with bootstrapping, gene lists were derived that define the VxInsight clusters. The 50 most significant discriminating genes for each cluster are provided in the Supplement,[44] with a summary of these lists, including the most significantly up-regulated and down-regulated genes, given in Table 4. Gene expression patterns for a subset of these genes are highlighted in Figure 7. The top 50 ranked genes for clusters B, D, and F are primarily up-regulated (90%, 92%, 98% of genes, respectively) in comparison to the down-regulation of several significant differentiating genes for clusters A, C, and E (36%, 54%, 14% of genes, respectively). Cluster D, containing virtually all of the cases with favorable cytogenetic abnormalities and a large percentage of intermediate and unfavorable karyotypes, is defined by high expression (top 46 characterizing genes are overexpressed) of a number of genes involved in DNA replication (*GART, MCM3, PCNA*), control of cell proliferation (*CDK4, ODC1, STMN1*), transcription (*POLR2H, EIR2S1, HTATSF1*) and DNA repair (*UNG, CHEK2, APEX1, ADPRT*). This gene expression signature may be reflective of high "proliferative" activity. An interesting finding is the decreased expression of homeobox A9 (HoxA9) and A10 (HoxA10) in cluster D compared to the other AMLs. This may relate in part to the low incidence of NPM1 mutations.[39]

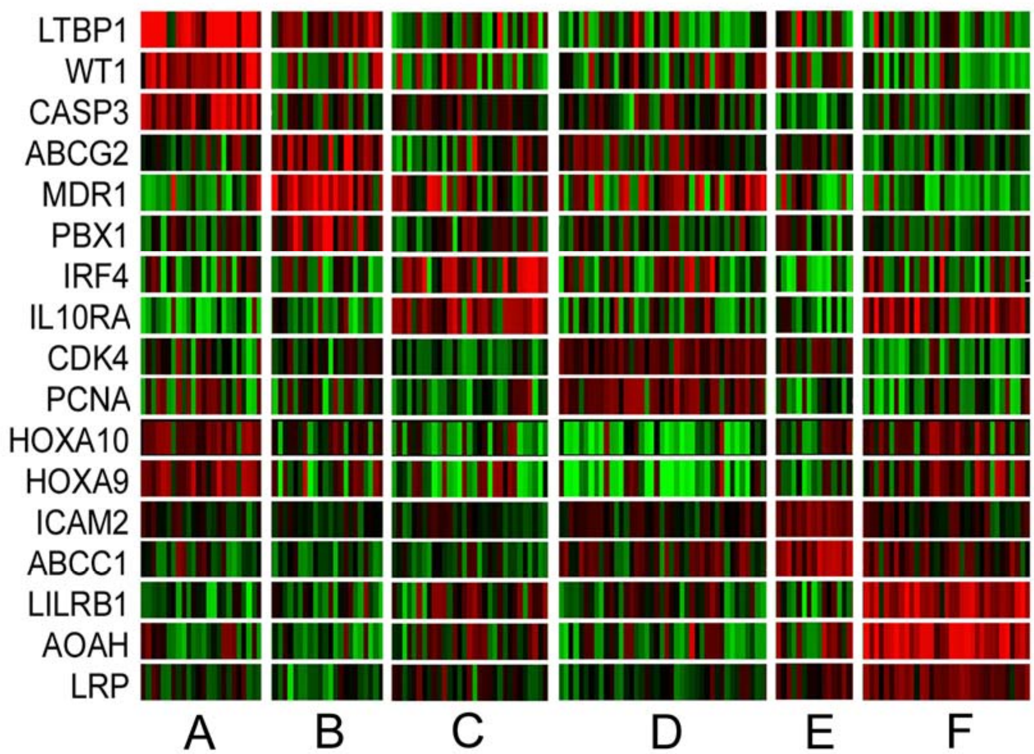

Figure 7

Genes associated with cell signaling (*IL12*-ranked 29), apoptosis (*LTBP1*, caspase 3), leukemic transformation (*MEIS*-ranked 30, *WT1*-ranked 22 , *FOXC1*), and multidrug resistance (*MRP2*-ranked 40) are overexpressed by cluster A. The top ranking gene, latent transforming growth factor (TGF) beta binding protein (*LTBP1*) activates latent TGF-beta, a modulator of apoptosis that is independent of caspase 3 mediated mechanisms.[57-59] *FOXC1* is a TGF-beta1 responsive gene that possibly functions as a tumor suppressor gene.[60] Notably absent in cluster A is expression of the MHC II alleles.

Cluster B with the poorest clinical outcomes, shows increased expression of the multi-drug resistance gene *ABCG2* (ranked 18). The multi-drug resistance membrane transporter (*MDR1*) is concurrently overexpressed (Figure 7). Additional genes of interest are *PBX1* and serine/threonine protein kinase 17A (*STK17A*-ranked 23). *STK17A* plays a role in the regulation of apoptosis; *PBX1* is a cofactor in genetic mechanisms that prevent myeloid differentiation but appears to lack inherent transformation ability in isolation.[61,62] Cluster C shows expression of genes involved in immunoregulation (IRF4, IL10R, MALT), including several probe sets for gamma interferon and interferon inducible genes.

Inhibitors of apoptotic function (*ICAM2*-ranked 34*, DFFA/DFF45*-ranked 33) are over-expressed among cluster E members. This cluster showed variable expression of genes related to immune function with up-regulation of some genes (*SPN, IRF3, IFITM2*) and down regulation of others (*MCP,CHUK*). Finally, 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 utilizing different statistical and normalization methods are provided in Supplement.[44] 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,[35] would have been harder to detect with a more restrictive supervised approach. The result is an interesting separation of the AML cases into six distinct clusters with outcome differences.

In contrast to previous studies using unsupervised computational methods alone,[32,34,35] we found significant outcome differences between the clusters defined by gene expression for RD after induction therapy (P<.0001), 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 was absent regarding prognostic factors, in particular 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.

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. [22,24-27] 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 (see Supplement for more detail). [39,44] For example, 8 of the top 15 ranked genes (53%) for cluster A were also genes found to be predictive of NPM1 mutations.[39] 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 Valk et al's AML dataset; their cluster group 6 showed a similar gene expression pattern to cluster A as well as a high incidence of NPM1 mutations (100%) (see Supplement).[33,44]

Gene expression profiles associated with NPM1 mutations are dominated by a stem-cell molecular signature [39]. Activation of HOX genes and TALE partner genes (i.e. *MEIS*) are found in NPM1 gene signatures [39]. The reportedly favorable impact of NPM1 mutations on survival has included higher CR rates [23,26], and a trend to longer OS and EFS [26]. However, other studies have observed either no significant effect, [22,25] or an impact only when NPM1 mutated cases are also FLT3-ITD negative.[24,26,27] While AML with NPM1 mutations are associated with increased FLT3 mutations,[22-24] 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.[63] IL-12 has anti-angiogenic and anti-tumor effects and unless offset by an increased level of pro-angiogenic regulators, may have a role in improving outcomes.[63,64]

Cluster A members had overexpression of Wilms tumor (*WT1*) gene; this gene is overexpressed at variable levels in 75-100% of AMLs at diagnosis.[18,65] A lower level of expression of *WT1* has been seen among the more differentiated AMLs in most but not all series.[18,66] 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.[14,66] One potential problem is that a number of the MHC class II genes were down-regulated in cluster 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 over-expression of genes that promote apoptosis (LTBP1, CASP3), with LTBP1 being a particular important gene for predicting cluster A membership (ranked 1) and for predicting NPM1 mutations.[24,39]

Patients in clusters B and C had the worst DFS, with estimated probabilities of 0% and 6%, respectively at five 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 three remitting patients all relapsed within 16 months. This group of patients might be considered prone to disease resistance, since they had the highest median age (68 years) and highest incidence of secondary disease (32%); yet these factors did not vary significantly in the six 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.[67] *ABCG2* is expressed by approximately one-third of adult AMLs when measured using semi-quantitative RT-PCR or flow cytometric analysis.[68-71] 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 to the 21 patients who achieved remission.[72] Similar and discrepant results have been reported by others using varying and sometimes discordant analytical methods and study designs.[71,73,74]

The significant *ABCG2* over-expression among members of our high induction failure cluster, B, supports a role for ABCG2 in chemoresistance, possibly in combination with *MDR1*.[75,76] 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,[69] while ABCG2 expressing cells from AML patients are resistant to daunorubicin *in vitro*.[77] 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 (i.e. idarubicin or newer agents in development).[78]

The other poor outcome cluster, C, had the highest rate of complete response 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 six clusters. Many high-ranking genes defining this cluster were down-regulated (54% of the top 50 genes). Among the significantly over-expressed genes were genes involved in immunoregulation including; interferon regulatory factor-4 (*IRF4*), a gene regulated by NF-kappaβ member *c-rel*;[79] *IL-10RA*, a member of the interferon receptor family; and *MALT1*, a factor required for NF-kappaβ activation. Immune mediated anti-tumor 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 the majority of patients in the favorable cytogenetic risk group [10/12, 83%, including all t(8;21)], as well as the largest percentage of patients with unfavorable karyotypes (15/44, 34%; 44% of members). A study of 116 adult AML patients, initially using an unsupervised clustering approach, also found karyotypic diversity within cluster groups.[35] 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. The majority of top ranked genes in cluster D were associated with DNA proliferation and repair. It is unclear if the “high proliferative” signature led to an increase in detectable cytogenetic abnormalities, as *in vitro* proliferation is required to detect such karyotypic abnormalities; or if 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 to unfavorable,[33,80,81] and higher among patients with NPM1 mutations,[39] normal karyotypes or subsets of patients with intermediate risk karyotypes.[33,82] 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 (<56 years). Cluster F (n=33) was defined by AML with monocytic differentiation (97% of members), the highest pre-treatment white blood cell 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.[25,26,27] When our gene lists were compared to those in the study by Valk et al, 23% of the top 40 genes defining cluster F were similar to those seen in Valk's cluster containing monocytic leukemias (see Supplement).[34,44] Similarly, when significant cluster defining genes in our study were analyzed using Valk et al and Bullinger et al datasets, the strongest gene expression relationships were found among the monocytic groups in all the studies (see Supplement).[34,35,44] 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.[35] 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.[39]

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 if 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 (manuscript in preparation). The gene signatures identified in this study will hopefully 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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Table 1: Clinical, morphologic, cytogenetic and mutation characteristics of adult acute myeloid leukemia cohort (N=170 patients)

		Patients	%
Age	< 56	34	20
	56+	136	80
Sex	Female	85	50
	Male	85	50
FAB classification	M1	40	24
	M2	60	35
	M4	42	25
	M5	13	8
	M6	1	1
	M7	2	1
	M0	10	6
	Other	2	1
Evaluable cytogenetics	No	29	17
	Yes	141	83
Cytogenetic risk category [1] (N=141)	Favorable	12	9
	Intermediate	83	59
	Unfavorable	44	31
	Not Assigned	2	1
Specific cytogenetic features (N=141)	Normal	65	46
	t(8;21)	8	6
	inv(16)	4	3
NPM1 mutation status (N=165)	Type A	45	27
	Non-Type A[2]	5	3
	Type A or non-A	50	30
FLT3 mutation[3] status (N=105)	ITD	46	27
	TKD	13	12

	Median	Min – Max
Age	65	20 – 84
WBC (1000/mm^3)	22.9	0.7 – 272.5
Peripheral Blasts (%)	43	0 – 99
Marrow Blasts (%)	70	5 – 99
Platelets (1000/mm^3)	53	2 – 1052
Hemoglobin (gm/dl)	9.1	4.3 – 14.4

[1] Cytogenetic risk categories are defined by the following cytogenetic abnormalities:
Favorable - inv(16)/t(16;16)/del(16q), t(8;21) or t(15;17) with any additional abnormalities (abn).
Intermediate - +8, -Y, +6, del(12p), or normal karyotype.
Unfavorable - -5/del(5q), -7/del(7q), inv(3q), abn of 11q, t(6;9), t(9;22), or abn 17p; or complex karyotype defined as >3 abn. Other findings are listed as not assigned or nonevaluable. [12]

[2] non-Type A includes 4 cases that hybridized to probes for 13 known variants (B-Q) [26,44] and 1 case that was sequenced

[3] ITD = internal tandem duplication; TKD= point mutations of intracellular tyrosine-kinase domain

Table 2. Treatment outcomes of 170 adult AML patients, by VxInsight cluster.

		A (N = 24)	B (N = 22)	C (N = 31)	D (N = 42)	E (N = 18)	F (N=33)	P-value[1]
Disease-free survival	Events	8	3	17	12	5	13	0.023
	5 yr[2]	27%	0%	6%	23%	19%	32%	
	95% CI	6 – 61%	0 – 71%	0 – 29%	2 – 45%	0 – 52%	10 – 54%	
	HR[4]	1.00	2.56	3.58	1.73	1.50	1.25	
	95% CI	---	0.67 – 9.81	1.52 – 8.44	0.71 – 4.25	0.49 – 4.59	0.52 – 3.03	
Overall survival	Deaths	18	21	29	34	15	28	0.40
	5 yr[2]	25%	5%	6%	18%	15%	17%	
	95% CI	8 – 42%	0 – 23%	0 – 15%	6 – 30%	0 – 32%	4 – 30%	
	HR[3]	1.00	1.75	1.62	1.21	1.73	1.39	
	95% CI	---	0.93 – 3.29	0.90 – 2.94	0.68 – 2.15	0.87 – 3.44	0.77 – 2.51	
Resistant disease	No. (%)	8 (33%)	17 (77%)	5 (16%)	19 (45%)	6 (33%)	5 (15%)	<0.0001
	95% CI	16 – 55%	55 – 92%	5 – 34%	30 – 61%	13 – 59%	5 – 32%	
	OR[4]	1.00	6.80	0.39	1.65	1.00	0.36	
	95% CI	---	1.94 – 27.4	0.10 – 1.35	0.59 – 4.86	0.27 – 3.66	0.09 – 1.25	
Complete response	No. (%)	11 (46%)	3 (14%)	18 (58%)	16 (38%)	7 (39%)	18 (55%)	0.023
	95% CI	26 – 67%	3 – 35%	39 – 75%	24 – 54%	17 – 64%	36 – 72%	
	OR[4]	1.00	0.19	1.64	0.73	0.75	1.42	
	95% CI	---	0.04 – 0.80	0.56 – 4.79	0.26 – 2.01	0.22 – 2.60	0.49 – 4.08	

[1] *P*-value for heterogeneity among six clusters, based on Pearson's chi-square test for independence (CR, RD) or log rank test (OS, DFS).

[2] Kaplan- Meier estimate of probability of OS or DFS at 5 years.

[3] Hazard ratio, relative to cluster A.

[4] Odds ratio, relative to cluster A.

Table 3: Clinical and laboratory correlates of VxInsight clusters derived from 170 adult acute myeloid leukemias

VxInsight Clusters

	A	B	C	D	E	F	P-value
Number of patients	24	22	31	42	18	33	
Median age in years (range)	67 (22-76)	68 (58-76)	65 (44-84)	62 (20-83)	60 (21-81)	64 (34-83)	0.27
N = secondary AML (%)	5 (25)	7 (32)	5 (17)	6 (20)	2 (22)	7 (27)	0.87
Median pretreatment lab values:							
WBC ($x10^3$)	29	6	14	20	33	57	<.0001
% PB blasts	76	28	38	48	85	11	<.0001
% BM blasts	82	59	55	71	80	70	.0039
Platelet ($x10^3$)	36	91	62	42	52	62	.0018
Hemoglobin (gm/dl)	9.8	8.7	9.4	8.7	9.5	9.3	.20
Cytogenetic risk group (N=139):							
Favorable - t(8;21)	0 (0%) 0 (0%)	0 (0%) 0 (0%)	1 (3%) 0 (0%)	10 (29%) 8 (24%)	1(8%) 0 (0%)	0 (0%) 0 (0%)	.0001 <.0001
Intermediate - abnormal - normal	16 (80%) 1 (5%) 15 (75%)	15(79%) 7 (37%) 8 (42%)	19(66%) 3 (10%) 16(55%)	9 (27%) 1 (3%) 8 (24%)	7 (54%) 1 (8%) 6 (46%)	17(71%) 5 (21%) 12(50%)	.0002 .011
Unfavorable*	4 (20%)	4 (21%)	9 (31%)	15 (44%)	5 (38%)	7(29%)	.41
NPM1 mutation status (N=165)							
NPM1+	18/23 (78%)	1/22 (5%)	4/30 (13%)	2/41 (5%)	6/18 (33%)	19/31 (61%)	<.0001
FLT3 mutation status (ITD, N=169; TKD, N=105)							
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 *NPM1+* and *FLT3 ITD*	7/23 (30%)	1/22 (5%)	2/30 (7%)	2/41 (5%)	3/18 (17%)	10/31 (32%)	.003

PB – peripheral blood; BM – bone marrow; CR – complete remission; RD – resistant disease
P-values determined using χ^2-test
* 11q23 abnormalities were seen in 9 cases and distributed in Clusters A-F respectively: 0,1,2,1,1,3.

Table 4: Rank ordered gene lists characterizing each cluster. Genes are up-regulated or down-regulated in relationship to the other clusters.[1]

	Up-regulated genes					Down-regulated genes				
Cluster	Order	*P-value	Gene	Probe set	Description	Order	*P-value	Gene	Probe set	Description
A	1	.002	LTBP1	1495_at	latent transforming growth factor beta binding protein 1	3	.004	HLA-DPB1	38095_i_at	major histocompatibility complex, class II, DP beta 1
	2	.003	CASP3	36143_at	caspase 3, apoptosis-related cysteine protease	7	.010	HLA-DMA	37344_at	major histocompatibility complex, class II, DM alpha
	4	.011	FTO	37242_at	fatso	8	.012	HLA-DPB1	38096_f_at	major histocompatibility complex, class II, DP beta 1
	5	.015	FOXC1	41027_at	forkhead box C1	9	.007	CD74	35016_at	CD74 antigen (invariant polypeptide of MHC, class II antigen-associated)
	6	.003	COL4A5	32667_at	collagen, type IV, alpha 5 (Alport syndrome)	12	.001	HLA-DRB3	41723_s_at	major histocompatibility complex, class II, DR beta 3
	11	.015	RASGRP3	34748_at	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	16	.009	HLA-DRA	37039_at	major histocompatibility complex, class II, DR alpha
	19	.025	MYCN	35158_at	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived	20	.007	RAB31	33371_s_at	RAB31, member RAS oncogene family
B	1	.005	BIA2	36713_at	BIA2	12	.01	LGALS1	33412_at	lectin, galactoside-binding, soluble, 1 (galectin 1)
	2	.003	CXorf6	38916_at	chromosome X open reading frame 6	34	.046	STX4A	37911_at	syntaxin 4A (placental)
	3	.014	PLOD2	34795_at	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2	37	.032	PTPRC	40520_g_at	protein tyrosine phosphatase, receptor type, C
	4	.011	OPTN	41744_at	optineurin	40	.036	EMP3	39182_at	epithelial membrane protein 3
	5	.016	CLIC2	40013_at	chloride intracellular channel 2	44	.026	CORO1A	38976_at	coronin, actin binding protein, 1A
	6	.017	RHD	37164_at	Rhesus blood group, D antigen	51	.048	INPPL1	36598_s_at	inositol polyphosphate phosphatase-like 1
	7	.021	CDC42BPA	39962_at	CDC42 binding protein kinase alpha (DMPK-like)					
	8	.038	ANK3	36965_at	ankyrin 3, node of Ranvier (ankyrin G)					
C	1	.003	SDR1	40782_at	short-chain dehydrogenase/reductase 1	3	.015	DNCL1	34891_at	dynein, cytoplasmic, light polypeptide 1
	2	.01	SDS	40390_at	serine dehydratase	4	.022	RAB9P40	109_at	Rab9 effector p40
	6	.058	SERPINF1	40856_at	serine (or cysteine) proteinase inhibitor, clade F	5	.035	BDH	37211_at	3-hydroxybutyrate dehydrogenase (heart, mitochondrial)
	8	.092	MALT1	38575_at	mucosa associated lymphoid tissue lymphoma translocation gene 1	7	.018	CGI-87	41590_at	CGI-87 protein
	9	.051	HERPUD1	39733_at	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	12	.035	BCS1L	31842_at	BCS1-like (yeast)
	10	.056	IRF4	37625_at	interferon regulatory factor 4	14	.027	POP5	39516_at	RNase MRP/RNase P protein-like
	11	.034	IL10RA	1062_g_at	interleukin 10 receptor, alpha					
	12	.035	BCS1L	31842_at	BCS1-like (yeast)					

	13	.054	RAB9A	39628_at	RAB9A, member RAS oncogene family					
D	1	.001	RNASEP1	37471_at	ribonuclease P1	24	.047	HOXA10	41448_at	homeo box A10
	2	.006	PGDS	35523_at	prostaglandin D2 synthase, hematopoietic	34	.028		32021_at	H. sapiens transcribed sequence with weak similarity to protein ref:NP_060265.1 (H.sapiens) hypothetical protein FLJ20378 [
	3	.006	NHP2L1	41746_at	NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae)	40	.046	KIAA0669	41788_i_at	KIAA0669 gene product
	4	.010	UNG	37686_s_at	uracil-DNA glycosylase	42	.056	HOXA9	37809_at	homeo box A9
	5	.011	POP1	38513_at	processing of precursors 1					
	6	.008	HSU79274	31838_at	protein predicted by clone 23733					
	7	.005	CGI-51	34845_at	CGI-51 protein					
	8	.010	NASP	33255_at	nuclear autoantigenic sperm protein (histone-binding)					
	9	.010	CDK4	1942_s_at	cyclin-dependent kinase 4					
E	1	.002	CAPN1	33908_at	calpain 1, (mu/I) large subunit	4	.002	DPM1	34879_at	dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit
	2	.010	HSF1	40200_at	heat shock transcription factor 1	16	.010	MCP	38441_s_at	membrane cofactor protein (CD46, trophoblast-lymphocyte cross-reactive antigen)
	3	.005	ACTN4	41753_at	actinin, alpha 4	19	0.019	STXBP3	37962_r_at	syntaxin binding protein 3
	5	.007	TNRC11	40998_at	trinucleotide repeat containing 11	25	0.007	PSMC6	949_s_at	proteasome (prosome, macropain) 26S subunit, ATPase, 6
	6	.007	G2AN	37040_at	alpha glucosidase II alpha subunit	35	0.033	CHUK	33770_at	conserved helix-loop-helix ubiquitous kinase
	7	.005	NFIC	440_at	nuclear factor I/C (CCAAT-binding transcription factor)	36	0.019	COPB	34326_at	coatomer protein complex, subunit beta
F	1	.001	EPB41L3	41385_at	erythrocyte membrane protein band 4.1-like 3	50	0.019	CCND2	36650_at	cyclin D2
	2	.001	FCGR2A	37688_f_at	Fc fragment of IgG, low affinity IIa, receptor for (CD32)	57	0.020	ERG	914_g_at	v-ets erythroblastosis virus E26 oncogene like (avian)
	3	.001	HK3	36372_at	hexokinase 3 (white cell)	80	0.035	IMPDH2	36624_at	IMP (inosine monophosphate) dehydrogenase 2
	4	.002	CSPG2	31682_s_at	chondroitin sulfate proteoglycan 2 (versican)	82	0.022	6-Sep	38826_at	septin 6
	5	0.005	PGAM1	41221_at	phosphoglycerate mutase 1 (brain)	84	0.025	RPL17	32440_at	ribosomal protein L17
	6	0.003	LILRB1	32475_at	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1					
	7	0.006	CYBB	37975_at	cytochrome b-245, beta polypeptide (chronic granulomatous disease)					
	9	0.004	CD86	36270_at	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)					

[1] Analysis of variance was used to identify rank ordered gene lists with bootstrap resampling to estimate the stability of these lists.

* P-value represents the estimated fraction of time that a gene was ranked at or above its observed position after tabulation of rankings from 1,000 bootstrap resamplings (see Supplement).[44]

FIGURE LEGENDS:

Figure 1. **VxInsight clusters in adult AML.** Six distinct clusters of AML patients are identified based on gene expression profiles and designated A-F. The data are visualized as a three dimensional terrain map with two dimensional distances reflecting gene expression profile correlates and the third dimension representing cluster membership density. Additional information on VxInsight is provided at http://www.cs.sandia.gov/projects/VxInsight.html.[44]

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 three dimensions. The largest sources of gene expression signal variance are represented as principal components (labeled PC1, PC2, PC3).[51] (**B)** Two-dimensional unsupervised clustering dendrograms and "heat map" of gene expression data from the 170 AML cases using 9,463 genes. Pearson's 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 >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, $P < .0001$) (see Supplement).[44]

Figure 3. Estimated distributions for disease free survival and overall survival of AML patients, by VxInsight cluster membership. (A) DFS varied significantly among the six clusters (log rank $P = .023$). (B) OS showed a trend that paralleled the DFS findings among the six clusters (log rank $P = .40$). Tickmarks 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 in the legends below the associated columns. Distribution of FAB classification varied significantly among clusters ($P < .0001$).

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 8-10). All 165 AML samples contained wild type NPM1. (B) Patient specimen (1,3,4,5,7) hybridized to NPM1 variant A probe consistent with a NPM1 variant type A mutation (see supplement). [44]

Figure 6. Estimated odds ratios for resistant disease in each of clusters B through F, relative to cluster A. For each cluster three estimates are shown: based on all 170 patients, without adjustment for other factors (bottom); based on 113 patients with known AML onset (de novo vs 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 vs favorable/intermediate) (top). Bars indicate 95% confidence intervals. Results of the three analyses are generally consistent, and in particular the heterogeneity of RD rates among the six clusters remained statistically significant after adjusting for possible confounding ($P < .0001$).

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 (high expression relative to the mean); green (low expression relative to the mean).