Review Article

The Prognostic and Functional Role of MicroRNAs in Acute Myeloid Leukemia

Guido Marcucci,1* Krzysztof Mrózek,1* Michael D. Radmacher,1 Ramiro Garzon,1 and Clara D. Bloomfield1

1. Division of Hematology, Department of Internal Medicine, The Comprehensive Cancer Center, The Ohio State University, Columbus, OH

* G.M and K.M. contributed equally to this work.

Running head: MicroRNAs in AML

Correspondence to Guido Marcucci, MD, The Ohio State University, The Comprehensive Cancer Center, Biomedical Research Tower, 460 W. 12th Ave Avenue, Columbus, OH 43210, USA. Phone: 614-366-2261; fax: 614-293-7527; e-mail: guido.marcucci@osumc.edu; or Krzysztof Mrózek, MD, PhD, The Ohio State University, The Comprehensive Cancer Center, James Cancer Hospital and Solove Research Institute, Room 1232A, 300 West 10th Avenue, Columbus, OH 43210-1228, USA. Phone: 614-293-3150; fax: 614-366-1637; e-mail: krzysztof.mrozek@osumc.edu

This work is supported in part by National Cancer Institute, Bethesda, MD, grants CA101140, CA16058 and CA102031, Kimmel Cancer Research Foundation and the Coleman Leukemia Research Foundation, Harry T Mangurian Jr Foundation.

Abstract word count: 197; text word count: 5,009; references: 116.
ABSTRACT

Expression of microRNAs, a new class of non-coding RNAs that hybridize to target messenger RNA and regulate their translation into proteins, has been recently demonstrated to be altered in acute myeloid leukemia (AML). Distinctive patterns of increased expression and/or silencing of multiple microRNAs (microRNA signatures) have been associated with specific cytogenetic and molecular subsets of AML. Changes in the expression of several microRNAs altered in AML have been shown to have functional relevance in leukemogenesis, with some microRNAs acting as oncogenes and others as tumor suppressors. Both microRNA signatures and a single microRNA (ie, miR-181a) have been shown to supply prognostic information complementing that gained from cytogenetics, gene mutations and altered gene expression. Moreover, it has been demonstrated experimentally that antileukemic effects can be achieved by modulating microRNA expression by pharmacologic agents and/or increasing low endogenous levels of microRNAs with tumor suppressor function by synthetic microRNA oligonucleotides, or down-regulating high endogenous levels of leukemogenic microRNAs by antisense oligonucleotides (antagomirs). Therefore, it is reasonable to predict the development of novel microRNA-based therapeutic approaches in AML. We review herein results of current studies analyzing changes of microRNA expression in AML and discuss their potential biologic, diagnostic and prognostic relevance.
INTRODUCTION

MicroRNAs are naturally occurring 18- to 25-nucleotide RNAs that are cleaved from 70 to 100 nucleotide hairpin precursors by a complex protein system that includes the RNase III Drosha and Dicer, members of the Argonaute family.\(^1,2\) Mature microRNAs typically hybridize to the 3' untranslated regions (UTRs) of protein-coding messenger RNAs (mRNAs) and cause their posttranscriptional repression and/or degradation in proliferating cells,\(^1,2\) although emerging data indicate that microRNAs can also regulate gene expression through binding to "seedless" 3' UTR microRNA recognition elements\(^3\) or increase translation of target mRNAs upon cell cycle arrest.\(^4\) MicroRNAs, whose nomenclature is described by Griffiths-Jones et al,\(^5\) regulate normal cell homeostasis and are involved in many physiological processes including hematopoiesis.\(^6-8\) Recently, dysregulation of microRNAs has been shown in many types of solid tumors and leukemia.\(^9\) Direct involvement of microRNAs in cancer has been suggested by a study demonstrating that several microRNAs are localized in genomic regions associated with cancer, such as breakpoint regions in chromosome aberrations involving oncogenes or tumor suppressor genes, minimal regions of loss of heterozygosity, minimal regions of amplification, and at loci close to fragile sites and integration sites of the human papilloma virus.\(^10\) Supporting their role in carcinogenesis, microRNAs mapped to the chromosomal regions deleted in cancer samples were found expressed at low levels,\(^9\) and several functional studies confirmed the important role of microRNA deregulation in hematologic malignancies, including acute myeloid leukemia (AML).\(^9,11-19\)
Patterns of microRNA expression differ between normal cells and AML blasts

Profiling and functional experiments revealed that several microRNAs play a role in the regulation of hematopoiesis.\textsuperscript{6-8} Hence, it was not surprising that many microRNAs are differentially expressed between AML blasts and normal cells.\textsuperscript{20-23} However, microRNA signatures derived by comparing microRNA expression between normal and malignant cells have shared few similarities among different studies. This lack of uniformity could be explained by the use of normal CD34+ cells obtained under different conditions, such as after growth factor mobilization versus collected directly from the bone marrow (BM) with no mobilization. It is possible that the use of growth factors could change microRNA expression in CD34+ cells, since similar changes have been observed for mRNA expression.\textsuperscript{24} Additionally, some studies used unselected BM mononuclear cells from healthy donors instead of CD34+ cells as controls.\textsuperscript{20} The use of different platforms for microRNA profiling and differences in the frequency of cytogenetic and/or molecular genetic alterations, shown to affect microRNA expression (see below), within AML patient cohorts analyzed could also explain discordant results.\textsuperscript{20-23} Although a comparison of microRNA expression between normal CD34+ cells and leukemic stem cells instead of AML blasts could potentially be more informative, to our knowledge, no such study has yet been reported.

MicroRNA signatures distinguish AML from acute lymphoblastic leukemia (ALL)

Similar to a pioneering study, which demonstrated that gene-expression profiling (GEP) can distinguish between AML and ALL,\textsuperscript{25} changes in microRNA expression were also shown to define lineage-specific leukemia. Using a bead-based microRNA-expression profiling assay, Mi et al.\textsuperscript{20} separated patients with AML from those with ALL based on 27
differentially expressed microRNAs (21 up-regulated and six down-regulated in AML compared with ALL), four of which, the up-regulated let-7b and miR-223, and down-regulated miR-128a and miR-128b, were the most discriminatory.

**Correlations of microRNA expression with cytogenetics in AML**

Cytogenetically, AML is a very heterogeneous disease, with approximately 200 chromosome abnormalities identified as recurrent.\textsuperscript{26-28} While many of these aberrations are very rare, being detected in a few patients worldwide, others occur more frequently and have been associated with response to treatment and survival.\textsuperscript{29,30} Among the more frequent abnormalities, t(8;21)(q22;q22) and inv(16)(p13q22) [and its variant t(16;16)(p13;q22)], whose presence characterizes patients with core-binding factor AML (CBF-AML), and t(15;17)(q22;q12~q21) or its variants, a translocation pathognomonic for acute promyelocytic leukemia (APL), bestow a relatively favorable outcome. In contrast, patients with balanced translocations involving band 11q23 and the MLL gene [t(11q23)/MLL] other than t(9;11)(p22;q23), inv(3)(q21q26) or t(3;3)(q21;q26), t(6;9)(p23;q34), deletion or loss of 5q, monosomy 7, structural abnormalities of 17p or a complex karyotype [defined as ≥3 chromosome aberrations in the absence of t(15;17), t(8;21), inv(16) or t(16;16), t(9;11), t(11q23), t(6;9), and inv(3) or t(3;3)\textsuperscript{31}] have a very poor prognosis.\textsuperscript{26,27,29-34} Patients with other chromosome aberrations or those with cytogenetically normal AML (CN-AML) have better outcome, but not as good as patients with CBF-AML and APL, and are classified as having an intermediate cytogenetic risk.\textsuperscript{26,27,29-33,35}
Recent studies have shown using microRNA-expression profiling that several of the aforementioned cytogenetic subtypes of AML are associated with microRNA signatures. Each of the favorable cytogenetic-risk subtypes, namely CBF-AML with t(8;21), CBF-AML with inv(16)/t(16;16), and APL with t(15;17), has had a unique microRNA-expression profile that distinguished them from each other and from other AML subtypes.\textsuperscript{21,23,36,37} Dixon-McIver et al\textsuperscript{37} identified a characteristic signature in APL patients with t(15;17), which comprised seven up-regulated microRNAs transcribed from genes located in the imprinted domain at chromosome band 14q32 (\textit{miR-127}, \textit{miR-154}, \textit{miR-154*}, \textit{miR-299}, \textit{miR-323}, \textit{miR-368} and \textit{miR-370}), and nine down-regulated microRNAs (\textit{miR-17-3p}, \textit{miR-185}, \textit{miR-187}, \textit{miR-194}, \textit{miR-200a}, \textit{miR-200b}, \textit{miR-200c}, \textit{miR-330}, and \textit{miR-339}). In another study,\textsuperscript{21} a signature from a cluster that consisted of samples from t(15;17)-positive APL patients displayed a strong up-regulation of \textit{miR-127}, \textit{miR-134}, \textit{miR-299–5p}, \textit{miR-323}, \textit{miR-376a} and \textit{miR-382}. In a third study,\textsuperscript{36} three of the seven microRNAs identified as class-discriminatory, \textit{miR-224}, \textit{miR-368}, and \textit{miR-382}, were almost exclusively overexpressed in the t(15;17) samples, whereas \textit{miR-17-5p}, \textit{miR-20a}, \textit{miR-126} and \textit{miR-126*} were down-regulated.

In contrast, overexpression of \textit{miR-126} and \textit{miR-126*} occurred in CBF-AML samples, both those with t(8;21)\textsuperscript{21,23,36} and those with inv(16).\textsuperscript{36} A colony-forming and replating assay revealed cooperation between \textit{miR-126} and t(8;21)/\textit{RUNX1-RUNXT1(AML1/ETO)} in enhancing proliferation of mouse BM progenitor cells.\textsuperscript{36} Overall, similarly to GEP studies, total concordance has not been achieved among the microRNA signatures from separate studies. Nevertheless, the up-regulation of microRNAs transcribed from genes located at 14q32 in APL with t(15;17) and the down-
regulation of miR-133a in CBF-AML with t(8;21) were found in two or more studies (Table 1).

Distinct patterns of microRNA expression in AML with t(11q23)/MLL were reported. Garzon et al identified eight microRNAs up-regulated (miR-326, miR-219, miR-194, miR-301, miR-324, miR-339, miR-99b, miR-328) and 14 down-regulated (miR-34b, miR-15a, miR-29a, miR-29c, miR-372, miR-30a, miR-29b, miR-30e, miR-196a, let-7f, miR-102, miR-331, miR-299, miR-193) in patients with t(11q23)/MLL versus all other AML patients. Many of the microRNAs down-regulated in t(11q23)/MLL-positive patients target critical oncogenes, eg, miR-34b targets CDK4 and CCNE2, miR-15a → BCL2, the let-7 family → RAS, the miR-29 family → DNMT3A, DNMT3B, MCL1 and TCL1, miR-372 → LATS2. In another study, AML with t(11q23)/MLL was characterized by significant overexpression of miR-196b and seven microRNAs from a unique polycistronic microRNA cluster miR-17-92: miR-17–5p, miR-17–3p, miR-18a, miR-19a, miR-19b, miR-20a and miR-92. A follow-up report demonstrated that aberrant overexpression of the miR-17-92 cluster was caused by its up-regulation by the MLL gene fusions via their direct binding to the promoter region of C13orf25, the miR-17-92 host gene, and the resulting chromatin modification. Additionally, the miR-17-92 cluster was up-regulated as a result of DNA amplification of the 13q31 locus, where the cluster is located. Mi et al identified 363 potential target genes of miR-17-92 whose expression were inversely correlated with the microRNAs' expression and, using gene ontology, found that these genes were significantly enriched in cell differentiation (myeloid and B-cell differentiation), cell cycle, hematopoiesis, and cell death and apoptosis, suggesting that miR-17-92 contributes to leukemogenesis by down-
regulating target genes promoting cell differentiation and apoptosis and those inhibiting cell proliferation. Recently, *CDKN1A* (*p21*) was identified as a direct target through which *miR-17-92* polycistron modulates cell cycle and differentiation status, and self-renewal of t(11q23)/*MLL* leukemia stem cells.\(^{39}\)

The t(11q23)/*MLL*-rearranged AML category is very heterogeneous cytogenetically, with over 50 different translocation partners of 11q23/*MLL* characterized at the molecular level to date.\(^{40}\) Preliminary data show that this heterogeneity also characterizes microRNA expression. When patients with t(6;11)(q27;q23) were compared with those with t(9;11)(p22;q23), sixteen microRNAs were up-regulated in patients with t(6;11), including the antiapoptotic *miR-21*, which targets the tumor suppressor *PTEN*, and *miR-26a* and *miR-26b*, which target the *TGFB1* regulator *SMAD1*.\(^{41,42}\) Studies on other specific t(11q23)/*MLL* rearrangements are needed to further characterize their microRNA patterns.

Garzon et al\(^{22}\) identified a signature comprised of 42 up-regulated and no down-regulated microRNAs in patient samples with isolated trisomy 8 (+8), which were compared with AML patients with other karyotypes that did not contain secondary +8. Among the up-regulated microRNAs, *miR-124a* and *miR-30d* are located at 8p21 and 8q23, respectively, suggesting that a gene dosage effect may play a role in their up-regulation. Interestingly, *miR-124a* targets the myeloid transcription factor C/EBP\(\alpha\).\(^{43}\) In contrast, Dixon-Mclver et al\(^{37}\) did not find correlation between +8 and microRNA expression, but they studied samples with an additional +8 rather than those with +8 as a sole cytogenetic abnormality.
Finally, a signature composed of 10 up-regulated (miR-10a, miR-10b, miR-26a, miR-30c, let-7a-2, miR-16–2, miR-21, miR-181b, miR-368, and miR-192) and 13 down-regulated microRNAs (miR-126, miR-203, miR-200c, miR-182, miR-204, miR-196b, miR-193, miR-191, miR-199a, miR-194, miR-183, miR-299, and miR-145) was identified in CN-AML. In the same subset of patients, miR-10a, miR-10b, and miR-196a-1 were correlated with expression of homeobox (HOX) genes. This is consistent with a high incidence of NPM1 mutations in CN-AML, between 46% and 62%, and the reported NPM1 mutation-associated gene-expression signature known to encompass HOX gene overexpression. However, as discussed in the next section, CN-AML consists of several molecular subsets characterized by the presence of recurrent gene mutations and expression changes. Hence, instead of a single microRNA-expression signature, CN-AML is rather associated with several microRNA-expression signatures denoting particular gene alterations occurring in this cytogenetic category.

Although microRNA-profiling appears to differentiate among distinct cytogenetic groups, the specific signatures differ among studies (Table 1). This is likely due to the lack of standardization of the analytic methods used by different groups, and currently precludes using microRNA-expression profiles as a diagnostic criterion. Nevertheless, microRNA profiling might become a diagnostic tool because the stability of microRNAs over time is better than that of longer, coding mRNAs used in GEP analyses, and the diagnostic accuracy of microRNA profiling might also be better. For example, using a combination of any two of a set of four microRNAs, it was possible to discriminate ALL from AML cases with an overall diagnostic accuracy of 97-98%. This was dramatically
different from the results obtained with GEP, where a much larger number of probes were necessary to classify ALL and AML patients correctly.25

**Correlations of microRNA expression with molecular markers in CN-AML**

Although most AML patients harbor at least one chromosome aberration at diagnosis, 40-49% of AML patients have a normal karyotype, and thus constitute the largest single cytogenetic subset of AML.29 During last 15 years, CN-AML has been found to be very heterogeneous molecularly, with several gene mutations and gene expression alterations demonstrated to affect clinical outcome of CN-AML patients.44,46 Molecular alterations shown repeatedly to confer adverse prognosis include an internal tandem duplication of the **FLT3** gene (**FLT3-ITD)**,46-50 partial tandem duplication of the **MLL** gene (**MLL-PTD**),46,51 mutations of the **WT1**,52-54 **IDH1** and **IDH2** genes,55,56 and high expression of the **BAALC**,57 **ERG**58 and **MN1**59,60 genes, whereas favorable prognosis is bestowed by mutations in the **NPM1**46,61-63 and **CEBPA** genes46,64; in the case of the latter especially biallelic mutations.65,66 Moreover, combinations of markers have been found useful for outcome prediction of CN-AML patients. For example, patients with **NPM1** mutations but no **FLT3-ITD** have a significantly better outcome than patients who harbor **FLT3-ITD** (with or without **NPM1** mutations) or have **FLT3** and **NPM1** wild-type alleles.61

Recently, several of the aforementioned recurrent molecular rearrangements in CN-AML have been correlated with microRNA-expression signatures (Table 1).
Mutations of the *NPM1* gene have been linked to a distinctive microRNA-expression signature that includes the up-regulation of *miR-10a*, *miR-10b*, and *miR-196a*, all of which reside in the genomic cluster of *HOX* genes. Notably, *HOX* gene family up-regulation is a prominent characteristic of gene-expression signatures identified in patients with *NPM1* mutations. Additionally, Garzon et al. reported down-regulation of *miR-204* and *miR-128a* in AML with *NPM1* mutations, and showed in cell line experiments that *miR-204* inhibited expression of *MEIS1* and *HOXA10*, members of the *HOX* gene cluster. Recently, Becker et al. reported a microRNA-expression signature, comprising 68 microRNAs, associated with *NPM1* mutations in patients aged ≥60 years with de novo AML. Thirty-four microRNA-probes were up-regulated and 34 down-regulated in *NPM1* mutated patients. Similar to previous studies, the prominent feature of the signature associated with *NPM1* mutations was up-regulation of microRNAs embedded in the *HOX* cluster, *miR-10a*, *miR-10b*, *miR-196a* and *miR-196b*. Down-regulated were *miR-126*, whose expression was recently positively correlated with *MN1* expression in younger CN-AML patients, and *miR-130a* and *miR-451* that are involved in, respectively, megakaryocytic differentiation and erythropoiesis. Importantly, *NPM1* mutation status could be predicted with high accuracy based on microRNA-expression profiles. In leave-one-out cross-validated analysis, the mutation status of 92.5% of patients was correctly predicted (sensitivity=96.2%, specificity=87.5%).

Two groups reported independently that *miR-155* was up-regulated in AML patients with *FLT3-ITD*, suggesting that *miR-155* contributes to the highly proliferative phenotype of this molecular subset of AML. This hypothesis is supported by data from animal models showing that forced *miR-155* expression can drive granulocyte/monocyte
expansion resulting in myeloid proliferation with dysplastic changes.\textsuperscript{68} MiR-155 was also the most overexpressed microRNA in the first FLT3-ITD-associated microRNA-expression signature derived in primary CN-AML patients aged \( \geq 60 \) years that comprised 32 differentially expressed probes.\textsuperscript{50} Also overexpressed in this signature was \textit{miR-125b-2*}, and underexpressed were \textit{miR-144} and \textit{miR-451}, normally upregulated during erythropoiesis,\textsuperscript{69} and \textit{miR-488} and \textit{miR-486-5p}; the latter is underexpressed in multiple cancers.\textsuperscript{70}

\textit{CEBPA} mutations have been associated with an up-regulation of \textit{miR-181a} and \textit{miR-335} in two AML studies,\textsuperscript{21,64} one of which was performed exclusively on samples from CN-AML patients.\textsuperscript{64} Notably, in this study,\textsuperscript{64} \textit{CEBPA} mutations were associated with up-regulation of several genes involved in erythroid differentiation, in agreement with the results by Choong et al\textsuperscript{71} reporting an increase of \textit{miR-181a} and \textit{miR-181b} levels during erythroid differentiation. Consequently, high expression of the \textit{miR-181} family members seems to contribute to the partial erythroid differentiation of leukemic blasts harboring \textit{CEBPA} mutations.\textsuperscript{64} Down-regulated in \textit{CEBPA}-mutated patients were \textit{miR-194} and \textit{miR-34a}.\textsuperscript{64} \textit{MiR-34a} was recently shown to be a target of C/EBP\( \alpha \) during granulopoiesis, and its low expression in leukemic blasts with \textit{CEBPA} mutations was demonstrated to elevate levels of E2F3 and its transcriptional target, E2F1, that in turn increased proliferation and inhibited granulocytic differentiation of the blasts.\textsuperscript{72} Forced expression of \textit{miR-34a} resulted in granulocytic differentiation of AML blasts carrying \textit{CEBPA} mutations suggesting that increasing \textit{miR-34a} levels might become useful therapeutically,\textsuperscript{72} as could manipulation of expression of \textit{miR-223}, which is part of a molecular network regulating granulopoiesis that also includes E2F1 and C/EBP\( \alpha \).\textsuperscript{73}
Finally, Hackanson et al\textsuperscript{43} showed that CEBPA mRNA is targeted by \textit{miR-124a in vitro}, and that \textit{miR-124a} is epigenetically silenced in leukemia cell lines. Epigenetic treatment with the DNA-demethylating agent 5-aza-2’deoxyctydine caused up-regulation of \textit{miR-124a}.\textsuperscript{43}

Our group has recently demonstrated the existence of a striking inverse correlation between \textit{BAALC} expression and expression of \textit{miR-148a} in younger CN-AML patients.\textsuperscript{57} This suggests that \textit{miR-148a} might act as a negative regulator of \textit{BAALC} expression, and that low or absent \textit{miR-148a} expression may increase expression of \textit{BAALC} and the resulting aggressive phenotype.\textsuperscript{57} In patients aged ≥60 years, we derived a microRNA-expression signature comprising 18 microRNAs, 10 up-regulated and eight down-regulated in low \textit{BAALC}-expressing patients.\textsuperscript{74} Consistent with higher expression of \textit{HOX} genes in low \textit{BAALC}-expressers, microRNAs embedded in the \textit{HOX} cluster, \textit{miR-10a, miR-10b} and \textit{miR-9}, were up-regulated in low \textit{BAALC} expressers, whereas underexpressed were \textit{miR-126}, positively correlated with \textit{MN1} expression (see below), and \textit{miR-222}, linked with hematological lineage differentiation and known to target \textit{KIT}.\textsuperscript{74}

We have also reported an \textit{MN1}-associated microRNA-expression signature comprising 15 microRNAs (Table 1).\textsuperscript{60} Of those, expression of eight microRNAs was positively and expression of seven negatively correlated with \textit{MN1} expression. Five of the eight microRNA probes positively associated with \textit{MN1} expression were members of the \textit{miR-126} family (including \textit{miR-126} and \textit{miR-126\textsuperscript{*}}). This microRNA family was recently reported to enhance the proangiogenic activity of vascular endothelial growth factor and
regulate new blood vessel formation.\textsuperscript{75} We also noted up-regulation of \textit{miR-424}, a regulator of monocyte and macrophage differentiation.\textsuperscript{76} Among the microRNAs negatively correlated with \textit{MN1}, we found microRNAs involved in apoptosis (\textit{miR-16}),\textsuperscript{77} malignant transformation (\textit{miR-19a} and \textit{miR-20a}, members of the \textit{miR-17-92} polycistron)\textsuperscript{11} or regulation of \textit{ERG} expression (\textit{miR-196a}).\textsuperscript{78}

Schwind et al\textsuperscript{74} reported recently the first microRNA-expression signature associated with \textit{ERG} expression. The signature consisted of 11 microRNAs, five up-regulated and six down-regulated in low \textit{ERG} expressers. Among the former was \textit{miR-107}, targeting \textit{NFIX}, a gene involved in a regulatory feedback loop involving \textit{miR-223} and \textit{CEBPA} during granulocytic differentiation,\textsuperscript{79} \textit{miR-148a}, and \textit{miR-208}, predicted \textit{in silico} to target \textit{ERG} itself. Down-regulated was \textit{miR-302d}, a microRNA associated with early developmental stages and “stemness”.\textsuperscript{74}

To our knowledge, only one study has derived a microRNA-expression signature associated with R172 mutation in \textit{IDH2}.\textsuperscript{55} Among the most up-regulated microRNAs in patients with R172 \textit{IDH2} mutations were \textit{miR-1} and \textit{miR-133}, hitherto not associated with human cancer but involved in embryonal stem-cell differentiation,\textsuperscript{80} and \textit{miR-125a-5p} and \textit{miR-125b}, which target \textit{TP53}. \textit{MiR-125b-1} is strongly up-regulated in AML with t(2;11)(p21;q23) and inhibits myeloid differentiation,\textsuperscript{81} whereas \textit{miR-125b-2} cooperates with \textit{GATA1} mutations during leukemogenesis of Down syndrome-associated acute megakaryoblastic leukemia.\textsuperscript{82} Moreover, overexpression of both \textit{miR-125b-1} and \textit{miR-125b-2} causes a dose-dependent myeloproliferative disorder progressing to a lethal myeloid leukemia in mice.\textsuperscript{83} None of the most down-regulated microRNAs in R172
IDH2-mutated patients, miR-194-1, miR-526, miR-520a-3p, or miR-548b, have yet been associated with AML, although related miR-520h promotes hematopoietic stem-cell differentiation. Importantly, the microRNA-expression signature appeared to predict the R172 IDH2 mutational status with high accuracy, suggesting that patients with R172 IDH2 mutations differ biologically from patients with wild-type IDH2.

Correlations of microRNA expression with marrow morphology in CN-AML

Debernardi et al were first to demonstrate that miR-181a was more highly expressed in leukemic blasts with FAB M1 and M2 marrow morphology compared with FAB M4 and M5. A subsequent study confirmed these results with regard to both miR-181a and miR-181b. Another study, comparing the microRNA expression profiles of FAB M1 and FAB M5 subtypes, found the higher expression of not only miR-181a and miR-181b but also miR-181a*, miR-181d, miR130a, miR-135b, miR-146a, miR-146b and miR-663 in FAB M1. In FAB M5 samples, miR-21, miR-193a and miR-370 were overexpressed. Lutherborrow et al demonstrated significant down-regulation of miR-181a, miR-181b, miR-181d, miR130a, miR-135b and miR-146a during induced monocytic differentiation of AML cell lines, suggesting direct involvement of these microRNAs in monocytic differentiation. They also identified key myeloid factors such as MAFB, IRF8 and KLF4 as targets of these microRNAs.

Correlations of microRNA expression with clinical outcome in AML

Recent studies have shown that changes in microRNA expression can influence clinical outcome of AML patients. Dixon-McIver et al correlated microRNA expression with cytogenetic-risk groups: patients with favorable cytogenetic findings [ie, t(8;21), inv(16)
and t(15;17)] had low expression of miR-let7b and miR-9 whereas high expression of these microRNAs was detected in samples from patients classified in the adverse or intermediate cytogenetic-risk groups.

Garzon et al\textsuperscript{22} reported that in a cytogenetically heterogeneous cohort of 122 newly diagnosed AML patients with predominantly intermediate- and poor-risk cytogenetics, overexpression of miR-20a, miR-25, miR-191, miR-199a and miR-199b, assessed using microRNA microarrays, adversely affected overall survival. Validation of these results in a separate cohort of AML patients using a different profiling method (real-time RT-PCR) confirmed that high expression levels of miR-191 and miR-199a conferred inferior event-free and overall survival. In multivariable analyses, the two microRNAs remained independently predictive of both survival endpoints after adjustment for cytogenetics.\textsuperscript{22}

A recent Cancer and Leukemia Group B (CALGB) study identified a microRNA-expression signature with prognostic significance in CN-AML patients with high-risk molecular features, that is FLT3-ITD, wild-type NPM1 alleles, or both.\textsuperscript{87} The signature, comprising 12 microRNA probes derived from a training set of patients treated on CALGB protocol 19808, was associated with event-free survival. It was afterwards tested in a validation set of similarly treated patients enrolled onto CALGB protocol 9621. Increased expression of five probes representing miR-181a and miR-181b was associated with decreased risk of an event [failure to achieve complete remission (CR), relapse or death], whereas higher expression of miR-124, miR-128-1, miR-194, miR-219-5p, miR-220a and miR-320 associated with an increased risk.
In addition, the genome-wide microRNA-expression profile was integrated with a gene-expression signature to identify genes regulated by microRNAs whose altered expression contributed to leukemogenesis in CN-AML with molecular high-risk features. Expression of 452 genes significantly correlated with the prognostic microRNA-expression signature and included genes encoding proteins involved in innate immunity such as intracellular sensors of microbial components and cell injury that are part of the “inflammasome” regulating activation of caspase-1 and interleukin-1β. Among these genes, TLR4, CARD12, CASP1 and IL1B were predicted targets of members of the miR-181 family, and their expression was inversely correlated with the expression levels of miR-181 family members. These data support a functional relationship between microRNA and gene expression, and suggest that down-regulation of members of the miR-181 family may contribute to the aggressive leukemia phenotype. Conversely, high expression of miR-181 family members may reduce the aggressiveness of the disease. The latter was confirmed by a subsequent study showing that up-regulated miR-181a and miR-181b were part of a microRNA-expression signature associated with prognostically favorable CEBPA mutations.

The aforementioned data suggest that measurements of microRNA expression may be useful for risk-assessment in CN-AML. However, microRNA expression has been determined in these studies by genome-wide microarray profiling, which is relatively difficult to apply for prospective assessment of the molecular risk in individual patients. Therefore, Schwind et al investigated whether expression of a single microRNA, miR-181a, could provide independent prognostic information. Indeed, higher expression of miR-181a was found to be associated with a higher CR rate, longer overall survival and
a trend for longer disease-free survival in all CN-AML patients studied, and with higher CR rates, and longer overall and disease-free survival in a subset with high-risk molecular features. Importantly, in both patient cohorts, higher miR-181a expression was significantly associated with better outcome in multivariable analyses. These data suggest that miR-181a expression may refine molecular risk classification of CN-AML and be used for molecular risk assessment in individual patients. Moreover, novel therapeutic options might be developed, such as delivery of miR-181a directly to the leukemia cells or the use of agents capable of increasing endogenous levels of miR-181a, which might benefit patients with prognostically adverse low miR-181a expression.89

**Functional aspects of microRNA expression in myeloid leukemogenesis**

Over the past three years, several studies have defined patterns of microRNA expression associated with cytogenetics, molecular subgroups and clinical outcome in AML. Current efforts are now concentrated on performing biological validation of these findings. Many questions remain unanswered. Do microRNAs contribute to myeloid leukemogenesis? If they do, what are the mechanisms? Below, we discuss the currently most relevant microRNAs involved in myeloid leukemogenesis (Table 2).

**miR-155**

Human miR-155, mapped to 21q21.3, resides in the non-coding BIC transcript and its expression is elevated in many solid tumors, lymphomas and acute leukemias.6,9,21,22,90 In AML, miR-155 expression is higher in patients carrying FLT3-ITD mutations.21,22,67 However, blocking FLT3 phosphorylation activity with a FLT3 inhibitor or overexpressing
FLT3-ITD in mouse myeloid precursors cells did not change miR-155 expression, suggesting that miR-155 expression is independent from FLT3-ITD signaling.\textsuperscript{67} Functionally, overexpression of miR-155 in human CD34+ stem cells results in myeloid proliferation with blockage of erythroid and megakaryocytic colony formation.\textsuperscript{91} Moreover, enforced up-regulation of miR-155 in normal murine hematopoietic stem cells induced myeloproliferation, although overt AML was not observed, indicating that transformation to an overt AML requires cooperation of additional genetic alterations.\textsuperscript{92} In contrast, the ectopic up-regulation of miR-155 in mouse lymphocyte precursors induced not only polyclonal lymphocytosis but was also sufficient to induce high-grade leukemia/lymphoma.\textsuperscript{93} However, the mechanistic basis for miR-155 biological effects on the myeloid and lymphoid lineages remains largely unresolved. Recently, two independent reports identified Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1) as a direct target of miR-155.\textsuperscript{92,93} SHIP1 is a negative regulator of cell signaling in the immune system.\textsuperscript{94} This phosphatase is also implicated in B-cell maturation because it has a differential expression in the pro-B compared with the pre-B stage.\textsuperscript{94} Mice with global SHIP1 deficiency develop a myeloproliferative disease characterized by increased granulocyte-monocyte populations and decreased B-lymphocyte numbers.\textsuperscript{95} Thus, SHIP1-deficient mice resemble the phenotype of miR-155 transgenic mice. Interestingly, SHIP1 is mutated in a small subset of AML patients, in whom loss of function of SHIP1 has been implicated in the oncogenic process.\textsuperscript{96} In addition to SHIP1, miR-155 targets CEBPB,\textsuperscript{93} encoding a protein with a critical function in granulopoiesis,\textsuperscript{97} suggesting that leukemogenic function of miR-155 results in deregulation of SHIP1 and CEBPB (Table 2).\textsuperscript{92,93}
**miR-196b**

The *miR-196b*, located between HOXA9 and HOXA10 genes at 7p15, has been found up-regulated in AML patients with t(11q23)/MLL. Popovic et al\(^9\) demonstrated that *miR-196b* overexpression in BM progenitor cells resulted in a partial block of their differentiation and increased proliferative capacity and survival, and that expression of *miR-196b* was induced by leukemogenic MLL fusion proteins. Importantly, treatment of BM cells transformed with the *MLL-AF9* fusion gene with the antagomir targeting *miR-196b* decreased proliferative capacity of the cells (Table 2).\(^9\) This suggests that *miR-196b* might become a novel therapeutic target in AML with t(11q23)/MLL.

**miR-223**

*miR-223*, located at Xq12, is expressed at low levels in hematopoietic stem cells but its expression increases dramatically during granulocytic differentiation.\(^6\) Hence, it was suggested that *miR-223* induces granulocytic differentiation. To answer this question, Johnnidis et al\(^9\) engineered a *miR-223* knockout mouse and found, rather surprisingly, that these mice exhibit granulocytosis and a hyperinflammatory state. Further experiments demonstrated that *miR-223* exhibits its antiproliferative effects in granulopoiesis by targeting the transcription factor *MEF2C*. A recent study reported that *MEF2C* mRNA levels are up-regulated in highly proliferative leukemic granulocyte–monocyte progenitors and that *MEF2C* ectopic expression enhanced their proliferation.\(^10\) Thus, *miR-223* acts normally as a negative regulator of granulocytic proliferation (Table 2) as part of an autoregulatory negative feedback loop also involving C/EBPα and E2F1.\(^7\) It has been reported that AML patients with t(8;21) exhibit lower *miR-223* levels than other AMLs.\(^10\) Furthermore, *miR-223* was shown to be a direct
transcriptional target of RUNX1/RUNX1T1 through the recruitment of chromatin remodeling enzymes. Treatment of AML blasts with hypomethylating agents, RUNX1/RUNX1T1 inhibitors or miR-223 oligonucleotides enhanced miR-223 levels and restored cell differentiation.101

miR-29

The miR-29 family comprises three isoforms arranged in two clusters: miR-29b-1/miR-29a located at 7q32 and miR-29b-2/miR-29c located at 1q23. Interestingly, band 7q32 is frequently deleted in AML and myelodysplastic syndromes (MDS) with del(7q).26 MiR-29 family members are down-regulated in high-risk chronic lymphocytic leukemia, lung cancer, invasive breast cancer, and cholangiocarcinoma.102-105 We and others reported down-regulation of miR-29 family members in primary AML samples with t(11q23)/MLL22,106 and those with del(7q),22,106,107 whereas miR-29a expression was increased in AML samples from (mostly) FLT3-ITD-negative CN-AML patients.108 Moreover, lower levels of miR-29 were detected in CN-AML samples with wild-type NPM1 compared with CN-AML with NPM1 mutations.67 Recently, ectopic overexpression of miR-29b in AML cell lines and primary AML blasts was shown to induce apoptosis and inhibit blast proliferation.106 Furthermore, direct inoculation of synthetic miR-29b oligonucleotides into xenograft tumors dramatically decreased tumor growth (Table 2). By analyzing mRNA expression changes in AML cell lines after miR-29a and miR-29b overexpression, several targets were identified, including oncogenes MCL1, CDK6, IGFR and JAK2. Notably, anti-apoptotic genes, including MCL1, were enriched among the mRNAs inversely correlated with miR-29 expression (Table 2).106 Our group also reported that miR-29b modulates DNA methylation by targeting directly
DNMT3a and DNMT3b and indirectly DNMT1 through SP1. Blum et al observed that those AML patients aged ≥60 years treated with single-agent decitabine, a DNMT inhibitor, who responded to treatment had higher pretreatment levels of miR-29b. Moreover, a recent study showed that miR-29b is involved in a protein-microRNA network, which includes SP1, NFκB, and HDAC, whose deregulation results in KIT overexpression in AML and is associated with adverse clinical outcome. Treatment with ectopic miR-29b or compounds capable of increasing levels of endogenous miR-29b (eg, bortezomib, mithramycin A or bay11-7082) resulted in KIT down-regulation.

These data support a tumor suppressor function of miR-29b and provide a rationale for the use of synthetic miR-29b oligonucleotides or agents increasing miR-29b expression as novel therapeutic options in AML.

**Future directions**

The development and increasing use of high-throughput genome-scale technologies is changing the way we approach the study of leukemia. By performing array-based comparative genomic hybridization, single nucleotide polymorphism array analyses, mRNA- and microRNA-expression profiling studies, and whole genome sequencing, it is possible to attain a comprehensive, multidimensional view of leukemia. Knowledge gained from these approaches is critical to better understanding of leukemogenesis and development of novel therapies. Two principal challenges to this approach exist. The first concerns technical problems inherent in the platforms used to analyze biological samples, such as reproducibility, sensitivity and background noise. The second relates to difficulty of processing large amounts of data and includes standardization in the analysis and reporting of results. Finally, this comprehensive integrative analysis will
require experimental validation. In particular, in vivo studies using transgenic and conditional “knockout” or “knockin” animal models should be performed.

The emergence of novel technologies, especially high-throughput small RNA sequencing methods, will allow more sensitive and efficient microRNA detection in patient samples, and identification of novel microRNAs. Additionally, sequence information will be available and mutations or polymorphisms could be discovered. However, standardization of these new methods is necessary to overcome the variability observed when different microRNA-expression detection platforms are used.

There is also the potential to develop microRNA-based therapeutic strategies. It is reasonable to restore lost tumor suppressor microRNA in AML blasts such as miR-29 or miR-181a by using synthetic oligonucleotides that mimic the endogenous mature microRNA or compounds that indirectly up-regulate microRNA expression. For example, miR-223 and miR-29b expression could be restored in RUNX1/RUNX1T1-positive blasts by using hypomethylating agents, such as decitabine. Conversely, silencing a highly expressed microRNA such as miR-155 or miR-196a with antisense oligonucleotides against mature microRNA sequences could also provide clinical benefit. Preliminary data indicate that using antagonirs is an effective approach to silence microRNA expression in mice. Elmén et al recently reported that the simple systemic delivery of an unconjugated locked–nucleic acid modified oligonucleotide (LNA-antimiR) effectively antagonized miR-122 expressed in the liver of nonhuman primates. Furthermore, LNA-antimiR-122 (SPC3649) is the first microRNA-targeted drug to enter human clinical trials; a phase one clinical trial testing the safety of
intravenous administration of LNA-antimiR-122 in healthy volunteers is ongoing in Denmark (www.santaris.com).

**Authorship**

Contribution: All authors participated in writing this article and approved its final version.

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


56. Paschka P, Schlenk RF, Gaidzik VI, et al. IDH1 and IDH2 mutations are frequent


76. Rosa A, Ballarino M, Sorrentino A, et al. The interplay between the master transcription factor PU.1 and miR-424 regulates human monocyte/macrophage


84. Debernardi S, Skoulakis S, Molloy G, Chaplin T, Dixon-Mclver A, Young BD. MicroRNA *miR-181a* correlates with morphological sub-class of acute myeloid
leukaemia and the expression of its target genes in global genome-wide analysis. 


predicted microRNAs in human embryonic stem cells and neural precursors. 


Arabidopsis and rapeseed microRNAs by comprehensive real-time PCR profiling

transcriptome of normal and malignant human B cells identifies hundreds of
novel microRNAs. *Blood.* Prepublished on August 23, 2010, as DOI
10.1182/blood-2010-05-285403.

113. Yang M, Mattes J. Discovery, biology and therapeutic potential of RNA

114. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale,


116. Elmén J, Lindow M, Schütz S, et al. LNA-mediated microRNA silencing in non-
**Table 1.** Most significant microRNAs associated with cytogenetics and molecular features in AML

<table>
<thead>
<tr>
<th>Chromosome aberration</th>
<th>Up-regulated microRNA</th>
<th>Down-regulated microRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(15;17)*21,36,37</td>
<td>miR-127</td>
<td>miR-17</td>
</tr>
<tr>
<td></td>
<td>miR-299</td>
<td>miR-126</td>
</tr>
<tr>
<td></td>
<td>miR-323</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-368</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-382</td>
<td></td>
</tr>
<tr>
<td>t(8;21)*21,23,36,37</td>
<td>miR-126/miR-126*</td>
<td>miR-133</td>
</tr>
<tr>
<td>inv(16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t(11q23)/MLL 21,22,23,36,37</td>
<td>miR-326</td>
<td>miR-29s</td>
</tr>
<tr>
<td></td>
<td>miR-219</td>
<td>miR-34a</td>
</tr>
<tr>
<td></td>
<td>miR-17-92</td>
<td>miR-16</td>
</tr>
<tr>
<td></td>
<td>miR-196a</td>
<td></td>
</tr>
<tr>
<td>Trisomy 8*22</td>
<td>miR-124a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-30d</td>
<td></td>
</tr>
<tr>
<td>Cytogenetically Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene alteration</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>NPM1 mutation</em> 21,63,67,78</td>
<td>miR-10a/-10b</td>
<td>miR-204</td>
</tr>
<tr>
<td></td>
<td>miR-196a</td>
<td>miR-128</td>
</tr>
<tr>
<td></td>
<td>miR-196b</td>
<td>miR-126</td>
</tr>
<tr>
<td></td>
<td></td>
<td>miR-130a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>miR-451</td>
</tr>
<tr>
<td><em>FLT3-ITD</em>21-23,5/6</td>
<td>miR-155</td>
<td></td>
</tr>
<tr>
<td><em>CEBPA mutation</em>21,64,72</td>
<td>miR-181a</td>
<td>miR-34a</td>
</tr>
<tr>
<td></td>
<td>miR-335</td>
<td></td>
</tr>
<tr>
<td><em>BAALC overexpression</em>57</td>
<td></td>
<td>miR-148a</td>
</tr>
<tr>
<td><em>MN1 overexpression</em>60</td>
<td>miR-126</td>
<td>miR-16</td>
</tr>
<tr>
<td></td>
<td>miR-126*</td>
<td>miR-17-92</td>
</tr>
<tr>
<td></td>
<td>miR-424</td>
<td>miR-100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>miR-196a</td>
</tr>
</tbody>
</table>
**Table 2. MicroRNAs with a documented functional role in myeloid leukemogenesis**

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Location</th>
<th>Expression</th>
<th>Regulated by</th>
<th>Targets</th>
<th>In vitro effects</th>
<th>In vivo effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-155</td>
<td>21q21.3</td>
<td>Up-regulated in FLT3-ITD&lt;sup&gt;21,22,67&lt;/sup&gt;</td>
<td>NFκB&lt;sup&gt;8&lt;/sup&gt;</td>
<td>SHIP1&lt;sup&gt;91&lt;/sup&gt;</td>
<td>Blocks megakaryopoiesis&lt;sup&gt;91&lt;/sup&gt;</td>
<td>Induces myeloproliferation with MDS changes in mice&lt;sup&gt;68&lt;/sup&gt;</td>
</tr>
<tr>
<td>miR-196b</td>
<td>7p15</td>
<td>Up-regulated in t(11q23)/MLL&lt;sup&gt;36,98&lt;/sup&gt;</td>
<td>MLL&lt;sup&gt;38&lt;/sup&gt;</td>
<td>HOXB8&lt;sup&gt;98&lt;/sup&gt;</td>
<td>Increases cell survival and proliferation of progenitors&lt;sup&gt;98&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>miR-223</td>
<td>Xq12</td>
<td>Down-regulated in t(8;21)&lt;sup&gt;101&lt;/sup&gt;</td>
<td>RUNX1/RUNX1T1, CEBPA, NFκA&lt;sup&gt;1,101&lt;/sup&gt;</td>
<td>MEF2C&lt;sup&gt;99&lt;/sup&gt;</td>
<td>Induces granulocytic differentiation&lt;sup&gt;99&lt;/sup&gt;</td>
<td>Granulocytosis in miR-223 KO mice&lt;sup&gt;99&lt;/sup&gt;</td>
</tr>
<tr>
<td>miR-29b-1</td>
<td>7q32</td>
<td>Down-regulated in wild-type NPM1, t(11q23)/MLL&lt;sup&gt;22,106,107&lt;/sup&gt; and -7 and del(7q)&lt;sup&gt;22,106,107&lt;/sup&gt;</td>
<td>NFκB, MYC&lt;sup&gt;9&lt;/sup&gt;</td>
<td>MCL1&lt;sup&gt;105,106&lt;/sup&gt;</td>
<td>Induces apoptosis&lt;sup&gt;105,106&lt;/sup&gt;</td>
<td>Reduces tumorigenicity&lt;sup&gt;106&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YY&lt;sup&gt;13&lt;/sup&gt;</td>
<td>CDK6, SP1&lt;sup&gt;105,106&lt;/sup&gt;</td>
<td>Inhibits proliferation&lt;sup&gt;105,106&lt;/sup&gt;</td>
<td>Regulates negatively DNA methylation&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
The prognostic and functional role of microRNAs in acute myeloid leukemia

Guido Marcucci, Krzysztof Mrózek, Michael D. Radmacher, Ramiro Garzon and Clara D. Bloomfield