Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin

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Acute myeloid leukemia (AML) carrying *NPM1* mutations and cytoplasmic nucleophosmin (NPMc+ AML) accounts for about one-third of adult AML and shows distinct features, including a unique gene expression profile. MicroRNAs (miRNAs) are small noncoding RNAs of 19–25 nucleotides in length that have been linked to the development of cancer. Here, we investigated the role of miRNAs in the biology of NPMc+ AML. The miRNA expression was evaluated in 85 adult *de novo* AML patients characterized for subcellular localization/mutation status of *NPM1* and *FLT3* mutations using a custom microarray platform. Data were analyzed by using univariate *t* test within BRB tools. We identified a strong miRNA signature that distinguishes NPMc+ mutated ($n = 55$) from the cytoplasmic-negative (*NPM1* unmutated) cases ($n = 30$) and includes the up-regulation of *miR-10a*, *miR-10b*, several *let-7* and *miR-29* family members. Many of the down-regulated miRNAs including *miR-204* and *miR-128a* are predicted to target several *HOX* genes. Indeed, we confirmed that *miR-204* targets *HOXA10* and *MEIS1*, suggesting that the *HOX* up-regulation observed in NPMc+ AML may be due in part by loss of *HOX* regulators-miRNAs. *FLT3*-ITD+ samples were characterized by up-regulation of *miR-155*. Further experiments demonstrated that the up-regulation of *miR-155* was independent from *FLT3* signaling. Our results identify a unique miRNA signature associated with NPMc+ AML and provide evidence that support a role for miRNAs in the regulation of *HOX* genes in this leukemia subtype. Moreover, we found that *miR-155* was strongly but independently associated with *FLT3*-ITD mutations.

FLT3-ITD | HOX | NPM1

Acute myeloid leukemia (AML) arises from multiple and sequential genetic alterations involving hematopoietic precursors (1). In ≈25% of cases, specific chromosomal translocations like the t(8;21), inv(16) or t(15;17) represent the initial events leading to malignant transformation (1) and are associated with a good outcome. In contrast, 40–50% of AMLs have normal karyotype by conventional banding analysis and are characterized by great molecular and clinical heterogeneity (2). Recent work has identified novel molecular abnormalities in normal karyotype AML (NK-AML) that has improved the classification and risk stratification of this large subgroup of patients. Among them, internal tandem duplications in the juxta-membrane domain or mutations in the second tyrosine kinase domain (TKD) of the *FLT3* gene have been found in 30–45% of NK-AML (3). Both types of mutations constitutively activate *FLT3* and *FLT3*-ITD mutations have been associated with increased risk of relapse (4). Mutations in the myeloid transcription factor *CEBPA* have been detected in 10–15% of NK-AML (5) and are associated with favorable prognosis (5, 6).

Mutations of the nucleophosmin (*NPM1*) gene, usually occurring at exon-12 (7) and more rarely at exon-11 (8) represent the most common genetic alteration in AML-NK (50–60% of cases) and account for about one-third of all adult AML (7). This gene encodes for a ubiquitously expressed nucleolar protein (*NPM1 or B23*) that shuttles between the nucleus and cytoplasm and is implicated in multiple functions, including ribosomal protein assembly and transport, control of centrosome duplication and regulation of *Arf* tumor suppressor gene integrity (9). *NPM1* mutations result in the relocalization of *NPM1* from the nucleus into the cytoplasm (7), hence the term NPMc+ (cytoplasmic-positive) AML. NPMc+ AML with displays distinctive features including mutual exclusion with AML with recurrent genetic abnormalities, multilineage involvement, unique gene expression profile [up-regulation of Homeobox (*HOX*) genes and CD34 negativity], increased frequency of *FLT3*-ITD mutations, and favorable prognosis (in the absence of *FLT3*-ITD) (10).

Despite this progress, little is known about how *NPM1* mutants promote leukemia. The integration of a whole genomic approach including non coding RNAs may lead to an improved understanding of the *NPM1* biology. MicroRNAs (MiRNAs) are noncoding RNAs of 19–25 nucleotides in length that regulate gene expression by inducing cleavage or translational inhibition of their targets mRNA through base pairing to partially complementary sites (11). MiRNAs are involved in controlling cell development and differentiation, as well as apoptosis and proliferation (11). Recently, miRNAs expression has been linked to hematopoiesis and cancer (12–15). Here, we asked whether NPMc+ (mutated) and NPMc− (unmutated) AML cases may differ in their miRNA signature and whether the distinctive gene expression profiling of NPMc+ AML (*CD34* negativity and *HOX* genes overexpression) (7, 16, 17) may be dictated by a specific miRNA signature. The miRNAs expression in AML with *FLT3*-ITD and *FLT3* active loop mutations was also explored. Hereby, we report a unique miRNA signature associated with NPMc+ AML and provide evidence that support a role for miRNAs in the regulation of *HOX* genes in this leukemia subtype. Moreover, we found that *miR-155* was strongly but independently associated with *FLT3*-ITD mutations.

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B.F. and C.M. have applied for a patent on clinical use of NPM1 mutants. The authors declare no other conflict of interest.

Data deposition: The microarray dataset has been deposited in the ArrayExpress database, www.ebi.ac.uk/arrayexpress (accession no. E-TABM-429).

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Results

A Unique miRNA Signature Is Associated with *NPMc+ AML*. To identify miRNAs associated with *NPMc+* AML we profiled 85 *de novo* AML patients (55 NPMc+ and 30 NPMc−) [see supporting information (SI) Table 2 for patient characteristics] by using our previously validated miRNA platform (18) (SI Table 2). We first compared NPMc+ to NPMc− AML patients using the univariate *t* test within BRB tools (Class comparison). We found 36 up-regulated and 21 down-regulated miRNAs in NPMc+ NK-AML patients (Table 1). Among the up-regulated miRNAs in NPMc+ samples, we identified miR-*10a* and -*b*, several *let-7* and *miR-29* family members along with the *miR-15a-16-1* and *miR-17-18a-19a-20a* cluster. Additionally, to validate the results of the microarray platform we performed qRT-PCR for the most differentially expressed miRNAs (*miR-10a*, *miR-10b*, and *miR-22*) in 44 AML patients randomly chosen from the initial cohort (28 NPMc+ and 16 NPMc−). As shown in SI Fig. 5, the qRT-PCR data reproduced the chip results with accuracy. Unsupervised analyses of the data revealed that the AML samples segregated mainly in two main clusters (Fig. 1). Samples within the cluster 1 (Fig. 1 *Left*) had a higher frequency of NPMc+ than cluster 2 (Fig. 1 *Right*) (χ^2 P = 0.007). It is noteworthy that *miR-10a* and *miR-10b* expression clearly differentiates NPMc+ vs. NPMc− cases.

***MiR-10a* Correlates Positively with *HOXB4* Expression.** Notable the two most up-regulated miRNAs in NPMc+ AML (*miR-10a* and *miR-10b*) are embedded in *HOX* gene clusters (*miR-10a* is located between the *HOXB4* and *HOXB5* gene in chromosome 17q21 and *mir-10b* between the *HOXD3 and HOXD4* gene in chromosome 2q31). It has been reported that in mouse embryos, *miR-10a* and *-10b* expression closely follows their host *HOX* cluster expression during development (19), suggesting that these miRNAs may be regulated by the same CIS elements that also regulates HOX genes. To investigate whether *miR-10a* correlated with its flanking *HOXB4* gene, we measured *miR-10a* and *HOXB4* in 18 AML patients from the original cohort (NPMc+ = 9 and NPMc− = 9) by qRT-PCR. Indeed, we identified a positive correlation between *miR-10a* and *HOXB4* expression (R = 0.57, P = 0.01, Pearson correlation test). However, no correlation was found between *miR-10b* and its flanking *HOXD3* gene (data not shown).

***MiR-204* Targets *HOXA10* and *MEIS1*.** Among the down-regulated miRNAs in patients with *NPMc+* AML, there are several miRNAs predicted to interact with *HOX* genes according to three available "in silico" target prediction software [Targetscan (20), Pictar (21), and RNAhybrid (22)] (SI Table 3). Indeed, few miRNAs have been shown to regulate *HOX* genes and play important roles during early development (14, 19). We hypothesize that the high *HOX* genes expression in NPMc+ AML may be due in part to the down-regulation of *HOX* regulators miRNAs in this subgroup. To start unraveling the role for miRNAs in *HOX* regulation in NPMc+ AML, we validated the predicted *HOX* targets for the down-regulated miRNAs in NPMc+ AML by performing Western blotting using HOXA9, HOXA10, and MEIS1 antibodies in AML cell lines after transfection of the candidate oligonucleotides miRNAs or scrambled oligonucleotides (SI Table 3 and SI Fig. 6). To perform this screening, we used the OCI-AML3 cell line (which harbors a *NPM1* mutation) (23) and the MEG-01 cell line (both cell lines with high expression of *HOXA9*, *HOXA10*, and *MEIS1*). As shown in Fig. 2 *A* and B, there was a robust down-regulation of HOXA10 and MEIS1 protein in both AML cells lines transfected with *miR-204* but not with the other miRNAs or scrambled oligonucleotides. As an additional control, we transfected antisense oligonucleotides against *miR-204* in K562 and MEG-01 cells and measured *MEIS1* and *HOXA10* protein expression after 48 h (Fig.

Table 1. miRNAs differentially expressed between NPMc+ (mutated) vs. NPMc− (unmutated) AML patients

miRNA	Parametric *P* value	FDR*	Fold change
Up-regulated in NPMc+			
hsa-miR-10a	<1e-07	<1e-07	20
hsa-miR-10b	<1e-07	<1e-07	16.67
hsa-miR-100	<1e-07	<1e-07	4.35
hsa-let-7a-3	<1e-07	<1e-07	3.45
hsa-miR-21	3.16E-05	4.24E-04	3.33
hsa-let-7f	2.63E-04	1.97E-03	3.33
hsa-let-7c	4.00E-07	1.45E-05	3.33
hsa-miR-16b	2.14E-04	1.80E-03	3.23
hsa-miR-16a	7.21E-04	4.16E-03	2.94
hsa-let-7a-2	2.00E-07	8.50E-06	2.94
hsa-miR-19b	1.29E-04	1.31E-03	2.94
hsa-miR-18a	8.60E-06	1.97E-04	2.86
hsa-miR-29c	2.59E-04	1.97E-03	2.78
hsa-miR-29a	3.97E-04	2.59E-03	2.78
hsa-let-7a-1	1.60E-06	4.52E-05	2.71
hsa-miR-16-1	5.07E-04	3.07E-03	2.63
hsa-miR-29b	1.11E-03	5.64E-03	2.56
hsa-miR-24	2.32E-04	1.84E-03	2.51
hsa-miR-20	1.21E-05	2.20E-04	2.44
hsa-miR-17	3.83E-03	1.52E-02	2.33
hsa-miR-369	1.23E-03	6.11E-03	2.27
hsa-let-7 g	1.02E-03	5.30E-03	2.27
hsa-let-7d	7.60E-06	1.93E-04	2.13
hsa-miR-19a	9.30E-06	1.97E-04	2.13
hsa-miR-106	5.52E-05	6.48E-04	2.13
hsa-miR-16-2	3.35E-04	2.30E-03	2.04
hsa-miR-195	1.50E-03	7.32E-03	2.04
hsa-miR-102	8.10E-03	2.66E-02	2.00
hsa-miR-152	3.17E-03	1.30E-02	1.89
hsa-miR-9	2.03E-03	9.09E-03	1.85
hsa-miR-142	2.04E-03	9.09E-03	1.82
hsa-miR-378	3.14E-03	1.30E-02	1.82
hsa-miR-98	1.69E-03	8.12E-03	1.64
hsa-miR-374	3.15E-04	2.22E-03	1.64
hsa-miR-15a	1.91E-03	8.99E-03	1.61
hsa-miR-155	6.78E-03	2.39E-02	1.54
Down-regulated in NPMc+			
hsa-miR-22	1.10E-05	2.15E-04	0.31
hsa-miR-192	2.43E-05	3.86E-04	0.67
hsa-miR-128a	2.93E-05	4.24E-04	0.65
hsa-miR-383	9.20E-05	1.02E-03	0.52
hsa-miR-373	2.07E-04	1.80E-03	0.57
hsa-miR-324	2.20E-04	1.80E-03	0.58
hsa-miR-127	2.74E-04	1.99E-03	0.45
*hsa-miR-373**	4.61E-04	2.85E-03	0.58
hsa-miR-139	5.73E-04	3.38E-03	0.49
hsa-miR-193b	9.32E-04	5.01E-03	0.55
hsa-miR-145	9.38E-04	5.01E-03	0.65
hsa-miR-498	2.23E-03	9.78E-03	0.56
hsa-miR-135a	2.40E-03	1.02E-02	0.57
hsa-miR-299	2.42E-03	1.02E-02	0.66
hsa-miR-429	3.90E-03	1.52E-02	0.63
hsa-miR-493	3.97E-03	1.53E-02	0.53
hsa-miR-326	4.65E-03	1.76E-02	0.63
hsa-miR-204	4.89E-03	1.80E-02	0.61
hsa-miR-198	8.20E-03	2.66E-02	0.65
hsa-miR-486	8.23E-03	2.66E-02	0.45

MiRNAs are sorted by *P* value of the univariate test (BRB tools). The first 56 genes are significant at the nominal 0.01 level of the univariate test.
*FDR, False discovery rate or *q* value is the expected percentage of genes identified by chance.

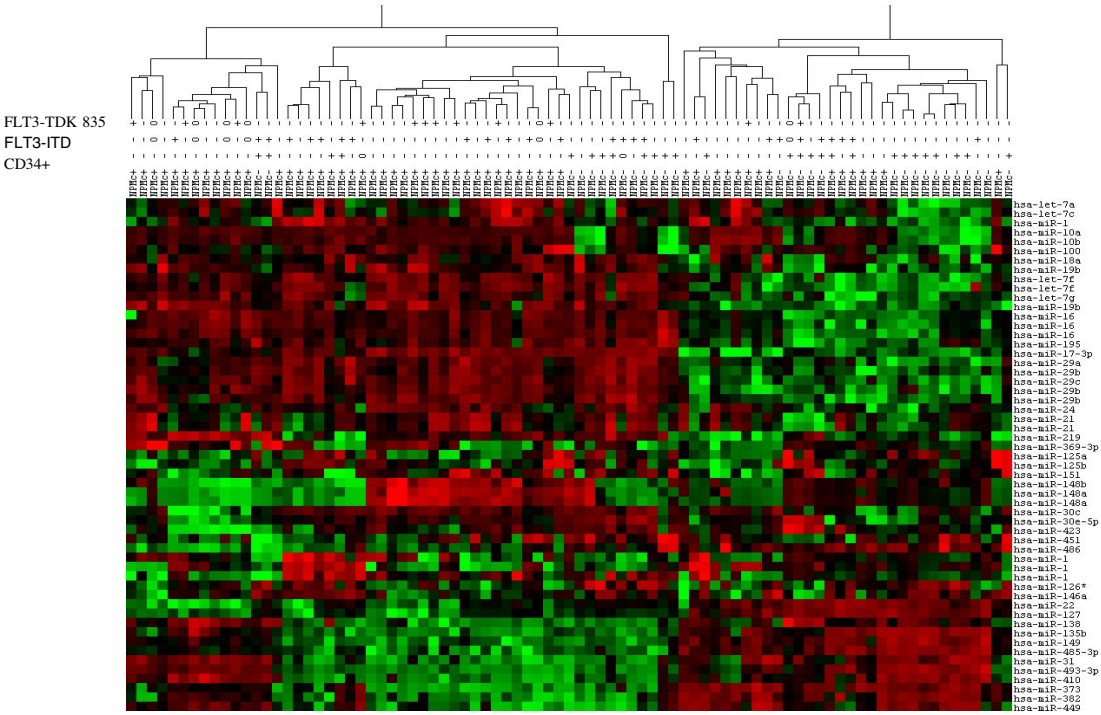

Fig. 1. Hierarchical cluster analysis of NK-AML patients. Overview of two-way (genes against samples) hierarchical cluster (Euclidean distance) of 85 AML samples using the genes that varies the most between samples. Mean centered signal intensities of gene-expression are depicted by a log-transformed (2 scale). Color areas indicate relative expression of each gene with respect to the gene median expression (red above, green below the median value and black, samples with signal intensity to background of 2 or less). Some miRNAs are represented by more than one probe. As shown, the clustering is mainly determined by the presence of cytoplasmic nucleophosmin (NPMc+). Among the *NPMc*+ positive samples, we identified also many subgroups. Patients with *FLT3*-ITD+ segregated among the different clusters.

2*C*). We observed up-regulation of *MEIS1* and *HOXA10* in both cell lines after transfection with *miR-204* antisense oligonucleotides with respect to scrambled oligonucleotides. To validate further these interactions, we cloned the 3′ untranslated region (3′ UTR) of *MEIS1* predicted to interact with *miR-204* into a luciferase reporter vector and cotransfected with *miR-204* or scrambled

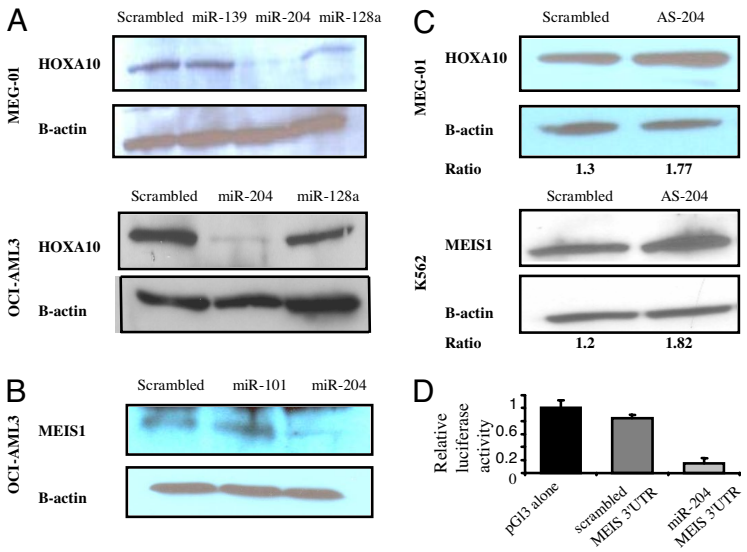

Fig. 2. *MiR-204* targets *HOXA10* and *MEIS1* in AML. (*A*) Western blotting showing HOXA10 protein using whole cell lysates from MEG-01 and OCI-AML3 cell lines transfected with scrambled oligonucleotides or *miR-139*, *miR-128a*, and *miR-204*. The blots were stripped and reprobed with β-*actin* for loading control. (*B*) Western blotting showing MEIS1 protein expression in OCI-AML3 cell lines transfected with scrambled oligonucleotides or sense *miR-204* and *miR-101*. (*C*) Western blotting showing HOXA10 and MEIS1 protein expression in MEG-01 and K562 cells transfected with scrambled oligonucleotides or antisense oligonucleotides against *miR-204*. The numbers below the immunoblot images represent the intensity of the HOXA-10/MEIS bands relative to the β-*actin* expression. (*D*) Relative luciferase activity in MEG01 cells transiently cotransfected with the luciferase reporter vector containing the 3′ UTR of *MEIS1* with *miR-204* or scrambled oligonucleotides. The results are presented as a fold difference in the luciferase/*Renilla* ratios with respect to the luciferase reporter constructs transfected with scrambled oligonucleotides.

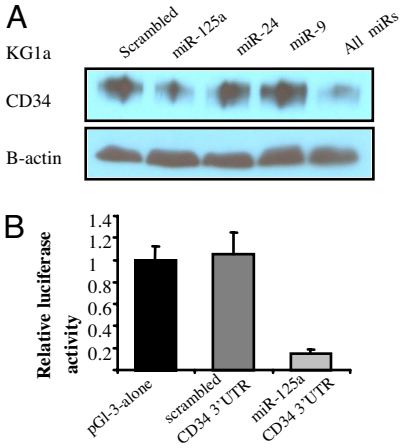

Fig. 3. *MiR-125a* targets CD34. (*A*) Western blotting showing CD34 protein expression in KG1a cells 48 h after transfection with scrambled oligonucleotides or sense *miR-125a*, *mIR-24*, *miR-9*, or all combined oligonucleotides (*B*) Relative luciferase activity in KG1a cells transiently cotransfected with the luciferase reporter vector containing the 3′ UTR of CD34 predicted to interact with *miR-125a* or scrambled oligonucleotides. The results are presented as a fold difference in the luciferase/*Renilla* ratios with respect to the empty reporter (pGl3 alone).

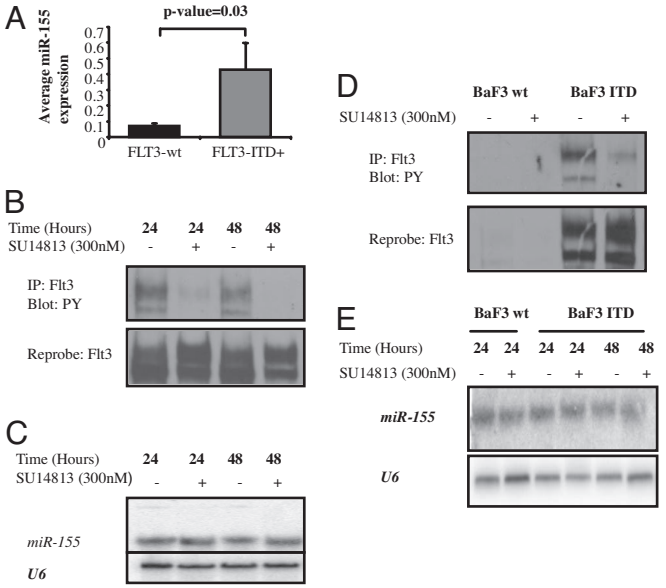

Fig. 4. *MiR-155* is overexpressed in *FLT3*-ITD+ AML. (*A*) Average *miR-155* expression in 32 NK-AML patients with *FLT3*-ITD+ ($n = 10$) and *FLT3*-wt ($n = 22$) after normalization with *18s* and $2\Delta C_t$ calculations. The average *miR-155* expression values between the two groups were compared by using *t* test. (*B*) IP/Western blot. MV4-11 cells were incubated for 24 and 48 h with SU14813 (300 nM) or DMSO (vehicle control). Lysates were generated and an immunoprecipitation was performed with an antibody for FLT3. SDS/PAGE was performed, followed by a Western blot with an antibody for phosphotyrosine. Subsequently, the blot was stripped and reprobed with anti-FLT3. (*C*) Northern blotting of *miR-155* in MV4-11 cells incubated for 24 and 48 h with SU14813 (300 nM) or DMSO (vehicle control). Total RNA was obtained with TRIzol and Northern blotting was performed with a probe antisense to *miR-155*. The blot was stripped and reprobed with U6 for loading control. (*D*) IP/Western blot. BaF3 and BaF3 *FLT3*-ITD cells were incubated for 24 h with SU14813 (300 nM) or DMSO (vehicle control). Lysates were generated and an immunoprecipitation was performed with an antibody for FLT3. SDS/PAGE was performed, followed by a Western blot with an antibody for phosphotyrosine. Subsequently, the blot was stripped and reprobed with anti-FLT3. (*E*) Northern blotting for *miR-155* in BaF3 and Baf3 *FLT3*-ITD clones incubated for 24 and 48 h with SU14813 or DMSO (control) as described. The blot was stripped and reprobed with U6 for loading control.

oligonucleotides into the MEG-01 cell line. A marked reduction in the luciferase/*Renilla* ratio was seen for *MEIS1* constructs transfected with *miR-204* (Fig. 2*D*).

CD34 Regulation by miRNAs. We have previously shown that AML blasts from *NPMc+* patients are frequently *CD34* negative (7, 16, 17). Interestingly, many miRNAs up-regulated in *NPMc+* AML are predicted to target *CD34*, including *miR-9*, *miR-24*, and *miR-125a*. To confirm these interactions, we transfected *miR-9*, *miR-24*, and *miR-125a* oligonucleotides or control oligonucleotides into the CD34+ AML cell line KG1a using nucleoporation and measured CD34 protein expression using Western blotting. As shown in Fig. 3*A*, a marked reduction of CD34 protein was evident in the *miR-125a* transfected cells compared with the scrambled oligonucleotides. To further validated this interaction, we performed luciferase reporter assays, where the 3′ UTR of the *CD34* gene predicted to interact with *miR-125a*, was cloned in to the luciferase reporter assay and cotransfected with *miR-125a* oligonucleotide or scrambled oligonucleotides into MEG-01 cell lines using lipofectamine. As shown in Fig. 3*B*, 85% of reduction in the luciferase normalized ratios was observed in the cells transfected with *miR-125a* compared with the controls.

***MiR-155* Is Up-Regulated in *FLT3*-ITD+ AML.** Using the univariate *t* test (BRB), we identified three up-regulated miRNAs (*miR-155*, *miR-302a*, and *miR-133a*) in *FLT3*-ITD+ as compared with *FLT3-wt* AML patients. This signature had a FDR of 0% at a significance level of $P < 0.01$. Then, we compared the miRNAs expression in *FLT3*-ITD+ to *FLT3-wt* in NPMc+ patients. Only *miR-155* was up-regulated at a significance level of $P < 0.01$. We validated the *miR-155* up-regulation in *FLT3*-ITD+ patients by measuring *miR-155* using a different method (qRT-PCR) in a group of 32 randomly chosen AML patients from the original cohort (Fig. 4*A*). AML patients with *FLT3*-TKD mutations were not associated with any distinctive miRNA signature. To further obtain insights about the relationship among *FLT3*-ITD and *miR-155*, we carried out an experiment to investigate whether blocking *FLT3* signaling using a potent *FLT3* inhibitor (SU14813) in two *FLT3*-ITD positive AML cell lines (MV4-11 and Molt-13) will impact on *miR-155* expression (Fig. 4*B*). As shown in Fig. 4*C* and SI Fig. 7, the *miR-155* expression remained unchanged after 48 h of successful *FLT3* signaling inhibition in the two cell lines tested. Furthermore we performed an additional control experiment where we generated stable Baf3 cell clones harboring the human *FLT3* gene with ITD by using retroviral infection, and measured *miR-155* expression in Baf3 wt and in Baf3 *FLT3*-ITD by Northern blotting (Fig. 4 *D* and *E*). We did not observe any significant difference in *miR-155* expression between Baf3 wt and Baf3 *FLT3*-ITD. In addition, *miR-155* expression did not change after treatment of Baf3 *FLT3*-ITD cells with the *FLT3* inhibitor (SU14813) for 48 h (Fig. 4 *D* and *E*).

Discussion

Employing a microarray platform, we systematically analyzed the miRNA expression of AML patients with known subcellular localization/mutation status of *NPM1* and identified a strong signature associated with the presence of cytoplasmic mutated nucleophosmin. Among the miRNAs up-regulated in NPMc+ AML, there were three families of tumor suppressor miRNAs: *miR-15-a*/*miR-16-1*, *miR-29*s (a/b/c), and the *let-7* (*let-7a*, *let-7b*, and *let-7f*). Low level of *let-7a* has been associated with short survival in lung cancer after surgery (24), and low level of *miR-29b* has been associated with shorter survival in CLL (25). Previous reports have shown that NK-AML with *NPM1*-mutated/*FLT-3*-ITD negative genotype have a good prognosis (reviewed in ref. 10). This clinical observation is in

keeping with our finding of several up-regulated tumor suppressors miRNAs in NPMc+ AML.

Consistent with previous reports, we have shown that *HOXB4* expression correlated positively with its embedded miRNA *miR-10a*, suggesting that this miRNA may be regulated by CIS elements that also regulate *HOX* genes (14, 19, 26). However, no correlation was found for *miR-10b* and its flanking *HOXD3* gene. This observation raises the question about the possibility of microarray cross hybridization, because the two miRNAs differ only in one nucleotide. Nevertheless, *miR-10b* up-regulation in NPMC+ was also detected by qRT-PCR, which is more specific. Finally, whether the *HOX* embedded miRNAs are innocent bystanders or have a critical role during leukemogenesis remained to be explored.

High expression of *HOX* genes is one of the most distinguishing features of NPMc+ AML (16, 17, 27). Remarkably, the pattern of perturbed *HOX* gene dysregulation in *NPM1*-mutated AML clearly differ from that observed in AML carrying rearrangements of *MLL* gene, because up-regulation of group-B *HOX* genes (especially *HOXB2* and *B6*) is observed in *NPM1*-mutated but not *MLL*-rearranged AML (17). This finding suggests that dysregulation of *HOX* gene expression in *NPM1* mutated AML occurs via a different mechanism than in AML with *MLL* rearrangement. Up-regulation of *HOX* expression in *MLL*-rearranged leukemia has been related to the direct binding of *MLL* fusion proteins to *HOX* gene promoters (28), but this is unlikely to be the case for *NPM1*-mutated AML. A major goal of our study was to assess whether miRNAs may contribute to the *HOX* up-regulation in NPMc+ AML, and, indeed, we demonstrated that *miR-204*, which is down-regulated in NPMc+ AML targets *HOXA10* and *MEIS-1*. Over-expression of *HOXA10* in murine hematopoietic stem cells perturbs myeloid differentiation and leads to AML (29). Likewise, enforced expression of *HOXA9* and *MEIS1* in mice induces AML after a short latency (30). This report links miRNAs in the regulation of *HOX* genes involved in leukemia. However, it remains to be clarified whether this finding reflects derivation from hematopoietic progenitors that physiologically tune *HOX* gene expression through their miRNA apparatus, or whether the NPM1 mutant protein contributes to leukemogenesis by inducing down-regulation of specific miRNAs that control *HOX* gene expression. Hypothetically, the mutant could target a myeloid committed progenitor and confer to this cell self-renewal capability through up-regulation of *HOX* genes, thus explaining why, despite frequent multilineage involvement (8), lymphoid lineage is typically not involved in NPMc+ AML (31).

It is of interest that several miRNAs up-regulated in NPMc+ AML are predicted to target CD34, a gene whose expression is frequently down-modulated in these leukemias (7, 16). Of the several miRNAs up-regulated in NPMc+ AML, we have validated that only *miR-125a* targets CD34. However, *miR-125a* is not consistently up-regulated in NPMc+ AML (only identified in some cases by using the unsupervised clustering), and its contribution in the CD34 regulation of NPMc+ AML remains to be clarified.

The analysis of miRNA expression in the whole group of NK-AML patients with known *FLT3* status revealed a distinct signature. A surprising result was the identification of *miR-155* as overexpressed in *FLT3*-ITD+ patients. Previous reports have established that *miR-155* overexpression halts myeloid development (32) and induced B cell lymphoma/leukemia in a transgenic mice model (33). Here, we also demonstrated, by inhibiting *FLT3* signaling in *FLT3*-ITD+ cell lines, that *miR-155* expression seems to be independent from *FLT3* signaling. Previous work showed that transgenic mice models for *FLT3*-ITD do not develop acute leukemia, but a chronic myeloproliferative disorder (34). Given the clear independent association of *miR-155* with *FLT3*-ITD mutations in AML and the known myeloid blocking effects that result from *miR-155* overexpression, it is tempting to hypothesize that, perhaps, *miR-155* could play a role in leukemogenesis.

Our findings have also potential clinical and therapeutic implications. The demonstration that NPMc+ AML carries a distinct microRNA signature further supports the view that *NPM1* mutations identify a distinct AML genetic entity (showing a normal karyotype in >90% of cases). Finally, our studies identify at least one miRNA (*miR-204*) to be explored for therapeutic intervention.

Patients and Methods

Patient Samples. The study was carried out on diagnostic bone marrow samples from 85 adult AML patients: 25 patients from the Institute of Hematology, University of Perugia; 29 cases from the Gruppo Italiano Malattie Ematologiche dell' Adulto (GIMEMA); and 31 cases from the Munich Leukemia Laboratory (MLL). The characteristics of these patients are shown in SI Table 2. All patients gave informed consent for the bone marrow biopsy (used for immunohistochemistry) and aspirate for cryopreservation (used for molecular studies). Approval was obtained from the institutional board review of each institution.

***NPM1* and *FLT3* Mutation Analysis.** *NPM1* mutations status was assessed by using immunohistochemistry/Western blotting, or mutation analysis. A bone marrow biopsy was available in 54 cases: 37 showed aberrant cytoplasmic NPM (NPMc+) that is fully predictive of *NPM1* mutations (35) whereas 19 cases showed the nucleus-restricted reactivity (NPMc−) typical of cases carrying a wild-type *NPM1* gene (35). In 19 of 54 cases, the immunohistochemical findings were also confirmed by Western blotting with specific anti-NPM mutants antibodies (36). *NPM1* mutations analysis carried out as previously described was available in 59 cases, including 28 patients who were previously evaluated by immunohistochemistry (37). *FLT3*-ITD and *FLT3*-TKD mutations were investigated in 79 cases, as described in ref. 37.

MiRNA Microarrays Experiments. RNA extraction and miRNA microchip experiments were performed as described in detail elsewhere (18). The miRNA microarray is based on a one-channel system (18). Five micrograms of total RNA was used for hybridization on the OSU custom miRNA microarray chips (OSU_CCC version 3.0), which contains ≈1,100 miRNA probes, including 345 human and 249 mouse miRNA genes, spotted in duplicates.

Real-Time Quantification of microRNAs. The single tube TaqMan miRNA assays were used to detect and quantify mature miRNAs as described in ref. 38, using PCR 9700 Thermocycler ABI Prism 7900HT and the sequence detection system (Applied Biosystems). Normalization was performed with *18s*. Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated by using the comparative $2^{\Delta}C_t$ method (39).

Data Analysis. Microarray images were analyzed by using GenePix Pro. Average values of the replicate spots of each miRNA were background-subtracted. Quantiles normalization was implemented by using the Bioconductor package/function. Differentially expressed microRNAs were identified by using the univariate *t* test within the BRB tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). Cluster and Java TreeView were used to build the unsupervised tree. The filtering was based on the variance for the gene across the arrays. Additional details are reported in *SI Methods*. The microarray dataset is deposited in Array-Express. Fisher's exact test, *t* test, and χ^2 were used to compare baseline patient characteristics and average miRNA expression between groups of patients. All reported *P* values were two-sided and obtained by using the SPSS software package (SPSS 10.0).

Cell culture, transfections, and luciferase reporter assays are described in detail in *SI Methods*.

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