The prognostic and functional role of microRNAs in acute myeloid leukemia

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Expression of microRNAs, a new class of noncoding 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. (Blood. 2011;117(4):1121-1129)

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 of protein-coding messenger RNAs (mRNAs) and cause their post-transcriptional 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'-untranslated region microRNA recognition elements3 or increase translation of target mRNAs on cell cycle arrest.4 MicroRNAs, whose nomenclature is described by Griffiths-Jones et al,5 regulate normal cell homeostasis and are involved in many physiologic 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

Profil ing and functional experiments revealed that several microRNAs play a role in the regulation of hematopoiesis.6-8 Hence, it was not surprising that many microRNAs are differentially expressed between AML blasts and normal cells.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 collection 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 because similar changes have been observed for mRNA expression.24 In addition, some studies used unselected BM mononuclear cells from healthy donors instead of CD34+ cells as controls.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 “Correlations of microRNA expression with cytogenetics in AML” and “Correlations of microRNA expression with molecular markers in CN-AML”), within AML patient cohorts analyzed could also explain discordant results.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 ALL

Similar to a pioneering study, which demonstrated that gene-expression profiling (GEP) can distinguish between AML and acute lymphoblastic leukemia (ALL),25 changes in microRNA expression were also shown to define lineage-specific leukemia. Using a bead-based microRNA-expression profiling assay, Mi et al26 separated patients with AML from those with ALL based on

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27 differentially expressed microRNAs (21 up-regulated and 6 down-regulated in AML compared with ALL), 4 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 chromosomal abnormalities identified as recurrent.26-28 Whereas 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.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)(q21q26), t(6;9)(p23q34), deletion or loss of 5q, monosomy 7, structural abnormalities of 17p or a complex karyotype [defined as more than or equal to 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)] have a very poor prognosis.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.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) or t(16;16), and APL with t(15;17), has had a unique microRNA-expression profile that distinguished them from each other.26,27,36,37 Dixon-McIver et al36 identified a characteristic signature in APL patients with t(15;17), which comprised 7 up-regulated microRNAs transcribed from genes located in the imprinted domain at chromosome band 14q32 (miR-127, miR-154, miR-154*, miR-299, miR-323, miR-368, and miR-370), and 9 down-regulated microRNAs (miR-17-3p, miR-185, miR-187, miR-194, miR-200a, miR-200b, miR-200c, miR-330, and miR-339). In another study,21 a signature from a cluster that consisted of samples from t(15;17)-positive APL patients displayed a strong up-regulation of miR-127, miR-134, miR-299-5p, miR-323, miR-376a, and miR-382. In a third study,6 3 of the 7 microRNAs identified as class-discriminatory, miR-224, miR-368, and miR-382, were almost exclusively overexpressed in the t(15;17) samples, whereas miR-17-5p, miR-20a, miR-126, and miR-126* were down-regulated.

In contrast, overexpression of miR-126 and miR-126* occurred in CBF-AML samples, both those with t(8;21)21,23,36 and those with inv(16).36 A colony-forming and replating assay revealed cooperation between miR-126 and t(8;21)/RUNX1-RUNXIT1(AML/ETO) in enhancing proliferation of mouse BM progenitor cells.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 2 or more studies (Table 1).21,36,37

Distinct patterns of microRNA expression in AML with t(11q23)/MLL were reported.22 Garzon et al22 identified 8 microRNAs up-regulated (miR-326, miR-219, miR-194, miR-301, miR-324, miR-339, miR-99b, and miR-328) and 14 down-regulated (miR-34b, miR-15a, miR-29a, miR-29c, miR-372, miR-30a, miR-29b, miR-30c, miR-196a, let-7f, miR-102, miR-331, miR-299, and 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, for instance, 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 → LAT52.22 In another study,36 AML with t(11q23)/MLL was characterized by significant overexpression of miR-196b and 7 microRNAs from a unique polycistronic microRNA cluster miR-17-92: miR-17-5p, miR-185, miR-187, miR-194, miR-200a, miR-323, miR-368, and miR-382. With the 13q31 locus, where the cluster is located. Mi et al36 identified 363 potential target genes of miR-17-92 cluster was up-regulated as a result of DNA amplification of the 13q31 locus, where the cluster is located. Mi et al36 identified 363 potential target genes of miR-17-92, whose expression was 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

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**Table 1. Most significant microRNAs associated with cytogenetics and molecular features in AML**

<table>
<thead>
<tr>
<th>Chromosome aberration</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
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<tr>
<td>t(15;17)21,36,37</td>
<td>miR-127</td>
<td>miR-17</td>
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<td></td>
<td>miR-299</td>
<td>miR-126</td>
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<td></td>
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<tr>
<td></td>
<td>miR-382</td>
<td>miR-126</td>
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<tr>
<td>t(8;21)21,36,37</td>
<td>miR-126/miR-126*</td>
<td>miR-133</td>
</tr>
<tr>
<td>inv(16)</td>
<td></td>
<td></td>
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<tr>
<td>t(11q23)/MLL21,22,36-38</td>
<td>miR-326</td>
<td>miR-29a</td>
</tr>
<tr>
<td></td>
<td>miR-219</td>
<td>miR-34a</td>
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<td></td>
<td>miR-17-92</td>
<td>miR-16</td>
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<td></td>
<td>miR-196a</td>
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<tr>
<td>Trisomy B21</td>
<td>miR-124a</td>
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<td></td>
<td>miR-30d</td>
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**Cytogenetically normal**

<table>
<thead>
<tr>
<th>Gene alteration</th>
<th>microRNA</th>
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<tr>
<td>NPM1 mutation21,39-41</td>
<td>miR-10a-10b</td>
</tr>
<tr>
<td>miR-196a</td>
<td>miR-128</td>
</tr>
<tr>
<td>miR-196b</td>
<td>miR-126</td>
</tr>
<tr>
<td>miR-130a</td>
<td>miR-451</td>
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</tbody>
</table>

| FLT3-ITD21,23,33  | miR-155 |
| CEBPA mutation21,42,43 | miR-181a | miR-34a |
| miR-335       |          |

| BAALC overexpression44 | miR-126 |
| miR-126* | miR-17-92 |
| miR-424 | miR-100 |
| miR-196a |        |

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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.47

The t(11q23)/MLL-rearranged AML category is very heterogeneous cyogenetically, with more than 50 different translocation partners of 11q23/MLL characterized at the molecular level to date.48 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), 16 microRNAs were up-regulated in patients with t(6;11), including the antiapoptotic mir-21, which targets the tumor suppressor PTEN, and mir-20a and mir-20b, which target the TGFβ1 regulator SMAD1.59,60 Studies on other specific t(11q23)/MLL rearrangements are needed to further characterize their microRNA patterns.

Garzon et al22 identified a signature composed 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/EBPα.51 In contrast, Dixon-McIver et al37 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.22 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%,4,2 and the reported NPM1 mutation-associated gene expression signature known to encompass HOX gene overexpression.53 However, as discussed in “Correlations of microRNA expression with molecular markers in CN-AML,” 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 probably the result of 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 2 of a set of 4 microRNAs, it was possible to discriminate ALL from AML cases with an overall diagnostic accuracy of 97% to 98%.20 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 chromosomal aberration at diagnosis, 40% to 49% of AML patients have a normal karyotype and thus constitute the largest single cytogenetic subset of AML.29 During the past 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.52,54 Molecular alterations shown repeatedly to confer adverse prognosis include an internal tandem duplication of the FLT3 gene (FLT3-ITD),54,58 partial tandem duplication of the MLL gene (MLL-PTD),54,59 mutations of the WT1,90,92 IDH1 and IDH2 genes,53,64 and high expression of the BAALC.54,65 and MN1 genes,56,68 whereas favorable prognosis is bestowed by mutations in the NPM1,54,67,68 and CEBPA genes,52,54, in the case of the latter, especially biallelic mutations.69,70 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.67

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,59 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.53 In addition, Garzon et al39 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 al40 reported a microRNA-expression signature, composed of 68 microRNAs, associated with NPM1 mutations in patients 60 years of age or older 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.40 Down-regulated were miR-126, whose expression was recently positively correlated with MNL1 expression in younger CN-AML patients,4,4 and miR-130a and miR-451, which are involved, respectively, megakaryocytic differentiation and erythropoiesis.6 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%).40

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.21,22 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.71 Mir-155 was also the most overexpressed microRNA in the first FLT3-ITD–associated microRNA-expression signature derived in primary CN-AML patients 60 years of age or older that encompassed 32 differentially

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expressed probes. In agreement with this signature was miR-125b-2*, and underexpressed were miR-144 and miR-451, normally up-regulated during erythropoiesis, and miR-488 and miR-486-5p; the latter is underexpressed in multiple cancers.

CEBP A mutations have been associated with an up-regulation of miR-181a and miR-335 in 2 AML studies, one of which was performed exclusively on samples from CN-AML patients. Notably, in this study, CEBPA mutations were associated with up-regulation of several genes involved in erythroid differentiation, in agreement with the results by Choong et al reporting an increase of miR-181a and miR-181b levels during erythroid differentiation. Consequently, high expression of the miR-181 family members seems to contribute to the partial erythroid differentiation of leukemic blasts harboring CEBPA mutations. Down-regulated in CEBPA-mutated patients were miR-194 and miR-34a. MiR-34a was recently shown to be a target of CEBPα during granulopoiesis, and its low expression in leukemia blasts with CEBPA mutations was demonstrated to elevate levels of E2F3 and its transcriptional target, E2F1, which in turn increased proliferation and inhibited granulocytic differentiation of the blasts. Forced expression of miR-34a resulted in granulocytic differentiation of AML blasts carrying CEBPA mutations, suggesting that increasing miR-34a levels might become useful therapeutically, as could manipulation of expression of miR-222, which is part of a molecular network regulating granulopoiesis that also includes E2F1 and CEBPα. Finally, Hackanson et al showed that CEBPA mRNA is targeted by miR-124a in vitro, and that miR-124a is epigenetically silenced in leukemia cell lines. Epigenetic treatment with the DNA-demethylating agent 5-aza-2′-deoxycytidine caused up-regulation of miR-124a.

Our group has recently demonstrated the existence of a striking inverse correlation between BAALC expression and expression of miR-148a in younger CN-AML patients. This suggests that miR-148a might act as a negative regulator of BAALC expression and that low or absent miR-148a expression may increase expression of BAALC and the resulting aggressive phenotype. In patients 60 years of age or older, we derived a microRNA-expression signature composed of 18 microRNAs, 10 up-regulated and 8 down-regulated, in low BAALC-expressing patients. Consistent with higher expression of HOX genes in low BAALC-expressers, microRNAs embedded in the HOX cluster, miR-10a, miR-10b, and miR-9, were up-regulated in low BAALC expressers, whereas underexpressed were miR-126, positively correlated with MN1 expression (see the next paragraph), and miR-222, linked with hematologic lineage differentiation and known to target KIT.

We have also reported an MN1-associated microRNA-expression signature composed of 15 microRNAs (Table 1). Of those, expression of 8 microRNAs was positively, and expression of 7 negatively, correlated with MN1 expression. Five of the 8 microRNA probes positively associated with MN1 expression were members of the miR-126 family (including miR-126 and miR-126*). This microRNA family was recently reported to enhance the proangiogenic activity of vascular endothelial growth factor and regulate new blood vessel formation. We also noted up-regulation of miR-424, a regulator of monocyte and macrophage differentiation. Among the microRNAs negatively correlated with MN1, we found microRNAs involved in apoptosis (miR-16), malignant transformation (miR-19a and miR-20a, members of the miR-17-92 polycistron), or regulation of ERG expression (miR-196a).

Schwind et al reported recently the first microRNA-expression signature associated with ERG expression. The signature consisted of 11 microRNAs, 5 up-regulated and 6 down-regulated, in low ERG expressers. Among the former was miR-107, targeting NFIX, a gene involved in a regulatory feedback loop involving miR-223 and CEBPA during granulocytic differentiation. MiR-148a, and miR-208, predicted in silico to target ERG itself. Down-regulated was miR-30d, a microRNA associated with early developmental stages and “stemness.”

To our knowledge, only one study has derived a microRNA-expression signature associated with R172 mutation in IDH2. Among the most up-regulated microRNAs in patients with R172 IDH2 mutations were miR-1 and miR-133, hitherto not associated with human cancer but involved in embryonal stem-cell differentiation, and miR-125a-5p and miR-125b, which target TP53. MiR-125b-1 is strongly up-regulated in AML with t(2;11)(p21;q23) and inhibits myeloid differentiation, whereas miR-125b-2 cooperates with GATA1 mutations during leukemogenesis of Down syndrome–associated acute megakaryoblastic leukemia. Moreover, overexpression of both miR-125b-1 and miR-125b-2 causes a dose-dependent myeloproliferative disorder progressing to a lethal myeloid leukemia in mice. None of the most down-regulated microRNAs in R172 IDH2-mutated patients, miR-194-1, miR-526, miR-520a-3p, or miR-548b, has 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 the 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, miR-130a, 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, miR-130a, 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 (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 cyogenetically 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 reverse-transcription polymerase chain reaction) confirmed that high expression levels of miR-191 and miR-199a conferred inferior event-free and overall survival. In multivariable analyses, the 2 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: FLT3-ITD, wild-type NPM1 alleles, or both.\textsuperscript{58} The signature, composed of 12 microRNA probes derived from a training set of patients treated on CALGB protocol 19808, was associated with event-free survival. It was afterward tested in a validation set of similarly treated patients enrolled onto CALGB protocol 9621. Increased expression of 5 probes representing miR-181a and miR-181b was associated with decreased risk of an event (failure to achieve complete remission, 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.\textsuperscript{38} 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 microbialial cells and cell injury, which are part of the “inflammasome” regulating activation of caspase-1 and interleukin-1β.\textsuperscript{39} 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.\textsuperscript{38} 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.\textsuperscript{42}

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\textsuperscript{19} 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 complete remission rate, longer overall survival, and a trend for longer disease-free survival in all CN-AML patients studied, and with higher complete remission 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.\textsuperscript{90}

### Functional aspects of microRNA expression in myeloid leukemogenesis

Over the past 3 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 biologic validation of these findings. Many questions remain unanswered. Do microRNAs contribute to myeloid leukemogenesis? If they do, what are the mechanisms? In the next 4 sections, 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 noncoding BIC transcript, and its expression is elevated in many solid tumors, lymphomas, and acute leukemias.\textsuperscript{6,9,21,22,99} In AML, miR-155 expression is higher in patients carrying FLT3-ITD mutations.\textsuperscript{21,22} However, blocking FLT3 phosphorylation activity with a FLT3 inhibitor or overexpressing FLT3-ITD in mouse myeloid...
precursor cells did not change miR-155 expression, suggesting that miR-155 expression is independent from FLT3-ITD signaling. Functionally, overexpression of miR-155 in human CD34+ stem cells results in myeloid proliferation with blockage of erythroid and megakaryocytic colony formation. 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. 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. However, the mechanistic basis for miR-155 biologic effects on the myeloid and lymphoid lineages remains largely unresolved. Recently, 2 independent reports identified Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1) as a direct target of miR-155.91,92 SHIP1 is a negative regulator of cell signaling in the immune system. This phosphatase is also implicated in B-cell maturation because it has a differential expression in the pre-B compared with the pre-B stage.90 Mice with global SHIP1 deficiency develop a myeloproliferative disease characterized by increased granulocyte-monocyte populations and decreased B-lymphocyte numbers.101 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.102 In addition to SHIP1, miR-155 targets CEBPB,93 encoding a protein with a critical function in granulopoiesis,103 suggesting that leukemogenic function of miR-155 results in deregulation of SHIP1 and CEBPB (Table 2).91,92

**miR-196b**

miR-196b, located between HOXA9 and HOXA10 genes at 7p15, has been found up-regulated in AML patients with t(11q23)/MLL.21,22,36-38 Popovic et al 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 antagonir targeting miR-196b decreased proliferative capacity of the cells (Table 2). It 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. Hence, it was suggested that miR-223 induces granulocytic differentiation. To answer this question, Johnnidis et al 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. 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. It has been reported that AML patients with t(8;21) exhibit lower miR-223 levels than other AMLs. Moreover, 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, RUNXI/RUNXI T1 inhibitors, or miR-223 oligonucleotides enhanced miR-223 levels and restored cell differentiation.

**miR-29**

The miR-29 family is composed of 3 isoforms arranged in 2 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 with del(7q). MiR-29 family members are down-regulated in high-risk chronic lymphocytic leukemia, lung cancer, invasive breast cancer, and cholangiocarcinoma.98,105-107 We and others reported down-regulation of miR-29 family members in primary AML samples with t(11q23)/MLL,99,100 and those with del(7q),22,96,97 whereas miR-29a expression was increased in AML samples from (mostly) FLT3-ITD–negative CN-AML patients. Moreover, lower levels of miR-29 were detected in CN-AML samples with wild-type NPM1 compared with CN-AML with NPM1 mutations.98 Recently, ectopic overexpression of miR-29b in AML cell lines and primary AML blasts was shown to induce apoptosis and inhibit blast proliferation. 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, antiapoptotic genes, including MCL1, were enriched among the miRNAs inversely correlated with miR-29 expression (Table 2). Our group also reported that miR-29b modulates DNA methylation by targeting directly DNMT3a and DNMT3b and indirectly DNMT1 through SPI. Blum et al observed that these AML patients 60 years of age or older treated with single-agent decitabine, a DNMT inhibitor, who responded to treatment had higher pretreatment levels of miR-29b. More recently, a study showed that miR-29b is involved in a protein-microRNA network, which includes SPI, 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, thiaramycin 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 are 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 biologic 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. In addition, 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 lose tumor suppressor microRNA in AML blasts, such as miR-29 or miR-181a, 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/RUNXIT1-positive blasts 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 antagonists 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 1 clinical trial testing the safety of intravenous administration of LNA-antimiR-122 in healthy volunteers is ongoing in Denmark (www.santaris.com).

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