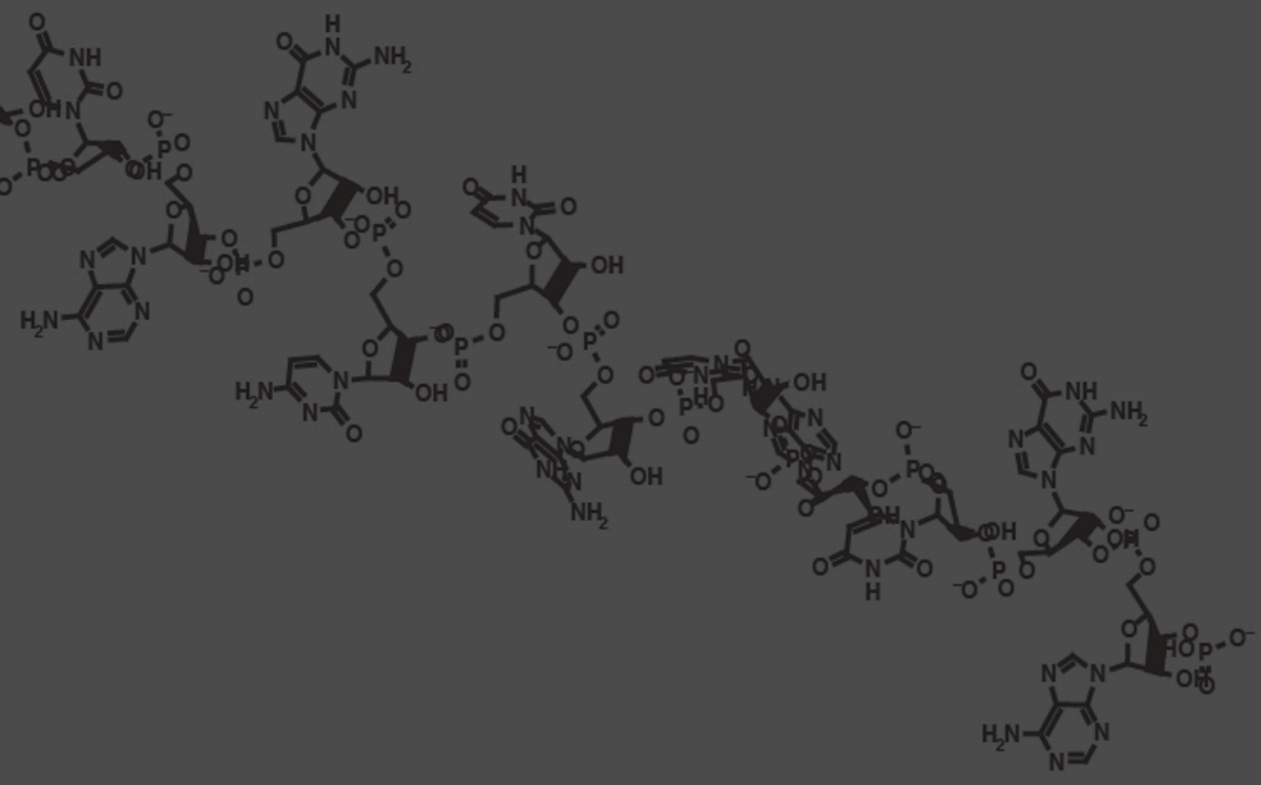
Prognostic and Functional Relevance of microRNAs in Acute Myeloid Leukemia



Su Ming Sun

Prognostic and Functional Relevance of microRNAs in Acute Myeloid Leukemia

De prognostische en functionele relevantie van microRNAs in acute myeloïde leukemie

proefschrift
ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus
Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
dinsdag 12 maart 2013 om 13:30 uur

door

Su Ming Sun

geboren te Delft

Promotiecommissie

Promotor:	Prof.dr. B. Löwenberg
Overige leden:	Prof.dr. H.R. Delwel
	Prof.dr. L.H.J. Looijenga
	Prof.dr. G.J. Ossenkoppele
Copromotor:	Dr. M. Jongen-Lavrencic

ISBN: 978-94-6169-358-7

Cover design: Su Ming Sun
Layout: Egied Simons
Printing: Optima Grafische Communicatie, Rotterdam

CONTENTS

Aan mijn ouders

1

General introduction

Acute Myeloid Leukemia

The process in which the various blood cells are produced is called hematopoiesis. In adult humans this takes place in the bone marrow. All functional hematopoietic cells develop from hematopoietic stem cells. These cells have a self-renewal capacity and can give rise to the different mature blood lineages. This differentiation process follows a highly hierarchical order, through various specific progenitor stages and maturation steps towards the different highly specialized mature blood cells (Figure 1). The mature blood cells have a limited lifespan and need to be constantly produced. This process is strictly regulated by specific hematopoietic growth factors to ensure that appropriate numbers of blood cell types are available to maintain homeostasis.[1]

Acute myeloid leukemia (AML) is a type of blood cancer where the maturation in the myeloid lineage, i.e. towards granulocytes and monocytes, is impaired, while the survival and proliferation of these aberrant cells is enhanced. These functional deficiencies are caused by cellular defects in various pathways, for example transcription machinery and the receptor signaling pathway, which control the physiological processes of cell survival, proliferation and differentiation. Genetic and epigenetic abnormalities may underline these regulatory anomalies. As a consequence of the maturation capability of the leukemic progenitor cells, the number of relatively immature cells increases and this eventually leads to bone marrow failure. The lack of functional mature granulocytes, monocytes, erythrocytes and platelets is responsible for the clinical symptoms, such as infections, fatigue and hemorrhage.[2]

From a molecular biology point of view, AML is a heterogeneous disease. Each AML is characterized by different underlying genetic and epigenetic abnormalities, such as particular chromosomal rearrangements or other genetic abnormalities of which many are recurrent (Table 1) and are involved in the pathogenesis/biology of AML.

Biology of AML

Many genetic and cytogenetic aberrations involve specific hematopoietic transcription factors and genes that play a role in signal transduction. Over the years a lot of these aberrations have been extensively studied for their role in the pathogenesis of AML.

Studies with various mouse models have demonstrated that most single aberrations do not lead to a full AML phenotype in mice. This has led to the postulation of the multi-hit model, where multiple hits are required for the development of AML. Based on this model, and in a somewhat simplified concept, the great variety of genetic aberrations has

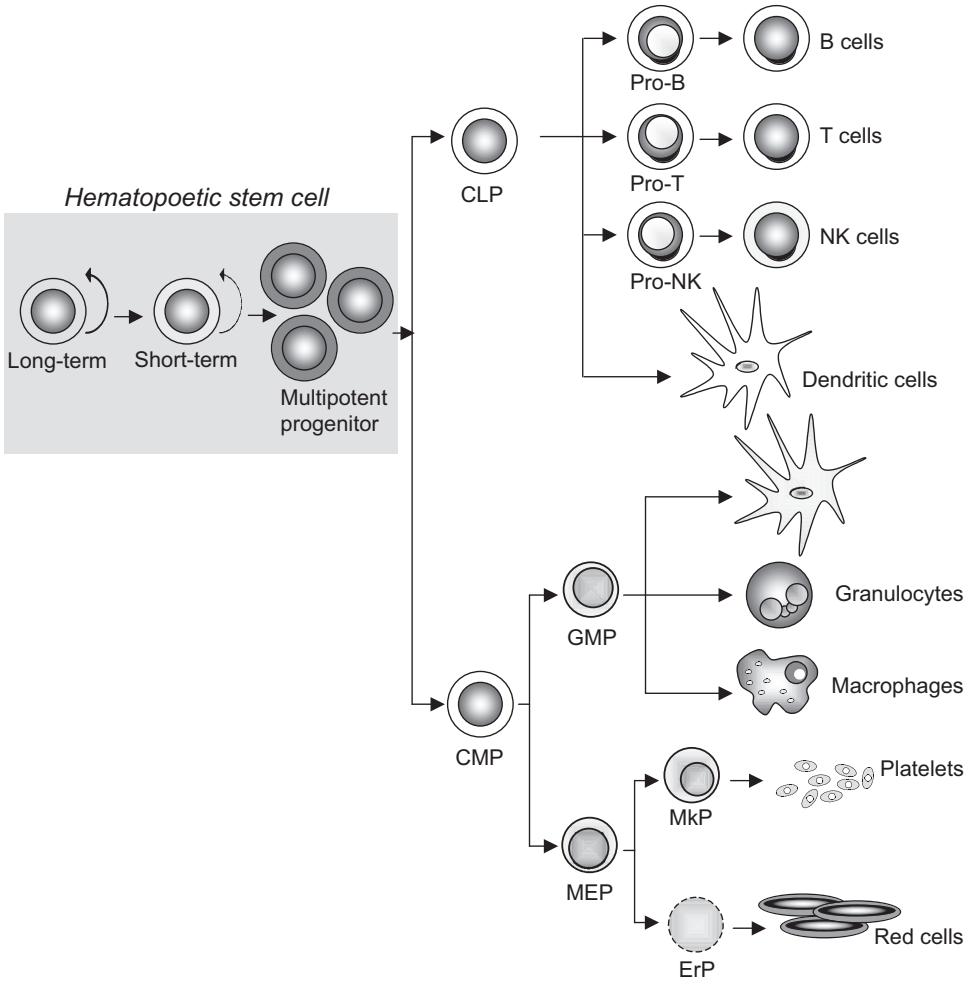


Figure 1. Schematic representation of the hematopoiesis.
Scheme is adapted from Reya.[123] The hematopoietic stem cells (HSCs) can be divided into long-term HSCs, short term HSCs and multipotent progenitors. They branch off to give rise to common lymphoid progenitors and the common myeloid progenitor. The common lymphoid progenitor gives rise to all lymphoid cells, i.e. B-cells, T-cells, natural killer cells (NK cells) and dendritic cells. The common myeloid progenitor branches off again into granulocyte macrophage precursors (GMPs) and megakaryocyte erythrocyte precursors (MEPs). Subsequently GMPs give rise to granulocytes, macrophages and dendritic cells and MEPs to megakaryocyte precursors (MkPs) and erythrocyte precursors (ErPs), which differentiate further into platelets and red blood cells respectively.

Table 1.

Recurrent cytogenetic and genetic aberrations	Related fusion gene	Class	Frequency	References
Chromosomal aberrations		**u.d.**		
Normal karyotype		u.d.	45%	
Complex*		u.d.	11%	
t(15;17)(q22;21)	PML-RAR	II	10%	87
plus 8		u.d.	9%	
7/7q-		u.d.	8%	
5/5q		u.d.	7%	
Y		u.d.	4%	
abn(12p)		u.d.	3%	
plus 21		u.d.	3%	
del(9q)		u.d.	2%	
Inv(16)(p13q22)/t((16;16)(p13;q22)	CBFB-MYH11	II	6%	
t(8;21)	AML1-ETO	II	5%	3,88
11q23 abnormalities	MLL fusions	II	2%	
t(9;11)	MLLT3-MLL	II	2%	
inv(3)(q21q26)/t(3;3)(q21;q26)		u.d.	1%	
t(6;9)	DEK-CAN	u.d.	1%	
Molecular aberrations				
NPM1		u.d.	25-35%	89
DNMT3A		u.d.	20-22%	16,90
FLT3-ITD		I	21-24%	11,91
ASXL1		u.d.	17-23%	92,93
CEBPA		II	5-14%	94,95
TET2		u.d.	7.6-13.2%	14,15
WT1		u.d.	10%	90,96
IDH1/IDH2		u.d.	6-10%	97,98
KIT		I	3-9%	99
NRAS		I	10%	100,101
FLT3-TKD		I	5-7%	24,90
KRAS		I	5%	101
JAK2		u.d.	2%	102

u.d., undetermined

* Complex karyotype has been defined as the presence of 3 or more chromosome abnormalities in the absence of t(8;21), inv(16) or t(16;16), and t(15;17)

† Frequencies based on Mrozek et al, Dohner et al and Takahashi et al 2011[22,103,104]

been classified into two different cooperating classes.[17-19] Class I aberrations lead to a proliferative advantage and Class II aberrations result in an impairment of differentiation. The model hypothesizes that mutations in both classes (Table 1) are needed to develop AML. In line with this model, many of these aberrations are mutually exclusive from each other. To illustrate the biology of AML, some of the most frequent aberrations in AML are now described.

Core-binding factor leukemia, involving translocation t(8;21) or inv(16) is characterized by a fusion between the genes coding for members of the core binding transcription factor and other proteins. In the case of translocation t(8;21), these genes are runt-related transcription factor 1 (*RUNX1*) with eight twenty one (*ETO*). In inv(16) translocation, however, these involve fusions of the core binding factor beta (*CBFB*) with myosin heavy chain 11 (*MYH11*). The core-binding factor transcription complex is an important master hematopoietic regulator expressed in hematological tissues. The gene fusions lead to an altered function of the master regulator. ETO is normally a repressor that interacts with nuclear co-repressors and histone deacetylases. Fusion with RUNX1 alters the function of RUNX1 from an enhancer to a suppressor.[3] In the case of inv(16), the binding capacity of the fusion product is enhanced due to an additional RUNX domain. The fusion gene disturbs the function of normal CBF complex by interacting with repressor proteins and by sequestering the wildtype RUNX1 in the cytoplasm, thereby preventing binding to DNA.[4]

In 14% of the AML patients, CAATC binding protein alpha (*CEBPA*), another key gene coding for an important hematopoietic transcription factor, is mutated. Mutations sometimes occur in a single allele but in most cases both alleles are affected. Mutations in *CEBPA* in AML are generally located at the N-terminus or at the C-terminus. The N-terminal mutations result in shortened protein that acts as a dominant negative. C-terminal mutations are located in the basic-leucine zipper region and are suggested to have a loss of function effect on the DNA binding of CEBPA.[56]

Another example of recurrent aberration in AML is translocation t(15;17), which occurs in about 10% of the AML cases (Table 1). Translocations involving t(15;17) result in the fusion of the genes coding for promyelocytic leukemia *(PML*) and retinoic acid receptor-alpha (*RARA*). Normally PML is the main constituent of nuclear bodies, which are involved in various biological processes. RARA is a nuclear receptor that forms a complex with retinoid X receptor (RXR) and in the absence of a ligand the complex acts as a repressor. Upon binding with a ligand, gene-activating proteins are recruited resulting in gene activation. The fusion product PML-RARA is suggested to result in an altered

response to the physiological ligands and is thought to act as a constitutive repressor.[7] In addition, the fusion also changes the localization of the nuclear bodies in a more micro-speckled localization pattern.[8] Leukemia with translocation t(15;17) is a prime example of how biological understanding can lead to better treatment. Namely, treatment with high dose all-trans retinoic acid (ATRA) induces differentiation of malignant cells, which significantly contributes to improved survival outcome of AML patients with PML-RARA.[9,10]

Another type of mutation implicated in the biology of AML is found in the gene coding for FMS-like tyrosine kinase 3 (*FLT3),* which is a membrane-bound receptor kinase. *FLT3* is normally expressed in myeloid and lymphoid progenitors and the expression is lost when the cells differentiate. Upon ligand binding, FLT3 attenuates the proliferation and differentiation of normal progenitor cells, through a series of signaling cascades. An internal tandem repeat mutation (*FLT3*-ITD) in the juxtamembrane domain results in constitutive activation of the receptor and subsequent downstream signaling, leading to increased proliferation.[11,12]

Recently, a new class of mutations has been identified, including mutations in Ten-Eleven-Translocation 2 (*TET2*)[13-15] and DNA (cytosine 5) methyl transferase 3A (*DNMT3A*).[14,16] These genes are involved in the regulation of the methylation status of DNA, by exchanging the 5-methyl cytosine for a 5-hydroxy methyl cytosine and *denovo* methylation of DNA respectively. This suggests that epigenetic changes also play a role in the pathogenesis of these AMLs.

Classification and Prognosis

In the past AML was classified solely by morphology, as described by the French-American-British classification. The current World Health Organization (WHO) classification also considers recurrent genetic aberrations.[21] In terms of survival outcome, there is considerable variation between different AML subtypes. Identifying disease-related prognostic risk factors at diagnosis is useful for determining the proper treatment decision strategy for each individual AML patient. Many of the recurrent genetic aberrations involved in the pathogenesis of AML, predict survival outcome in patients with AML (Table 2). These aberrations with prognostic relevance are used for risk stratification, such as the method recommended by the European Leukemia Net[22] or another risk stratification approach involving monosomal karyotype in AML.[23] Various other leukemia-related prognostic factors have been identified, for example altered gene expression levels that are specific for particular AML subtypes (Table 2).

Table 2 .

Prognostic Markers	Favorable/Adverse	Subset	References
Cytogenetic			
inv(16)	Favorable		105
t(15;17)	Favorable		105
t(8;21)	Favorable		105
FLT3-TID	Adverse		106
MLL fusion	Adverse		107
t(6;9)	Adverse		108
t(9;11)	Adverse		105
Mutation			
CEBPA	Favorable	Normal Karyotype	109
DNMT3A	Favorable		14,90
NPM1	Favorable	Normal Karyotype	110
IDH1/2	Adverse		98,111
TET2	Adverse		14,15
TP53	Adverse		112,113
Polymorphism			
WT1	Favorable		114
Expression			
miR-181a	Favorable	Normal Karyotype	115
miR-3151	Favorable	Normal Karyotype	116
BAALC	Adverse	Normal Karyotype	117
CD34+	Adverse	Intermediate	118
ERG	Adverse	Normal Karyotype	119
EVI1	Adverse		120
MN1	Adverse	Normal Karyotype	121
ID1	Adverse	Normal Karyotype	122

miRNAs

In 2001, the human genome project reached its first milestone with the sequencing of the human genome. About 2% of the human genome was found to encode around 20,000 genes. The function of the remaining 98% of DNA that does not code for protein was unknown and was previously designated as junk DNA.[24] With the completion of the human genome Encyclopedia of DNA Elements (ENCODE) project it became clear that 76% of the DNA in the full genome was transcribed, even though only 2% encodes for proteins, suggesting a large fraction of non-coding RNA.[25] Among the different classes of non-coding RNA, a subclass has been identified and designated microRNAs (miRNAs).

miRNAs comprise a class of conserved negative regulators, consisting of single-stranded non-coding RNA of 20~22 nt (nucleotides) long. Initially the first miRNA, *lin-4* was discovered in *Caenorhabditis elegans* (*C. elegans*) in 1993.[26,27] *Lin-4* is an essential gene for the normal temporal control of different developmental stages. Worms carrying *lin-14* mutations, which lead to elevated levels of *lin-14*, display almost identical phenotypes as *lin-4 null* worms. In addition,*lin-4* null mutations result in increased levels of *lin-14*, suggesting a role of *lin-14* as a negative regulator for *lin-4*. Surprisingly, the interaction between *lin-4* and *lin-14* was not mediated by a protein, but was mapped to an antisense RNA-RNA interaction in the 3'UTR of *lin-14*.

Although at first they were considered to be a fluke of nature specific to worms, wider interest in miRNAs was sparked by the discovery of a second miRNA, let-7 in the same organism *C. elegans*.[28] The discovery that let-7 was conserved across species triggered a revolution in the field of miRNAs.[28] In 2001, more of these tiny RNA fragments were discovered and were assigned potential regulatory roles. To accommodate the growing number of miRNAs, the repository database "miRbase" was established. At the start of the database, there were 555 human mature miRNAs. This number increased rapidly to the 2154 mature miRNAs currently deposited in the database (miRbase v18).[29]

Biogenesis and function

miRNAs are processed in a specific manner (Figure 2). miRNAs are located in introns or exons of protein coding genes. They are either transcribed via their own promoter[30-32] or they appear as by-products following the transcription of protein coding genes.[33,34] When initially transcribed from the genome, the transcripts start of as primary-miRNA (pri-miR) transcripts characterized by a unique hairpin structure. Subsequently they have to undergo successive processing steps to become a fully fledged mature miRNA. The first processing step takes place in the nucleus, where the unique hairloop structure of the pri-miRNA is recognized and cleaved by the DROSHA/DGCR8 complex. This yields a precursor-miRNA (pre-miR), which has a stemloop structure of about 60 nt with a 3'overhang.[35,36] Next the pre-miR is transported into the cytoplasm and is recognized and cleaved again by DICER, with a RNase III activity, at about 22 nt away from 3'overhang. This results in a duplex about 22 nt long called miRNA-miRNA* complex. One or both mature miRNAs are then selected to be loaded on to the RNA induced silencing complex (RISC) complex.[37,38] The miRNA then guides the RISC complex to a specific 3'UTR of mRNAs.

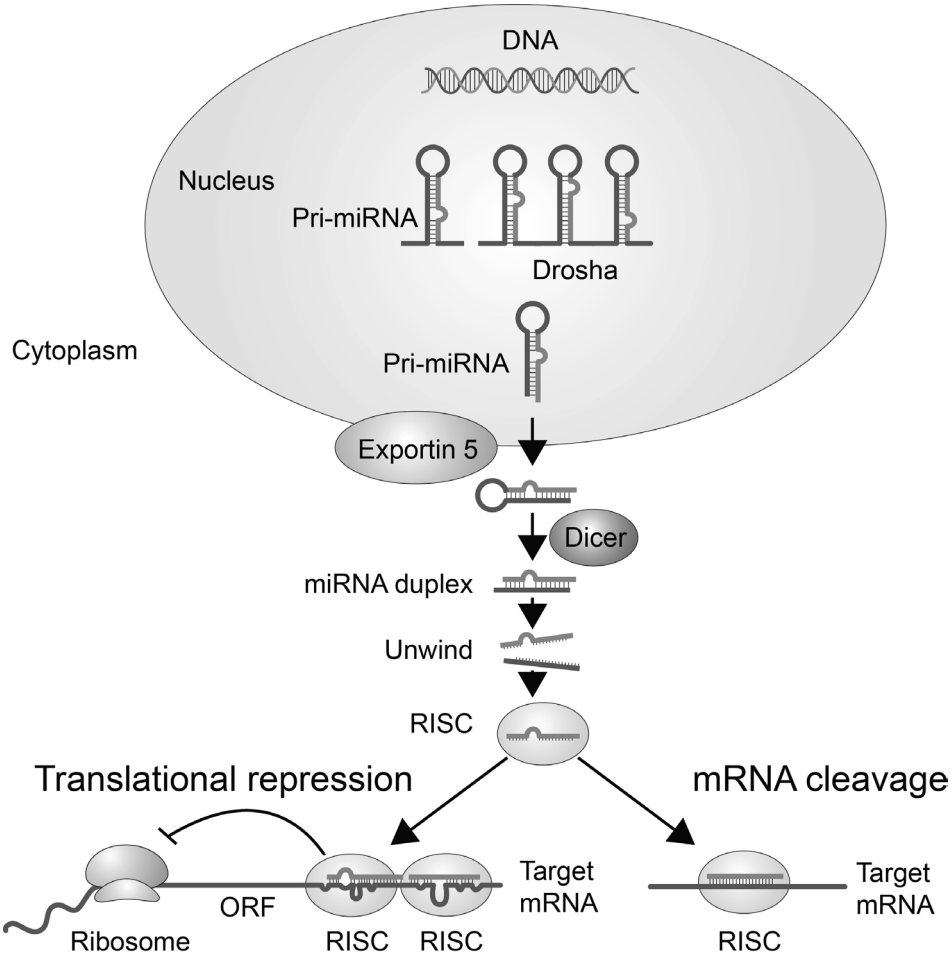


Figure 2. Schematic representation of the miRNA biogenesis.
miRNA genes are transcribed to generate primary miRNA transcripts (pri-miRNAs). The transcripts are recognized and cleaved by the Drosha–DGCR8 complex, generating a structure of about 70 nt with a stemloop and a ~2 nt 3'overhang called pre-miRNAs. The pre-miRNAs are subsequently exported out of the nucleus into the cytoplasm. RNase III member protein, DICER is involved in the second processing step and cleaves the pre-miRNAs, yielding duplex RNA. Selection of the mature miRNA occurs and the other RNA strand is degraded. The mature miRNA is loaded into the RISC complex. The miRNA-RISC complex consequently acts on target mRNAs and either degrades the mRNA transcript or inhibit its translation.

The targeting of the miRNA to the 3'UTR is based on partial complementation between the miRNA and the 3'UTR, which is mainly determined by a seed sequence at the position 2-8 bp from the 3'.[39,40] The RISC complex can exert its effect on mRNA in two different ways. In the case of a perfect sequence complementation between the miRNA and the target 3'UTR, the mRNA transcript will be cleaved. In the case of partial complementarily, the translation from the mRNA transcript will be inhibited.[41,42]

Due to the nature of a seed sequence of 6-8 nt long, each miRNA can potentially interact with a large number (~1000) of targets and conversely a 3'UTR of a single mRNA can contain multiple binding sites for the same but also other miRNAs.[43] However, it is highly unlikely that a certain miRNA in each cell will truly downregulate all predicted targets. The presence of specific RNA binding proteins can mask the miRNA binding site in a cell-context-specific manner and interfere with miRNA function.[44,45]

Moreover, multiple miRNAs can have the same seed sequence and belong to the same miRNA family; together they potentially can regulate the same set of targets. Furthermore, some miRNAs are located in clusters in the genome and are transcribed together. These miRNA families and genomic clusters can act together to regulate mRNA in a combinatorial fashion.[46]

In addition, miRNA sequencing studies show that miRNAs are prone to modifications as well that result in so-called iso-miRs.[47,48] Iso-miRs add additional complexity by either influencing the processing of miRNAs or by changing the target set of miRNAs through alterations of the seed sequence. One class of modifiers is adenosine deaminases acting on RNA (ADAR), which modify adenosine to inosine in long double-stranded RNA structures like pri-miRNA structures.[49-51] Another example of a post-transcription modifier is terminal uridylyltransferase 4 (*TUT4*) that recognizes, for example, the GCAG sequence of let-7 and consequently adds an uridine tail to the pre-let-7.This prevents processing of pre-miRNA transcription by DICER.[52,53]

The current prevailing consensus is that miRNAs downregulate protein levels by binding to the 3' UTR regions of mRNA together with the RISC complex and that miRNAs have a 'fine tune' function, which comes on top of the regulation provided by the transcription factors. To achieve this fine control, miRNA and transcription factors, act together in regulatory loops.[54,55] Some other modes of miRNA action have been reported as well. These include, for example, the regulation of transcripts by binding to 5'UTR and even protein coding region.[56-58]

miRNA in biological processes

An increasing body of evidence shows that miRNAs are important for the normal functioning of a cell. Studies that deplete most of the mature miRNAs in specific lineages, by conditional knockout of *DICER*, suggest that miRNAs are essential for normal lineage development.[59,60] Many miRNAs have a function in biological processes and are involved in important regulatory networks. For example, miR-34a is involved in the feedback loop with P53. It regulates P53 protein levels but is also regulated by P53. In addition, miR-34a

also directly regulates the protein levels of a subset of P53 targets, like B-cell lymphoma 2 (BCL2), sirtuin1 (SIRT1) and c-MYC.

In other cases, miRNAs have been shown to be important for cell fate decisions. In the megakaryocyte-erythrocyte progenitor, miR-150 expression drives the cell differentiation toward megakaryocytes at the cost of erythrocytes, by regulating the transcription factor myeloblastosis (MYB).[61] The miRNAs miR-1 and miR-133 are specifically expressed in skeletal and cardiac muscle tissues. This tissue specificity is conserved between species. In the genome, both miR-1 and miR-133 are located next to each other and transcribed from a single primary miRNA transcript. Both contribute to muscle differentiation but in a different manner. Expression of miR-1 in myoblast cells induces myogenesis. However, expression of miR-133 promotes the proliferation, by regulating the levels of histone deacytelase 4 (HDAC4) and serum response factor (SRF).[62] miRNAs are also important for the self-renewal capacity of hematopoietic stem cells. One example is miR-125a that is highly expressed in long-term hematopoietic stem cells. Increased expression of miR-125a causes an increased number of hematopoietic stem cells.[63] Another prime example of miRNA function is their ability to generate induced pluripotent stem cells (iPSc). Anokye-Danso *et al* reported an efficient induction of pluripotent stem cells by the introduction of the miR-307/367 cluster, which is partially due to induction of octamer-binding transcription factor 4 (OCT4) by miR-367.[64]

miRNAs are clearly involved in many different physiological processes. Deregulation of miRNA expression may have many undesired effects and contribute to the development of disease.

miRNAs in cancer

miRNAs have been proposed to have a role in the development of cancer. The first two miRNAs identified in cancer were miR-15 and miR-16. These microRNAs are often deleted or downregulated in chronic lymphocytic leukemia (CLL) and were shown to regulate apoptosis by down regulating *BCL2*.[65,66] Since then, an increasing number of studies have reported the deregulation of miRNAs in cancer. For example miR-34a, which has been described to be involved in apoptosis, is downregulated in many solid tumors. The miRNAs of the let-7 family and the miR-17-92 cluster are often found deregulated in various types of cancer and have been shown to target classical oncogenes such as *RAS* and *MYC*.[67-69] Conversely, many of the oncogenes like *MYC* and nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-kB*) regulate the expression of these oncomiRs.[67,70] A single oncomiR, miR-21 is sufficient to induce the cancer following the

introduction of miR-21 in bone marrow cells and results in pre-B-cell lymphoma *in vivo*.[71] miRNAs may also promote tumor metastasis. miR-10b is upregulated in breast cancer by the transcription factor TWIST related transcription factor 1 (TWIST1). miR-10b then upregulates homeobox D10 (*HOXD10*), which in turn upregulates the pro-metastatic gene *RAS* homologue gene family member C (*RHOC)*.[72] Another example is miR-9, which is upregulated in breast cancer cell lines and regulates the levels of E-cadherin, which promotes cell mobility via the β-catenin pathway. E-cadherin in turn contributes to elevated vascular endothelial growth factor A (VEGFA) levels, consequently leading to stimulation of angiogenesis.[73] The diverged homeodomain gene (*GAX*) is important for promoting angiogenesis in vascular endothelial cells. It contains two miR-130a binding sites. miR-130a can negatively regulate GAX levels as well as the levels of anti-angiogenic homeodomain gene 5A (HOX5A).[74]

Classification/Prediction with miRNAs

New techniques that allow the high throughput determination of miRNA expression levels have made it possible to perform miRNA profiling studies. The first large study, which was performed by Lu *et al* in a panel of normal tissues and tumor cell lines, has demonstrated characteristic miRNA expression patterns of the different tissues and tumor cell lines.[75] Since then many miRNA profiling studies have been performed in different cancer types, adding further support to the idea that miRNAs are deregulated in cancer.[76-78] Mi *et al* showed that acute lymphoblastic leukemia (ALL) can be distinguished from AML by differences in miRNA expression profiles.[79] Likewise, miRNA expression profiling studies have shown that miRNAs can be used to distinguish cancer from normal tissue.[80,81]

In terms of prognosis and association with survival, Takamizawa *et al* showed that high expression of let-7 in human lung cancer was associated with better survival. In lung cancer patients who underwent potential curative resection, unsupervised clustering based on miRNA expression revealed two clusters. Interestingly, the cluster of patients with low let-7 expression levels had unfavorable survival when compared to the cluster of patients with high let-7 expression.[82] In breast cancer, high miR-21 expression is associated with several unfavorable clinical features, such as lymph node metastasis, late stage breast cancer and unfavorable survival outcome.[81] Since the publication of these first studies a variety of miRNAs and miRNA signatures have been reported to be associated with survival in cancers.[83-86]

Aim and outline of this thesis

It is becoming increasingly clear that miRNAs are important in biological processes of normal and malignant tissues. The aim of this thesis was to explore the role of miRNAs in AML.

Our first question was whether specific miRNAs expression patterns correlate with AML subtypes and subsequently whether miRNAs can be used to classify AML. We also determined the miRNA expression patterns in normal human granulocytic subsets (Chapter 2). This provided a reference for the AML studies and a possible lead to the potential functional importance of these expression patterns. In Chapter 3 we set out to conduct a miRNA expression study in a cohort of 215 patients with recently diagnosed AML. The aim was to assess the potential of miRNAs to classify and class predict AML subsets. We focused on two specific miRNAs that might have a biological functional role in AML. In Chapter 4, we studied miR-9 and miR-9* in greater detail. The choice for these miRNAs was based on the results obtained in Chapters 2 and 3, as these two miRNAs were not expressed during normal myeloid differentiation but were highly expressed in AML. We studied their association with clinical outcomes and examined the functional impact of ectopic miR-9 and miR-9* expression on myeloid differentiation *in vitro*.

In Chapter 5 we addressed the question as to whether certain selected individual miRNAs might predict clinical outcome, i.e. survival, among patients with AML. Finally Chapter 6 discusses the results described in this thesis and their future perspectives.

REFERENCES

1. Metcalf D. Concise review: hematopoietic stem cells and tissue stem cells: current concepts and unanswered questions. *Stem Cells*. 2007;25(10):2390-2395.
2. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. *N Engl J Med*. 1999;341(14):1051-1062.
3. Wang J, Hoshino T, Redner RL, Kajigaya S, Liu JM. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. *Proc Natl Acad Sci U S A*. 1998;95(18):10860-10865.
4. Lam K, Zhang DE. RUNX1 and RUNX1-ETO: roles in hematopoiesis and leukemogenesis. *Front Biosci*. 2012;17:1120-1139.
5. Fos J, Pabst T, Petkovic V, Ratschiller D, Mueller BU. Deficient CEBPA DNA binding function in normal karyotype AML patients is associated with favorable prognosis. *Blood*. 2011;117(18):4881-4884.
6. Paz-Priel I, Friedman A. C/EBPalphadysregulation in AML and ALL. *Crit Rev Oncog*. 2011;16(1-2):93-102.
7. Saeed S, Logie C, Stunnenberg HG, Martens JH. Genome-wide functions of PML-RARalpha in acute promyelocytic leukaemia. *Br J Cancer*. 2011;104(4):554-558.
8. Brown NJ, Ramalho M, Pedersen EW, Moravcsik E, Solomon E, Grimwade D. PML nuclear bodies in the pathogenesis of acute promyelocytic leukemia: active players or innocent bystanders? *Front Biosci*. 2009;14:1684-1707.
9. Huang ME, Ye YC, Chen SR, et al. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood*. 1988;72(2):567-572.
10. Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. *Blood*. 2008;111(5):2505-2515.
11. Nakao M, Yokota S, Iwai T, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia*. 1996;10(12):1911-1918.
12. Yamamoto Y, Kiyoi H, Nakano Y, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood*. 2001;97(8):2434-2439.
13. Ito S, Shen L, Dai Q, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science*. 2011;333(6047):1300-1303.
14. Shen Y, Zhu YM, Fan X, et al. Gene mutation patterns and their prognostic impact in a cohort of 1185 patients with acute myeloid leukemia. *Blood*. 2011;118(20):5593-5603.
15. Gaidzik VI, Paschka P, Spath D, et al. TET2 mutations in acute myeloid leukemia (AML): results from a comprehensive genetic and clinical analysis of the AML study group. *J ClinOncol*. 2012;30(12):1350-1357.
16. Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med*. 2010;363(25):2424-2433.
17. Dohner K, Dohner H. Molecular characterization of acute myeloid leukemia. *Haematologica*. 2008;93(7):976-982.
18. Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*. 1996;84(2):321-330.
19. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. *Annu Rev Genomics Hum Genet*. 2002;3:179-198.
20. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol*. 1976;33(4):451-458.

21. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*. 2009;114(5):937-951.
22. Dohner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood*. 2010;115(3):453-474.
23. Breems DA, Van Putten WL, De Greef GE, et al. Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. *J ClinOncol*. 2008;26(29):4791-4797.
24. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409(6822):860-921.
25. Consortium EP, Dunham I, Kundaje A, et al. An integrated encyclopedia of DNA elements in the human genome. *Nature*. 2012;489(7414):57-74.
26. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. 1993;75(5):843-854.
27. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell*. 1993;75(5):855-862.
28. Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR, Ruvkun G. The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. *Mol Cell*. 2000;5(4):659-669.
29. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res*. 2006;34(Database issue):D140-144.
30. Borchert GM, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. *Nat StructMol Biol*. 2006;13(12):1097-1101.
31. Lee Y, Kim M, Han J, et al. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J*. 2004;23(20):4051-4060.
32. Morlando M, Ballarino M, Gromak N, Pagano F, Bozzoni I, Proudfoot NJ. Primary microRNA transcripts are processed co-transcriptionally. *Nat StructMol Biol*. 2008;15(9):902-909.
33. Kim YK, Kim VN. Processing of intronic microRNAs. *EMBO J*. 2007;26(3):775-783.
34. Ruby JG, Jan CH, Bartel DP. Intronic microRNA precursors that bypass Drosha processing. *Nature*. 2007;448(7149):83-86.
35. Han J, Lee Y, Yeom KH, et al. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell*. 2006;125(5):887-901.
36. MacRae IJ, Zhou K, Doudna JA. Structural determinants of RNA recognition and cleavage by Dicer. *Nat* 37. Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science*. 2001;293(5531):834-838.
38. Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev*. 2001;15(20):2654-2659.
39. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-297.
40. Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell*. 2007;27(1):91-105.
41. Pillai RS, Bhattacharyya SN, Filipowicz W. Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol*. 2007;17(3):118-126.
42. Wu L, Fan J, Belasco JG. MicroRNAs direct rapid deadenylation of mRNA. *ProcNatlAcadSci U S A*. 2006;103(11):4034-4039.

43. Farh KK, Grimson A, Jan C, et al. The widespread impact of mammalian MicroRNAs on mRNA repression and evolution.*Science*. 2005;310(5755):1817-1821.
44. Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E. The role of site accessibility in microRNA target recognition. *Nat Genet*. 2007;39(10):1278-1284.
45. Kedde M, Strasser MJ, Boldajipour B, et al. RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell*. 2007;131(7):1273-1286.
46. Yu J, Wang F, Yang GH, et al. Human microRNA clusters: genomic organization and expression profile in leukemia cell lines. *BiochemBiophys Res Commun*. 2006;349(1):59-68.
47. Lee LW, Zhang S, Etheridge A, et al. Complexity of the microRNA repertoire revealed by next-generation sequencing. *RNA*. 2010;16(11):2170-2180.
48. Zhou H, Arcila ML, Li Z, et al. Deep annotation of mouse iso-miR and iso-moR variation. *Nucleic Acids Res*. 2012;40(13):5864-5875.
49. Amariglio N, Rechavi G. A-to-I RNA editing: a new regulatory mechanism of global gene expression. *Blood Cells Mol Dis*. 2007;39(2):151-155.
50. Borchert GM, Gilmore BL, Spengler RM, et al. Adenosine deamination in human transcripts generates novel microRNA binding sites. *Hum Mol Genet*. 2009;18(24):4801-4807.
51. Peng Z, Cheng Y, Tan BC, et al. Comprehensive analysis of RNA-Seq data reveals extensive RNA editing in a human transcriptome. *Nat Biotechnol*. 2012;30(3):253-260.
52. Jones MR, Quinton LJ, Blahna MT, et al. Zcchc11-dependent uridylation of microRNA directs cytokine expression. *Nat Cell Biol*. 2009;11(9):1157-1163.
53. Heo I, Joo C, Kim YK, et al. TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell*. 2009;138(4):696-708.
54. Feng M, Yu Q. miR-449 regulates CDK-Rb-E2F1 through an auto-regulatory feedback circuit. *Cell Cycle*. 2010;9(2):213-214.
55. Zhao C, Sun G, Li S, Shi Y. A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination.*Nat StructMol Biol*. 2009;16(4):365-371.
56. DuursmaAM, Kedde M, Schrier M, le Sage C, Agami R. miR-148 targets human DNMT3b protein coding region. *RNA*. 2008;14(5):872-877.
57. Orom UA, Nielsen FC, Lund AH. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell*. 2008;30(4):460-471.
58. Lee I, Ajay SS, Yook JI, et al. New class of microRNA targets containing simultaneous 5'-UTR and 3'-UTR interaction sites. *Genome Res*. 2009;19(7):1175-1183.
59. O'Carroll D, Mecklenbrauker I, Das PP, et al. A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway.*Genes Dev*. 2007;21(16):1999-2004.
60. *Raaijmakers MH, Mukherjee S, Guo S, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. Nature*. 2010;464(7290):852-857.
61. Lu J, Guo S, Ebert BL, et al. MicroRNA-mediated control of cell fate in megakaryocyte-erythrocyte progenitors. *Dev Cell*. 2008;14(6):843-853.
62. Chen JF, Mandel EM, Thomson JM, et al. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet*. 2006;38(2):228-233.
63. Guo S, Lu J, Schlanger R, et al. MicroRNA miR-125a controls hematopoietic stem cell number. *ProcNatlAcadSci U S A*. 2010;107(32):14229-14234.
64. Anokye-Danso F, Trivedi CM, Juhr D, et al. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell*. 2011;8(4):376-388.
65. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *ProcNatlAcadSci U S A*. 2005;102(39):13944-13949.

66. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *ProcNatlAcadSci U S A*. 2002;99(24):15524-15529.

67. Mu P, Han YC, Betel D, et al. Genetic dissection of the miR-17~92 cluster of microRNAs in Myc-induced B-cell lymphomas. *Genes Dev*. 2009;23(24):2806-2811.

68. Akao Y, Nakagawa Y, Naoe T. let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol Pharm Bull*. 2006;29(5):903-906.

69. He XY, Chen JX, Zhang Z, Li CL, Peng QL, Peng HM. The let-7a microRNA protects from growth of lung carcinoma by suppression of k-Ras and c-Myc in nude mice. *J Cancer Res ClinOncol*. 2010;136(7):1023-1028.

70. Sun Y, Wu J, Wu SH, et al. Expression profile of microRNAs in c-Myc induced mouse mammary tumors. *Breast Cancer Res Treat*. 2009;118(1):185-196.

71. Medina PP, Nolde M, Slack FJ. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature*. 2010;467(7311):86-90.

72. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature*. 2007;449(7163):682-688.

73. Ma L, Young J, Prabhala H, et al. miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol*. 2010;12(3):247-256.

74. Chen Y, Gorski DH. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5.*Blood*. 2008;111(3):1217-1226.

75. *Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature*. 2005;435(7043):834-838.

76. Garofalo M, Quintavalle C, Di Leva G, et al. MicroRNA signatures of TRAIL resistance in human non-small cell lung cancer. *Oncogene*. 2008;27(27):3845-3855.

77. Schetter AJ, Leung SY, Sohn JJ, et al. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA*. 2008;299(4):425-436.

78. Corthals SL, Sun SM, Kuiper R, et al. MicroRNA signatures characterize multiple myeloma patients. *Leukemia*. 2011;25(11):1784-1789.

79. Mi S, Lu J, Sun M, et al. MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia. *ProcNatlAcadSci U S A*. 2007;104(50):19971-19976.

80. Iorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res*. 2005;65(16):7065-7070.

81. Yan LX, Huang XF, Shao Q, et al. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA*. 2008;14(11):2348-2360.

82. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res*. 2004;64(11):3753-3756.

83. Budhu A, Jia HL, Forgues M, et al. Identification of metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology*. 2008;47(3):897-907.

84. Jung M, Mollenkopf HJ, Grimm C, et al. MicroRNA profiling of clear cell renal cell cancer identifies a robust signature to define renal malignancy. *J Cell Mol Med*. 2009;13(9B):3918-3928.

85. Raponi M, Dossey L, Jatkoe T, et al. MicroRNA classifiers for predicting prognosis of squamous cell lung cancer. *Cancer Res*. 2009;69(14):5776-5783.

86. Yanaihara N, Caplen N, Bowman E, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell*. 2006;9(3):189-198.

87. Kakizuka A, Miller WH, Jr., Umesono K, et al. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell*. 1991;66(4):663-674.

88. Reikvam H, Hatfield KJ, Kittang AO, Hovland R, Bruserud O. Acute myeloid leukemia with the t(8;21) translocation: clinical consequences and biological implications. *J Biomed Biotechnol*. 2011;2011:104631.

89. Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med*. 2005;352(3):254-266.

90. Thol F, Damm F, Ludeking A, et al. Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. *J ClinOncol*. 2011;29(21):2889-2896.

91. Abu-Duhier FM, Goodeve AC, Wilson GA, et al. FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. *Br J Haematol*. 2000;111(1):190-195.

92. Carbuccia N, Trouplin V, Gelsi-Boyer V, et al. Mutual exclusion of ASXL1 and NPM1 mutations in a series of acute myeloid leukemias. *Leukemia*. 2010;24(2):469-473.

93. Rocquain J, Carbuccia N, Trouplin V, et al. Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leukemias. *BMC Cancer*. 2010;10:401.

94. Pabst T, Mueller BU, Zhang P, et al. Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet*. 2001;27(3):263-270.

95. Wouters BJ, Lowenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. *Blood*. 2009;113(13):3088-3091.

96. Paschka P, Marcucci G, Ruppert AS, et al. Wilms' tumor 1 gene mutations independently predict poor outcome in adults with cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study. *J ClinOncol*. 2008;26(28):4595-4602.

97. Mardis ER, Ding L, Dooling DJ, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med*. 2009;361(11):1058-1066.

98. Abbas S, Lugthart S, Kavelaars FG, et al. Acquired mutations in the genes encoding IDH1 and IDH2 both are recurrent aberrations in acute myeloid leukemia: prevalence and prognostic value. *Blood*. 2010;116(12):2122-2126.

99. Care RS, Valk PJ, Goodeve AC, et al. Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. *Br J Haematol*. 2003;121(5):775-777.

100. Valk PJ, Bowen DT, Frew ME, Goodeve AC, Lowenberg B, Reilly JT. Second hit mutations in the RTK/RAS signaling pathway in acute myeloid leukemia with inv(16). *Haematologica*. 2004;89(1):106.

101. Bowen DT, Frew ME, Hills R, et al. RAS mutation in acute myeloid leukemia is associated with distinct cytogenetic subgroups but does not influence outcome in patients younger than 60 years. *Blood*. 2005;106(6):2113-2119.

102. Levine RL, Loriaux M, Huntly BJ, et al. The JAK2V617F activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia. *Blood*. 2005;106(10):3377-3379.

103. Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. *Blood Rev*. 2004;18(2):115-136.

104. Takahashi S. Current findings for recurring mutations in acute myeloid leukemia. *J HematolOncol*. 2011;4:36.

105. Fenaux P, Preudhomme C, Lai JL, Morel P, Beuscart R, Bauters F. Cytogenetics and their prognostic value in de novo acute myeloid leukaemia: a report on 283 cases. *Br J Haematol*. 1989;73(1):61-67.

106. Schlenk RF, Dohner K, Krauter J, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med*. 2008;358(18):1909-1918.

107. Ziemin-van der Poel S, McCabe NR, Gill HJ, et al. Identification of a gene, MLL, that spans the breakpoint in 11q23 translocations associated with human leukemias. *ProcNatlAcadSci U S A*. 1991;88(23):10735-10739.

108. *Garcon L, Libura M, Delabesse E, et al. DEK-CAN molecular monitoring of myeloid malignancies could aid therapeutic stratification. Leukemia*. 2005;19(8):1338-1344.

109. Taskesen E, Bullinger L, Corbacioglu A, et al. Prognostic impact, concurrent genetic mutations, and gene expression features of AML with CEBPA mutations in a cohort of 1182 cytogenetically normal AML patients: further evidence for CEBPA double mutant AML as a distinctive disease entity. *Blood*. 2011;117(8):2469-2475.

110. Dohner K, Schlenk RF, Habdank M, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood*. 2005;106(12):3740-3746.

111. Boissel N, Nibourel O, Renneville A, et al. Prognostic impact of isocitrate dehydrogenase enzyme isoforms 1 and 2 mutations in acute myeloid leukemia: a study by the Acute Leukemia French Association group. *J ClinOncol*. 2010;28(23):3717-3723.

112. Bowen D, Groves MJ, Burnett AK, et al. TP53 gene mutation is frequent in patients with acute myeloid leukemia and complex karyotype, and is associated with very poor prognosis. *Leukemia*. 2009;23(1):203-206.

113. Stirewalt DL, Kopecky KJ, Meshinchi S, et al. FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood*. 2001;97(11):3589-3595.

114. Damm F, Heuser M, Morgan M, et al. Single nucleotide polymorphism in the mutational hotspot of WT1 predicts a favorable outcome in patients with cytogenetically normal acute myeloid leukemia. *J ClinOncol*. 2010;28(4):578-585.

115. Schwind S, Maharry K, Radmacher MD, et al. Prognostic significance of expression of a single microRNA, miR-181a, in cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *J ClinOncol*. 2010;28(36):5257-5264.

116. Eisfeld AK, Marcucci G, Maharry K, et al. miR-3151 interplays with its host gene BAALC and independently affects outcome of patients with cytogenetically normal acute myeloid leukemia. *Blood*. 2012;120(2):249-258.

117. Langer C, Radmacher MD, Ruppert AS, et al. High BAALC expression associates with other molecular prognostic markers, poor outcome, and a distinct gene-expression signature in cytogenetically normal patients younger than 60 years with acute myeloid leukemia: a Cancer and Leukemia Group B (CALGB) study. *Blood*. 2008;111(11):5371-5379.

118. Rockova V, Abbas S, Wouters BJ, et al. Risk stratification of intermediate-risk acute myeloid leukemia: integrative analysis of a multitude of gene mutation and gene expression markers. *Blood*. 2011;118(4):1069-1076.

119. Marcucci G, Baldus CD, Ruppert AS, et al. Overexpression of the ETS-related gene, ERG, predicts a worse outcome in acute myeloid leukemia with normal karyotype: a Cancer and Leukemia Group B study. *J ClinOncol*. 2005;23(36):9234-9242.

120. Groschel S, Lugthart S, Schlenk RF, et al. High EVI1 expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. *J ClinOncol*. 2010;28(12):2101-2107.

121. Langer C, Marcucci G, Holland KB, et al. Prognostic importance of MN1 transcript levels, and biologic insights from MN1-associated gene and microRNA expression signatures in cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study. *J ClinOncol*. 2009;27(19):3198-3204.

122. Tang R, Hirsch P, Fava F, et al. High Id1 expression is associated with poor prognosis in 237 patients with acute myeloid leukemia. Blood. 2009;114(14):2993-3000.

123. Reya T, Morrison SJ, Clarke MF, Weissman I L. Stem cells, cancer, and cancer stem cells. *Nature*. 2001;414(6859):105-111.

2

Su Ming Sun[1]
Menno K. Dijkstra[1]
André C. Bijkerk[2]
Rik A. Brooimans[2]
Peter J.M. Valk[1]
Stefan J. Erkeland[1]
Bob Löwenberg[1]
Mojca Jongen-Lavrencic[1]

[1]Department of Hematology, [2]Department of Medical Tumor Immunology, Erasmus University Medical Center, Rotterdam, The Netherlands

Transition of highly specific microRNAs patterns in association with discrete maturation stages of human granulopoiesis

British journal of haematology. 2011;155(3):395-8. Epub 2011/05/11.

ABSTRACT

Normal human neutrophil development is a complex biological process, where the balance between cell proliferation, differentiation and apoptosis is tightly regulated by a transcriptional program that results in the production of appropriate numbers of circulating mature neutrophils. MicroRNAs (miRNAs) are small non-coding RNAs of 18~25 nt that affect cellular protein levels. Only limited data is available on miRNA expression patterns during normal granulocytic differentiation of primary human cells.

We have examined miRNA expression patterns in distinct stages of granulocytic differentiation sorted from human bone marrow and identified highly stage-specific expressed miRNAs. Some of these differentially expressed miRNA are related to each other in terms of seed sequence (miRNA families) and location on the genome (miRNA clusters). These data reveal distinct expression patterns of specific miRNA sets that closely follow the transition of discrete maturation stages along the myeloblast-neutrophil pathway.

INTRODUCTION

The process of granulopoiesis involves the development of terminally differentiated segmented granulocytes from morphologically defined myeloblasts via promyelocytes, myelocytes and metamyelocytes. The regulation of granulopoiesis is tightly controlled by external stimuli and transcription factors.[1,2]

MicroRNAs (miRNAs) are a class of small non coding RNAs that regulate cellular protein levels by either inhibition of translation or cleavage of the mRNA transcripts.[3] Not much is known about miRNA expression in neutrophil development. The expression of limited number of miRNAs was previously determined in various cell lines[4-7] and primary human neutrophils.[8,9] Evidence for the importance of miRNAs in granulocytic differentiation has recently been put forward. CEPBA and PU.1 are implicated in the regulation of miR-223[10] that is specifically up-regulated in the granulocytic differentiation[11] and functions as a negative regulator of the myeloid transcription factor MEF2C.[12] Furthermore, GFI1 is another important granulocytic transcription factor that regulates the expression miR-21 and miR-196b.[13]

In this study we set out to systematically characterize the miRNA expression patterns in successive morphologically defined stages of normal human neutrophilic development. We sorted myeloblasts, promyelocytes, metamyelocytes and mature neutrophils from normal human bone marrow using specific cell surface markers and determined the miRNA expression profiles of 365 miRNAs.

MATERIAL AND METHODS

Isolation of consecutive morphologically distinct cellular stages of normal human granulopoiesis

Bone marrow samples were collected from healthy individuals following the declaration of Helsinki principles. Different successive stages of terminal granulocytic differentiation, myeloblasts, promyelocytes, metamyelocytes and neutrophils, were FACS-sorted using cell type specific markers[14] i.e. CD10-APC, CD11b-APC-Cy7, CD34-Pe-Cy7, CD45-PerCP, CD117-PE (Becton Dickinson) and CD36-FITC (Beckman Coulter). The purity of sorted samples was determined by immunophenotyping and morphology of the cytospins, stained with May Grünwald Giemsa.

RNA isolation and Quality control

Total RNA was isolated from cells using Trizol (Invitrogen) and the quality of the RNA was checked with the RNA 6000 Nanoassay on the Agilent 2100 Bioanalyzer (Agilent).

MiRNA expression profiling

The miRNA TAQMAN low density array (TLDA) v1.0 panel, a quantitative reversed transcriptase polymerase chain reaction (RQ-PCR) platform, was used for the detection of 365 miRNAs (Applied Biosystems).[15]

Data analysis

miRNAs with Ct values above 35 and miRNAs with non determined calls were considered as not expressed and their ΔCt values were set to minimum value of 16.[16] $2^{-\Delta CT}$ was used with RNU48 as endogenous control.[16] The expression values were geometric mean centred and log2 transformed. MiRNAs with standard deviation of 0 were left out. Further analyses were done with Partek Genomic suite software version 6.4 including principal component analysis and ANOVA, corrected by false discovery rate.[17] Myeloblast-, metamyelocyte- and neutrophil-specific miRNAs were identified by significant pairwise comparison between the subsequent maturation stages. Chromosomal locations were obtained from miRBase release 13[18] and miRNAs within 500 bp were considered as genomic cluster. MiRNA families were obtained from TargetScan release 5.1.[19] OmniViz Desktop version 6.0.1 was used to generate the heatmap figures.

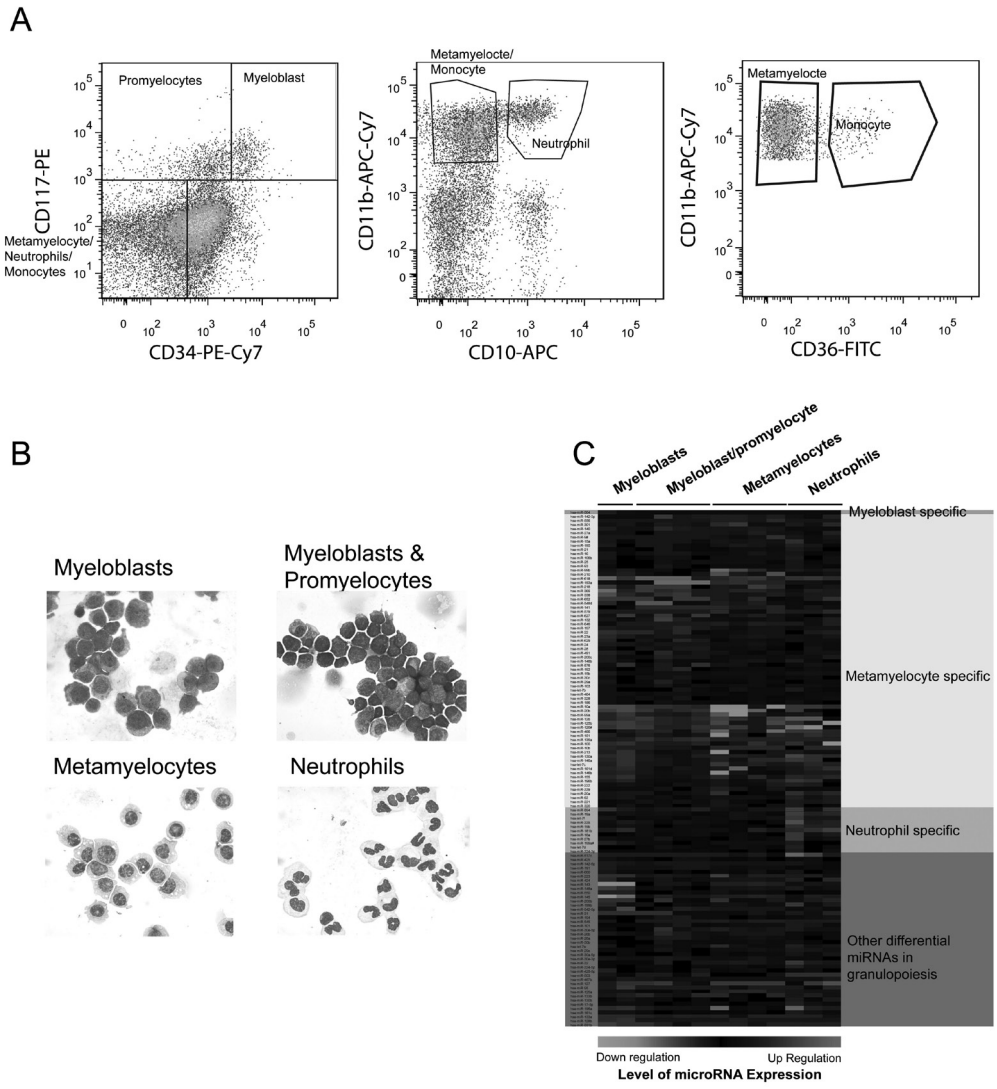


Figure 1. miRNA expression in cell sorted neutrophilic differentiation stages of normal human bone marrow (A) Gating strategy for FACS cell sorting of bone marrow cellular morphological maturation stages (myeloblasts, promyelocytes, metamyelocytes and neutrophils). The following different cell surface markers were used, CD117, CD34, CD11b, CD10 and CD36. DAPI was used to distinguish living from dead cells. (B) Cytospins, stained with May Grünwald Giemsa, of cells taken from the different sorted maturation stages. (C) Heatmap of log2 transformed geometric mean centred miRNA expression showing significantly differentially expressed miRNA determined by ANOVA and corrected for multiple testing of the sorted neutrophil stages of maturation, depicted on the rows and columns respectively. MiRNA expression was represented by colour scale, where green reflects lower expression, the black colour reflects mean expression and red reflects higher expression.

Table 1. Differential expression of miRNA families

miRNA family (no. miRNAs measured)	miRNA	Stage specific change
miR-15/16/195/424/497 (5)	hsa-miR-15b	Metamyelocyte, ↑
	hsa-miR-15a, hsa-miR-16, hsa-miR-195	Metamyelocyte , ↑
	hsa-miR-424	Various, ↑
miR-17-5p/20/93.mr/106/519.d (6)	hsa-miR-20a, hsa-miR-20b	Metamyelocyte, ↓
	hsa-miR-106b, hsa-miR-93	Metamyelocyte, ↑
	hsa-miR-17-5p	Various, ↓
miR-181 (6)	hsa-miR-181d	Metamyelocyte, ↓
	hsa-miR-181b	Neutrophiles, ↓
	hsa-miR-181c	Various, ↓
miR-25/32/92/92ab/363/367 (4)	hsa-miR-92	Metamyelocyte, ↓
	hsa-miR-25	Metamyelocyte, ↑
	hsa-miR-32	Various, ↑
miR-30a/30a-5p/30b/30b-5p/30cde/384-5p (5)	hsa-miR-30c	Metamyelocyte, ↓
	hsa-miR-30e-5p	Various, ↑
	hsa-miR-30b	Various, ↑
	hsa-miR-30a-5p	Various, ↑
let-7/98 (8)	hsa-let-7b	Metamyelocyte, ↑
	hsa-let-7c	Metamyelocyte, ↓
	hsa-let-7f, hsa-let7d	Neutrophil, ↓
	hsa-let-7a	Various, ↓
miR-130/301 (3)	hsa-miR-130a, hsa-miR-301	Metamyelocyte, ↑
	hsa-miR-130b	Various, ↓
miR-99ab/100 (3)	hsa-miR-99a, hsa-miR-100	Metamyelocyte, ↓
	hsa-miR-99b	Metamyelocyte, ↓
miR-10 (2)	hsa-miR-10a, hsa-miR10b	Metamyelocyte, ↓
miR-125/351 (2)	hsa-miR-125b	Metamyelocyte, ↓
	hsa-miR-125a	Various, ↓
miR-133 (2)	hsa-miR-133a, hsa-miR-133b	Various, ↓
miR-146 (2)	hsa-miR-146a, hsa-miR-146b	Metamyelocyte, ↓
miR-148/152 (2)	hsa-miR-148b	Metamyelocyte, ↑
	hsa-miR-148a	Various, ↑
miR-19 (2)	hsa-miR-19a, hsa-miR-19b	Neutrophil, ↓
miR-196ab (2)	hsa-miR-196b	Metamyelocyte, ↓
	hsa-miR-196a	Various, ↓
miR-200bc/429 (2)	hsa-miR-200c	Metamyelocyte, ↑
	hsa-miR-200b	Various, ↑
miR-221/222 (2)	hsa-miR-221, hsa-miR-222	Metamyelocyte, ↓
miR-26ab/1297 (2)	hsa-miR-26a, hsa-miR-26b	Various, ↑
miR-27ab (2)	hsa-miR-27b	Neutrophil, ↓
	hsa-miR-27a	Metamyelocyte, ↑
miR-29abc (2)	hsa-miR-29a	Metamyelocyte, ↑
	hsa-miR-29c	Various, ↑

Note: miRNA family as determined in TargetScan version 5.1, the number of members measured in this study has been depicted between ( ). Up- and down- regulation have been depicted as ↑ and ↓.

RESULTS AND DISCUSSION

Myeloblasts, promyelocytes, metamyelocytes and neutrophils have characteristic miRNA expression profiles

Normal bone marrow cells were sorted into fractions of different granulocytic maturation stages using specific cell surface markers (Figure 1A). Morphologic examination together with immunophenotyping confirmed the various granulocytic maturation stages with average purity > 80%. (Figure 1B, Supplementary Table 1).

The miRNA expression of the different cellular stages was profiled by RQ-PCR platform. One third (118/365) of the miRNAs was not expressed in any subset (Supplemental Figure 1 and Supplemental Table 2). Unsupervised PCA analysis of the expressed miRNAs revealed sharp distinctions between the sequential granulocytic maturation stages (Supplemental Figure 2) Hundred and twenty-five miRNAs were significantly differentially expressed between two or more granulocytic maturation stages (Figure 1C). Some miRNAs showed entirely granulocytic maturation stage specific expression (Figure 1C and Supplementary Table 3). Specific sets of miRNA were found differentially expressed in myeloblast (1 miRNA), metamyelocytes (46 miRNAs) and neutrophils (11 miRNAs).

It is of particular interest to note that defined sets of miRNAs in a coordinated way are subject to up-regulation or down-regulation at these discrete maturation stages, indicating that regulation of expression of miRNAs is subject to switch control along the maturation pathway. These changes in miRNA expression patterns mark highly characteristic transitions between subsequent cellular stages and suggest an important function of miRNAs in protein level regulation, necessary for normal neutrophilic development.

Differential expression of miRNA-families and genomic miRNA-clusters in granulopoiesis

MiRNAs are related to each other in terms of sharing the seed sequence (miRNA-families) and in terms of same chromosomal location (miRNA-clusters). miRNAs sharing the same seed, potentially target the same set of targets and therefore combined expression of miRNA family members can enhance down regulation of targets.

The miRNA families, let-7/98 (5 out of 8), miR-15/16/195/424/497 (5 out of 6), miR-30a/30a-5p/30b/30n-5p/30cde/384-5p (4 out of 5), miR-17-5p/20/93.mr/106/519.d (5 out of 6), miR-130/301 (3 out of 3), miR-181 (3 out of 3) and miR-99ab/100 were differentially expressed in neutrophil maturation (Table 1). The majority of those

miRNA family members showed coordinated expression in the same direction and at the same maturation stage. One example is the miR-181 family with 3 family members, showed down-regulation in a sequential manner, leading to a consistent decline of the cumulative miR-181 seed expression. The miR-15/16/195/424/497 family have functions in cellular apoptosis and cell cycle regulation.[20-22] Concomitant up- regulation of miR-15/16/195/424/497 family member may enhance the down-regulation of apoptosis and cell cycle related targets needed for maturation towards to neutrophils. Furthermore, miR-17-5p/20/93.mr/106/519.d family is down-regulated in neutrophils. This is in concordance with other studies where miR-17-5p/20/93.mr/106/519.d family have been implicated in the regulation of stem cell differentiation.[23]

Combined expression of the miRNAs in a cluster might regulate different targets involved in the same pathway.[24] We found 12 significantly differentially expressed genomic miRNA clusters (Supplemental Table 4). Besides cluster members that displayed similar expression behaviour, we observed that the expression pattern of miRNA from same cluster was not always synchronised such as miR-17-92 cluster on chromosome 13 and the clusters found on chromosome 17, 19, 22 and the X chromosome. For example the miR-17-92 cluster, contained 6 significantly differentially expressed miRNAs in granulopoiesis with different expression patterns. MiRNA-20a, miR-92 expression was switched down in metamyelocytes, miR-18a, miR-19a and miR-19b were down-regulated in neutrophils, whereas the miR-17-5p expression gradually decreased from myeloblasts onwards.

This is the first comprehensive study of miRNA expression in normal human granulocytic differentiation that provides support for a critical and complex role of miRNAs in the regulation of granulocytic maturation. These data provide a basic framework for the molecular characterisation of stage-specific granulocytic cells, further investigations of the functional role of the individual miRNAs, miRNA families and clusters in neutrophilic differentiation and the identification of abnormal miRNA expression and function in case of myeloid disease.

ACKNOWLEDGEMENTS

We would like to thank Peter van Geel, Arie Prins, Elwin Rombouts for their assistance in sorting the cells and Kirsten van Lom for her assistance in assessing the morhology of the cells.

Supplementary information is available in the Appendix and on the the British Journal of Haematology website.

REFERENCES

1. Friedman AD. Transcriptional regulation of granulocyte and monocyte development. *Oncogene*. 2002;21(21):3377-3390.
2. Panopoulos AD, Watowich SS. Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis. *Cytokine*. 2008;42(3):277-288.
3. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-297.
4. Fatica A, Rosa A, Ballarino M, De Marchis ML, Rasmussen KD, Bozzoni I. Role of microRNAs in myeloid differentiation. *Biochem Soc Trans*. 2008;36(Pt 6):1201-1205.
5. Kasashima K, Nakamura Y, Kozu T. Altered expression profiles of microRNAs during TPA-induced differentiation of HL-60 cells. *Biochem Biophys Res Commun*. 2004;322(2):403-410.
6. Garzon R, Pichiorri F, Palumbo T, et al. MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. *Oncogene*. 2007;26(28):4148-4157.
7. Ramkissoon SH, Mainwaring LA, Ogasawara Y, et al. Hematopoietic-specific microRNA expression in human cells. *Leuk Res*. 2006;30(5):643-647.
8. Merkerova M, Vasikova A, Belickova M, Bruchova H. MicroRNA expression profiles in umbilical cord blood cell lineages. *Stem Cells Dev*. 2009.
9. Merkerova M, Belickova M, Bruchova H. Differential expression of microRNAs in hematopoietic cell lineages. *Eur J Haematol*. 2008;81(4):304-310.
10. Fukao T, Fukuda Y, Kiga K, et al. An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. *Cell*. 2007;129(3):617-631.
11. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science*. 2004;303(5654):83-86.
12. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature*. 2008;451(7182):1125-1129.
13. Velu CS, Baktula AM, Grimes HL. Gfi1 regulates miR-21 and miR-196b to control myelopoiesis. *Blood*. 2009;113(19):4720-4728.
14. van Lochem EG, van der Velden VH, Wind HK, te Marvelde JG, Westerdaal NA, van Dongen JJ. Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts. *Cytometry B Clin Cytom*. 2004;60(1):1-13.
15. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res*. 2005;33(20):e179.
16. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. *Blood*. 2008;111(10):5078-5085.
17. Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A*. 2003;100(16):9440-9445.
18. Griffiths-Jones S. The microRNA Registry. *Nucleic Acids Res*. 2004;32(Database issue):D109-111.
19. Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell*. 2007;27(1):91-105.
20. Linsley PS, Schelter J, Burchard J, et al. Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. *Mol Cell Biol*. 2007;27(6):2240-2252.
21. Liu Q, Fu H, Sun F, et al. miR-16 family induces cell cycle arrest by regulating multiple cell cycle genes. *Nucleic Acids Res*. 2008;36(16):5391-5404.

22. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A*. 2005;102(39):13944-13949.

23. Foshay KM, Gallicano GI. miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation. *Dev Biol*. 2009;326(2):431-443.

24. Chow TF, Mankaruos M, Scorilas A, et al. The miR-17-92 cluster is over expressed in and has an oncogenic effect on renal cell carcinoma. *J Urol*. 2010;183(2):743-751.

3

Mojca Jongen-Lavrencic
Su Ming Sun
Menno K. Dijkstra
Peter J.M. Valk
Bob Löwenberg

Department of Hematology, Erasmus Medical Center, Rotterdam, The Netherlands

MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia

Blood. 2008;111(10):5078-85. Epub 2008/03/14.

ABSTRACT

Acute myeloid leukemia (AML) is a highly diverse disease characterized by various cytogenetic and molecular abnormalities. MicroRNAs are small non-coding RNAs that show variable expression during myeloid differentiation.

MicroRNAs expression in marrow blasts in 215 cases of newly diagnosed and (cyto) genetically defined AML was assessed using quantitative RT-PCR for 260 known human microRNAs. In the same series mRNA gene expression profiles were established, allowing a direct comparison between microRNA and mRNA expression.

We show that microRNA expression profiling following unsupervised analysis reveals distinctive microRNA signatures that correlate with cytogenetic and molecular subtypes of AML i.e AMLs with t(8;21), t(15;17), inv(16), *NPM1* and *CEBPA* mutations. Significantly differentially expressed microRNAs for genetic subtypes of AML were identified. Specific microRNAs with established oncogenic and tumor suppressor functions, such as microRNA-155, microRNA-21, let-7 appear to be associated with particular subtypes. Combinations of selected sets of microRNAs could predict cytogenetically normal AML with mutations in the genes of *NPM1* and *CEBPA* and *FLT3-ITD* with similar accuracy as mRNA probe set combinations defined by gene expression profiling (GEP). MicroRNA expression apparently bears specific relationships to the heterogeneous pathobiology of AML. Distinctive microRNA signatures appear of potential value in the clinical diagnosis of AML.

INTRODUCTION

The pathogenesis of acute myeloid leukemia (AML) is a heterogeneous multi-step process affecting cell differentiation, proliferation and apoptosis, which ultimately leads to malignant transformation of hematopoietic progenitors. Deregulated gene-expression, disrupting cellular pathways, has been used for the classification of AML.[1-5] The prognosis of AML depends on well-defined leukemia-specific prognostic factors, such as the cytogenetic abnormalities t(15;17), t(8;21), inv(16) with a relatively favorable prognosis and the 3q26 abnormalities, -5/-5q, -7/-7q with an unfavorable prognosis.[6-8] Furthermore, various molecular abnormalities in AML with normal karyotype have apparent prognostic significance, such as the somatic gene mutations in nucleophosmin-1 (*NPM1),* FMS-like tyrosine kinase 3 *(FLT3* (internal tandem duplications (ITD*))*, and CCAAT/enhancer binding protein alpha (*CEBPA*).[9-13] *NPM1* mutations frequently occur in association with *FLT3*-ITD mutations. Several studies on the clinical impact in AML subgroups revealed that the subset of AML with *NPM1* mutations lacking *FLT3*-ITD mutations have a significantly better overall survival.[14]

MicroRNAs are a class of small non-coding RNAs that regulate translation of protein coding mRNAs and thereby protein expression, by translation inhibition or cleavage of the mRNA transcripts.[15] There is an accumulating body of evidence indicating that microRNAs play important roles in cellular growth and differentiation.[16] MicroRNA expression profiles of tumor samples have recently been shown to provide phenotypic signatures of particular cancer types.[17-22] MicroRNAs can act as tumor suppressors and oncogenes. For instance, expression of some microRNAs, such as let-7[23] and the microRNA15a/16-1 cluster,[24] have been reported to be reduced in lung cancer and chronic lymphocytic leukemia (CLL) respectively, suggesting tumor suppressor activities. In contrast, microRNA-17-92 cluster[25] and microRNA-155/BIC[26,27] have been shown to be overexpressed in B-cell lymphomas, indicative of their oncogenic potential. A characteristic microRNA expression signature may aid in the diagnosis of certain types or subtypes of cancers. It has been shown that microRNA profiles of bone marrow samples from patients with acute lymphoblastic leukemia (ALL) discriminated subsets of ALL with different molecular aberrancies.[17]

In AML, information about microRNA expression has only been gathered in a limited series of patients so far. Debernardi *et al*.[28] reported in 30 AML patients with normal cytogenetics, that microRNA-181a correlates with cytological subclass. They also reported that the expression of microRNA-10a, microRNA-10b and microRNA-196a, that are located in intergenic regions in the *HOX* gene clusters, in AML correlates positively

with *HOXA* and *HOXB* gene expression, suggesting a role of these microRNAs in aberrant regulation of proliferation and differentiation in leukemogenesis[28]. It was recently reported that microRNA expression signatures discriminate ALL from AML[29] and AML from normal bone marrow CD34+ cells.[30]

Here we addressed the question whether microRNA expression signatures could be used to classify a heterogeneous disease such as AML. We used a microRNA quantification method based on reverse transcriptase (RT) reaction using stem-loop primers followed by real-time quantitative PCR analysis[31] for 260 known human microRNAs to study the expression profile in 215 clinically and molecularly well-characterized *de novo* AML bone marrow samples. Furthermore, we sought to identify microRNAs associated with known AML cytogenetic and molecular abnormalities. Finally, we set out to directly compare microRNA based class predictors of AML subgroups with predictors identified by mRNAs GEP in the same patient cohort.

MATERIALS AND METHODS

Patients and cell samples

Patients used in this study had a newly diagnosed AML determined by cytological examination of bone marrow (Table 1A-B). All patients provided written informed consent and were treated according to the protocols of the Dutch-Belgian-Hematology-Oncology-Cooperative group (available at www.hovon.nl).[32-34] A total of 215 patients provided bone marrow aspirates at the time of diagnosis. Blast and mononuclear cells were purified by Ficoll-Hypaque (Nygaard) centrifugation and cryopreserved. After thawing the AML samples contained 80-100% blast cells, regardless of the blast count at diagnosis. CD34+ cells from four healthy control subjects were sorted from bone marrow aspirates using a fluorescence-activated cell sorter.

RNA isolation and quality control of RNA

After thawing, total RNA was isolated from cells using Trizol reagent according to manufacturer's protocol (Invitrogen, Breda, NL). Quality control of RNA was done by using RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent). All RNA samples showed high quality (without RNA degradation or DNA contamination).

Table 1A. Clinical characteristics of the cohort of 215 patients with newly diagnosed AML

Characteristics	Number of cases	%
Sex- number of cases and %		
Male	117	54,4%
Female	98	45,6%
Total	215	100,0%
Age (years) – number of cases and % distribution		
Median	49,7	
Range	18-77,2	
<35	45	20,9%
35-60	118	54,9%
>60	52	24,2%
Total	215	100,0%
White cell count - x 10^9/l		
Median	33	
Range	0,3-510	
Marrow blasts - %		
Median	60	
Range	0-93	
Platelet count - x 10^9/l		
Median	64,5	
Range	8-931	
French-American-British classification - number of cases and %		
M0	6	2,8%
M1	47	21,9%
M2	55	25,6%
M3	6	2,8%
M4	37	17,2%
M5	44	20,5%
M6	2	0,9%
Not determined	18	8,4%
Total	215	100,0%

Table 1B. Cytogenetic and molecular characteristics of the cohort of 215 patients with newly diagnosed AML

Characteristics	Number of cases	%
Cytogenetic abnormalities		
-9q	5	2.3%
-8	11	5.1%
11q23	6	2.8%
-5/-5(q)/-7/-7(q)	16	7.4%
Complex	5	2.3%
Inv(16)	15	7.0%
normal karyotype (NK)	100	46.5%
t(15;17)	6	2.8%
t(6;9)	2	0.9%
t(8;21)	12	5.6%
t(9;22)	2	0.9%
-X/Y	3	1.4%
Other	30	14.0%
Not determined	2	0.9%
Total	215	100.0%
Molecular abnormalities		
FLT3-TKD	20	9.3%
FLT3- ITD	62	28.8%
N-RAS	22	10.2%
K-RAS	1	0.5%
CEBPA	22	10.2%
NPM1	68	31.6%
Total patients	215	100.0%
Normal Karyotype	100	100.0%
with FLT3-TKD	10	10.0%
with FLT3-ITD	45	45.0%
with N-RAS	11	11.0%
with K-RAS	0	0.0%
with CEBPA	19	19.0%
with NPM1	55	55.0%

Mutation analyses

Sequence analyses for *FLT3*-ITD, *FTL3*-TKD (tyrosine kinase domain), NPM1 and CEBPA mutations were performed as described previously.[13,35]

MicroRNA profiling using Multiplex real-time quantitative RT-PCR

A multiplexing RT-PCR method was used for the detection of multiple microRNAs.[36] MicroRNA specific RT primers for 260 mature human microRNAs including several small nuclear RNAs (RNU19, RNU24, RNU38B, RNU43, RNU44, RNU48, RNU49, RNU49 and RNU66) that were used for internal normalization were divided into 7 pools containing 32-47 primers per pool (Applied Biosystems) and used for multiplexing RT reaction. RT reactions of 10 µl contained: 1 µl 10x RT buffer (Applied Biosystems), 0.2 µl dNTPs (100 mM each), 1 µl MultiScribe Reverse Transcriptase (50 U/µl), 0.125 µl AB RNase Inhibitor (20 U/µl), 2 µl 5X multiplex RT primers, 2 µl of 50 ng/µl total RNA and 3.675 µl H2O. Reactions were incubated in a Primus HT Thermocycler (Biotech) in a 96-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C.

Single real-time quantitative PCR reactions of 10 µl were set up in 384-well plates for each of the 260 microRNAs. Real-time quantitative PCR reactions contained 2.5 µl of 4X Universal Master Mix® (Applied Biosystems, Nieuwerkerk a/d IJssel, NL), 1.6 µl of 20-fold diluted multiplex RT product, 2.5 µl of 5-fold H2O diluted 20X Taqman MicroRNA primer/probe Assay® (Applied Biosystems) and 3.4 µl H2O. Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA, USA) was used to dispense 2.5 µl primer/probe assays into 384-well plates. This facility was kindly provided by A.G. Uitterlinden (Internal Medicine, Erasmus MC Rotterdam, the Netherlands). Real-time quantitative RT-PCR was performed on an AB 7900 HT Sequence Detection System (SDS) (Applied Biosystems, Nieuwerkerk a/d IJssel, NL) in a 384-well plate format, under following conditions: first a hot start of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. A Twister robot (Applied Biosystems, Nieuwerkerk a/d IJssel, NL) was used to load 384-well plates automatically into the 7900 HT SDS. Reproducibility was tested studying microRNA expression in 10 AML samples in duplicate. RNA was isolated in two independent experiments and subsequently cDNA synthesis and real-time RT-PCR steps were also performed in two separate experiments. High correlation in microRNAs expression between two independent experiments was found, with correlation coefficient R of 0.97. (Supplementary Figure 1).

Normalization and filtering

SDS 2.3 software (Applied Biosystems) was used to analyze Real-time RT-PCR data. The geometric mean of RNU24, RNU43, RNU48 and RNU66 was used for internal normalization[37] since these RNUs showed the most consistent expression in our cohort (Supplementary Figure 2). The relative quantification method, 2^{-dCt} was used to calculate the expression relative to the mean of the RNUs.[38] The relative expression (2^{-dCt}) of the microRNAs was normalized with the geometric mean of each microRNA for all patients. To obtain a normal linear distribution, a log2 transformation was performed. MicroRNAs that were unreliably quantifiable or not expressed (Ct value of 35 or higher) or not differentially expressed as defined by inter-quartile range < 0.1 were excluded from further analysis, leaving a set of 178 microRNAs.

mRNA gene expression profiling (GEP)

A total of 215 AML cases were analyzed for mRNA GEP using Affymetrix Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA) as described previously.[2]

Unsupervised cluster analyses

We used the unsupervised cluster analyses as described previously.[2] A total of 215 AML patient bone marrow specimens were grouped based on Pearson correlation of the microRNA expression using the Correlation View visualization tool of the OmniViz package version 4.0.0. The groups of patients were ordered based on absolute correlation. Clinical and molecular data was projected next to the correlation plot using the software program Heatmapper.[39]

Significance Analysis of Microarrays (SAM)

All supervised analyses were done using SAM with the statistical program R version 2.4.1 with the bioconductor package version 2.0 implemented.[40] The differentially expressed microRNAs were identified by comparing specimens with specific cytogenetic (e.g. t(15;17), t(8;21), inv(16)) or molecular abnormalities (*NPM1, CEBPA* and *FLT3*-ITD mutations) with all remaining AML patients in the cohort using SAM with a threshold of minimal 2-fold change in expression and a false discovery rate (FDR) of 0%.

Prediction Analysis of Microarrays (PAM)

Supervised class prediction of AML with cytogenetic abnormalities such as t(15;17), t(8;21) and inv(16) and molecular aberrations such as *NPM1, CEBPA* and *FLT3*-ITD

mutations, using microRNAs and mRNA expression data was performed using PAM.[41] The total cohort of 215 different AML patients as described in Table 1, was randomly divided in to a training set of 143 patients to build the class predictor and a validation set of 72 patients to test the predictor independent from the training set. The 10-fold method of cross-validation was applied to assess the class predictor in the training set. The minimum numbers of microRNAs and probe sets of genes (mRNA) that were identified were subsequently tested on the validation set. The accuracy of the specific class predictor was described by the number of truly predicted and false positively predicted cases in the validation set.

RESULTS

Unsupervised analysis of microRNA expression reveals signatures that correlate with particular cytogenetic and molecular subgroups of AML

Using an approach of unsupervised ordering (i.e. a priori not taking account of morphologic subtype, cytogenetics, molecular abnormalities or other external information) 215 cases of AML were classified into the subgroups with similar expression patterns of microRNAs. The correlation view of Figure 1 (right section) shows the clusters with distinctive microRNA signatures. Figure 1 (left section) visualizes the distinct signatures of up- and down-regulated microRNAs. Certain microRNA expression patterns correlated with specific cytogenetic or molecular abnormalities (also shown in Figure 1). Thus microRNA clustering appears representative of particular (cyto)genetic subtypes of leukemia. The microRNA clustering that was evident from unsupervised clustering, was frequently driven by a selected number of microRNAs. Detailed information about cluster distribution, and clinical, cytogenetic and molecular data are presented in the Supplementary Table 1.

The 6 cases of AML with t(15;17) aggregated in cluster #16. One of the AML cases, that clustered together with specimens expressing t(15;17) in cluster #16, had not been reported to carry the t(15;17). However, a subsequent PCR analysis showed the presence of the PML-RARA fusion gene suggesting that the t(15;17) had been missed by cytogenetic analysis. MicroRNA signature of cluster #16 signature was characterized by a set of strongly upregulated microRNAs (microRNA-382, -134, -376a, -127, -299-5p, and -323). Most AMLs with inv(16) were found in microRNA expression cluster #10 (60%; 9/15) and the other six cases showed variable signatures scattered over various clusters. The majority (83%; 10/12 cases) of AML with t(8;21) shared the microRNA signature of cluster #7 that was characterized by mainly downregulated microRNAs.

Interestingly, some AML cases carrying a inv(16) clustered into the cluster characterized by t(8;21) and vice versa. A similar global downregulation of microRNAs was evident in microRNA expression cluster #8 that contained AMLs with *CEBPA* mutations. Three of six AMLs with 11q23 chromosomal abnormalities shared the microRNA expression profile of cluster #18. The microRNA expression profiles of all AMLs with *NPM1* mutations (n=68 cases) were restricted to the 6 clusters #2, #4, #6, #9, #11 and #13 that appeared following unsupervised analysis. Clustering in these instances was mainly determined by a limited set of overexpressed microRNAs (microRNA-10a, -10b -196a and -196b). No characteristic clusters of AMLs with *FLT3*-ITD nor *FLT3*-TKD mutations appeared following unsupervised analysis nor were they apparent for the subsets of AML's with dual *FLT3*-ITD and *NPM1* mutations. Several AML's aggregated together in clusters #1, #14 and #15 and thus shared three characteristic microRNAs expression signatures. However no currently known cytogenetic or known molecular abnormalities were associated with these clusters. Normal bone marrow CD34+ specimens revealed a specific microRNA signature (cluster #20).

Significantly differentially expressed microRNAs in relation to cytogenetic translocations and molecular abnormalities

In order to identify the most significantly differentially expressed microRNAs that are associated with specific subtypes of AML, we performed a supervised analysis using SAM. MicroRNA expression for each of the cytogenetic, t(15;17), t(8;21), inv(16) and molecular (*NPM1, CEBPA* and *FLT3*-ITD mutations) subtypes was compared to the microRNAs expression patterns of the remaining AMLs of the cohort. Several discriminating microRNAs were identified for each subtype. None of these microRNAs was localized in the chromosomal region specific for the corresponding cytogenetic translocation such as t(15;17), t(8;21) and inv(16). The detailed data on SAM are presented in the Supplementary Table 2.

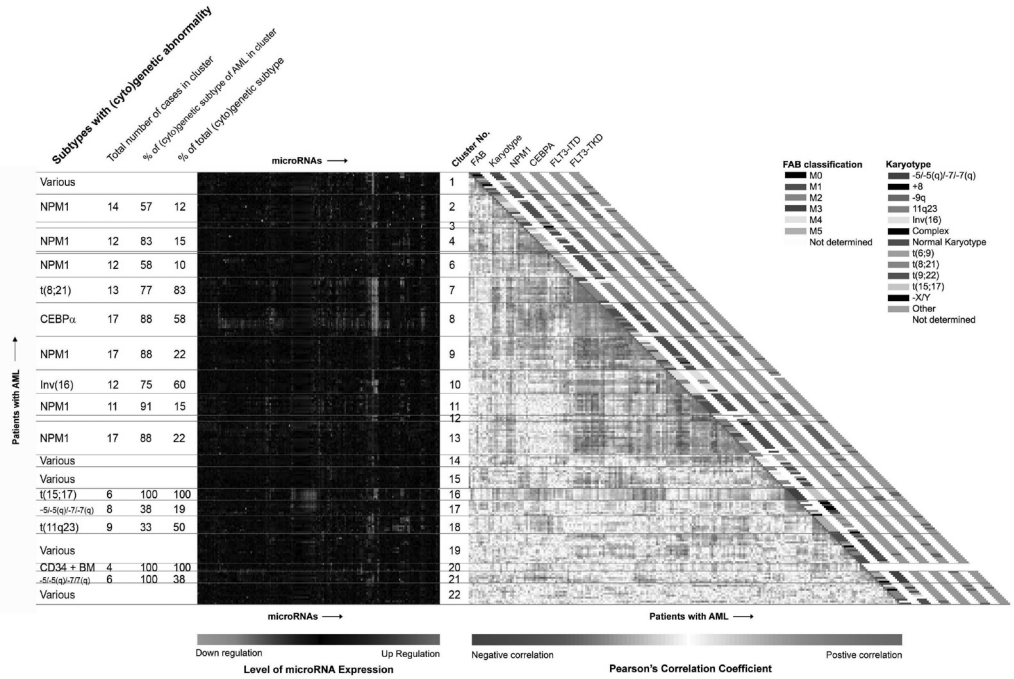


Figure 1.
Correlation view based on unsupervised clustering of 215 AML specimens involving 178 differentially expressed mature human microRNAs (right section) and the relative expression levels of microRNAs that characterize each of the individual clusters (left section)In the Pearson's correlation view the red squares indicate a positive correlation and the blue squares a negative pairwise correlation between the microRNAs expression signatures. Using the program Heatmapper [39] cytological, cytogenetic and molecular data were plotted along the diagonal. Cytogenetic abnormalities and FAB classifications are indicated with different colors. The presence of molecular abnormalities, such as *NPM1, CEBPA, FLT3*-ITD and *FLT3*-TKD mutations are indicated in red and absence in green. On the left part of the Figure 1 each individual microRNA expression cluster with a particular (cyto)genetic AML subtype is indicated ('subtypes with (cyto)genetic abnormality'). The total number of cases in each cluster and % of (cyto)genetic subtype of AML in cluster are presented in the columns. In addition, also the percentages of these (cyto)genetic subtypes present within each microRNA expression cluster (thus also indicating relative proportion of cases with the abnormality that are located outside the cluster) is given ('% of total (cyto)genetic subtype). The levels of expression of 178 microRNAs of each of the 22 clusters including normal bone marrow CD34+ cells are presented in the Heatmap on the left side of the Correlation view. The scale bar indicates an increase (red) or decrease (green) in the level of expression by a factor of 7 relative to the geometric mean of all samples.

Class prediction of subgroups of AML with cytogenetic or molecular abnormalities by microRNA expression profiling, GEP (mRNA) or the combination of both methods

Using PAM we determined the minimal set of microRNAs and transcripts (mRNA) that could predict a particular (cyto)genetic subtype of AML in the cohort. Furthermore, we performed PAM by combining microRNA and mRNA expression data, to investigate whether microRNAs would improve the accuracy of the prediction. The number of correctly predicted (true positive) and number of false positive cases in the 10-fold cross validation of the training set for individual subtypes as well as the numbers of microRNAs and transcript probe sets (mRNA) in the class predictors are presented in Table 2 and Supplementary Table 3.

A class predictor of 10 microRNAs could predict AML with t(8;21) and a set of 7 microRNAs predicted AML with t(15;17). We constructed a predictor of 72 microRNAs for AML with inv(16). Class prediction of AML subgroups with cytogenetic abnormalities using mRNA-GEP was more accurate since there were no errors in the validation set and it required only 2 mRNA probe sets for t(8;21), 1 mRNA probe set for inv(16) and 8 probe sets for t(15;17).

As regards AML with molecular aberrations, sets of microRNAs were derived that were characteristic for AML with *NPM1, CEBPA* and *FLT3*-ITD mutations, respectively. Defined sets of 28 microRNAs could predict AML with *CEBPA* mutations and 10 microRNAs were predictive of AML with *NPM1* mutations. A selection of as many as 48 microRNAs were required to predict AML with *FLT3*-ITD mutations. MicroRNA and mRNA-GEP based class predictors of AML molecular subtypes showed comparable accuracies. MicroRNA-10a and microRNA-10b were included in the mixed microRNA-mRNA expression predictor of AML with *NPM1* mutations (Supplementary Table 3). In addition, two microRNAs (microRNA-155, microRNA-10b) were included in the combined microRNA-mRNA class predictor of AML with *FLT3*-ITD (Supplementary Table 3). However, the combination did not improve the overall accuracy of prediction of AML with mutant *NPM1* nor mutant *FLT3*-ITD as compared to the mRNA predictor alone.

Table 2. microRNA and mRNA class predictors for subsets of AML with (cyto)genetic abnormalities identified using Prediction Analysis of Microarrays (PAM)*

microRNA Class Predictor							
	Training set (N=143)			Validation set (N=72)			Class Predictor
(Cyto)genetic subset	# of Cases	True Prediction	False positive	# of Cases	True Prediction	False positive	# micro-RNAs
t(15;17)	5	5	3	1	1	4	7
t(8;21)	8	7	8	4	3	3	10
Inv(16)	6	5	10	9	4	2	72
NPM1	47	46	21	21	20	6	10
CEBPA	17	2	3	5	4	1	28
FLT3-ITD	46	33	22	16	14	5	48
mRNA Class Predictor							
	Training set (N=143)			Validation set (N=72)			Class Predictor
(Cyto)genetic subset	# of Cases	True Prediction	False positive	# of Cases	True Prediction	False positive	# Probe sets
t(15;17)	5	4	0	1	1	1	8
t(8;21)	8	8	0	4	4	0	2
Inv(16)	6	6	0	9	9	1	2
NPM1	47	23	11	21	20	2	7
CEBPA	17	13	4	5	3	2	20
FLT3-ITD	46	31	7	16	13	2	36

* PAM was performed to define microRNA and mRNA class predictors containing a minimal number of microRNAs and probe sets (mRNAs), respectively, that could predict whether a case belonged to a particular (cyto)genetic subset (first column). The cohort was randomly segregated into a training set and a validation set. The number of cases in the Training set (column 2) and the Validation set (column 5) are given. The 10-fold method of cross-validation, applied on the training set was used to compute truly predicted cases and errors. The cases that were truly predicted (column 3) or were false positive (column 4) in the Training set are indicated. The minimal number of microRNAs or probe sets (mRNAs) that were identified in the Training set were subsequently tested on the Validation set. The number of cases correctly predicted ie true prediction, this set (column 6) or were false positively predicted (column 7) are shown. The characteristics of the microRNAs and probe sets (mRNAs) are presented in Supplementary Table 3.

DISCUSSION

Our study demonstrates that the heterogeneity of AML can be resolved according their variable microRNAs expression signatures. We used quantitative real-time RT-PCR to study the expression of 260 known human microRNAs.

The cohort of 215 AML cases represents a cross section of a mixed diversity of clinical AML. Others[42,43] and we[3] have previously established that gene expression profiling can comprehensively classify the disorder. We now demonstrate that the expression profiling of a limited set of known human small non-coding microRNAs can also be used to identify subgroups with distinctive microRNA expression patterns, that notably correlate with particular cytogenetic and molecular AML entities.

The unsupervised clustering method revealed an association between the majority of the AMLs with t(8;21), t(15;17), inv(16) and characteristic microRNAs expression profiles (clusters # 7, #16, #10) (Figure 1, Supplementary Table 1). However, not all AMLs with the latter cytogenetic abnormalities clustered together. For example, several cases of AML with inv(16) and t(8;21) expressed microRNA patterns that were discrepant from the more common clusters #10 and #7. Interestingly 2 cases with t(8;21) clustered together with AMLs with inv(16) and 1 case with inv(16) was found in the cluster # 7 mainly containing cases with t(8;21). Another study [43] had reported that some cases with inv(16) clustered together with cases with t(8;21) and vice versa using mRNA expression profiling. Whether these variations of microRNA and mRNA profiles among the latter cytogenetic subtypes are to be explained by the presence of other coexisting underlying genetic abnormalities in these not yet elucidated leukemias, remains to be seen.

Using supervised analysis, we identified the microRNAs signatures characteristic of AMLs with cytogenetic abnormalities: t(15;17), t(8;21) and inv(16). A prominent signature (microRNA-382, -134, -376a, -127, -299-5p, and -323), predominantly comprised of upregulated microRNAs, was identified for AML with t(15;17). Most of these microRNAs were found to be located on chromosome 14 and therefore appear not directly implicated in chromosomal translocation of AML with t(15;17) itself. The functions of the latter microRNAs in AML with t(15;17) are currently not known and the predicted targets are yet to be experimentally validated.

AML with t(8;21) was characterized mainly by a set of downregulated microRNAs. We found two members of a known tumor suppressor microRNA family, let-7b and let-7c to be downregulated in AML with t(8;21) and also in AML with inv(16). Previous reports have linked let-7 microRNAs to human cancer[23,44-47] showing that they regulate

the expression of oncogenes *RAS*[48] and *HMGA2*.[44,46] Reduced expression of the let-7 in lung cancer is associated with poor prognosis.[49] It is tempting to speculate that, similar to the gain of function of *RAS* due to gene mutations[50-52] *RAS* overexpression due to downregulation of let-7 may be a cooperating event in the pathogenesis of AML with inv(16). Combining our microRNA and mRNA expression data sets we did not find a significant negative correlation between the expression levels of let-7 and its targets *RAS* and *HMGA*. However, microRNAs mainly regulate gene expression by translational inhibition and to a lesser extent by degradation or cleavage of mRNA targets. Therefore, despite a lack of a negative correlation of microRNA and mRNA expression levels, we cannot exclude the possibility of downregulation of let-7 in AML with inv(16) and t(8;21) resulting in increased protein levels of RAS and thereby contributing to malignant transformation in CBF leukemias. SAM also showed microRNA-127 to be significantly downregulated in AML with inv(16). MicroRNA-127 has been reported to function as a tumor suppressor and may be silenced by hypermethylation in cancer models. Epigenetic activation of microRNA-127 results in a downregulation of the *BCL6*-proto-oncogene.[53] Thus altogether it is conceivable that let-7 and microRNA-127 would contribute to the pathobiology of core binding factor leukemias with inv(16) and AML with t(8;21).

SAM also revealed differentially expressed microRNAs in certain molecular subgroups of AML. One prominent signature, predominantly containing upregulated microRNA-10a, microRNA-10b, microRNA-196a and microRNA-196b was identified in AML carrying *NPM1* mutations. *NPM1*-mutations are present in approximately 35% of patients with AML and are the most common molecular abnormality in patients with AML. They are specially prevalent in AML with a normal karyotype.[14] It has been demonstrated that *NPM1* mutations are associated with specific gene expression signatures.[35,54-56] These transcript signatures are characterized by overexpression of homeobox genes (*HOXA, HOXB* families and *TALE* genes). Interestingly, the microRNAs (-10a, -10b, -196a, -196b) that are upregulated in AML with mutant *NPM1*, are all located within the genomic cluster of *HOX* genes. Yekta et al have reported that microRNA-196a directs translational inhibition (cleavage) of *HOXB8* mRNA.[57] These observations are consistent with an aberrant regulatory circuit including *NPM1*, *HOX* genes and microRNAs, which might be engaged in the arrest of cellular differentiation of hematopoietic progenitors and development of AML with mutant *NPM1*. Obviously, functional studies are required to provide formal evidence about the pathogenetic role of these candidate microRNAs.

In AMLs with *FLT3*-ITD we found oncogenic microRNA-155 to be significantly upregulated. Apart from the oncogenic potential of microRNA-155 in lymphomas[27,58] and

its role in immune function[59,60] this particular microRNA has been postulated to have a role in myeloid differentiation.[61]

AML with *CEBPA* mutations was associated with a distinct set of mainly downregulated microRNAs. The possible cause of the phenomenon of downregulation remains entirely elusive. One possibility to be considered is that differential regulation of microRNA processing, i.e. expression levels of enzymes Drosha and Dicer, would result in a global downregulation of microRNA expression in one subtype of AML and upregulation in another. However, no differential expression of Drosha and Dicer was observed in AML patients with *CEBPA* mutation (data not shown), therefore other players may be implicated in this regulation.

Since microRNAs may have independent roles in the pathobiology of AML, not related to the underlying cytogenetic and molecular abnormalities, we performed a supervised analysis to identify differentially expressed microRNA between all AML samples and normal CD34+ bone marrow samples. We found microRNA-21 to be consistently upregulated in AML. In earlier studies this microRNA has been demonstrated to be associated with various cancers. Several tumor suppressors have experimentally been validated as targets of microRNA-21[62,63]. Furthermore, microRNA-21 was found to contribute to cancer growth by modulating *PTEN* expression[64]. Conditional deletion of *PTEN* in adult hematopoietic cells produced myeloproliferative disease and transplantable leukemias.[65,66] Potential function of microRNA-21 in relation to *PTEN* and AML deserves further investigation.

The microRNAs expression profiles were used for the class prediction of various subtypes of AML. We were able to predict AMLs with t(8;21), t(15;17) and inv(16) by limited sets of selected microRNAs. We also used PAM to compare the ability of class prediction by microRNA expression with that by transcript expression profiles in the same series of patients. Prediction of AML with t(8;21), t(15;17) and inv(16) using microRNAs required a greater number of microRNAs than mRNA probe sets. That the mRNA predictor required a smaller set of probes than the classifier constructed by microRNAs, is not at all surprising. MicroRNA analysis made use of a restricted number of only 260 microRNA's which is in sharp contrast to 54675 probes that were included on the microarray employed for mRNA profiling. As a matter of fact, the observed predictability of AML subtypes on the basis of a limited number of microRNAs demonstrates the power and potential of the approach of microRNA expression profiling for AML subtypes. The number of known human microRNAs continuously increases and more than 1000 of them are predicted *in*

silico. Therefore, in the future, new microRNAs with specific expression in the subsets of leukemia will likely enhance the possibilities for classification and class prediction of AML by microRNA expression.

To this end, the study presented here demonstrates that AML can be classified according their variable and distinctive microRNA expression profiles. Application of SAM and PAM methods revealed significant differentially expressed microRNAs that characterize and categorize the subgroups of AML with different cytogenetic and molecular aberrations, in particular AML with t(8;21), inv(16), t(15;17) and AML with *NPM1* and *CEBPA* mutations. In comparison with mRNAs the microRNAs class predictor was less accurate in predicting AML with t(15;17), t(8;21) and inv(16). Combinations of selected sets of microRNAs could predict AMLs with mutations in the genes of *NPM1, CEBPA* and *FLT3*-ITD with similar accuracy as mRNA probe set combinations. Furthermore, several established oncogenic and tumor suppressor microRNAs, such as microRNA-155, microRNA-21, let-7 appear to be associated with specific subtypes of AML. These data suggest that microRNAs are implicated in the pathogenesis of AML and may have a useful role in future diagnostics.

ACKNOWLEDGMENTS

We want to thank Andre Uitterlinden (Department of Internal Medicine) for providing robot facility and Pascal Arp (Department of Internal Medicine) for his technical support in high-throughput experiments using a robot. Furthermore, Berna Beverloo (Department of Clinical Genetics) for reviewing cytogenetic abnormalities and Mars van 't-Veer and HRC (Hematology Review Committee) for reviewing AML bone marrow clinical diagnostics. We also thank Bas Wouters for his support in gene expression data analysis and Ruud Delwel for helpful discussions. This work was financially supported by Erasmus University Medical Center grant to M.J.L.

Supplementary information is available in the Appendix or on the Blood website

REFERENCES

1. Ebert BL, Golub TR. Genomic approaches to hematologic malignancies. Blood. 2004;104:923-932.
2. Valk PJ, Delwel R, Lowenberg B. Gene expression profiling in acute myeloid leukemia. *Curr Opin Hematol.* 2005;12:76-81.
3. Valk PJ, Verhaak RG, Beijen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med.* 2004;350:1617-1628.
4. Bullinger L, Valk PJ. Gene expression profiling in acute myeloid leukemia. *J Clin Oncol.* 2005;23:6296-6305.
5. Bullinger L, Dohner K, Bair E, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med.* 2004;350:1605-1616.
6. Grimwade D, Walker H, Harrison G, et al. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. *Blood.* 2001;98:1312-1320.
7. Slovak ML, Kopecky KJ, Cassileth PA, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. *Blood.* 2000;96:4075-4083.
8. Byrd JC, Mrozek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). *Blood.* 2002;100:4325-4336.
9. Dohner K, Schlenk RF, Habdank M, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood.* 2005;106:3740-3746.
10. Thiede C, Koch S, Creutzig E, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood.* 2006;107:4011-4020.
11. Frohling S, Schlenk RF, Breitruck J, et al. Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. *Blood.* 2002;100:4372-4380.
12. Preudhomme C, Sagot C, Boissel N, et al. Favorable prognostic significance of CEBPA mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). *Blood.* 2002;100:2717-2723.
13. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, Meijer J, et al. Biallelic mutations in the CEBPA gene and low CEBPA expression levels as prognostic markers in intermediate-risk AML. *Hematol J*. 2003;4:31-40.
14. Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biologic and clinical features. *Blood.* 2007;109:874-885.
15. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281-297.
16. Plasterk RH. Micro RNAs in animal development. *Cell.* 2006;124:877-881.
17. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature.* 2005;435:834-838.
18. Kent OA, Mendell JT. A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. *Oncogene*. 2006;25:6188-6196.
19. Xi Y, Shalgi R, Fodstad O, Pilpel Y, Ju J. Differentially regulated micro-RNAs and actively translated messenger RNA transcripts by tumor suppressor p53 in colon cancer. *Clin Cancer Res.* 2006; 12:2014-2024.

20. Cummins JM, Velculescu VE. Implications of micro-RNA profiling for cancer diagnosis. *Oncogene.* 2006;25:6220-6227.

21. Cummins JM, He Y, Leary RJ, et al. The colorectal microRNAome. *Proc Natl Acad Sci U S A.* 2006;103:3687-3692.

22. Iorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res.* 2005;65:7065-7070.

23. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res.* 2004;64:3753-3756.

24. Calin GA, Ferracin M, Cimmino A, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med.* 2005;353:1793-1801.

25. Hayashita Y, Osada H, Tatematsu Y, et al. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res.* 2005;65:9628-9632.

26. Kluiver J, van den Berg A, de Jong D, et al. Regulation of pri-microRNA BIC transcription and processing in Burkitt lymphoma. *Oncogene.* 2007;26:3769-3776.

27. Eis PS, Tam W, Sun L, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A.* 2005;102:3627-3632.

28. Debernardi S, Skoulakis S, Molloy G, Chaplin T, Dixon-McIver A, Young BD. MicroRNA miR-181a correlates with morphological sub-class of acute myeloid leukaemia and the expression of its target genes in global genome-wide analysis. *Leukemia.* 2007;21:912-916.

29. Mi S, Lu J, Sun M, et al. MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia. *Proc Natl Acad Sci U S A.* 2007;104:19971-19976.

30. Isken F, Steffen B, Merk S, et al. Identification of acute myeloid leukaemia associated microRNA expression patterns. *Br J Haematol.* 2008;140:153-161.

31. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* 2005;33:e179.

32. Lowenberg B, Suciu S, Archimbaud E, et al. Use of recombinant GM-CSF during and after remission induction chemotherapy in patients aged 61 years and older with acute myeloid leukemia: final report of AML-11, a phase III randomized study of the Leukemia Cooperative Group of European Organisation for the Research and Treatment of Cancer and the Dutch Belgian Hemato-Oncology Cooperative Group. *Blood.* 1997;90:2952-2961.

33. Lowenberg B, van Putten W, Theobald M, et al. Effect of priming with granulocyte colony-stimulating factor on the outcome of chemotherapy for acute myeloid leukemia. *N Engl J Med.* 2003;349:743-752.

34. Ossenkoppele GJ, Graveland WJ, Sonneveld P, et al. The value of fludarabine in addition to ARA-C and G-CSF in the treatment of patients with high-risk myelodysplastic syndromes and AML in elderly patients. *Blood.* 2004;103:2908-2913.

35. Verhaak RG, Goudswaard CS, van Putten W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood.* 2005;106:3747-3754.

36. Lao K, Xu NL, Sun YA, Livak KJ, Straus NA. Real time PCR profiling of 330 human micro-RNAs. *Biotechnol J.* 2007;2:33-35.

37. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002;3:RESEARCH0034.

38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25:402-408.

39. Verhaak RG, Sanders MA, Bijl MA, et al. HeatMapper: powerful combined visualization of gene expression profile correlations, genotypes, phenotypes and sample characteristics. *BMC Bioinformatics.* 2006;7:337.
40. Carey VJ, Gentry J, Whalen E, Gentleman R. Network structures and algorithms in Bioconductor. *Bioinformatics.* 2005;21:135-136.
41. Tibshirani R, Hastie T, Narasimhan B, Chu G. Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci U S A*. 2002;99:6567-6572.
42. Golub TR, Slonim DK, Tamayo P, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science.* 1999;286:531-537.
43. Bullinger L, Rucker FG, Kurz S, et al. Gene-expression profiling identifies distinct subclasses of core binding factor acute myeloid leukemia. *Blood.* 2007;110:1291-1300.
44. Shell S, Park SM, Radjabi AR, et al. Let-7 expression defines two differentiation stages of cancer. *Proc Natl Acad Sci U S A.* 2007;104:11400-11405.
45. Zhang HH, Wang XJ, Li GX, Yang E, Yang NM. Detection of let-7a microRNA by real-time PCR in gastric carcinoma. *World J Gastroenterol.* 2007;13:2883-2888.
46. Lee YS, Dutta A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes Dev.* 2007;21:1025-1030.
47. Brueckner B, Stresemann C, Kuner R, et al. The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. *Cancer Res.* 2007;67:1419-1423.
48. Johnson SM, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. *Cell.* 2005;120:635-647.
49. Yanaihara N, Caplen N, Bowman E, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell.* 2006;9:189-198.
50. Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer.* 2007;7:295-308.
51. Bacher U, Haferlach T, Schoch C, Kern W, Schnittger S. Implications of NRAS mutations in AML: a study of 2502 patients. *Blood.* 2006;107:3847-3853.
52. Valk PJ, Bowen DT, Frew ME, Goodeve AC, Lowenberg B, Reilly JT. Second hit mutations in the RTK/RAS signaling pathway in acute myeloid leukemia with inv(16). *Haematologica.* 2004;89:106.
53. Saito Y, Liang G, Egger G, et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell*. 2006;9:435-443.
54. Alcalay M, Tiacci E, Bergomas R, et al. Acute myeloid leukemia bearing cytoplasmic nucleophosmin (NPMc+ AML) shows a distinct gene expression profile characterized by up-regulation of genes involved in stem-cell maintenance. *Blood.* 2005;106:899-902.
55. Baldus CD, Mrozek K, Marcucci G, Bloomfield CD. Clinical outcome of de novo acute myeloid leukaemia patients with normal cytogenetics is affected by molecular genetic alterations: a concise review. *Br J Haematol.* 2007;137:387-400.
56. Wilson CS, Davidson GS, Martin SB, et al. Gene expression profiling of adult acute myeloid leukemia identifies novel biologic clusters for risk classification and outcome prediction. *Blood.* 2006;108:685-696.
57. Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. *Science.* 2004;304:594-596.
58. Kluiver J, Poppema S, de Jong D, et al. BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *J Pathol.* 2005;207:243-249.

59. Thai TH, Calado DP, Casola S, et al. Regulation of the germinal center response by microRNA-155. *Science.* 2007;316:604-608.

60. Rodriguez A, Vigorito E, Clare S, et al. Requirement of bic/microRNA-155 for normal immune function. *Science.* 2007;316:608-611.

61. Georgantas RW, Hildreth R, Morisot S, Alder J, Civin CI. MicroRNA hsa-mir-155 Blocks Myeloid and Erythroid Differentiation of Human CD34+ Cells. *ASH Annual Meeting Abstracts*. 2006;108:1337-.

62. Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem.* 2007;282:14328-14336.

63. Asangani IA, Rasheed SA, Nikolova DA, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene.* 2007.

64. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology.* 2007;133:647-658.

65. Yilmaz OH, Valdez R, Theisen BK, et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature.* 2006;441:475-482.

66. Zhang J, Grindley JC, Yin T, et al. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature.* 2006;441:518-522.

4

Su Ming Sun[1]
Katarzyna Nowek[1]
Lars Bullinger[2]
Menno K. Dijkstra[1]
Peter J.M. Valk[1]
Hartmut Döhner[2]
Stefan J. Erkeland[1]
Bob Löwenberg[1]
Mojca Jongen-Lavrencic[1]

[1]Department of Hematology, Erasmus University Medical Center, Rotterdam, the Netherlands; [2]Department of Internal Medicine III, University of Ulm, Ulm, Germany

Prognostic and functional relevance of aberrant microRNA-9/9* expression in acute myeloid leukemia

Submitted

5

Su Ming Sun[1*]
Veronika Rockova[1,2*]
Lars Bullinger[3]
Menno K. Dijkstra[1]
Hartmut Döhner[3]
Bob Löwenberg[1#]
Mojca Jongen-Lavrencic[1#]

[1]Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands; [2]Department of Biostatistics, Erasmus University Medical Center, Rotterdam, The Netherlands; [3]Department of Internal Medicine III, University of Ulm, Ulm, Germany, *authors equally contributed, #authors equally contributed

The prognostic relevance of miR-212 expression with survival in cytogenetically and molecularly heterogeneous AML

Leukemia. 2012. Epub 2012/06/14.

ABSTRACT

Acute myeloid leukemia (AML) is a highly heterogeneous disease, characterized by various cytogenetic and molecular abnormalities, many of which may express prognostic value. MicroRNAs (miRNAs) are a class of small regulatory RNAs. The prognostic value of miRNAs in AML is yet to be determined.

Here we set out to identify miRNAs that are consistent significant prognostic determinants, independent from other known prognostic factors. A discovery cohort (n=167) and a validation cohort (n=409) of a heterogeneous AML population were used to reliably identify miRNAs with prognostic value.

We report miR-212 as an independent prognostic factor, significantly associated with a prolonged overall survival (OS) and also event-free and relapse-free survival in a discovery cohort (HR=0.77, P=0.015 for OS) which was subsequently confirmed in an independent validation cohort of 409 cases (HR=0.83, P=0.016). The prognostic significance and the prevalence of high miR-212 did not correlate with specific (cyto)genetic subtypes of AML. High miR-212 expression levels are associated with a gene expression profile which is significantly enriched for genes involved in the immune response.

MiR-212 may improve the current prognostic risk stratification of mixed AML including normal karyotype AML and AML with cytogenetic and molecular abnormalities.

INTRODUCTION

Acute myeloid leukemia (AML) is a complex heterogeneous disease, caused by a chain of events involving genetic and epigenetic changes. These alterations lead to a disease phenotype that is characterized by an accumulation of immature progenitors due to an increased proliferation and block in differentiation. Several of these molecular and genetic changes have been shown to have prognostic relevance and are useful for risk classification in treatment protocols.[1]

Currently the panel of prognostic markers known in AML include clinical characteristics and cytogenetic abnormalities, such as t(8;21)[2,3], inv(16)[4], t(11q23)[5] as well as, molecular aberrations, including somatic gene mutations in nucleophosmin (*NPM1*)[6,7], CCAAT/enhancer binding protein α (*CEBPA*)[8,9], internal tandem duplications in the FMS-like tyrosine kinase 3 gene (*FLT3*-ITD)[10] and DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*).[11-13] In addition, overexpression of particular genes, e.g. high transcript levels of ecotropic virus integration site 1 (*EVI1*)[14] , v-ets erythroblastosis virus E26 oncogene homolog (*ERG*), brain and acute leukemia, cytoplasmic *(BAALC*), meningioma (disrupted in balanced translocation) 1 (*MN1*) or cluster of designation 34 (*CD34)* have recently been proposed as prognostic biomarkers.[15-18]

MicroRNAs (miRNAs) are short non coding RNAs (20-25 nt) capable of regulating protein levels by either cleavage of mRNA transcript or inhibition of translation.[19] There is an increasing body of evidence supporting the important role of miRNAs in hematopoiesis and cellular processes such as differentiation, proliferation and apoptosis.[20] Previously others and we have shown that different miRNA expression patterns reflect the cytogenetic and molecular heterogeneity of AML.[21-23] Recent findings have shown that several miRNAs have functional importance in the development of AML.[24-27] In a cohort of 122 AML patients, Garzon *et al* showed association of miR-199a, miR-199b, miR-191, miR-25 and miR-20 with adverse survival and were able to validate the association of miR-199 and miR-191 in an independent cohort of 60 AML patients.[21] Marcucci *et al* reported the prognostic value of a miRNA signature in normal karyotype AML (NK-AML).[23] Subsequently in a cohort of 187 normal karyotype AMLs, the expression of miR-181a, one of the miRNAs of the signature, predicted better survival.[28]

In the current study we identified miR-212 in a discovery cohort (n=167) as a prognostic relevant miRNA that is widely expressed in AML and that can be confirmed as predictor in an independent validation cohort of heterogeneous AML patients (n=409). We report here that miR-212 expression positively correlates with survival, independently

of known clinical, molecular and cytogenetic prognostic markers. Thus the study reveals a profound independent prognostic impact of a single miRNA in a large heterogeneous cohort of AML including leukemia with normal karyotype as well as those with various cytogenetic and molecular abnormalities.

MATERIALS AND METHODS

Patients, treatment and mutational analysis

All patients included in this study had a newly diagnosed AML according the 2001 WHO classification. All patients provided written informed consent in accordance with the Declaration of Helsinki and accord with assurance filled with and approved by the institutional review board of the Erasmus University Medical Center. The analysis was done in an initial discovery cohort consisting of 167 patients, treated according the protocols 04, 04A, 29 and 42 of the Dutch-Belgian-Hematology-Oncology-Cooperative group (HOVON) (www.hovon.nl). The validation cohort originated from two different sources, i.e. 183 patients of AML treated according to protocol HOVON-42A and 226 AML patients treated according the AML German-Austrian Study Group (AMLSG) protocol 98A[29]. The two cohorts showed similar distribution in miR-212 expression and some differences in patient characteristics. (Supplemental Figure 1, Supplemental Table1). To increase power and to minimize differences, the latter two series were combined resulting in a validation cohort of 409 patients. Demographic, hematological and genetic features of the discovery cohort and the validation cohort are given in Table 1. Both cohorts showed similar overall survival (OS) (Supplemental Figure 2).

Samples, from either bone marrow aspirates or blood at the time of diagnosis, were purified with Ficoll-Hypaque (Nygaard, Oslo, Norway) centrifugation resulting in >80% of blast cells and subsequently cryopreserved.[9,30] Mutation analyses were performed as described previously.[7,9,31]

RNA isolation, miRNA expression, data normalization and miRNA selection

After thawing total RNA isolation, quantitative RT-PCR and analysis were performed as described previously.[22,32] Briefly, total RNA was extracted using Trizol according to manufacturer's protocol (Invitrogen, Breda, The Netherlands). The miR-212 expression in the discovery cohort was determined by real time quantitative RT-PCR assays for miRNAs (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands) in multiplex manner as described previously[22] and for the validation cohort real time quantitative

Table 1. Patients characteristics in the discovery and validation cohorts

	Discovery cohort (n=167)		Validation cohort (n=409)	
	No	% of cohort	No	% of cohort
Clinical parameters				
Age (mean)	43		44	
Range (min-max)	15	60	18	60
WBC, x 10^9 /L (mean)	54		45	
Range (min-max)	0.8	263.4	0.8	427
Sex				
male	92	(55%)	205	(50%)
female	75	(45%)	204	(50%)
ELN genetic risk				(0%)
Favorable	51	(31%)	143	(35%)
Intermediate-I	53	(32%)	128	(31%)
Intermediate-II	36	(22%)	69	(17%)
Adverse	27	(16%)	69	(17%)
Cytogenetics				
+8	12	(7%)	16	(4%)
-5 or -5q	0	(0%)	3	(1%)
-7 or -7q	7	(4%)	9	(2%)
-9q	4	(2%)	8	(2%)
11q23	5	(3%)	10	(2%)
t(6;9)	2	(1%)	2	(0%)
t(8;21)	9	(5%)	28	(7%)
t(9;11)	4	(2%)	9	(2%)
inv(3) or t(3;3)	0	(0%)	3	(1%)
inv(16)	9	(5%)	36	(9%)
Normal karyotype	84	(50%)	204	(50%)
Complex karyotype	14	(8%)	45	(11%)
Other	18	(11%)	37	(9%)
Molecular genetics*				
CEBPA single	4	(2%)	6	(1%)
CEBPA double	12	(7%)	20	(5%)
FLT3-ITD	39	(23%)	71	(17%)
FLT3-TKD	7	(4%)	28	(7%)
NPM1	49	(29%)	109	(27%)

* Molecular data determined in NK-AML

RT-PCR was performed in a singleplex manner according to manufacturer protocol. MiR-212 expression was determined centrally at one location and validation cohorts from the two different trials showed similar distribution in expression. A shift in distribution was

observed between discovery and validation cohort, which correlates with the difference of single and multiplex RT-PCR reaction (Supplemental Figure 1)

Data was normalized using the endogenous control RNU48, which was found to be stable expressed in AML samples[22,32] and other cell types.[32-34] A minimal threshold was set for Ct values above 35 to a ΔCt value of 16.5. The relative quantification method, $2^{-\Delta Ct\ 35}$ was used to calculate the expression relative to RNU48. Finally the expression data was log transformed to obtain symmetrical distribution. The 167 cases of the discovery cohort were analyzed for gene expression profiling (GEP) using Affymetrix Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA) as described previously.[30] The gene expression data are available at http://www.ncbi.nlm.nih.gov/geo/ as accession GSE6891.

Definition of survival endpoints, molecular cytogenetic risk and statistical analysis

Overall survival (OS), event-free survival (EFS), relapse-free survival (RFS) and complete remission (CR) were defined according to recommendation of the European LeukemiaNet (ELN).[1] Genetic risk groups are reported according to the ELN criteria[1] with one minor adjustment, namely, instead of any mutation of *CEBPA* only double mutations in *CEBPA* were considered favorable: Favorable group includes inv(16)(p13.1q22), t(16;16)(p13.1;q22), t(8;21)(q22;q22), NK-AML with mutation in *NPM1* without *FLT3*-ITD[6,7] and NK-AML with *CEBPA* double mutation.[8,9] Intermediate-I refers to remainder of the NK-AML. Intermediate-II involves t(9;11)(p22;q23) and various other cytogenetic abnormalities not classified as favorable or adverse. Adverse risk designates inv(3)(q21q26.2) or t(3;3)(q21;q26.2), t(v;11q23), t(6;9)(p23;q34), -5 or 5q-, -7, abnl(17p) and complex karyotype.[1]

To test for the association to the various survival outcomes, i.e. OS, EFS and RFS, the miRNA expression was used as a continuous variable in both univariable and multivariable analyses using Cox proportional hazards model (reported p-values correspond to the Wald test). The proportional hazards assumption was tested using scaled Schoenfeld residuals. To represent graphically the prediction capabilities of single miRNAs we used Kaplan Meier curves dichotomizing the expression based on the median expression value. Cox proportional hazards model with a penalized spline for miR-212 (df=4) was fitted to assess, whether the miR-212 expression has a linear effect on the hazard function. The test for the nonlinearity (OS: P=0.28/0.6 (univariable analysis, discovery/ validation cohort) and P=0.18/0.69 (multivariable analysis, discovery/ validation cohort)) suggests that it is appropriate to treat the miR-212 expression as a continuous linear variable.

Similar conclusion was obtained from an analogous analysis with EFS and RFS and a logistic regression model for CR. The shapes of the fitted spline curves did not indicate a better referral cutoff value than the median value. Logrank test was then used to test for differences in survival distributions. Logistic regression was used to assess the association between the miRNA expression and CR, univariably and in a multivariable fashion correcting for known prognostic markers (age, log(WBC) and ELN genetic risk). Treatment was added to the multivariable analysis to adjust for the differences in treatment protocols. Andersen-Gill model was used to model the association of survival in relation to a time dependent covariate of transplantation. Spearman correlation analysis was performed to determine the association between two continuous variables, Wilcoxon rank sum test between a continuous and a categorical variable (two categories) and Fisher's exact test between two categorical variables.

To identify genes differentially expressed in miR-212 high/low groups (as determined by the median value), Wilcoxon rank sum analysis was performed, controlling for the false discovery rate (FDR) by the Benjamini-Hochberg procedure (FDR<0.05). The expression values for every gene were determined from either a single probe or a combination of multiple probes. Only probes expressed above lower detection limit (30) in more than 20% of the cases were taken into account in the analysis. In case of multiple probes per gene, log-transformed MAS5 normalized expression intensities were combined by performing a principal component analysis using the first principal component. The expression values were then standardized to have zero mean and standard deviation one.

All performed statistical tests were two-sided. Survival and association analyses were done using Stata/Se v11.1 and the gene expression data analysis was performed in R (version R-2.13.0). Ingenuity systems was used to determine overrepresented pathways in the selected genes.

RESULTS

High miR-212 expression is predictive for favorable survival

Using univariable analysis in the discovery cohort (n=167), we observed high miR-212 expression to be highly predictive for better OS (HR=0.78, P=0.007). Higher miR-212 expression was also found to be strongly associated with higher CR rate, better EFS and RFS (Table 2). These findings were confirmed in the independent validation cohort (n=409), for OS (HR=0.81, P=0.001), EFS and RFS. The relationship between high miR-212 and higher CR rate could not be reproduced in the confirmatory cohort (Table 2). Kaplan-Meier curves for OS (Figure 1), EFS and RFS (Supplemental Figure 3) depict the estimates of survival distribution in patients with high versus low miR-212 expression, as determined by a median expression value.

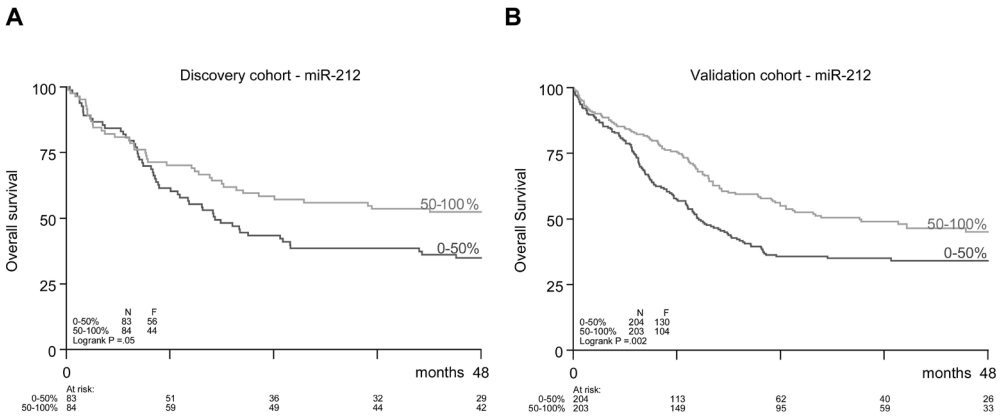


Figure 1. Kaplan-Meier survival curves of patients with high and low miR-212 expression. Patients were dichotomized into high miR-212 and low miR-212 groups based on median expression value of miR-212. Patients with high miR-212 expression had significantly better survival in both the discovery cohort (A) and the validation cohort (B).

Independent prognostic value of miR-212

To correct for established prognostic factors, cytogenetic abnormalities and molecular mutations in the genes *NPM1*, *FLT3*-ITD according to ELN criteria and were included in the multivariable Cox proportional hazard model together with age and log(WBC). A minor modification was made with regards to the ENL criteria, normal karyotype with *CEBPA,* only double mutations were considered favorable instead of any mutated *CEBPA*.

Table 2. Association of miR-212 expression levels with achievements of complete response and survival in the discovery and validation cohorts

	Discovery cohort						Validation cohort					
	HR/ OR	Std. Err	z	P-value	[95% Conf.	Interval]	HR/ OR	Std. Err	z	P-value	[95% Conf.	Interval]
Univariable analysis												
CR	1.74	0.46	2.32	**0.020**	1.10	2.99	1.04	0.15	0.35	0.727	0.80	1.38
EFS	0.75	0.07	-3.14	**0.002**	0.59	0.89	0.86	0.06	-2.67	**0.008**	0.73	0.95
RFS	0.73	0.10	-2.55	**0.011**	0.52	0.92	0.82	0.07	-2.70	**0.007**	0.67	0.94
OS	0.78	0.08	-2.72	**0.007**	0.60	0.92	0.81	0.06	-3.36	**0.001**	0.69	0.91
Multivariable analysis*												
CR	1.78	0.48	2.03	**<0.001**	1.02	2.98	0.91	0.14	-0.67	0.501	0.67	1.21
EFS	0.78	0.08	-2.86	**0.004**	0.61	0.91	0.89	0.06	-1.87	0.062	0.76	1.01
RFS	0.77	0.10	-2.32	**0.020**	0.55	0.95	0.85	0.07	-2.07	**0.038**	0.70	0.99
OS	0.79	0.08	-2.44	**0.015**	0.62	0.95	0.85	0.06	-2.42	**0.016**	0.72	0.97

*Multivariable Cox proportional hazard model adjusted for treatment, included known prognostic factors, i.e. age, log(WBC) and molecular genetics (ENL genetic risk)
Univariable/multivariable logistic regression used for CR and Cox proportional hazards model for EFS, RFS and OS. Hazard ratio (HR) >1 or <1 indicate an increased or decreased risk. Odds ratio (OR) >1 or <1 indicate increased or decreased odds for reaching complete remission. The sample sizes for OS and EFS of the discovery cohort and validation cohorts were n=167 and n=409, respectively. For RFS the sample sizes were n=141 and n=319 for discovery and validation, respectively. P values <0.05 depicted in bold, P values<0.1 & >0.05 depicted in italic.

Difference in treatment protocols were taken along to adjust for treatment related differences. The overall proportionality hazards assumption test was not significant (Supplementary Table 2). In the discovery cohort, high miR-212 remained highly significantly associated with better OS (HR=0.79, P=0.015) as well as with the other survival endpoints, EFS and RFS. In the validation cohort we were able to confirm the independent prognostic value of miR-212 for OS (HR=0.79, P=0.016) (Table 2, Supplementary Table 2). Additionally, miR-212 was also significant as a favorable determinant of RFS in both the discovery (HR=0.77, P=0.020) and the validation cohort (HR=0.85, P=0.038). It was borderline significant in the validation cohort for EFS (HR=0.89, P=0.062) (Table 2, Supplementary Table 2). The interaction term between miR-212 expression and the different genetic risk group was found insignificant for the discovery (P=0.733) and validation cohort (P=0.320) (data not shown).

To determine whether the prognostic value of miR-212 is also independent of the allogeneic stem cell transplantation treatment that is known as a common factor to impact on relapse probability, we considered the Andersen-Gill model with transplantation

as a time dependent covariate for the discovery cohort. After addition of allogeneic transplantation in the model, miR-212 retained its significance (OS: HR= 0.77, P= 0.014; EFS: HR=0.75, P= 0.004; RFS: HR=0.70, P=0.009).

Relationship of high miR-212 expression with other clinical and hematological features

The associations of the clinical and genetic characteristics with the expression levels of miR-212 in the discovery and validation cohorts are summarized in Table 3. Overall, there was no consistent relationship between the distribution of miR-212 expression levels and clinical parameters. Among the genetic subsets only the presence of inv(16) was consistently associated with higher miR-212 in the discovery cohort and in the validation cohort.

Table 3. Relationship of miR-212 expression levels with clinical and genetic features of AML patients in the discovery and validation cohorts.

	Discovery cohort (n=167)		Validation cohort (n=409)	
	P-value	Median difference / Spearman correlation	P-value	Median difference / Spearman correlation
Clinical parameters				
Age	0.345	rho= 0.073	0.467	rho= -0.036
WBC	0.695	rho= 0.030	0.022	rho= -0.113
Sex*	0.820	-0.043	0.179	-0.086
Cytogenetics				
+8	0.202	-0.093	0.250	0.192
t(8;21)	0.966	-0.053	0.642	-0.167
inv(16)	0.059	-0.501	0.004	-0.432
Normal karyotype	0.851	0.033	0.541	-0.060
Complex karyotype	0.961	0.103	0.009	0.220
Other	0.392	0.093	0.052	-0.279
Molecular Genetics**				
CEBPA double	0.072	0.417	0.242	-0.280
FLT3-ITD	0.976	0.042	0.187	-0.433
NPM1	0.012	-0.060	0.684	0.212

Table reports P-values of Spearman correlation test (together with Spearman correlation coefficients) or Wilcoxon rank sum test (together with median differences in positive vs negative groups) to assess the association between the miRNA expression and different clinical and molecular parameters for the subtypes consisting of more than 5% of the cohort.

* Median difference between females vs males

** Molecular data only determined in patients with normal karyotype

Genes involved in immune response are enriched in patients with high miR-212 expression

To gain more biological insight in AML characterized by high miR-212 expression we studied differential gene expression in the discovery cohort (n=167). We found 867 genes/probes to be differentially expressed in between cases with high and low miR-212 expression (as determined by the median expression value) (Supplemental Table 3). Analysis using Ingenuity pathway analysis showed that most enriched pathways among the molecular and cellular functions belong to cell to cell interaction ($P=5.44\cdot10^{-8}$), cell death ($P= 6.69\cdot10^{-8}$), antigen presentation ($P=8.52\cdot10^{-8}$) and cellular movement ($P=8.52\cdot10^{-8}$). At the level of physiological system development and function the pathways most significantly enriched pathways were hematological system development and function ($P=8.52\cdot10^{-8}$), hummoral immune response ($P=8.52\cdot10^{-8}$), immune cell trafficking ($P=8.52\cdot10^{-8}$) and hematopoiesis ($P=3.64\cdot10^{-4}$) (Figure 2).

Strikingly many of the genes in the differentially expressed genes between AML patients with high and low miR-212, in these enriched pathways are involved in immune response which largely was found upregulated in high mir-212 expression cases. Among these involved in chemotaxis of immune cells such as chemokine (C-C motif) ligand 3 (*CCL3*), chemokine (C-C motif) ligand 4 (*CCL4*), chemokine (C-C motif) ligand 5 (*CCL5*). We identified other genes like *MN1*, *BAALC* and urokinase plasminogen activator receptor (*PLAUR*) being differentially expressed between AMLs with high and low miR-212 expression.

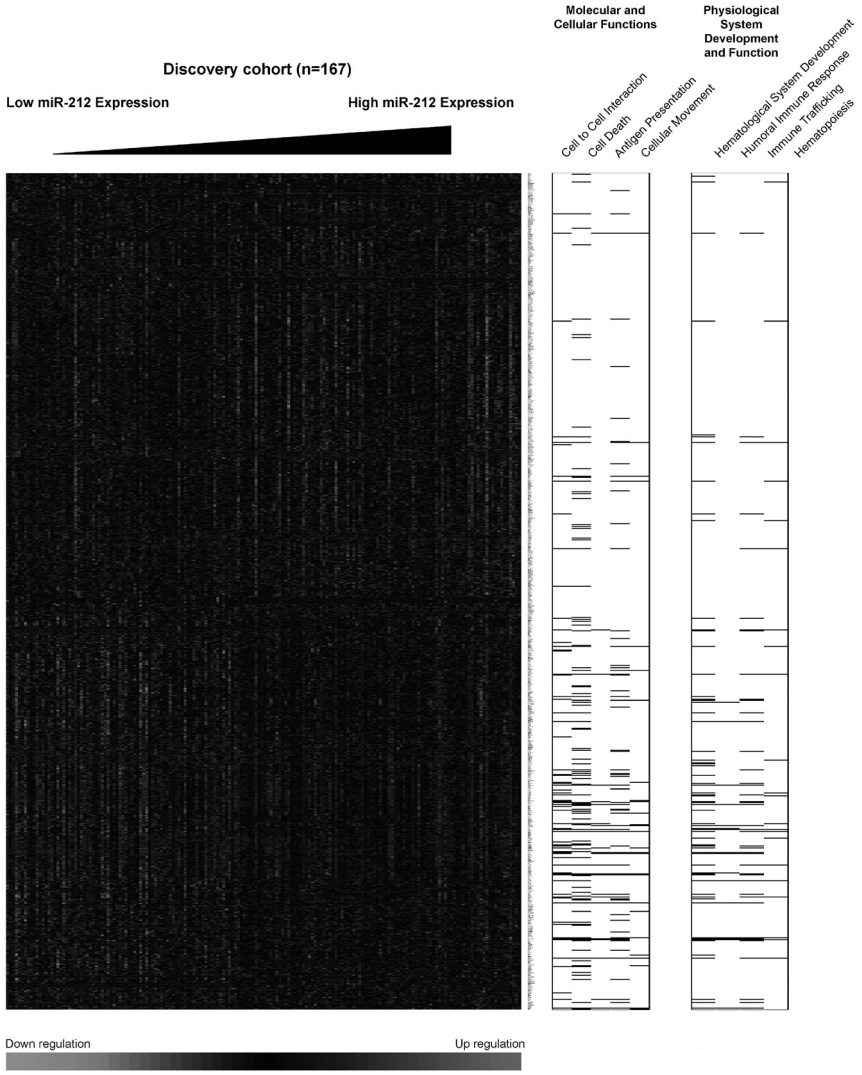


Figure 2. Differentially expressed probes between patients with high and low miR-212 expression in AML.

The left panel shows a heatmap of 867 genes differentially expressed (FDR < 0.05) between high (above median) and low (below median) miR-212 expression groups (845 of these genes were also detected by Spearman correlation analysis (FDR < 0.05) using continuous miR-212 expression). Patients (columns) are ordered from the left to the right by increasing miR-212 values. Genes (rows) have been sorted according to their expression patterns by hierarchical clustering. Green color indicates expression values lower than the mean expression value (black) and red color indicates expression values higher than the mean expression value. The enriched pathways of the selected genes are depicted in the right panel. Columns represent top enriched pathways, where the black color indicates the involvement of the particular gene in the above mentioned category.

DISCUSSION

The relevance of miRNAs as prognostic markers in heterogeneous patient population of AML is largely unknown. In this study we have investigated the role of miR-212 as prognostic factor in AML that are independent of established confounders and can be used as predictors for survival. In the present study we were able to validate the strong prognostic value of miR-212 in an independent validation cohort.

MiR-212 shows significant association with various survival endpoints using univariable and multivariable analysis including well established prognostic confounders in two independent cohorts. Thus miR-212 emerged from the analysis as a notably robust prognostic determinant of overall survival, event-free survival and relapse-free survival. MiR-212 expression level predicts better prognosis and adds to the prognostic effect of various previously established molecular and cytogenetic markers in a highly mixed population of AML. Previously, several other molecular prognostic markers have been identified in AML, such as mutations in the genes *NPM1*, *FLT3*-ITD, *CEBPA*, and high expression of the transcripts of *ERG, BAALC, CD34* and miR-181. However these molecular biomarkers express prognostic impact which is mainly restricted to leukemia without cytogenetic abnormalities, so called normal karyotype AML. Furthermore, a portion of these molecular markers like *ERG, BAALC, CD34* and *NPM1* show strong correlations with each other and or interact with other specific molecular subtypes.[15-18,28]

The expression level of miR-212 does not associate with any particular known AML subtype. It presents an independent prognostic marker that may contribute to the current risk classification of AML patients. In addition, the present study involves patients of age 60 and younger, additional studies in independent cohorts with younger and elderly patients are warranted to determine the overall prognostic relevance of miR-212.

To derive some biological insights in AML characterized by high miR-212 expression, we identified genes differentially expressed between patients with the highest and lowest miR-212 expression. Interestingly, we found a significant enrichment of genes involved in the immune response, in particular those involved in chemotaxis of immune cells. For example, *CCL3* and *CCL4*, upregulated in high miR-212 expression cases, belong to the CCL2-4/CXCL1/8 class of chemokines and the release of these chemokines, influence the T- and NK-cells chemotaxis.[36,37] Conceivably stronger chemotaxis of immune cells by leukemic cells might contribute to their anti-leukemic effects as part of the immune response resulting in a better response to therapy in patients with high miR-212 expression.[38,39]

Among the differentially expressed genes, we also find genes with previously reported prognostic significance in AML. *BAALC*[15] and *MN1*[16] were downregulated and

PLAUR[40] were upregulated in patients with high miR-212 expression. These markers were not included in the multivariable analysis. Following the addition of these factors to other previously indicated prognostic markers in the multivariable analysis miR-212 remained significant indicating the prognostic ability of miR-212 independent of those molecular prognostic factors (Supplemental Table 4).

The function of miR-212 in AML and other hematopoietic malignancies is unknown. An increased expression of miR-212 was observed in pancreatic cancer where it has been suggested to act as an oncomiR by down modulation of retinoblastoma tumor suppressor.[41] In gastric cancer miR-212 has been reported to be differentially expressed in certain subtypes with a possible function in regulation of methylation via methyl-CpG-binding protein.[42-44] In head and squamous cell carcinoma and non-small cell lung cancer cell lines miR-212 is shown to modulate the cell survival, either by down regulating *PED* and thereby causing an increase in tumor necrosis factor alpha (TNFa) related apoptosis[45] or overcoming cetuximab resistance by targeting heparin-binding EGF-like growth factor (*HB-EGFR)*.[46] This indicates the importance of miR-212 in regulating cell survival, the role of the aforementioned targets in AML is unclear as the targets are not among the genes found to be differentially expressed between patients with high and low miR-212 expression. Further research is required necessary to elucidate the biological role of miR-212 in AML.

In conclusion, we identified and validated that high expression of miR-212 predicts for better survival independently of other reported prognostic factors among molecularly and cytogenetically heterogeneous AML

ACKNOWLEDGMENTS

Authors thank Wim van Putten from the HOVON data center, for contribution to the statistical analysis and Peter Valk for molecular characterization of the HOVON AML samples and critical reviewing of the manuscript.

This study was supported in part by the Erasmus MC grant (to MJL), the NutsOhra fund (project 1004-195 to MJL), the Deutsche José Carreras Stiftung e.V. (DJCLS R 06/41v to LB), and LB was supported in part by the Deutsche Forschungsgemeinschaft (Heisenberg-Stipendium BU 1339/3-1).

Supplementary information is available in the Appendix and on the Leukemia website

REFERENCES

1. Dohner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood*. 2010;115(3):453-474.
2. Rowley JD. Identificaton of a translocation with quinacrine fluorescence in a patient with acute leukemia. *Ann Genet*. 1973;16(2):109-112.
3. Mrozek K, Prior TW, Edwards C, et al. Comparison of cytogenetic and molecular genetic detection of t(8;21) and inv(16) in a prospective series of adults with de novo acute myeloid leukemia: a Cancer and Leukemia Group B Study. *J Clin Oncol*. 2001;19(9):2482-2492.
4. Arthur DC, Bloomfield CD. Association of partial deletion of the long arm of chromosome 16 and bone marrow eosinophilia in acute non-lymphocytic leukemia. *Blood*. 1983;62(4):931.
5. Ziemin-van der Poel S, McCabe NR, Gill HJ, et al. Identification of a gene, MLL, that spans the breakpoint in 11q23 translocations associated with human leukemias. *Proc Natl Acad Sci U S A*. 1991;88(23):10735-10739.
6. Dohner K, Schlenk RF, Habdank M, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood*. 2005;106(12):3740-3746.
7. Verhaak RG, Goudswaard CS, van Putten W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood*. 2005;106(12):3747-3754.
8. Green CL, Koo KK, Hills RK, Burnett AK, Linch DC, Gale RE. Prognostic significance of CEBPA mutations in a large cohort of younger adult patients with acute myeloid leukemia: impact of double CEBPA mutations and the interaction with FLT3 and NPM1 mutations. *J Clin Oncol*. 2010;28(16):2739-2747.
9. Taskesen E, Bullinger L, Corbacioglu A, et al. Prognostic impact, concurrent genetic mutations, and gene expression features of AML with CEBPA mutations in a cohort of 1182 cytogenetically normal AML patients: further evidence for CEBPA double mutant AML as a distinctive disease entity. *Blood*. 2011;117(8):2469-2475.
10. Nakao M, Yokota S, Iwai T, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia*. 1996;10(12):1911-1918.
11. Thol F, Damm F, Ludeking A, et al. Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. *J Clin Oncol*. 2011;29(21):2889-2896.
12. Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med*. 2010;363(25):2424-2433.
13. Yan XJ, Xu J, Gu ZH, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nat Genet*. 2011;43(4):309-315.
14. Groschel S, Lugthart S, Schlenk RF, et al. High EVI1 expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. *J Clin Oncol*. 2010;28(12):2101-2107.
15. Langer C, Radmacher MD, Ruppert AS, et al. High BAALC expression associates with other molecular prognostic markers, poor outcome, and a distinct gene-expression signature in cytogenetically normal patients younger than 60 years with acute myeloid leukemia: a Cancer and Leukemia Group B (CALGB) study. *Blood*. 2008;111(11):5371-5379.

16. Langer C, Marcucci G, Holland KB, et al. Prognostic importance of MN1 transcript levels, and biologic insights from MN1-associated gene and microRNA expression signatures in cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study. *J Clin Oncol*. 2009;27(19):3198-3204.
17. Marcucci G, Maharry K, Whitman SP, et al. High expression levels of the ETS-related gene, ERG, predict adverse outcome and improve molecular risk-based classification of cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B Study. *J Clin Oncol*. 2007;25(22):3337-3343.
18. Rockova V, Abbas S, Wouters BJ, et al. Risk stratification of intermediate-risk acute myeloid leukemia: integrative analysis of a multitude of gene mutation and gene expression markers. *Blood*. 2011;118(4):1069-1076.
19. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-297.
20. Havelange V, Garzon R. MicroRNAs: emerging key regulators of hematopoiesis. *Am J Hematol*. 2010;85(12):935-942.
21. Garzon R, Garofalo M, Martelli MP, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc Natl Acad Sci U S A*. 2008;105(10):3945-3950.
22. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. *Blood*. 2008;111(10):5078-5085.
23. Marcucci G, Radmacher MD, Maharry K, et al. MicroRNA expression in cytogenetically normal acute myeloid leukemia. *N Engl J Med*. 2008;358(18):1919-1928.
24. Bousquet M, Quelen C, Rosati R, et al. Myeloid cell differentiation arrest by miR-125b-1 in myelodysplastic syndrome and acute myeloid leukemia with the t(2;11)(p21;q23) translocation. *J Exp Med*. 2008;205(11):2499-2506.
25. Garzon R, Heaphy CE, Havelange V, et al. MicroRNA 29b functions in acute myeloid leukemia. *Blood*. 2009;114(26):5331-5341.
26. Popovic R, Riesbeck LE, Velu CS, et al. Regulation of mir-196b by MLL and its overexpression by MLL fusions contributes to immortalization. *Blood*. 2009;113(14):3314-3322.
27. Russ AC, Sander S, Luck SC, et al. Integrative nucleophosmin mutation-associated microRNA and gene expression pattern analysis identifies novel microRNA - target gene interactions in acute myeloid leukemia. *Haematologica*. 2011.
28. Schwind S, Maharry K, Radmacher MD, et al. Prognostic significance of expression of a single microRNA, miR-181a, in cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin On*29. Schlenk RF, Dohner K, Mack S, et al. Prospective evaluation of allogeneic hematopoietic stem-cell transplantation from matched related and matched unrelated donors in younger adults with high-risk acute myeloid leukemia: German-Austrian trial AMLHD98A. *J Clin Oncol*. 2010;28(30):4642-4648.
30. Verhaak RG, Wouters BJ, Erpelinck CA, et al. Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. *Haematologica*. 2009;94(1):131-134.
31. Schlenk RF, Dohner K, Krauter J, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med*. 2008;358(18):1909-1918.
32. Sun SM, Dijkstra MK, Bijkerk AC, et al. Transition of highly specific microRNA expression patterns in association with discrete maturation stages of human granulopoiesis. *Br J Haematol*. 2011;155(3):395-398.
33. Corthals SL, Sun SM, Kuiper R, et al. MicroRNA signatures characterize multiple myeloma patients. *Leukemia*. 2011;25(11):1784-1789.

34. Merkerova M, Vasikova A, Belickova M, Bruchova H. MicroRNA expression profiles in umbilical cord blood cell lineages. *Stem Cells Dev*. 2010;19(1):17-26.

35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408.

36. Bruserud O, Ryningen A, Olsnes AM, et al. Subclassification of patients with acute myelogenous leukemia based on chemokine responsiveness and constitutive chemokine release by their leukemic cells. *Haematologica*. 2007;92(3):332-341.

37. Maghazachi AA. Role of chemokines in the biology of natural killer cells. *Curr Top Microbiol Immunol*. 2010;341:37-58.

38. Kittan NA, Hildebrandt GC. The chemokine system: a possible therapeutic target in acute graft versus host disease. *Curr Top Microbiol Immunol*. 2010;341:97-120.

39. Costello RT, Mallet F, Chambost H, et al. Acute myeloid leukaemia triggering via CD40 induces leukocyte chemoattraction and cytotoxicity against allogenic or autologous leukemic targets. *Leukemia*. 2000;14(1):123-128.

40. Atfy M, Eissa M, Salah HE, El Shabrawy DA. Role of urokinase plasminogen activator receptor (CD87) as a prognostic marker in acute myeloid leukemia. *Med Oncol*. 2011.

41. Park JK, Henry JC, Jiang J, et al. miR-132 and miR-212 are increased in pancreatic cancer and target the retinoblastoma tumor suppressor. *Biochem Biophys Res Commun*. 2011;406(4):518-523.

42. Im HI, Hollander JA, Bali P, Kenny PJ. MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. *Nat Neurosci*. 2010;13(9):1120-1127.

43. Wada R, Akiyama Y, Hashimoto Y, Fukamachi H, Yuasa Y. miR-212 is downregulated and suppresses methyl-CpG-binding protein MeCP2 in human gastric cancer. *Int J Cancer*. 2010;127(5):1106-1114.

44. Xu L, Wang F, Xu XF, et al. Down-regulation of miR-212 expression by DNA hypermethylation in human gastric cancer cells. *Med Oncol*. 2011;28 Suppl 1:S189-196.

45. Incoronato M, Garofalo M, Urso L, et al. miR-212 increases tumor necrosis factor-related apoptosis-inducing ligand sensitivity in non-small cell lung cancer by targeting the antiapoptotic protein PED. *Cancer Res*. 2010;70(9):3638-3646.

46. Hatakeyama H, Cheng H, Wirth P, et al. Regulation of heparin-binding EGF-like growth factor by miR-212 and acquired cetuximab-resistance in head and neck squamous cell carcinoma. PLoS One. 2010.

6

General discussion

Almost two decades have passed since the discovery of the first miRNA. Knowledge about miRNAs has since increased rapidly. The interest in miRNAs in relation to cancer was sparked by initial publications in 2006 that demonstrated deregulation of miRNA expression in cancer.[1,2] Functional studies with miRNAs in biological processes and diseases have led to the discovery that miRNAs are important regulators of protein levels. Knockout studies of the miRNA machinery show that miRNAs are essential in many cell types and cellular processes.[3-5]

AML is a heterogeneous disease, characterized by a block in cell differentiation and increased proliferation abilities of the cells. Many cytogenetic, genetic and epigenetic types of aberrations underlie this disease. The role of miRNAs in human AML remains largely elusive, i.e. in AML as a whole as well as in the distinct AML subtypes. The aim of this thesis was to study the expression profiles of miRNAs in AML, their relevance as predictors of clinical outcomes and their possible function in the biology of AML.

miRNA expression in granulocytic development: Can we identify miRNAs that discriminate between distinct myeloid differentiation stages?

Chapter 2 illustrates that myeloid cells at distinct maturation stages show different miRNA expression signatures. Some miRNAs are specifically expressed in immature cells, whereas others are particularly elevated in more mature subsets. We also found stage specific miRNAs, i.e. miRNAs that are selectively expressed in metamyelocytes, for example. Similar observations are made for miRNA families and clusters, where it is striking that some families are upregulated progressively in concert during maturation. One apparent example in this regard is the miR-181 family. Progressive downregulation of targets of miR-181 family may be functionally important for myeloid differentiation. So although the distinct differentiation stages belong to the same myeloid lineage, the miRNA expression patterns change between the successive stages.

The precise function of these unique miRNAs expressed in particular stages remains an interesting question for future studies. Recently, unique locked nucleic acid (LNA) inhibitors, that are capable of reducing the expression of specific miRNAs in a particular matter became available. The use of LNA inhibitors in functional studies might make it possible to unravel which miRNAs restrictedly expressed in myeloid progenitors, i.e. myeloblasts, are essential for development towards neutrophils. Furthermore, revealing the functional role of particular miRNA signatures in normal myelopoiesis could provide insights into the role of these regulatory RNAs in the development of leukemia.

miRNA expression in AML: Can we identify miRNA expression profiles that associate with the distinct cytogenetically and genetically defined AML subtypes?

Cytogenetic and other genetic changes in AML distinguish different AML subtypes. The discovery that AML cases with similar cytogenetic and genetic abnormalities correlated strongly with their miRNA expression pattern (Chapter 3) suggested that miRNAs are related to the different AML subtypes.[6-10] In fact, we identified limited sets of selected miRNAs that are able to predict AML subtypes, for example AML with translocations t(8;21), t(15;17), and inv(16). This finding is reminiscent of the previously reported pattern of expression of coding genes (mRNA) with the ability to predict AML subsets.[11,12] In Chapter 3 we directly compared the mRNA and miRNA expression predictive abilities for cytogenetic and genetic AML subtypes. The miRNA information does not result in markedly improved AML subtype prediction suggesting that most of the information of the miRNA expression is also contained in the coding gene expression. Thus either the miRNA expression or the mRNA expression has similar value in terms of predicting the specific subtypes.

miRNAs associated with distinct subtypes of AML can be identified. However AML is phenotypically characterized by impairment in the myeloid differentiation. The maturation arrest in AML happens at various stages of myeloid differentiation, which is reflected by different morphologically defined AML subtypes. The miRNAs that are differentially expressed between AML subtypes might in part be a reflection of distinct myeloid differentiation stages rather than AML specific miRNA expression. Identifying AML-specific changes in miRNA expression proved to be a challenge. We profiled the miRNA expression of different stages of granulopoiesis, with the idea of using this as reference set to identify AML-specific miRNAs. It is not known if the morphological properties of AML cells are a result of the pathogenic event or a reflection of the cell type in which the event occurred. In addition, the different miRNA expression profiling procedures and the relative quantification methods used for miRNA profiling in Chapters 2 and 3 make a direct comparison of the expression difficult. Identifying strictly AML-specific miRNAs by combining expression profiling data of normal myeloid differentiation and AML datasets is therefore still a challenge. miR-125b, for instance, was found to be upregulated in AML cases with translocation t(15;17) and t(2;11).[6,13] Similarly, miR-196b is highly upregulated in AML with upregulated *HOX* genes. miR-451 is upregulated in AML compared to normal myeloblast (CD34+) cells. In normal granulocytic differentiation the expression of these miRNAs is high in immature cells and is downregulated in more mature cells. Based on this observation it can be speculated that these miRNAs might be

associated with an immature differentiation stage rather that reflecting pathobiology of particular AML subset. A more direct inclination of AML-specific miRNAs are those not expressed in normal granulocytic differentiation but expressed aberrantly in AML. miR-9 and miR-9* (miR-9/9*), described in Chapter 4, are two examples of such miRNAs.

Unique sets of miRNA can be distinguished for both AML subtypes as well as distinct stages of granulocytic differentiation. Our current knowledge can be improved, as the development of next-generation sequencing techniques allows more in-depth analyses in terms of quantitative data of a complete pool of miRNAs and information on miRNA modifications. This is likely to improve classification and to allow more quantitative comparisons between various cell subsets in the future.

miRNAs as prognostic factors in AML: Is there a correlation of miRNAs expression levels with clinical outcome in AML?

The expression levels of specific miRNAs in AML at diagnosis may also be used as prognostic information for relapse and post-treatment survival. This information could be useful for classifying AML according to risk. It is well established that certain cytogenetic and genetic abnormalities correlate strongly with treatment outcome and predict survival values. Recently the monosomal karyotype index was identified as an independent prognostic factor that improved classic cytogenetic risk stratification.[14] Furthermore, a significant amount of data has been generated in recent years that showed prognostic relevance of a range of gene mutations and aberrant expression of protein-coding genes.[15-19] The question arises as to whether the assessment of the expression levels of particular miRNAs would contribute to the improvement of the existent risk classification.

There is limited knowledge about the prognostic relevance of single miRNAs. High expression levels of one particular miRNA, i.e. miR-181, predict for better prognosis in normal karyotype AML.[20] Recently, miR-3151, located in intron1 of the *BAALC* gene, was identified by RNA deep sequencing. In AML with normal karyotype, the expression of miR-3151 is strongly correlated with *BAALC* expression and like *BAALC,* high miR-3151 expression associates with adverse survival outcomes.[21] In Chapter 4 we described how AML cases with high expression of the aberrantly expressed miRNAs miR-9/9* are associated with favorable survival in subset of patients with AML aged 60 years and less. In Chapter 5 we demonstrated that high miR-212 expression predicts for better survival in AML, independently from other established clinical parameters and cytogenetic and genetic markers included in the ENL prognostic risk score. Many prognostic factors in AML appear to have a biological role in AML as well. The difference observed in

expression of genes involved in immune response between patients with high and low miR-212 expression makes miR-212 an interesting candidate for further functional studies.

In conclusion, miRNAs add prognostic value and may further refine the current risk stratification of AML. Of the 2154 miRNAs currently registered in miRbase version 18, only a fraction have been investigated so far for their potential prognostic value in AML. Therefore, the full potential of miRNAs to improve risk stratification in AML needs to be further established.

The function of miRNAs in AML: What are the functional contributions of miRNAs to the biology of AML?

miRNA profiling studies in AML have revealed an association of specific miRNA patterns with different subtypes of AML. This has raised the question as to whether miRNAs are functionally involved in the biology of AML.[6,7,10,22]

Many of the miRNAs have been studied functionally in MLL-fusion-related AMLs due to the access of various models to study the leukemogenic effect of MLL fusions. For instance, in a subset of clinical AML with MLL-AF9 fusions, miR-196b is found upregulated. Using specific miRNA antisense inhibitors against miR-196b in *in vitro* colony assays, it has been demonstrated that miR-196b contributes to MLL-AF9 induced transforming capacities by increasing cell proliferation, partially blocking cell differentiation and enhancing cell survival.[23] Similarly miR-155 was found upregulated in AML with MLL-fusions.[6,8] In addition, *in vivo* experiments using mouse models with high miR-155 expression in hematopoietic stem cells showed that miR-155 results in a myeloproliferative phenotype in the bone marrow.[24] In another study involving AML-bearing MLL rearrangements, miR-495 was found to be downregulated. Importantly, the data pointed to a mechanism in which the downregulation of miR-495 is a consequence of MLL fusion proteins. Restoration of miR-495 expression in MLL-AF9 transduced bone marrow cells, suppressed colony forming capacities *in vitro* and delayed leukemogenesis *in vivo*. This suggests that miR-495 acts as a tumor suppressor *in vitro* and *in vivo*. In human cell lines and MLL-AF9 induced leukemic bone marrow cells, miR-495 directly targets Pre-B-cell leukemia transcription factor 3 (*PBX3*) and Homeobox protein Meis1 (*MEIS1*) and contributes to the MLL-AF9 induced leukemogenesis by promoting cell growth and cell viability.[25]

In AML with chromosomal translocation t(2;11), miR-125b, which is located near the chromosomal breakpoint, is found to be specifically upregulated. Introduction of miR-125b into the myeloid cell lines or normal CD34+ primary hematopoietic cells interferes

with cellular differentiation *in vitro* towards granulocytes and monocytes.[13,26] In addition, miR-125a and miR-125b are highly expressed in hematopoietic stem cells and appear to regulate the stem cell numbers.[27,28] Transplantation of fetal liver cells overexpressing miR-125b causes the generation of leukemia, although mainly B and T cell leukemias were found rather than AML.[29]

These studies provide strong evidence indicative of the functional impact of miRNAs in the development of AML. However a few remarks can be made. These miRNAs are generally not restricted to one subtype. Many of these miRNAs, as shown in Chapter 3, are also found differentially expressed in other AML subtypes, like miR-196b, which besides MLL abnormalities is also highly associated with AML carrying mutation in *NPM1*. It remains unclear if miR-196, for example, has the same function in other than MLL-fusion AMLs. In addition, the up/down regulation of the miRNAs in the overexpression or knockdown experimental models generally have a much larger magnitude than observed in AML patients. Recently Mukherji *et al* demonstrated using a reporter assay that the effect of a miRNA is dependent on the levels of its targets.[30] Below a certain threshold value the downregulating effect of the miRNA increase dramatically, thereby shifting from fine-tuning to a switch like function. It is likely that this phenomenon is also dependent on the miRNA levels and therefore the functional effect of miRNAs is probably dosage dependent.

Another important miRNA is miR-223, a miRNA that is predominantly expressed in hematopoiesis.[31] In Chapter 2 we showed that expression of miR-223 is greatly enhanced in mature granulocytes. These data may suggest a role of miR-223 in granulocytic differentiation. Surprisingly miR-223 was found to be dispensable for granulocytic development, but it appeared important for maintaining the homeostasis as a negative regulator, as miR-223 knockout mice show an expanded granulocytic compartment.[32] In addition, miR-223 is reported to be downregulated in specific AML subtypes[33,34], in particular the subtypes where CEBPA is deregulated. Downregulation of miR-223 might contribute to leukemogenesis by enhancing proliferation through the upregulation of E2F1 and MEF2C. As a matter of fact miR-223 appears to play a key role in an autoregulatory loop involving CEBPA, E2F1 and NF-A.[32,34,35] Nevertheless, it remains difficult to envision what the anti-proliferative effect of miR-223 in the remaining AML subtypes is, as the differential expression of miR-223 might be a reflection of morphological differences embedded in the different AML subtypes.

By combining the information we obtained on miRNA expression levels from the studies in Chapters 2 and 3, we aimed to identify candidate miRNAs with a potential role in

leukemogenesis, irrespective of granulocytic differentiation. We selected miRNAs, miR-9 and miR-9*(miR-9/9*), which have low expression or are expressed below detection level during normal granulocytic differentiation. Although AML with translocation t(8;21) and AML with double mutations in CEBPA gene lack measurable miR-9/9* expression levels , miR-9/9* are highly expressed in the other AML subtypes (Chapter 4). The expression in the latter group therefore seems abnormal. Our *in vitro* studies show that enforced expression of miR-9/9* results in the inhibition of myeloid cell differentiation (Chapter 4). The high expression found of miR-9/9* in AML is similar to the levels obtained in the overexpression studies. Interestingly miR-9/9* overexpression also resulted in inhibition of myeloid differentiation. This is similar to *AML1-ETO* fusion and mutant *CEBPA* that both disrupt the myeloid maturation process. In addition, *AML1-ETO* downregulates *CEBPA*[36] and recent data show that *AML1-ETO*, which acts as a repressor, shares the same DNA binding sites as *ERG.*[37] We identified *ERG* as a miR-9/9* target and showed its downregulation in AML with high miR-9/9* expression. We hypothesize that miR-9/9*, *CEBPA* and *AML1-ETO* interrupt myeloid development by disrupting the same pathways in different manners, thereby playing a critcal role in the pathogenesis of AML.

Future perspective and directions

Studies from the past two decades on the role of miRNAs have revealed their regulation of cellular processes in hematopoietic development, such as proliferation and differentiation, and added an extra dimension to the complexity of normal hematopoiesis and leukemogenesis. The results described in this thesis contribute to a better understanding of the role of miRNAs in the prognosis and biology of AML. Nevertheless, the endeavor to understand the role of miRNA in AML has only just begun. In our studies a comprehensive miRNA expression profiling revealed AML subtypes with specific miRNA signatures. We also selected miRNA candidates to further study their role in risk stratification and their biological function in the development of AML. Many questions remain unanswered. We would like to address several of these with respect to potential approaches and the future studies.

miRNA targets identification and experimental validation

How should we identify miRNA target genes experimentally? In Chapters 4 and 5 we selected potential relevant targets of specific miRNA of interest by studying the inversed correlation of miRNA and mRNAs expression levels in AML using information provided by target prediction algorithms. Target identification is important to further distinguishes

the functions of miRNAs. The further experimental validation of *in silico* predicted miRNA targets is therefore essential. Although information obtained at the transcriptional level may be a good reflection of the effect of miRNAs, some miRNA targets are unchanged at transcript level but altered at the protein level only. A proteomics approach should be used to include those potential miRNA targets, such as stable isotope labeling with amino acids in cell culture (SILAC).[38] SILAC is a highly efficient and quantitative labeling method based on metabolic incorporation of heavy amino acids in cell culture that is followed by tandem mass spectrometry analysis of the whole proteome. For miR-9/9*, for example, *in vitro* model miR-9/9* targets may be identified in the 32D cell line by studying proteome changes upon enforced expression of these miRNAs. The identification of direct miRNA targets relies on the different target algorithms. A more unbiased and direct approach would be the isolation of the RISC complex together with the miRNA-mRNA complexes using high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation followed by sequencing (HITS-CLIP).[39]

After putative target identification, studies are warranted to elucidate the direct functional contribution of candidate targets to the observed miR-9/9* induced inhibition in myeloid cell differentiation. One approach would be to apply *in vitro* shRNAs to knockdown the expression of identified candidate target genes and thereby phenocopy the miR-9/9* inhibitory effect on cell differentiation. This would functionally verify the role of these targets in the observed phenotype. *In vivo* studies are essential for gaining an understanding of the functions of miRNAs under more physiological conditions. In addition, *in vivo* studies with appropriate mouse models harboring specific leukemic genetic aberrations will address potential interactions of miRNAs with known cytogenetic and genetic aberrations in AML and would provide insights into the functional contribution of miRNA in the biology of AML. It would be interesting to study the contribution of miR-9/9* in a pre-leukemic *in vivo* model characterized by cell proliferative advantage. In primary human AML, overexpression of miR-9/9* is also associated with *FLT3*-ITD abnormality. *FLT3*-ITD knock-in murine model shows a myeloproliferative phenotype. These mice develop chronic myeloproliferative disease with a 100% penetrance that does not progress to acute leukemia. We hypothesize that introducing miR-9/9*, in hematopoietic progenitors of *FLT3*-ITD knock-in mice, will result in the development of AML.

miRNAs for treatment of AML

The development of new drugs currently focuses on the discovery of small molecules and other biological inhibitors of protein-coding genes.

Modulation of miRNAs expression and function offers a new therapeutic window. An example of miRNA-based therapy is locked nucleic acid (LNA) inhibitor against miR-122. This has been shown to be effective against the replication of the hepatitis C virus *in vivo* in mice and non-primate monkeys. Currently one miR-122 inhibitor is in a clinical phase 2 trial that aims to study the efficacy of this approach in the treatment of patients with hepatitis C.[40,41] A similar therapeutic possibility could be based on increasing miRNA expression, for example by applying miRNA mimics.[42] miR-29b has been reported to be downregulated in certain subtypes of AML and ectopic expression of this miRNA resulted in induced apoptosis and inhibition of cell proliferation in AML *in vitro* models.[43] Interestingly, treatment of xenograft AML tumors with miRNA-29b mimic resulted in decreased tumor growth indicating a potential opportunity for miRNA based therapeutic targeting. Performing functional screens using currently available miRNA inhibitor libraries in appropriate AML models may further enable miRNA based treatment discovery and development in AML.

REFERENCES

1. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6(11):857-866.
2. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435(7043):834-838.
3. Alemdehy MF, van Boxtel NG, de Looper HW, et al. Dicer1 deletion in myeloid-committed progenitors causes neutrophil dysplasia and blocks macrophage/dendritic cell development in mice. *Blood*. 2012;119(20):4723-4730.
4. O'Carroll D, Mecklenbrauker I, Das PP, et al. A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. *Genes Dev*. 2007;21(16):1999-2004.
5. Raaijmakers MH, Mukherjee S, Guo S, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature*. 2010;464(7290):852-857.
6. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. *Blood*. 2008;111(10):5078-5085.
7. Garzon R, Garofalo M, Martelli MP, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc Natl Acad Sci U S A*. 2008;105(10):3945-3950.
8. Garzon R, Volinia S, Liu CG, et al. MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood*. 2008;111(6):3183-3189.
9. Li Z, Lu J, Sun M, et al. Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. *Proc Natl Acad Sci U S A*. 2008;105(40):15535-15540.
10. Wang Y, Li Z, He C, et al. MicroRNAs expression signatures are associated with lineage and survival in acute leukemias. *Blood Cells Mol Dis*. 2010;44(3):191-197.
11. Bullinger L, Dohner K, Bair E, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med*. 2004;350(16):1605-1616.
12. Valk PJ, Verhaak RG, Beijen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med*. 2004;350(16):1617-1628.
13. Bousquet M, Quelen C, Rosati R, et al. Myeloid cell differentiation arrest by miR-125b-1 in myelodysplastic syndrome and acute myeloid leukemia with the t(2;11)(p21;q23) translocation. *J Exp Med*. 2008;205(11):2499-2506.
14. Breems DA, Van Putten WL, De Greef GE, et al. Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. *J Clin Oncol*. 2008;26(29):4791-4797.
15. Langer C, Radmacher MD, Ruppert AS, et al. High BAALC expression associates with other molecular prognostic markers, poor outcome, and a distinct gene-expression signature in cytogenetically normal patients younger than 60 years with acute myeloid leukemia: a Cancer and Leukemia Group B (CALGB) study. *Blood*. 2008;111(11):5371-5379.
16. Marcucci G, Baldus CD, Ruppert AS, et al. Overexpression of the ETS-related gene, ERG, predicts a worse outcome in acute myeloid leukemia with normal karyotype: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2005;23(36):9234-9242.
17. Langer C, Marcucci G, Holland KB, et al. Prognostic importance of MN1 transcript levels, and biologic insights from MN1-associated gene and microRNA expression signatures in cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study. *J Clin Oncol*. 2009;27(19):3198-3204.
18. Pabst T, Mueller BU, Zhang P, et al. Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet*. 2001;27(3):263-270.

19. Dohner K, Dohner H. Molecular characterization of acute myeloid leukemia. *Haematologica*. 2008;93(7):976-982.

20. Schwind S, Maharry K, Radmacher MD, et al. Prognostic significance of expression of a single microRNA, miR-181a, in cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2010;28(36):5257-5264.

21. Eisfeld AK, Marcucci G, Maharry K, et al. miR-3151 interplays with its host gene BAALC and independently affects outcome of patients with cytogenetically normal acute myeloid leukemia. *Blood*. 2012;120(2):249-258.

22. Marcucci G, Radmacher MD, Maharry K, et al. MicroRNA expression in cytogenetically normal acute myeloid leukemia. *N Engl J Med*. 2008;358(18):1919-1928.

23. Popovic R, Riesbeck LE, Velu CS, et al. Regulation of mir-196b by MLL and its overexpression by MLL fusions contributes to immortalization. *Blood*. 2009;113(14):3314-3322.

24. O'Connell RM, Rao DS, Chaudhuri AA, et al. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med*. 2008;205(3):585-594.

25. Jiang X, Huang H, Li Z, et al. miR-495 is a tumor-suppressor microRNA down-regulated in MLL-rearranged leukemia. *Proc Natl Acad Sci U S A*. 2012;109(47):19397-19402.

26. Bousquet M, Nguyen D, Chen C, Shields L, Lodish HF. MicroRNA-125b transforms myeloid cell lines by repressing multiple mRNA. *Haematologica*. 2012;97(11):1713-1721.

27. Guo S, Lu J, Schlanger R, et al. MicroRNA miR-125a controls hematopoietic stem cell number. *Proc Natl Acad Sci U S A*. 2010;107(32):14229-14234.

28. Ooi AG, Sahoo D, Adorno M, Wang Y, Weissman IL, Park CY. MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. *Proc Natl Acad Sci U S A*. 2010;107(50):21505-21510.

29. Bousquet M, Harris MH, Zhou B, Lodish HF. MicroRNA miR-125b causes leukemia. *Proc Natl Acad Sci U S A*. 2010;107(50):21558-21563.

30. Mukherji S, Ebert MS, Zheng GX, Tsang JS, Sharp PA, van Oudenaarden A. MicroRNAs can generate thresholds in target gene expression. *Nat Genet*. 2011;43(9):854-859.

31. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science*. 2004;303(5654):83-86.

32. Johnnidis JB, Harris MH, Wheeler RT, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature*. 2008;451(7182):1125-1129.

33. Eyholzer M, Schmid S, Wilkens L, Mueller BU, Pabst T. The tumour-suppressive miR-29a/b1 cluster is regulated by CEBPA and blocked in human AML. *Br J Cancer*. 2010;103(2):275-284.

34. Pulikkan JA, Dengler V, Peramangalam PS, et al. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood*. 2010;115(9):1768-1778.

35. Eyholzer M, Schmid S, Schardt JA, Haefliger S, Mueller BU, Pabst T. Complexity of miR-223 regulation by CEBPA in human AML. *Leuk Res*. 2010;34(5):672-676.

36. Pabst T, Mueller BU, Harakawa N, et al. AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t(8;21) myeloid leukemia. *Nat Med*. 2001;7(4):444-451.

37. Martens JH, Mandoli A, Simmer F, et al. ERG and FLI1 binding sites demarcate targets for aberrant epigenetic regulation by AML1-ETO in acute myeloid leukemia. *Blood*. 2012.

38. Vinther J, Hedegaard MM, Gardner PP, Andersen JS, Arctander P. Identification of miRNA targets with stable isotope labeling by amino acids in cell culture. *Nucleic Acids Res*. 2006;34(16):e107.

39. Chi SW, Zang JB, Mele A, Darnell RB. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature*. 2009;460(7254):479-486.

40. Elmen J, Lindow M, Schutz S, et al. LNA-mediated microRNA silencing in non-human primates. *Nature*. 2008;452(7189):896-899.

41. Lanford RE, Hildebrandt-Eriksen ES, Petri A, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science*. 2010;327(5962):198-201.

42. Kota J, Chivukula RR, O'Donnell KA, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell*. 2009;137(6):1005-1017.

43. Garzon R, Heaphy CE, Havelange V, et al. MicroRNA 29b functions in acute myeloid leukemia. *Blood*. 2009;114(26):5331-5341.

NEDERLANDSE SAMENVATTING

Acute myeloïde leukemie (AML) is een vorm van bloedkanker, waarbij de aanmaak en rijping van witte bloedcellen uit stamcellen in het beenmerg verstoord is. De cellen die zich ontwikkelen tot AML cellen hebben verstoringen in twee verschillende processen. Een toegenomen aanmaak in combinatie met verstoorde cel uitrijping, differentiatie, heeft als gevolg dat de abnormale cellen, de normale cellen in het beenmerg overgroeien een tekort komt aan normale rijpe bloed cellen.

AML kan onder worden verdeeld in verschillende subtypes. Dit kan aan de hand van hun uiterlijk (cytomorfologie), waarop de Franse - Amerikaanse - Britse (FAB) classificatie gebaseerd is, of aan de hand van een gezamenlijke (cyto)genetische afwijking. Dat zijn afwijkingen in de chromosomen of in genen. Deze afwijkingen leiden tot een verstoorde functie van bepaalde genen en zijn waarschijnlijk ook betrokken bij het ontstaan van deze specifieke subtypes van AML. In het beenmerg van een AML patiënt kunnen meerdere afwijkingen gevonden worden, die samen bijdragen aan de ziekte beeld. Verschillende (cyto)genetische afwijkingen zijn vaak ook geassocieerd met de overleving kansen van de patiënt. Het is dus belangrijk om de specifieke afwijkingen in kaart te brengen, om zo tot een juiste prognose en therapie te komen voor een individuele patiënt met AML.

MicroRNAs (miRNAs) zijn kleine fragmenten van RNA, van ongeveer 20-25 nucleotiden lang en vooralsnog relatief weinig bestudeerd. Deze kleine miRNAs coderen niet voor eiwitten, zoals RNA vaak doet, maar reguleren de hoeveelheid van specifieke eiwitten. Dat doen ze door samen met het RISC complex aan het uiteinde (de 3 ‘UTR) van lange stukken coderend RNA te binden (zogenaamde messenger transcripten). Na de binding wordt ofwel de translatie tot eiwit tegen gehouden of het gebonden RNA afgebroken. In beide gevallen leidt dit tot een verlaagde hoeveelheid eiwit.

De precieze functie van miRNAs in AML was voorheen niet bekend. Het doel van dit proefschrift is om meer inzicht te krijgen in de expressie patronen van miRNAs en de mogelijke bijdrage van miRNAs in het ontstaan van AML. Tevens willen we bestuderen of miRNAs gebruikt kunnen worden voor de voorspelling van de verschillende AML subtypes en de prognose van patiënt.

Voordat we de expressie van miRNAs in een abnormale situatie bestudeerden, in het geval van AML, hebben we eerst het expressie profiel van miRNAs tijdens de normale rijping van neutrofielen, in kaart gebracht (**hoofdstuk 2**). Hiermee is duidelijk geworden dat elk rijping stadium een specifiek miRNA expressie profiel vertoond en dat miRNAs expressie dus de verschillende cel types reflecteert. Dit suggereert dat miRNAs een functie

hebben binnen de normale differentiatie van neutrofielen. Verder laten we zien hoe sommige miRNA families, miRNAs die de dezelfde eiwitten kunnen reguleren, trapsgewijs tot expressie komen tijdens de differentiatie, zoals de miR-181 familie, waarbij in sequentiële stadia, een miRNA familie lid verhoogd tot expressie komt. Het is dus duidelijke dat verschillende stadia van rijping, ook een verschillende miRNA expressie patroon hebben en dat de verscheidene miRNAs waarschijnlijk een rol spelen in de differentiatie proces.

In **hoofdstuk 3** hebben we gekeken hoe het expressie profiel is van ongeveer 260 verschillende miRNAs in AML cellen van 215 goed gekarakteriseerde verschillende AML patiënten. De belangrijkste bevinding is dat op basis van de miRNA expressie patronen er groepen van AML patiënten ontstaan die gekenmerkt zijn met dezelfde al bekende (cyto) genetische afwijkingen. Dit duidt erop dat miRNAs in AML niet willekeurig verstoord zijn, maar dat er specifieke miRNA patronen te herkennen zijn. Zo is een hoge expressie van miR-10a, miR-10b, miR-196a en miR-196b geassocieerd met AML met een mutatie in NPM1 gen en hebben AML patiënten met t(15;17) en deel van de patiënten met een -5/-7 deletie een verhoogt expressie van een groep miRNAs afkomstig van chromosome 14q32. AML patiënten met t(8;21) en mutaties in *CEBPA* gen lijken een al gehele verlaging van miRNA expressie te vertonen. Verder blijkt het mogelijk om de verschillende subtypes aan de hand van de expressie niveaus van een kleine set miRNAs te voorspellen. Hieruit blijkt dat miRNAs de verschillende AML subtypes reflecteren en mogelijk betrokken zijn bij het ontstaan van AML.

In **hoofdstuk 4** hebben we meer in detail gekeken naar miR-9 en miR-9* (miR-9/9*). In **hoofdstuk 3** vinden we dat miR-9/9* een hogere expressie heeft in AML ten opzichte van jonge normale beenmerg cellen. In **hoofdstuk 2** blijkt ook dat miR-9/9* expressie, naast jonge beenmerg cellen, ook laag blijven in expressie in de rest van de gemeten neutrofielen rijpings stadia. We kunnen dus constateren dat miR-9/9* abnormale expressie vertoont in de meerderheid van de subtypes van AML. Dit hebben we bevestigd in **hoofdstuk 4**, waar we de expressie van miR-9/9* hebben bepaald in groot aantal AML patiënten (n=647). Verder hebben we de expressie van miR-9/9* gekarakteriseerd, in het bijzonder de relatie met de verschillende AML subtypes. Hier vonden we dat miR-9/9* een specifieke expressie vertoont in het geval van AML patiënten met een dubbele mutatie in *CEBPA* gen of t(8;21) tonen een lagere miR-9/9* expressie en gevallen met een *FLT*-ITD mutatie en mutaties in *NPM1* tonen een hogere miR-9/9* expressie. Verder is gebleken dat hoge miR-9/9* expressie is geassocieerd met een betere overleving in een specifieke AML subset. Om de functie van abnormale hoge miR-9 en miR-9* expressie in AML te onderzoeken hebben we in het 32D cel-lijn model de miR-9 en miR-9* expressie

niveau verhoogd. Dit had als gevolg dat de cellen, die normaal gesproken zich kunnen ontwikkelen tot volwassen neutrofiele cellen, een verstoring in de rijping proces krijgen. Uit ons onderzoek blijkt dat dit mogelijk gebeurt door het remmen van de productie van het *ERG* gen product en eiwit, een belangrijke regulator van de hematopoiese. Dit toont aan dat miRNAs direct kunnen bijdragen aan het proces dat leidt tot AML.

Hiernaast wilden we graag weten of de expressie niveaus van miRNAs die we beschrijven in **hoofdstuk 3**, een voorspellende waarde heeft voor de overleving van patiënten met AML en dus een klinische relevantie hebben. In **hoofdstuk 5** hebben we een aantal miRNAs gevonden die waren geassocieerd met overleving, daarvan hebben we gekeken of deze hun associatie met overleving behouden in een onafhankelijke groep. Het blijkt dat hoge miR-212 expressie een robuuste voorspeller is van een betere overleving in AML. Daarnaast blijkt deze relatie onafhankelijk te zijn van andere bekende prognostische factoren. Patiënten met een hogere miR-212 expressie reageren beter op gegeven behandeling en leven daardoor langer dan patiënten met lage expressie van miR-212. Gen expressie analyses tussen patiënten met een hoge en lage miR-212 expressie, wijst uit dat dit mogelijk komt door een beter immune response.

Tot slot wordt er in het afsluitende **hoofdstuk 6** de belangrijkste bevinden in een breder context gezet en de bijdrage van miRNAs aan de verschillende aspecten van AML besproken. Met het onderzoek door ons en anderen staat vast dat miRNAs betrokken zijn met het normale neutrofiele rijpings proces als mede de processen die betrokken zijn in AML en dat miRNAs zowel in staat zijn patiënten in verschillende AML subgroepen te classificeren, als het schatten van een prognose.

DANKWOORD

Tot slot, in het laaste en belangrijkste deel van het proefschrift, wil ik iedereen bedanken die een bijdrage hebben geleverd aan het tot stand komen van dit proefschrift en met name een aantal personen in het bijzonder.

Allereerst gaat mijn dank uit naar mijn promotor en co-promotor. Beste Prof.dr. Bob Löwenberg, bedankt voor de mogelijkheid om onderzoek te doen op de hematologie afdeling, de interesse, alle kritische noten tijdens mijn onderzoek en de begeleiding tijdens het schrijven van de manuscripten.

Mojca Jongen-Lavrencic, ik wil je bedanken de mogelijkheid die je mij gegeven hebt om mijn promotieonderzoek te doen, voor je dagelijkse begeleiding, je drive en toewijding. We hebben de afgelopen jaren intensief samen gewerkt, wat niet altijd even makkelijk ging, maar we hadden beide hetzelfde doel voor ogen. Succes met je toekomstige onderzoek.

Prof. dr. Ossekoppele en Prof.dr. Looijenga, bedankt voor het lezen van dit proefschrift en jullie deelname aan mijn verdediging. Prof.dr. Delwel, Ruud, bedankt voor het deelnemen aan de lees commissie, maar ook voor de nodigde discussies en inzichten de afgelopen jaren.

Menno, mijn paranimf, ik ben blij dat je me bij wilt staan mijn verdediging. Ik ben samen met jou het traject begonnen en ben blij dat ik deze met jou ook kan eindigen. Ik ben blij dat we altijd eerlijk tegen elkaar konden zijn. Bedankt voor al het werk wat je verzet hebt en voor je gezelschap over de jaren heen.

Kasia, my other paranimf, your energetic attitude towards life always made me feel old. Although very briefly, I enjoyed the time we worked together. I am happy that you came to the lab to continue with the miR-9 story. As my successor, you are in luck, I have small feet, so it very easy to fill my shoes.

Team ‘Mojca’ is niet compleet zonder Stefan Havik, bedankt voor je korte bijdrage en gezelschap tijdens lunches.

Promoveren is een lange en moeizame weg met veel obstacles, werken op de 13e heeft daarbij veel geholpen deze obstacles te overwinnen. Het was een plezier om op de 13e te werken, mede dankzij de aanwezigheid van vele collega’s.

Alle leden van de Delwel/Valk/Jongen werkbespreking bedankt, een onmiskenbaar succes, uiteraard dankzij de lekker broodjes en de hoogwaardige discussies.Voor mij waren de werkbesprekingen erg motiverend en interessant. Peter bedankt voor je eerlijke, heldere en down to earth suggesties en opmerkingen. Eric, onze labexpert, Claudia en Marije, bedankt voor jullie technische hulp.

Ivo , Marieke, Stefan E. en Jan C. bedankt voor de wetenschappelijke impulsen tijdens de vrijdag ochtend besprekingen.
Simone en Renée, met jullie heb ik het langst op een kamer gezeten. Ik heb veel aan jullie gehad en van jullie beide heb ik veel geleerd.
Rasti, my first roommate, I enjoyed our work related interactions, and for sure also non work related issues. We should definitely drink more beers together in the future.
Mijn andere voormalige kopkamergenoten, Eric V, mijn (soms verlepte) dimsum partner, Lucila, Kerim en Erdogan, het was een leuke tijd, met veel interessante discussies.
My other ex-colleagues, who went ahead and left the lab before me, Tanja, Sophie, Bas, Sanne, Annemarie, Karishma, Andrjez, Jurgen, Arturo, Jo, Saman and Elnaz, all the best to you guys and good luck with your careers.
En natuurlijk de rest van de 13e, Joyce, Ono, Paulette, Marijke, BMT team, de dames van de diagnostiek, de dames van Secreteriaat en Jan. Egied bedankt voor de lessen photoshop and illustrator lessen en het opmaken van mijn boekje.
Xiwen, I have to thank you for help and support me during a difficult period. Life is more meaningful, when shared with another person. I am happy that I can do this with you.
Ten slotte kan ik niet anders dan mijn ouders, familie en vrienden te bedanken, zonder hun steun heb ik dit alles niet kunnen bereiken.

CURRICULUM VITAE

Su Ming Sun was born on May 28th in Delft. In 2000 he obtained his VWO diploma on the Grotius College in Delft. After his high school he continued with his bachelor study Life Science and Technology at the Technical University of Delft and the Leiden University and finished his bachelor in 2003. Afterwards he continued with the specialization Functional Genomics of the study Life Science and Technology and obtained his master degree with honors in 2006. During his studies, he worked at the department of molecular genetics on the project "Unraveling the function of YML011c (RAD33), a putative Nucleotide Excision Repair protein" under supervision of Prof. dr. Jaap Brouwer, Dr. Riekje Brandsema and Dr. Ben den Dulk. In 2007 he started as graduate student in the hematology department at the Erasmus University Medical Center in Rotterdam working on the project "The role of microRNAs in the pathogenesis of Acute Myeloid Leukemia" under the supervision of Dr. Mojca Jongen-Lavrencic (Co-promotor) and Prof. dr. Bob Löwenberg (Promotor), which resulted in this thesis. In March 2012 he started at the Toxicogenetics department in LUMC where he works on the project "Cohesion networks in sister-chromatid and DNA repair" in the group of Dr. Haico van Attikum.

CURRICULUM VITAE

Su Ming Sun werd geboren op 28 mei 1982 te Delft. In 2000 behaalde hij zijn VWO diploma aan het Grotius College in Delft. Aansluitend ging hij Life Science and Technology studeren aan de Technische Universiteit Delft en Universiteit Leiden, waar hij in 2003 zijn bachelor of science behaald heeft. Aansluitend heeft hij zijn studie Life Science and Technology vervolgend met de specialisatie Funtional Genomics, waar hij in 2006 zijn master of science met lof behaalde. Tijdens zijn studie heeft hij gewerkt aan het onderzoek met de titel "Het ontrafelen van de functie YML011C (RAD33), een onbekend eiwit betrokken bij het nucleotide excisie herstel mechanisme" onder supervisie van Prof. dr. Jaap Brouwer, Dr. Riekje Brandsema en Dr. Ben den Dulk op de moleculaire genetica groep van de universiteit Leiden in Leiden. Na het behalen van zijn ingenieurs diploma is hij als promovendus begonnen in februari 2007 op de afdeling Hematologie van de ErasmusMC en heeft zich bezig gehouden met het project "de functie van miRNAs in het ontstaan van acute myeloïde leukemie" onder supervisie van Dr. Mojca Jongen-Lavrencic (co-promotor) en Prof. dr. Bob Löwenberg (Promotor), aldaar dit proefschrift tot stand is gekomen. Na het voltooien van de promotie traject is hij begonnen als postdoctorale fellow op de afdeling Toxicogenetica van LUMC waar hij werkt aan het project "Cohesie netwerk in zuster chromatid en DNA schade herstel" onder leiding van Dr. Haico van Attikum.

PUBLICATIONS

• **Sun SM**, Nowek K, Bullinger L, Dijkstra MK, Valk PJM, Dohner H, Erkeland SJ, Löwenberg B. Prognostic and functional relevance of aberrant miR-9/9* expression in Acute Myeloid Leukemia. Submitted

• **Sun SM**, Rockova V, Bullinger L, Dijkstra MK, Dohner H, Lowenberg B, Jongen-Lavrencic M. et al. The prognostic relevance of miR-212 expression with survival in cytogenetically and molecularly heterogeneous AML. *Leukemia.* 2012. Epub 2012/06/14.

• Corthals SL, **Sun SM**, Kuiper R, de Knegt Y, Broyl A, van der Holt B, Beverloo HB, Peeters JK, El Jarari L, Lokhorst HM, Zweegman S, Jongen-Lavrencic M, Sonneveld P. MicroRNA signatures characterize multiple myeloma patients. *Leukemia*. 2011;25(11):1784-9. Epub 2011/06/28.

• **Sun SM**, Dijkstra MK, Bijkerk AC, Brooimans RA, Valk PJ, Erkeland SJ, Lowenberg B, Jongen-Lavrencic M. Transition of highly specific microRNA expression patterns in association with discrete maturation stages of human granulopoiesis. B*ritish journal of haematology*. 2011;155(3):395-8. Epub 2011/05/11.

• Meenhuis A, van Veelen PA, de Looper H, van Boxtel N, van den Berge IJ, **Sun SM**, Taskesen E, Stern P, de Ru AH, van Adrichem AJ , Demmers J, Jongen-Lavrencic M, Touw IP, Sharp PA, Erkeland SJ. MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. *Blood*. 2011;118(4):916-25. Epub 2011/06/02.

• Jongen-Lavrencic M, **Sun SM**, Dijkstra MK, Valk PJ, Lowenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. *Blood*. 2008;111(10):5078-85. Epub 2008/03/14.

• den Dulk B, **Sun SM,** de Ruijter M, Brandsma JA, Brouwer J. Rad33, a new factor involved in nucleotide excision repair in Saccharomyces cerevisiae. *DNA repair.* 2006;5(6):683-92. Epub 2006/04/06.

PHD PORTFOLIO SUMMARY

Summary of PhD training and teaching activities

Name PhD student: Su Ming Sun Erasmus MC Department: Hematology Research School: Molecular Medicine	PhD period: 1/02/2007 – 30/08/2011 Promotor(s): Prof.dr. Bob Löwenberg Supervisor: Dr. Mojca Jongen-Lavrencic

1. PhD training		
	Year	**Workload (ECTS)**
General academic skills		
- Laboratory animal science	2008	4.2
In-depth courses (e.g. Research school, Medical Training)		
- Logistic Regression	2007	1.4
- Basic and Translational Oncology	2007	1.8
- Basic data Analysis Gene expr. Arrays	2008	1.1
- Partek	2009	1.3
- Matlab Fundamentals and Statistical Methods	2007	1.3
Presentations		
- 8 Hematology Presentations	2007-2011	5.0
- 3 Journal Club	2007-2011	2.0
(Inter)national conferences		
- 4th Dutch Hematology Congress	2009	1.0
- 51th American Society of Hematology (ASH) (Poster)	2009	2.0
- Affymetrix Core Lab Directors meeting (Invited Oral)	2009	1.0
- Small RNA and Cancer (Keystone) (Poster)	2011	2.0
Seminars and workshops		
- Hematology lectures	2011-2012	3.0
- InDesign workshop	2011	0.5
- The Workshop on Photoshop and Illustrator	2010	0.3
- Browsing Genes and Genomes with Ensemble IV	2009	0.6
Teaching activities		
- Supervising Master student	2011-2012	4.0
Other		
- Organizing Hematology PhD lunch with the Invited speaker	2009-2010	1.0

APPENDIX

Chapter 2

Supplemental Table 1. Purity cell sorted granulocytic maturation stages

Sample	Blast	Myeloblast & Promyeloblast	Metamyelocytes	Neutrophils
1	>90 %	91%	95%	92%
2	>90 %	86%	97%	83%
3	>90 %	94%	94%	90%
4	-	96%	84%	-

Supplemental table 2. Nonexpressed miRNAs

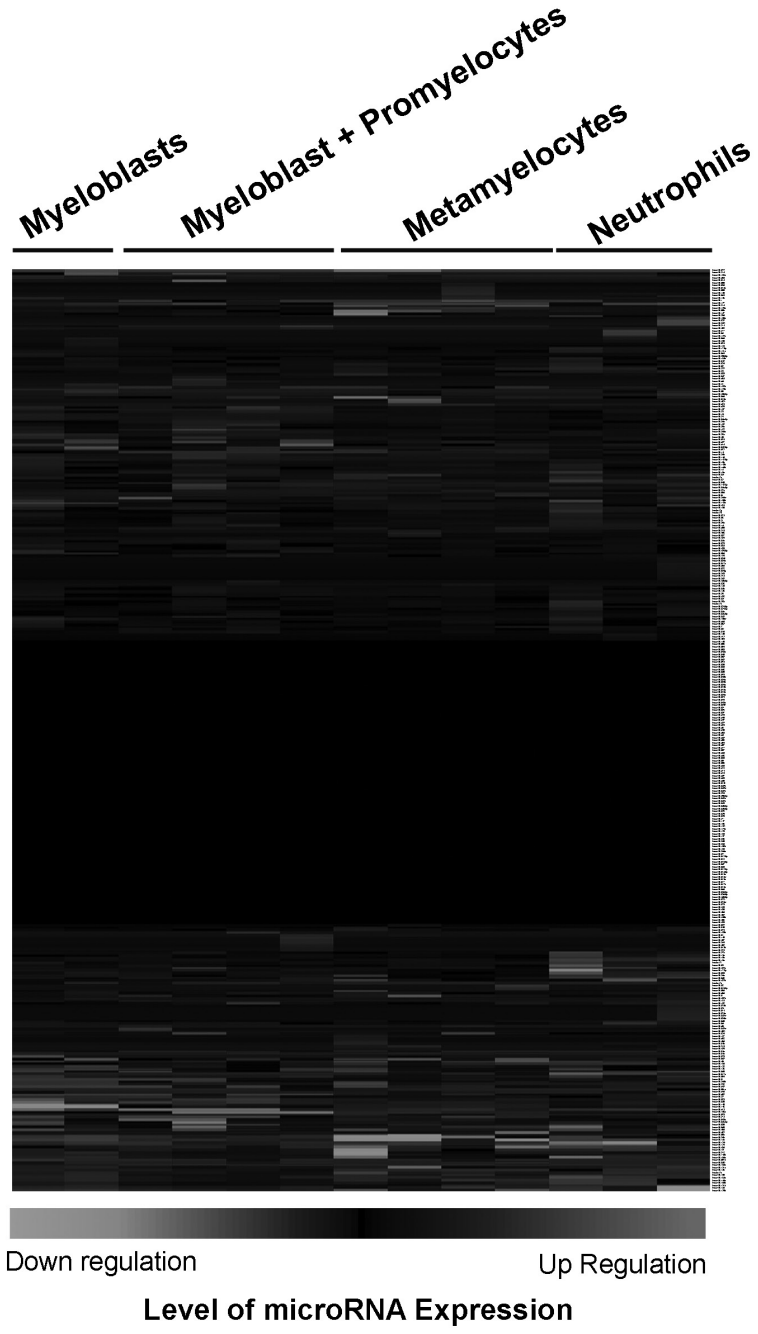
miRNA not expressed in granulopoiesis			
hsa-miR-105	hsa-miR-369-3p	hsa-miR-518f	hsa-miR-587
hsa-miR-124a	hsa-miR-369-5p	hsa-miR-519b	hsa-miR-588
hsa-miR-129	hsa-miR-371	hsa-miR-519d	hsa-miR-591
hsa-miR-139	hsa-miR-373*	hsa-miR-519e	hsa-miR-593
hsa-miR-147	hsa-miR-376a*	hsa-miR-520a	hsa-miR-597
hsa-miR-153	hsa-miR-379	hsa-miR-520b	hsa-miR-600
hsa-miR-182*	hsa-miR-380-3p	hsa-miR-520c	hsa-miR-603
hsa-miR-184	hsa-miR-380-5p	hsa-miR-520d	hsa-miR-606
hsa-miR-184	hsa-miR-409-5p	hsa-miR-520h	hsa-miR-607
hsa-miR-187	hsa-miR-448	hsa-miR-521	hsa-miR-608
hsa-miR-193b	hsa-miR-449b	hsa-miR-523	hsa-miR-609
hsa-miR-198	hsa-miR-453	hsa-miR-524	hsa-miR-613
hsa-miR-200a	hsa-miR-488	hsa-miR-544	hsa-miR-614
hsa-miR-203	hsa-miR-489	hsa-miR-548a	hsa-miR-615
hsa-miR-205	hsa-miR-490	hsa-miR-548b	hsa-miR-617
hsa-miR-206	hsa-miR-492	hsa-miR-548c	hsa-miR-622
hsa-miR-211	hsa-miR-506	hsa-miR-549	hsa-miR-626
hsa-miR-220	hsa-miR-507	hsa-miR-552	hsa-miR-631
hsa-miR-299-3p	hsa-miR-508	hsa-miR-553	hsa-miR-644
hsa-miR-299-5p	hsa-miR-512-3p	hsa-miR-554	hsa-miR-645
hsa-miR-299-5p	hsa-miR-512-5p	hsa-miR-558	hsa-miR-647
hsa-miR-302a	hsa-miR-513	hsa-miR-562	hsa-miR-649
hsa-miR-302b	hsa-miR-515-5p	hsa-miR-563	hsa-miR-653
hsa-miR-302b*	hsa-miR-516-5p	hsa-miR-569	hsa-miR-654
hsa-miR-302c	hsa-miR-517	hsa-miR-570	hsa-miR-657
hsa-miR-302d	hsa-miR-517b	hsa-miR-570	hsa-miR-658
hsa-miR-323	hsa-miR-518a	hsa-miR-575	hsa-miR-661
hsa-miR-325	hsa-miR-518c	hsa-miR-578	hsa-miR-662
hsa-miR-329	hsa-miR-518c*	hsa-miR-585	
hsa-miR-368	hsa-miR-518d	hsa-miR-586	

Supplemental Table 3. is available on the British Journal of Haematology website.

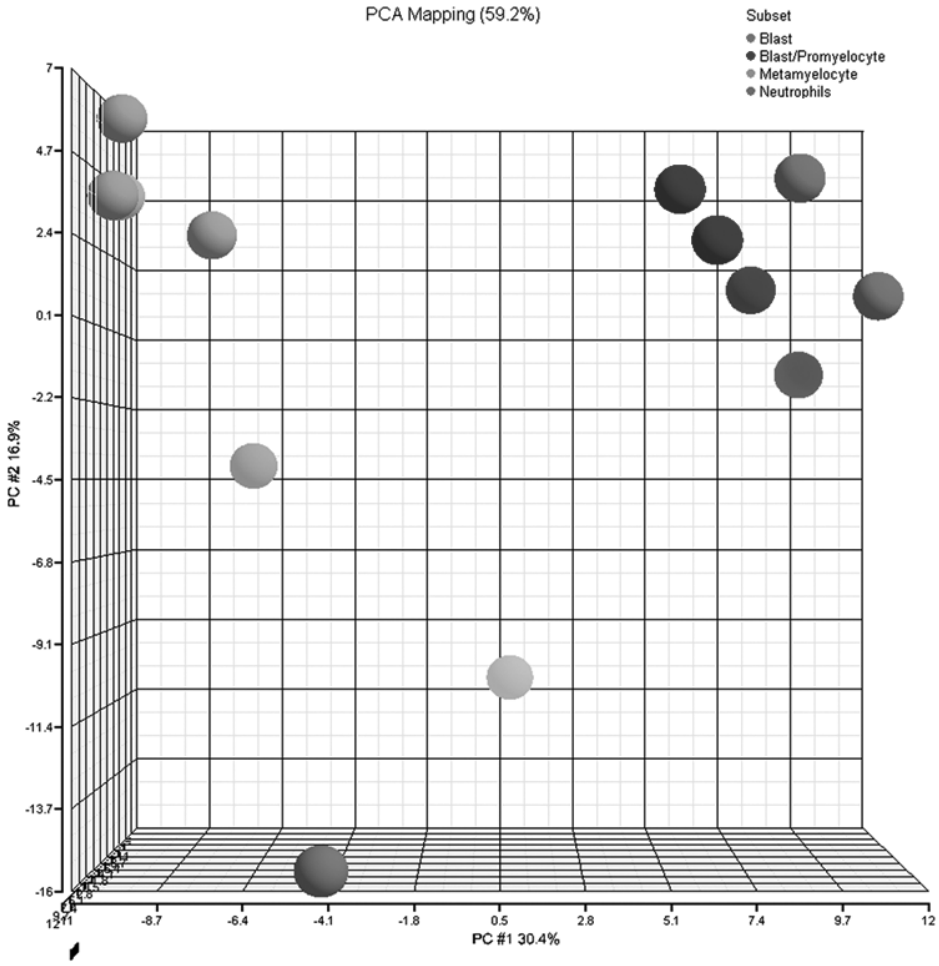
Supplemental Table 4. Differential expressed genomic miRNA clusters

Chromosome	Start	miRNA
3	49032585	hsa-miR-425, ↑
	49032585	hsa-miR-425-5p, ↑
	49033055	hsa-miR-191, ↑
3	161605070	hsa-miR-15b, ↑
	161605227	hsa-miR-16, ↑
7	99529119	hsa-miR-25, ↑
	99529327	hsa-miR-93, ↑
	99529552	hsa-miR-106b, ↑
9	95978060	hsa-let-7a, ↑
	95978450	hsa-let-7f, ↑
9	96887311	hsa-miR-23b,-
	96887548	hsa-miR-27b, ↑
11	64415185	hsa-miR-192, ↑
	64415403	hsa-miR-194, ↑
13	49521110	hsa-miR-16, ↑
	49521256	hsa-miR-15a, ↑
13	90800860	hsa-miR-17-3p, -
	90800860	hsa-miR-17-5p, ↑
	90801006	hsa-miR-18a, ↑
	90801146	hsa-miR-19a, ↑
	90801320	hsa-miR-20a, ↑
	90801447	hsa-miR-19b, ↑
	90801569	hsa-miR-92, ↑
17	6861658	hsa-miR-195, ↑
	6861954	hsa-miR-497, -
19	56887677	hsa-miR-99b, ↑
	56887851	hsa-let-7e, -
	56888319	hsa-miR-125a, ↑
22	20337270	hsa-miR-301, ↑
	20337593	hsa-miR-130b, ↑
X	73423664	hsa-miR-545, ↑
	73423846	hsa-miR-374, -
X	133131074	hsa-miR-363*, -
	133131234	hsa-miR-92, ↑
	133131367	hsa-miR-19b, ↑
	133131505	hsa-miR-20b , ↑
	133131737	hsa-miR-18b, -
X	133508024	hsa-miR-503, ↑
	133508310	hsa-miR-424, ↑

Note: Chromosomal location are obtained from miRBase release 13. Genomic miRNA clusters has been defined as miRNA within chromosomal distance of 500 kb with eachother. Up- and down- regulation have been depicted as ↑ and ↓ and - as non-differential expressed .



Supplemental figure 1. miRNA expression of different stages of granulocytic differentiation.
Heatmap of geometric mean relative miRNA expression showing all miRNAs of the different granulocytic maturation stages, depicted on the rows and columns respectively. Expression was represented by colour scale, where green reflects lower expression; black reflects mean expression and red reflects higher expression.



Supplemental Figure 2. Principal Component Analysis.
Principal Component Analysis plot, depicting the coordinates of the samples in three principal components space derived from their miRNA expression.

Chapter 3

Supplementary table 1 can be found on the Blood website.

Supplemental Table 2.

Abnormality	Gene ID	Score(d)	Fold Change	Chromosome	Start	End	Strand
11q23	hsa-miR-9	2.97	8.63	1	153203206	153203294	-
11q23	hsa-miR-9	2.97	8.63	5	87998427	87998513	-
11q23	hsa-miR-9	2.97	8.63	15	87712252	87712341	+
11q23	hsa-miR-429	2.49	2.37				
11q23	hsa-miR-213	-1.88	0.35	1	195559830	195559939	-
11q23	RNU49	-2.10	0.19				
11q23	hsa-miR-146a	-2.90	0.17	5	159844937	159845035	+
5/7(q)	hsa-miR-125a	2.99	2.16	19	56888319	56888404	+
5/7(q)	hsa-let-7e	2.79	2.11	19	56887851	56887929	+
5/7(q)	hsa-miR-130a	2.62	2.14	11	57165247	57165335	+
5/7(q)	hsa-miR-203	1.93	6.40	14	103653495	103653604	+
5/7(q)	hsa-miR-99a	-1.48	0.27	21	16833280	16833360	+
5/7(q)	hsa-miR-148a	-1.52	0.43	7	25762779	25762846	-
5/7(q)	hsa-miR-10b	-1.52	0.24	2	176840538	176840647	+
5/7(q)	hsa-miR-182	-1.52	0.37	7	129004174	129004283	-
5/7(q)	hsa-miR-9	-2.38	0.15	5	87998427	87998513	-
5/7(q)	hsa-miR-9	-2.38	0.15	1	153203206	153203294	-
5/7(q)	hsa-miR-9	-2.38	0.15	15	87712252	87712341	+
5/7(q)	hsa-miR-223	-2.53	0.36	X	65021733	65021842	+
8	hsa-miR-124a	2.55	3.40	8	65454260	65454368	+
8	hsa-miR-124a	2.55	3.40	8	9798308	9798392	-
8	hsa-miR-124a	2.55	3.40	20	61280297	61280383	+
8	hsa-miR-9	2.28	2.00	1	153203206	153203294	-
8	hsa-miR-9	2.28	2.00	5	87998427	87998513	-
8	hsa-miR-9	2.28	2.00	15	87712252	87712341	+
8	hsa-miR-335	-2.24	0.28	7	129729903	129729996	+
8	hsa-miR-181a	-2.49	0.41	9	124534275	124534384	+
8	hsa-miR-181b	-2.59	0.40	1	195559659	195559768	-
8	hsa-miR-181b	-2.59	0.40	9	124535543	124535631	+
8	hsa-miR-213	-2.75	0.30	1	195559830	195559939	-
CEPBA	hsa-miR-335	3.01	2.26	7	129729903	129729996	+
CEPBA	hsa-miR-181a	2.26	2.02	9	124534275	124534384	+
CEPBA	hsa-miR-497	-1.26	0.47				
CEPBA	hsa-miR-224	-1.45	0.07	X	150797618	150797698	-
CEPBA	hsa-miR-218	-1.51	0.40	5	168127729	168127838	-
CEPBA	hsa-miR-218	-1.51	0.40	4	20206167	20206276	+

Abnormality	Gene ID	Score(d)	Fold Change	Chromosome	Start	End	Strand
CEPBA	hsa-miR-511	-1.75	0.36				
CEPBA	hsa-miR-214	-1.76	0.31	1	168839595	168839704	-
CEPBA	hsa-miR-193a	-1.88	0.48	17	26911128	26911215	+
CEPBA	hsa-miR-365	-1.91	0.36				
CEPBA	hsa-miR-99b	-2.32	0.46	19	56887677	56887746	+
CEPBA	hsa-miR-500	-2.55	0.42				
CEPBA	hsa-miR-21	-3.38	0.40	17	55273409	55273480	+
CEPBA	RNU49	-3.40	0.44				
CEPBA	hsa-miR-149	-3.50	0.44	2	241115408	241115496	+
CEPBA	hsa-miR-196a	-3.73	0.50	17	44064851	44064920	-
CEPBA	hsa-miR-196a	-3.73	0.50	12	52671789	52671898	+
CEPBA	hsa-miR-9	-3.95	0.17	5	87998427	87998513	-
CEPBA	hsa-miR-9	-3.95	0.17	1	153203206	153203294	-
CEPBA	hsa-miR-9	-3.95	0.17	15	87712252	87712341	+
Flt3-ITD	hsa-miR-155	4.92	2.43	21	25868163	25868227	+
Flt3-ITD	hsa-miR-10b	4.71	2.26	2	176840538	176840647	+
Flt3-ITD	hsa-miR-511	2.78	3.31				
Flt3-ITD	hsa-miR-135a	1.97	2.14	12	96460058	96460157	+
Flt3-ITD	hsa-miR-135a	1.97	2.14	3	52303275	52303364	-
Flt3-ITD	hsa-miR-203	-1.18	0.50	14	103653495	103653604	+
Flt3-ITD	hsa-miR-214	-1.30	0.48	1	168839595	168839704	-
Flt3-ITD	hsa-miR-130a	-1.83	0.49	11	57165247	57165335	+
Flt3-ITD	hsa-miR-145	-2.41	0.36	5	148790402	148790489	+
Flt3-ITD	hsa-miR-182	-2.51	0.28	7	129004174	129004283	-
Flt3-ITD	hsa-miR-30a-3p	-2.63	0.50	6	72169975	72170045	-
Flt3-ITD	hsa-miR-338	-2.71	0.47	17	76714278	76714344	-
Flt3-ITD	hsa-miR-143	-2.74	0.41	5	148788674	148788779	+
Inv16	hsa-miR-365	3.23	2.51				
Inv16	hsa-miR-511	3.20	2.36				
Inv16	hsa-miR-424	3.03	2.63	X	133406164	133406261	-
Inv16	hsa-miR-199b	2.96	2.57	9	128086554	128086663	-
Inv16	hsa-miR-193a	2.75	2.12	17	26911128	26911215	+
Inv16	hsa-miR-335	2.40	2.47	7	129729903	129729996	+
Inv16	hsa-miR-432	-1.38	0.11				
Inv16	hsa-miR-296	-1.40	0.45				
Inv16	hsa-miR-218	-1.41	0.34	5	168127729	168127838	-
Inv16	hsa-miR-218	-1.41	0.34	4	20206167	20206276	+
Inv16	hsa-miR-135b	-1.55	0.26	1	202149087	202149183	-
Inv16	hsa-miR-148a	-1.56	0.35	7	25762779	25762846	-
Inv16	hsa-miR-135a	-1.63	0.08	12	96460058	96460157	+
Inv16	hsa-miR-135a	-1.63	0.08	3	52303275	52303364	-
Inv16	hsa-miR-155	-1.77	0.41	21	25868163	25868227	+

Abnormality	Gene ID	Score(d)	Fold Change	Chromosome	Start	End	Strand
Inv16	hsa-let-7b	-1.86	0.34	22	44830085	44830167	+
Inv16	hsa-miR-127	-2.01	0.02	14	100419069	100419165	+
Inv16	hsa-miR-192	-2.18	0.47	11	64415185	64415294	-
Inv16	hsa-miR-196b	-2.76	0.18	7	26982339	26982422	-
Inv16	hsa-miR-196a	-3.00	0.17	12	52671789	52671898	+
Inv16	hsa-miR-196a	-3.00	0.17	17	44064851	44064920	-
Inv16	hsa-miR-10a	-3.33	0.01	17	44012199	44012308	-
Inv16	hsa-miR-10b	-3.80	0.02	2	176840538	176840647	+
NPM1	hsa-miR-10b	10.08	5.19	2	176840538	176840647	+
NPM1	hsa-miR-10b	10.08	5.19	2	176840538	176840647	+
NPM1	hsa-miR-10a	9.63	5.31	17	44012199	44012308	-
NPM1	hsa-miR-10a	9.63	5.31	17	44012199	44012308	-
NPM1	hsa-miR-196a	5.83	2.25	12	52671789	52671898	+
NPM1	hsa-miR-196a	5.83	2.25	17	44064851	44064920	-
NPM1	hsa-miR-196a	5.83	2.25	17	44064851	44064920	-
NPM1	hsa-miR-196a	5.83	2.25	12	52671789	52671898	+
NPM1	hsa-miR-196b	5.76	2.31	7	26982339	26982422	-
NPM1	hsa-miR-196b	5.76	2.31	7	26982339	26982422	-
NPM1	hsa-let-7b	4.85	2.25	22	44830085	44830167	+
NPM1	hsa-let-7b	4.85	2.25	22	44830085	44830167	+
NPM1	hsa-miR-152	3.63	2.30	17	43469526	43469612	-
NPM1	hsa-miR-152	3.63	2.30	17	43469526	43469612	-
NPM1	hsa-miR-135a	2.77	2.41	3	52303275	52303364	-
NPM1	hsa-miR-135a	2.77	2.41	12	96460058	96460157	+
NPM1	hsa-miR-135a	2.77	2.41	12	96460058	96460157	+
NPM1	hsa-miR-135a	2.77	2.41	3	52303275	52303364	-
NPM1	hsa-miR-323	-0.99	0.50	14	100561822	100561907	+
NPM1	hsa-miR-203	-1.02	0.31	14	103653495	103653604	+
NPM1	hsa-miR-193b	-1.04	0.41				
NPM1	hsa-miR-224	-1.12	0.15	X	150797618	150797698	-
NPM1	hsa-miR-494	-1.22	0.43				
NPM1	hsa-miR-99b	-1.30	0.50	19	56887677	56887746	+
NPM1	hsa-miR-485-5p	-1.34	0.28				
NPM1	hsa-miR-370	-1.46	0.17	14	100447229	100447303	+
NPM1	hsa-miR-299-5p	-1.47	0.23	14	100559884	100559946	+
NPM1	hsa-miR-134	-1.48	0.18	14	100590777	100590849	+
NPM1	hsa-miR-382	-1.57	0.10	14	100590396	100590471	+
NPM1	hsa-miR-497	-1.66	0.48				
NPM1	hsa-miR-379	-1.87	0.16	14	100558156	100558222	+
NPM1	hsa-miR-379	-1.87	0.16	14	100558156	100558222	+
NPM1	hsa-miR-511	-1.93	0.46				
NPM1	hsa-miR-511	-1.93	0.46				

Abnormality	Gene ID	Score(d)	Fold Change	Chromosome	Start	End	Strand
NPM1	hsa-miR-376a	-1.96	0.09	14	100576872	100576939	+
NPM1	hsa-miR-376a	-1.96	0.09	14	100576872	100576939	+
NPM1	hsa-miR-143	-2.03	0.49	5	148788674	148788779	+
NPM1	hsa-miR-143	-2.03	0.49	5	148788674	148788779	+
NPM1	hsa-miR-432	-2.07	0.15				
NPM1	hsa-miR-432	-2.07	0.15				
NPM1	hsa-miR-433	-2.26	0.23				
NPM1	hsa-miR-433	-2.26	0.23				
NPM1	hsa-miR-146a	-2.37	0.48	5	159844937	159845035	+
NPM1	hsa-miR-146a	-2.37	0.48	5	159844937	159845035	+
NPM1	hsa-miR-151	-2.48	0.43	8	141811845	141811934	-
NPM1	hsa-miR-151	-2.48	0.43	8	141811845	141811934	-
NPM1	hsa-miR-127	-2.49	0.03	14	100419069	100419165	+
NPM1	hsa-miR-127	-2.49	0.03	14	100419069	100419165	+
NPM1	hsa-miR-451	-2.95	0.22				
NPM1	hsa-miR-451	-2.95	0.22				
NPM1	hsa-miR-450	-3.03	0.44	X	133399891	133399981	-
NPM1	hsa-miR-450	-3.03	0.44	X	133399891	133399981	-
NPM1	hsa-miR-365	-3.14	0.40				
NPM1	hsa-miR-365	-3.14	0.40				
NPM1	hsa-miR-126#	-4.31	0.25				
NPM1	hsa-miR-126#	-4.31	0.25				
NPM1	hsa-miR-130a	-4.64	0.28	11	57165247	57165335	+
NPM1	hsa-miR-130a	-4.64	0.28	11	57165247	57165335	+
NPM1	hsa-miR-335	-5.11	0.31	7	129729903	129729996	+
NPM1	hsa-miR-335	-5.11	0.31	7	129729903	129729996	+
NPM1	hsa-miR-320	-5.23	0.47	8	22158420	22158501	-
NPM1	hsa-miR-320	-5.23	0.47	8	22158420	22158501	-
NPM1	hsa-miR-424	-7.25	0.08	X	133406164	133406261	-
NPM1	hsa-miR-424	-7.25	0.08	X	133406164	133406261	-
t(15;17)	hsa-miR-193b	8.84	35.58				
t(15;17)	hsa-miR-379	7.56	39.99	14	100558156	100558222	+
t(15;17)	hsa-miR-382	7.34	41.86	14	100590396	100590471	+
t(15;17)	hsa-miR-134	6.85	35.64	14	100590777	100590849	+
t(15;17)	hsa-miR-376a	6.81	33.05	14	100576872	100576939	+
t(15;17)	hsa-miR-485-5p	6.81	26.56				
t(15;17)	hsa-miR-452	6.48	15.39				
t(15;17)	hsa-miR-127	6.48	72.73	14	100419069	100419165	+
t(15;17)	hsa-miR-299-5p	6.46	20.75	14	100559884	100559946	+
t(15;17)	hsa-miR-224	6.39	22.29	X	150797618	150797698	-
t(15;17)	hsa-miR-100	6.36	72.75	11	121528147	121528226	-
t(15;17)	hsa-miR-432	5.96	26.19				

Abnormality	Gene ID	Score(d)	Fold Change	Chromosome	Start	End	Strand
t(15;17)	hsa-miR-370	5.53	28.64	14	100447229	100447303	+
t(15;17)	hsa-miR-99a	5.48	62.54	21	16833280	16833360	+
t(15;17)	hsa-miR-494	5.42	12.23				
t(15;17)	hsa-miR-433	5.12	21.58				
t(15;17)	hsa-miR-203	4.89	9.52	14	103653495	103653604	+
t(15;17)	hsa-miR-323	4.85	10.12	14	100561822	100561907	+
t(15;17)	hsa-miR-125b	4.31	23.80	21	16884428	16884516	+
t(15;17)	hsa-miR-125b	4.31	23.80	11	121475675	121475762	-
t(15;17)	hsa-miR-365	3.88	11.36				
t(15;17)	hsa-miR-369-5p	3.85	5.52	14	100601688	100601757	+
t(15;17)	hsa-miR-154	3.67	4.49	14	100595845	100595928	+
t(15;17)	hsa-miR-497	3.23	4.75				
t(15;17)	hsa-miR-424	3.12	5.91	X	133406164	133406261	-
t(15;17)	hsa-miR-450	2.87	3.41	X	133399891	133399981	-
t(15;17)	hsa-miR-181a	2.82	4.49	9	124534275	124534384	+
t(15;17)	hsa-miR-181b	2.71	4.19	1	195559659	195559768	-
t(15;17)	hsa-miR-181b	2.71	4.19	9	124535543	124535631	+
t(15;17)	hsa-miR-199b	2.52	3.29	9	128086554	128086663	-
t(15;17)	hsa-miR-193a	2.43	3.52	17	26911128	26911215	+
t(15;17)	hsa-miR-181d	2.40	3.35				
t(15;17)	hsa-miR-213	2.36	3.89	1	195559830	195559939	-
t(15;17)	hsa-miR-496	2.04	2.23				
t(15;17)	hsa-miR-146a	1.91	2.23	5	159844937	159845035	+
t(15;17)	hsa-miR-130a	1.89	2.35	11	57165247	57165335	+
t(15;17)	hsa-miR-409-5p	1.86	2.10				
t(15;17)	hsa-let-7e	1.82	2.13	19	56887851	56887929	+
t(15;17)	hsa-miR-335	1.77	2.29	7	129729903	129729996	+
t(15;17)	hsa-miR-125a	1.75	2.14	19	56888319	56888404	+
t(15;17)	hsa-miR-130b	1.73	2.33	22	20332147	20332228	+
t(15;17)	hsa-miR-222	1.67	2.69	X	45362675	45362784	-
t(15;17)	hsa-miR-196b	-3.11	0.00	7	26982339	26982422	-
t(15;17)	hsa-miR-196a	-3.41	0.01	12	52671789	52671898	+
t(15;17)	hsa-miR-196a	-3.41	0.01	17	44064851	44064920	-
t(8;21)	hsa-miR-126#	4.51	5.48				
t(8;21)	hsa-miR-187	-1.52	0.34	18	31738779	31738887	-
t(8;21)	hsa-miR-224	-1.58	0.03	X	150797618	150797698	-
t(8;21)	hsa-miR-338	-1.59	0.41	17	76714278	76714344	-
t(8;21)	hsa-miR-221	-1.66	0.47	X	45361839	45361948	-
t(8;21)	hsa-miR-188	-1.79	0.45	X	49471145	49471230	+
t(8;21)	hsa-miR-188	-1.79	0.45	X	49471145	49471230	+
t(8;21)	hsa-let-7b	-1.82	0.26	22	44830085	44830167	+
t(8;21)	hsa-miR-135a	-1.82	0.11	3	52303275	52303364	-

Abnormality	Gene ID	Score(d)	Fold Change	Chromosome	Start	End	Strand
t(8;21)	hsa-miR-135a	-1.82	0.11	12	96460058	96460157	+
t(8;21)	hsa-miR-19a	-1.83	0.50	13	90801146	90801227	+
t(8;21)	hsa-miR-107	-1.84	0.47	10	91342484	91342564	-
t(8;21)	hsa-miR-182	-1.95	0.19	7	129004174	129004283	-
t(8;21)	hsa-miR-342	-2.00	0.38	14	99645745	99645843	+
t(8;21)	hsa-miR-20b	-2.34	0.35	13	90801320	90801390	+
t(8;21)	hsa-miR-501	-2.34	0.26				
t(8;21)	hsa-miR-1	-2.47	0.06	18	17662963	17663047	-
t(8;21)	hsa-miR-1	-2.47	0.06	20	60561958	60562028	+
t(8;21)	hsa-miR-100	-2.49	0.07	11	121528147	121528226	-
t(8;21)	hsa-miR-152	-2.70	0.16	17	43469526	43469612	-
t(8;21)	hsa-miR-210	-2.70	0.24	11	558089	558198	-
t(8;21)	hsa-miR-500	-2.90	0.16				
t(8;21)	hsa-miR-502	-2.94	0.23				
t(8;21)	hsa-miR-339	-2.96	0.25	7	835810	835903	-
t(8;21)	hsa-miR-125b	-3.22	0.07	11	121475675	121475762	-
t(8;21)	hsa-miR-125b	-3.22	0.07	21	16884428	16884516	+
t(8;21)	hsa-miR-148a	-3.27	0.09	7	25762779	25762846	-
t(8;21)	hsa-miR-99a	-3.29	0.07	21	16833280	16833360	+
t(8;21)	hsa-miR-133a	-3.40	0.04	20	60572564	60572665	+
t(8;21)	hsa-miR-133a	-3.40	0.04	18	17659657	17659744	-
t(8;21)	hsa-miR-10b	-3.59	0.01	2	176840538	176840647	+
t(8;21)	hsa-let-7c	-3.67	0.20	21	16834019	16834102	+
t(8;21)	hsa-miR-133b	-3.73	0.03	6	52121680	52121798	+
t(8;21)	hsa-miR-9	-3.80	0.01	15	87712252	87712341	+
t(8;21)	hsa-miR-9	-3.80	0.01	5	87998427	87998513	-
t(8;21)	hsa-miR-9	-3.80	0.01	1	153203206	153203294	-
t(8;21)	hsa-miR-10a	-4.72	0.00	17	44012199	44012308	-
t(8;21)	hsa-miR-196a	-5.66	0.01	17	44064851	44064920	-
t(8;21)	hsa-miR-196a	-5.66	0.01	12	52671789	52671898	+
t(8;21)	hsa-miR-196b	-6.16	0.00	7	26982339	26982422	-

Supplemental Table 3

t(15;17)				
miRNA	**mRNA (GEP)**		**miRNA + GEP**	
miRNA	**Probe**	**Genesymbol**	**Probe/miRNA**	**Genesymbol**
hsa-miR-382	241975_at	LOC399959	hsa-miR-382	
hsa-miR-379	233364_s_at	---	hsa-miR-193b	
hsa-miR-193b	210794_s_at	MEG3	hsa-miR-379	
hsa-miR-485-5p	225381_at	LOC399959	hsa-miR-485-5p	
hsa-miR-134	204537_s_at	GABRE	hsa-miR-134	
hsa-miR-376a	226210_s_at	MEG3	hsa-miR-299-5p	
hsa-miR-299-5p	227390_at	MEG3	hsa-miR-127	
hsa-miR-127	210997_at	HGF	hsa-miR-376a	
	205110_s_at	FGF13	hsa-miR-494	
			hsa-miR-323	
			241975_at	LOC399959
			233364_s_at	---
			210794_s_at	MEG3
			225381_at	LOC399959

t(8;21)				
miRNA	**mRNA (GEP)**		**miRNA + GEP**	
miRNA	**Probe**	**Genesymbol**	**Probe/miRNA**	**Genesymbol**
hsa-miR-196b	205529_s_at	RUNX1T1	205529_s_at	RUNX1T1
hsa-miR-196a	228827_at	---	228827_at	---
hsa-miR-126#				
hsa-miR-10a				
hsa-miR-133b				
hsa-miR-9				
hsa-miR-10b				
hsa-miR-148a				
hsa-miR-133a				
hsa-let-7c				

Inv16				
miRNA	**mRNA (GEP)**		**miRNA + GEP**	
miRNA	**Probe**	**Genesymbol**	**Probe/miRNA**	**Genesymbol**
hsa-miR-10a	201497_x_at	MYH11	201497_x_at	MYH11
hsa-miR-10b	207961_x_at	MYH11	207961_x_at	MYH11
hsa-miR-196b				
hsa-miR-196a				

hsa-miR-511
hsa-miR-335
hsa-miR-365
hsa-miR-155
hsa-miR-424
hsa-miR-491
hsa-miR-145
hsa-miR-378
hsa-miR-21
hsa-miR-199b
hsa-miR-135a
hsa-miR-103
hsa-miR-149
hsa-miR-1
hsa-miR-24
hsa-miR-151
hsa-miR-133a
hsa-miR-221
hsa-miR-133b
hsa-miR-193a
hsa-miR-301
hsa-miR-340
hsa-miR-422b
hsa-miR-98
hsa-miR-148b
hsa-miR-331
hsa-miR-422a
hsa-miR-192
hsa-miR-28
hsa-miR-22
hsa-miR-143
hsa-miR-423
hsa-miR-345
hsa-miR-338
hsa-miR-320
hsa-miR-30d
hsa-miR-93
hsa-miR-324-5p
hsa-miR-107
hsa-miR-212

hsa-let-7i
hsa-miR-374
hsa-miR-505
hsa-miR-127
hsa-miR-23b
hsa-miR-142-3p
hsa-miR-30b
RNU38B
hsa-miR-31
hsa-miR-9
hsa-miR-142-5p
hsa-miR-126#
hsa-miR-25
hsa-miR-99b
hsa-miR-152
hsa-miR-214

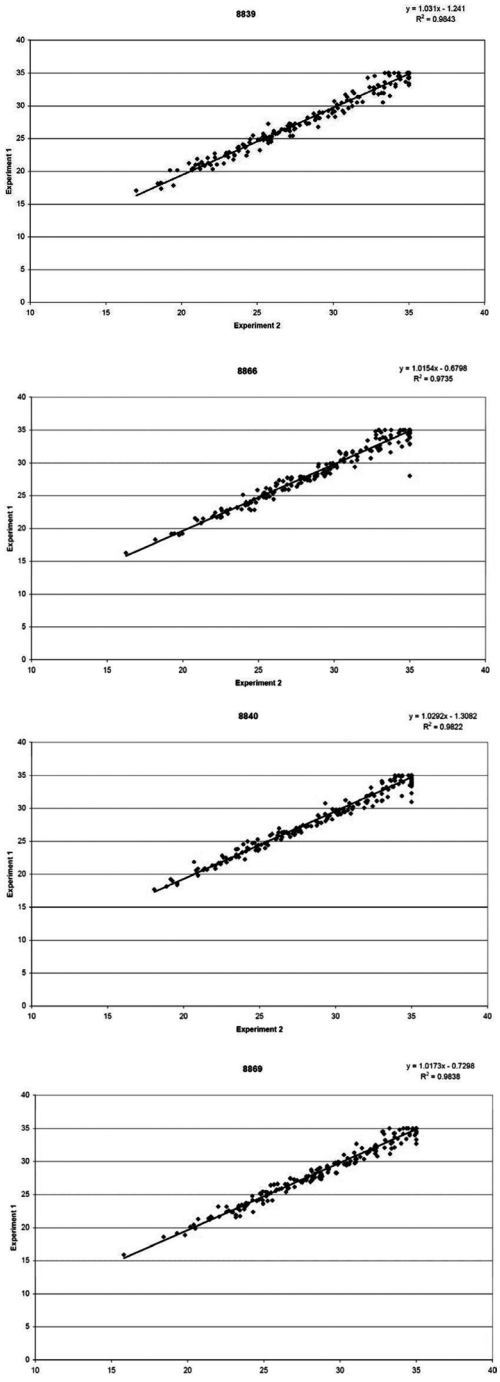
NPM1				
miRNA	**GEP**		**miRNA+ GEP**	
miRNA	**Probe**	**Genesymbol**	**Probe/miRNA**	**Genesymbol**
hsa-miR-10b	236892_s_at	---	hsa-miR-10b	
hsa-miR-10a	228904_at	HOXB3	hsa-miR-10a	
hsa-miR-424	205366_s_at	HOXB6	236892_s_at	---
hsa-miR-196b	209543_s_at	CD34	228904_at	HOXB3
hsa-miR-196a	235521_at	HOXA3	205366_s_at	HOXB6
hsa-miR-335	213844_at	HOXA5	209543_s_at	CD34
hsa-let-7b	230743_at	LOC404266	235521_at	HOXA3
hsa-miR-9			213844_at	HOXA5
hsa-miR-320			230743_at	LOC404266
hsa-miR-130a			235753_at	HOXA7

CEBPA				
miRNA	**GEP**		**miRNA+ GEP**	
miRNA	**Probe**	**Genesymbol**	**Probe/miRNA**	**Genesymbol**
hsa-miR-196b	202252_at	RAB13	202252_at	RAB13
hsa-miR-9	223095_at	MARVELD1	223095_at	MARVELD1
hsa-miR-196a	211682_x_at	UGT2B28	211682_x_at	UGT2B28
hsa-miR-149	222423_at	NDFIP1	222423_at	NDFIP1
hsa-miR-422b	224822_at	DLC1	224822_at	DLC1
RNU49	217800_s_at	NDFIP1	217800_s_at	NDFIP1

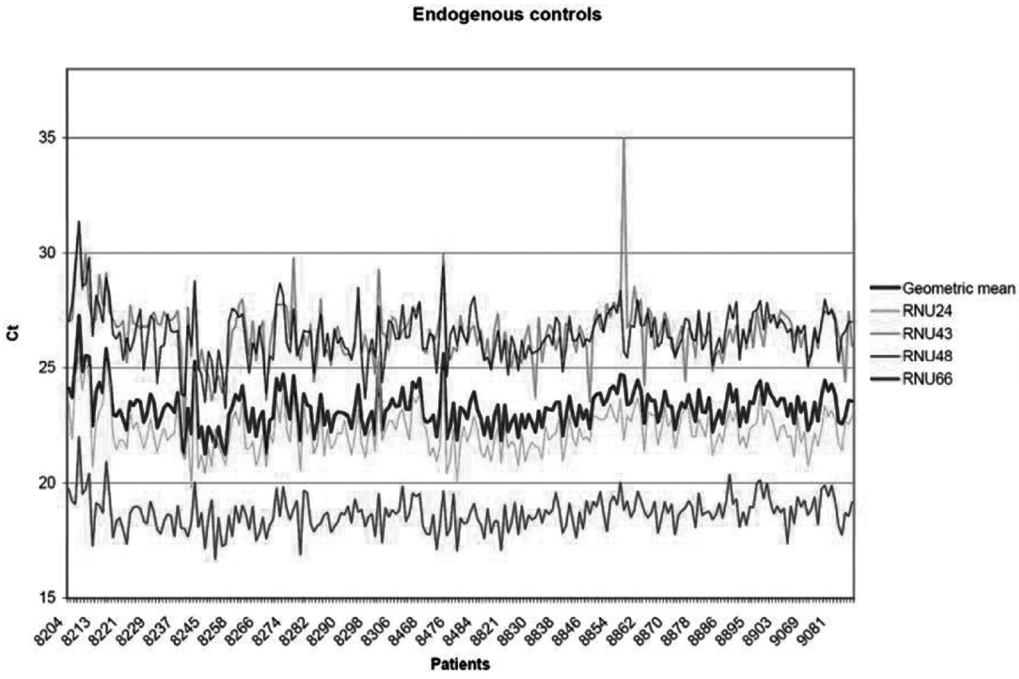
hsa-miR-130b	217853_at	TNS3	217853_at	TNS3
hsa-miR-501	241383_at	LOC201181	241383_at	LOC201181
hsa-miR-335	200765_x_at	CTNNA1	200765_x_at	CTNNA1
hsa-miR-500	1555630_a_at	RAB34	1555630_a_at	RAB34
hsa-miR-21	210762_s_at	DLC1	210762_s_at	DLC1
hsa-miR-99b	201518_at	CBX1	201518_at	CBX1
hsa-miR-378	206726_at	PGDS	206726_at	PGDS
hsa-miR-148a	210448_s_at	P2RX5	210448_s_at	P2RX5
hsa-miR-34a				
hsa-miR-135a				
hsa-miR-10a				
hsa-miR-125a				
hsa-let-7b				
hsa-miR-152				
hsa-miR-23a				
hsa-miR-22				
hsa-let-7e				
hsa-miR-17-3p				
hsa-miR-339				
hsa-let-7i				

Flt3-ITD				
miRNA	**GEP**		**miRNA+ GEP**	
miRNA	**Probe**	**Genesymbol**	**Probe/miRNA**	**Genesymbol**
hsa-miR-155	235391_at	FAM92A1	hsa-miR-155	
hsa-miR-10b	209392_at	ENPP2	hsa-miR-10b	
hsa-miR-10a	228011_at	FAM92A1	235391_at	FAM92A1
hsa-miR-424	229437_at	BIC	209392_at	ENPP2
hsa-miR-511	210839_s_at	ENPP2	228011_at	FAM92A1
hsa-miR-451	215388_s_at	CFH /// CFHR1	229437_at	BIC
hsa-miR-151	213800_at	CFH	210839_s_at	ENPP2
hsa-miR-22	201664_at	SMC4	215388_s_at	CFH /// CFHR1
hsa-miR-193a	227461_at	STON2	213800_at	CFH
hsa-miR-182	206341_at	IL2RA	201664_at	SMC4
hsa-miR-30e-5p	201663_s_at	SMC4	227461_at	STON2
hsa-miR-32	211269_s_at	IL2RA	206341_at	IL2RA
hsa-miR-338	1553808_a_at	NKX2-3	201663_s_at	SMC4
hsa-miR-30a-3p	228904_at	HOXB3	211269_s_at	IL2RA
hsa-miR-143	228372_at	C10orf128	1553808_a_at	NKX2-3
hsa-miR-133a	205227_at	IL1RAP	228904_at	HOXB3

hsa-miR-150	216920_s_at	TARP /// TRGC2 /// TRGV9	228372_at	C10orf128
hsa-miR-200c	1555037_a_at	IDH1	205227_at	IL1RAP
hsa-miR-191	209014_at	MAGED1	216920_s_at	TARP /// TRGC2 /// TRGV9
hsa-miR-146b	215806_x_at	TARP /// TRGC2 /// TRGV9	1555037_a_at	IDH1
hsa-miR-197	203373_at	SOCS2	209014_at	MAGED1
hsa-miR-141	205453_at	HOXB2	215806_x_at	TARP /// TRGC2 /// TRGV9
hsa-miR-365	232979_at	---	203373_at	SOCS2
hsa-miR-140	213217_at	ADCY2	205453_at	HOXB2
hsa-miR-30b	211144_x_at	TARP /// TRGC2 /// TRGV9	232979_at	---
hsa-miR-103	1555600_s_at	APOL4	213217_at	ADCY2
hsa-miR-30d	201069_at	MMP2	211144_x_at	TARP /// TRGC2 /// TRGV9
hsa-miR-335	209813_x_at	TRGV9	1555600_s_at	APOL4
hsa-miR-425	204438_at	MRC1 /// MRC1L1	201069_at	MMP2
hsa-miR-1	219615_s_at	KCNK5	209813_x_at	TRGV9
hsa-miR-345	236892_s_at	---	204438_at	MRC1 /// MRC1L1
hsa-miR-126#	203372_s_at	SOCS2	219615_s_at	KCNK5
hsa-miR-30c	230743_at	LOC404266	236892_s_at	---
hsa-miR-101	202455_at	HDAC5	203372_s_at	SOCS2
hsa-miR-26b	200923_at	LGALS3BP	230743_at	LOC404266
hsa-miR-192	237108_x_at	FLJ42875	202455_at	HDAC5
hsa-let-7b			200923_at	LGALS3BP
hsa-miR-30a-5p				
hsa-miR-196a				
hsa-miR-145				
hsa-miR-194				
hsa-miR-339				
hsa-miR-342				
hsa-miR-142-5p				
hsa-miR-26a				
hsa-miR-31				
hsa-miR-135a				
hsa-miR-98				



Supplemental Figure 1.
Four AML samples presented here show high correlation coefficient (R=0.97 to 0.99) when comparing microRNA expression levels in two independent experiments



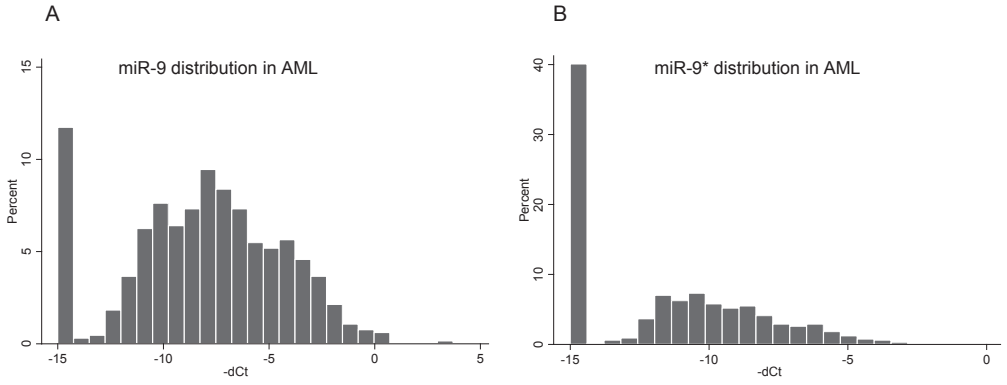
Supplemental Figure 2.
Geometric mean of these four RNUs was used for internal normalization.

Chapter 4

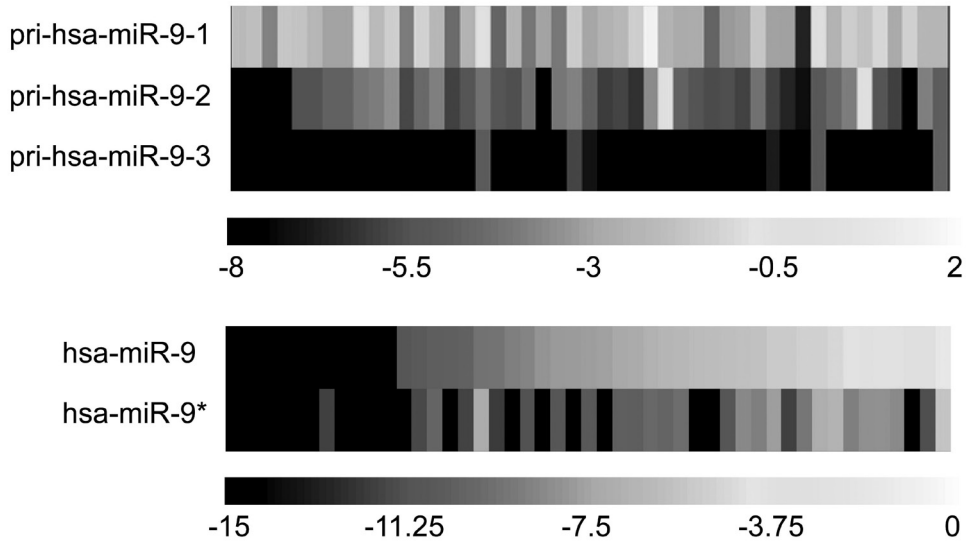
Supplementary Table 1. Multivariable analysis with continous miR-9/9* expression

		HR/OR	Std. Err	z	P-value	[95% Conf.	Interval]
miR-9	OS	0.958	0.022	-1.90	0.057	0.916	1.001
	EFS	0.971	0.021	-1.39	0.164	0.931	1.012
	CR	1.033	0.049	0.69	0.489	0.942	1.134
	RFS	0.979	0.028	-0.76	0.447	0.926	1.034
miR-9*	OS	0.957	0.021	-2.00	0.046	0.917	0.999
	EFS	0.968	0.020	-1.58	0.114	0.931	1.008
	CR	0.973	0.044	-0.60	0.549	0.891	1.063
	RFS	0.956	0.025	-1.71	0.088	0.907	1.007

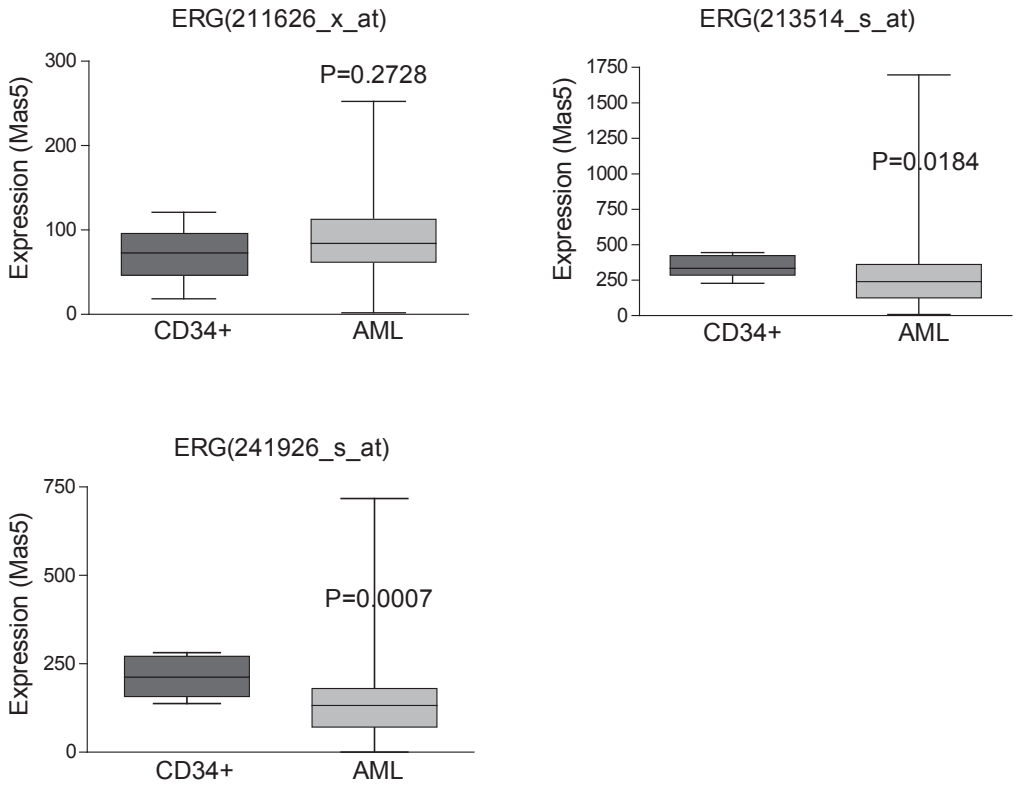
Multivariable models adjusted for treatment, included known prognostic factors, i.e. age, log(WBC), molecular genetics (ELN genetic risk) and treatment protocol.
Multivariable logistic regression used for CR and Cox proportional hazards model for EFS, RFS and OS.
log2 transformed expression data (-dCT) was used for analysis.
Hazard ratio (HR) >1 or <1 indicate an increased or decreased risk. Odds ratio (OR) >1 or <1 indicate increased or decreased odds for reaching complete remission. The sample sizes for OS and EFS (n=496) and RFS (n=387) for miR-9 and miR-9*, respectively. P values <0.05 depicted in bold, P values<0.1 & >0.05 depicted in italic.



Supplemental Figure 1. Expression distribution.
Histogram showing the distribution of AML patients in percentages with increasing miR-9 (A) and miR-9* (B) expression.



Supplemental Figure 2. Primary miRNA transcript levels.
The upper panel shows the expression, depicted as –dCt values, of the pri-miR-9-1, pri-miR-9-2 and pri-miR-9-3.Expression relative to the endogenous control (PBGD). Lower panel shows the mature miR-9 and miR-9* expression of the same patients. Expression depicted as - dCt values relative to endogenous control (RNU24). With black indicating low/below detection, whereas yellow/white indicates higher expression.



Supplemental Figure 3. Average ERG expression in AML.
Boxplot of MAS5 expression value for probesets representing ERG expression of CD34+ (n=11) and AML (n=215), showing lower average ERG expression in AML compared to CD34+ cells in two out of three probsets. Mann-Whitney U test was used to determine significance between the two means.

Chapter 5

Supplemental Table 1. Molecular Characteristics different cohorts

	Cohort1 (n=167)		Hovon42a (n=181)		AMLGS (n=216)	
	No	% of cohort	No	% of cohort	No	% of cohort
Cytogenetics						
8	12	7.2%	6	3.3%	10	4.6%
-5 or -5q	0	0.0%	1	0.6%	2	0.9%
-7 or -7q	7	4.2%	4	2.2%	5	2.3%
-9q	4	2.4%	5	2.8%	3	1.4%
11q23	5	3.0%	7	3.9%	3	1.4%
t(6;9)	2	1.2%	0	0.0%	2	0.9%
t(8;21)	9	5.4%	13	7.2%	15	6.9%
t(9;11)	4	2.4%	2	1.1%	7	3.2%
inv(3) or t(3;3)	0	0.0%	2	1.1%	1	0.5%
inv(16)	9	5.4%	10	5.5%	26	12.0%
Normal karyotype	84	50.3%	81	44.8%	123	56.9%
Complex karyotype	14	8.4%	35	19.3%	10	4.6%
Other	18	10.8%	18	9.9%	19	8.8%
Molecular genetics*						
CEBPA single	4	2.4%	1	0.6%	5	2.3%
CEBPA double	12	7.2%	7	3.9%	13	6.0%
FLT3-ITD	39	23.4%	26	14.4%	45	20.8%
FLT3-TKD	7	4.2%	11	6.1%	17	**7.9%**
NPM1	49	29.3%	48	26.5%	61	28.2%

* Molecular data determined in NK-AML

Supplemental Table 2. Full description of multivariable Cox proportional hazards models and multivariable logistic regression models

Discovery Cohort (n=167): Complete Remission (CR)						
	Logistic Regression Model					
	OR	Std. Err	z	P-value	[95% Conf.	Interval]
miR-212	1.74	0.48	2.03	0.043	1.02	2.98
age	1.00	0.02	0.21	0.834	0.96	1.05
log(WBC)	0.67	0.15	-1.82	0.069	0.43	1.03
ENL genetic risk*						
Favorable	1.66	1.07	0.79	0.432	0.47	5.84
Intermediate II	0.99	0.68	-0.02	0.987	0.26	3.79
Adverse	1.05	0.79	0.07	0.944	0.24	4.58
Treatment protocol**				0.197		
Constant	948.84	3090.71	2.10	0.035	1.60	562147.40

* ENL genetic risk group, Intermediate I as reference

Discovery Cohort (n=167): Event Free Survival (EFS)						
	Cox Proportional Hazards Model					
	HR	Std. Err	z	P-value	[95% Conf.	Interval]
miR-212	0.75	0.08	-2.86	0.004	0.61	0.91
age	1.01	0.01	0.77	0.441	0.99	1.02
log(WBC)	1.19	0.09	2.20	0.028	1.02	1.38
ENL genetic risk*						
Favorable	0.35	0.10	-3.75	0.000	0.21	0.61
Intermediate II	1.13	0.28	0.47	0.636	0.69	1.84
Adverse	1.20	0.33	0.66	0.512	0.70	2.07
Treatment protocol**				0.042		

* ENL genetic risk group, Intermediate I as reference
** P value determined by LR test of model with and without treatment protocol
Overall test of proportional hazard assumption: P 0.5811

Discovery Cohort (n=141): Relapse Free Survival (RFS)						
	Cox Proportional Hazards Model					
	HR	Std. Err	z	P-value	[95% Conf.	Interval]
miR-212	0.72	0.10	-2.32	0.020	0.55	0.95
age	1.02	0.01	1.46	0.144	0.99	1.04
log(WBC)	1.12	0.12	1.02	0.305	0.90	1.38
ENL genetic risk*						
Favorable	0.25	0.11	-3.27	0.001	0.11	0.57
Intermediate II	1.13	0.38	0.35	0.725	0.58	2.19
Adverse	1.19	0.45	0.47	0.640	0.57	2.52
Treatment protocol**				0.160		

* ENL genetic risk group, Intermediate I as reference
** P value determined by LR test of model with and without treatment protocol
Overall test of proportional hazard assumption: P 0.350

Discovery Cohort (n=167) : Overall Survival (OS)						
	Cox Proportional Hazards Model					
	HR	Std. Err	z	P-value	[95% Conf.	Interval]
miR-212	0.77	0.08	-2.44	0.015	0.62	0.95
age	1.02	0.01	1.70	0.089	1.00	1.03
log(WBC)	1.24	0.11	2.46	0.014	1.04	1.46
ENL genetic risk*						
Favorable	0.39	0.11	-3.21	0.001	0.22	0.70
Intermediate II	0.99	0.27	-0.05	0.961	0.58	1.68
Adverse	1.27	0.37	0.83	0.406	0.72	2.24
Treatment protocol**				0.357		

* ENL genetic risk group, Intermediate I as reference
** P value determined by LR test of model with and without treatment protocol
Overall test of proportional hazard assumption: P 0.669

Validation Cohort (n=407): Complete Remission (CR)						
	Logistic Regression Model					
	OR	Std. Err	z	P-value	[95% Conf.	Interval]
miR-212	0.88	0.14	-0.83	0.407	0.65	1.19
age	0.99	0.01	-0.58	0.560	0.97	1.02
log(WBC)	0.74	0.08	-2.88	0.004	0.60	0.91
ENL genetic risk*						
Favorable	6.34	2.68	4.37	0.000	2.77	14.51
Intermediate II	1.45	0.54	1.01	0.310	0.71	2.99
Adverse	0.38	0.13	-2.74	0.006	0.19	0.76
Treatment protocol	0.53	0.15	-2.26	0.024	0.31	0.92
Constant				0.0214		

* ENL genetic risk group, Intermediate I as reference
** P value determined by LR test of model with and without treatment protocol

Validation Cohort (n=409): Event Free Survival (EFS)						
	Cox Proportional Hazards Model					
	HR	Std. Err	z	P-value	[95% Conf.	Interval]
miR-212	0.88	0.06	-1.87	0.062	0.76	1.01
age	1.01	0.01	1.54	0.124	1.00	1.02
log(WBC)	1.20	0.06	3.69	0.000	1.09	1.32
ENL genetic risk*						
Favorable	0.30	0.05	-7.43	0.000	0.22	0.41
Intermediate II	0.51	0.10	-3.57	0.000	0.36	0.74

Adverse	1.51	0.25	2.52	0.012	1.10	2.09
Treatment protocol				0.018		

* ENL genetic risk group, Intermediate I as reference
** P value determined by LR test of model with and without treatment protocol
Overall test of proportional hazard assumption: P .111

Validation Cohort (n=319): Relapse Free Survival (RFS)						
	Cox Proportional Hazards Model					
	HR	Std. Err	z	P-value	[95% Conf.	Interval]
miR-212	0.83	0.07	-2.07	0.038	0.70	0.99
age	1.01	0.01	1.48	0.138	1.00	1.03
log(WBC)	1.30	0.09	3.95	0.000	1.14	1.48
ENL genetic risk*						
Favorable	0.31	0.06	-5.80	0.000	0.21	0.46
Intermediate II	0.38	0.10	-3.75	0.000	0.23	0.63
Adverse	1.54	0.36	1.87	0.062	0.98	2.43
Treatment protocol				0.010		

* ENL genetic risk group, Intermediate I as reference
** P value determined by LR test of model with and without treatment protocol
Overall test of proportional hazard assumption: P .049

Validation Cohort (n=409): Overall Survival (OS)						
	Cox Proportional Hazards Model					
	HR	Std. Err	z	P-value	[95% Conf.	Interval]
miR-212	0.83	0.06	-2.42	0.016	0.72	0.97
age	1.02	0.01	3.05	0.002	1.01	1.03
log(WBC)	1.31	0.07	5.11	0.000	1.18	1.45
ENL genetic risk*						
Favorable	0.29	0.05	-6.64	0.000	0.21	0.42
Intermediate II	0.53	0.11	-3.20	0.001	0.36	0.78
Adverse	1.88	0.33	3.65	0.000	1.34	2.65
Treatment protocol				0.686		

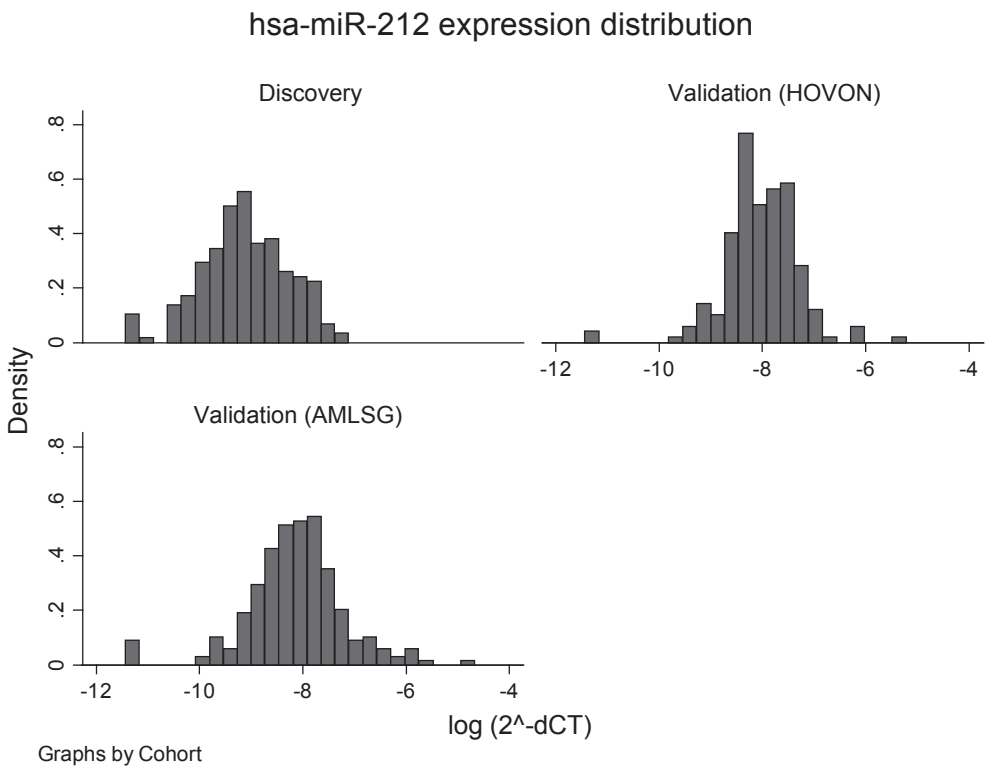
* ENL genetic risk group, Intermediate I as reference
** P value determined by LR test of model with and without treatment protocol
Overall test of proportional hazard assumption: P 0.6125

Supplemental Table 3 shows differential expressed genes between patients with high and low miR-212 expression of the discovery cohort and can be found on the Leukemia website.

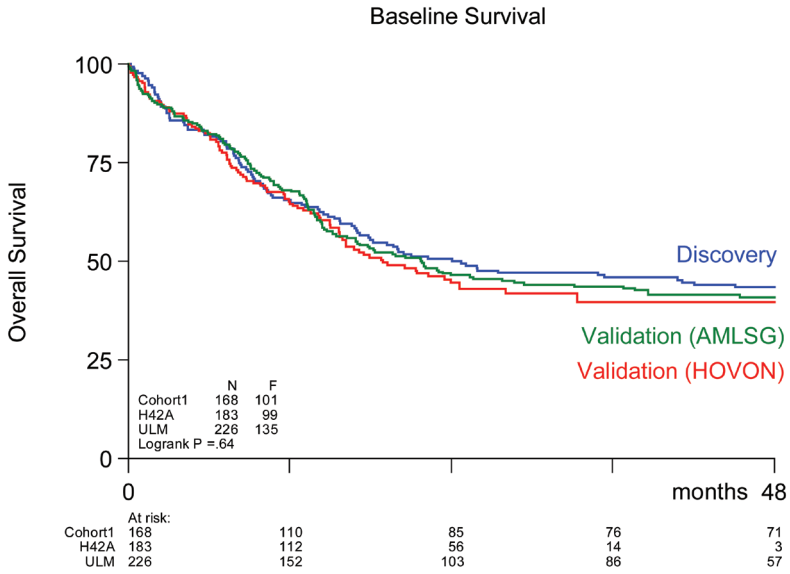
Supplemental Table 4. Multivariable Cox proportional hazard model of the discovery cohort (n=167), including selected differentially expressed factors

Factors	HR/OR	Std.Err	z	P-value	[95% Conf.	Interval]
AGE	1.01	0.01	0.72	0.472	0.99	1.02
log(WBC)	1.30	0.12	2.99	0.003	1.10	1.55
ELN genetic risk*						
Favorable	0.33	0.10	-3.66	<0.001	0.18	0.60
Intermediate II	1.01	0.27	0.04	0.967	0.59	1.72
Adverse	1.10	0.32	0.34	0.733	0.62	1.96
miR-212	0.76	0.09	-2.26	0.024	0.60	0.96
BAALC	1.55	0.27	2.52	0.012	1.10	2.18
MN1	0.76	0.13	-1.65	0.100	0.55	1.05
PLAUR	0.93	0.13	-0.54	0.586	0.70	1.22
S100A8	1.38	0.18	2.53	0.012	1.08	1.77

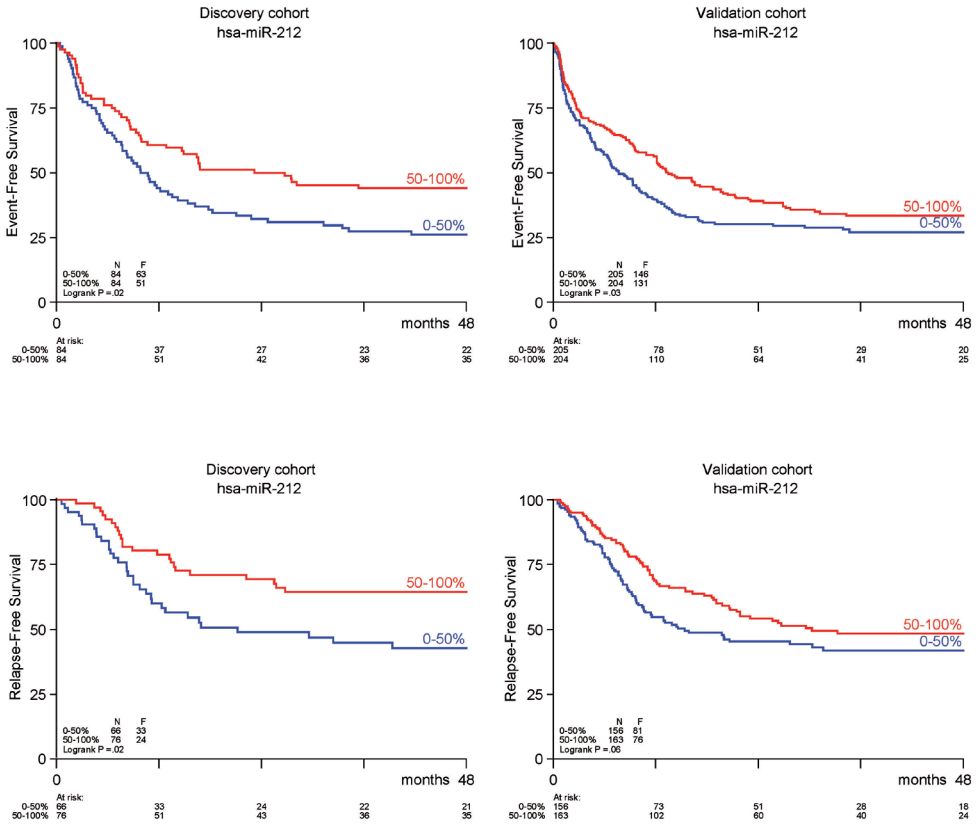
* ENL risk group intermediate: NK was used as reference
Log transformed summarized (PC) expression values were used as continuous variable



Supplemental Figure 1 displays distribution of miR-212 expression in the discovery and validation sub cohorts.



Supplemental Figure 2. The base line overall survival of discovery and validation sub cohorts.

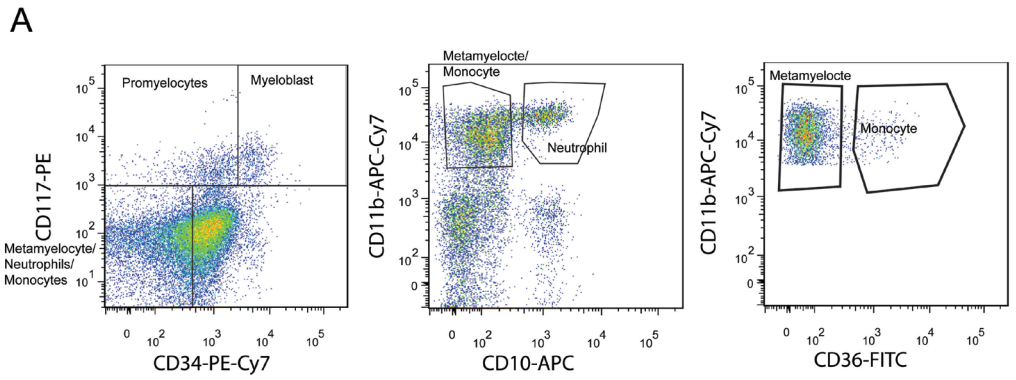


CR rate	Discovery cohort	Validation cohort
High miR-212	92.86%	80.30%
Low miR-212	80.72%	76.96%

Supplemental Figure 3. The Kaplan-Meier plots for EFS and RFS of the discovery and validation cohort, dichotomized by the median miR-212 expression. In addition the percentage of patients reaching complete remission of the discovery and validation cohort is listed for patients above and below the median miR-212 expression.

COLOR SECTION

Chapter 2



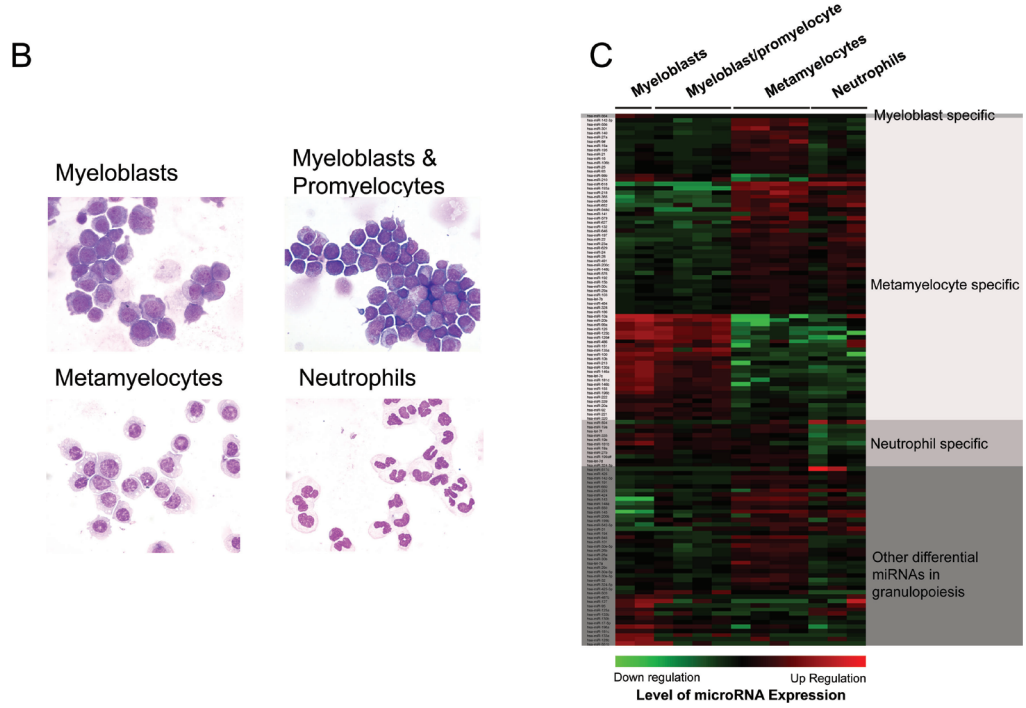


Figure 1. miRNA expression in cell sorted neutrophilic differentiation stages of normal human bone marrow (A) Gating strategy for FACS cell sorting of bone marrow cellular morphological maturation stages (myeloblasts, promyelocytes, metamyelocytes and neutrophils). The following different cell surface markers were used, CD117, CD34, CD11b, CD10 and CD36. DAPI was used to distinguish living from dead cells. (B) Cytospins, stained with May Grünwald Giemsa, of cells taken from the different sorted maturation stages. (C) Heatmap of log2 transformed geometric mean centred miRNA expression showing significantly differentially expressed miRNA determined by ANOVA and corrected for multiple testing of the sorted neutrophil stages of maturation, depicted on the rows and columns respectively. MiRNA expression was represented by colour scale, where green reflects lower expression, the black colour reflects mean expression and red reflects higher expression.

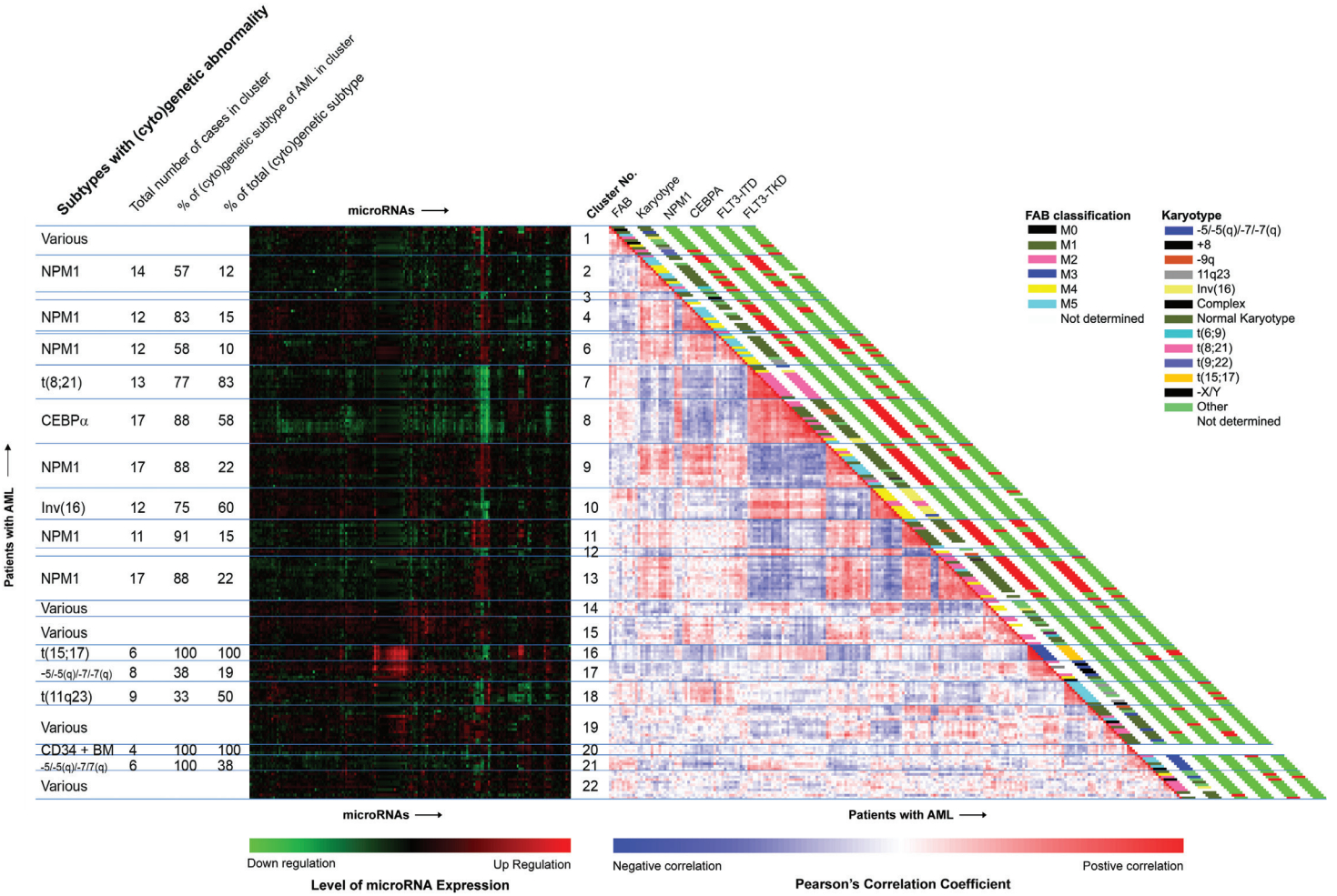

Subtypes with (cyto)genetic abnormality
Total number of cases in cluster
% of (cyto)genetic subtype of AML in cluster
% of total (cyto)genetic subtype
microRNAs
Cluster No.
FAB
Karyotype
NPM1
CEBPA
FLT3-ITD
FLT3-TKD
Various
NPM1 14 57 12
NPM1 12 83 15
NPM1 12 58 10
t(8;21) 13 77 83
CEBPα 17 88 58
NPM1 17 88 22
Inv(16) 12 75 60
NPM1 11 91 15
NPM1 17 88 22
Various
Various
t(15;17) 6 100 100
-5/-5(q)/-7/-7(q) 8 38 19
t(11q23) 9 33 50
Various
CD34 + BM 4 100 100
-5/-5(q)/-7/7(q) 6 100 38
Various
1
2
3
4
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Patients with AML
FAB classification
M0
M1
M2
M3
M4
M5
Not determined
Karyotype
-5/-5(q)/-7/-7(q)
+8
-9q
11q23
Inv(16)
Complex
Normal Karyotype
t(6;9)
t(8;21)
t(9;22)
t(15;17)
-X/Y
Other
Not determined
microRNAs
Down regulation
Up Regulation
Level of microRNA Expression
Patients with AML
Negative correlation
Postive correlation
Pearson's Correlation Coefficient

Figure 1..Correlation view based on unsupervised clustering of 215 AML specimens involving 178 differentially expressed mature human microRNAs (right section) and the relative expression levels of microRNAs that characterize each of the individual clusters (left section)In the Pearson's correlation view the red squares indicate a positive correlation and the blue squares a negative pairwise correlation between the microRNAs expression signatures. Using the program Heatmapper [39] cytological, cytogenetic and molecular data were plotted along the diagonal. Cytogenetic abnormalities and FAB classifications are indicated with different colors. The presence of molecular abnormalities, such as *NPM1, CEBPA, FLT3*-ITD and *FLT3*-TKD mutations are indicated in red and absence in green. On the left part of the Figure 1 each individual microRNA expression cluster with a particular (cyto)genetic AML subtype is indicated ('subtypes with (cyto)genetic abnormality'). The total number of cases in each cluster and % of (cyto) genetic subtype of AML in cluster are presented in the columns. In addition, also the percentages of these (cyto)genetic subtypes present within each microRNA expression cluster (thus also indicating relative proportion of cases with the abnormality that are located outside the cluster) is given ('% of total (cyto) genetic subtype).
The levels of expression of 178 microRNAs of each of the 22 clusters including normal bone marrow CD34+ cells are presented in the Heatmap on the left side of the Correlation view. The scale bar indicates an increase (red) or decrease (green) in the level of expression by a factor of 7 relative to the geometric mean of all samples.

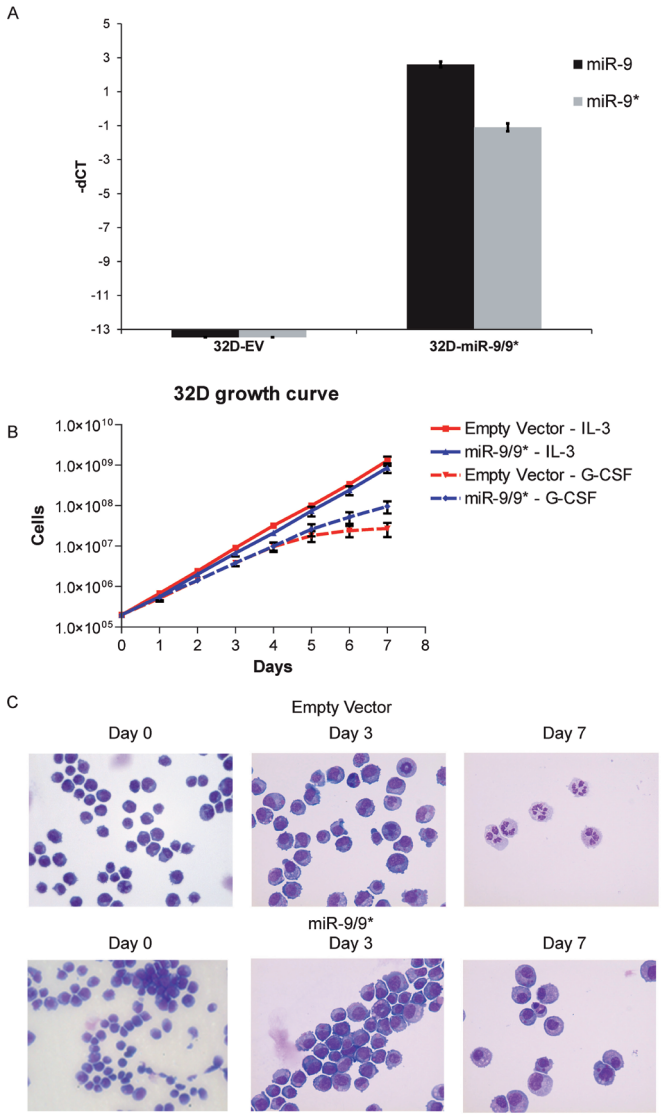


Figure 3. Enforced expression of miR-9/9* leads to a defect in granulocytic maturation. Empty vector or vector containing miR-9/9* precursor was retroviral transduced in 32D cells, transduced cells were purified using FACS sorting of GFP positive cells. Three independent experiments were performed. (A) Average relative expression of miR-9 and miR-9* was measured using quantitative RT-PCR using sno224 and sno234 as endogenous control. Expression is presented as - dCt values. Measurements with values below detection were set to -13. The error bars represent the standard deviation. (B) Average growth curves of cells containing no miRNA (EV) and miR-9/9* in red and blue respectively. Growth curve of 32D cells supplemented with IL-3 (solid line) or G-CSF (dashed lined) for 7 days. Error bars represent the standard deviation. (C) Representative micrographs of cytospins stained with May Grünwald Giemsa for EV and miR-9/9* growing on IL-3 (day 0) and on day 3 and day 7 of G-CSF induced differentiation of 32D cells.

Chapter 5

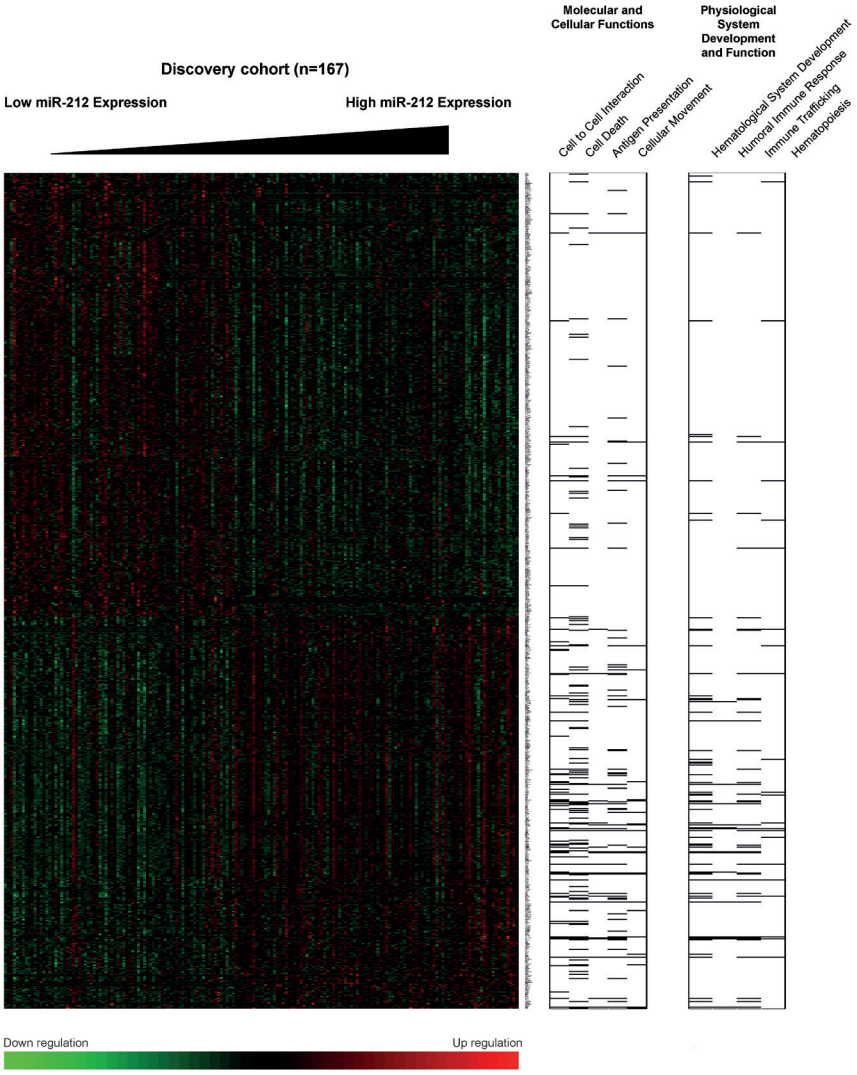
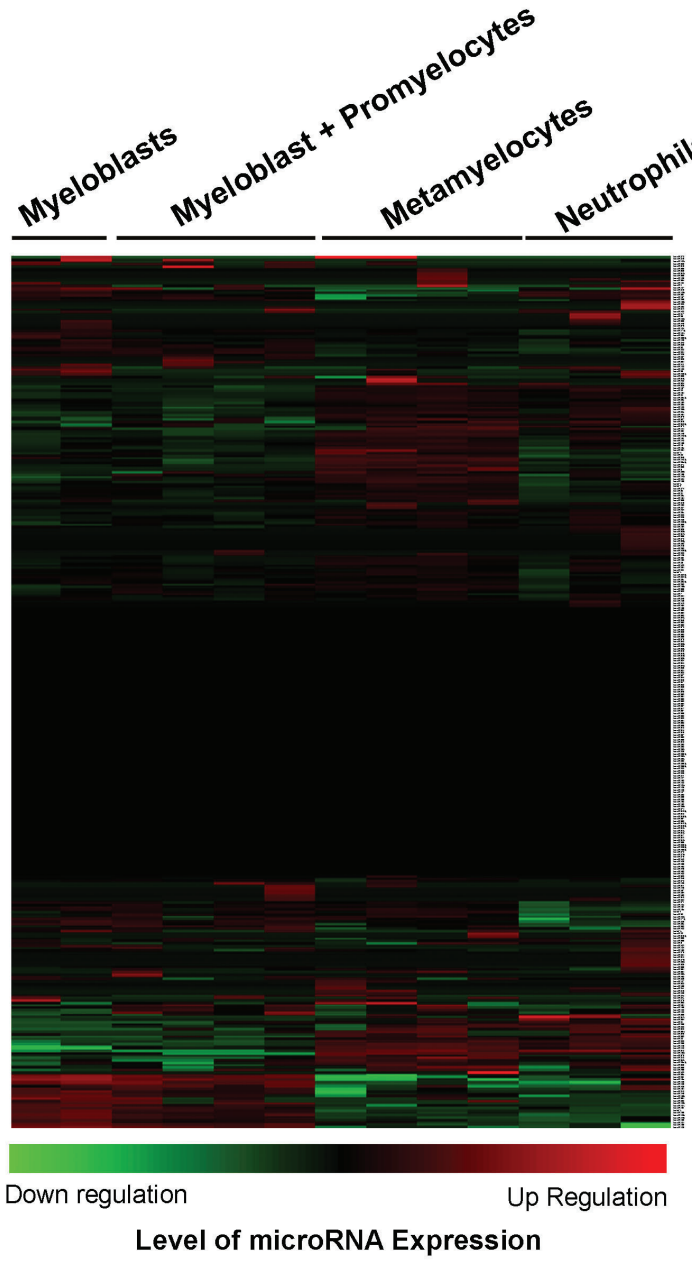


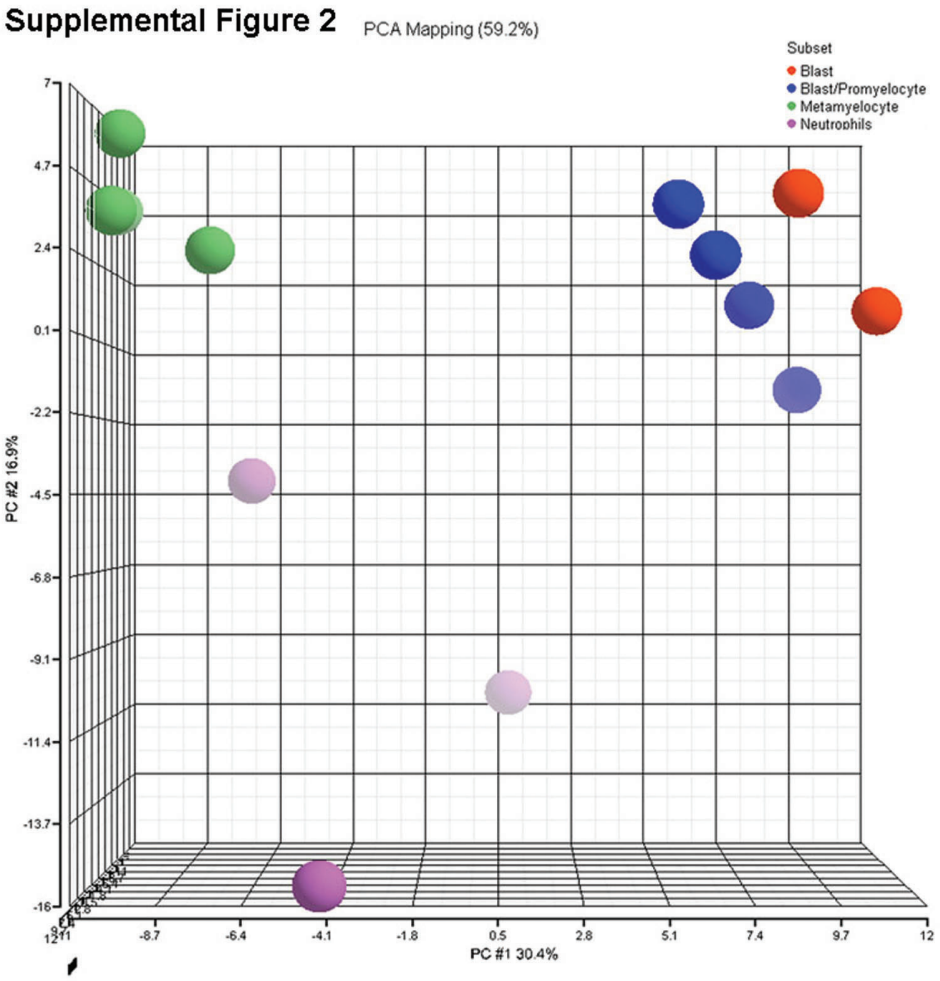
Figure 2. Differentially expressed probes between patients with high and low miR-212 expression in AML.

The left panel shows a heatmap of 867 genes differentially expressed (FDR < 0.05) between high (above median) and low (below median) miR-212 expression groups (845 of these genes were also detected by Spearman correlation analysis (FDR < 0.05) using continuous miR-212 expression). Patients (columns) are ordered from the left to the right by increasing miR-212 values. Genes (rows) have been sorted according to their expression patterns by hierarchical clustering. Green color indicates expression values lower than the mean expression value (black) and red color indicates expression values higher than the mean expression value. The enriched pathways of the selected genes are depicted in the right panel. Columns represent top enriched pathways, where the black color indicates the involvement of the particular gene in the above mentioned category.

Chapter 2
Supplemental figures



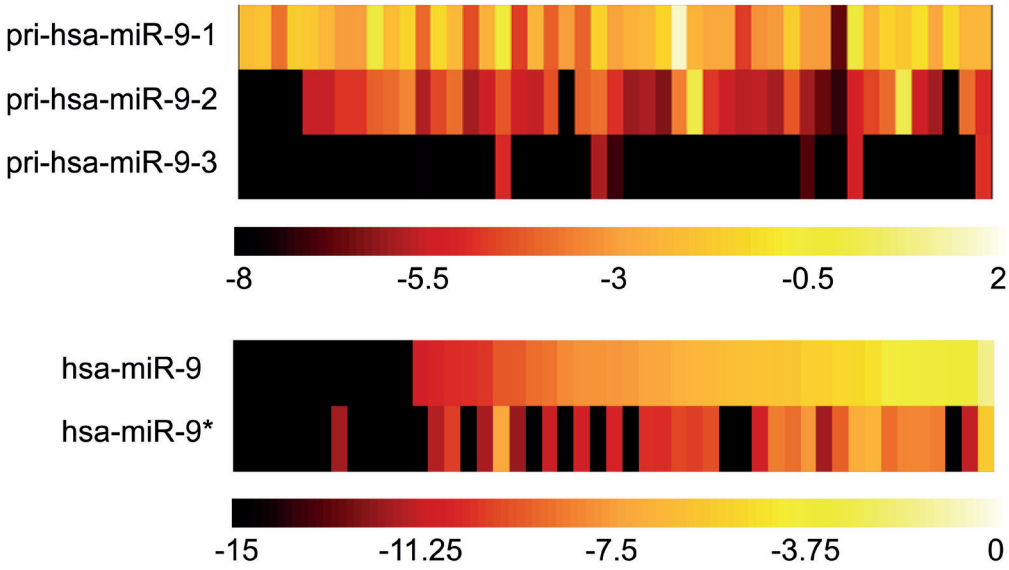
Supplemental figure 1. miRNA expression of different stages of granulocytic differentiation.
Heatmap of geometric mean relative miRNA expression showing all miRNAs of the different granulocytic maturation stages, depicted on the rows and columns respectively. Expression was represented by colour scale, where green reflects lower expression; black reflects mean expression and red reflects higher expression.



Supplemental Figure 2. Principal Component Analysis.
Principal Component Analysis plot, depicting the coordinates of the samples in three principal components space derived from their miRNA expression.

Chapter 2

Supplemental figure 2



Supplemental Figure 2. Primary miRNA transcript levels.
The upper panel shows the expression, depicted as –dCt values, of the pri-miR-9-1, pri-miR-9-2 and pri-miR-9-3.Expression relative to the endogenous control (PBGD). Lower panel shows the mature miR-9 and miR-9* expression of the same patients. Expression depicted as - dCt values relative to endogenous control (RNU24). With black indicating low/below detection, whereas yellow/white indicates higher expression.