Distinct microRNA expression profiles in acute myeloid leukemia with common translocations

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MicroRNAs (miRNAs) are postulated to be important regulators in cancers. Here, we report a genome-wide microRNA expression analysis in 52 acute myeloid leukemia (AML) samples with common translocations, including t(8;21)/AML1(RUNX1)-ETO(RUNX1T1), inv(16)/CBFβ-MYH11, t(15;17)/PML-RARA, and MLL rearrangements. Distinct microRNA expression patterns were observed for t(15;17), MLL rearrangements, and core-binding factor (CBF) AMLs including both t(8;21) and inv(16) samples. Expression signatures of a minimum of two (i.e., miR-126/126*), three (i.e., miR-224, miR-368, and miR-382), and seven (miR-17-5p and miR-20a, plus the aforementioned five) miRNAs could accurately discriminate CBF, t(15;17), and MLL-rearrangement AMLs, respectively, from each other. We further showed that the elevated expression of miR-126/126* in CBF AMLs was associated with promoter demethylation but not with amplification or mutation of the genomic locus. Our gain- and loss-of-function experiments showed that miR-126/126* inhibited apoptosis and increased the viability of AML cells and enhanced the colony-forming ability of mouse normal bone marrow progenitor cells alone and particularly, in cooperation with AML1-ETO, likely through targeting Polo-like kinase 2 (PLK2), a tumor suppressor. Our results demonstrate that specific alterations in microRNA expression distinguish AMLs with common translocations and imply that the deregulation of specific miRNAs may play a role in the development of leukemia with these associated genetic rearrangements.

Acute myeloid leukemia (AML) is a heterogeneous group of genetically diverse hematopoietic malignancies with variable response to treatment (1). Chromosome translocations are frequently observed in AML (2). Four major rearrangements in AML are the t(8;21), inv(16), t(15;17), and MLL/11q23 translocations, which account for ~30% of all AML cases (3), and have been incorporated in the WHO classification as the criteria for subclassification of AML (4). The t(8;21), t(15;17), and inv(16) have been established as molecular indicators for favorable clinical outcome in AML, whereas MLL-rearrangement AML is classified as a disease of intermediate or poor prognosis (1, 5). Both the t(8;21) and inv(16) rearrangements result in the disruption of core-binding factor (CBF), a heterodimeric transcriptional regulator of normal hematopoiesis (6, 7) and, therefore, are collectively referred to as CBF AMLs, which represent 10–20% of primary AMLs (8, 9). Many expression profiling studies of protein-coding genes have been performed on AML by using DNA microarray analysis (e.g., refs. 10–13). However, the results of analyses of AML by different laboratories are not always consistent. For example, there is a relatively small overlap between genes relevant for prognosis reported by Bullinger et al. (11) and those reported by Valk et al. (12). Therefore, further validation of these observations in large cohorts and in independent studies is required before clinical application becomes feasible, and it seems unlikely that mRNA expression profiling alone can reveal the entire pathobiology of AML (5).

Recently, Lu et al. (14) described a new, bead-based flowcytometric microRNA (miRNAs, miRs) expression profiling method that could successfully classify tumors and showed that identification of poorly differentiated tumors by using microRNA expression profiles was more accurate than using mRNA expression profiles. This finding indicates that miRNA profiling could be superior to mRNA profiling in certain clinical circumstances and suggests that microRNA profiling is an important tool in molecular classification. miRNAs are endogenous ~22-nt noncoding RNAs that can play important regulatory roles in development, cell proliferation, cell survival, and apoptosis (15). Moreover, evidence is emerging that miRNAs can function as oncogenes and tumor suppressor genes (16, 17).

To provide insights into the complex genetic alterations in leukemogenesis and to identify possible markers for the diagnosis and treatment of AML in the future, we performed a genome-wide bead-based microRNA expression analysis (14). The profiling data were analyzed with unsupervised and supervised clustering analyses, and a minimal set of miRNAs that can accurately predict the AML subtypes was identified. The expression pattern of the class-discriminator miRNAs was further validated by quantitative real-time PCR (qPCR). Finally, the mechanism of gene-expression regulation and function of miR-126, a class-discriminator microRNA for CBF AMLs, as well as its potential targets, was carefully studied.

Results

miRNA Expression Profiling in AMLs. We performed a large-scale microRNA expression profiling analysis of 435 mammalian miRNAs on 57 samples, including 47 primary AML specimens, 7 AML cell lines, and 3 normal control samples (see Materials and Methods). The 47 primary AML specimens included 10 t(8;21)/AML1(RUNX1)-ETO(RUNX1T1), 7 inv(16)/CBFβ-MYH11, 10 t(15;17)/PML-RARA, and 20 MLL-rearrangement AMLs (including one t(6;11)/MLL-AF6(MLLT4), 9 t(9;11)/MLL-AF9(MLLT3), and one t(1;19)).

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The seven AML cell lines included one t(8;21), one inv(16), and five MLL-rearrangement AMLs. Three normal bone marrow samples from healthy donors were used as normal controls, which included two mononuclear cell (MNC) samples and one purified CD15- myeloid progenitor cell sample. Bead-based miRNA expression profiling detection was performed as described (14). Two samples with total fluorescence <15,000 were discarded as unsuccessful labeling/sample quality. After normalization and filtering [see supporting information (SI) Text], a total of 55 samples (including 52 AML and 3 normal controls) and 112 human miRNA genes with confidently detectable expression values were selected for further analyses.

We first performed an unsupervised, two-way (genes against samples), hierarchical cluster analysis (HCA). Remarkably, leukemia samples clearly grouped into three clusters: (i) t(8;21) and inv(16) samples grouped together as Cluster 1 with three exceptions; (ii) t(15;17) samples grouped together as Cluster 2 with two exceptions; and (iii) all of the MLL-rearrangement AML samples grouped together and formed Cluster 3. Normal control samples also grouped together and formed a subcluster within Cluster 3 (see Fig. S1A). The clustering was not associated with patient’s age, gender, blast cell percentage, clinical state, or sample source (peripheral blood or bone marrow). Clustering of cases with MLL rearrangement did not correlate completely with either MLL partner genes or French–American–British (FAB) subtypes (Fig. S1A), which is consistent with observations from the miRNA-based expression assays (18, 19). In a three-dimensional view of a principal-component analysis (PCA), the three clusters are clearly separate (Fig. S1B).

**miRNAs Differentially Expressed Between the Subtypes of AMLs.** Gene expression data were further analyzed for significance by using ANOVA multiple-groups test and permutation tests. The ANOVA analysis identified 32 miRNAs that were significantly, differentially expressed between the subgroups (Fig. 1A). We also used significance analysis of microarray (SAM) (20) and permutation tests to identify differentially expressed genes with a “one-vs.-all of the others” approach. We identified 24, 19, 11, and 10 (including 52 AML and 3 normal controls) and 112 human miRNA genes with confidently detectable expression values were selected for further analyses.

Overexpression of miR-126/126* in CBF AMLs Is Not a Consequence of DNA Amplification. To reveal the mechanism underlying the overexpression of miR-126/126* in CBF AMLs, we further used the prediction analysis of microarray (PAM) method (24) to determine the minimal number of miRNAs that can predict the AML subtypes accurately (see Materials and Methods). As shown in Table S1, the expression signature of a minimal number of two (i.e., miR-126, and miR-126*), three (i.e., miR-224, miR-368, and miR-382), and seven (i.e., miR-17–5p and miR-20a plus the aforementioned five) miRNAs can best characterize CBF, t(15;17), and MLL-rearrangement AMLs, respectively, resulting in a diagnostic accuracy >94%. The detailed cross-validated probabilities of the entire set of 52 AML samples is shown in Fig. S2; and see Table S2. A similar level of accuracy was achieved by using a different supervised learning algorithm, namely SVM (data not shown).
Differential Expression of miR-126/126* Is Not Associated with Mutation.

Calin et al. (26) stated that mutations in miRNA sequences are common and may have functional importance. We therefore investigated whether differential expression of miR-126/126* is also associated with DNA sequence mutations. We sequenced the region of the 128-bp CpG island in the above 21 samples used for the methylation analysis. We did not identify any mutations in this region except for a known SNP at nucleotide 151 (A-to-G transition) of the CpG island fragment in one or both alleles of all of the samples. Although the biological function of this SNP needs to be clarified, it does not appear to be associated with differential expression of miR-126/126*, because this SNP existed in both CBF and non-CBF leukemia samples.

**Fig. 1.** Expression profiling analysis and validation of miRNAs in AMLs with common translocations. (A) Expression profiling of the 41 differentially expressed miRNAs in 55 samples as detected by the bead-based miRNA expression assay. Unsupervised, average linkage hierarchical clustering was performed. Expression data were mean centered, and the relative value for each sample was represented by a color, with red representing a high expression and green representing a low expression (scale shown in the upper right). The three clusters of AMLs and the subcluster with normal controls are indicated by color bars across the top of the figure (four colors). (B) Expression profiling of the seven class-discriminatory miRNAs in 56 AML and 9 control samples as detected by TaqMan qPCR. Data are presented as ΔΔCt. "OLD" samples are those included in the bead-based miRNA expression assay, whereas "NEW" samples are independent samples that were not included in that assay. "cl." cell line; "Nc.," normal control; "MNCc.," mononuclear cells; "CD34c.," CD34+ hematopoietic stem/progenitor cells; "CD15c.," CD15+ myeloid progenitor cells; "j-miR-Zc.," a miRNA identified by L.L., H.Z., and T.R.G. (unpublished data).
MiR-126 Inhibits Cell Apoptosis and Increases Cell Viability. To examine the functional role of miR-126 in AML cells, we performed gain- and loss-of-function experiments. As shown in Fig. 2, forced expression of miR-126 significantly inhibited apoptosis in THP-1 and ME-1 cells with or without Etoposide treatment. Forced expression of miR-126 significantly increased cell viability in THP-1 and ME-1 cells with or without Etoposide treatment. Furthermore, knockdown of miR-126 by anti-miR-126 inhibitor significantly increased apoptosis. As expected, we also observed that forced expression of miR-126 significantly increased cell viability in THP-1 and ME-1 cells, whereas the opposite effect was observed when miR-126 was down-regulated (Fig. 2B). The forced expression and knockdown of miR-126 were confirmed by qPCR, respectively (see Fig. S5a). Thus, miR-126 may function as an oncogene in leukemogenesis.

MiR-126 Enhances Proliferation of Mouse Bone Marrow Progenitor Cells Alone and Particularly, in Cooperation with the t(8;21) Fusion Gene. The AML1-ETO (AE) fusion gene resulting from the t(8;21) cannot cause leukemia alone in vivo (27). To investigate whether miR-126 has a synergistic action with AE in oncogenicity, we performed a colony-forming and replating assay. Mouse bone marrow progenitor cells transduced with MSCV-puro (empty vector; as a control), MSCVpuro-miR-126, MSCVpuro-AE, and MSCVpuro-AE-miR-126, respectively, were plated on methylcellulose medium (see SI Text for details). The forced expression of miR-126 and AML1-ETO were confirmed by qPCR (see Fig. S5b). The colonies were replated every 7 days under the same conditions. As shown in Fig. 2C, transduction of miR-126 or AE alone or both of them together caused significantly more colonies (4- to 36-fold; \( P < 0.001, \) t test) than transduction of MSCVpuro empty vector (i.e., control) after replating (i.e., in the second and third rounds of plating). Remarkably, after replating, the number of colonies of MSCVpuro-AE-miR-126 (\( > 300 \)) is significantly greater (\( > 3 \)-fold; \( P < 0.001, \) t test) than the sum of the colonies (\( < 100 \)) of MSCVpuro-AE and MSCVpuro-miR-126 (Fig. 2C), indicating that there is a very significantly synergistic effect between miR-126 and AE.

Identification of Potential Targets of miR-126 in AMLs. Recent findings indicate that animal miRNAs may not only repress protein synthesis but also induce mRNA degradation of a large portion of targets (28, 29). Thus, we used qPCR to determine whether a candidate target exhibits a significant inverse correlation of expression at RNA level with miR-126. From 674 predicted targets of miR-126 by at least one of four currently available major prediction programs, including TargetScanS (30), Miranda (31), PicTar (32), and MAMI (http://mami.med.harvard.edu/), we selected all of the 12 genes (i.e., CRK, FBX033, IRS1, DIP2C, GOLPH3, PHF15, PLK2, PTPN9, RGS3, SLC7A5, SPRED1, and TOM1) that were predicted by at least three different programs for qPCR (Table S3). We found that only PLK2 and SPRED1 exhibited a significant inverse correlation (for PLK2: \( rs = -0.42, P < 0.01; \) for SPRED1: \( rs = -0.66, P < 0.001; \) Spearman’s rank correlation test) with the expression of miR-126 in the whole set of 41 samples tested (see Fig. S6 a–c). We then performed a luciferase reporter assay to validate the potential regulatory relationship. A significantly negative effect (\( P < 0.01; \) paired t test) on luciferase activity was observed in the presence of miR-126 on the 3′ UTR of PLK2 but not on that of SPRED1; such repression disappeared when the predicted target site in the 3′ UTR of PLK2 was mutated (Fig. S7 a and b). Moreover, the down- and up-regulation of PLK2 was associated with overexpression and down-regulation of miR-126, respectively (Fig. S7c). These results indicate that PLK2, but not SPRED1, is a bona fide target of miR-126.

Discussion

Here, we demonstrate that the expression signatures of as few as seven miRNAs accurately distinguish subtypes of AMLs with common translocations (Table S1). Interestingly, our findings that t(8;21) and inv(16) samples grouped together in both unsupervised and supervised analyses (see Fig. S1a and Fig. 1A) and that two miRNAs (i.e., miR-126 and miR-126*) could be used as predictors to discriminate CBF AMLs from non-CBF AMLs (Table S1 and Fig. S2), provide compelling evidence to support the notion that there is a common leukemogenic pathway existing in CBF AMLs (9, 33, 34). In fact, clinical studies have usually stratified t(8;21) and inv(16) patients together into...
one favorable-risk prognosis category, and treated them similarly (35, 36).

Several differentially expressed miRNAs identified here, including miR-10a, miR-10b, miR-17, miR-20, miR-29a, miR-29c, miR-126, miR-191, miR-181, and let-7d (see Fig. 1A) were also reported previously in the studies of hematopoietic cells (37–39). leukemia cell lines (39, 40), acute promyelocytic leukemia (41), or morphological subgroups of AMLs (42). During the preparation of this article, we became aware that several other articles regarding miRNAs in AML were published. Fazi et al. (43) observed a down-regulation of miR-223 in (8;21) AML samples and showed that there was an epigenetic silencing of the myelopoiiesis regulator miR-223 by the AML1-ETO oncoprotein. We also found that miR-223 was expressed at a lower level in the majority of (8;21) samples than in other AML-M2 and AML-M4 samples, but it was significantly differentially expressed between AML and ALL (44), not between the subtypes of AML we studied. Fontana et al. (45) reported that miRNAs 17-5p-20a-106a control monocytopoiesis through suppressing AML1 protein expression, leading to M-CSF receptor down-regulation and thereby enhancing blast proliferation and inhibition of monocytic differentiation and maturation. Therefore, their study together with our finding (see Fig. 1A) that the miR-17-92 cluster is particularly overexpressed in MLL-rearrangement AMLs (M4/M5, with the majority of leukemic cells being monoblasts) suggest that miR-17-92 may play an important role in the development of such AML. Using a qPCR method, Isken et al. (46) identified that miR-23b was down-regulated, whereas miR-221/222 and miR-34a were overexpressed in AMLs compared with normal controls. In a large-scale miRNA microarray study, Garzon et al. (47) found that miRNA expression in AML is closely associated with cytogenetics such as t1q23) and FLT3-ITD mutations. Jongen-Lavrencic et al. (48) recently analyzed 215 AML cases including those bearing the common translocations using qPCR for 260 miRNAs, and notably, consistent with our findings, they also identified miR-126 and miR-382 as a class predictor for CBF and t(15;17) leukemia, respectively.

A current challenge is to clarify the mechanism(s) underlying regulation of miRNA expression (17). Here, we found that the overexpression of miR-126/126* in CBF AML is not a consequence of amplification or mutation of the genomic locus but is associated with partial de-methylation of the CpG island in which miR-126/126* is embedded. In addition, as shown in Fig. S5b, forced expression of AML1-ETO can significantly up-regulate expression of endogenous miR-126 (2–2-fold), suggesting that, besides being related to DNA demethylation, overexpression of miR-126 in CBF leukemia might be also attributed to a direct up-regulation of AML1-ETO or CBFB-MYH11.

It is believed that the deregulation of miRNAs contributes to tumorigenesis by negatively regulating expression of their targets (15, 17). We identified PLK2 as a valid target of miR-126. PLK2 is one of the Polo-like kinase (PLK) family members that function in regulation of the cell cycle and DNA damage-induced checkpoints in mammals (49, 50). PLK2 expression is up-regulated directly by wild-type p53 after DNA damage and activates a G2 checkpoint in these circumstances (50, 51). Down-regulation of PLK2 was frequently observed in B-cell malignancies, and ectopic expression of PLK2 in Burkitt lymphoma cells resulted in apoptosis (49), suggesting that PLK2 may function as a tumor suppressor gene in hematologic malignancies (49, 50). Thus, miR-126 may play a role in leukemogenesis through negatively regulating PLK2. Our finding that miR-126 may function as an oncogene is supported further by an observation that miR-126 is significantly up-regulated in acute megakaryoblastic leukemia cell lines compared with in vitro differentiated megakaryocytes and CD34+ hematopoietic progenitors (39).

Acute leukemias, like other human cancers, occur as the consequence of more than one mutation. Primary oncogenic events, such as those triggered by chromosomal rearrangements, are generally insufficient by themselves to cause leukemia and require secondary cooperating mutations to generate a fully transformed cell (52). For example, conditional expression of knockin CBFβ-MYH11 or AML1-ETO fusion gene, or expression of AML1-ETO after retroviral transduction/transplantation does not result in leukemia without a “second hit,” such as those induced by N-ethyl-N-nitrosourea (ENU) (27, 53). Because miR-126/126* were almost uniquely overexpressed in CBF AMLs, we hypothesize that their overexpression may contribute to the development of CBF AML as a second hit by cooperating with the primary oncogenic events (i.e., CBFβ-MYH11 or AML1-ETO), which is supported by our finding that miR-126 inhibits apoptosis and increases cell viability in AML cells and enhances proliferation of mouse normal bone marrow progenitor cells alone and particularly, in cooperation with the t(8;21) fusion genes. Clearly, further in vitro and in vivo assays are necessary to validate such a hypothesis. Similarly, miR-224, miR-368, and miR-382 may contribute to the development of APL in cooperation with the t(15;17), whereas the miR-17-92 cluster may contribute to MLL-rearrangement AML through cooperating with the MLL rearrangements.

Materials and Methods

See SI Text for more details on materials and methods used.

Bead-Based miRNA Expression Profiling Assay and Data Analysis. Bead-based miRNA expression profiling assay was performed as described (14) with some modifications (44). The TIGR Multiplet Array Viewer software package (54) was used to perform data analysis and to visualize the results. The class prediction analysis was performed by using PAM software (24).

qPCR Assays of miRNAs, mRNAs, and DNA Locus Copy Number. The TaqMan qPCR method (25) was used to validate the differential expression patterns of miRNAs by using kits from Applied Biosystems: qPCR with SYBR green dye (Qiagen) was used to determine expression of miRNA genes. U6 RNA and PGK1 (or GAPDH) were used as endogenous controls for qPCR of miRNA and mRNA, respectively. PCRs were performed in an Applied Biosystems 7900HT system according to the relevant manufacturer’s recommendation, and each sample was detected in triplicate. We followed a method described by He et al. (23) to analyze DNA copy number of the miR-126/126* locus with modification as described (44).

Bisulfite Genomic Sequencing and Mutation Screening. Genomic DNA (1 μg) from each of sample was treated with sodium bisulfite (55). Thereafter, the relevant genomic DNA regions were PCR amplified. After PCR amplification, DNA methylations levels were analyzed by bisulfite genomic sequencing with M13 primer as described (56). For mutation screening, the genomic regions were PCR amplified from genomic DNA samples with no sodium bisulfite treatment, and PCR products were then purified and sequenced directly.

Apoptosis Assay. THP-1 or ME-1 cells were plated at a concentration of 10,000 cells per well in triplicate for each condition in a 96-well plate 24 h after transfection with MSCV/puro-miR-126, the control plasmid MSCVpuro, anti-miR126 inhibitor (Dharmacon), or inhibitor control (i.e., scrambled oligonucleotides; Dharmaconcomx). Etoposide (5 μM; Sigma) or the same volume of DMSO (mock treatment) was added to each well, and caspase-3 and caspase-7 activation was detected by using ApoONE Homogenous Caspase 3/7 Assay (Promega) 24 h later, by following the manufacturer’s manual.

Cell Viability Assay. THP-1 or ME-1 cells were plated at a concentration of 10,000 cells per well in triplicate for each condition in a 96-well plate 24 h after transfection with MSCV/puro-miR-126, MSCVpuro, anti-miR126 inhibitor, or inhibitor control. Metabolic activity of the cells was determined by using CellTiter-blue Reagent (Promega) 24 h later by following the manufacturer’s manual.

 Colony-Forming and Replating Assay. In vitro colony-forming (i.e., immortalization) assays were performed as described (57) with some modifications.
Luciferase Reporter and Mutagenesis Assays. MiR-126 expression plasmid (i.e., MSCVpuro-miR-126) or its control plasmid (i.e., MSCVpuro-PG) (23) was co-transfected into HEK293T cells with a single report plasmid (pMIR-Report plasmid; Ambion) containing either the wild-type or mutated 3’ UTR of an individual predicted target gene. Luciferase was measured 42 h after transfection. The firefly luciferase activity was then normalized to β-galactosidase activity. Experiments were repeated three times independently.

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