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RESEARCH ARTICLE

AML M3 and AML M3 Variant Each Have a Distinct Gene Expression Signature but also Share Patterns Different from other Genetically Defined AML Subtypes

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Acute promyelocytic leukemia (APL) with t(15;17) appears in two phenotypes: AML M3, with abnormal promyelocytes showing heavy granulation and bundles of Auer rods, and AML M3 variant (M3v), with non- or hypogranular cytoplasm and a bilobed nucleus. We investigated the global gene expression profiles of 35 APL patients (19 AML M3, 16 AML M3v) by using high-density DNA-oligonucleotide microarrays. First, an unsupervised approach clearly separated APL samples from other AMLs characterized genetically as t(8;21) ($n = 35$), inv(16) ($n = 35$), or t(11q23)/MLL ($n = 35$) or as having a normal karyotype ($n = 50$). Second, we found genes with functional relevance for blood coagulation that were differentially expressed between APL and other AMLs. Furthermore, a supervised pairwise comparison between M3 and M3v revealed differential expression of genes that encode for biological functions and pathways such as granulation and maturation of hematologic cells, explaining morphologic and clinical differences. Discrimination between M3 and M3v based on gene signatures showed a median classification accuracy of 90% by use of 10-fold CV and support vector machines. Additional molecular mutations such as FLT3-LM, which were significantly more frequent in M3v than in M3 ($P < 0.0001$), may partly contribute to the different phenotypes. However, linear regression analysis demonstrated that genes differentially expressed between M3 and M3v did not correlate with FLT3-LM.

INTRODUCTION

Acute myeloid leukemia (AML) is a morphologically and genetically heterogeneous disease. One subtype with specific morphology and a specific cytogenetic and molecular genetic aberration is acute promyelocytic leukemia (APL). On the basis of morphology alone, APL can be separated into two distinct subtypes according to the FAB classification: AML M3 and AML M3 variant (M3v) (Bennett et al., 1976; Testa et al., 1978; Golomb et al., 1979). The latter is also called microgranular (hypogranular) APL in the new WHO classification (Jaffe et al., 2001). The cytomorphology of APL blasts is obviously different in the two subtypes: in AML M3, the abnormal promyelocytes show heavy granulation and bundles of Auer rods; in AML M3v, blasts have a non- or hypogranular cytoplasm or contain fine, dustlike cytoplasmic granules that may not be apparent by light microscopy. Furthermore, M3v blasts show a typical bilobed nuclear configuration. This latter morphologic phenotype, together with missing granulation, often resulted in a misleading diagnosis of acute monocytic or myelomonocytic leukemia prior to the cytogenetic correlation of both AML M3 and M3v with t(15;17)(q22;q12) being observed (Testa et al., 1978; Golomb et al., 1979; Bennett et al., 1980). Although the morphology of typical APL versus the microgranular variant is mostly discriminating, a few patients have shown features of both phenotypes in peripheral blood or bone marrow. In cytochemistry, all APLs demonstrate a very strong myeloperoxidase reaction and are negative for nonspecific esterase. A diagnosis of typical APL often is accompanied by leukopenia, whereas the variant form is characterized by leukocytosis. Both subtypes are frequently associated with severe bleeding episodes because of disseminated intravascular coagulation (DIC). This leads to early death, even

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before a specific treatment can be started, in at least 10% of patients.

On the genetic level, a translocation t(15;17) is detectable in almost all cases, resulting in two different *PML–RARA* fusion transcripts: the so-called L and S transcripts. Only very rare cases show a t(11;17) with its molecular counterpart the *PLZF–RARA*, or a t(5;17) with the *NPM* and *RARA* genes fused. These two subtypes also may differ in morphology from cases with a typical t(15;17) (Bennett et al., 2000; Grimwade et al., 2000). In both APL subtypes that have a t(15;17), the fusion protein PML–RARA induces arrest at different stages of granulocytic differentiation. In the presence of high concentrations of all-*trans* retinoic acid (ATRA), the arrest of differentiation is overcome, leading to maturation of abnormal promyelocytic blasts into polymorphonuclear cells, finally inducing apoptosis. The introduction of ATRA into treatment of AML with t(15;17) in combination with chemotherapy has improved survival outcome, from less than 50% to 80% of patients surviving long-term (Lengfelder et al., 2000; Degos and Wang, 2001).

However, it has been shown in animal models that the expression of *PML–RARA* alone is not sufficient to induce APL. Therefore, additional molecular genetic mechanisms of full-blown leukemia need to be proposed (Downing and Shannon, 2002; Gilliland and Griffin, 2002; Kelly et al., 2002). The observation of a higher frequency of FLT3-length mutations (FLT3-LMs) in M3v than in classical APL (75% vs. 23%) provided a first hint of additional molecular mutations in AML with t(15;17) in adults and also of molecular differences between the two subtypes (Schnittger et al., 2002). Because the FLT3-LM has not been found in all AML M3 variants and also has been detected in typical APL, additional partners in a molecular network need to be identified. However, so far, no single cytogenetic or molecular genetic marker or specific pattern that would clearly distinguish between these two very morphologically different subtypes of APL has been identified. Recent microarray studies were able to demonstrate that in its transcriptome, AML with t(15;17) is clearly distinct from AML with t(8;21), inv(16), or t(11q23)/*MLL* (Schoch et al., 2002a; Kohlmann et al., 2003).

In the present study, we investigated the global gene expression profiles of 19 patients with AML M3 and 16 patients with AML M3v, using HG-U133, a set of high-density DNA-oligonucleotide microarrays (Affymetrix, Santa Clara, CA) representing more than 33,000 transcripts. The gene expression profiles of the APL cases were compared to those of AML samples with other phenotypic and cytogenetic subtypes ($n = 155$), demonstrating that the two APL subtypes share a characteristic pattern of deregulated genes that makes them distinct from other AML subtypes. To our knowledge, this was the first identification of highly discriminative genes that are correlated with typical APL and its microgranular variant form. Furthermore, a number of differentially expressed genes encoding for different biological functions and involved in several pathways such as granulation and maturation of hematologic cells were detected, which may explain the morphologic and even clinical differences between these two subtypes of APL.

MATERIALS AND METHODS

We analyzed bone marrow aspirates of 35 untreated adult APL patients at diagnosis:

t(15;17)(q22;q12) and FAB AML M3 ($n = 19$)
t(15;17)(q22;q12) and FAB AML M3v, microgranular variant ($n = 16$)

We also included 155 additional AML samples of the following subtypes: t(8;21) ($n = 35$), inv(16) ($n = 35$), t(11q23)/*MLL* ($n = 35$), and normal karyotype ($n = 50$).

All bone marrow samples were referred to the Laboratory for Leukemia Diagnostics between 1999 and 2003 and were registered in our leukemia database (Dugas et al., 2001). Samples were received either locally or by overnight express mail. APL patients were treated within a prospective randomized multicenter trial (Lengfelder et al., 2000). The studies were conducted according to the rules of the local internal review board and the tenets of the revised Helsinki protocol. In all patients who were included in this investigation, diagnosis was performed by the investigators with an individualized combination of cytomorphology, cytogenetics, fluorescence in situ hybridization (FISH), immunophenotyping, and molecular genetics as described previously (Haferlach and Schoch, 2002; Schoch et al., 2002b; Kern et al., 2003a; Schnittger et al., 2003). At the time each patient was diagnosed, mononuclear cells from the bone marrow aspirate were purified by Ficoll density centrifugation, and 5×10^6 or 1×10^7 cells were lysed (RLT lysis buffer; Qiagen, Hilden, Germany). For subsequent molecular analyses, these stabilized lysates were frozen and stored at -80°C. Gene expression analyses were performed as previously described (Schoch et al., 2002a; Kern et al., 2003b; Kohlmann et al., 2003).

Data Analysis

Gene expression data were preprocessed according to Affymetrix recommendations and analyzed as follows. For the differentially expressed genes in each disease entity, a *t*-test statistic (two-sample *t* test, unequal variances) was calculated in the context of a one-versus-all (OVA) approach, using the software package R version 1.7.1 (http://www.r-project.org/). To address the problem of multiple testing, the false discovery rates (FDRs) of genes were calculated (Tusher et al., 2001; Storey and Tibshirani, 2003).

Class prediction was performed by use of support vector machines (SVMs; Vapnik, 1998). SVM models were built with libsvm (www.csie.ntu.edu.tw/~cjlin/libsvm/). Briefly, the complete data set was randomly split into training and independent test cohorts. Then differentially expressed genes were identified in the training set, and a learning model was built that included the top differentially expressed genes. With this approach, the algorithm would learn to discriminate between the respective subtypes on the basis of gene expression data in the given cohort of training patients. Having learned the expression features of the classes, the algorithm could recognize new samples as class members on the basis of their expression patterns in the test cohort.

We performed linear regression analyses using SPSS 11.5 (Chicago, IL) to assess the relations between expression levels of distinct differentially expressed genes and other clinical and biological parameters such as M3 or M3v morphology, white blood cell (WBC) count, and FLT3-LM status.

Functional Gene Annotation

By use of the NetAffx™ Analysis Center, the probe sets were functionally annotated and grouped according to their biological function, for example, according to GeneOntology biological process descriptions (Ashburner et al., 2000; Liu et al., 2003). We were especially interested in genes coding for proteins that have functional relevance in blood coagulation. As such, all U133 microarray probe sets corresponding to the GO category blood coagulation (Accession: GO:0007596; release date March 22, 2004) were identified, and the expression data were filtered for further analyses.

Data Visualization

To assess similarity of gene expression patterns, we applied hierarchical cluster analyses and principal component analyses (PCA; Mardia et al., 1979; Eisen et al., 1998). Using GeneMaths 2.01 software (Applied Maths, St-Martens-Latem, Belgium) we analyzed the transformed gene expression data (cluster algorithm: Ward; selected coefficient: Euclidean distance).

Biological Networks Analysis

Biological networks have been generated through the use of Ingenuity Pathways Analysis, a Web-delivered application that generates networks using differentially expressed genes from expression array data analyses (Ingenuity, Mountain View, CA). Networks were generated addressing two questions: (i) discrimination of APL from other genetically defined AML subtypes and (ii) discrimination of FAB M3 from FAB M3v samples. In both analyses, a data set containing the gene identifiers in probe set format with the corresponding characteristics on magnitude of change was uploaded as a tab-delimited text file into the Ingenuity Pathway database. A detailed description of the procedure is available in the supplemental material.

Quantification of Candidate Genes by Real-Time RT-PCR

The microarray data were validated in 55 patients who had APL with a t(15;17), of whom 35 had the FAB M3 variant and 20 had the FAB M3v variant. Sixteen of the patients (9 M3, 7 M3v) also were analyzed by microarray technology in this study. Thirteen transcripts (*PACIN2*, *LGALS1*, *IQGAP1*, *GSN*, *CORO1A*, *CAPG*, *ARPC1B*, *SEPQ*, *PTPRE*, *IL3RA*, *HTM4*, *CD45*, and *BMI1*) were selected for quantification by real-time RT-PCR (LightCycler technology; Roche Applied Science, Mannheim, Germany). Detailed information on primer and hybridization probe design, respective sequences, and procedure temperatures, as well as validation results, is given in the supplemental material.

RESULTS

Discrimination of APL from Other Genetically Defined AML Subgroups

Using an unsupervised class discovery approach, we first aimed at separating patients with APL and t(15;17) from three other distinct and genetically defined AML subgroups with recurrent chromosomal aberrations: AML with t(8;21), with inv(16), and with t(11q23)/*MLL*. In contrast to our previous studies, we now analyzed whether these AML subclasses could be discovered by analysis of their

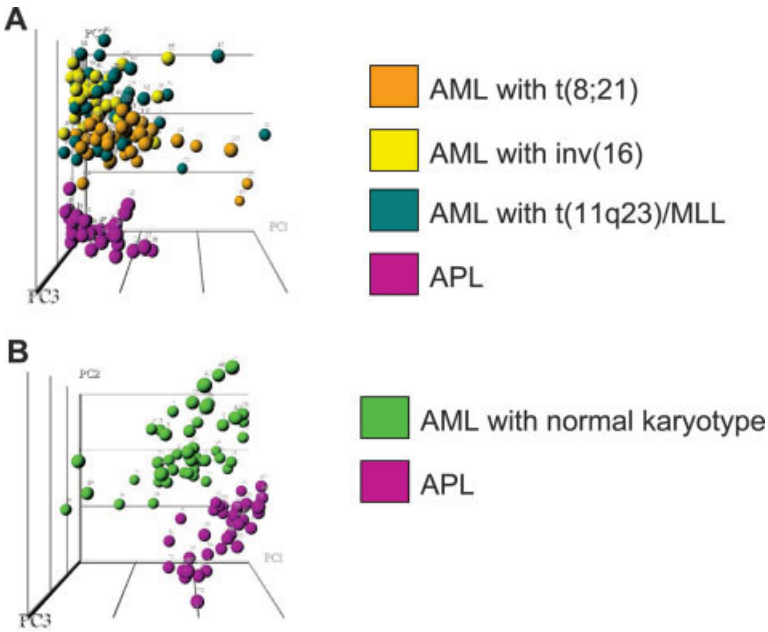

Figure 1. Class discovery: APL versus other AML subclasses. Without any gene selection or filtering, each patient's global gene expression pattern is represented by a single color-coded sphere. The feature space consisted of all measured expression data, that is, all of the approximately 44,000 probes represented on the two microarrays. An unsupervised PCA discovered AML subclasses and segregated the 35 APL samples from (A) those with AML with a t(8;21) ($n = 35$), an inv(16) ($n = 35$), or a t(11q23)/*MLL* ($n = 35$) and (B) those with AML with a normal karyotype ($n = 50$).

global gene expression signatures. Therefore, the leukemia samples used in this analysis were subjected to an unsupervised algorithm (PCA).

The respective global gene expression profiles derived from these samples were presented to the algorithm without any class information attached. As the visualization in Figure 1A shows, APL samples clearly clustered distinctly from AML with t(8;21), inv(16), or t(11q23)/MLL. In a second step, we intended to discriminate the APL patients ($n = 35$) from the AML patients with a normal karyotype ($n = 50$). Again, through the use of an unsupervised approach as described above, it could be observed that the APL profiles were clearly distinct from AML with a normal karyotype (Fig. 1B).

Moreover, using a supervised analysis approach, we identified genes that were differentially expressed between APL and AML with t(8;21), or inv(16), or t(11q23)/MLL aberrations as well as between APL and AML with a normal karyotype. When both lists containing the top 1,000 differentially expressed probe sets were matched, 505 probe sets were found to be overlapping. With the use of network analysis software, we were able to observe and confirm a known finding: that genes with functional relevance in MHC-II antigen presentation are expressed less in APL (Fig. 2) (Watts, 1997; Masternak et al., 2000; Villadangos and Ploegh, 2000; Orfao et al., 2004). Eight additional networks and gene expression signal intensities for all genes included in the networks are available online. In addition, details of supervised analyses on classification accuracies in APL and other genetically defined AML subgroups are given in the supporting online document.

Expression Signatures of Genes Coding for Proteins with Functional Relevance for Blood Coagulation

Both subtypes of APL are frequently associated with severe bleeding episodes characterized by a combination of DIC and hyperfibrinolysis. Thus, in a next step, gene expression signatures relevant to blood clotting were analyzed further.

The probe sets were functionally annotated and grouped according to their biological function, for example, GeneOntology process descriptions. A total of 132 microarray probe sets, representing 61 genes, corresponded to the Biological Process GO category of blood coagulation (Accession number GO:0007596). The corresponding gene expression data were visualized by PCA. More detailed information on the genes and expression signal intensities is given as supporting online material.

When all 35 APL patients were compared to 50 AML cases with a normal karyotype on the basis of genes involved in coagulation, two distinct clusters were observed (Fig. 3A). In addition, when compared to other AML subclasses with recurrent chromosomal aberrations, that is, cases with t(8;21), inv(16), or *MLL* gene rearrangements, all APL cases could be separated on the basis of their specific gene expression pattern by genes correlated with coagulation (Fig. 3B). For further validation of these findings, we then compared AML M3 samples with AML M3v samples, using the same

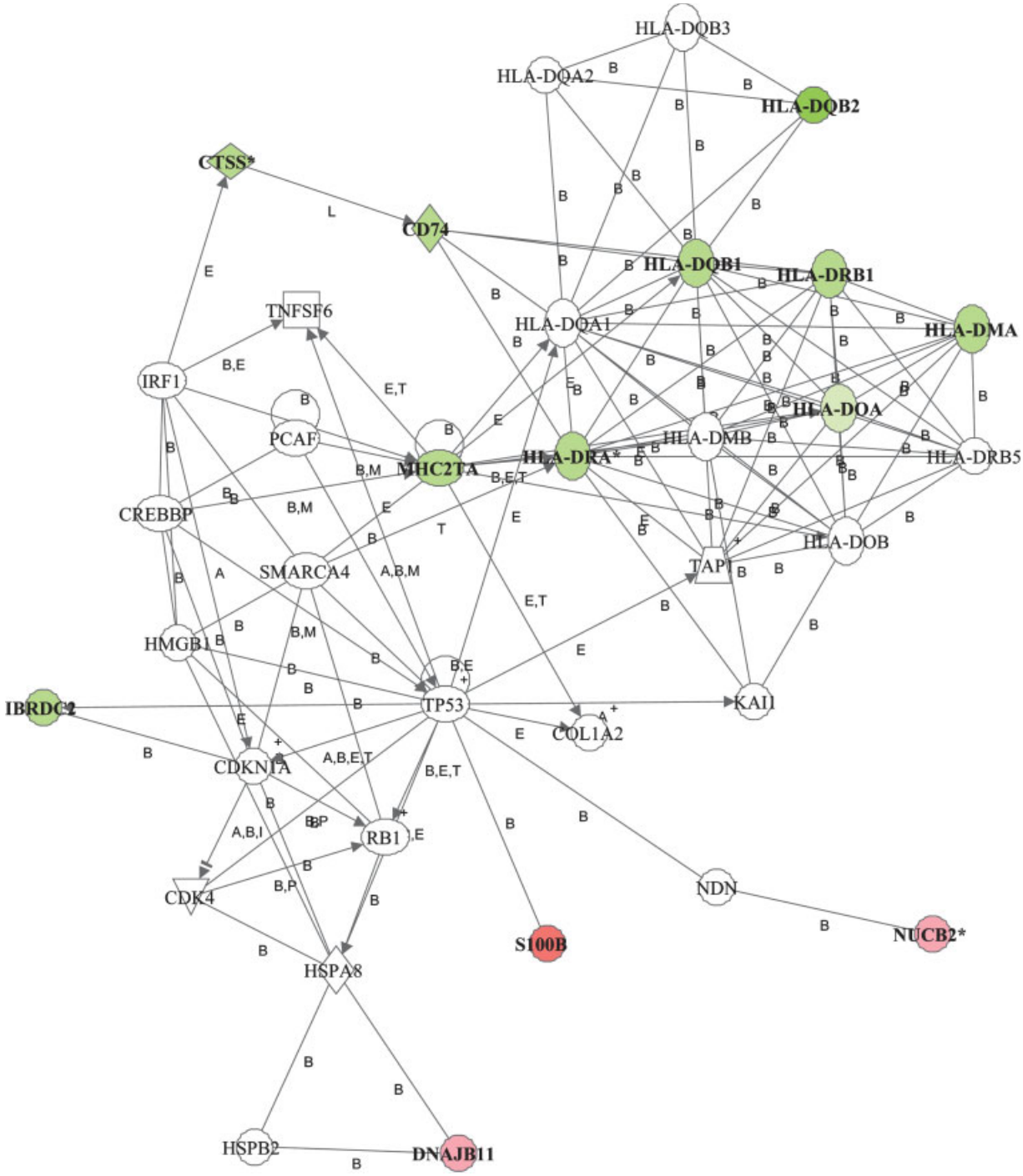

Figure 2. Biological network distinguishing APL from other AML subclasses. The network is graphically displayed, with genes/gene products as nodes and the biological relationships between the nodes as edges. The intensity of the node color indicates the degree of differential gene expression. A node that is green or red indicates lower or higher expression, respectively, in APL cases relative to AML cases with a t(8;21), an inv(16), a t(11q23)/*MLL*, or a normal karyotype. Note: Focus genes were included in the original text format file derived from the list of differentially expressed genes ($n = 505$ overlapping APL-specific probe sets). Nonfocus genes were derived from queries for interactions between focus genes and all other gene objects stored in the Ingenuity knowledge database.

genes encoding for clotting of the relevant proteins. As anticipated, no clear distinction was observed between the two groups (Fig. 3C). In both types of comparisons, which were based on a preselected set of genes with functional relevance in blood clotting, all APL samples demonstrated a

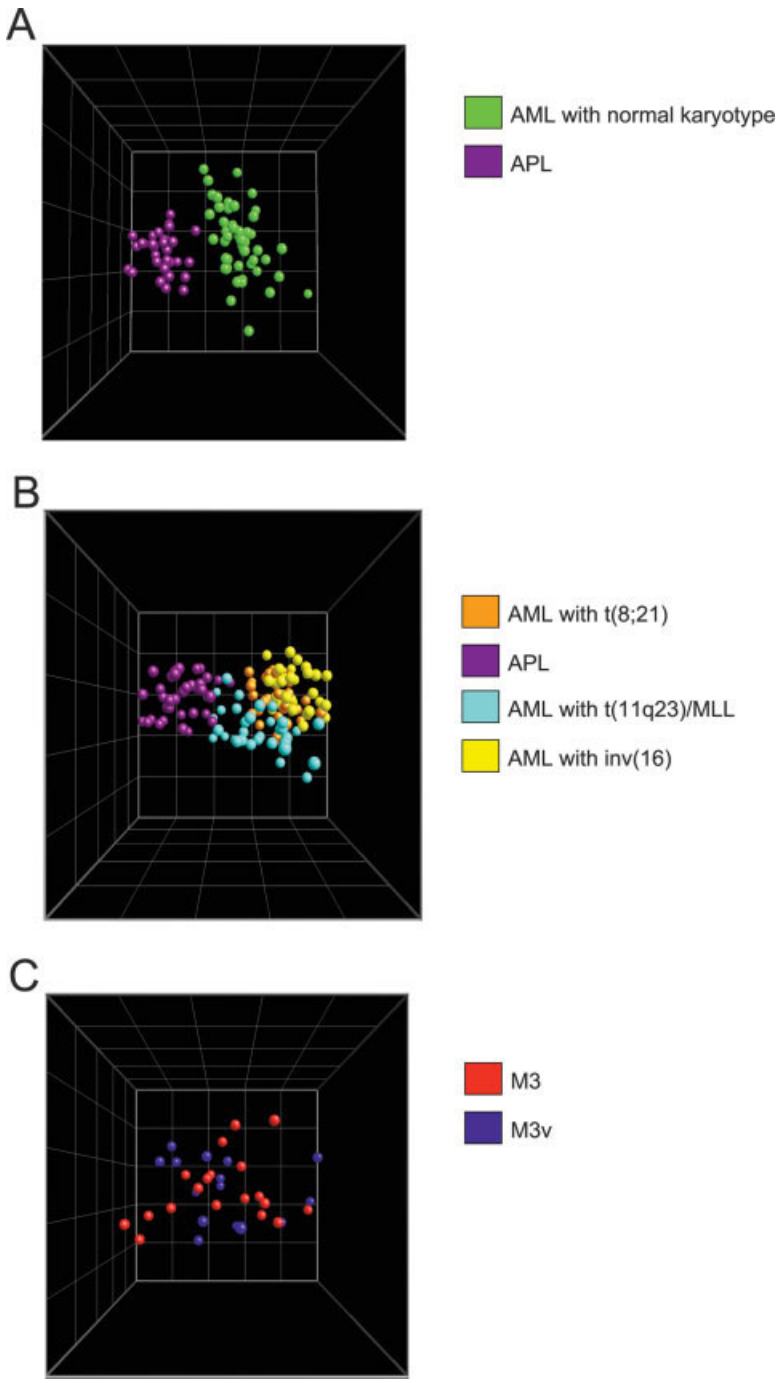

Figure 3. PCA based on genes from the GO category blood coagulation. The three-dimensional PCA feature space visualizes measured expression data on genes from the Gene Ontology Biological Process category blood coagulation (accession number GO:0007596). (A) Analysis of patients with APL comparing them to those with AML with a normal karyotype ($n = 83$ probe sets). (B) Analysis of patients with APL comparing them to those with AML with a t(8;21), an inv(16), or a t(11q23)/MLL ($n = 75$ probe sets). (C) Comparison of M3 and M3v ($n = 62$ probe sets).

distinct expression pattern different from that of all other AML subclasses.

Discrimination Between FAB M3 and FAB M3v Samples

Genes differentially expressed in the two morphologic APL subtypes were then identified. The profiles of 19 AML M3 cases were directly compared to those of 16 AML M3v cases. Morphologic classification was done routinely at diagnosis, and no further reevaluation was performed prior to all the samples being processed for this microarray study. This was decided, although we were aware of several rare cases that show a mixture of the two morphologic phenotypes (see Fig. 4).

A common supervised data analysis algorithm was chosen for identification of significantly differentially expressed genes. First, M3 profiles were

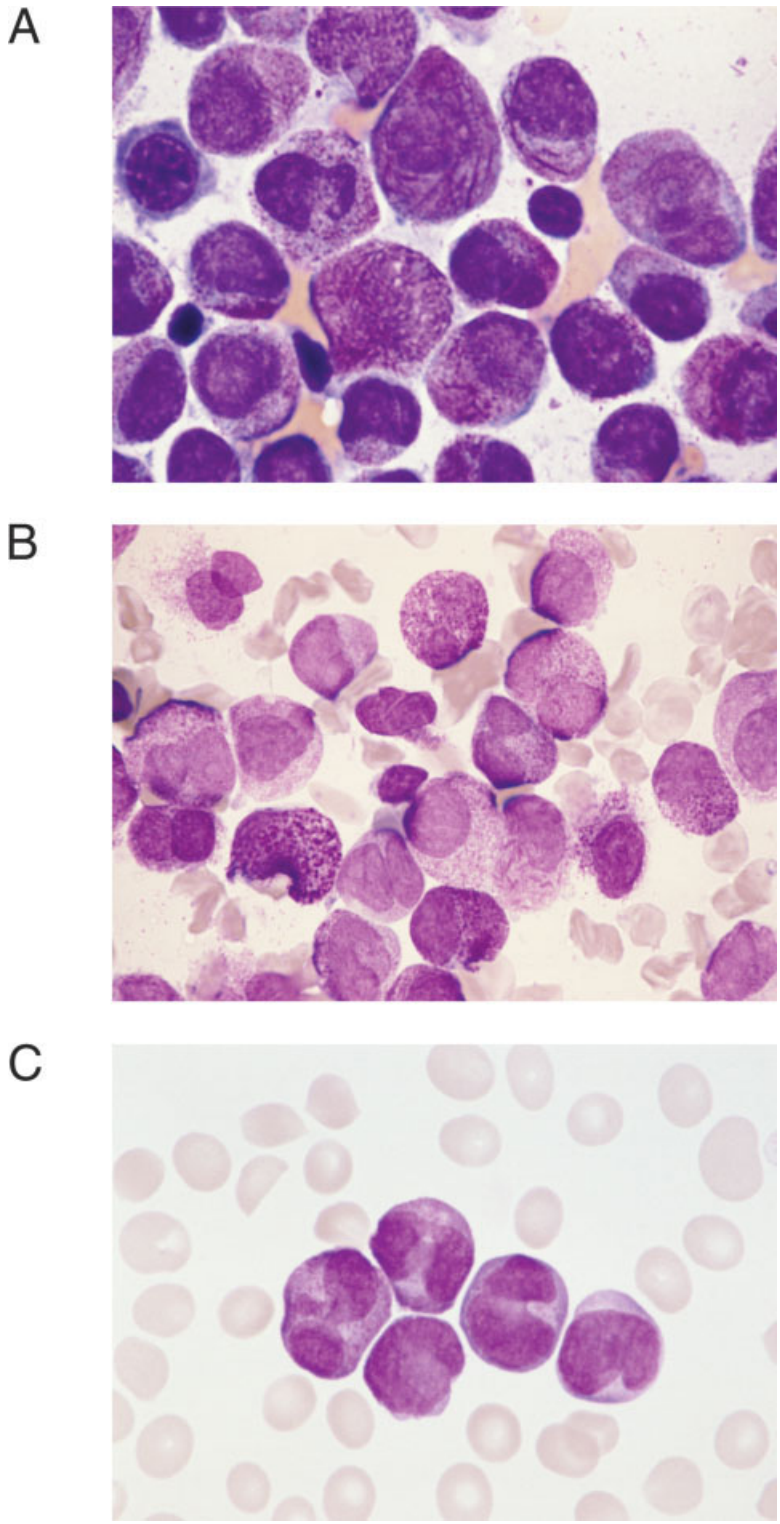

Figure 4. Morphology of APL samples. Three Pappenheim stains of bone marrow smears from APL patients represent a light microscopic view at ×630 magnification of (A) typical FAB M3 case with heavy granulation and bundles of Auer rods; (B) case demonstrating a mixture of the two morphologic phenotypes; (C) typical FAB M3v case with non- or hypogranular cytoplasm and bilobed nuclear configuration (figures by T.H.).

compared to M3v profiles, and the FDR of differentially expressed genes was estimated.

First, an FDR of 5% (q value < 0.05) was chosen, that is, among all features considered significant, on average 5% were truly null. According to this criterion, 186 probe sets were significant. Figure 5A shows the hierarchical cluster and principal-components analyses using these 186 probe sets. The M3 samples clustered distinctly from the M3v cases, with few exceptions. Figure 4B shows

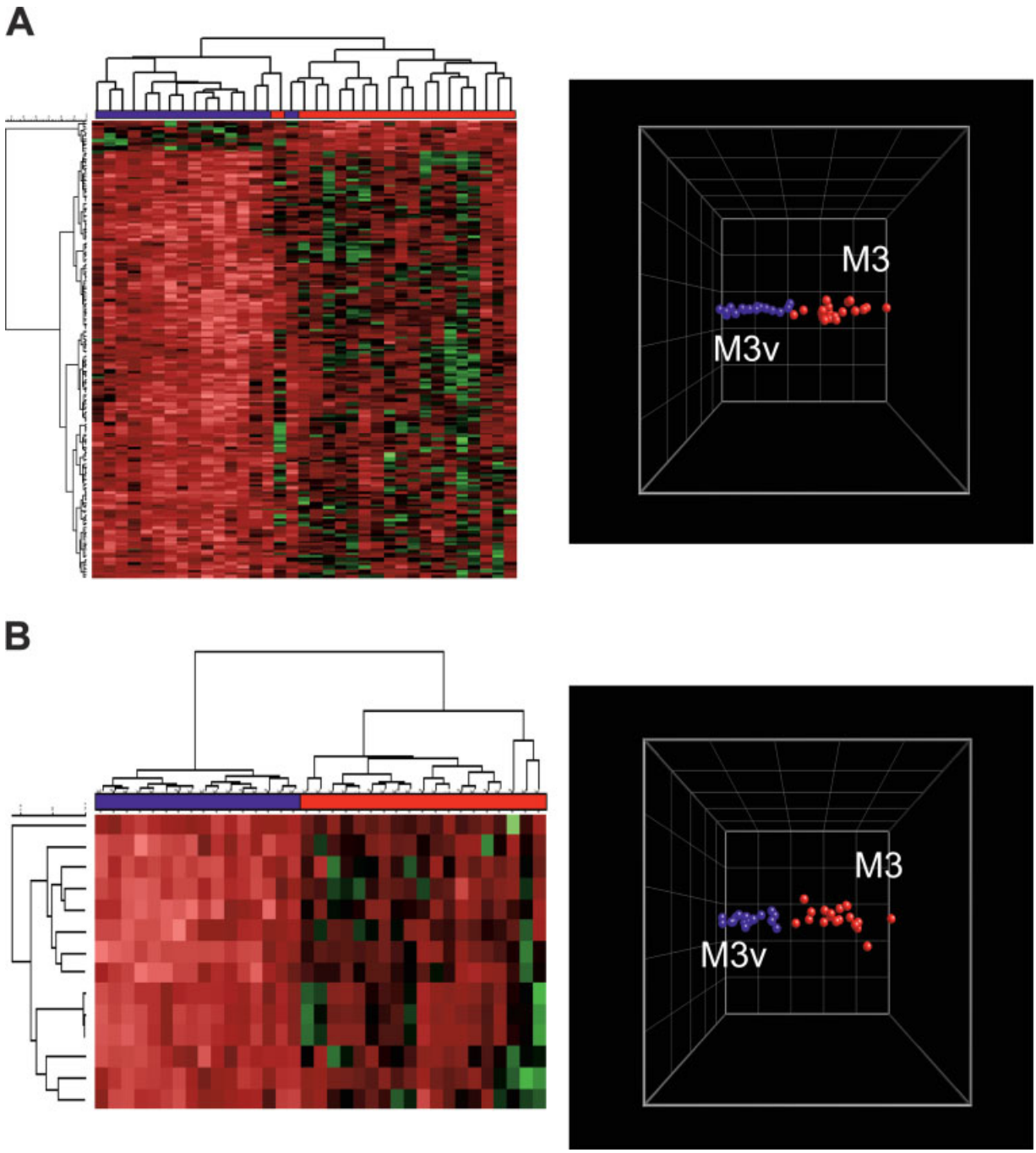

Figure 5. Visualization of genes differentially expressed in M3 and M3v. In the hierarchical cluster analysis and the PCA, the feature space consisted of measured expression data on genes differentially expressed between FAB M3 and M3v. In the hierarchical clustering, the normalized expression value for each gene was coded by color (standard deviation from mean). Red indicates high expression and green low expression in a cell. (A) Visualization of data based on $n = 186$ probe sets discovered at 5% FDR. (B) Visualization of data based on $n = 14$ probe sets discovered at 1% FDR.

the corresponding morphology of the blasts of a borderline case.

We next applied a more stringent criterion, that is, an FDR of 1%. With this criterion, the number of significant probe sets shrank to 14. As shown in Figure 5B, now all M3 cases could be separated robustly from the M3v samples on the basis of the expression pattern of those 14 probe sets, which represented the genes *ANXA2*, *CAP1*, *CAPN2*, *CD97*, *GLUD1*, *LGALS1*, *LMNA*, *MMP19*,

PLXNB2, *S100A10*, *TAGLN2*, and *TGFB1*. All genes demonstrated higher expression in the M3v samples. The supplemental material contains bar graphs that visualize the absolute expression signal intensities for each gene.

Subsequently, we aimed at predicting the respective morphologic APL subtype by using SVMs. The complete data set was randomly split, although balanced by the morphological APL subtypes, into training and independent test cohorts. Then differentially expressed genes in the training set were identified, a *t*-test statistic was calculated, and an SVM model was built that was based on the 50 genes in the training set that demonstrated the greatest difference in expression between the FAB M3 and FAB M3v subtypes. We used this SVM to predict a subtype of the samples in the test cohort.

By using a 10-fold cross-validation approach (9 of 10 for training and 1 of 10 for testing, 10 iterations), APL morphologic subtypes were predicted on the basis of their gene expression signature with a median of accuracy of 91%. To assess the robustness of class prediction, we applied a resampling approach, that is, the complete classification procedure was repeated 100 times (training set: two thirds of patients; test set: one third). By this means, 95% confidence intervals for prediction accuracy, sensitivity, and specificity were estimated. In this study, we have demonstrated that APL morphologic subtypes can be predicted on the basis of their gene expression signature with a median accuracy of 91% (95% confidence interval of accuracy: 73%, 100%). The algorithm chose the *S100A10*, *TAGLN2*, *LGALS1*, *CAP1*, *GLUD1*, *ANXA2*, *TGFB1*, *LMNA*, and *MMP19* genes in more than 90% of the runs.

Next, biological networks were generated. The networks were based on genes whose expression was significantly differentially regulated between FAB M3 and FAB M3v samples. Figure 6 shows one such biological network. Five additional networks are included in the supporting material, available online.

Fourteen focus and 21 nonfocus genes constitute network 1 (Fig. 6). In this network, several members of the SMN complex, functioning in the assembly of spliceosomal small nuclear ribonucleoproteins, are represented (Pellizzoni et al., 2002). Two of them, *SNRPB* and *GEMIN7*, demonstrated higher expression in FAB M3v profiles than in FAB M3 profiles. A similar pattern was shown for *CDC42*, a small GTPase of the Rho subfamily, which regulates signaling pathways that control diverse cellular functions including cell morphology, migration, endocytosis, and cell-cycle progression (Etienne-Manneville, 2004), and its substrates, *MAP3K11* and *IQGAP1* (Bock et al., 2000; Fukata et al., 2002). Other genes that had lower expression in FAB M3 samples included two members of the calpain system, *CAPN2* and *CAPNS1* (Goll et al., 2003); the antiapoptotic *BAG1* (Takayama et al., 1995); *SREBF2*, a sterol regulatory element–binding transcription factor (Miserez et al., 1997); and *CTNNA1*, a component of the E-cadherin/catenin cell adhesion complex (Furukawa et al., 1994).

Granulation Pattern in APL Subtypes

It has been shown that different patterns of granulation are associated with different levels of defensin and transcobalamin (Boxer and Smolen, 1988; Hirata et al., 1993; Jandl, 1996). Therefore, we evaluated genes known to be relevant to granulation. Higher expression of defensin alpha 1 (*DEFA1*) was observed in M3 cases, in accordance with a higher number of primary granules. On the other hand, transcobalamin II (*TCN2*), a cobalamin transport protein, was found to have higher expression in secondary granules and also to be elevated in cases with M3v morphology. The supplemental material contains bar graphs visualizing the absolute expression signal intensities for each gene.

Genes Reflecting Different Stages of Maturation Between AML M3 and AML M3v

Five genes involved in maturation were found to be differentially expressed. *PTPRC* (CD45 antigen) demonstrated elevated expression in FAB M3v cases compared to M3 samples. The APL cases also differed in expression of *ALOX5*, which is involved in the leukotriene pathway, with greater expression in M3v than in M3 cases. Furthermore, the interleukin-3 receptor alpha chain gene (*IL3RA*) was more highly expressed in M3v cases. In addition, we observed differences in expression of the *CD2* gene, which was more highly expressed in M3v. A similar pattern of expression was observed for *CD2BP2*. The corresponding bar graphs visualizing the absolute expression signal intensities for each gene are available in the supplemental online section.

Gene Expression Can Also Differentiate Between AML M3 and AML M3v, Explaining Differences in Their Respective Phenotypic Characteristics

From the morphologic and clinical points of view, differences between typical APL and its variant are known to occur in the granulation pattern, in WBC numbers, in Auer rods, and especially in the frequency of faggot cells and the detection of

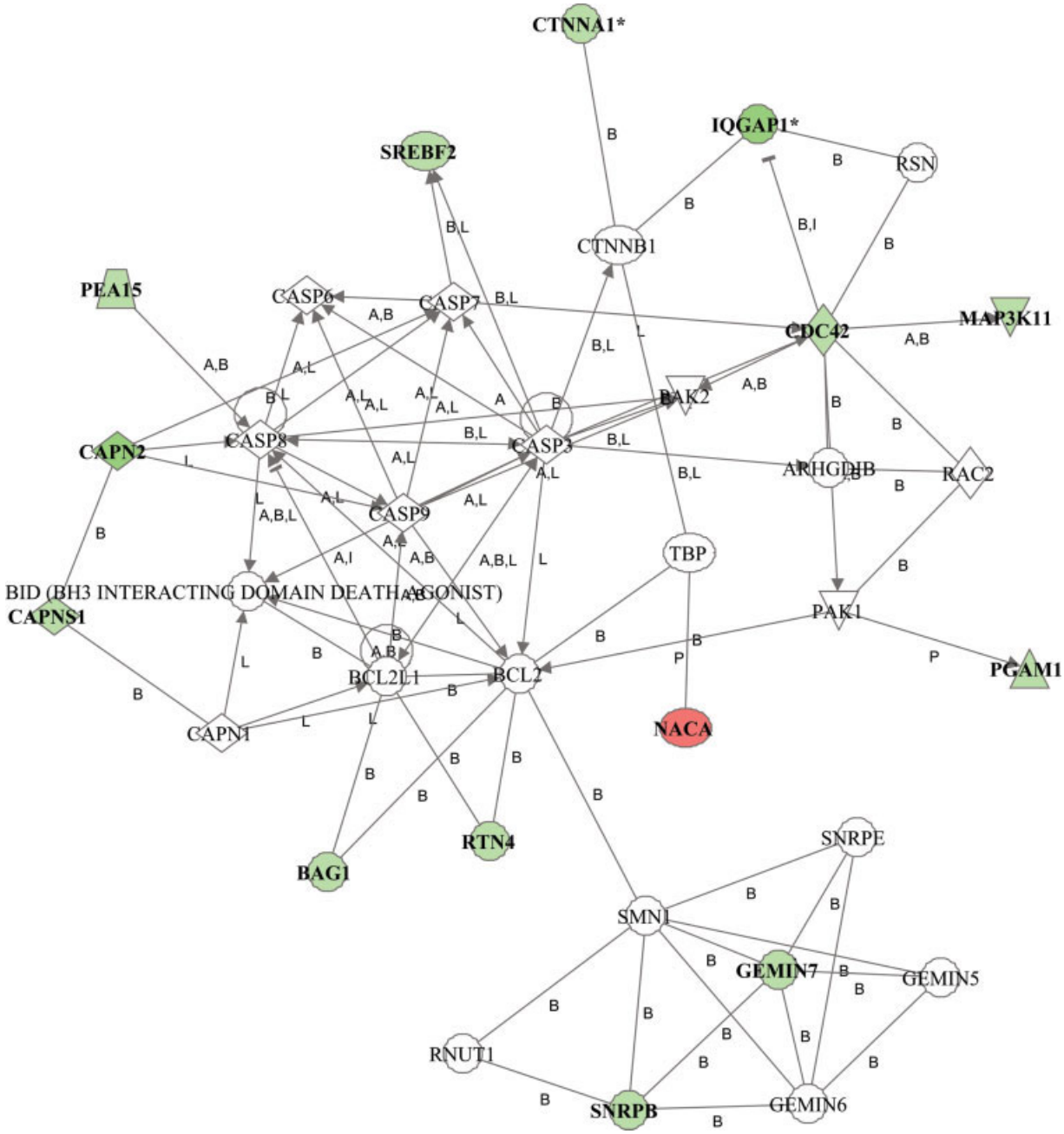

Figure 6. Network of genes differentially expressed in M3 and M3v. As an example, biological network 1 is displayed graphically as nodes (genes/gene products) and edges (the biological relationships between the nodes). The intensity of the node color indicates the degree of differential gene expression. Green and red intensities correspond to lower and higher expression, respectively, in FAB M3 cases compared to FAB M3v cases. Edge length reflects evidence supporting that node-to-node relationship, in that edges supported by more articles from the literature are shorter. Note: Focus genes were included in the original text format file derived from the list of differentially expressed genes (5% FDR cutoff). Nonfocus genes were derived from queries for interactions between focus genes and all other gene objects stored in the Ingenuity knowledge data base.

FLT3-LM. In this respect, we analyzed all AML M3 ($n = 118$) and AML M3v cases ($n = 46$) that were diagnosed in our laboratory by the morphologic, cytogenetic, and molecular genetic techniques as described above. Data on these 164 APL patients are shown in Table 1. We detected no MLL–PTD or KIT mutations in any of our AML M3 or AML M3v cases, respectively.

Regarding the breakpoint type of the *PML–RARA* fusion of 124 cases with t(15;17), bcr1 was found in 55 cases (44.4%), bcr2 in 4 cases (3.2%), and bcr3 in 65 cases (52.4%). The bcr1 breakpoint

TABLE 1. Morphological and Genetic Differences in Our APL Cohort

	AML M3 ($n = 118$)	AML M3v ($n = 46$)	*P*
Age (median, range)	51.4 (17–82)	47.9 (18–83)	n.s.
Male:female	59:59	21:24	n.s.
WBC at diagnosis (μ/l)	1,400 (200–70,900)	15,300 (500–332,000)	**0.000000005**
Hemoglobin (g/dl)	9.4 (4–15)	9.0 (4.8–14.1)	n.s.
Platelets (μ/l)	31,000 (7,000–187,000)	26,000 (8,000–115,000)	n.s.
Auer rods leading to faggot cells	68/78 (87.2%)	20/31 (64.5%)	**0.0055**
Additional chromosomal aberrations	46/118 (39%)	17/46 (37%)	n.s.
FLT3-LM	20/80 (25%)	34/46 (73.9%)	**<0.0001**
FLT-TKD*	3/66 (4.5%)	4/41 (10.8%)	n.s.
NRAS	3/63	0/30	n.s.

*TKD = mutations in the tyrosine kinase domain; n.s. = not significant.

was found more often in M3 cases ($P = 0.022$), and the bcr3 breakpoint was found more frequently in M3v cases ($P = 0.018$, Table 2, personal communications of Susanne Schnittger).

To prove that the genes identified to distinguish between AML M3 and AML M3v were not simply related to length mutations of the *FLT3* gene (FLT3-LM) or to the WBC count, both known to differ between the two disease subtypes, linear regression analyses, with AML M3v, FLT3-LM, and WBC count as covariates, were performed for each of the top 20 genes. The results indicated that for nearly all the top 20 genes, the relation was strongest to AML M3v (Table 3). Significant relationships with FLT3-LM and WBC count were present for only two and six genes, respectively. Therefore, the identified genes do not simply reflect the WBC count or the occurrence of FLT3-LM, but truly are related to the presence of an AML M3 or AML M3v morphology, respectively.

Correlation of the Expression of Genes as Measured on the Microarray and Quantitative RT-PCR

The microarray data were further validated in 55 patients with APL and t(15;17): 35 FAB M3 and 20 FAB M3v. Sixteen of these (M3, $n = 9$; M3v, $n = 7$) also had been analyzed in this study by microarray technology. Thirteen transcripts (*PACIN2*, *LGALS1*, *IQGAP1*, *GSN*, *CORO1A*, *CAPG*, *ARPC1B*, *SEPQ*, *PTPRE*, *IL3RA*, *HTM4*, *CD45*, and *BMI1*) were selected for quantification by real-time RT-PCR. Good correlation was observed between microarray and quantitative PCR data. In 11 of the 13 genes, a highly significant correlation was found. The correlation coefficients for these genes varied between 0.64 and 0.91, and *P* values ranged from 0.04 to <0.001. The additional patients measured only by quantitative PCR showed levels of expression in the range of expression measured in the corresponding group of patients analyzed both by microarray and quantitative PCR. More information is provided in the supplemental material.

DISCUSSION

It was postulated that distinct features of AML as defined by cytomorphology, immunophenotype, cytogenetics, and molecular genetic parameters could be reproduced by gene expression patterns (Haferlach et al., 2003). In a first approach, it was demonstrated that three AML subtypes with reciprocal rearrangements, that is, AML with t(8;21), inv(16), or t(15;17), could be distinguished by their unique gene expression profiles (Schoch et al., 2002a). In addition, AML with 11q23 aberrations also showed a distinct gene expression pattern (Kohlmann et al., 2003). Thus, all four subgroups defined genetically in the first step of the new hierarchy of the current WHO classification (Jaffe et al., 2001) showed unique underlying gene expression signatures. This was confirmed by others, emphasizing that gene expression patterns are robust and reproducible (Armstrong et al., 2002; Miller et al., 2002; Staudt, 2002; Yeoh et al., 2002; Debernardi et al., 2003; Ross et al., 2003; Kohlmann et al., 2005). It was further published recently that subdiscrimination in AML focusing on molecular markers such as FLT3-LM or CEPBA (Valk et al., 2004) and on prognostication in patients with normal karyotypes also seemed possible on the basis of specific gene expression profiles (Yagi et al., 2003; Bullinger et al., 2004; Grimwade and Haferlach, 2004; Liu and Karuturi, 2004).

In the present study, we first confirmed that APL showed a specific expression signature shared by M3 and M3v, distinguishing both subtypes from other genetically defined AMLs. We used unsupervised data analysis approaches to discriminate APL (FAB M3 and M3v combined) from AML with t(8;21), inv(16), or t(11q23)/*MLL* aberrations and

TABLE 2. *PML–RARA* Gene Fusion Breakpoints in AML M3 and AML M3v

	bcr1 (n)	bcr2 (n)	bcr3 (n)
M3 ($n = 86$)	44 (51.1%)*	3 (3.7%)	39 (45.2%)**
M3v ($n = 38$)	11 (28.9%)*	1 (2.6%)	26 (68.4%)**
Total ($n = 124$)	**55 (44.4%)**	**4 (3.2%)**	**65 (52.4%)**

*$P = 0.022$.
**$P = 0.018$.

TABLE 3. Linear Regression Analyses of 20 Most Discriminative Genes of Morphology of M3 versus M3v

	Covariates		
Gene	AML M3v	WBC count	FLT3-LM
NFE2L1	0.004	n.s.	n.s.
S100A10	0.00006	0.002	n.s.
TAGLN2	0.00007	n.s.	n.s.
GLUD1	0.0004	n.s.	n.s.
LGALS1	0.0001	0.005	0.034
ANXA2	0.00001	0.025	n.s.
CD97	0.00004	n.s.	n.s.
TGFB1	0.0001	n.s.	n.s.
LMNA	0.018	0.010	0.025
MMP19	0.00002	n.s.	n.s.
CRIP1	0.0003	0.00002	n.s.
CAPN2	0.00001	n.s.	n.s.
CDC42	0.0002	n.s.	n.s.
PLXNB2	0.00002	n.s.	n.s.
ANXA2	0.00006	n.s.	n.s.
LMNA	0.0004	0.034	n.s.
ANXA2	0.00002	n.s.	n.s.
CAP1	0.00005	n.s.	n.s.
MGC10997	0.0001	n.s.	n.s.
CAMK1D	0.001	n.s.	n.s.

P values are given; n.s. = not significant.

also from AML with a normal karyotype. Thus, repeatedly we added proof to the finding that t(15;17) fusion transcripts lead to a unique and highly reproducible gene expression signature. In addition, using a supervised analysis approach, we identified genes in APL that were differentially expressed in AML with t(8;21), inv(16), or t(11q23)/*MLL* aberrations as well as in AML with a normal karyotype. When overlapping APL-specific genes were examined using pathway analysis software, a phenomenon known from immunophenotyping of APL was confirmed on a transcriptional level, that is, that genes with functional relevance in MHC-II antigen presentation are expressed less in APL (Orfao et al., 2004). Other overlapping APL-designating genes/probe sets that were found to be consistently more highly expressed in APL in these two analyses encoded for candidate genes including *AGRN*, *ANXA8*, *CTSW*, *HGF*, *LAMC1*, *LGALS12*, *MST1*, *PTGDS*, *SERPING1*, *SLC24A3*, *STAB1*, and *TPM4*. In a recent study, some of these probes also were listed as highly correlated with a t(15;17)-specific gene signature (Valk et al., 2004). Similarly, genes with consistently lower expression genes in APL included *CD86*, *CDW52*, *CSPG2*, *CTSS*, *DEFA4*, *HOXA9*, *HOXA10*, *MEIS1*, *MARCKS*, *MS4A6A*, *S100A9*, and *SCAP2*. This confirms recently reported data on global down-regulation of *HOX* gene expression in APL (Thompson et al., 2003).

Patients with APL suffer from severe bleeding at diagnosis, and before ATRA was introduced into induction protocols, there was an early death rate of up to 30%, which is still as high as 10% with the inclusion of ATRA (Sanz et al., 1999; Tallman et al., 2002). Therefore, through the use of the GeneOntology annotation, we performed an additional supervised analysis that included genes known to be involved in clotting. Several genes including *ANXA5*, *CD59*, *THBS1*, *SERPINE1*, *LMAN1*, and *THBD* were found to be more highly expressed in APL than in other AML subtypes. This confirmed a previous finding in patients with DIC of elevated plasma levels of *SERPINE1*, also known as the plasminogen activator inhibitor-I (Watanabe et al., 2001).

An additional major focus of the present study was a more detailed analysis of the transcriptome of APL subtypes. We have demonstrated for the first time that AML M3 and its morphologic variant can be discriminated and also can be predicted on the basis of their specific gene expression signatures.

At diagnosis, 56% of AML patients were measured as having a WBC count of >10,000/μl, 15% a WBC within the normal range, and 29% a leukocyte count of <4,000/μl (own data on 1,155 unselected AML patients). However, a striking difference was observed for the WBC counts of APL patients. Most patients with M3 showed leukopenia, whereas M3v patients had normal or elevated WBC counts. This is surprising because in AML M3 cases, the bone marrow also was mostly packed and did not demonstrate a pattern different from that in other AML subtypes or AML M3v. In addition, the phenotype of M3 was characterized by heavy granulation of promyelocytic blasts. In contrast, the granulation in the M3v cases was mostly invisible. In the myeloperoxidase reaction, both APL subtypes were strongly positive. This makes it obvious that the granules in M3v cases were so-called secondary granules that can be found in more mature cells of granulocytic differentiation. We therefore correlated these phenotypic findings

with the expression levels of candidates known to be present in primary and/or in secondary granules and found highly significant correlations between phenotype and gene expression profiles regardless of shared cytogenetic genotype. Differences in gene expression were detected for *DEFA1*, known to be more highly expressed in primary granules, and for *TCN2*, more highly expressed in secondary granules.

Another very important phenotypic difference between M3 and M3v is the shape of the nucleus. We speculated that genes encoding for nuclear envelope proteins might be differentially expressed between the two morphologic APL subtypes. Indeed, a close examination of an APL-specific network revealed that *LMNA*, encoding for a member of the intermediate filament family, was up regulated in M3v compared to M3 cases (see M3-M3v network no. 6 in the supplemental document). Lamins are components of the nuclear lamina, a fibrous layer on the nucleoplasmic side of the inner nuclear membrane that is thought to provide a framework for the nuclear envelope (McKeon et al., 1986). That lamins A and C were lacking in some undifferentiated cells might point FAB M3v toward a more mature APL subtype (Wydner et al., 1996).

These observations suggest that each of the two subtypes of APL arises from a different level of immature stem cells, leading to individual programming and maturation controls and stops (Grimwade and Enver, 2004). Therefore, we evaluated the different stages of differentiation of several genes. For example, interleukin-3 receptor alpha chain, *IL3RA*, was more highly expressed in M3v cases. Abnormalities of *IL3RA* are frequently observed in AMLs and may contribute to the proliferative advantage of leukemic blasts (Testa et al., 2002). In another study, it was demonstrated that activation of human interleukin 3 (IL-3) receptor stimulates self-renewal or myeloid differentiation (Evans et al., 2002).

However, we also detected a difference in expression of the *CD2* gene. There was a tendency for greater expression of *CD2* in M3v than in M3 samples. This is in line with the observed expression of CD34 and lymphoid markers in the hypogranular APL variant (Foley et al., 2001; Mistry et al., 2003; Grimwade and Enver, 2004). Interestingly, a similar pattern of expression also was observed for *CD2BP2*. This intracellular protein binds to a site within the cytoplasmic region of CD2 (Nishizawa et al., 1998). Thus, that lymphoid markers also were expressed in our group of M3v patients may point to the M3v blasts having a more immature/less committed nature.

FAB M3 and M3v cases might further differ in their leukotriene pathway because of changes in *ALOX5* expression and thereby represent different stages of myeloid differentiation (Scoggan et al., 1996; Busse, 1998). It also has been shown that isoforms of *PTPRC* (CD45 antigen) play an important role in the proliferation and differentiation of hematopoietic cells (Craig et al., 1994). In our study, the expression of *PTPRC* was elevated in FAB M3v cases compared to M3 samples (for expression intensities plotted as bar graphs, see the supporting online document).

With respect to molecular markers, we and others have described a highly significant difference in FLT3-LM status between M3 and M3v. In only 25% of M3 cases was an FLT3-LM detected, but 73.9% of M3v cases demonstrated an FLT3-LM ($P < 0.0001$; Schnittger et al., 2002; Mistry et al., 2003; Grimwade and Enver, 2004). It was demonstrated previously that AML with FLT3-LM is associated with an elevated WBC count in comparison to AML without FLT3-LM (Schnittger et al., 2002). We found a highly significant correlation among morphology, WBC count, and FLT3-LM in AML M3 and AML M3v. To test the independence of the genes identified by microarray analyses, we performed linear regression analysis of the 20 genes with the greatest differential in expression between M3 and M3v as well as in WBC count and FLT3-LM status. Only six genes were found to be dependent on the other two parameters. This again underlines the multifactorial background of the M3 and M3v subtypes. Differences between the two phenotypes in breakpoint cluster regions bcr1 and bcr3 also do not explain gene expression differences and their respective phenotypes.

To confirm the differences in gene expression, 13 differentially expressed genes that discriminated between M3 and M3v were evaluated. A highly significant reproducibility of expression level could be demonstrated when expression was assessed with individual real-time PCR assays on an expanded set of APL patients.

In conclusion, in comparison to other AML subgroups, patients with APL and t(15;17) showed specific gene expression signatures. Some of these APL-specific genes could be correlated with clotting disorders. By using supervised approaches, we were able to discriminate between AML M3 and M3v with very high accuracy on the basis of the parts of genes responsible for maturation, granulation, and nucleus configuration. Therefore, these genes may help to explain the known differences between these two leukemia entities.

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