

Molecular signatures in acute myeloid leukemia

Krzysztof Mrózek^a, Michael D. Radmacher^{a,b}, Clara D. Bloomfield^a and Guido Marcucci^{a,c}

^aDivision of Hematology and Oncology, Department of Internal Medicine, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio, ^bThe Cancer and Leukemia Group B (CALGB) Statistical Center, Duke University Medical Center, Durham, North Carolina and ^cDivision of Human Cancer Genetics, Department of Microbiology, Virology, Immunology and Medical Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio, USA

Correspondence to Krzysztof Mrózek, Division of Hematology and Oncology, the Comprehensive Cancer Center, Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, Room 1248B, The Ohio State University, 300 West Tenth Avenue, Columbus, OH 43210-1228, USA
Tel: +1 614 293 3150; fax: +1 614 293 3575;
e-mail: krzysztof.mrozek@osumc.edu

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Purpose of review

Acute myeloid leukemia (AML) is characterized by a high degree of heterogeneity with respect to chromosome abnormalities, gene mutations and changes in expression of multiple genes and microRNAs. In this article, we review the results of recent studies of AML that used microarray-based genome-wide gene-expression and microRNA-expression profiling.

Recent findings

Genome-wide analyses of gene expression and microRNA expression have revealed AML signatures that are closely associated with some, but not all, cytogenetic and molecular genetic subsets, helped in identification of novel biologic subtypes and led to characterization of molecular pathways involved in leukemogenesis. For some AML categories, namely core-binding factor AML and/or cytogenetically normal AML, gene-expression and microRNA-expression profiling provided prognostic information additional to that obtained from cytogenetics and analyses of gene mutations and single gene expression changes.

Summary

Gene-expression and microRNA-expression profiling not only has the potential to enhance our understanding of the disease biology, but also appears to constitute an applicable approach for outcome prediction and identification of novel therapeutic targets.

Keywords

acute myeloid leukemia, gene-expression profiling, microRNA

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Introduction

Acute myeloid leukemia (AML) arises as a result of accumulation of acquired genetic alterations occurring in hematopoietic progenitor cells and altering their growth, differentiation and proliferation [1–4,5^{**},6,7,8^{**}]. The AML heterogeneity at both the cytogenetic and molecular genetic levels has been associated with pretreatment features and clinical outcome of AML patients. The cytogenetic and molecular genetic aberrations associated with AML are not mutually exclusive and often coexist in the leukemic cells and concurrently affect the expression of downstream target genes that encode proteins involved in complex biologic networks supporting leukemogenesis. It is anticipated that microarray genome-wide gene-expression profiling (GEP) and microRNA-expression profiling assays are well suited to reveal characteristic patterns (signatures) of activation or silencing or both of multiple genes or microRNAs that reflect the underlying biology of the disease subtypes, provide diagnostic and prognostic information, and potentially reveal novel molecular targets for therapeutic intervention. Recent reports have shown high interlaboratory reproducibility and standardization of

GEP of leukemia samples, thereby demonstrating the routine applicability of this approach for prospective studies of AML patients [9,10].

Usefulness of gene-expression profiling for the diagnosis of patients with acute myeloid leukemia

Several recurrent chromosome abnormalities and their molecular equivalents, such as t(8;21)(q22;q22)/*RUNX1-RUNX1T1*, inv(16)(p13;q22) or t(16;16)(p13;q22)/*CBFB-MYH11*, t(15;17)(q22;q12)/*PML-RARA*, t(9;11)(p22;q23)/*MLLT3-MLL* and other balanced abnormalities involving 11q23/*MLL*, t(6;9)(p23;q34)/*DEK-NUP214*, inv(3)(q21;q26.2) or t(3;3)(q21;q26.2)/*RPN1-EVII* and t(1;22)(p13;q13)/*RBM15-MKL1*, are now included in the 2008 World Health Organization (WHO) classification of de-novo AML, and together with morphology, immunophenotype and clinical features are being used to define different disease entities [11]. Moreover, de-novo AML with mutations in distinct genes such as *CEBPA* and *NPM1* have been included as provisional entities in the 2008 WHO classification.

The aforementioned recurrent chromosome aberrations and gene mutations are also recognized to be prognostically relevant in addition to other molecular alterations such as *FLT3* internal tandem duplication (*FLT3*-ITD) and *FLT3* tyrosine kinase domain (*FLT3*-TKD) and *KIT*, *WT1* and *MLL* mutations, and *CXCR4*, *ERG*, *BAALC* and *MNI* overexpression [2,12–18]. To date, in order to identify and prioritize all these alterations for diagnosis and risk-adapted stratification of AML patients, pretreatment samples are subjected to a variety of assays. Therefore, it is reasonable to raise the question of whether GEP as a single assay is readily applicable for diagnosis and outcome class prediction in AML and can substitute for the current multitest diagnostic and prognostic work-up.

GEP has been proven successful in distinguishing AML patients from those with acute lymphoblastic leukemia [19] and, among the AML patients, those with prognostically favorable inv(16)/t(16;16), t(8;21) and t(15;17) from other cytogenetic/molecular subsets. Patients with prognostically favorable karyotypes were accurately identified with 100% sensitivity and specificity in most recent studies [20–22,23[•]]. Notably, in a study by Verhaak *et al.* [23[•]], four of 37 patients with inv(16) and four of 25 patients with t(15;17) were correctly identified by GEP as confirmed by reverse transcriptase-PCR, but not routine cytogenetic analysis. However, the accuracy of prediction for other cytogenetic AML subsets, such as those with abnormalities involving band 11q23, abnormalities involving 3q, -5/5q-, -7/7q- or t(9;22), has been much lower [22,23[•],24]. Similarly, the prediction accuracy for specific molecular subsets of patients such as those harboring *FLT3*-ITD, *FLT3*-TKD and mutations in the *NRAS* and *KRAS* genes [23[•]] was quite disappointing. In another study, restricted to patients with cytogenetically normal AML (CN-AML) [25[•]], a 20-gene classifier signature for *FLT3*-ITD mutation status had only moderate classification accuracy, with 73% sensitivity and 85% specificity. Furthermore, although patients with *NPM1* mutations were recognized based on an *HOX* and *TAL1* gene-specific signature [26,27], this signature was prone to generate false-positive results. Patients with 11q23/*MLL* abnormalities, known to be associated with increased expression of *HOX* genes [28], were often misclassified, as were all patients with t(6;9) [23[•]]. In pediatric AML, however, it seems possible to distinguish patients with *NPM1* mutations from those with 11q23/*MLL* translocations based on different patterns of *HOX* gene expression [29].

Altogether, these results support the notion that although genome-wide gene-expression analyses can accurately predict AML with t(8;21), inv(16)/t(16;16) and t(15;17), they are less successful in predicting other cytogenetic and molecular genetic subsets of AML. Therefore, at present, the usefulness of GEP for AML diagnosis

appears limited, although large, multiinstitutional collaborative studies are planned to develop, refine and validate diagnostic GEP-based classifiers for less frequent cytogenetic or distinct molecular AML subtypes [30^{••}].

Gene-expression profiling to identify distinct prognostic subsets in specific acute myeloid leukemia subtypes

Microarray GEP has been applied to study specific AML categories to derive signatures that would identify subsets of patients with differing outcomes. Initial studies showed that gene-expression signatures predictive of outcome among AML patients have strong associations with other cytogenetic or molecular prognostic factors, thereby raising the question of whether GEP could provide predictive information in addition to that already offered by standard cytogenetics or single gene assays or both. More recently, GEP has been adapted to identify subsets of AML patients with diversified outcome within subgroups of patients with already specified cytogenetic and molecular alterations. One of these subgroups is core-binding factor (CBF) AML that includes patients with t(8;21) or inv(16)/t(16;16) [31,32]. Bullinger *et al.* [33] used unsupervised two-way hierarchical cluster analysis to stratify 93 CBF-AML patients into two groups with dissimilar gene-expression profiles and significantly different survival. Patients who clustered in the group with worse survival more often harbored inv(16) and had higher white blood counts (WBC) than the remaining patients. Although four patients found to carry the prognostically adverse *FLT3*-ITD were included in the group with worse outcome, in general patients did not seem to cluster based on known prognostic factors in CBF-AML such as *KIT* mutations or secondary trisomy 22 in inv(16) patients [34]. Patients with worse outcome showed overexpression of genes controlling cell proliferation and downregulation of those involved in apoptosis [33]. In another GEP study, Paschka *et al.* [35] dichotomized patients with inv(16)/t(16;16) as a sole chromosome abnormality and no mutations in the *KIT* gene, and, separately, t(8;21) patients without *KIT* mutations into subsets with significantly different event-free survival (EFS) based on two different gene-expression signatures. These results, however, require corroboration.

Another AML subset in which GEP has been relatively successful for prognostication is CN-AML, the largest subset of de-novo AML comprising 40–45% of patients [36]. The first study showing that GEP can divide CN-AML patients into prognostically relevant subgroups was from Bullinger *et al.* [24]. Subsequently, Radmacher *et al.* [37] validated the prognostic significance of the ‘Bullinger’ signature in a larger group of CN-AML patients dissimilarly treated, with a longer follow-up and using a different microarray platform. Despite these

differences, the signature was validated using a class prediction algorithm and supported the clinical applicability of this approach for AML prognostication. However, the prediction accuracy of the classifier was overall modest, with approximately 60% of the patients having their outcome predicted correctly [37].

A more recent GEP study [38] reported a signature capable of predicting outcome in CN-AML. The signature was developed using supervised principal component analysis from GEP data obtained from a training set of 163 intensively treated German CN-AML patients, and consisted of 86 probe sets representing 66 different genes that were significantly associated with survival. A prognostic score based on this signature was defined and then applied to an independent test set of 79 German CN-AML patients. Despite clinical and treatment differences, the prognostic score was shown to be an independent predictor for outcome in multivariable analyses of the test set after adjustments for age, *FLT3*-ITD/*FLT3*-wild-type allelic ratio and *NPM1* mutation status. Likewise, the prognostic score retained its predictive significance in a multivariable model for survival of a validation cohort of 64 younger (age <60 years) CN-AML patients enrolled on Cancer and Leukemia Group B protocols. These data demonstrated that the performance of the GEP-based predictor was robust in CN-AML patient populations even when they came from different continents (Europe and America) and differed with regard to pretreatment features and treatment received [38].

Interestingly, both the signatures developed by Metzeler *et al.* [38] and that validated by Radmacher *et al.* [37] strongly correlated with *FLT3*-ITD status. However, the fact that in multivariable analyses, the 'Metzeler' signature was found to be independently associated with outcome after adjusting for *FLT3*-ITD status suggests that additional prognostic information can be provided by the GEP. Indeed, this finding is consistent with a report by Bullinger *et al.* [25*] showing that a GEP-based predictor of *FLT3*-ITD status in CN-AML misclassified roughly only 20% of patients and outperformed *FLT3*-ITD status in its ability to predict outcome.

Gene-expression profiling to delineate biologic pathways

GEP has also been instrumental for the discovery of novel, biologic subgroups of AML. *CEBPA* encodes a transcription factor involved in myeloid differentiation, and mutations of this gene have been associated mostly with CN-AML and predict favorable outcome. Wouters *et al.* [39] identified a subset of AML patients who did not harbor *CEBPA* mutations, but were characterized by a

GEP signature resembling that of AML patients with these mutations. Further investigations revealed that in these cases, *CEBPA* was silenced epigenetically, mostly through promoter hypermethylation, and the leukemic blasts exhibited expression of genes associated with aberrantly activated Notch signaling and *NOTCH1* mutations.

In another recent study, Marcucci *et al.* [40] derived a gene-expression signature associated with *CEBPA* mutations in a high-risk molecular group of CN-AML, that is, in patients with wild-type *NPM1* genes with or without *FLT3*-ITD or those with *NPM1* mutation and *FLT3*-ITD. In addition to overexpression of *CD34*, *CD38* and *CD7*, a prominent characteristic of this signature was the upregulation of genes involved in erythroid differentiation (e.g. *GATA1*, *ZFPM1*, *HEMGN*, *EPOR*, *TFRC*), and genes encoding erythrocyte membrane proteins and hemoglobin chains. These findings were consistent with higher hemoglobin levels observed in patients with *CEBPA* mutations at diagnosis. In contrast, genes involved in myeloid differentiation (e.g. *RUNX1*, *SPI1*, *ID1*) were downregulated in patients with *CEBPA* mutations, as were several members of the homeobox family. Concurrent downregulation of *HOX* genes and upregulation of *CD34* and *CD38* and genes involved in erythroid lineage differentiation suggest that cells harboring *CEBPA* mutations represent a more functionally mature type of malignant blasts and, perhaps, this may explain their increased sensitivity to chemotherapy [40]. Furthermore, these findings were consistent with the reported association of *CEBPA* mutations with *NPM1* wild-type rather than *NPM1* mutations, which are usually accompanied by *HOX* gene overexpression.

MicroRNA-expression profiling to identify cytogenetic acute myeloid leukemia subtypes

Recent studies analyzed genome-wide expression of microRNAs for both biology discovery and clinical outcome prediction. MicroRNAs are naturally occurring, small RNAs, 19–25 nucleotides in length, cleaved from 70–100 nucleotide hairpin precursors, that hybridize to complementary mRNA targets and inhibit their translation or cause degradation [41]. Initially discovered as regulators of normal cell homeostasis, microRNAs have recently been shown to be frequently located at cancer-associated genomic regions [42] and to represent a new class of genes that play a role in malignant transformation [43,44].

MicroRNA-expression profiling has been shown to be able to readily distinguish between AML and acute lymphoblastic leukemia [45] or AML blasts from normal CD34-positive blasts [46]. Furthermore, microRNA-expression profiling studies have demonstrated that

microRNA signatures can also distinguish between cytogenetic subtypes of AML, with AML patients who carry t(8;21), inv(16) or t(15;17) having unique microRNA-expression signatures [7,47]. Similar to GEP studies, although there was not a perfect concordance between the signatures derived from various studies, some commonalities emerged, such as upregulation of several microRNAs transcribed from genes located in the imprinted region within the 14q32 band in acute promyelocytic leukemia with t(15;17) [7,47,48] and the downregulation of *miR-133a* in CBF-AML with t(8;21) [7,47]. Patterns of microRNA expression associated with trisomy 8, rearrangements of 11q23/*MLL*, and CN-AML have also been reported [6,48].

Abnormal microRNA-expression signatures have also been associated with distinct molecular aberrations in AML. Upregulation of *miR-155* in patients with *FLT3*-ITD has been independently reported by two research groups [6,47,49]. This observation is consistent with the reported high blast proliferation and decreased survival in *FLT3*-ITD-positive AML; in a mouse model, sustained expression of *miR-155* was shown to drive granulocyte/monocyte expansion and cause pathological features characteristic of myeloid neoplasia [50*].

Mutations of the *NPM1* gene have been associated with a characteristic microRNA-expression signature that includes the upregulation of *miR-10a*, *miR-10b* and *miR-196a* [49]. Interestingly, these microRNAs reside in the genomic cluster of *HOX* genes, whose upregulation is a prominent feature of gene-expression signatures in patients with *NPM1* mutations. Consistent with this observation, expression of *miR-10a*, *miR-10b* and *miR-196a-1* has been correlated with *HOX* gene expression in CN-AML by Debernardi *et al.* [51]. Interestingly, among the downregulated microRNAs in the *NPM1*-associated microRNA signature, *miR-204* and *miR-128* were found. Both of these microRNAs are predicted to target *HOX* genes, as confirmed by Garzon *et al.* [49] in cell line experiments showing that *miR-204* inhibits expression of *HOXA10* and *MEIS1*.

Upregulation of *miR-181a* and *miR-335* in AML patients with *CEBPA* mutations was observed in two studies [40,47], one of which was restricted to CN-AML [40]. In another study [52], *miR-124a* was demonstrated to target *CEBPA* mRNA *in vitro*, and it was shown to be epigenetically silenced in leukemic cell lines.

Finally, expression of *miR-181a* [51] and *miR-181b* [46] has been associated with French American British (FAB) marrow morphology of CN-AML patients, with higher expression of these microRNAs detected in FAB M1 and M2 compared with FAB M4 and M5 subtypes.

Associations of microRNA-expression profiles with clinical outcome

Recently, microRNA expression in AML has begun to be associated with clinical outcome. Overexpression of *miR-199a* and *miR-191* negatively affected survival and EFS across all cytogenetic subgroups in univariable analyses and retained its adverse prognostic influence in a multivariable model together with cytogenetics [6]. Dixon-McIver *et al.* [7] reported that expression of *miR-9* and *miR-let7b* was correlated with cytogenetic risk groups: the expression was low in the favorable and high in intermediate and adverse cytogenetic risk groups.

In CN-AML with high-risk molecular features (i.e. with *FLT3*-ITD, wild-type *NPM1*, or both), we have recently identified a microRNA-expression signature associated with EFS and validated its prognostic relevance in an independent patient set [8**]. The important characteristic of this signature was an inverse association between expression of *miR-181a* and *miR-181b* and risk of an event. In this study, the genome-wide microRNA-expression profile was also correlated with gene expression data in an attempt to identify microRNA-regulated genes that contribute to leukemogenesis in the high-risk CN-AML subset. Expression levels of 452 genes significantly correlated with the prognostic microRNA signature. Several genes involved in mechanisms of innate immunity, including genes encoding Toll-like receptors (*TLR2*, *TLR4*, *TLR8*), interleukin-1- β (*IL1B*) and its regulators (*CARD8*, *NLRC4*, *NOD2*, *PYCARD*, *CASP1*) [53**], were overly represented in the microRNA-dependent gene-expression signature. Proteins encoded by some of these genes have been reported to maintain growth and proliferation of AML blasts and may represent suitable therapeutic targets [54,55].

Conclusion

Recent genome-wide analyses of gene expression and microRNA expression have revealed signatures that are closely associated with some, but not all, cytogenetic and molecular genetic subsets of AML. For CBF-AML and CN-AML, GEP has provided prognostic information additional to that obtained from analyses of leukemia karyotypes, gene mutations and changes in expression of specific genes. However, further studies should prospectively validate many of the results reviewed in this article. Importantly, GEP and microRNA-expression profiling, in addition to or in combination with such other techniques as single nucleotide polymorphism arrays [56], array-based comparative genomic hybridization [57] and genome sequencing [58], are invaluable for discovery of the molecular pathways involved in leukemogenesis that merit further investigation as potential targets for development of novel, personalized therapeutic

approaches that would depend on the genetic make-up of the disease in individual patients.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 148).

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