MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia

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Acute myeloid leukemia (AML) is a highly diverse disease characterized by various cytogenetic and molecular abnormalities. MicroRNAs are small noncoding RNAs that show variable expression during myeloid differentiation. MicroRNA expression in marrow blasts in 215 cases of newly diagnosed and (cyto)genetically defined AML was assessed using quantitative reverse-transcription–polymerase chain reaction (RT-PCR) for 260 human microRNAs. In the same series, mRNA gene expression profiles were established, allowing a direct comparison between microRNA and mRNA expression. We show that microRNA expression profiling following unsupervised analysis reveals distinctive microRNA signatures that correlate with cytogenetic and molecular subtypes of AML (ie, AMLs with t(8;21), t(15;17), inv(16), *NPM1,* and *CEBPA* mutations). Significantly differentially expressed microRNAs for genetic subtypes of AML were identified. Specific microRNAs with established oncogenic and tumor suppressor functions, such as microRNA-155, microRNA-21, and let-7, appear to be associated with particular subtypes. Combinations of selected sets of microRNAs could predict cytogenetically normal AML with mutations in the genes of *NPM1* and *CEBPA* and *FLT3-ITD* with similar accuracy as mRNA probe set combinations defined by gene expression profiling. MicroRNA expression apparently bears specific relationships to the heterogeneous pathobiology of AML. Distinctive microRNA signatures appear of potential value in the clinical diagnosis of AML. (Blood. 2008;111:5078-5085)

Introduction

The pathogenesis of acute myeloid leukemia (AML) is a heterogeneous multistep process affecting cell differentiation, proliferation, and apoptosis, which ultimately leads to malignant transformation of hematopoietic progenitors. Deregulated gene expression, disrupting cellular pathways, has been used for the classification of AML.[1-5] The prognosis of AML depends on well-defined leukemia-specific prognostic factors, such as the cytogenetic abnormalities t(15;17), t(8;21), and inv(16) with a relatively favorable prognosis and the 3q26 abnormalities, −5/−5q and −7/−7q with an unfavorable prognosis.[6-8] Furthermore, various molecular abnormalities in AML with normal karyotype have apparent prognostic significance, such as the somatic gene mutations in nucleophosmin-1 (*NPM1),* FMS-like tyrosine kinase 3 (*FLT3,* internal tandem duplications [ITDs]), and CCAAT/enhancer binding protein alpha *(CEBPA).*[9-13] *NPM1* mutations frequently occur in association with *FLT3*-ITD mutations. Several studies on the clinical impact in AML subgroups revealed that the subset of AML with *NPM1* mutations lacking *FLT3*-ITD mutations has a significantly better overall survival.[14]

MicroRNAs are a class of small noncoding RNAs that regulate translation of protein coding mRNAs and thereby protein expression, by translation inhibition or cleavage of the mRNA transcripts.[15] There is an accumulating body of evidence indicating that microRNAs play important roles in cellular growth and differentiation.[16] MicroRNA expression profiles of tumor samples have recently been shown to provide phenotypic signatures of particular cancer types.[17-22] MicroRNAs can act as tumor suppressor. For instance, expression of some microRNAs, such as let-7[23] and the microRNA15a/16-1 cluster,[24] has been reported to be reduced in lung cancer and chronic lymphocytic leukemia (CLL) respectively, suggesting tumor suppressor activities. In contrast, microRNA-17-92 cluster[25] and microRNA-155/BIC[26,27] have been shown to be overexpressed in B-cell lymphomas, indicative of their oncogenic potential.

A characteristic microRNA expression signature may aid in the diagnosis of certain types or subtypes of cancers. It has been shown that microRNA profiles of bone marrow samples from patients with acute lymphoblastic leukemia (ALL) discriminated subsets of ALL with different molecular aberrancies.[17]

In AML, information about microRNA expression has been gathered only in a limited series of patients so far. Debernardi et al[28] reported in 30 AML patients with normal cytogenetics that microRNA-181a correlates with cytologic subclass. They also reported that the expression of microRNA-10a, microRNA-10b, and microRNA-196a, which are located in intergenic regions in the *HOX* gene clusters, in AML correlates positively with *HOXA* and *HOXB* gene expression, suggesting a role of these microRNAs in aberrant regulation of proliferation and differentiation in leukemogenesis.[28] It was recently reported that microRNA expression signatures discriminate ALL from AML[29] and AML from normal bone marrow $CD34^+$ cells.[30]

Here we addressed the question whether microRNA expression signatures could be used to classify a heterogeneous disease such as AML. We used a microRNA quantification method based on reverse-transcriptase (RT) reaction using stem-loop primers followed by real-time quantitative polymerase chain reaction (PCR)

Submitted January 10, 2008; accepted March 5, 2008. Prepublished online as *Blood* First Edition paper, March 12, 2008; DOI 10.1182/blood-2008-01-133355.

The online version of this article contains a data supplement.

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Table 1. Clinical characteristics of the cohort of 215 patients with newly diagnosed AML

Characteristic	Median (range)	No. of cases (%)
Sex		
Male		117 (54.4)
Female		98 (45.6)
Total		215 (100.0)
Age	49.7 y (18-77.2 y)	
Younger than 35 y		45 (20.9)
35 to 60 y		118 (54.9)
Older than 60 y		52 (24.2)
Total		215 (100.0)
White cell count, $\times 10^9$/L	33 (0.3-510)	
Marrow blasts, %	60 (0-93)	
Platelet count, $\times 10^9$/L	64.5 (8-931)	
French-American-British classification		
M0		6 (2.8)
M1		47 (21.9)
M2		55 (25.6)
M3		6 (2.8)
M4		37 (17.2)
M5		44 (20.5)
M6		2 (0.9)
Not determined		18 (8.4)
Total		215 (100.0)

Table 2. Cytogenetic and molecular characteristics of the cohort of 215 patients with newly diagnosed AML

Characteristic	No. of cases	%
Cytogenetic abnormalities		
−9q	5	2.3
−8	11	5.1
11q23	6	2.8
−5/−5(q)/−7/−7(q)	16	7.4
Complex	5	2.3
Inv(16)	15	7.0
Normal karyotype (NK)	100	46.5
t(15;17)	6	2.8
t(6;9)	2	0.9
t(8;21)	12	5.6
t(9;22)	2	0.9
−X/Y	3	1.4
Other	30	14.0
Not determined	2	0.9
Total	215	100.0
Molecular abnormalities		
FLT3-TKD	20	9.3
FLT3-ITD	62	28.8
N-RAS	22	10.2
K-RAS	1	0.5
CEBPA	22	10.2
NPM1	68	31.6
Total patients	215	100.0
Normal karyotype	100	100.0
With FLT3-TKD	10	10.0
With FLT3-ITD	45	45.0
With N-RAS	11	11.0
With K-RAS	0	0.0
With CEBPA	19	19.0
With NPM1	55	55.0

analysis[31] for 260 known human microRNAs to study the expression profile in 215 clinically and molecularly well-characterized de novo AML bone marrow samples. Furthermore, we sought to identify microRNAs associated with known AML cytogenetic and molecular abnormalities. Finally, we set out to directly compare microRNA-based class predictors of AML subgroups with predictors identified by mRNAs GEP in the same patient cohort.

Methods

Patients and cell samples

Patients used in this study had a newly diagnosed AML determined by cytologic examination of bone marrow (Tables 1, 2). All patients provided written informed consent in accordance with the Declaration of Helsinki and were treated according to the protocols of the Dutch-Belgian-Hematology-Oncology-Cooperative group (available at http://www.hovon.nl).[32-34] The ethics committee of Erasmus University Medical Center, Rotterdam, The Netherlands, approved the study. A total of 215 patients provided bone marrow aspirates at the time of diagnosis. Blast and mononuclear cells were purified by Ficoll-Hypaque (Nygaard, Oslo, Norway) centrifugation and cryopreserved. After thawing, the AML samples contained 80% to 100% blast cells, regardless of the blast count at diagnosis. $CD34^+$ cells from 4 healthy control subjects were sorted from bone marrow aspirates using a fluorescence-activated cell sorter.

RNA isolation and quality control of RNA

After thawing, total RNA was isolated from cells using Trizol reagent according to manufacturer's protocol (Invitrogen, Breda, The Netherlands). Quality control of RNA was done using RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). All RNA samples showed high quality (without RNA degradation or DNA contamination).

Mutation analyses

Sequence analyses for *FLT3*-ITD, *FTL3*-TKD, *NPM1,* and *CEBPA* mutations were performed as described previously.[13,35]

MicroRNA profiling using multiplex real-time quantitative RT-PCR

A multiplexing RT-PCR method was used for the detection of multiple microRNAs.[36] MicroRNA-specific RT primers for 260 mature human microRNAs including several small nuclear RNAs (RNU19, RNU24, RNU38B, RNU43, RNU44, RNU48, RNU49, and RNU66) that were used for internal normalization were divided into 7 pools containing 32 to 47 primers per pool (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and used for multiplexing RT reaction. RT reactions of 10 μL contained: 1 μL 10× RT buffer (Applied Biosystems), 0.2 μL dNTPs (100 mM each), 1 μL MultiScribe Reverse Transcriptase (50 U/μL), 0.125 μL AB RNase Inhibitor (20 U/μL), 2 μL 5× multiplex RT primers, 2 μL of 50 ng/μL total RNA, and 3.675 μL H_2O. Reactions were incubated in a Primus HT Thermocycler (MWY Biotech, High Point, NC) in a 96-well plate for 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and then held at 4°C.

Single real-time quantitative PCR reactions of 10 μL were set up in 384-well plates for each of the 260 microRNAs. Real-time quantitative PCR reactions contained 2.5 μL 4× Universal Master Mix (Applied Biosystems), 1.6 μL 20-fold diluted multiplex RT product, 2.5 μL 5-fold H_2O diluted 20× Taqman MicroRNA primer/probe Assay (Applied Biosystems), and 3.4 μL H_2O. Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA) was used to dispense 2.5 μL primer/probe assays into 384-well plates. This facility was kindly provided by A.G. Uitterlinden (Internal Medicine, Erasmus MC Rotterdam, The Netherlands). Real-time quantitative RT-PCR was performed on an ABI 7900 HT Sequence Detection System (SDS; Applied Biosystems) in a 384-well plate format, under following conditions: first a hot start of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. A Twister robot (Applied Biosystems) was used to load 384-well plates automatically into the 7900 HT SDS. Reproducibility was tested studying

microRNA expression in 10 AML samples in duplicate. RNA was isolated in 2 independent experiments, and subsequently cDNA synthesis and real-time RT-PCR steps were also performed in 2 separate experiments. High correlation in microRNA expression between 2 independent experiments was found, with correlation coefficient R of 0.97 (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Normalization and filtering

SDS 2.3 software (Applied Biosystems) was used to analyze real-time RT-PCR data.

The geometric mean of RNU24, RNU43, RNU48, and RNU66 was used for internal normalization[37] since these RNUs showed the most consistent expression in our cohort (Figure S2). The relative quantification method, 2^{-dCt} was used to calculate the expression relative to the mean of the RNUs.[38] The relative expression (2^{-dCt}) of the microRNAs was normalized with the geometric mean of each microRNA for all patients. To obtain a normal linear distribution, a log2 transformation was performed. MicroRNAs that were unreliably quantifiable or not expressed (Ct value of 35 or higher) or not differentially expressed as defined by interquartile range less than 0.1 were excluded from further analysis, leaving a set of 178 microRNAs.

mRNA gene expression profiling

A total of 215 AML cases were analyzed for mRNA gene expression profiling (GEP) using Affymetrix Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA) as described previously.[2] Gene expression data are available at http://www.ncbi.nlm.nih.gov/geo/ as accession GSE6891.[39]

Unsupervised cluster analyses

We used the unsupervised cluster analyses as described previously.[3] A total of 215 AML patient bone marrow specimens were grouped based on Pearson correlation of the microRNA expression using the Correlation View visualization tool of the OmniViz package version 4.0.0 (Omniviz, Maynard, MA). The groups of patients were ordered based on absolute correlation. Clinical and molecular data were projected next to the correlation plot using the software program Heatmapper (http://www1.erasmusmc.nl/hematologie//heatmapper/, Erasmus University Medical Center).[40]

Significance analysis of microarrays

All supervised analyses were done using significance analysis of microarray (SAM) with the statistical program R version 2.4.1 with the Bioconductor package version 2.0 implemented (http://www.bioconductor.org/, Bioconductor).[41]

The differentially expressed microRNAs were identified by comparing specimens with specific cytogenetic (eg, t(15;17), t(8;21), inv(16)) or molecular abnormalities (*NPM1, CEBPA* and *FLT3*-ITD mutations) with all remaining AML patients in the cohort using SAM with a threshold of minimal 2-fold change in expression and a false discovery rate (FDR) of 0%.

Prediction analysis of microarrays

Supervised class prediction of AML with cytogenetic abnormalities, such as t(15;17), t(8;21), and inv(16) and molecular aberrations such as *NPM1, CEBPA,* and *FLT3*-ITD mutations, using microRNAs and mRNA expression data were performed using prediction analysis of microarrays (PAM).[42] The total cohort of 215 different AML patients as described in Table 1 was randomly divided into a training set of 143 patients to build the class predictor and a validation set of 72 patients to test the predictor independent from the training set. The 10-fold method of cross-validation was applied to assess the class predictor in the training set. The minimum numbers of microRNAs and probe sets of genes (mRNA) that were identified were subsequently tested on the validation set. The accuracy of the specific class predictor was described by the number of truly predicted and false positively predicted cases in the validation set.

Results

Unsupervised analysis of microRNA expression reveals signatures that correlate with particular cytogenetic and molecular subgroups of AML

Using an approach of unsupervised ordering (ie, a priori not taking account of morphologic subtype, cytogenetics, molecular abnormalities, or other external information), 215 cases of AML were classified into the subgroups with similar expression patterns of microRNAs. The correlation view of Figure 1 (right section) shows the clusters with distinctive microRNA signatures. Figure 1 (left section) visualizes the distinct signatures of up- and down-regulated microRNAs. Certain microRNA expression patterns correlated with specific cytogenetic or molecular abnormalities (also shown in Figure 1). Thus microRNA clustering appears representative of particular (cyto)genetic subtypes of leukemia. The microRNA clustering that was evident from unsupervised clustering was frequently driven by a selected number of microRNAs. Detailed information about cluster distribution and clinical, cytogenetic, and molecular data is presented in Table S1.

The 6 cases of AML with t(15;17) aggregated in cluster no. 16. One of the AML cases, which clustered together with specimens with t(15;17) in cluster no. 16, had not been reported to carry the t(15;17) However, a subsequent PCR analysis showed the presence of the *PML-RARA* fusion gene suggesting that the t(15;17) may have been missed by cytogenetic analysis due to cytogenetically cryptic fusion. MicroRNA signature of cluster no. 16 signature was characterized by a set of strongly up-regulated microRNAs (microRNA-382, -134, -376a, -127, -299–5p, and -323). Most AMLs with inv(16) were found in microRNA expression cluster no. 10 (60%; 9/15), and the other 6 cases showed variable signatures scattered over various clusters. The majority (83%; 10/12 cases) of AML with t(8;21) shared the microRNA signature of cluster no. 7 that was characterized by mainly down-regulated microRNAs. Interestingly, some AML cases carrying a inv(16) clustered into the cluster characterized by t(8;21) and vice versa. A similar global down-regulation of microRNAs was evident in microRNA expression cluster no. 8 that contained AMLs with *CEBPA* mutations. Three of 6 AMLs with 11q23 chromosomal abnormalities shared the microRNA expression profile of cluster no. 18. The microRNA expression profiles of all AMLs with *NPM1* mutations (n = 68 cases) were largely restricted to the 6 clusters (no. 2, no. 4, no. 6, no. 9, no. 11, and no. 13) that appeared following unsupervised analysis. Clustering in these instances was determined mainly by a limited set of overexpressed microRNAs (microRNA-10a, -10b, -196a, and -196b). No characteristic clusters of either AMLs with *FLT3*-ITD or *FLT3*-TKD mutations appeared following unsupervised analysis nor were they apparent for the subsets of AMLs with dual *FLT3*-ITD and *NPM1* mutations. Several AMLs aggregated together in clusters no. 1, no. 14, and no. 15 and thus shared 3 characteristic microRNA expression signatures. However no currently known cytogenetic or known molecular abnormalities were associated with these clusters. Normal bone marrow $CD34^+$ specimens revealed a specific microRNA signature (cluster no. 20).

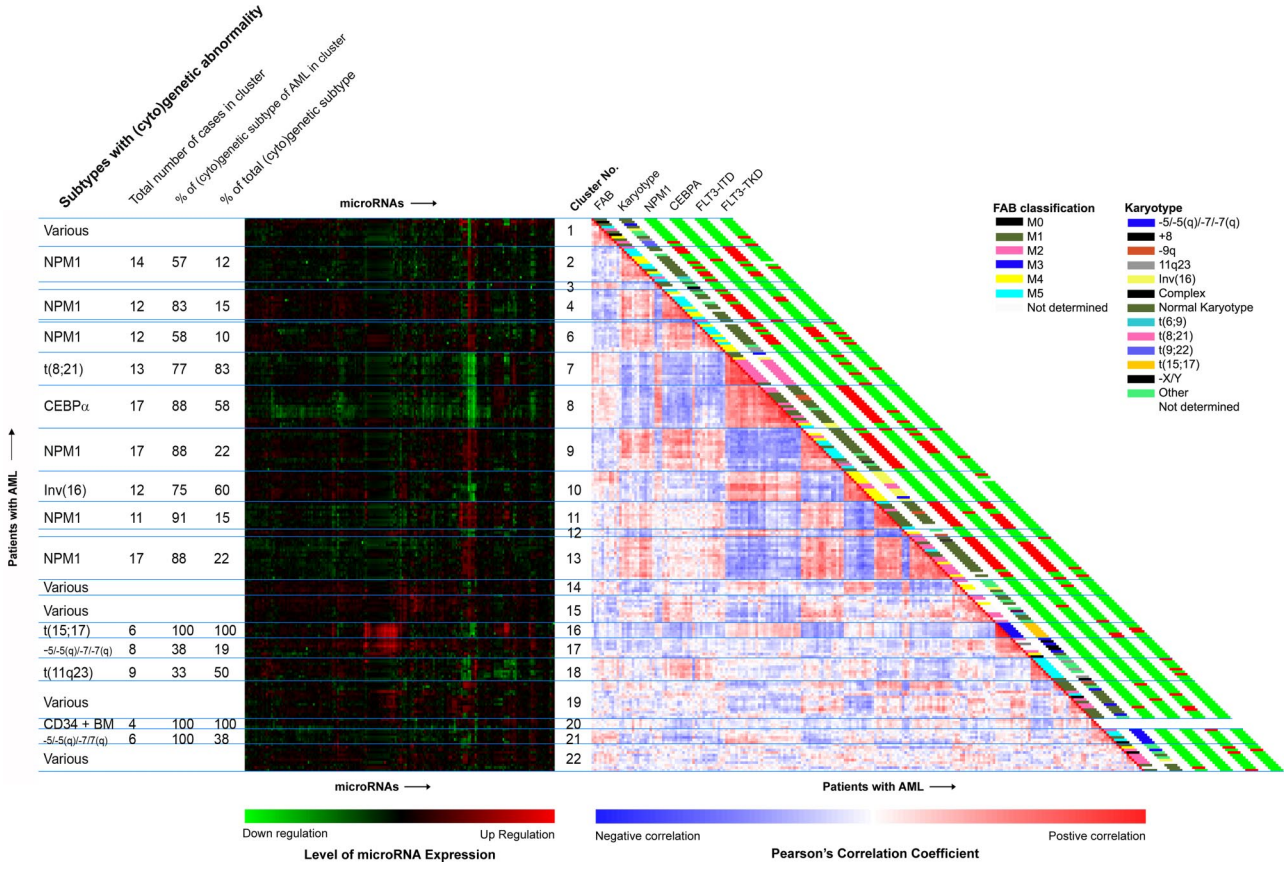

Figure 1. Correlation view based on unsupervised clustering of 215 AML specimens involving 178 differentially expressed mature human microRNAs (right section) and the relative expression levels of microRNAs that characterize each of the individual clusters (left section). In the Pearson correlation view, the red squares indicate a positive correlation and the blue squares a negative pairwise correlation between the microRNA expression signatures. Using the program Heatmapper,[40] cytologic, cytogenetic, and molecular data were plotted along the diagonal. Cytogenetic abnormalities and FAB classifications are indicated with different colors. The presence of molecular abnormalities, such as *NPM1, CEBPA, FLT3*-ITD, and *FLT3*-TKD mutations, is indicated in red and absence in green. On the left part of the figure, each individual microRNA expression cluster with a particular (cyto)genetic AML subtype is indicated (subtypes with [cyto]genetic abnormality). The total number of cases in each cluster and percentage of (cyto)genetic subtype of AML in cluster are presented in the columns. In addition, the percentages of these (cyto)genetic subtypes present within each microRNA expression cluster (thus also indicating relative proportion of cases with the abnormality that are located outside the cluster) is given (% of total [cyto]genetic subtype). The levels of expression of 178 microRNAs of each of the 22 clusters including normal bone marrow CD34$^+$ cells are presented in the heatmap on the left side of the correlation view. The scale bar indicates an increase (red) or decrease (green) in the level of expression by a factor of 7 relative to the geometric mean of all samples.

Significantly differentially expressed microRNAs in relation to cytogenetic translocations and molecular abnormalities

To identify the most significantly differentially expressed microRNAs that are associated with specific subtypes of AML, we performed a supervised analysis using SAM. MicroRNA expression for each of the cytogenetic (t(15;17), t(8;21), inv(16)) and molecular (*NPM1, CEBPA,* and *FLT3*-ITD mutations) subtypes was compared with the microRNA expression patterns of the remaining AMLs of the cohort. Several discriminating microRNAs were identified for each subtype. None of these microRNAs was localized in the chromosomal region specific for the corresponding cytogenetic translocation such as t(15;17), t(8;21), and inv(16). The detailed data on SAM are presented in the Table S2.

Class prediction of subgroups of AML with cytogenetic or molecular abnormalities by microRNA expression profiling, GEP (mRNA), or the combination of both methods

Using PAM we determined the minimal set of microRNAs and transcripts (mRNA) that could predict a particular (cyto)genetic subtype of AML in the cohort. Furthermore, we performed PAM by combining microRNA and mRNA expression data, to investigate whether microRNAs would improve the accuracy of the prediction. The number of correctly predicted (true positive) and number of false-positive cases in the 10-fold cross validation of the training set for individual subtypes as well as the numbers of microRNAs and transcript probe sets (mRNA) in the class predictors are presented in Table 3 and Table S3.

A class predictor of 10 microRNAs could predict AML with t(8;21) and a set of 7 microRNAs predicted AML with t(15;17). We constructed a predictor of 72 microRNAs for AML with inv(16). Class prediction of AML subgroups with cytogenetic abnormalities using mRNA-GEP was more accurate since there were no errors in the validation set and it required only 2 mRNA probe sets for t(8;21) and for inv(16) and 8 probe sets for t(15;17).

As regards AML with molecular aberrations, sets of microRNAs were derived that were characteristic for AML with *NPM1, CEBPA,* and *FLT3*-ITD mutations, respectively. Defined sets of 28 microRNAs could predict AML with *CEBPA* mutations, and 10 microRNAs were predictive of AML with *NPM1* mutations. A selection of as many as 48 microRNAs was required to predict AML with *FLT3*-ITD mutations. MicroRNA- and mRNA-GEP–based class predictors of AML molecular subtypes showed comparable accuracies. MicroRNA-10a and microRNA-10b were included in the mixed microRNA-mRNA expression predictor of AML with *NPM1* mutations (Table S3). In addition, 2 microRNAs (microRNA-155,

Table 3. microRNA and mRNA class predictors for subsets of AML with (cyto)genetic abnormalities identified using prediction analysis of microarrays (PAM)

	Training set, N = 143			Validation set, N = 72			Class predictor
(Cyto)genetic subset	No. of cases	True prediction	False positive	No. of cases	True prediction	False positive	No. microRNAs
microRNA class predictor							
t(15;17)	5	5	3	1	1	4	7
t(8;21)	8	7	8	4	3	3	10
Inv(16)	6	5	10	9	4	2	72
NPM1	47	46	21	21	20	6	10
CEBPA	17	2	3	5	4	1	28
FLT3-ITD	46	33	22	16	14	5	48
	No. of cases	**True prediction**	**False positive**	**No. of cases**	**True prediction**	**False positive**	**No. probe sets**
mRNA class predictor							
t(15;17)	5	4	0	1	1	1	8
t(8;21)	8	8	0	4	4	0	2
Inv(16)	6	6	0	9	9	1	2
NPM1	47	23	11	21	20	2	7
CEBPA	17	13	4	5	3	2	20
FLT3-ITD	46	31	7	16	13	2	36

PAM was performed to define microRNA and mRNA class predictors containing a minimal number of microRNAs and probe sets (mRNAs), respectively, that could predict whether a case belonged to a particular (cyto)genetic subset (first column). The cohort was randomly segregated into a training set and a validation set. The number of cases in the training set (column 2) and the validation set (column 5) are given. The 10-fold method of cross-validation, applied on the training set was used to compute truly predicted cases and errors. The cases that were truly predicted (column 3) or were false positive (column 4) in the training set are indicated. The minimal number of microRNAs or probe sets (mRNAs) that were identified in the training set was subsequently tested on the validation set. The number of cases correctly predicted (ie, true prediction) in the validation set (column 6) or were false positively predicted (column 7) are shown. The characteristics of the microRNAs and probe sets (mRNAs) are presented in Table S3.

microRNA-10b) were included in the combined microRNA-mRNA class predictor of AML with *FLT3*-ITD (Table S3). However, the combination did not improve the overall accuracy of prediction of AML with mutant *NPM1* or mutant *FLT3*-ITD compared with the mRNA predictor alone.

Discussion

Our study demonstrates that the heterogeneity of AML can be resolved according to their variable microRNA expression signatures. We used quantitative real-time RT-PCR to study the expression of 260 known human microRNAs.

The cohort of 215 AML cases represents a cross section of a mixed diversity of clinical AML. Others[43,44] and we[3] have previously established that gene expression profiling can comprehensively classify the disorder. We now demonstrate that the expression profiling of a limited set of known human small noncoding microRNAs can also be used to identify subgroups with distinctive microRNA expression patterns, which notably correlate with particular cytogenetic and molecular AML entities.

The unsupervised clustering method revealed an association between the majority of the AMLs with t(8;21), t(15;17), inv(16), and characteristic microRNAs expression profiles (clusters no. 7, no. 16, no. 10) (Figure 1; Table S1). However, not all AMLs with the latter cytogenetic abnormalities clustered together. For example, several cases of AML with inv(16) and t(8;21) expressed microRNA patterns that were discrepant from the more common clusters no. 10 and no. 7. Interestingly, 2 cases with t(8;21) clustered together with AMLs with inv(16) and 1 case with inv(16) was found in the cluster no. 7 containing mainly cases with t(8;21). Another study[44] had reported that some cases with inv(16) clustered together with cases with t(8;21) and vice versa using mRNA expression profiling. Whether these variations of microRNA and mRNA profiles among the latter cytogenetic subtypes are to be explained by the presence of other coexisting underlying genetic abnormalities in these not yet elucidated leukemias remains to be seen.

Using supervised analysis, we identified the microRNA signatures characteristic of AMLs with cytogenetic abnormalities: t(15;17), t(8;21), and inv(16). A prominent signature (microRNA-382, -134, -376a, -127, -299–5p, and -323), predominantly composed of up-regulated microRNAs, was identified for AML with t(15;17). Most of these microRNAs were found to be located on chromosome 14 and therefore appear not directly implicated in chromosomal translocation of AML with t(15;17) itself. The functions of the latter microRNAs in AML with t(15;17) are currently not known and the predicted targets are yet to be experimentally validated.

AML with t(8;21) was characterized mainly by a set of down-regulated microRNAs. We found 2 members of a known tumor suppressor microRNA family, let-7b and let-7c, to be down-regulated in AML with t(8;21) and also in AML with inv(16). Previous reports have linked let-7 microRNAs to human cancer[23,45-48] showing that they regulate the expression of oncogenes *RAS*[49] and *HMGA2*.[45,47] Reduced expression of the let-7 in lung cancer is associated with poor prognosis.[50] It is tempting to speculate that, similar to the gain of function of *RAS* due to gene mutations,[51-53] *RAS* overexpression due to down-regulation of let-7 may be a cooperating event in the pathogenesis of AML with inv(16). Combining our microRNA and mRNA expression data sets, we did not find a significant negative correlation between the expression levels of let-7 and its targets *RAS* and *HMGA*. However, microRNAs regulate gene expression mainly by translational inhibition and to a lesser extent by degradation or cleavage of mRNA targets. Therefore, despite a lack of a negative correlation of microRNA and mRNA expression levels, we cannot exclude the possibility of down-regulation of let-7 in AML with inv(16) and t(8;21) resulting in increased protein levels of RAS and thereby contributing to malignant transformation in CBF leukemias. SAM also showed microRNA-127 to be significantly down-regulated in AML with inv(16). MicroRNA-127 has been reported to function as a tumor suppressor and may be silenced by hypermethylation in cancer models. Epigenetic activation of microRNA-127 results in a down-regulation of the *BCL6* proto-oncogene.[54] Thus altogether it is conceivable that let-7 and microRNA-127 would

contribute to the pathobiology of core-binding factor leukemias with inv(16) and AML with t(8;21).

SAM also revealed differentially expressed microRNAs in certain molecular subgroups of AML. One prominent signature, predominantly containing up-regulated microRNA-10a, microRNA-10b, microRNA-196a, and microRNA-196b, was identified in AML carrying *NPM1* mutations. *NPM1* mutations are present in approximately 35% of patients with AML and are the most common molecular abnormality in patients with AML. They are especially prevalent in AML with a normal karyotype.[14] It has been demonstrated that *NPM1* mutations are associated with specific gene expression signatures.[35,55-57] These transcript signatures are characterized by overexpression of homeobox genes (*HOXA, HOXB* families and *TALE* genes). Interestingly, the microRNAs (-10a, -10b, -196a, -196b) that are up-regulated in AML with mutant *NPM1,* are all located within the genomic cluster of *HOX* genes. Yekta et al have reported that microRNA-196a directs translational inhibition (cleavage) of *HOXB8* mRNA.[58] These observations are consistent with an aberrant regulatory circuit including *NPM1, HOX* genes, and microRNAs, which might be engaged in the arrest of cellular differentiation of hematopoietic progenitors and development of AML with mutant *NPM1*. Obviously, functional studies are required to provide formal evidence about the pathogenetic role of these candidate microRNAs.

In AMLs with *FLT3*-ITD, we found oncogenic microRNA-155 to be significantly up-regulated. Apart from the oncogenic potential of microRNA-155 in lymphomas[27,59] and its role in immune function[60,61] this particular microRNA has been postulated to have a role in myeloid differentiation.[62]

AML with *CEBPA* mutations was associated with a distinct set of mainly down-regulated microRNAs. The possible cause of the phenomenon of down-regulation remains entirely elusive. One possibility to be considered is that differential regulation of microRNA processing (ie, expression levels of enzymes Drosha and Dicer) would result in a global down-regulation of microRNA expression in one subtype of AML and up-regulation in another. However, no differential expression of Drosha and Dicer was observed in AML patients with *CEBPA* mutation (data not shown), therefore other players may be implicated in this regulation.

Since microRNAs may have independent roles in the pathobiology of AML, not related to the underlying cytogenetic and molecular abnormalities, we performed a supervised analysis to identify differentially expressed microRNA between all AML samples and normal $CD34^+$ bone marrow samples. We found microRNA-21 to be consistently up-regulated in AML. In earlier studies, this microRNA has been demonstrated to be associated with various cancers. Several tumor suppressors have experimentally been validated as targets of microRNA-21.[63,64] Furthermore, microRNA-21 was found to contribute to cancer growth by modulating *PTEN* expression.[65] Conditional deletion of *PTEN* in adult hematopoietic cells produced myeloproliferative disease and transplantable leukemias.[66,67] Potential function of microRNA-21 in relation to *PTEN* and AML deserves further investigation.

The microRNA expression profiles were used for the class prediction of various subtypes of AML. We were able to predict AMLs with t(8;21), t(15;17), and inv(16) by limited sets of selected microRNAs. We also used PAM to compare the ability of class prediction by microRNA expression with that by transcript expression profiles in the same series of patients. Prediction of AML with t(8;21), t(15;17), and inv(16) using microRNAs required a greater number of microRNAs than mRNA probe sets. That the mRNA predictor required a smaller set of probes than the classifier constructed by microRNAs is not at all surprising. MicroRNA analysis made use of a restricted number of only 260 microRNAs, which is in sharp contrast to 54 675 probes that were included on the microarray used for mRNA profiling. As a matter of fact, the observed predictability of AML subtypes on the basis of a limited number of microRNAs demonstrates the power and potential of the approach of microRNA expression profiling for AML subtypes. The number of known human microRNAs continuously increases, and more than 1000 of them are predicted in silico. Therefore, in the future, new microRNAs with specific expression in the subsets of leukemia will likely enhance the possibilities for classification and class prediction of AML by microRNA expression.

To this end, the study presented here demonstrates that AMLs can be classified according to their variable and distinctive microRNA expression profiles. Application of SAM and PAM methods revealed significant differentially expressed microRNAs that characterize and categorize the subgroups of AML with different cytogenetic and molecular aberrations, in particular AML with t(8;21), inv(16), and t(15;17) and AML with *NPM1* and *CEBPA* mutations. In comparison with mRNAs, the microRNA class predictor was less accurate in predicting AML with t(15;17), t(8;21), and inv(16). Combinations of selected sets of microRNAs could predict AMLs with mutations in the genes of *NPM1, CEBPA,* and *FLT3*-ITD with similar accuracy as mRNA probe set combinations. Furthermore, several established oncogenic and tumor suppressor microRNAs, such as microRNA-155, microRNA-21, and let-7, appear to be associated with specific subtypes of AML. These data suggest that microRNAs are implicated in the pathogenesis of AML and may have a useful role in future diagnostics.

Acknowledgments

We thank Andre Uitterlinden (Department of Internal Medicine) for providing robot facility and Pascal Arp (Department of Internal Medicine) for his technical support in high-throughput experiments using a robot. Furthermore, we thank Berna Beverloo (Department of Clinical Genetics) for reviewing cytogenetic abnormalities and Mars van 't-Veer and HRC (Hematology Review Committee) for reviewing AML bone marrow clinical diagnostics. We also thank Bas Wouters for his support in gene expression data analysis and Ruud Delwel for helpful discussions.

This work was supported by a grant from Erasmus University Medical Center (M.J.-L.).

Authorship

Contribution: M.J.-L. designed the study, designed and supervised the experiments and data analysis, and wrote the paper; S.M.S. and M.K.D. performed experiments and data analysis; P.J.M.V. and B.L. codesigned the study, cowrote the paper, and established bio-bank and database of AML specimens containing all clinical, cytogenetic, and molecular data.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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