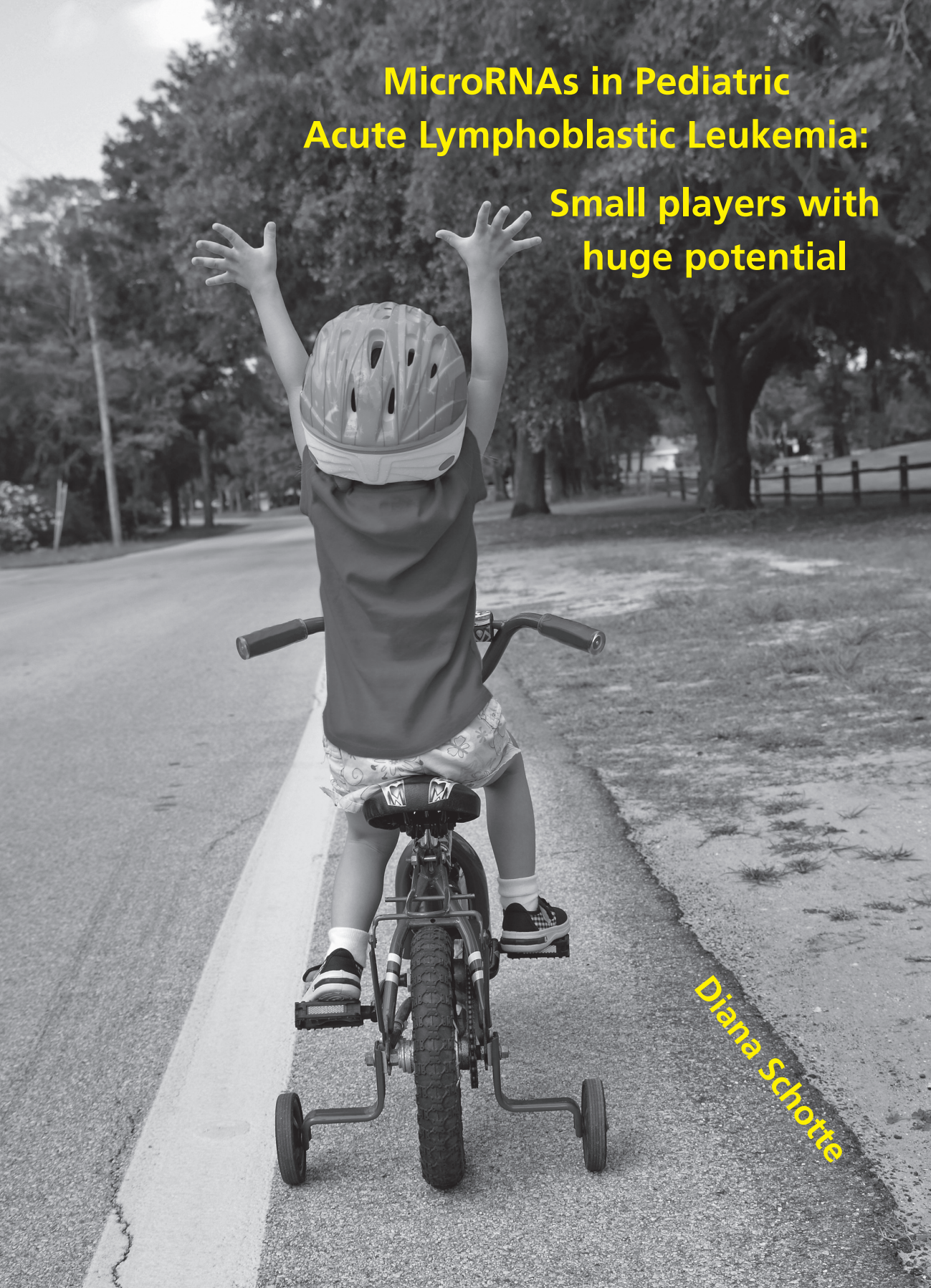
MicroRNAs in Pediatric Acute Lymphoblastic Leukemia:

Small players with huge potential



Diana Schotte

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MicroRNAs in Pediatric Acute Lymphoblastic Leukemia: Small players with huge potential

MicroRNAs in acute lymfatische leukemie bij kinderen:
kleine spelers met grote potentie

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Prof.dr. R. Agami

Copromotor: Dr. M.L. den Boer

"If you add a little to a little and do this often, soon the little will become great"

Hesiod (700 BC)

Voor alle kinderen met leukemie en hun ouders

CONTENTS

Chapter 1

General Introduction

"Life is like riding a bicycle. To keep your balance you must keep moving"
by Albert Einstein

Hematopoiesis is a dynamic balance of cellular proliferation, survival, apoptosis and differentiation in which the pluripotent hematopoietic stem cell gives rise to lymphoid and myeloid precursors of blood cells. The B-lymphoid precursor sequentially differentiates from proB-cells into common/preB-cells and finally yields mature B-lymphocytes. The T-lymphoid precursor generates thymocytes or proT-cells that further differentiate into T-lymphocytes. The myeloid precursor gives rise to granulocytes, monocytes, platelets and erythrocytes. This process is under tight surveillance by regulators including transcription factors, cytokines and growth factors. In case of defects in surveillance and/or oncogenic hits in the maturing hematopoietic cell, cells may arrest at different levels of maturation. Leukemia cells grow exponentially with limited differentiation thereby suppressing the development of normal blood cells in the bone marrow. As a consequence leukemia patients suffer from fatigue (shortage of erythrocytes or anemia), infections (shortage of mature lymphocytes and granulocytes) and bleeding (low platelet numbers). Despite the fact that multiple consecutive events are required for the development of leukemia, the clinical symptoms associated with acute leukemia occur rather abrupt and the progression of the disease is very fast if not treated instantly.

PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

Approximately 80% of children with acute leukemia suffer from acute lymphoblastic leukemia (ALL), whereas 15-20% has acute myeloid leukemia (AML) and less than 5% has other types of leukemia, mainly chronic myeloid leukemia. This thesis focuses on ALL which is the most common type of childhood cancer with an annual incidence of newly diagnosed cases of approximately 5 cases per 100,000 population of children[1]. In the Netherlands about 120 new cases between 0-18 years of age are diagnosed each year with a peak incidence between 2 and 5 years of age.

Of all children with ALL, approximately 80% are cured with current treatment protocols based on a combination of multiple classes of drugs including a.o. glucocorticoids (prednisolone, dexamethasone), vincristine, anthracyclines (a.o. daunorubicin), L-asparaginase, methotrexate and thiopurines (6-thioguanine and 6-mercaptopurine).

SUBTYPES OF PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

ALL is a heterogeneous disease characterized by various genetic abnormalities with distinct prognostic features. Immunophenotypically, ALL is divided into B-cell and T-cell lineage. B-cell lineage accounts for 85% of pediatric ALL cases whereas the remaining 15% are of T-lineage origin (Figure 1).

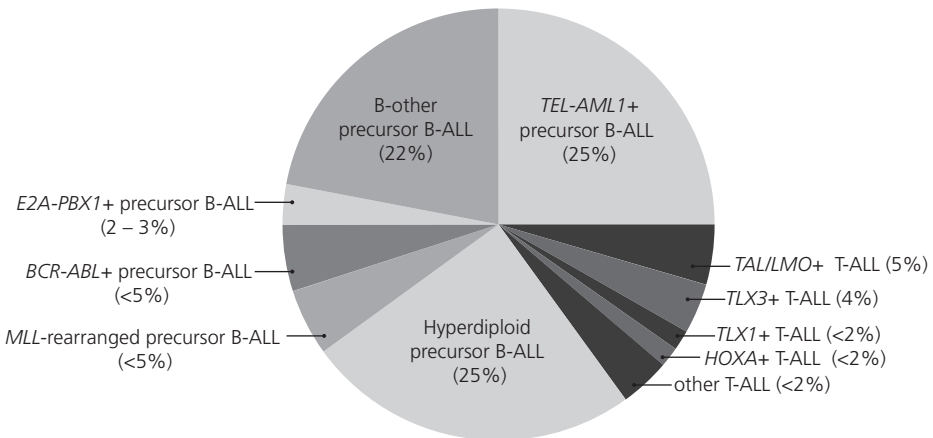

Figure 1. Frequency of subtypes in Dutch children with acute lymphoblastic leukemia.
Abbreviations: ALL: acute lymphoblastic ALL; B-other: precursor B-ALL negative for the major cytogenetic aberrations, i.e. *TEL-AML1, BCR-ABL, E2A-PBX1, MLL*-translocations and hyperdiploidy; *TAL/LMO*+ T-ALL: *TAL-LMO*-activated subtype of T-ALL; *TLX3*+*: TLX3*-activated; *TLX1*+*: TLX1*-activated; *HOXA*+*; HOXA*-activated, other T-ALL: T-ALL without activation of *TAL/LMO, TLX3, TLX1* and *HOXA* genes.

Genetic subtypes of precursor B-ALL

One of the two main genetic subtypes of precursor B-ALL is characterized by the presence of more than 50 chromosomes (or a DNA index >1.16). This **hyperdiploid** subtype is found in approximately 25% of children with precursor B-ALL (Figure 1) and is associated with a favorable outcome, especially when extra copies of chromosome 4, 10 or 17 are present[2-4]. The fact that hyperdiploid-positive leukemia cells relatively easy undergo apoptosis and are highly sensitive to anti-metabolites, L-asparaginase and other drugs may, contribute to the good prognosis[4,5].

The second genetic subtype is characterized by the ***TEL-AML1*** translocation or **t(12;21) (p13;q22)** which creates a fusion between the *TEL* (or *ETV6*) gene encoding a nuclear phosphoprotein of the ETS family of transcription factors and the *AML1* (or *RUNX1*) gene, encoding almost the complete transcription factor regulating myeloid and lymphoid lineage-

specific genes[3,6,7]. This subtype accounts for 25% of precursor B-ALL cases (Figure 1) and shares L-asparaginase sensitivity[8] and a 5-year disease-free survival of more than 85% with the hyperdiploidy-positive subtype[4,9].

In contrast to the previous two genetic subtypes, the overall survival of children carrying a rearrangement of the mixed lineage leukemia (***MLL***) gene on chromosome **11q23** does not exceed 50%[10-13]. The frequency of *MLL*-rearranged ALL is less than 5% in children (Figure 1), and is mainly found in infants below the age of 1 year[3,13] . The unfavorable prognosis of *MLL*-rearranged precursor B-ALL patients has been linked to resistance to prednisolone and L-asparaginase[9]. Over 50 fusion partner genes have been identified so far, with *MLL-AF4*/t(4;11)(q21;q23), *MLL-ENL*/t(11;19)(q23;p13.3) and *MLL-AF9*/t(9;11)(p22;q23) being the most frequently found in childhood ALL[14]. These fusion genes may convert normal hematopoietic cells into leukemia cells through multiple interactions of its fusion products with chromatin regulatory factors and potentially via the aberrant upregulation of *HOXA* genes[15,16].

A second prognostically unfavorable group is formed by ***BCR-ABL*/t(9;22)(q34;q11)** -positive ALL. These patients have a 5-year event-free survival of up to 50%[17,18]. The translocation t(9;22) fuses the *BCR* gene to the *ABL* gene resulting into a constitutively activated tyrosine kinase fusion product that drives proliferation and reduces the rate of apoptosis[3]. Also *BCR-ABL*-positive ALL accounts for less than 5% of children with ALL (Figure 1)[3,4,9].

An intermediate prognostic group is characterized by the ***E2A-PBX1*/t(1;19)(q23;p13.3)** translocation[3] (~2-3% of all childhood ALL cases in the Netherlands, Figure 1). The t(1;19) fuses the HOX DNA-binding co-factor PBX1 to the transactivation domain of the basic-Helix-Loop-Helix (bHLH) transcription factor E2A[19]. Since aberrant activation of *HOX* genes is associated with malignant transformation and E2A is crucial for lymphocyte development, the *E2A-PBX1* translocation may contribute to leukemogenesis.

Precursor B-ALL cases with genetic lesions that are less frequently occurring than the above-mentioned genetic subtypes, are in this thesis referred to as **B-other**. Patients with this subtype account for ~22% of all children with ALL in the Netherlands (Figure 1). Most of the above-mentioned genetic aberrations are mutually exclusive. For the two high-risk genetic subtypes, i.e. infant *MLL*-rearranged and *BCR-ABL*-positive ALL, separate treatment protocols exist. The remaining cases (that is *TEL-AML1*-positive, hyperdiploid and *E2A-PBX1*-positive) are risk-stratified based upon clinical features including white blood cell count, age at diagnosis and initial treatment response.

Genetics in T-ALL

Risk-adapted therapy has improved the outcome of pediatric **T-ALL** towards ~75% 5-years event-free survival, albeit this outcome still remains inferior compared with the >80% event-free survival of precursor B-ALL[12,20]. The relative adverse effect on outcome may be due to

the fact that T-ALL cases are more resistant to different classes of drugs compared with precursor B-ALL and hence, higher dosages and/or other drugs are needed to overcome drug resistance[3,21]. Originally, T-ALL was taken as a 'homogenous' group whereas evidence was recently provided that many different genetic abnormalities exist comprising at least four subtypes of T-ALL[19,22]. The largest genetic subtype is characterized by rearrangements and deletions affecting the *TAL* or *LMO* oncogenes thereby frequently inhibiting the translational activity of *E2A/HEB* transcription factors. The *TAL/LMO*-activated subtype occurs in 15-30% of pediatric T-ALL cases and roughly represents 5% of the total number of pediatric ALL cases (Figure 1)[9]. The second largest genetic subtype is presented by rearrangements of the *TLX3* (or *HOX11L2*) oncogene. This subtype represents 20-25% of pediatric T-ALL (4% of all cases) and induces the expression of *TLX3* whereas this gene is usually not expressed during normal T-cell development. Chromosomal translocations affecting the *TLX1* (or *HOX11*) oncogene or promoting *HOXA* transcription form two subgroups. Both subgroups account each for ~8-10% of pediatric T-ALL cases which corresponds to <2% of all pediatric ALL patients (Figure 1). *HOXA* activation is triggered by the fusion genes *MLL-ENL, CALM-AF10* and *SET-NUP214* as well as by an inversion of chromosome 7 [inv (7) (p15q35)] which put the *HOXA* gene cluster in the vicinity of the T-cell receptor-beta enhancer locus. At present, the genetic subtype of T-ALL is not used for risk-stratification of children with ALL, albeit studies have demonstrated favorable (*TAL/LMO*- and *TLX1*-subgroups) and unfavorable (*TLX3*- and *HOXA*-activated subgroups) prognosis in children[9,23]. Most prognostic studies are hampered by retrospective analysis of patients compiled from different treatment protocols with different success rates. Ongoing studies are currently addressing the prognostic value of these subtypes in contemporary treatment protocols which may yield evidence for assignment of T-ALL to risk-adapted treatment protocols in the future.

IS ACUTE LYMPHOBLASTIC LEUKEMIA A DISEASE OF DYSREGULATED PROTEIN-CODING GENES *ONLY*?

The genetic aberrations described above include chromosomal translocations that affect the expression of proto-oncogenes (e.g. *TLX3*[24]) and create fusion genes encoding altered transcription factors (e.g. *TEL-AML1*) or yield constitutively activated kinases (e.g. *BCR-ABL*). These genetic abnormalities contribute to the disruption of normal hematopoiesis and stimulate leukemic transformation of hematopoietic progenitor cells. This suggests that mainly protein-coding genes are affected by the specific genetic aberrations. But what about the role of non-protein coding genes in the biology of ALL?

In the early nineties it was believed that non-protein coding DNA was non-functional and a waste of nucleotides. In the last decennium this concept was shown to be wrong by the discovery that a class of small non-protein coding RNAs, i.e. microRNAs (miRNAs)

regulate the expression of protein-coding genes . Thereby miRNAs control important cellular processes like proliferation, apoptosis and differentiation. Since these processes are essential for a well balanced hematopoiesis, we hypothesized that besides protein-coding genes also miRNAs may be involved in leukemogenesis. This thesis is aimed to identify those miRNAs that are dysregulated in – and may therefore contribute to the biology underlying- different subtypes of pediatric ALL.

OUTLINE OF THIS THESIS

Chapter 2 contains a review summarizing the current knowledge about the function of miRNAs and their potential importance to acute leukemias. In **chapter 3** and **chapter 4** we hypothesized that many miRNAs relevant to ALL are yet unknown. This can be explained by the cell-type dependent expression and function of miRNAs and by the fact that many of currently known miRNAs have been identified in non-leukemia/non-hematopoietic cell types. To discover miRNAs in ALL, we initially used a conventional small RNA concatemer-cloning method followed by Sanger sequencing (**chapter 3**). In **chapter 4** we applied the more recently developed and more sensitive technique of high-throughput sequencing. Both strategies resulted in the identification of known and novel miRNAs that are expressed in ALL. In total we identified 470 known and 36 novel miRNAs of which many were uniquely expressed in ALL or normal hematopoietic cells. The expression signature of mature miRNAs in ALL subtypes was confirmed by real-time quantitative PCR using a miRNA-specific reverse transcription stem-loop primer (**chapter 5**). Besides specific for the ALL subtype, miRNA expression levels were also found discriminative for resistance to chemotherapeutic drugs used in the treatment of pediatric ALL, i.e. vincristine, daunorubicin and L-asparaginase. In addition, the expression profile of 14 miRNAs determined in leukemic cells taken at initial diagnosis of ALL was predictive for the long-term clinical outcome of children with ALL. These data support the hypothesis that leukemia-specific miRNA expression patterns exist. These findings also emphasize that functional studies of the biology of these miRNAs in leukemia need to be addressed in the appropriate cellular context. In **chapter 6** we showed that overexpression of *miR-196b* in *MLL*-rearranged precursor B-ALL and *HOXA*-regulated T-ALL patients coincided with upregulation of *HOXA* cluster genes. In contrast, co-expression of these genes was absent in other types of ALL. In **chapter 7** we focused on the cause of downregulated miRNAs in *MLL*-rearranged precursor B-ALL. We observed that increased levels of DNA methylation occurred in the vicinity of miR-genes with reduced expression in *MLL*-rearranged ALL. This expression level could be increased by exposure of leukemic cells to the demethylating agent zebularine. These findings are challenging since this may point to epigenetic targets for therapy.

Finally, the results are summarized in **chapter 8** and the work is further discussed and perspectives for future studies are given in **chapter 9**. **Chapter 10** contains a summary in Dutch or "Nederlandse samenvatting".

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Chapter 2

MicroRNAs in acute leukemia: from biological players to clinical contributors

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ABSTRACT

MicroRNAs (miRNAs) are involved in the management of hematopoiesis. As a consequence, miRNA dysregulation causes disruption of the hematopoietic system and leukemia may arise. We here comprehensively discuss microRNAs found discriminative for cytogenetic and molecular subtypes of acute leukemia. These miRNAs are either known microRNAs involved in leukemogenesis with proven tumor suppressor or oncogenic activities or are newly identified by high-throughput sequencing with yet unknown function. Furthermore, forces are outlined that drive aberrant miRNA function which include genetic abnormalities (e.g. deletions, translocations and mutations) and epigenetic aberrations (e.g. aberrant DNA methylation or histone modifications). Interestingly, leukemia-silenced miRNAs can be re-expressed upon treatment with de-methylating agents. Targeting miRNA expression may serve a therapeutical role, albeit at present this way of targeted therapy is in its infancy. However, emerging knowledge about the biology of miRNAs in leukemia may result into a role for these miRNAs in the diagnosis and treatment of acute leukemia.

DISCOVERY AND FUNCTION OF MICRORNAS: BACK TO THE BASICS

In 1993 Ambros and Ruvkun discovered the first miRNA (miRNA) (lin-4) that repressed *messenger*RNA (mRNA) lin-14 through complementary basepairing in *C. elegans*[1,2]. It took till 2000 to recognize that this phenomenon was not restricted to worms, but also occurred in mammals[3]. Today miRNAs are known as highly conserved 18-25 nucleotides non-protein coding small RNAs that regulate the expression of over 60% of all human genes[4]. MiRNAs achieve this regulation by binding with their seed sequence (usually covering nucleotide position 2 to 8 from the 5' miRNA) to a (partially) complementary sequence in the 3' untranslated region (3'UTR) of the targeted mRNA (Figure 1). This leads to inhibition of translation or destabilization of the mRNA by deadenylation[4]. MiRNAs are processed from a larger stem-loop precursor (pre-miRNA) by the enzyme Dicer, generating double-stranded RNA (dsRNA) intermediates. One strand encodes the mature miRNA that forms a complex with Argonaute (Ago) proteins which is mandatory for the miRNA in order to bind to its target-mRNA (Figure 1). In contrast, the other strand (denoted as miRNA*-strand) is believed to be non-functional and is degraded upon its release[5]. The processing and final action of miRNAs shares similarities with RNA interference (RNAi)[6]. This new pathway of translational regulation coincides with the discovery of miRNAs and was awarded with the Nobel Prize (2006) for its promise as therapeutic tool[7]. RNAi is triggered by exogenous dsRNA as part of a defence mechanism against viruses. Similar to endogenous miRNAs, dsRNAs are processed by Dicer and require binding to Ago proteins in order to silence genes[6].

Our understanding of miRNA functioning probably reflects a tip of the iceberg. MiRNA-mRNA basepairing may besides translational silencing also promote the expression of target genes. A pioneering study showed that miR-328 silences survival factor proviral integration site 1 (*PIM1*) through mRNA 3'UTR -basepairing and simultaneously interacted with the CCAAT/enhancer binding protein alpha (*C/EBPα)*-regulating protein, poly(rC)-binding protein hnRNP E2 in myeloid progenitor cells[8]. The interaction between miR-328 and hnRNP E2 was independent from the miRNA seed sequence, Dicer and Ago proteins and prevented hnRNP E2 from repressing *C/EBPα* translation. Although confirmatory studies are needed, these data imply that miRNAs can also regulate gene expression by interference with the function of regulatory translational factors. Other studies show that miRNA function is dependent upon the specific cell type. On one hand miR-221 and miR-222 function as tumor suppressors in erythroblastic leukemia cells, on the other hand the same miRNAs act as oncogenes in solid tumors[9]. All together, the dual abilities for miRNAs like the mRNA decay/protein decoy activity for miR-328 (Figure 1) and the dual function for miR-221/222 indicate that the role of miRNAs in diseases like acute leukemia may be more complex than previously appreciated.

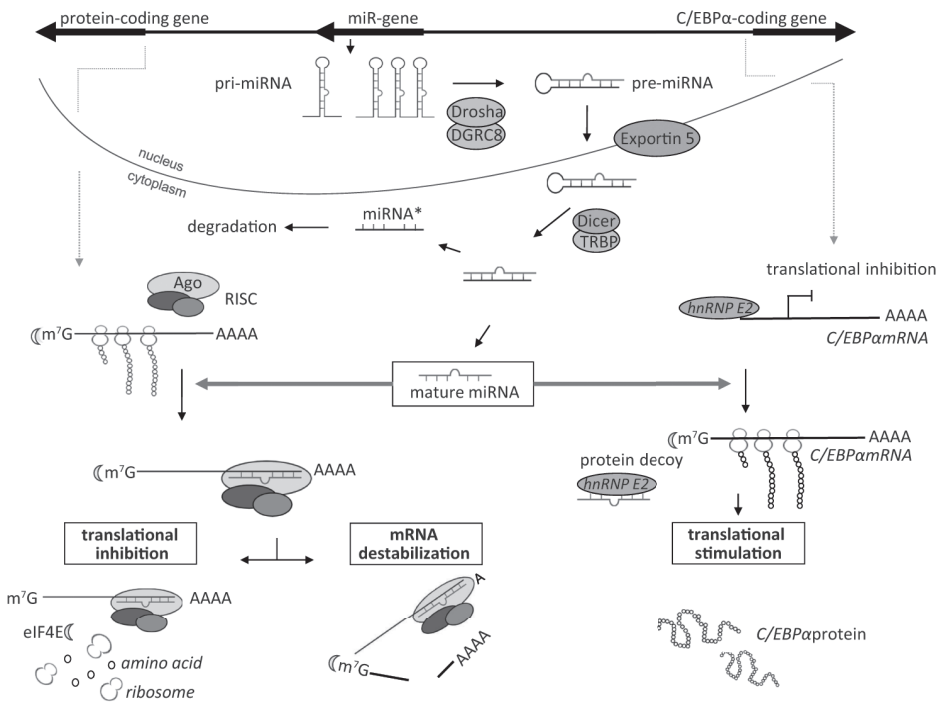

Figure 1. MiRNAs regulate the translation of protein-coding genes.
miRNA gene (miR-gene) is transcribed in a primary miRNA (pri-miRNA) transcript that is further processed into a stem-loop structured miRNA precursor (pre-miRNA) by the RNase III enzyme Drosha facilitated by the DiGeorge critical region 8 (DGRC8) protein. After export to the cytoplasm by Exportin-5, the pre-miRNA is cut by the RNase III enzyme Dicer into 18-25 nucleotide long double-stranded miRNA/miRNA* intermediates. Whereas the miRNA* strand is rapidly degraded, the mature miRNA either interacts with Argonaute (Ago) proteins as part of the RNA-induced silencing (RISC) complex or acts as protein decoy (e.g. scavenging of the poly(rC)-binding protein hnRNP E2 by miR-328[8]). If bound to Ago the miRNA hybridizes to (partially) complementary sites typically in the 3′ untranslated region (UTR) of target messenger RNA (mRNA). The 3′ UTR binding inhibits further translation of proteins via competition of miRNA-bound Ago for 5′ cap (m7G) binding with the initiation factor of translation eIF4E or by destabilizing the mRNA via deadenylation. If miR-328 binds to hnRNP E2, hnRNP E2 is prevented from inhibiting C/EBPα with translational re-activation of C/EBPα as a result[8].

MICRORNA (MIS)MANAGEMENT OF HEMATOPOIESIS

MiRNAs direct different biological processes including development, differentiation and hematopoiesis by regulating the expression of target genes. MiRNAs fine-tune the hematopoietic system and manage the control of both lymphoid and myeloid lineages[10]. Amongst others, miR-181 was one of the first miRNAs demonstrated to be preferentially

expressed in hematopoietic tissue. Enforced expression in hematopoietic stem cells stimulated the differentiation into B-lineage cells[11]. MiR-328 stimulated differentiation of myeloid cells by promoting the expression of the master regulator of myeloid differentiation *C/EBPα* through scavenging of the translational inhibitor hnRNP E2[8]. Conversely, miR-124a prevents cells from differentiation towards the myeloid lineage via direct inhibition of *C/EBPα* protein expression[12]. The down-regulation of *miR-15a/16-1* due to a genomic deletion in chronic lymphocytic leukemia (CLL) patients provided the first link between disturbed miRNA expression and (hematopoietic) cancer[13]. Recently it was confirmed that *miR-15a/16-1*-deletion contributes to the development of CLL. Mice with this deletion developed CLL or a related leukemia type, possibly by accelerating B-cell proliferation through regulating cell cycle-associated genes[14]. Enhanced B-cell proliferation was also observed in miR-155 transgenic mice which finally developed acute lymphoblastic leukemia (ALL) and high grade lymphoma [15]. Deletion of miR-145 and miR-146a genes on chromosome 5q was associated in patients with myelodysplastic syndrome[16]. Both miRNAs are abundantly expressed in normal hematopoietic stem/progenitor cells. Silencing of miR-145 and miR-146a in mouse hematopoietic stem/progenitor cells resulted into a myelodysplastic syndrome-like phenotype which progressed into a myeloid-like leukemia in mouse models[16,17]. These studies exemplify that dysregulated expression of miRNAs may contribute to leukemogenesis by disturbing the tight control of normal hematopoietic processes.

ABERRANT MICRORNA EXPRESSION CHARACTERIZES DIFFERENT CYTOGENETIC AND MOLECULAR SUBTYPES IN ACUTE LEUKEMIA

To reveal which miRNAs are dysregulated in acute leukemia, miRNA genome-wide expression studies have been conducted. These studies showed that leukemic cells of acute leukemia patients have different miRNA expression patterns than in normal hematopoietic cells[18-21]. Quantitative analysis of 397 miRNAs revealed that leukemic cells of pediatric precursor B-ALL patients have different miRNA expression profiles than samples of normal bone marrow and $CD34^+$ sorted cells[22]. Moreover, miRNAs were significantly differentially expressed between T-ALL cases and normal thymocytes[22]. Cytogenetically relevant types of ALL in children display characteristic miRNA expression signatures as visualized in Figure 2 (adapted from Schotte et al, Haematologica 2011[22]). Most striking differences were found for 11q23/*MLL*-rearranged precursor B-ALL cases which displayed downregulation of miR-708 and upregulation of miR-196b and t(12;21)/*TEL-AML1*- positive precursor B-ALL cases which distinguished by upregulation of miR-383, -125b, -99a and -100 (Table 1)[22]. Remarkably, the microRNA signature of *TEL-AML1*-positive and hyperdiploid (>50 chromosomes) ALL cases partly overlapped similar to what has been observed for mRNA expression profiles[22,23], which may suggest a common underlying biology. High expression of miR-33, miR-215, miR-369-5p,

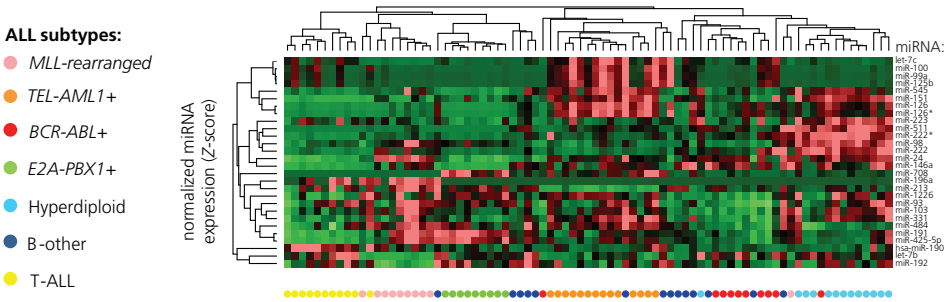

Figure 2. Clustering of ALL subtypes by miRNA expression pattern.
Subtypes of pediatric ALL cluster together based on similarities in miRNA expression patterns. The hierarchical clustering of cases is based on the expression level of miRNAs most discriminative for 7 subtypes of ALL, as determined by stem-loop RT-quantitative PCR (see Schotte, Haematologica 2011 for experimental details[22]). The 28 miRNAs represent the top 5 most discriminative miRNAs per subtype of which two miRNAs being discriminative for more than one subtype. The heatmap shows which miRNAs are overexpressed (in red) and which are underexpressed (in green) relative to snoRNA. Expression levels are plotted as standardized Z-scores per miRNA. Figure is adapted from data presented by Schotte et al, Haematologica 2011[22].

miR-496, miR-518d and miR-599 was associated with an unfavorable prognosis whereas high expression of other miR-genes (i.e. miR-10a, miR-134, miR-214, miR-484, miR-572, miR-580, miR-624 and miR-627) was linked to a favorable prognosis in pediatric ALL. A combined profile of these 14 miR-genes further improved the predictive value for long-term clinical outcome in children with ALL[22].

Adult acute myeloid leukemia (AML) patients with t(8;21)/*AML1-ETO* or inv(16)/*CBF*β-*MYH11* were distinguished from other AML patients by high and low expression of miR-126/126*[21] and let-7b/c[20], respectively (Table 2). High expression of another set of miRNAs including miR-382 was unique for t(15;17)/*PML-RAR*α-positive AML[18,20]. In 11q23/*MLL*-rearranged AML cases the oncogenic miR-17-92 cluster was upregulated[21,24,25] whereas the tumor suppressor miR-29 was downregulated (Table 2)[19,26]. MiRNA expression levels are also associated with molecular subtypes of AML, the mutational status and associated gene expression pattern in AML (Table 2). Adult cases with cytogenetically normal (CN)-AML and favourable *C/EBP*α-mutations demonstrated higher levels of miR-181[27] whereas CN-AML patients with unfavorable isocitrate dehydrogenase 2 *(IDH2)*-mutations displayed high levels of miR-1 and miR-133a [28] compared to CN-AML cases without these additional mutations. CN-AML patients with favorable nucleophosmin *(NPM1)*-mutations overexpressed several miRNAs including miR-10a, -10b, -100, -196a, -196b and let-7 whereas other miRNAs i.e. miR-126, -130a and -451 were downregulated compared to *NPM1*-wildtype cases[29]. Interestingly, *NPM1*-wildtype patients with relatively high expression of miR-126 often display increased transcription of the meningioma (*MN1*) gene, which is associated with an

Table 1. MiRNAs characterizing different cytogenetic and molecular subtypes in *childhood* acute leukemia.

miRNA	aberrant expression	fold-change	ALL or AML	control group	reference
let-7b	downregulation	- 69	precursor B-ALL with 11q23/*MLL*-translocation	precursor B-ALL without 11q23/*MLL*-translocation	22
let-7c	downregulation	- 16	precursor B-ALL with 11q23/*MLL-translocation*	precursor B-ALL without 11q23/*MLL*-translocation	22
miR-29a, -b, -c	downregulation	- 8-50	AML with 11q23/*MLL*-translocation	AML without 11q23/*MLL*-translocation	19,26
miR-99a	upregulation	+ 30-38	precursor B-ALL with t(12;21)/*TEL-AML1*	precursor B-ALL without t(12;21)/*TEL-AML1*/ ALL with HH, DS ALL, iAMP21	22,42
miR-100	upregulation	+ 30	precursor B-ALL with t(12;21)/*TEL-AML1*	precursor B-ALL without *TEL-AML-1*	22
miR-125b	upregulation	+ 30-35	precursor B-ALL with t(12;21)/*TEL-AML1*	precursor B-ALL without t(12;21)/*TEL-AML1*/	22,42
miR-155	upregulation	+ 2-5	AML with *FLT3*-ITD	AML with *FLT3*-wildtype	26,35
miR-196a	upregulation	+ 2283	precursor B-ALL with 11q23/*MLL*-translocation	precursor B-ALL without 11q23/*MLL*-translocation	22
		+ 45	AML with 11q23/*MLL*-translocation	AML patients without 11q23/*MLL*-translocation	26
miR-196b	upregulation	+ 500	precursor B-ALL with 11q23/*MLL*-translocation	precursor B-ALL without 11q23/*MLL*-translocation	66
		+ 212	AML with 11q23/*MLL*-translocation	AML patients without 11q23/*MLL*-translocation	26
		+ 800	T-ALL	precursor B-ALL	66
miR-320a	downregulation	-3*	precursor B-ALL with t(12;21)/*TEL-AML1*	precursor B-ALL without *TEL-AML-1*	70
miR-383	upregulation	+ 1671	precursor B-ALL with t(12;21)/*TEL-AML1*	precursor B-ALL without t(12;21)/*TEL-AML1*	22
miR-494	downregulation	-5*	precursor B-ALL with t(12;21)/*TEL-AML1*	precursor B-ALL without *TEL-AML-1*	70
miR-708	downregulation	- 288	precursor B-ALL with 11q23/*MLL*-translocation	precursor B-ALL without 11q23/*MLL*-translocation	22
		- 3884	T-ALL	precursor B-ALL	22
novel sol-miR-23$^{\Delta}$	downregulation	- 6-10	precursor B-ALL	normal bone marrow/ normal CD34+	65

Acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), normal CD34+ hematopoietic progenitor cells (normal CD34+), high hyperdiploidy with more than 50 chromosomes (HD), ALL in Down Syndrome patients (DS ALL), subtype of ALL characterized by intrachromosomal amplification of small region within the long arm of chromosome 21 (iAMP21). *) P-value unknown: all others $P<0.05$, unknown: unknown fold-change. ΔNovel sol-miR-23 is recently annotated by miRBase as hsa-mir-4474.

Table 2. MiRNAs characterizing different cytogenetic and molecular subtypes in *adult* acute leukemia.

miRNA	aberrant expression	fold-change	ALL or AML	control group	reference
let-7a	upregulation	+ 2	CN-AML with *NPM1*-mutation	CN-AML with *NPM1*-wildtype	29
let-7b	downregulation	- 3*	AML with inv (16)/*CBF*β-*MYH11*	AML without inv (16)/*CBF*β-*MYH11*	20
		- 4*	AML with t(8;21)/*AML1-ETO*	AML without t(8;21)/*AML1-ETO*	20
let-7c	downregulation	- 4*	AML with inv (16)/*CBF*β-*MYH11*	AML without inv (16)/*CBF*β-*MYH11*	20
		- 5*	AML with t(8;21)/*AML1-ETO*	AML without t(8;21)/*AML1-ETO*	20
	upregulation	+ 2	CN-AML with *NPM1*-mutation	CN-AML with *NPM1*-wildtype	29
miR-1	upregulation	+ 4-10	CN-AML with *IDH2*-mutation	CN-AML with IDH1/IDH2 -wildtype	28
miR-10a	upregulation	+ 10	CN-AML with *NPM1*-mutation	CN-AML with *NPM1*-wildtype	29
miR-10b	upregulation	+ 2-8	CN-AML with *NPM1*-mutation	CN-AML with *NPM1*-wildtype	29
miR-17-92 cluster	upregulation	+ 5-10*	ALL with 11q23/*MLL*-translocation	ALL/AML without 11q23/*MLL*-translocation, normal CD34+, normal MNCs	25
		+ 5-17*	T-ALL	normal tonsil lymphocytes	58
		+ unknown*	AML with 11q23/*MLL*-translocation	AML without 11q23/*MLL*-translocation, normal CD34+, normal MNCs	21,24
miR-24a, -b	upregulation	unknown*	AML with t(8;21)/*AML1-ETO*	normal CD34+ myeloid cells	40
miR-29a, -b, -c	downregulation	- 8-50	AML with 11q23/*MLL*-translocation	AML without 11q23/*MLL*-translocation	19,26
		- 3	CN-AML with *NPM1*-wildtype	CN-AML with *NPM1*-mutation	35
miR-125b	upregulation	+ 4-5	CN-AML with *IDH2*-mutation	CN-AML with IDH1/IDH2 -wildtype	28
miR-126/126*	upregulation	+ unknown*	AML with inv (16)/*CBF*β-*MYH11* or t(8;21)/*AML1-ETO*	AML without inv (16)/*CBF*β-*MYH11* or t(8;21)/*AML1-ETO*	21
	downregulation	- 3	CN-AML with *NPM1*-mutation	CN-AML with *NPM1*-wildtype	29
mir-130a	downregulation	- 3	CN-AML with *NPM1*-mutation	CN-AML with *NPM1*-wildtype	29
miR-133a	upregulation	+ 4-7	CN-AML with *IDH2*-mutation	CN-AML with IDH1/IDH2 -wildtype	28

Continuation of Table 2. MiRNAs characterizing different cytogenetic and molecular subtypes in *adult* acute leukemia.

miRNA	aberrant expression	fold-change	ALL or AML	control group	reference
miR-181a, -b, -c, -d	upregulation	+ 2	CN-AML with *C/EBPα* -mutation	CN-AML with C/EBPα -wildtype	27
miR-196a	upregulation	+ 2	CN-AML with *NPM1*-mutation	CN-AML with *NPM1*-wildtype	20,29
miR-196b	upregulation	+ 2*	AML with *NMP1*-mutation	AML with *NPM1*-wildtype	20
miR-382	upregulation	+ 58*	AML with t(15;17)/*PML-RARα*	AML without t(15;17)/*PML-RARα*	20
miR-451	downregulation	- 3	CN-AML with *NPM1*-mutation	CN-AML with *NPM1*-wildtype	29

Acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), cytogenetically normal AML (CN-AML), nucleophosmin (NPM1), isocitrate dehydrogenase 1/2 (IDH1/2), normal CD34+ hematopoietic progenitor cells (normal CD34+), normal mononuclear cells (normal MNCs). *) P-value unknown: all others P<0.05, unknown: unknown fold-change.

unfavorable prognosis[30]. The unique mRNA expression profiles associated with cytogenetic aberrations and mutational status of protein-coding genes and prognosis may reveal insight into the biology of these leukemia types.

KNOWN MICRORNAS INVOLVED IN LEUKEMOGENESIS

Tumor suppressor microRNAs

Let-7 is by far the most extensively studied miRNA and is known for its tumor suppressive capacities in a variety of malignancies by targeting different oncogenes including *NRAS*, *KRAS*, *MYC* and *HMGA2, IMP1* and *TRIM71*[31-34]. Besides in solid tumors, low levels of let-7b and let-7c were found in different types of acute leukemia e.g. t(8;21) and inv(16)-positive adult AML (Table 2)[20]. Moreover, let-7b downregulation in children with *MLL*-rearranged ALL patients was linked to an upregulation of oncoprotein c-Myc suggesting that dysregulation of let-7 is not restricted to solid tumors but may also play a role in leukemia[22].

The expression of miR-29 is downregulated in *MLL*-rearranged AML and in *NMP1*-wildtype CN-AML (Table 1 and 2)[35]. MiR-29 is known to inhibit genes involved in apoptosis (e.g. *MCL-1*)[36] and cell cycling (e.g. cyclin-dependent kinase 6 or *CDK6*)[36]. Luciferase 3'UTR binding assays confirmed that miR-29b binds to *MCL-1* mRNA. In correspondence, re-expression of miR-29b induced apoptosis by downregulating Mcl-1 expression in AML patient's samples and drastically reduced tumor growth in a leukemia xenograft model[36]. Another target of miR-29 (and miR-181) is the oncogene T-cell leukemia/lymphoma 1 (*TCL1*) in B-cell chronic lymphocytic leukemia (B-CLL)[37]. Activation of this oncogene in transgenic mice led to development of CD5+ leukemia resembling B-CLL in humans[38]. This mouse model was built with only the protein-coding part of the *TCL1* gene. Another model by Efanov et al comprised the total length of *TCL1* gene including the 3'UTR that contains binding sites for miR-29 (and miR-181)[39]. The full length *TCL1* transgenic mice developed leukemia with delayed onset compared with the model lacking the 3'UTR[38]. These findings suggest that negative regulation of *TCL1* by miR-29 and miR-181 inhibit the progression of B-CLL disease and supports a role for *miR-29* as tumor suppressor gene in leukemia. Whether, however, dysregulation of *TCL1* contributes to acute leukemias is yet unknown.

Oncogenic microRNAs

The high expression of miR-126/miR-126* in inv(16) and t(8;21)-positive AML patients (Table 2) prompted Li et al to functionally study these miRNAs[21]. Enforced expression of miR-126 and its star strand in AML cell lines inhibited the apoptotic potential and facilitated cell survival. In addition, proliferation of mouse bone marrow progenitors was preferentially enhanced by enforced expression of miR-126 in combination with the t(8;21) fusion[21]. Therefore miR-126/126* is considered as oncogene to be involved in leukemogenesis of t(8;21) AML.

MiR-24 is another miRNA activated in t(8;21)-positive AML patients (Table 2)[40]. Its up-regulation is caused by binding of the AML-ETO fusion product to the *miR-24-23-27* gene locus[40]. MiR-24 inhibits the synthesis of mitogen-activated protein kinase (MAPK) phosphatase 7, thereby facilitating cell growth through activation of c-jun and p38 kinases in myeloid leukemia cells[40].

MiR-125b and neighbouring miRNA genes *let-7c*, *miR-99a* and *miR-100* were aberrantly upregulated in *TEL-AML1*-positive ALL and different myeloid leukemias (Table 1 and 2)[41-43]. Pro-B cells gained a survival advantage[42] and differentiation of $CD34^+$ myeloid progenitors was disturbed upon miR-125b overexpression[41,43]. Moreover, the oncogenic activity of miR-125b was recently shown in mice which were transplanted with fetal liver cells that over-expressed miR-125b[44]. These xenografted mice developed B-ALL and T-ALL suggesting an active role for miR-125b in leukemogenesis[44]. Furthermore, overexpression of miR-125b in *BCR-ABL*-positive leukemia cells accelerated the development of leukemia suggesting that miR-125b facilitates the oncogenic action of the *BCR-ABL* fusion gene[44]. A potential pathway involved in miR-125b-facilitated-leukemogenesis may include the downregulation of *IRF4,* a transcription factor that inhibits proto-oncogene BCL-6 (B- cell CLL/lymphoma 6) in lymphoma[45]. Enforced expression of miR-125b also facilitated IL3-independent growth of Ba/F3 mouse cells[42]. In addition to its oncogenic role, miR-125b expression has been associated with drug resistance in *TEL-AML1*-positive pediatric ALL[22]. Functional studies showed that inhibition of miR-125b sensitized *TEL-AML1*-positive cells to doxorubicin[42]. These studies exemplify a role for miR-125b in leukemogenesis and points to miR-125b as potential therapeutic target.

MiR-155 is overexpressed in lymphomas, $FLT3/ITD^+$ AML and different pediatric ALL subtypes[26,35,46-49]. MiR-155 acts as an oncogene as its introduction in mouse bone marrow resulted in myeloproliferative disorders[50] and B-lineage ALL / lymphoma[51]. The oncogenic effect was mediated by downregulation of *SHIP* (Src homology 2 domain -containing inositol-5-phosphatase) and *C/EBP*β[15]. Both genes were predicted by TargetScan[52] and subsequent luciferase 3'UTR binding assays confirmed the binding of miR-155[15]. Transduction of miR-155 in natural killer-cell lymphoma/leukemia led apart from the downregulation of SHIP also to the inhibition of the tumor suppressor genes *PTEN* (phosphatase and tensin homologue) and *PDCD4* (programmed cell death 4)[49]. Since *SHIP* and *PTEN* are both negative regulators of the *PI(3)K* survival pathway this in turn resulted in upregulated phosphorylated Akt thereby rescuing cells from apoptosis and boosting proliferation[49].

Initially, miR-21 was found overexpressed in different types of tumors including leukemia and lymphoma[53]. Overexpression of miR-21 in transgenic mice caused precursor B-cell lymphoblastic leukemia/lymphoma[53]. These lymphoid malignancies disappeared by induction of apoptosis and proliferation arrest upon silencing of *miR-21*. This shows that miR-21 itself is capable to initiate, maintain and support survival of lymphoma/leukemia *in vivo*[53].

Upregulation of miR-196b coincides with *HOXA* overexpression in pediatric *MLL*-rearranged precursor B-ALL, *HOXA*-activated T-ALL[54] and in different subtypes of AML[20,26,29] (Table 1 and 2, Figure 2). The high level expression of miR-196b was not predictive for prednisolone resistance in pediatric ALL, albeit *MLL*-rearranged cases are more resistant to this drug[54,55]. Overexpression of miR-196b facilitated proliferation and blocked differentiation in mouse bone marrow progenitor cells[56] which may be indicative for a role in leukemogenesis. In contrast to promoting leukemogenesis, miR-196b may also inhibit this process as miR-196b and miR-196a were recently reported to downregulate the oncogenic transcription factor ERG in adult patients with AML and T-ALL[57]. Like discussed for miR-221/222, miR-196a/b may therefore have a dual role in leukemia depending on the genetic context of cells.

The miR-17-92 cluster located on chromosome 13q31 consists of six miRNAs i.e. miR-17/17*, -18a, -19a, -19b, -20a and -92a, and was found overexpressed in *MLL*-rearranged acute leukemias and T-ALL patients (Table 2)[20,21,24,25,58,59]. Together with the fact that this polycistronic cluster cooperated with c-*Myc* to accelerate B-cell lymphoma formation *in vivo*[60] suggests that *miR-17-92* acts as an oncogene in lymphoid tissue. Induced expression of the miRNA cluster enhanced the colony forming capacity of mouse bone marrow progenitors especially when *MLL* fusion genes were co-expressed[25,59]. Increased expression of the miR-17-92 cluster was associated with a downregulation of p21 inhibitor of cell cycle progression. Complete knockdown of p21 mimicked the oncogenic effect of miR-17-92 in *MLL*-rearranged leukemia cells[59]. p21 and 19 other genes are predicted targets for miR-17-92 by TargetScan[52], PicTar[61], MAMI[62] and/or Miranda[63,24]. These potential target genes were all downregulated in *MLL*-rearranged ALL[24,25,59]. Two genes i.e. *APP* and *RASSF2* were confirmed targets for miR-17-92 as shown by a luciferase 3'UTR binding assay[24] and the expression of one gene (i.e. *TNFRSF21*) inversely correlated with the expression of the miR-17-92 cluster[25]. The miR-17-92 cluster also has a oncogenic role in T-ALL. Mice transplanted with *miR-19/Notch1*-overexpressing hematopoietic progenitor cells developed ALL faster than mice receiving progenitor cells transfected with *Notch1*[58]. To identify the target genes of miR-19, a large-scale short hairpin screen was performed[58]. About 14,000 shRNAs were transduced into a cell line model to search for genes whose silencing resembled the proliferative effect of miR-19. Four human genes were identified that harboured miR-19-binding sites in their 3'UTR i.e. proapoptotic *Bim* (*BCL2L11*), tumor suppressor *PTEN,* AMP-activated kinase (*PRKAA1*), phosphatase *PP2A* (*PPP2r5e*) and dedicator of cytokinesis-5 (*DOCK5*). Except for *DOCK5* all identified target genes were confirmed by luciferase 3'UTR binding assays and other functional studies using miR-19-overexpression and -inhibition strategies. These protein-coding genes represent known negative regulators of the phosphatidylinositol-3- kinase (PI(3)K) survival pathway and silencing of these genes largely affected lymphocyte survival *in vitro* and accelerated leukemogenesis in *Notch1*-induced T-ALL *in vivo*[58]. This suggests that coordinated knockdown of these survival inhibitors is responsible for the oncogenic effect of miR-19 in *Notch1*-induced T-ALL.

NOVEL MICRORNAS POTENTIALLY INVOLVED IN LEUKEMOGENESIS

The major disadvantage of miRNA profiling studies is that they are focussed on known miRNAs. As miRNA function and expression is highly dependent upon its cellular context and most miRNAs have been discovered in non-leukemic tissue, profiling studies may have missed miRNAs important to acute leukemia and its subtypes. For this reason we and others cloned miRNAs from patient's leukemia cells[64-68]. Initially a small RNA concatemer-cloning procedure was used followed by Sanger sequencing and bio-informatic analysis[64,66]. This led to the identification of 8 novel miR-genes in *MLL*- and non-*MLL* rearranged ALL[66]. With the more sensitive high-throughput sequencing or deep sequencing method another set of 28 novel and 431 candidate novel miR-genes were identified in 7 subtypes of ALL[65]. Stem-loop quantitative RT-PCR demonstrated that these novel and candidate novel miRNAs were differentially expressed between different cytogenetic subtypes of ALL and between ALL and normal hematopoietic cells[65]. In total 186 novel (candidate) mature miRNAs and miRNA*-strands were found exclusively in ALL subtypes whereas 84 were found uniquely expressed in normal hematopoietic cells[65]. High-throughput sequencing of 3 pediatric ALL cases and 2 normal bone marrow samples by Zhang et al revealed 42 novel miRNAs of which 5 were unique to ALL and 22 exclusively detected in normal donor bone marrow cells[68]. The differential expression of these newly identified miRNAs between leukemic cells and normal hematopoietic cells warrants further studies for their potential role in leukemogenesis.

FORCES THAT DRIVE THE DYSREGULATION OF MICRORNAS IN ACUTE LEUKEMIA

MiRNA dysregulation may be due to genomic aberrations including amplifications and translocations, especially since many miRNAs are encoded on such fragile sites. E.g. amplification of the *miR-17-92* gene cluster contributed to the high level of expression of this cluster in *MLL*-rearranged patients[25]. In addition, *MLL*-fusion proteins[25,59] and oncogene *c-myc* may be responsible for *miR-17-92* cluster overexpression[59]. In T-ALL, the newly identified translocation t(13;14)(q32;q11) targeted the *miR-17-92* gene cluster and possibly thereby facilitates its expression[58]. Moreover this rearrangement coincided with an activating *Notch1* translocation i.e. t(9;14)(q14;q11)[58] , which even more supports the activation of the cluster since *Notch1* can target *c-Myc,* the direct regulator of miR-17-92[59,69]. Interestingly, miR-494 and miR-320a were downregulated by the TEL-AML1 fusion protein derived from the t(12;21)/(p13;q22) translocation found in ~25% of children with ALL (Table 1)[70]. Since both miRNAs were shown to target the anti-apoptosis protein survivin this may facilitate the survival and proliferation of *TEL-AML1*-positive ALL cells[70]. Also genomic deletions may dysregulate the expression of miRNAs. The chromosome 13q14 deletion is responsible for the inactivation of

miR-15/16-cluster in CLL patients[13] and a deletion of 7 Mb on chromosome 12 reduced the expression of miR-203 in murine T-cell leukemias/lymphomas[71]. MiR-128b sensitizes *MLL*-rearranged leukemia cells to prednisone[72] and a mutation in the *miR-128b* gene reduced the amount of mature miR-128b leading to prednisone resistance[73]. The occurrence of the mutation in *miR-128b* may indicate that other mutations exist which drive leukemogenesis and/or response to treatment. Recent studies show that mutations in pseudogenes may affect the expression of other genes by alternative scavenging of miRNAs[74]. For example pseudogene *PTENP1* acts as decoy for *PTEN* -targeting miRNAs as both *PTENP1* and *PTEN* share a highly similar 3'UTR[74]. Copy number losses and mutations affecting the binding of miRNAs to 3' UTR of *PTENP1* were found that may be responsible for the downregulation of the tumorsuppressor gene *PTEN* in leukemia[58,74].

Apart from genomic aberrations or aberrant activity of specific regulatory factors (e.g. *c-Myc)*, miRNA dysregulation may also be caused by aberrant expression of neighbouring protein-coding genes. E.g. miR-196b is co-expressed with its neighbouring genes *HOXA9* and *HOXA10* in *MLL*-rearranged ALL[54], T-ALL[54] and AML patients[26]. Similar co-activation may explain the high association for miR-10a (positioned in between *HOXB4* and *HOXB5*) as well as miR-196a (encoded in between *HOXB9* and *HOXB13*) with the *HOXB* cluster as observed in adult[35,75] and childhood AML[26]. It is however intriguing that aberrant expression of neighbouring genes, genomic aberrations and specific regulatory factors can only explain the minority of dysregulated miRNAs whereas an explanation is still lacking for the majority of aberrantly expressed miRNAs in acute leukemia.

MICRORNAS AS BYSTANDERS AND ACTORS OF EPIGENETICS

A deletion of *miR-203* affects one allele and is often accompanied by CpG hypermethylation of the second allele in mice[71]. This results in the complete silencing of *miR-203* in mice. Silencing of miR-203 was also found in human T-cell leukemias and illustrates that modification by epigenetics can be another regulatory level of miRNA expression. The genetic and epigenetic silencing of miR-203 is of clinical relevance since this miRNA controls the expression of the oncogene *c-ABL* and *BCR-ABL* fusion product in the prognostically unfavorable group of t(9;22) */BCR-ABL1*-translocated precursor B-ALL and chronic myeloid leukemia[71].

The epigenetic regulation of miRNA expression may be a bystander effect of the epigenetic control on protein-coding genes. The locus of the precursor of miR-126/miR-126* is embedded in a 287-bp CpG island and the high expression of miR-126/126* in AML patients carrying t(8;21)/AML1-ETO and inv(16)/CBFβ-MYH11 was associated with a lower level of methylation of this CpG island compared to the level in other subtypes of AML[21]. Similarly, high expression of *miR-196b* involved in leukemogenesis was associated with CpG island hypomethylation of the promoter region of the *miR-196b/HOXA* locus in

MLL-rearranged ALL which is on itself remarkable since *MLL*-rearranged ALL is characterized by genome-wide CpG island hypermethylation[54,76]. The MLL fusion product however may be directly involved in the mechanism underlying the transcriptional activation of the *miR-196b/HOXA* locus as it has been demonstrated that this fusion product recruits DOT1L histone methyltransferase leading to dimethylation of histone H3 lysine 79 residues (H3K79me2). This H3K79 dimethylation allows further chromatin remodelling and opens up the entire *HOXA* cluster including the embedded *miR-196b* gene[77]. The hypomethylation of *miR-196b/HOXA* locus is *MLL*-specific since in other non-*MLL* precursor B-ALL a closed chromatin structure was observed with concomitantly hypermethylation of *miR-196b/HOXA* locus and reduced expression of these genes[54].

The regulation of gene expression by epigenetics also involves modifications of histones by methylation (e.g. H3K79me2) and acetylation (e.g. H3K56 acetylation)[78]. The effect of the histone deacetylase inhibitor, trichostatin A was studied in an ALL cell line[79]. Trichostatin affected the expression of more than 40 miRNAs including *miR-22. MiR-22* was re-expressed upon reduction of H3K27 trimethylation of histones in close proximity of its promoter area, whereas DNA methylation was not affected. This illustrates that histone modifications can, independently from DNA methylation, contribute to regulation of miRNA expression in ALL. In addition, ChIP-on-Chip analyses of 27,800 CpG islands in two ALL cell lines revealed that *miR-124a* and 11 other miR-genes displayed both aberrant DNA methylation and histone modifications that resulted into a closed chromatin structure[80,81]. These epigenetic events contributed to the transcriptional silencing of *miR-124a* which in turn accelerated growth of ALL cells via upregulation of target *CDK6* and phosphorylation of retinoblastoma (*Rb*)[80].
Besides the fact that expression of miRNAs can be regulated by DNA methylation and histone modifications, miRNAs themselves can regulate the transcription of genes involved in the epigenetic machinery. Tumor suppressor miR-29b targets the DNA methyltransferases *DNMT1* and *DNMT3* which facilitate maintenance and *de novo* DNA methylation, respectively[82]. Enforced expression of miR-29b led to global DNA hypomethylation and re-expression of the tumor suppressor genes $p15^{INK4B}$ and estrogen receptor 1 (*ESR1)* in AML[82]. Recently we proposed that miR-152 may be another actor of the epigenetic machinery since the seed sequence of miR-152 shared sequence homology with the 3' UTR of the *DNMT1* transcript and expression levels of miR-152 inversely correlated to those of *DNMT1* in *MLL*-rearranged ALL[83]. Others have added miR-290 to this list of so-called "epi-miRNAs" upon the finding that miR-290 controlled DNA methylation and telomere recombination through retinoblastoma-like 2 (Rbl2)-dependent regulation of DNMTs[84]. Interestingly, a recent report demonstrated the discovery of a new class of miRNAs that directly (independent of target genes) mediated DNA methylation in plants[85]. After their loading into AGO4 clade proteins, these 24 nucleotide long miRNAs (lmiRNAs) directed DNA methylation at their own loci in *cis* as well as at their target genes in *trans* resulting a downregulation of target genes, likely via recruitment of de novo cytosine methyltransferase DRM2[85]. This fits in the hypothesis that

Dicer processed miRNAs are required to maintain DNA methylation in human cancer cells[86]. In summary, miRNAs may function both as bystander and as actor of the epigenetic control of gene expression.

MICRORNAS AS THERAPEUTICAL TOOL

Using epigenetic drugs

The actors of the epigenetic machinery are themselves under epigenetic control and therefore deregulated expression may be restored by epigenetic drugs. Treatment of ALL cells with trichostatin A resulted in re-expression of *miR-124a* by opening the chromatin structure via reduction of H3K27 trimethylation[79]. A similar result was established by 5-aza-2'-deoxycytidine that demethylated the promoter of *miR-124a*[80]. Patients may benefit from restoring miRNAs back to normal levels, especially since hypermethylation of *miR-124a* was linked to a higher relapse and mortality rate in ALL patients[80]. Despite the favourable effect of miR-124a re-expression in precursor B-ALL, the effect of treatment in AML is less clear. In AML both *miR-124a* and its target gene *C/EBPα* are hypermethylated[12]. Consequently, reactivation of *miR-124a* not only executes a tumor suppressive effect by downregulating *CDK6* but also prevents demethylated *C/EBPα* of being restored to normal levels[12]. *C/EBPα* is crucial for myeloid but not lymphoid differentiation and its low levels may contribute to the differentiation block underlying AML. Therefore, ALL patients may benefit more from miR-124 re-activation upon epigenetic drugs than AML patients.

Exposure to the de-methylating drug zebularine re-activated seven out of 11 miR-genes that were downregulated in children with t(4;11)-positive *MLL*-rearranged ALL due to CpG island hypermethylation. This is illustrated for *miR-152* in Figure 3 (adapted from Stumpel/Schotte et al. Leukemia 2011[83]). The silencing by DNA methylation suggests that miR-152 and the other six miRNAs (i.e. miR-200b, -200a, -429, -503, -432, and -10a) have tumor suppressive capacities similar to miR-124a. Accordingly, *miR-432* is part of the large *miR-127* tumor suppressor cluster that downregulates protein levels of *BCL-6*[83]. In addition, re-expression of miR-200b coincided with downregulation of the oncogene *ZEB2* which is highly expressed in t(4;11)*/AF4-MLL* -positive ALL[83,87] and led to disease progression in human cancer[88]. *MLL* wildtype, *AF4-MLL* and *DNMT1* were identified as potential target genes for miR-152 based on 3'UTR sequence homology[83]. This is of high interest because the co-expression of *MLL* wild-type is facilitating *MLL-AF9*-induced leukemogenesis[89] and the AF4-MLL reciprocal fusion protein contributes to leukemic transformation[90]. Moreover high levels of *DNMT1* may be responsible for the genome-wide hypermethylation that characterizes t(4;11)*/AF4-MLL* -positive ALL and was linked to an increased risk of relapse[76]. None of the re-activated miRNAs was associated with DNA hypermethylation in adult or pediatric non-*MLL* precursor B-ALL. Their hypermethylation-related reduced expression levels are therefore specific for *MLL*-rearranged ALL. The degree of methylation of *miR-152* was also predictive for a poor

clinical outcome of *MLL*-rearranged ALL[83] which points to the potential of de-methylating drugs as new therapeutic agents to treat this type of pediatric ALL.

Using non-epigenetic drugs

Epigenetic drugs like DNA-demethylating agents (e.g. 5-aza-2'-deoxycytidine and zebularine) and histone deacetylase inhibitors (e.g. trichostatin A) are indirect modulators of miRNA expression. More specific down-regulation may be achieved by antagomirs and locked nucleic acid (LNA)-anti-miR oligonucleotides that bind to miRNAs following the Watson-Crick complementarity rule. In leukemia cells the antagomir against miR-19 reactivated silenced tumor suppressors including *PTEN*, thereby opposing miR-19-driven leukemogenesis[58]. Treatment with a miR-196b-specific antagomir abrogated the growth advantage of *MLL-AF9* transformed/miR-196b overexpressing bone marrow cells[56] suggesting that inhibition of even a single miRNA may provide significant therapeutic benefit. Interestingly, the effectivity of antagomirs is not limited to the *in vitro* situation. Intravenous administration of antagomirs directed against miR-16, -122, -192 and -194 resulted in specific and longlasting reduction of miRNA levels in mice[91]. In addition, intravenous injections with LNA-anti-miR-122 formed stable heteroduplexes with liver-specific miR-122 leading to its depletion from liver in non-human primates[92]. Since miR-122 is essential for the hepatitis C virus[93], the LNA-anti-miR-122 is currently being explored as therapeutical tool for this disease as part of a worldwide clinical trial[94]. Anti-miRNA molecules are thus promising silencers of miRNAs. Hence, their effectivity to treat acute leukemia deserves further study.

In addition to the direct downregulation by anti-miRNA molecules, one could also consider the use of miR-masks[95] and miRNA sponges[96]. MiR-masks are usually 2'-O-methyl-modified oligoribonucleotides of 22-nucleotide length that compete with endogenous miRNA by blocking the complementary 3'UTR-binding site of its target mRNA[95]. The disadvantage of miR-masks is that they affect the expression of one target gene, whereas molecules that directly target a miRNA will affect all (multiple) targets of that specific miRNA[95]. As an alternative, miRNA sponges may be used. MiRNA sponges are short oligonucleotide sequences that contain multiple miRNA binding sites to which target miRNAs can bind. This reduces the number of free miRNA molecules that can bind to their (multiple) biological target mRNAs[97]. The sponges can be introduced into cells by adenoviral and lentiviral viruses in mice[96]. A lentiviral miR-326 sponge was delivered via intravenous injection in mice with experimental autoimmune encephalomyelitis and this resulted in the depletion of miR-326 in CD4+ T-cells[98]. As a consequence, the miRNA sponge reduced the development of the autoimmune disease in these mice[98]. The advantage of miRNA sponges over direct anti-miRNA molecules is that they can inhibit function of a complete miRNA family with similar seed sequence. On the other hand, their delivery needs a viral vehicle and requires integration in the host genome which makes the therapeutic controling and dosing in patients more difficult than in case of anti-miRNA molecules.

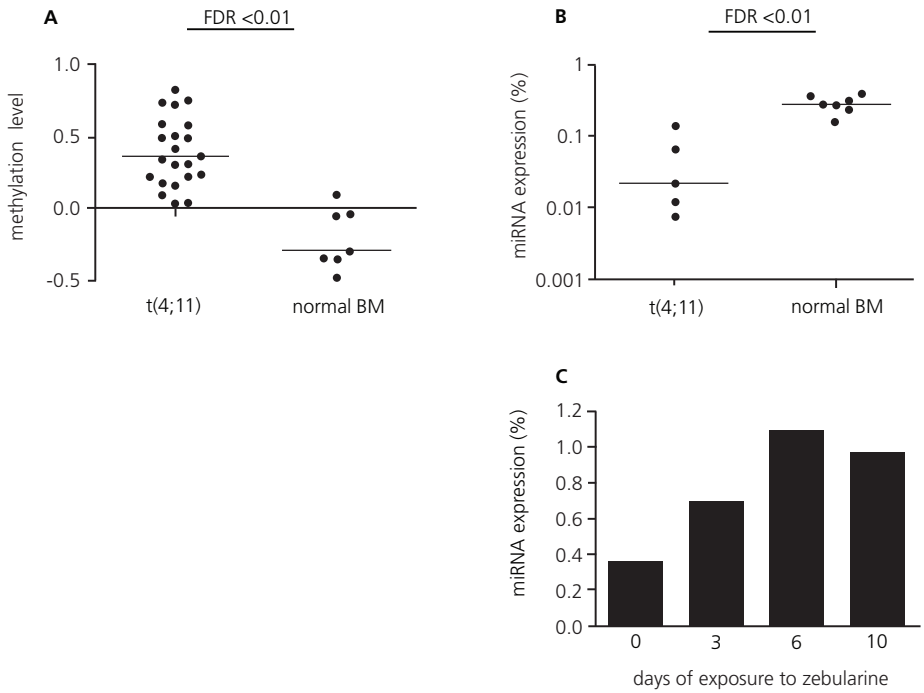

Figure 3. The effect of epigenetic drugs on miRNA expression.
(**A**) The methylation level of *miR-152* CpG islands is higher in t(4;11)-positive precursor B-ALL (n=22) than in normal bone marrow (n=7) samples, P_{FDR}<0.01. CpG methylation status was determined by a differential methylation hybridization procedure and methylation levels are presented as normalized log-ratios (see Stumpel/Schotte et al., Leukemia 2011 for experimental details[83]). (**B**) In correspondence to the methylation status, the expression level of miR-152 is lower in t(4;11)-positive infant ALL cases (n=5) compared to normal bone marrow samples (n=7), P_{FDR}<0.01. (**C**) Exposure to the demethylating agent zebularine resulted in upregulation of mature miR-152 levels in the *MLL*-rearranged ALL cell line SEMK2. Data presented are adapted from Stumpel/Schotte et al., Leukemia 2010[83].

On one hand miRNAs may be targeted by therapeutic agents, on the other hand miRNAs themselves may serve as 'targeted drug', especially those with tumor suppressive function. A variety of functional studies have overexpressed specific (precursor) miRNAs in cells of different origin, either via synthetic molecules or viral vectors, in order to study re-activation of tumor suppressive miRNAs that are lost during leukemia development. Tissue-specific expression requires a retroviral or adenoviral vector with a tissue-specific promoter. This approach led to high level of miR-26a expression in liver and dramatically inhibited local tumor formation[99]. A similar strategy may be used to target leukemia cells, e.g. by using the LCK-promoter to drive miRNA expression in T-ALL.

Challenges for microRNA-redirected treatment

Before using these manipulators of miRNA function in leukemia patients a few hurdles have to be taken. Administration of anti-miRNA molecules may have off-target effects on miRNAs to which they have a partial complementarity[100]. At first glance, off-target effects may be more manageable in case of (re)-introducing a (precursor) miRNA that is normally present in healthy non-leukemic cells. Unfortunately, this ectopically introduced miRNA may overload the RISC complex thereby affecting the processing of other miRNAs necessary for the survival of healthy (non-leukemic) cells[101]. Moreover, viral delivery of miRNAs may potentially activate the immune system[102]. Although the off-target toxicity associated with miRNA-specific replacement and depletion strategies is reported to be low[9], further research should first elucidate potential off-target effects before implementation in clinics.

The main challenge to overcome before applying miRNA-related drugs in the clinic is the delivery of these drugs to the affected tissue, e.g. bone marrow in acute leukemia. As mentioned above a viral vector with a tissue-specific promoter may be used. Synthetic miRNA mimics or anti-miRNA molecules do not have this benefit. Alternatively, aptamers (RNA oligonucleotides fused to the (anti-) miRNA) and liposomes may be used[100]. Aptamers and liposomal particles can direct the attached (anti)-miRNA molecule to specific cell surface receptors. As aptamers have only recently been discovered, liposomal particles have underwent more study so far. Liposomes direct the delivery of miRNAs to specific organs through modifications added to the liposomal surface[102]. Theoretically, such modifications like immunoconjugates directed against CD19 and CD33 could be applied in treatment of (precursor)-B-ALL and AML, respectively[102]. Moreover the lipid coating itself increases the size of the miRNA or anti-miRNA particle and increases the half-life of the molecule due to reduced renal clearance. Antagomirs are examples of anti-miRNA molecules that make use of the lipid coating as they have a cholesterol moiety at their 3' end. Antagomirs also have 2'O-methyl modifications for stability and a phosphorothioate backbone in order to make them resistant to exonuclease cleavage thereby increasing the half-life of the antagomir[100,102]. An example of a lipid-coated synthetic miRNA molecule is miR-34[101]. This miRNA mimic was intravenously delivered in mice with lung tumors and blocked growth of these tumors[101]. Moreover, its systemical delivery did not induce an immune system response or elevation of liver or kidney enzymes and was thus well tolerated. The uptake of synthetic miR-34 by normal cells had no effect as the affected pathways were already activated by the endogenous miRNAs in these cells[101].

CONCLUSIONS AND FUTURE PERSPECTIVES: BIOLOGICAL PLAYERS BECOMING CLINICAL CONTRIBUTORS

The discovery of miRNAs as powerful regulators of gene expression shed a new light on our understanding of acute leukemia. It became evident that leukemia is not only a disease of dysregulated protein-coding genes. In contrast, the biology of acute leukemia involves also dysregulation of the regulators of these genes i.e. miRNAs. Accordingly a number of miRNAs have been reported that serve a tumor suppressive and/or oncogenic role in acute leukemia (Table 1). Unique miRNA expression patterns were found for different genetic, prognostic, mutational and drug-resistant subtypes of acute leukemia. This implies that miRNAs have potential to classify patients and that specific subtypes may benefit from treatment strategies opposing the gain of oncogenic or loss of tumor suppressor function of miRNAs.

Besides being bystanders of epigenetics, miRNAs themselves act as epigenetic factors which make the consequences of their aberrant expression far more complex. Epigenetic drugs like DNA de-methylating agents and histone modifiers can reverse epigenetic changes and re-activate silenced miRNA. Therefore these drugs deserve further exploration in patients, particularly since the methylation degree of several miR-genes correlated to clinical outcome in acute leukemia. Direct manipulation of miRNA expression by anti-miRNA molecules or tissue-directed viral delivery is also a promising therapeutic options when safety has been proven.

In conclusion, miRNAs were discovered as small biological players but despite their small size their impact on diseases like acute leukemia seems to grow with every miRNA-related publication. Aberrant expression and function of oncogenic and tumor suppressive miRNAs may contribute to the biology of diverse leukemia subtypes. We are beginning to understand their clinical impact step by step since recent studies showed that expression and methylation levels are linked to clinical outcome and patients may thus benefit from miRNA-targeted therapeutic strategies. Therefore these small biological players may become clinical contributors in the diagnosis and treatment of acute leukemia.

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Chapter 3

Identification of new microRNA genes and aberrant microRNA profiles in childhood acute lymphoblastic leukemia

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ABSTRACT

MicroRNAs (miRNAs) control the expression of protein-coding genes in normal hematopoietic cells and, consequently, aberrant expression may contribute to leukemogenesis. To identify miRNAs relevant to pediatric acute lymphoblastic leukemia (ALL), we cloned 105 known and 8 new miRNA genes expressed in patients' leukemia cells. Instead of known miRNA genes, new miRNA genes were not evolutionary conserved. Quantification of 19 selected miRNA genes revealed an aberrant expression in ALL compared with normal CD34+ cells ($P \leq 0.02$); both upregulated (14/19) and downregulated (5/19) expressions were observed. Eight miRNAs were differentially expressed between *MLL* and non-*MLL* precursor B-ALL cases ($P < 0.05$). Most remarkable, miR-708 was 250- up to 6500-fold higher expressed in 57 *TEL-AML1*, *BCR-ABL*, *E2A-PBX1*, hyperdiploid and B-other cases than in 20 *MLL*-rearranged and 15 T-ALL cases ($0.0001 < P < 0.01$) whereas the expression of miR-196b was 500-fold higher in *MLL*-rearranged and 800-fold higher in 5 of 15 T-ALL cases as compared with the expression level in remaining precursor B-ALL cases ($P < 0.001$). The expression did not correlate with the maturation status of leukemia cells based on immunoglobulin and T-cell receptor rearrangements, immunophenotype or MLL-fusion partner. In conclusion, we identified new miRNA genes and demonstrated that miRNA expression profiles are ALL subtype-specific rather than linked to the differentiation stadium associated with these subtypes.

INTRODUCTION

Recently, a new class of ~22-nucleotides endogenous RNAs, called microRNAs (miRNAs), has been discovered. Despite their name, these small RNAs have a major biological function by affecting the translation of proteins encoded by many genes[1,2]. Although previous and current cancer research mainly focused on protein-coding genes, the function of miRNAs in gene regulation emphasizes the need to address the contribution of non-coding small RNAs in cancer cell physiology. MiRNAs are encoded by at least 710 genes in human, and are estimated to represent 1-5% of all predicted human genes[3,4]. They form one strand of the miRNA:miRNA* duplex that is processed from a larger stem-loop precursor[5-9]. Only the mature miRNA strand binds to its (imperfect) complementary *messenger*RNAs (mRNAs), resulting in translational repression of the targeted mRNA[10-13]. Currently characterized miRNAs inhibit translation of proteins that play important roles in fundamental biological processes, such as proliferation and differentiation[2,14,15], processes that are also affected in cancer cells. The expression profiles of miRNAs have been shown to be altered in various tumors including lung cancer[16], colon cancer[17], and different types of leukemia e.g. chronic lymphocytic leukemia (CLL)[18] and acute myeloid leukemia (AML)[19,20]. Oncogenic activity of miRNAs was shown by the fact that introduction of the *miR-17-92* cluster, which is upregulated in malignant lymphomas, accelerated c-myc-induced lymphomagenesis in mice[21].

MiRNA expression levels may be used in the classification of cancer. Lu et al. showed that poorly differentiated tumors could be more accurately identified using miRNA expression profiles compared to mRNA expression profiles[36]. More recently, miRNA signatures were also shown to classify different cytogenetic entities of adult AML and aberrant miRNA expression was shown to be linked to the prognosis of adult AML[19,20].

In contrast to several solid tumors, lymphomas, CLL and AML, the importance of miRNAs in acute lymphoblastic leukemia (ALL) is yet largely unknown. ALL represents a heterogenous disease characterized by various underlying genetic abnormalities. Intensive combination chemotherapy schedules have resulted in a 5-year event-free survival of ~80% in children whereas the treatment of adult ALL is much less successful resulting in ~40% 5-year event-free survival[22]. Genetically different subtypes of ALL have different clinical outcomes. For example, infants (children <1 year) with ALL cells bearing a rearrangement of the *MLL* gene have a highly unfavorable 4-year prognosis of <40%[22]. It is not unlikely that miRNAs also play a role in ALL since its subtypes are characterized by differentiation arrests at various stages in normal B- and T-cell development.

Present miRNA expression array-detection techniques are based on miRNA genes that have been published in the miRBase database. Consequently, one may miss yet unknown miRNAs that are especially expressed in leukemic cells. Hence, we first systematically cloned miRNA-genes that are expressed in leukemic cells of pediatric ALL patients with a poor prognostic *MLL* gene rearrangement and a prognostically more favorable precursor B-ALL subtype (B-other)

in order to identify "leukemia-specific miRNAs". In total, 105 known and 8 new miRNA genes were identified in these two subtypes. Quantification of miRNA expression by stem-loop real-time PCR analysis revealed that the expression of both known and newly identified miRNA genes differed between genetically and prognostically different subgroups of ALL and between these subtypes and normal $CD34^+$ progenitor cells. Our data show that the current number of miRNA genes relevant to ALL is underestimated and that systematic miRNA-gene cloning and subsequent miRNA expression analysis reveals ALL subtype-specific miRNA profiles. Our data also indicate that these subtype-related differences in miRNA expression levels cannot be explained by differences in the maturation status of individual ALL cases as judged upon immunoglobulin (Ig) and T-cell receptor (TCR)-rearrangement patterns and CD marker expression. Hence, the miRNA expression pattern seems more subtype-specific than B or T-cell differentiation-status specific in pediatric ALL.

MATERIALS AND METHODS

Patient samples

After obtaining informed consent, peripheral blood or bone marrow samples were obtained from children with ALL at primary diagnosis. *MLL* gene rearranged precursor B-ALL samples were collected from newly diagnosed infants (<1 years of age) who participated in the Interfant-study. All samples were screened for (specific) *MLL* gene rearrangements by reverse transcriptase-PCR (RT-PCR) and fluorescence *in situ* hybridization (FISH). A total of 20 *MLL*-rearranged samples included in this study are positive for t(4;11), n=8; t(11;19), n=8; t(9;11), n=3 and t(1;11), n=1. Fifteen T-ALL samples and 57 $CD19^+$ precursor B-ALL samples were obtained from the Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia study (COALL; Hamburg, Germany) and the Erasmus MC-Sophia Children's Hospital (Rotterdam, The Netherlands). All non-infant precursor B-ALL samples were negative for *MLL* translocation and were genetically characterized by presence of hyperdiploidy (more than 50 chromosomes, n=10), the *TEL-AML1* (n=10), *BCR-ABL* (n=10) and *E2A-PBX* (n=8) translocations. The remaining 19 precursor B-ALL samples were negative for hyperdiploidy, *MLL*, *TEL-AML1*, *BCR-ABL* and *E2A-PBX* translocations (B-other). $CD34^+$ control samples were obtained from granulocyte colony-stimulating factor (G-CSF)- mobilized peripheral blood stem cell harvests from two children with a brain tumor after informed consent.

Isolation of RNA and DNA out of leukemic cells

Mononuclear cells were isolated out of primary bone marrow and peripheral blood samples as previously described[23]. Non-malignant cells were removed using immunomagnetic beads. All processed leukemia samples contained >90% blast cells, as determined on May-Grünwald-Giemsa (Merck, Germany) stained cytospin preparations of isolated cells. $CD34^+$

control cells were enriched using magnetic beads (Miltenyi Biotec) specific for CD34. Purity (>90%) was assessed using flow cytometry. A minimum of 5 x 10^6 cells was lysed in TRIzol reagent (Invitrogen) in order to extract total RNA[23]. Quality of RNA was examined by the 2100 bioanalyzer (Agilent technologies).

Analysis of the maturation status of *MLL*-rearranged precursor B-ALL and T-ALL cases

Ig/TCR rearrangement patterns of *MLL*-translocated ALL samples were analyzed by PCR as described earlier[24]. Three stages of B-cell maturation were defined: immature (no or only incomplete *IGH* (DH-JH) rearrangements and no detectable TCR gene rearrangements), intermediate (incomplete (DH-JH) or complete (VH-JH) *IGH* rearrangements and/or incomplete (Dd2-Dd3 or Vd2-Dd3) *TCRD* rearrangements; no other Ig/TCR gene rearrangements detectable) and mature (Vδ2-Jα rearrangement and/or *IGK* rearrangement and/or *IGL* rearrangement and/or *TCRG* rearrangement and/or *TCRB* rearrangement). The maturation status of T-ALL samples was based on the expression of CD1 and surface-membrane- bound CD3 as determined by flow cytometry ($SmCD3^-/CD1^-$=immature, $SmCD3^-/CD1^+$=intermediate, $SmCD3^+$=mature).

Direct cloning of miRNAs

We used a slightly modified version of the direct cloning as previously described by Lau et al.[25]. Briefly, 1 pmol of ^{32}P-labeled 23-mer Carrier Oligo (5′ UGUCAGUUUGUUAAUUAACCCAA 3′) was spiked into a minimum of 1.5 µg total RNA extracted from purified leukemic cells. Subsequently, RNA size fractionation was performed on a 15% polyacrylamide 8 M urea gel (National Diagnostics, USA) and 18-26 nt small RNAs were isolated using the radiolabeled oligo as a reference. The 18-26-nt fractionated small RNAs were first ligated to a 3′adaptor oligonucleotide (5′pre-adenylated) without the presence of ATP, and then to a 5′adaptor oligonucleotide (not adenylated) in the presence of ATP. Final ligation products were amplified by RT-PCR, after which the PCR products were digested with PacI restriction enzyme (NEB, USA) to eliminate carrier oligo products. After precipitation, PacI-digested PCR products were purified on a 15% non-denaturing polyacrylamide gel (National Diagnostics) and according their size isolated from the gel and further amplified by 10-20 PCR cycles. PCR products were then digested with BanI restriction enzyme (NEB) and, if necessary, once more with PacI restriction enzyme. This was followed by concatemerization of BanI restricted products and subsequent ligation into the pCR 2.1-TOPO vector (Invitrogen, USA). Finally, the sequence of small RNA libraries was analyzed (Macrogen, Korea) and the data were bioinformatically analyzed to identify which miRNAs were expressed.

Bioinformatic analysis of small cloned RNAs

Cloned small RNA sequences were mapped to human and mouse genomes. Candidate precursor miRNA sequences were selected and computationally folded with the Vienna RNA Package[26]. Folded precursors were identified as miRNA candidates by applying a set of parameters derived from known miRNA genes as shown by Online Supplementary Figure S1. The following parameters were applied to human miRNA candidates: a loop length of 6-35 basepairs (bp); a 15-36 bp length of the miRNA* sequence, which is defined as the (imperfect) complementary strand of the miRNA within the folded precursor structure; a percentage of 55-100% of the miRNA that binds to the miRNA* sequence with 100% complementarity; and finally an energy from the precursor miRNA sequences between -42.5 and -6.8 kcal/mol. In case of mouse miRNA precursors a loop length of 7-35 bp, a miRNA* length of 15-28 bp, a pairing percentage between 67% and 100%, and an energy between -46.4 and -14.2 kcal/mol were applied.

Stem-loop RT-PCR

MiRNA expression was measured by stem-loop RT-PCR using primers and probes of the TaqMan MicroRNA assay for yet known miRNAs or newly developed primers and probes for newly identified miRNA candidates (Applied Biosystems, USA)[27]. Endogenous small nucleolar RNA 1 (snoRNA 1, 5' AUUUGCUAUCUGAGAGAUGGUGAUGACAUUUUAAACC-ACCAAGAUCGCUGAUGCA 3') or spiked synthetic miR-181c was used as a reference for RNA-input. In the latter case, synthetic miRNA-181c (Applied Biosystems) was added to total RNA before reverse transcription. Real-time PCR was performed in triplicate on an Applied Biosystems 7900HT RT-PCR system.

Statistical analysis

Mann Whitney U (MWU) Test was used to compare expression levels of miRNAs between two groups. The Fisher's exact test was used for frequency variables. Differences were considered statistically significant when $P<0.05$, 2-sided.

RESULTS

Identification of new miRNAs in two subtypes of pediatric ALL

A total of 1128 and 1080 small RNA (sRNA) sequences were cloned from the leukemic cells of an *MLL*-rearranged patient carrying a t(1;11) translocation and a precursor B-ALL patient negative for all known genetic subtypes in ALL (referred to as B-other), respectively (Figure 1A). Sequences of cloned sRNAs were mapped to the human genome and sequences encoding protein-coding mRNAs were excluded[28]. Precursor miRNA candidates were then extracted, folded to create the initial miRNA duplex and evaluated on the basis of features

derived from a set of known miRNAs present in the miRBase database as described in Material and Methods[4]. Ninety-five and 88 miRNA genes were found to be expressed in the leukemic cells of the *MLL*-rearranged and B-other cases, respectively. A number of these genes were present in both patients, resulting in the identification of a total of 113 unique miRNA genes (Figure 1A and Tables 1 and Online Supplementary Table S1). Eight of these 113 genes (7%) are newly discovered miRNA genes encoding 8 new mature miRNAs not previously reported in miRBase v11.0[4]. The 105 known genes (93%) encode 86 mature miRNAs and 15 miRNA* forms.

Table 1 and Online Supplementary Table S1 show the clone count within the constructed libraries for newly cloned and known miRNAs. Newly identified miRNAs were cloned with a lower frequency than that of the majority of known miRNAs (P<0.001). E.g., the maximum total clone count for newly found miRNAs is 4 (hsa-miR-1979), whereas the maximum clone count for known miRNAs is 667 (miR-142-3p). Another difference between newly found miRNAs and yet known miRNAs is the fact that the sequences of the newly identified miRNA genes are not conserved between human and mice. Ninety-two percent (79/86) and 80% (12/15) of the known mature miRNAs and miRNA*s share homology with mouse miRNAs, respectively (Figure 1B).

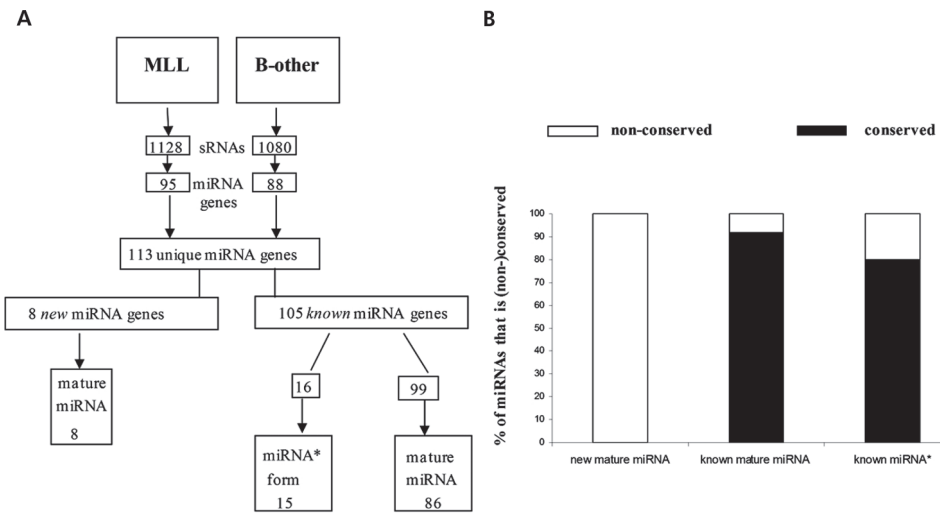

Figure 1. (A) Identification of miRNA genes expressed in leukemic cells of *MLL*-rearranged and other precursor B-ALL patients by direct cloning of small RNAs and subsequent bioinformatic analysis. See Materials and methods for details. **(B) Evolutionary conservation of miRNAs that are expressed in ALL.** Bars represent the percentage of miRNAs that share homology with mouse miRNAs (conserved; black) and of miRNAs without mouse homology (non-conserved; white). Conservation is shown for known mature and known star forms as well as for newly identified mature miRNAs.

Table 1. Characterization of newly cloned miRNAs in ALL patients.

miRNA gene	sequence of mature miRNA	size (nt)	MLL clone count[1]	Prec B-ALL clone count[1]	total clone count[1]	chrom loc[2]	start-end[3]	cons[4]	clust[5]	predicted stem-loop pre-miRNAs
hsa-miR-1972	TCAGGCCAGGCA-CAGTGGCTCA	22	0	1	1	(-) 16p13.11	15011709-15011687	–	–	U CACA GUGUC UAUAGGCAUG GCCAC CCUGGCUUAAAU A AUGUCCGUAC CGGUG GGACCGGACUUA U U ACAC AAAAUU
hsa-miR-1973	ACCGTGCAAAGG-TAGCATA	19	0	2	2	(+) 4q26	117440354-117440373	–	–	CAACG- AU 5′ UAUGUU GCCAUGGU C 3′ AUACGA CG-UGCCA C UGGAAA GU
hsa-miR-1974	TGGTTGTAGTCC-GTGCGAGAATA	23	0	1	1	(-) 5q15	93930950-93930928	–	–	GUU AA UUUU UA UGUUCUUGUA GA UACAACGAUGG UCA U AUAAGAGCGU CU AUGUUGGUGCU GGU C GC- G- ---- UA
hsa-miR-1975	CCCCCACAAC-CGCGCTTGAC-TAGCT	25	0	2	2	(+) 7q36.1	148269563-148269587	–	–	C --- UUAU------ U AGUUGGU CGAGUGU UGUGGG UGUUAAG U UCGAUCA GUUCGCG ACACCC ACAAUUU G - CCA CCCCCUUGUU A
hsa-miR-1976	CCTCCT-GCCCTCCTTGCT-GTAA	22	1	0	1	(+) 1p36.11	26753652-26753672	–	–	A UCCUAA GCAGCAAGGA GGCAGGGG G UGUCGUUCCU CCGUCCUC G C CUGUGU

Continuation of Table 1. Characterization of newly cloned miRNAs in ALL patients.

miRNA gene	sequence of mature miRNA	size (nt)	MLL clone count[1]	Prec B-ALL clone count[1]	total clone count[1]	chrom loc[2]	start-end[3]	cons[4]	clust[5]	predicted stem-loop pre-miRNAs
hsa-miR-1977	GATTAGGGTGCT-TAGCTGTTAA	22	0	1	1	(-) 1p36.33	556126 - 556105	–	–	GGU A AGUGUUU UU UUGAUUAG GCUUAGCUGUUA CUA GUGGG U AAUUAAUU CGAUUCGGGAAU GAU UACCC A --- CUGGU-- UG
hsa-miR-1978	GGTTTG-GTCCTAG-CCT-TTCTA	21	0	1	1	(-) 2q23.1	149355855-149355835	–	–	C CAC CC AUAAA UAGA GGGCU AUCA CC C AUCU UCCGA UGGU GG A U UCC UU AUAA-
hsa-miR-1979	CTCCCACTGCTT-CACTTGACTA	22-24	4	0	4	(-) 4q32.3	166541333-166541312	–	–	A C GC CUU AAA UCUUU CUC CACU UUCA GACUAGCCUUU A GGAAA GAG GUGG AAGU UUGGUUCGGAA A A A GU AGU AGA

[1] clone count within the library constructed with total RNA from the *MLL*-rearranged (1128 clones in total) and B-other ALL patient (1080 clones in total).
[2] chromosomal location of mature miRNA on either (+) or (-) strand .
[3] "start" and "end" coordinates of mature miRNA according to the UCSC genome browser, release March 2006.
[4] mature miRNA shows either homology with mice (conserved +) or not (conserved -).
[5] miRNA genes can be clustered based on their chromosomal location of less than 200 nucleotides apart from each other (+/- : yes/no).

MiRNA expression profiles differ between *MLL*-rearranged and other precursor B-ALL cases

Since the total clone count obtained by summation of clones containing identical DNA sequences is not an accurate quantification method, miRNA expression levels were measured by stem-loop RT-PCR[27]. Initially, the relative expression levels to spiked miR-181c of 8 novel and 86 known miRNAs/miRNA* forms were analyzed in the same samples that were used for direct miRNA cloning. MiRNA levels were compared to the level in $CD34^+$ normal cells (Online Supplementary Tables S2 and S3). Online Supplementary Table S2 shows that despite their low clone frequency within the library (Table 1), the expression of newly identified miRNAs can be detected using the stem-loop RT-PCR technique. For example, hsa-miR-1975 was detected by just a single clone count but this miRNA is 3.5-fold higher expressed in precursor B-ALL compared to $CD34^+$ using quantitative stem-loop RT-PCR.

Out of the total of 94 validated miRNAs, we selected a set of highly differentially regulated miRNA candidates for which quantitative stem-loop RT-PCR primers/probes were available (Online Supplementary Tables S2 and S3). These miRNAs were validated in a larger group of 16 *MLL*-rearranged and 19 other precursor B-ALL patients being negative for *MLL*, *TEL-AML1*, *BCR-ABL*, *E2A-PBX* and hyperdiploidy (B-other). In addition, five miRNAs were included that are located near the 11q23 chromosomal region involved in the *MLL* translocation (i.e. miR-100, miR-34b, miR-181c) or in the function of MLL-1 protein (i.e. miR-142-5p and miR-142-3p)[29]. As shown in Table 2, all 19 miRNAs are significantly differentially expressed in B-other ALL versus normal $CD34^+$ blood cells ($P \leq 0.001$), and 18 out of 19 miRNAs show a significant differential expression in *MLL*-rearranged ALL versus normal $CD34^+$ cells ($P \leq 0.02$). Expressions of 14 out of 19 miRNAs (miR-128a, miR-142, miR-150, miR-181, miR-30e-5p, miR-193, miR-34b, miR-365, miR-582, miR-708) were median 1.4- to 2534-fold *up*regulated in ALL compared with normal $CD34^+$ progenitor cells ($P \leq 0.001$). Most striking was the 2534-fold higher expression of the recently discovered miR-708 in the B-other group as compared with normal $CD34^+$ cells ($P < 0.001$). In contrast, only 5 out of 19 miRNAs (miR-100, miR-125b, miR-99a, miR-196b, miR-let-7e) are *down*regulated in B-other as compared with normal $CD34^+$ cells (median 8- to 233-fold; $P < 0.001$).

Intriguing is the 560-fold difference in expression of miR-196b between *MLL*-rearranged and B-other cases ($P < 0.001$); miR-196b is 233-fold lower expressed in B-other ALL versus normal $CD34^+$ cells ($P < 0.001$) whereas this miRNA is 2.4-fold higher expressed in *MLL*-rearranged patients (Table 2; $P = 0.02$). In addition to miR-196b, seven other miRNAs are differentially expressed between *MLL*-rearranged and other precursor B-ALL cases as shown in Figure 2 ($0.001 \leq P < 0.05$), e.g. miR-708 is 528-fold higher expressed in B-other than in *MLL*-rearranged ALL cases ($P < 0.001$; Figure 2, Table 2).

Only one out of five miRNAs tested for its potential linkage to the *MLL*-chromosomal region (11q23) and/or MLL1 function, i.e. *miR-34b*, showed a ~2-fold downregulation in *MLL*-rearranged ALL as compared with B-other ALL cases ($P = 0.02$; Figure 2, Table 2).

Table 2. Aberrantly expressed miRNAs in leukemic cells of *MLL*-rearranged and B-other ALL patients.

mature miRNA	location[1]	MLL vs CD34 (fold-change)[2]	P-value[3]	B-other ALL vs CD34 (fold-change)[2]	P-value[3]	CNV[4] MLL (%)	CNV[4] B-other (%)
hsa-miR-100	11q24.1	-89.3	< 0.001	-50.0	< 0.001	–	–
hsa-miR-125b	11q24.1; 21q21.1	-75.2	< 0.001	-52.6	< 0.001	–	–
hsa-miR-128a	2q21.3	36.8	< 0.001	33.9	< 0.001	–	–
hsa-miR-142-3p	17q23.2	2.9	< 0.001	4.6	< 0.001	–	–
hsa-miR-142-5p	17q23.2	2.0	< 0.001	3.3	< 0.001	–	–
hsa-miR-150	19q13.33	4.8	< 0.001	14.1	< 0.001	–	–
hsa-miR-151-5p	8q24.3	-1.1	0.52	3.2	< 0.001	–	–
hsa-miR-181a	1q31.3; 9q33.3	17.8	< 0.001	15.4	< 0.001	–	4
hsa-miR-181b	1q31.3; 9q33.3	14.3	< 0.001	13.4	< 0.001	–	4
hsa-miR-181c	19p13.12	7.8	< 0.001	6.4	< 0.001	–	–
hsa-miR-193a	17q11.2	9.7	< 0.001	30.4	< 0.001	–	–
hsa-miR-196b	7p15.2	2.4	0.021	-233	< 0.001	–	4
hsa-miR-30e-5p	1p34.2	4.1	< 0.001	7.1	< 0.001	–	–
hsa-miR-34b	11q23.1	1.4	0.001	2.3	0.001	–	–
hsa-miR-365	16p13.12; 17q11.2	7.5	< 0.001	14.0	< 0.001	–	–
hsa-miR-582	5q12.1	2.2	<0.001	4.6	< 0.001	–	–
hsa-miR-99a	21q21.1	-79.4	< 0.001	-75.2	< 0.001	–	–
hsa-let-7e	19q13.33	-18.2	< 0.001	-8.3	< 0.001	–	–
hsa-miR-708	11q14.1	4.8	< 0.001	2534	< 0.001	–	–

[1] chromosomal location(s) of precursor miRNA according to the UCSC genome browser, release March 2006.
[2] median fold-change in miRNA expression in *MLL*-rearranged ALL (n=16) or other precursor B-ALL (n=19) cases compared to normal CD34$^+$ cells.
[3] Mann-Whitney U-test.
[4] percentage of *MLL* or B-other precursor B-ALL patients with a copy number variation (CNV) resulting in deletion or amplification of specific miRNA precursor.[30]

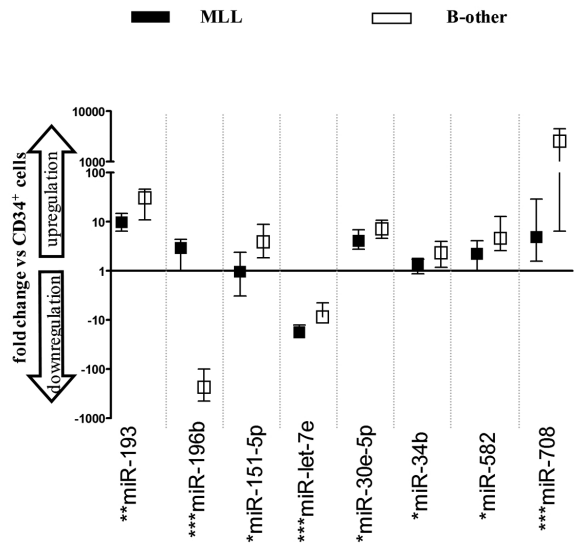

Figure 2. Differential expression of miRNAs in *MLL*-rearranged ALL and non-*MLL* rearranged precursor B-ALL patients.
Box-plots (median and interquartile range) show the distribution of fold-change of expression in 16 *MLL*-translocated and 19 B-other precursor B-ALL cases (negative for known genetic abnormalities) compared with CD34+ samples. *MLL*-rearranged ALL versus B-other: *P<0.05; **P<0.01; ***P<0.001 (MWU).

Except for *miR-196b*, none of the differentially expressed miRNAs are located within regions of copy number variation (CNV) identified by Mullighan et al[30]. However, since this CNV, i.e. a deletion of 7p, occurs in a minority of B-other patients (4%), it is unlikely that this deletion can explain the difference in miR-196b expression between B-other and *MLL*-translocated ALL patients.

Subtype-specific miRNA expression profiles in ALL

Since comparison between *MLL*-rearranged ALL and B-other ALL (negative for all major genetic abnormalities in ALL) revealed striking differences in miR-196b and miR-708 expression, we wondered whether this reflected a difference in maturation status between both subtypes or was indicative for the leukemia subtype itself. In order to address this question, miR-196b and miR-708 expression levels were determined in other ALL subtypes i.e. T-ALL (n=15) and precursor B-ALL patients positive for *TEL-AML1* (n=10), *BCR-ABL* (n=10) or *E2A-PBX* (n=8) or with hyperdiploidy (n=10). MiR-196b was median 500-fold higher expressed in *MLL*-rearranged ALL compared with all other precursor B-ALL cases ($P<0.001$) and differed 350-fold up to 600-fold between *MLL*-rearranged ALL and the different subtypes of precursor B-ALL separately (Figure 3A; $0.001 \leq P < 0.05$). As the differentiation status of *MLL*-positive ALL is less mature than *MLL*-negative precursor B subtypes, we hypothesized that miR-196b

expression levels may reflect the maturation status rather than the genetic subtype. However, analysis of immunoglobulin and T-cell receptor rearrangement patterns as measure for B-cell maturation status[24,31] revealed that 10 out of 16 *MLL*-rearranged samples with a high miR-196b expression have a mature immunogenotype, and, moreover, that one out of four *MLL*-rearranged samples with a low miR-196b expression has an immature immunogenotype. In addition, the high miR-196b expression level is not restricted to the proB immunophenotype as shown in Online Supplementary Table S4 and the expression level of miR-196b seems independent of the specific *MLL* gene rearrangement (Online Supplementary Table S5). Interestingly, 5 out of 15 T-ALL cases also show 800-fold higher miR-196b expression than the expression of miR-196b in non-*MLL*-precursor B-ALL cases (Figure 3A; $P<0.001$). However, only 2 out of these 5 cases have an immature phenotype based on CD1 and surface-membrane bound CD3 expression (Figure 3A) and the remaining cases are intermediate and mature T-ALL cases. Figure 3B shows that miR-708 is 250- to 6500-fold higher expressed in *TEL-AML1*, *BCR-ABL*, *E2A-PBX1*, hyperdiploid and B-other cases compared with *MLL*-rearranged and T-ALL patients ($P\leq0.01$). MiR-708 is also differentially expressed among different non-*MLL* rearranged cases. E.g. *E2A-PBX1*+ and *TEL-AML1*+ patients show an 8-to 9-fold higher miR-708 expression than *BCR-ABL*-translocated patients ($P<0.01$). Similar to miR-196b, the expression level of miR-708 does not reflect the differentiation status of *MLL*-rearranged and T-ALL cases (Online Supplementary Table S6). Taken together, our present data do not suggest that high expression of miR-196b or low expression of miR-708 is indicative for a less-differentiated (immature) lymphoid cell type.

MicroRNA genes belonging to the same cluster have a similar expression pattern

MiRNA genes such as *miR-181a* and *miR-181b* belong to the same cluster based on their close location of less than 200 nucleotides apart from each other on chromosome 9q33.3 (181a-cluster). Likewise, *miR-181c* and *miR-181d* are located next to each other on 19p13 (181c-cluster). These grouped genes are thought to be derived from a common ancestor during evolution and are generally co-transcribed[32,33]. In correspondence with this finding, miR-181a and miR-181b as well as miR-181c and miR-181d show a similar relative expression level compared to $CD34^+$ cells in *MLL*-rearranged, *TEL-AML*$^+$, *BCR-ABL*$^+$, *E2A-PBX*$^+$, hyperdiploid, B-other and T-ALL patients (Figure 4 and Online Supplementary Table S6, $P<0.002$). Another cluster of genes (miR-99a-cluster on 21q21.1) is represented here by miR-*125b-2* and *miR-99a*. These miRNAs are both lower expressed in *MLL*-translocated ALL and B-other ALL than in normal $CD34^+$ cells ($P<0.001$, Online Supplementary Table S7).

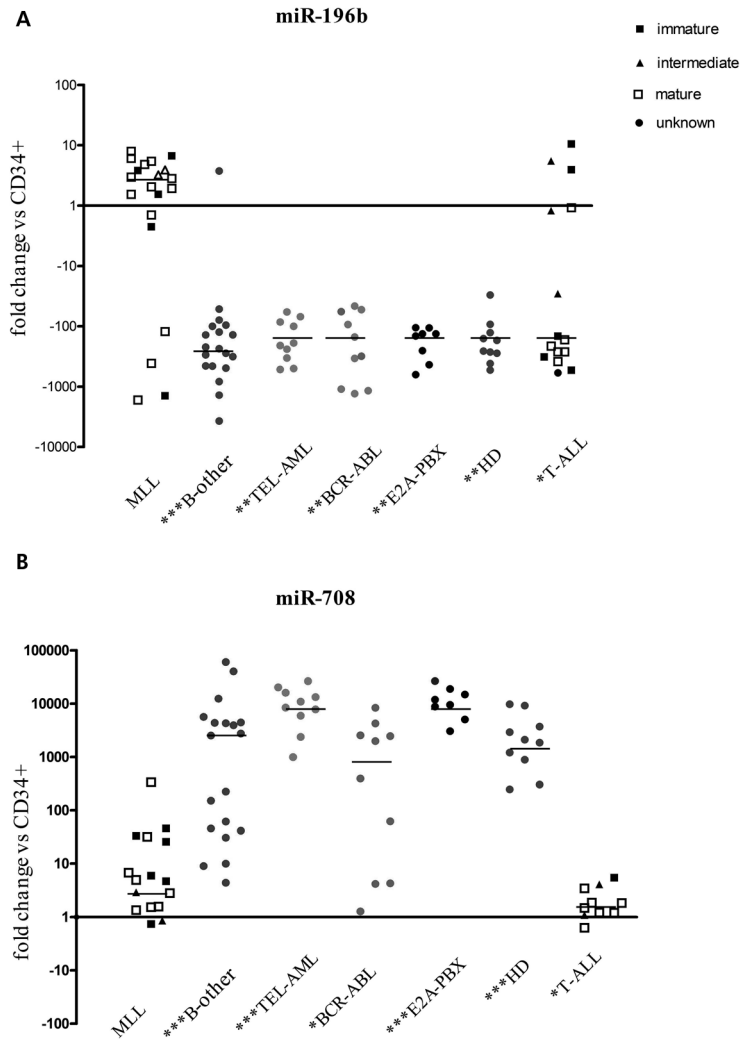

Figure 3. Subtype-specific miRNA expression profiles in pediatric ALL.
The expression levels of miR-196b (**A**) and miR-708 (**B**) were measured in leukemic cells of 92 patients, reflecting different subtypes of pediatric ALL as indicated at the X-axis. B-other cases are negative for *MLL*, *TEL/AML1*, *BCR-ABL*, *E2A-PBX* and hyperdiploidy. The fold-change of expression in individual leukemic samples compared with $CD34^+$ samples is shown. In addition, the maturation status of samples is indicated by a black square (immature), black triangle (intermediate) and open square (mature) as defined by Ig/TCR and CD marker expression (see Materials and methods). Samples without information about the Ig/TCR status are indicated by dots. Horizontal lines indicate the median fold-change. *MLL*-rearranged ALL versus indicated ALL subtypes: *P<0.05; **P<0.01; ***P<0.001 (MWU).

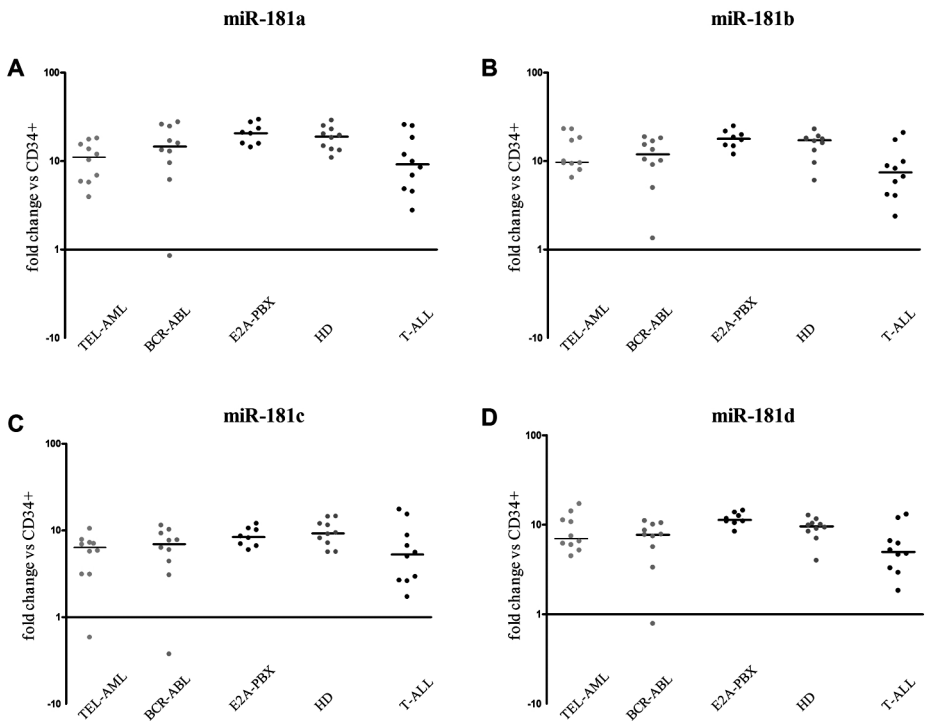

Figure 4. Clustered miRNA genes have a similar expression pattern in ALL subtypes.
The expression level of miR-181a (**A**), miR-181b (**B**), miR-181c (**C**) and miR-181d (**D**) was measured in leukemic cells of T-ALL (n=10) and precursor B-ALL patients positive for *TEL/AML1* (n=10), *BCR-ABL* (n=10), *E2A-PBX* (n=8) or hyperdiploidy (HD, n=10). Dots point to the fold change of expression in leukemic samples compared with normal $CD34^+$ samples. MWU-test determined significant expression in all ALL subtypes vs normal $CD34^+$ cells with $P<0.001$ for all miR-181 members. The expression levels did not significantly differ between subtypes (e.g. *MLL*-rearranged vs *TEL/AML1*-precursor B-cases, $P>0.05$).

DISCUSSION

By direct cloning of miRNAs expressed in leukemic cells of *MLL*-rearranged and B-other (precursor B-ALL phenotype without the known genetic abnormalities *MLL, TEL-AML1*, *BCR-ABL*, *E2A-PBX* or hyperdiploidy) subtypes, we identified 105 known and 8 new human miRNA genes. These miRNA genes encode 101 known and 8 new mature miRNAs and miRNA* forms. The newly cloned miRNAs are not conserved between human and mice and are less frequently cloned from ALL samples than yet known mature miRNAs. Recently Landgraf et al. postulated that predicted hairpin precursors with low clone counts and evolutionary less-conserved sequences are not cell-type specific and may originate from dsRNA[34]. However, using mature miRNA-specific stem-loop RT-PCR, the expression of these new and less-

conserved miRNAs was demonstrated in ALL patients, sometimes even at relative high levels; e.g. hsa-miR-1975 is 3.5-fold higher expressed in precursor B-ALL than in normal CD34+ cells (Online Supplementary Table S2). We also identified a low clone count miRNA that, by the time of submission of this paper, was indicated as miR-708[34,35]. Quantitative PCR revealed that miR-708 was differentially expressed in ALL subtypes (varying between 5- and 2500-fold compared with normal CD34+ cells, Table 2). This exemplifies that even low count miRNAs are of clinical interest. In addition, our data suggest that expression analysis of miRNA genes that are presently known in the miRBase database, results in an underestimation of miRNAs that may be important for leukemia and/or informative for genetic abnormalities underlying different ALL subtypes.

A set of 19 miRNAs was further validated in an extended group of patients using the above-mentioned stem-loop RT-PCR technique. This technique was chosen because current probe-based array techniques are hampered by the fact that only known miRNAs can be tested. The expression level of 18 miRNAs in *MLL*-rearranged ALL and all 19 miRNAs in B-other ALL differed from the expression level in normal CD34+ progenitor cells. In contrast with previous reports showing mainly downregulation of miRNAs in cancer including leukemia[19,36], we found both upregulated (14/19) and downregulated (5/19) expression of miRNAs in ALL compared with normal CD34+ cells. Altered miRNA expression levels may lead to an inappropriate expression of target-oncoproteins or target-tumor suppressors, thereby facilitating the development of leukemia. A typical example is found in CLL where a deletion of the *miR-15a/16-1* cluster abolishes the inhibition of the anti-apoptotic Bcl-2 target oncogene and, as a consequence, facilitates proliferation of these cells[37]. Overexpression of miR-21 in adult AML may be linked to reduced expression of tumor suppressor genes such as *PTEN*, a gene that is known to play a role in leukemogenesis[20,38].

The expression of miRNAs may be regulated by epigenetic modifications such as promoter hypermethylation. Interestingly, the leukemic fusion gene *AML1/ETO* has been reported to promote the hypermethylation (and hence reduced expression levels) of miR-223 in t(8;21)-positive AML[39,40]. We also identified expression differences between different genetic subtypes of precursor B-ALL that are characterized by leukemia-specific fusion genes such as MLL fusion genes in *MLL*/11q23-rearranged ALL, *TEL/AML1* in t(12;21)-positive ALL and *BCR/ABL* in t(9;22) positive ALL (see Table 2; Figure 3). Since miRNAs are known to be differentially regulated during development[41-44], subtype-specific expression of miRNAs like miR-196b (high in *MLL*-rearranged ALL) and miR-708 (high in other precursor B-subtypes) might reflect a difference in differentiation status of leukemic cells; e.g. *MLL*-rearranged ALL is linked to a more immature proB type compared to a more differentiated common/preB subtype often found in non-*MLL* precursor B-ALL subtypes[24,45]. However, no linkage between miR-196b and miR-708 expression and the maturation status of *MLL*-rearranged precursor B-ALL and T-ALL cases was detected in the present study. This observation suggests that the expression

level of both miRNAs reflects the differences between leukemic subtypes and is less likely to be associated with the differentiation status.

Recently the *MLL* gene-encoded MLL1 methyltransferase protein was found to extensively bind the *miR-142* gene on 17q23.2 resulting in dysregulation of *miR-142* expression[29]. However, miR-142 was not found to be differentially expressed between *MLL*-translocated ALL cases compared to other precursor B-ALL cases in the present study. In addition, no aberrant expression levels were found for other miR genes located in the proximity of the *MLL* gene, including miR-100 and miR-34b. In contrast, a striking difference in miR-196b expression of 500-fold was found between *MLL*-rearranged ALL and different non-*MLL* precursor B-ALL subtypes as well as a 800-fold difference between a subset of T-ALL cases and non-*MLL* precursor B-ALL cases. The *miR-196b* gene is located in the *HOXA* cluster at chromosome 7p15: an area reported to be affected by CNV in ≤5% of non-*MLL* precursor B-ALL cases and 0% of *MLL*-rearranged cases[30] (Table 2). Recently, it was suggested that transcriptional activation of this cluster is caused by MLL1 binding and subsequent H3-K4 trimethylation of associated histones[29]. Since *miR-196b* is mapped between *HOXA9* and *HOXA10*, the transcriptional activation of *HOXA* genes by MLL1 might also affect the expression of miR-196b. Indeed, we observed that miR-196b expression correlated with the expression of *HOXA9* and *HOXA10* in *MLL*-rearranged cases (data not shown). All together, these findings point to a possible co-regulation of the *HOXA* cluster and *miR-196b* in *MLL*- translocated ALL. Similar to miRNA-mRNA co-transcriptional regulation, also the transcription of miRNA genes can be co-regulated such as described for the miR-17-92 polycistron[21]. In the present study we demonstrated that also the family members of the 181a and 181c-clusters are co-expressed at similar levels in pediatric ALL.

Co-regulation of protein-encoding and/or miR-encoding genes may have important regulatory consequences in cell physiology by generating feedback-loops that avoid uncontrolled expression of protein-encoding genes (e.g. miR-17-92 cluster and E2F1-c-Myc loop)[46]. Interestingly, the miR-196 family may be involved in regulation of evolutionary conserved homeobox (*HOX*) genes, which are powerful regulators of (lymphoid) development. MiR-196a, which differs only in one nucleotide from miR-196b, has been shown to target the translation of *HOXB8* and *HOXC8*[47], the latter also predicted as potential target of miR-196b by three computer algorithms that base their prediction on sequence homology, i.e. miRanda, PicTar and Targetscan (Online Supplementary Table S8)[48-50]. Aberrant miR-196 expression may contribute to leukemogenesis since dysregulated *HOX* genes were shown to directly induce leukemia in mice[51]. According to the algorithm miRanda, miR-708 also might regulate two target candidate genes linked to leukemia (Online Supplementary Table S9)[48]. The first one, IKAROS family zinc finger 4 (IKZF4) associates with its family member IKAROS, a regulator of lymphocyte commitment and differentiation, which contributes to leukemia if dysregulated[52-54]. The second one, the Feline sarcoma (Fes) oncogene, is involved in cell survival and, interestingly, is found to be translocated in acute promyleocytic leukemia[55,56].

All together, both miR-708 and miR-196b might inhibit the translation of proteins associated with normal survival and development of lymphocytes. Dysregulation of these proteins by aberrant expression of miR-196b and/or miR-708 might therefore contribute to leukemogenesis. However, additional biological studies have to reveal whether these miRNAs effectively contribute to leukemogenesis or whether the observed upregulation in case of miR-196b is just a bystander-effect of the activated *HOXA* cluster in *MLL*-rearranged cases.

In summary, this study revealed novel miRNAs that are, in contrast to presently known miRNAs, not evolutionary conserved. We demonstrate that the expression of both known and newly identified miRNA genes varies in genetically and prognostically different subtypes of pediatric ALL and normal $CD34^+$ progenitor cells. At present, out data suggest that the differential expression of miRNAs such as miR-196b and miR-708, is more associated with the leukemic subtype than with the maturation status of cells. This phenomenon warrants further functional studies of the role of miRNAs in leukemia.

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Chapter 4

Discovery of new microRNAs by small RNAome deep sequencing in childhood acute lymphoblastic leukemia

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ABSTRACT

MicroRNAs (miRNAs) relevant to acute lymphoblastic leukemia (ALL) in children are hypothesized to be largely unknown since most miRNAs have been identified in non-leukemic tissues. In order to discover these miRNAs we applied high-throughput sequencing to pooled fractions of leukemic cells obtained from 89 pediatric cases covering seven well-defined genetic types of ALL and normal hematopoietic cells. This resulted into 78 million small RNA reads representing 554 known, 28 novel and 431 candidate novel miR-genes. In all, 153 known, 16 novel and 170 candidate novel mature miRNAs and miRNA-star strands were only expressed in ALL, whereas 140 known, 2 novel and 82 candidate novel mature miRNAs and miRNA-star strands were unique to normal hematopoietic cells. Stem-loop reverse transcriptase (RT)-quantitative PCR analyses confirmed the differential expression of selected mature miRNAs in ALL types and normal cells. Expression of 14 new miRNAs inversely correlated with expression of predicted target genes (-0.49≤Spearman's correlation coefficient (Rs)≤-0.27, P≤0.05); among others low levels of novel sol-miR-23 associated with high levels of its predicted (anti-apoptotic) target *BCL2* (*B-cell lymphoma 2*) in precursor B-ALL (Rs -0.36, P=0.007). The identification of more than 1000 miR-genes expressed in different types of ALL forms a comprehensive repository for further functional studies that address the role of miRNAs in the biology of ALL.

INTRODUCTION

In the early nineties, microRNAs (miRNAs) of ~21-nucleotide length were discovered in the nematode *Caenorhabditis elegans*[1,2]. Since miRNAs were initially regarded to be specific for the worm, it took geneticists till the next decade to recognize that this novel gene-regulatory mechanism was also part of humans[1]. The importance of this highly conserved, non-protein coding class of small RNA became evident upon studies showing that they regulate the activity of many protein-coding genes[3]. These miRNA-targeted genes include tumor suppressors and oncogenes which are regulated by basepairing of the mature miRNA with the complementary mRNA. This results in mRNA cleavage, translational repression or deadenylation[3,4].

MiRNAs are expressed in a tissue-specific fashion[5]. Hematopoietic cells display other miRNAs than other tissues. E.g. miR-142 is mainly expressed in hematopoietic cells[6], whereas miR-192, miR-194 and miR-215 are abundantly present in the gut[5] and miR-372 is highly characteristic for testis[7,8]. In leukemia, both lineage (e.g. myeloid and lymphoid) and genetic type specific (e.g. t(8;21), *MLL*-rearranged and *TEL-AML1*-positive) miRNA signatures have been found by us and others[5,9-13].

As miRNAs have tumor suppressor and oncogenic capacity, the discovery of leukemia-related miRNAs may give insight into the biology of disease. Detection of differences in expression levels of miRNAs is limited by the knowledge of previously identified miRNAs, often discovered in non-leukemic tissues. This type of expression analyses may miss miRNAs being relevant for leukemia. Today over 900 miRNAs[14] have been discovered – mostly by cloning followed by conventional sequencing and computational prediction[15]. High-throughput or deep sequencing of small RNA fractions may result in the discovery of many more miRNAs, since this technique has increased sequencing depth over the conventional method and current estimates predict as many as 1000 up to 25 000 miRNAs present in humans[16-18].

In this study we applied Solexa high-throughput sequencing[19] on small RNA fractions isolated out of 70 cases covering seven different types of pediatric ALL and 19 cases covering three types of normal hematopoietic cells. This technique yielded approximately 8 million small RNA reads in each leukemia type and control group. Bioinformatic analysis revealed that these reads correspond to 554 known, 28 novel and 431 candidate novel miR-genes that have not been previously published in miRBase[14]. A selection of 22 novel and candidate novel miRNAs were validated by stem-loop reverse transcriptase (RT) real-time quantitative PCR (stem-loop RT-qPCR). Seventeen of these miRNAs were differentially expressed between genetic types of ALL and normal cells. Moreover, expression levels of new miRNAs inversely correlated to mRNA expression levels of predicted target genes. This study provides expression signatures of known, novel and candidate novel miRNAs per subtype of leukemia which can serve as repository for further functional studies in the type of cells they were discovered.

MATERIALS AND METHODS

Patient samples

Bone marrow and peripheral blood was collected from children with newly diagnosed ALL. Mononuclear cells of these samples were isolated and enriched as previously described[20,21]. All leukemia samples contained ≥90% leukemic cells as determined by May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. Flow cytometry was used to determine the immunophenotype (precursor B-ALL or T-ALL). Fluorescence *in situ* hybridization (FISH) and RT-PCR techniques allowed the screening of the genetic type, and conventional karyotyping was used to analyze the ploidy status of ALL cases. For each ALL type, 10 patients were included, i.e. *MLL*-rearranged, *TEL-AML1*-positive, *BCR-ABL*-positive, *E2A-PBX1*-positive, hyperdiploid (>50 chromosomes), B-other (negative for the 5 mentioned genetic aberrations) and T-ALL. To study miRNAs in normal hematopoietic counterparts, sorted fractions representing different stages of B-cell and T-cell differentiation are preferred. However, these subpopulations are rare and require large amounts of starting material. Alternatively, sucrose-gradient processed normal bone marrow (nBM, n=10 children), CD34-positive cells (>90% purity) sorted from granulocyte colony-stimulating factor (G-CSF)-stimulated blood samples of children suffering from a solid tumor without bone marrow involvement (n=4) and thymocytes extracted from thymic lobes resected from children during surgery for their congenital heart disease (n=6) were included[11,22]. All samples were collected after approval of the institutional review board and informed consent from parents or legal guardians.

High-throughput sequencing of miRNAs

Total RNA was extracted with TRIzol reagent (Invitrogen, Leek, NL) according to the manufacturer's protocol. The quality of RNA samples was measured on the 2100 Bioanalyzer (Agilent, Amstelveen, NL). Only RNA samples with RNA Integrity Number ≥7.5 were used for further processing. Each ALL subtype-representing library was constructed using pooled RNA extracted from 10 individual patients. For each of the normal hematopoietic tissues, RNA of 3 (CD34+-sorted cells), 10 (normal bone marrow) and 6 (thymocytes) fractions was pooled. A total of 10 microgram of each RNA library was size-fractioned on a 15% Tris-borate-EDTA gel. Small RNAs of 18-30 nucleotides were excised out of gel and each fraction was amplified by RT-PCR using the Small RNA Sample Prep Kit (Illumina, San Diego, USA) according to manufacturer's instructions. DNA concentration and size was checked on a 2100 Bioanalyzer. Next, two to three pM of small DNA was loaded onto a flow cell and these fractions were sequenced using the Illumina genome analyzer GAII based on Solexa sequencing technology. Raw sequences of small RNAs were computational analyzed to identify miRNAs as described below.

Computational analysis of small RNA sequences

Following trimming of adaptor sequences, small RNA sequence reads were mapped to the genome of human and other species. Sequences were aligned to Ensembl (release 56/GRCh37 assembly), UCSC (GRCh37/hg 19) and miRBase version 14. If the small RNA sequence read appeared to be non-protein coding, flanking sequences of 100 nucleotides on either side of the small RNA were extracted for further computational analysis. The potential miRNA precursors were then computationally folded into hairpin structures and tested for a set of features derived from known miR-genes in order to identify putative novel miRNAs (Table 1 and Figure 1). The features used to identify miRNAs were based on experience in the identification of miRNAs by the previous work of Berezikov et al and Hannon et al[23-25]. Details can also be found on the website www.interna-genomics.com[23-25]. We have normalized the read frequencies of miRNAs by dividing the number of absolute read sequences (numerator) by the sum of total miRNA sequence reads (denominator) per subtype. The total sum of sequence reads represents the reads of known miRNAs, novel miRNAs and candidate novel miRNAs. Raw data sets can be found at the Short Read Archive (SRA) of the NCBI website (www.ncbi.nlm.nih.gov/sra, accession number SRP005294). Novel and candidate novel miRNA reads are designated as sol-miR (of Solexa) followed by a sequential number throughout this paper. For new sol-miRs that have been approved by miRBase, the official miRBase identification number has been added to Tables 2 and 3 and Online Supplementary Tables S2 and S5. For known miRNAs, the official miRBase identification number has been used.

Expression analysis of novel miRNAs and mRNA transcripts

The expression of miRNAs was validated by stem-loop RT-qPCR as described elsewhere[11]. Specific stem-loop RT-PCR primer and probe combinations were designed for 22 novel and candidate novel miRNAs by Applied Biosystems, USA[26]. Expression levels were validated in 7 *MLL*-rearranged patients and 8 cases of each of the following leukemia types: *TEL-AML1*-positive, *BCR-ABL*-positive, *E2A-PBX1*-positive, hyperdiploid, B-other and T-ALL cases. Expression levels in normal hematopoietic cells were determined in 6 normal bone marrow, 4 CD34+-sorted and 6 thymocyte fractions. Each RT-qPCR reaction was performed in duplicate with 5 ng of RNA as input[11]. Endogenous small nucleolar RNA 1 (RNU24) was used as reference for the RNA input as the expression of this reference showed limited variation among different types of ALL[12]. All RT-qPCR reactions were performed according to manufacturer's instructions on an Applied Biosystems 7900HT system.

Previously published datasets of pediatric ALL cases (using Affymetrix U133 plus 2.0 GeneChips, Santa Clara, CA, USA) were used to determine the transcript levels of predicted target genes . This dataset has been deposited at the NCBI's GEO[27] and is accessible through GEO series accession number GSE 13351. Data were extracted and normalized as described before[28].

Table 1. Features of the identified sequence used to determine the confidence level of novel miR-genes

A	Good features for precursor hairpin	Bad features for precursor hairpin
	Dicer/Drosha overhang 1-3 nt	Dicer/Drosha overhang <1 or >3 nt
	minimal 10 reads in ≥1 library	high variability in sequence of 5′ region of mature miRNA**
	folding-energy of permutated precursor with P≤0.01 *	distance from miRNA to stem base <5 nt
	–	encoded on >10 loci in the human genome
	–	length of mature miRNA <21 or >22 nt
	–	overlap with other small RNA (e.g. tRNA or rRNA) of non-human species
	–	miRNA sequence forms part of loop sequence
	–	>90% GC bonds within mature miRNA

B		
Confidence level	**Good feature for precursor hairpin**	**Bad feature for precursor hairpin**
novel	≥2	0
candidate novel	1	0
candidate novel	≥2	1
other	≤1	≥1

Abbreviations: miRNA, microRNA; rRNA, ribosomal RNA; tRNA, transfer RNA. Confidence levels for new miR-genes were defined as follows: **novel**: precursor hairpin with no bad features (bad=0) AND at least 2 good features (good feature ≥2); **candidate novel**: bad=0 AND good=1 OR bad=1 AND good ≥2; **other**: other miRNAs not belonging to the novel or candidate novel class. * In each of 1000 runs the precursor sequence was permutated and energy needed for folding into a hairpin was calculated. *P*-value was based on the number of permutated precursor hairpins with a folding-energy equal or smaller than that of the original precursor hairpin out of 1000 iterations. **Definition of high variability: the top read accounts for less than 30% of total sequences representing a unique miRNA. The top read sequence is defined as the sequence variant for a unique miRNA with the highest read frequency.

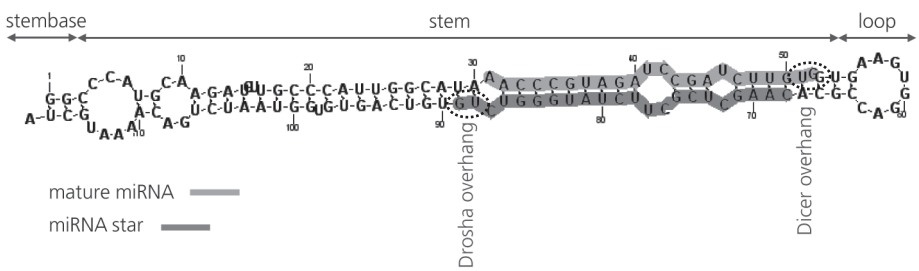

Figure 1. Parameters used to identify novel miRNAs.
Small cloned RNA sequence reads are mapped to the human genome and the putative precursor is extracted by taking the miRNA sequence and the 100 nucleotides flanking sequence on either side of the miRNA. The precursor sequence is then computational folded into a hairpin structure, from which features are deduced as mentioned in Table 1 to identify the likelihood for a novel miRNA.

Statistics

Sequence reads were normalized by dividing the absolute read number per miRNA by the total number of miRNA (novel, candidate novel and known) reads per library to obtain a read frequency (%). Read frequencies and levels of miRNA expression were compared between types of ALL patients using the Mann-Whitney U (MWU)-test. *P*-values were considered significant at $P \leq 0.05$ level (2-tailed).

RESULTS

MiRNAs identified by high-throughput sequencing in ALL and normal hematopoietic cells

High-throughput sequencing was performed on small RNA fractions representing seven types of pediatric ALL (T-ALL, *TEL-AML1*-positive, *MLL*-rearranged, *BCR-ABL*-positive, *E2A-PBX1*-positive, hyperdiploid and other precursor B-ALL patients negative for the major cytogenetic aberrations), and three normal tissues i.e. normal bone marrow, CD34-positive cells and thymocytes. The sequencing of these ten small RNA libraries yielded a total number of 78 million sequence reads which entered a computational pipeline. This pipeline was used to distinguish miRNA sequences from other small RNAs. The criteria used to identify miRNA sequences and predict precursor hairpin structures, were based on previous work by Berezikov et al and Hannon et al[23-25]. In short, sequences were mapped to the human genome available in Ensembl (release 56/GRCh37 assembly) and UCSC (GRCh37/hg19). Sequences that did not map to protein-coding mRNA or to known small RNAs (including transfer RNA, ribosomal RNA, small nuclear and small nucleolar RNA) were further explored. Flanking sequences of 100 nt on both sides of a potential miRNA were retrieved from Ensembl (release 56/GRCh37 assembly). The small RNA and its flanking sequences were computationally folded into a hairpin structure, which was tested for a set of features that was previously used to define yet published and mainly evolutionary conserved miR-genes (Table 1). The number of assigned good and bad features determined the likelihood (confidence level) of the small sequence to represent a true miR-gene. The highest likelihood that a small RNA sequence represented a novel miRNA was obtained if the predicted precursor had at least two out of three good features and lacked bad features: these are depicted as novel miRNAs and fulfilled highly stringent criteria covering among others the length of mature miRNA, a minimal number of reads in at least one library, and predicted Drosha and Dicer cut sites (Table 1). Second in line are those predicted precursor sequences with one bad and at least two good features, as well as those without bad features but with one good feature: depicted as candidate novel miRNAs. All other hairpins may include potential (novel) miRNAs but do not fit these stringent criteria and are therefore called miRNA-other (Table 1).

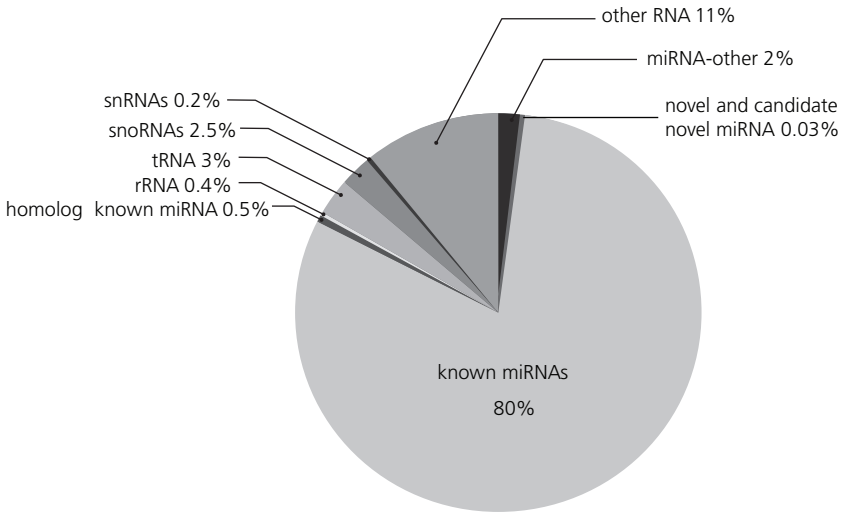

Figure 2. Composition of the small RNAome in leukemic cells of pediatric ALL patients.
Frequency of non-coding small RNAs identified by high-throughput sequencing of ALL patient samples is shown. Frequencies of novel and candidate novel miRNAs are taken together. Abbreviations: ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear (snRNAs), small nucleolar RNAs (snoRNAs). 'Homolog to known' miRNAs refers to sequences that map to novel genomic loci of yet known miRNAs. MiRNA-other category reflects new miRNAs with lower confidence level than those in category 'novel' and 'candidate novel'. Other RNA category represents all other small RNAs not belonging to the categories mentioned above. See Table 1 for details of used features to determine the confidence level of predicted miRNAs.

Eighty percent of the sequence reads were identified as known miRNAs which indicates that the pre-fractioning of 18-30 nucleotide RNAs by gel-electrophoresis is suitable to extract miRNAs from total RNA (Figure 2). 14 866 reads (0.03%) represented novel and candidate novel miRNAs. Some reads (0.5%) were mapped to new locations on the genome while they encoded known miRNAs or their antisense transcript (complementary to the mature miRNA but encoded on the other DNA strand and therefore ruled out as star strand). These reads were categorized as homolog to known miR-genes. Two percent of the sequences was assigned as miRNA-other structures (lowest confidence level). Other non-coding RNAs including transfer RNAs, ribosomal RNAs, small nuclear RNAs and small nucleolar RNAs represented 17% of all sequences.

In each ALL type, 5 to 10 million small RNA sequence reads were analyzed. Figure 3 summarizes the number of reads corresponding to known and newly identified miRNA sequences in different ALL types, e.g. 9 246 296 out of 10 315 285 of small RNA reads in *BCR-ABL*-positive ALL patients were representing known miRNA sequences whereas 194 and 2008 reads represented novel and candidate novel miRNAs, respectively. Strikingly, *TEL-AML1*-positive ALL patients displayed ~10-fold more reads of novel miRNAs than other

types of ALL. In total, high throughput sequencing revealed 895 unique miRNA sequences representing 470 known, 28 novel and 397 candidate novel mature miRNAs. Moreover, 372 known, 12 novel and 39 candidate novel miRNA-star forms (miRNA*) were detected (Figure 3). Both novel and candidate novel sequences have not yet been reported in miRBase version 14[14]. These mature miRNAs were encoded by 554 known (Online Supplementary Table S1), 28 novel (Online Supplementary Table S2) and 431 candidate novel miR-genes (Online Supplementary Table S3), respectively.

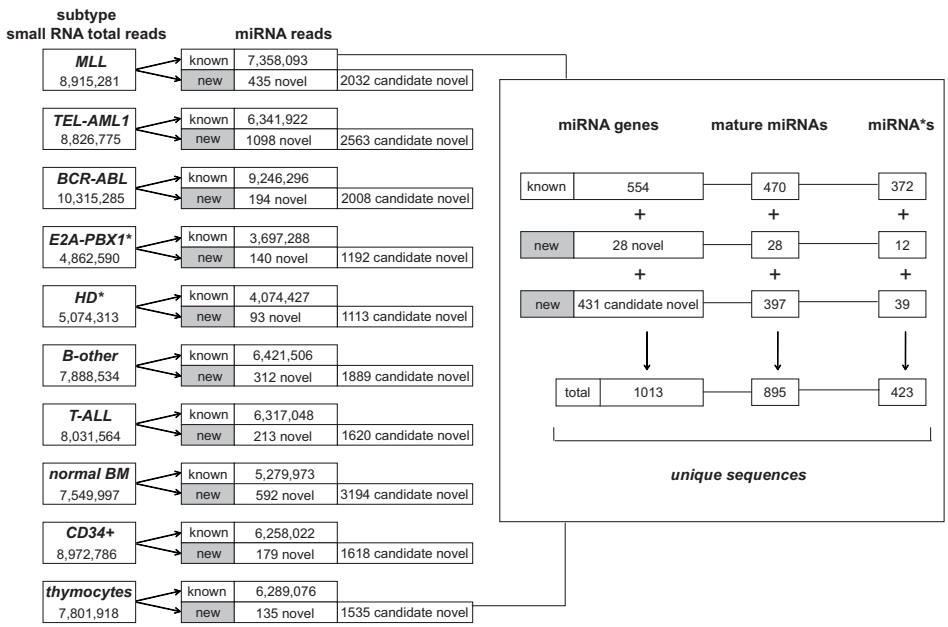

Figure 3. Overview of known and novel miRNAs in ALL types and normal hematopoietic cells. At the left, the total numbers of small RNA sequence reads are listed. At the right, the numbers of unique miR-genes encoding mature miRNAs and miRNA-star forms (miRNA*s) are depicted. New miRNAs in both panels represent novel and candidate novel sequences (see Materials and methods section for explanation of used features). * 2 pM was loaded for *E2A-PBX1* and HD (hyperdiploid) whereas for other libraries 3 pM was used.

The read frequency of the 28 novel mature miRNAs and 12 novel miRNA-star forms in ALL and normal hematopoietic cells is illustrated by Table 2 and Online Supplementary Table S2. The novel mature sol-miR-35 was detected in *TEL-AML1*-positive but not in other ALL types nor in normal hematopoietic cells, and therefore may be unique for this type of ALL (Table 2). Most miRNAs, however, are not restricted to one specific type of ALL (Table 2). In all, 4 out of 28 (14%) novel mature miRNAs and 6 out of 12 (50%) miRNA-star forms were detected in

Table 2. Read frequency of 28 mature miRNAs and 12 miRNA-star forms (encoded by 28 novel miR-genes) identified in different types of ALL and normal hematopoietic cells.

novel miRNA	annotation by miRBase	RT-qPCR	*MLL* (% x 10-4)	*TEL-AML1* (% x 10^{-4})	*BCR-ABL* (% x 10^{-4})	*E2A-PBX1* (% x 10^{-4})	Hyperdiploid (% x 10^{-4})	B-other (% x 10^{-4})	T-ALL (% x 10^{-4})	CD34+ (% x 10^{-4})	nBM (% x 10^{-4})	Thymocytes (% x 10^{-4})
novel sol-miR-5	hsa-mir-3154	**	26.01	6.18	2.58	6.19	6.36	6.26	1.19	10.31	7.48	0.16
novel sol-miR-6	hsa-mir-3150b	**	2.30	85.51	3.66	8.34	0.73	7.72	2.69	1.89	55.56	1.27
novel sol-miR-6*	hsa-mir-3150b*	–	0	0.63	0.11	0.81	0.49	0	0	0	0	0
novel sol-miR-11	hsa-mir-3136	**	3.65	9.22	2.05	5.38	0.98	4.94	5.69	1.26	6.73	2.69
novel sol-miR-11*	hsa-mir-3136*	–	0	0.16	0	0	0	0.15	0.16	0	0	0
novel sol-miR-13	hsa-mir-3117	–	0	39.55	0	0	0	0.62	0.47	0	0.19	2.85
novel sol-miR-13*	hsa-mir-3117*	–	0	0.63	0	0	0	0	0	0	0	0
novel sol-miR-14	hsa-mir-5187	–	2.97	2.50	1.51	2.15	0.98	2.01	1.58	5.04	13.09	1.11
novel sol-miR-14*	hsa-mir-5187*	–	0	0	0	0	0	0	0.16	0	0	0.32
novel sol-miR-15	hsa-mir-3151	**	9.86	1.41	4.63	0	1.96	1.08	0.95	6.92	0.75	0
novel sol-miR-18	hsa-mir-3190	**	2.97	1.72	1.29	1.08	1.71	2.47	0.95	0.63	6.36	0.16
novel sol-miR-18*	hsa-mir-3190*	–	0	0	0	0	0	0	0	0	0.19	0
novel sol-miR-23	hsa-mir-4474	**	0.14	0.16	0.00	0	0	1.39	1.74	2.20	2.43	3.01
novel sol-miR-24	hsa-mir-3177	**	0.54	2.50	0.32	0.27	0.24	1.85	0.63	0.16	3.18	1.27
novel sol-miR-24*	hsa-mir-3177*	–	0	0	0.00	0	0	0.15	0	0	0	0
novel sol-miR-27	hsa-mir-3942	**	0.68	0.63	0.22	0	0.24	0.77	2.06	0.79	1.50	2.53
novel sol-miR-27*	hsa-mir-3942*	–	0.14	0	0	0.27	0	0.31	0.32	0	0	0
novel sol-miR-35	hsa-mir-5186	**	0	2.50	0	0	0	0.00	0	0	0	0
novel sol-miR-36	hsa-mir-5188	–	0	0	0	0	0	0.93	0.16	0	0.56	0
novel sol-miR-37	hsa-mir-5006	–	0	0.16	0	0	0	0.15	1.11	0.63	1.87	0.79
novel sol-miR-38	hsa-mir-5189	–	0	0	0.11	0	0	0.31	0.47	0.47	2.62	0.16

Continuation of Table 2. Read frequency of 28 mature miRNAs and 12 miRNA-star forms (encoded by 28 novel miR-genes) identified in different types of ALL and normal hematopoietic cells

novel miRNA	annotation by miRBase	RT-qPCR	*MLL* (% x 10-4)	*TEL-AML1* (% x 10^{-4})	*BCR-ABL* (% x 10^{-4})	*E2A-PBX1* (% x 10^{-4})	Hyperdiploid (% x 10^{-4})	B-other (% x 10^{-4})	T-ALL (% x 10^{-4})	CD34+ (% x 10^{-4})	nBM (% x 10^{-4})	thymocytes (% x 10^{-4})
novel sol-miR-39	hsa-mir-3183	–	0.41	0.31	0.11	0.54	0	0.31	0	0.47	1 .50	0.32
novel sol-miR-40	hsa-mir-5190	–	0.14	0.57	0.29	0.27	0	1.39	1.26	0	0	1.27
novel sol-miR-41	hsa-mir-5191	–	0	0.16	1.08	1.08	0.86	1.54	0.32	0	0.19	0
novel sol-miR-42	hsa-mir-5192	–	0.41	0.00	0.43	0.27	0	0.77	0.32	0	0	0
novel sol-miR-43	hsa-mir-4774	–	0.81	1.25	0.22	0.81	0	1.08	0.47	0.47	0.94	0.47
novel sol-miR-44	hsa-mir-5193	–	0	0.86	0	0	0	0.31	0.16	0.16	0.19	0
novel sol-miR-45	hsa-mir-4637	–	0.41	0.16	0	0.54	0.49	0.62	0.47	0	0.37	0.47
novel sol-miR-46	hsa-mir-3936	–	0	0.16	0.65	0.27	0.24	0.46	0.32	0.47	0.37	0.32
novel sol-miR-47	hsa-mir-5194	–	1.22	0.78	0.11	0	0.49	0.15	0.47	0	1.12	0
novel sol-miR-48	hsa-mir-5195	–	0.27	6.41	0.54	2.69	1.71	2.63	0	0	0.37	0
novel sol-miR-48*	hsa-mir-5195*	–	0.14	0.78	0.22	1.35	0.49	0.93	0	0	0.19	0
novel sol-miR-49	hsa-mir-5196	–	0	0	0	0	0	0.31	0	0	0	0
novel sol-miR-49*	hsa-mir-5196*	–	0.27	0.99	0.43	1.35	0.73	0.51	0.47	0	0.06	0
novel sol-miR-50	hsa-mir-5000	–	0.14	0.31	0	0	0	0.46	0.47	0	0.19	0.47
novel sol-miR-50*	hsa-mir-5000*	–	0	0	0	0	0	0.15	0	0.16	0	0
novel sol-miR-51	hsa-mir-3140	–	3.65	2.50	0.32	2.96	3.26	3.24	2.85	1.42	2.81	1.58
novel sol-miR-51*	hsa-mir-3140*	–	0.14	0.31	0	0	0	0.15	0.16	0	0	0.16
novel sol-miR-52	hsa-mir-5197	–	0.95	1.33	0	0.27	0.12	1.31	0	0	0	0
novel sol-miR-52*	hsa-mir-5197*	–	0.54	1.25	0	0.81	0.73	0.77	0.16	0	0	0

Read frequency: the number encoding novel mature miRNAs and star miRNAs (miRNA*s) plotted as a percentage of total miRNA reads multiplied by a factor 10^4. Read frequencies that are shown have been normalized by dividing the absolute number of reads by the total number of miRNA reads for each specific subtype (total number equals the sum of known miRNA reads + novel miRNA reads + novel candidate miRNA reads) . ** indicates the miRNAs of which the expression in ALL and control samples was validated by stem-loop RT-qPCR (Online Supplementary Figure S1).

ALL but not in normal bone marrow and CD34+ fractions (Table 2 and Online Supplementary S5). Sol-miR-14* was exclusively found in T-lineage cells (T-ALL and thymocytes, Table 2). Six mature miRNAs and four miRNA-star forms were found in T-ALL but not in thymocytes and one mature miRNA (novel sol-miR-39) was present in thymocytes but not in T-ALL. Overall, 16 novel, 170 candidate novel and 153 known mature miRNAs/ miRNA-star strands were uniquely found in ALL whereas 2 novel, 82 candidate novel and 140 known mature miRNAs/miRNA-star forms were unique for normal hematopoietic counterparts (Online Supplementary Tables S5-S7). Hence, this indicates that miRNAs and miRNA-star forms are differentially expressed in ALL compared with normal hematopoietic cells.

Novel miRNAs with highest read frequencies are shown in Table 3 for each ALL type. Overall, the read frequency of novel miRNAs was 10-fold lower than of known miRNAs (Figure 4A, $P<0.0001$). Novel miRNAs also differ in evolutionary conservation from yet reported miRNAs. Online Supplementary Tables S1 and S2 report the detection of novel and known miRNAs in different species. Whereas novel miRNAs are often present in two other species besides human (e.g. chimpanzee, macaque, mouse, rat or zebrafish), yet known miRNAs are frequently present in four of these species (Figure 4B, Online Supplementary Tables S1 and S2, $P<0.0001$).

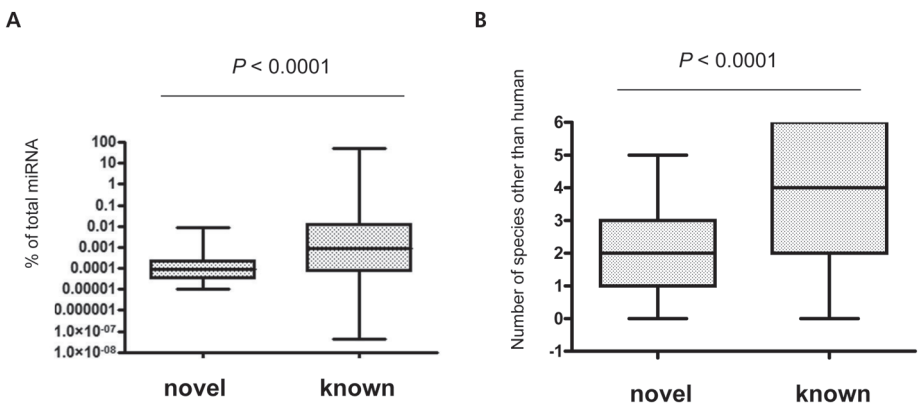

Figure 4. Abundance and evolutionary conservation of novel and known miRNAs in ALL.
(**A**) box plots represent the read frequency of novel and known miRNAs cloned from the seven different ALL types. Read frequency represents the percentage of total miRNA reads. (**B**) box plots reflect the number of species other than human in which novel and known miRNAs are present. MWU-test was used to compare the abundance and number of non-human species for which the miRNA sequence can also be aligned.

Table 3. Top 10 of novel miRNAs with highest read frequency per type ALL.

	novel miRNA	annotation by miRbase	read frequency % of total miRNAs x 10^{-4}	ALL type
1	novel sol-miR-5	hsa-mir-3154	26.0	
2	novel sol-miR-15	hsa-mir-3151	9.9	
3	novel sol-miR-11	hsa-mir-3136	3.6	
4	novel sol-miR-51	hsa-mir-3140	3.6	
5	novel sol-miR-14	hsa-mir-5187	3.0	***MLL***
6	novel sol-miR-18	hsa-mir-3190	3.0	
7	novel sol-miR-6	hsa-mir-3150b	2.3	
8	novel sol-miR-47	hsa-mir-5194	1.2	
9	novel sol-miR-52	hsa-mir-5197	0.9	
10	novel sol-miR-43	hsa-mir-4774	0.8	
1	novel sol-miR-6	hsa-mir-3150b	85.5	
2	novel sol-miR-13	hsa-mir-3117	40.0	
3	novel sol-miR-11	hsa-mir-3136	9.2	
4	novel sol-miR-48	hsa-mir-5195	6.4	
5	novel sol-miR-5	hsa-mir-3154	6.2	***TEL-AML1***
6	novel sol-mir-51	hsa-mir-3140	2.5	
7	novel sol-miR-14	hsa-mir-5187	2.5	
8	novel sol-miR-24	hsa-mir-3177	2.5	
9	novel sol-miR-35	hsa-mir-5186	2.5	
10	novel sol-miR-18	hsa-mir-3190	1.7	
1	novel sol-miR-15	hsa-mir-3151	4.6	
2	novel sol-miR-6	hsa-mir-3150b	3.7	
3	novel sol-miR-5	hsa-mir-3154	2.6	
4	novel sol-miR-11	hsa-mir-3136	2.0	
5	novel sol-miR-14	hsa-mir-5187	1.5	***BCR-ABL***
6	novel sol-miR-18	hsa-mir-3190	1.3	
7	novel sol-miR-41	hsa-mir-5191	1.1	
8	novel sol-miR-46	hsa-mir-3936	0.6	
9	novel sol-miR-48	hsa-mir-5195	0.5	
10	novel sol-miR-49*	hsa-mir-5196*	0.4	

Continuation of Table 3. Top 10 of novel miRNAs with highest read frequency per type ALL.

	novel miRNA	annotation by miRbase	read frequency % of total miRNAs x 10^{-4}	ALL type
1	novel sol-miR-6	hsa-mir-3150b	8.3	
2	novel sol-miR-5	hsa-mir-3154	6.2	
3	novel sol-miR-11	hsa-mir-3136	5.4	
4	novel sol-miR-51	hsa-mir-3140	3.0	
5	novel sol-miR-48	hsa-mir-5195	2.7	***E2A-PBX1***
6	novel sol-miR-14	hsa-mir-5187	2.2	
7	novel sol-miR-48*	hsa-mir-5195*	1.3	
8	novel sol-miR-49*	hsa-mir-5196*	1.3	
9	novel sol-miR-41	hsa-mir-5191	1.1	
10	novel sol-miR-18	hsa-mir-3190	1.1	
1	novel sol-miR-5	hsa-mir-3154	6.4	
2	novel sol-miR-51	hsa-mir-3140	3.3	
3	novel sol-miR-15	hsa-mir-3151	2.0	
4	novel sol-miR-48	hsa-mir-5195	1.7	
5	novel sol-miR-18	hsa-mir-3190	1.7	***hyper-diploid***
6	novel sol-miR-11	hsa-mir-3136	1.0	
7	novel sol-miR-14	hsa-mir-5187	1.0	
8	novel sol-miR-41	hsa-mir-5191	0.9	
9	novel sol-miR-6	hsa-mir-3150b	0.7	
10	novel sol-miR-49*	hsa-mir-5196*	0.7	
1	novel sol-miR-11	hsa-mir-3136	5.7	
2	novel sol-miR-51	hsa-mir-3140	2.8	
3	novel sol-miR-6	hsa-mir-3150b	2.7	
4	novel sol-miR-27	hsa-mir-3942	2.1	
5	novel sol-miR-23	hsa-mir-4474	1.7	***T-ALL***
6	novel sol-miR-14	hsa-mir-5187	1.6	
7	novel sol-miR-40	hsa-mir-5190	1.3	
8	novel sol-miR-5	hsa-mir-3154	1.2	
9	novel sol-miR-37	hsa-mir-5006	1.1	
10	novel sol-miR-18	hsa-mir-3190	0.9	

Ten novel miRNAs with the highest read frequency are shown for each ALL type. Read frequencies are presented as a percentage of the total number of identified miRNAs per ALL type multiplied by a factor 10^4. * Indicates the miRNA-star strand.

Newly discovered miRNAs are aberrantly expressed in different types of ALL

Stem-loop RT-qPCR confirmed that known miRNAs with high read frequencies, e.g. miR-361-3p, miR-196b and miR-708 were abundantly expressed in ALL. Moreover, similar to the read frequencies these miRNAs were differentially expressed between genetic types of ALL (Figure 5A-C); miR-361-3p was 3-fold higher (P<0.001) expressed in hyperdiploid cases than in other precursor B-ALL cases, miR-196b was ~500-fold higher (P<0.001) expressed in *MLL*-rearranged compared with non-*MLL*-rearranged precursor B-ALL cases and miR-708 was ~300 and 3000-fold downregulated (P≤0.002) in *MLL*-rearranged and T-ALL cases, respectively. The expression level of 10 novel and 12 candidate novel miRNAs (Online Supplementary Tables S2 and Table S3) was also measured with stem-loop RT-qPCR (Figure 5D-F and Online Supplementary Figure S1). The expression levels of 5 out of 22 selected new miRNAs were below detection limits of real-time quantitative PCR (i.e. comparative cycle threshold (Ct) larger than 40, corresponding to <0.001% of snoR-1 levels).

The remaining 17 (candidate) novel miRNAs were differentially expressed between ALL types (Online Supplementary Figure S1). Albeit novel sol-miR-6 had a relative low read frequency of 0.7×10^{-4} up to 86×10^{-4}% of total miRNAs in ALL (Table 2), RT-qPCR analysis showed that this miRNA was median nine-fold higher expressed in *TEL-AML1*-positive patients than in precursor B-ALL cases without this translocation (P=0.02, Figure 5D). Sol-miR-11 was three-fold upregulated in *E2A-PBX1*-positive cases compared with other precursor B-ALL patients, whereas this miRNA was undetectable (i.e. <0.001% of snoRNA-1 input) in *MLL*-rearranged cases (P=0.02, Figure 5E).

RT-qPCR analysis of novel miRNAs also confirmed the differential expression of miRNAs between ALL and normal hematopoietic cells: novel sol-miR-14 and sol-miR-23 were undetectable in most precursor B-ALL cases but expressed in normal bone marrow and CD34+ cells (P≤0.02, Figure 5F and Online Supplementary Figure S1-D). Similarly, sol-miR-30 was 4- to 17-fold lower expressed in precursor B-ALL than in CD34+-sorted cells and normal bone marrow (P<0.001, Online Supplementary Figure S1-N). In T-ALL, sol-miR-18 and sol-miR-16 were up to five-fold lower expressed than in healthy thymocytes (P<0.01, Online Supplementary Figures S1-F and -J).

As miRNAs may inhibit translation of proteins by cleaving mRNA, aberrant expression of new miRNAs may affect the expression level of their mRNA targets. Targetscan 5.1[29] was used to predict these targets based on their homology to the 2-8 nucleotide seed sequence of each miRNA. Table 4 shows that the expression levels of 14 out of total 17 differentially expressed (candidate) novel miRNAs inversely correlated with the mRNA levels of predicted target genes for these miRNAs (-0.49≤Spearman's correlation coefficients (Rs)≤-0.27, P≤0.05). Novel sol-miR-23 was predicted to target *BCL2* (*B-cell lymphoma 2*) and a lower expression level of this miRNA correlated with higher expression level of *BCL2* in patients (Rs -0.36, P=0.007, Table 4, Online Supplementary Figure S2).

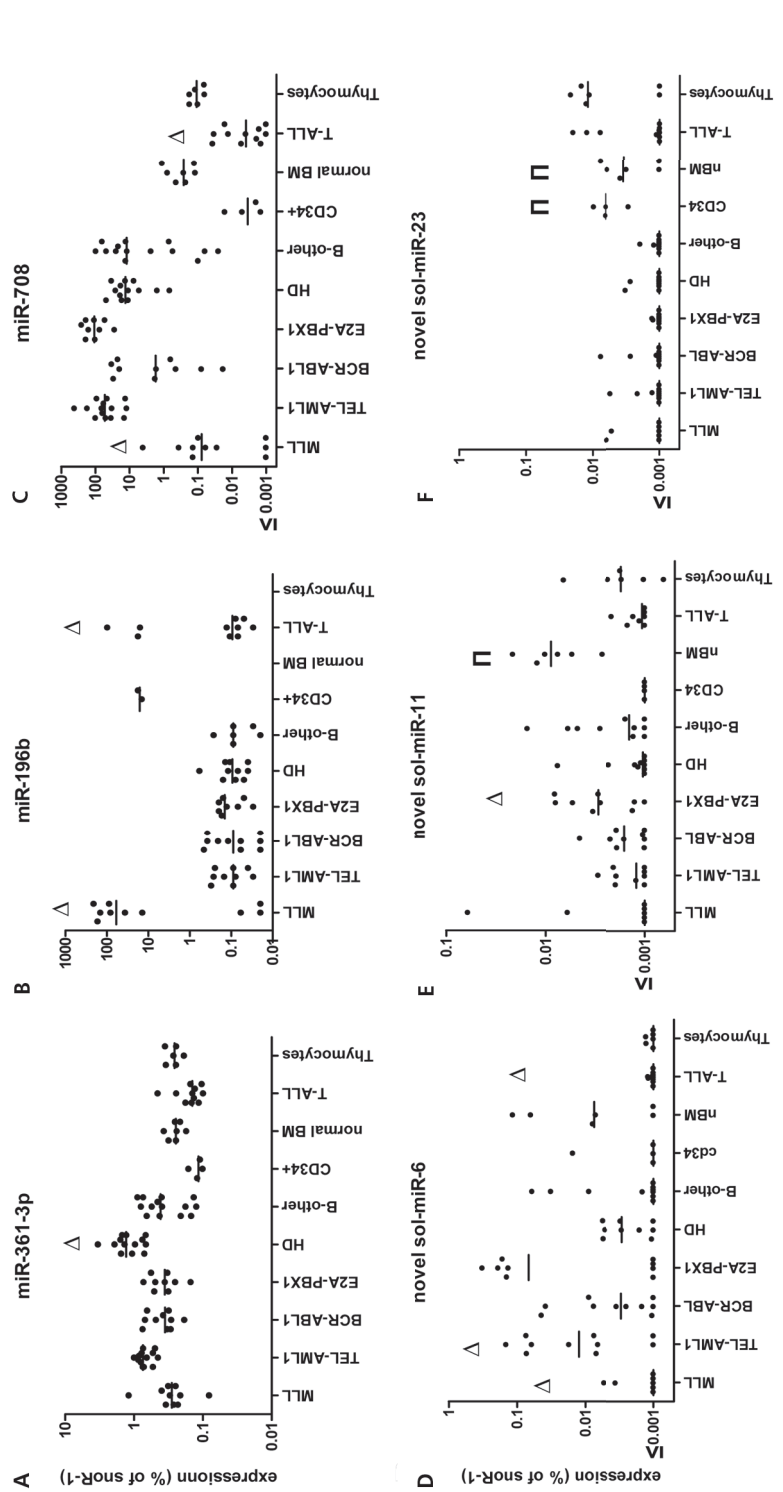

Figure 5. Expression levels of known and novel miRNAs in types of ALL and normal hematopoietic cells.
Expression levels of miR-361-3p (**A**), miR-196b (**B**), miR-708 (**C**), novel sol (of Solexa)-miR-6 (**D**), novel sol-miR-11 (**E**) and novel sol-miR-23 (**F**) were measured by stem-loop RT-qPCR. Dots represent the expression level in individual patients as a percentage of the expression level of the endogenous reference snoRNA-1. Levels below 0.001% are undetermined (Ct>40). The median in each group is indicated by a line. HD, hyperdiploid. Δ $P \leq 0.05$; based on the comparison between expression levels of the indicated type and the remaining ALL cases; Π $P \leq 0.05$; based on comparison between normal hematopoietic cell populations and ALL.

Table 4. Correlation between the expression level of the newly identified miRNAs and the expression level of their predicted targets.

novel sol-miR-5	hsa-mir-3154	GTF2I Rs -0.46 **	UBE3A Rs -0.45 **	PPP1R12A Rs -0.45 **	ZFAND5 Rs -0.43 **	RAD23B Rs -0.37 *
novel sol-miR-6	hsa-mir-3150b	SIT1 Rs -0.36 *	RLBP1 Rs -0.29 *	PAX2 Rs -0.28 *	CPLX2 Rs -0.27 *	–
novel sol-miR-11	hsa-mir-3136	MBNL1 Rs -0.29 *	ARL1 Rs -0.27 *	–	–	–
novel sol-miR-14	hsa-mir-5187	–	–	–	–	–
novel sol-miR-15	hsa-mir-3151	CAMK2G Rs -0.38 **	ABCE1 Rs -0.36 *	HNRNPA -0.32 *	RNF125 Rs -0.32 *	EIF5B Rs -0.31 *
novel sol-miR-18	hsa-mir-3190	AP3B1 Rs -0.49 **	FGF12 Rs -0.37 *	TMEM97 Rs -0.32 *	NLK Rs -0.3 *	ABCF2 Rs -0.3 *
novel sol-miR-23	hsa-mir-4474	NDRG4 Rs -0.4 **	MAGED1 Rs -0.39 **	BCL2 Rs -0.36 **	SLC16A2 Rs -0.29 *	IMPAD1 Rs -0.29 *
novel sol-miR-27	hsa-mir-3942	TNPO1 Rs -0.38 **	CLTC Rs -0.33 *	FBXW11 Rs -0.3 *	APC Rs -0.29 *	KPNA3 Rs -0.29 *
candidate novel sol-miR-9	–	ZNF576 Rs -0.33 *	GOSR2 Rs -0.31 *	RNF8 Rs -0.31 *	WDTC1 Rs -0.3 *	CASP2 Rs -0.29 *
candidate novel sol-miR-16	–	–	–	–	–	–
candidate novel sol-miR-19	–	RPGRIP1L Rs -0.43 **	VRK3 Rs -0.39 **	HS2ST1 Rs -0.37 **	CALML4 Rs -0.36 **	TAOK3 Rs -0.32 *
candidate novel sol-miR-22	–	SRP72 Rs -0.33 *	AAK1 Rs -0.29 *	–	–	–
candidate novel sol-miR-28	–	PHF15 Rs -0.45 **	LRP4 Rs -0.38 **	CACNA1A Rs -0.35 **	PARD6B Rs -0.34 *	BTBD3 Rs -0.3 *
candidate novel sol-miR-30	–	CCNL2 Rs -0.37**	CUL1 Rs -0.37 *	BRCA2 Rs -0.33 *	PBX1 Rs -0.32 *	SNX4 Rs -0.31 *
candidate novel sol-miR-31	–	RNF4 Rs -0.32 *	RANBP17 Rs -0.30 *	HSPA9 Rs -0.27 *	–	–
candidate novel sol-miR-33	–	–	–	–	–	–
candidate novel sol-miR-34	–	FUT9 Rs -0.43 **	BCAT1 Rs -0.42 **	LARP4 Rs -0.34 *	PTPLAD1 Rs -0.33 *	HNRNPC Rs -0.33 *

Target genes were predicted for novel and candidate novel miRNAs by Targetscan 5.1. MiRNA expression levels were determined by stem-loop RT-qPCR and compared with the expression of target genes as determined by Affymetrix U133 plus 2.0 GeneChips in the same patients (see Materials and methods section for more details). Table 4 summarizes the Spearman's correlation coefficients (Rs) of mRNA expression levels of a maximum of top 5 predicted target genes that inversely correlated to the expression level of the indicated miRNA. **P*-value ≤0.05 and ***P*-value ≤0.01. Annotation of novel miRNAs by miRBase is shown in the second column.

DISCUSSION

High-throughput Solexa deep sequencing followed by computational analyses identified 554 known, 28 novel and 431 candidate novel miR-genes being expressed in seven different types of childhood ALL and three types of normal hematopoietic cells. Validation of selected miRNAs by stem-loop RT-qPCR confirmed aberrant expression patterns in subtypes of ALL and normal cells. Expression levels of 14 newly discovered miRNAs were inversely correlated to the transcript level of predicted target genes. This points to new miRNAs that may contribute to the biology of ALL and are therefore plausible candidates for more detailed functional studies.

MiRNA expression levels and function highly depend on the cellular context in which they are studied, including type of tissue, hematopoietic lineage and/or the presence of genomic translocations. For example miR-221 and miR-222 are downregulated in erythroblastic leukemia but overexpressed in chronic lymphocytic leukemia[30]. MiR-221 and miR-222 inhibit growth of erythroblastic leukemia cells by targeting the oncogene *c-KIT*, suggesting a tumor suppressor function for both miRNAs. However, the same miRNAs were reported to stimulate proliferation in thyroid and hepatocellular carcinomas through dowregulation of the tumor supressor genes *PTEN* and *p27*, implying that both miRNAs can also serve a oncogenic role[30]. As the function of miRNAs is cell-type dependent and most known miRNAs have been discovered in non-leukemic/non-hematopoietic cell types, we hypothesized that many miRNAs of interest to ALL are yet unknown. Because array-based expression techniques are limited to known miRNAs we chose for sequencing of expressed miRNAs to address this hypothesis in well-defined types of pediatric ALL and normal hematopoietic cells. The contemporary deep sequencing technique enables simultaneous sequencing of millions of small RNA reads and is by far more sensitive to identify miRNAs than Sanger-based sequencing of conventional small RNA concatemer-cloning products like we and others previously used[11,31-33]. For example, 10 µg of total RNA input results in up to 10 million reads by high-throughput sequencing in contrast to ~1100 reads by conventional cloning methodology[11]. Therefore, high-throughput sequencing of small RNAs expressed in leukemic cells is currently the most sensitive approach to discover novel miRNAs that may be relevant to ALL.

In the present study we used well-defined and stringent criteria to define the confidence levels of identified miRNA sequences (Table 1, Figure 1). In general, 16 novel, 170 candidate novel and 153 known mature miRNA/miRNA-star strands were only expressed in ALL whereas 2 novel, 82 candidate novel and 140 known mature miRNA/miRNA-star strands were unique for normal hematopoietic cells (Online Supplementary Tables S5-S7). The number of novel and candidate novel miRNAs identified in this study is in correspondence with other high-throughput studies that used similar stringency criteria in melanoma, ovarian tissues and acute myeloid cell lines[34-36]. In addition, high-throughput sequencing of two libraries compiled

of 3 pediatric (genotypically not defined) ALL cases and 2 normal bone marrow samples revealed 42 novel miRNAs of which 5 were unique to ALL and 22 exclusively detected in normal donor bone marrow cells[37]. In a recent study seven new miRNAs were cloned from AML with normal karyotype[38]. These studies suggest that the number of novel miRNAs being identified is not expected to drastically increase upon additional high-throughput sequencing analyses of similar samples unless the criteria for identification of miRNAs are being altered upon new scientific insights into the structure of miRNAs. The ultimate proof for a genuine miRNA is given by the experimental evidence that a (candidate) novel miRNA precursor is being processed into a mature miRNA by an active Dicer machinery[39]. The stem-loop RT-qPCR can be used for this purpose since it selectively detects expression levels of processed mature miRNAs[26]. In the present study we confirmed 17 out of 22 tested novel miRNAs as being genuine. The other five tested were below detection limits of stem-loop RT-qPCR which can be indicative for a less efficient (stem-loop) primer design and/or the fact that a predicted miRNA is not a true miRNA.

In general, novel and candidate novel miRNAs were expressed at lower levels than known miRNAs (Figure 4). Despite this reduced average of expression, individual cases and/or specific subtypes can display relative high levels of newly identified miRNAs; e.g. sol-miR-6 in a subset of *E2A-PBX1*-positive and *TEL-AML1*-positive ALL cases (Figure 5 and Online Supplementary Figure S1). The fact that novel and candidate novel miRNAs were not previously detected in other tissue types may point to miR-genes being selectively expressed in genetic subtypes of leukemia and/or normal hematopoietic cells. A similar heterogeneity among patients was observed for the known miRNA-196b. Among *MLL*-rearranged and T-lineage ALL cases, miR-196b expression was specifically upregulated in cases with genetic lesions that affect *HOXA*-cluster gene activities which is an important leukemogenic event in these subtypes[13].

In correspondence to the relative low expression levels, the read frequency of novel miRNAs was much lower than those of known miRNAs (Figure 4). The most abundantly expressed miR-genes_across all studied types of ALL include let-7 family members. These let-7 family miRNAs were also highly expressed in normal bone marrow cells, CD34+ hematopoietic precursor cells and thymocytes (see Online Supplementary Table S1). Also in other tissues the let-7 family is abundantly expressed suggesting a general, non-cell type specific, function of let-7 miRNAs in gene transcription[5,40,41]. The fine-tuning may come from less abundantly but more cell-type specific miRNAs. Strikingly, we identified a higher frequency of sequence reads for novel miRNAs in *TEL-AML1*-positive patients compared with other precursor B-ALL types. This may suggest that miRNA-regulated gene expression is more active in *TEL-AML1*-positive patients compared with the other types of B-lineage ALL. Of particular interest are the known miR-125b, miR-126* and miR-383 (Online Supplementary Table S4) and the newly identified sol-miR-6 in *TEL-AML1*-positive ALL, the latter being recently also found in ovarian tissue and which is now called miR-3150b (Table 2 and 3)[34].

Based on the seed sequence of the newly identified miRNAs (e.g. UGUGGCU for sol-miR-23), Target scan 5.1 was used to predict the target genes of these miRNAs[29]. The expression of 14 newly identified miRNAs negatively correlated with the expression of 61 target genes, which may point to the functionality of these newly identified miRNAs (Table 4). Novel sol-miR-23 (recently annotated by miRBase as hsa-mir-4474[14]) was 6- and 10-fold lower expressed in precursor B-ALL than in normal bone marrow and CD34+-sorted cells, respectively (Figure 5F). A decrease in sol-miR-23 was linked to an increase of mRNA levels of its predicted target *BCL2* (Table 4, Online Supplementary Figure S2). High expression level of *BCL2* is shown to have potential oncogenic effects at critical stages of differentiation[42] and may affect resistance to cytotoxic drugs[43]. Although high level of *BCL2* expression may not cause resistance to chemotherapy in leukemia[44], *BCL2* expression may promote *BCR-ABL1*-dependent leukemogenesis[45].

MiRNAs can reduce protein expression levels both by mRNA degradation and by translational silencing (without need for mRNA degradation)[46]. Therefore, a lack of an inverse correlation between miRNA and mRNA levels of predicted target genes does not exclude an inhibitory role for the miRNA in protein translation. To discover targeted genes, proteome-wide screens of altered protein levels upon miRNA manipulation are wishfull but unfortunately these technologies are still in its infancy. Alternative methods based on interaction between miRNA/mRNA and/or RISC complex such as Ribonucleoprotein ImmunoPrecipitation-gene chip (RIP-chip)[47,48] and 3'UTR-binding luciferase assays[49] may be informative. However, these techniques often yield false positive information due to artificial (binding) conditions in the experimental procedures.

In conclusion, high-throughput sequencing of 7 well-characterized ALL types and 3 normal hematopoietic cell fractions (representing 70 and 19 cases, respectively) resulted in the discovery of 28 novel and 431 candidate novel miR-genes besides 554 yet described miR-genes. Subsequent stem-loop RT qPCR confirmed aberrant expression levels of newly discovered miRNAs in ALL types and normal hematopoietic cells. Hence, the presented data form a comprehensive basis for further functional studies in order to understand the role of miRNAs in pediatric ALL.

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Chapter 5

MicroRNAs characterize genetic diversity and drug resistance in pediatric acute lymphoblastic leukemia

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ABSTRACT

Background

MicroRNAs regulate activity of protein-coding genes including those involved in hematopoietic cancers. The aim of the current study was to explore which microRNAs are unique for seven different subtypes of pediatric acute lymphoblastic leukemia.

Design and methods

Expression levels of 397 microRNAs (including novel microRNAs) were measured by quantitative real-time polymerase-chain-reaction in 81 pediatric leukemia cases and 17 normal hematopoietic control cases.

Results

Except for *BCR-ABL*-positive and B-other acute lymphoblastic leukemia, all other major subtypes i.e. T-cell, *MLL*-rearranged, *TEL-AML1*-positive, *E2A-PBX1*-positive and hyperdiploid acute lymphoblastic leukemia have unique microRNA-signatures that differ from each other and from those in healthy hematopoietic cells. Strikingly, the microRNA signature of *TEL-AML1*-positive and hyperdiploid cases partly overlapped, which may suggest a common underlying biology. Moreover, aberrant downregulation of let-7b (~70-fold) in *MLL*-rearranged acute lymphoblastic leukemia was linked to upregulation of oncoprotein c-Myc (P_{FDR}<0.0001). Resistance to vincristine and daunorubicin was characterized by ~20-fold upregulation of miR-125b, miR-99a and miR-100 (P_{FDR}≤0.002). No discriminative microRNAs were found for prednisolone and only one microRNA was linked to L-asparaginase resistance. A combined expression profile based on 14 microRNAs that were individually associated with prognosis, was highly predictive for clinical outcome in pediatric acute lymphoblastic leukemia (5-year disease-free survival of 89.4%±7% versus 60.8±12%, *P*=0.001).

Conclusions

Genetic subtypes and drug resistant leukemic cells display characteristic microRNA signatures in pediatric acute lymphoblastic leukemia. Functional studies of discriminative and prognostic important microRNAs may provide new insights into the biology of pediatric acute lymphoblastic leukemia.

INTRODUCTION

Discovery of regulatory microRNA (miRNA) genes and their biological roles are part of extensive ongoing studies. These ~21-nucleotide miRNAs inhibit the activity of the majority of all human genes on the post-transcriptional level[1]. Usually, miRNAs establish such inhibition through imperfect basepairing with the 3′ untranslated region of a protein-coding *messenger*RNA (mRNA) resulting in inhibition of the translation itself or in degradation/destabilization of its target mRNA[2,3]. By doing so, they are involved in various biological processes like hematopoiesis, differentiation and apoptosis. For example, miR-181a is preferentially expressed in precursor B cells and stimulation of miR-181a in hematopoietic progenitors resulted in differentiation towards a more mature B-cell phenotype[4]. Family member miR-181b is mainly present in mature B-cells like germinal center B-cells and its expression is decreasing upon further differentiation into plasma or memory B-cells[5]. MiR-15a and miR-16 promote apoptosis by targeting anti-apoptotic Bcl-2 in hematopoietic cells[6]. Such miRNA-affected-processes are found to be dysregulated in different (hematopoietic) malignancies. Deletion or downregulation of miR-15a and miR-16 in patients with chronic lymphocytic leukemia protects the leukemic cells from apoptosis[6] and amplification of the miR-17-92 cluster accelerates the development of c-myc-induced lymphomas in mice[6,7].

Children with some subtypes of childhood acute lymphoblastic leukemia (ALL), such as hyperdiploid (>50 chromosomes) and *TEL-AML1*-translocated ALL, have a favorable 5-year disease-free survival (DFS) of >85% after combination chemotherapy[8]. However, children with other subtypes, such as *BCR-ABL*-positive and mixed lineage leukemia (*MLL*)-rearranged ALL, have an unfavorable outcome with a 5-year DFS of only ~50%[9,10]. Besides the genetic subtype, cellular drug resistance influences the outcome of children with ALL[11-14]. Understanding miRNA expression levels in different genetic subgroups of ALL may provide new insight into the biology of underlying genetic abnormalities and/or drug resistance. To this aim we used stem-loop-based reverse-transcriptase (RT) quantitative real-time polymerase chain reaction (stem-loop RT-qPCR) to measure the expression of 397 miRNAs in 81 pediatric ALL cases reflecting different genetic subtypes i.e. T-ALL, precursor B-ALL with hyperdiploidy, *MLL*-rearrangement, *TEL-AML1, BCR-ABL, E2A-PBX1* and precursor B-ALL not carrying these cytogenetic aberrations (B-other) and also in 17 control non-leukemic hematopoietic cell samples. Furthermore, we studied whether the miRNA expression profiles were related to resistance to four important drugs used in ALL treatment i.e. prednisolone, vincristine, L-asparaginase, and daunorubicin, and whether miRNAs were associated with the clinical outcome of patients.

DESIGN AND METHODS

Patient samples

Mononuclear cells were isolated from peripheral blood or bone marrow samples collected from 81 children with newly diagnosed ALL and 17 control cases using sucrose density centrifugation[11,15]. The percentage of leukemic cells was determined by May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. If the percentage of leukemic cells was below 90%, samples were enriched by eliminating non-malignant cells with immunomagnetic beads (see Online Supplementary text for more details)[11,15]. $CD34^+$ -cells (>90% purity) were sorted from granulocyte colony-stimulating factor (G-CSF)-stimulated blood cell samples of children with a brain tumor or Wilm's tumor (see Online Supplementary Text)[16]. Thymocytes were isolated from thymic lobes that were resected from children during surgery for their congenital heart disease (see Online Supplementary Text)[17]. Samples were collected with informed consent from parents or guardians according to local institutional reviewing board approval. The immunophenotype and genetic subtype were determined by routine diagnostic procedures including flow cytometry for lineage-detection (T-ALL or precursor B-ALL), fluorescence *in situ* hybridization (FISH) and RT-PCR for genetic subtype and conventional karyotyping to determine the ploidy status of ALL cases. A total of 10 *MLL*, 14 *TEL-AML1*, 10 *BCR-ABL*, 9 *E2A-PBX1*, 13 hyperdiploid, 14 B-other (negative for the 5 previously listed genetic abnormalities) and 11 T-ALL cases were included. These cases were retrospectively selected based on availability of material and cover different treatment protocols. Expression levels in normal hematopoietic cells were determined in seven normal bone marrow, four $CD34^+$-sorted fractions and six thymocyte fractions.

Drug resistance

To determine cellular drug resistance, the concentrations of prednisolone, vincristine, L-asparaginase or daunorubicin that was lethal to 50% (LC_{50}) of the ALL cells was measured by MTT drug resistance assay as described previously (see Online Supplementary Text)[11,18]. Median LC_{50} values were used to assign cases as sensitive (≤median LC_{50}) or resistant (>median LC_{50}) to each drug.

Expression analysis

Total RNA was extracted with TRIzol reagent (Invitrogen, Leek, NL) according to the manufacturer's protocol. The 2100 bioanalyzer (Agilent, Amstelveen, NL) was used to determine the quality of total RNA. All RNA samples had an RNA Integrity Number of ≥7.5.

Expression levels of 397 miRNAs were analyzed by stem-loop RT-qPCR microRNA arrays (Applied Biosystems, Foster City, CA, USA)[19]. Three hundred and sixty-five miRNAs were assayed using TaqMan MicroRNA arrays with 100 ng of RNA as input for each RT reaction according to the manufacturer's protocol. An additional 32 miRNAs (Online Supplementary

Table S1) were measured using miRNA assays that were custom designed by Applied Biosystems since these miRNAs were not covered by the TaqMan MicroRNA array platform and/or were recently identified by our cloning study[16]. RT reactions for custom miRNA assays were performed in duplicates, in a total volume of 15 µl containing 0.5 mM dNTPs, 10 U/µl RT, 1x RT buffer, 0.25 U/µl RNase inhibitor and 0.25x multiplex RT primer pool covering the 32 miRNAs (Applied Biosystems). RT reactions were incubated as previously described[19]. Next, cDNA samples were diluted 10-fold in water. Duplicate PCR reactions of 15 µl were performed in a 96-well plate for each of the 32 miRNAs. PCR reactions contained 1 µl of diluted cDNA sample in 1x Universal TaqMan Master Mix and 1x specific primer/probe mix. PCR reactions were performed on an ABI 7900HT Sequence Detection System. Duplicate measurements of two independent experiments were highly correlated (Rs=0.9, P<0.0001, Online Supplementary Figure S1-A). Moreover, multiple measurements for snoR-13 and snoR-14 were analysed within one TaqMan Microarray-plate which highly correlated (Rs≥0.96, *P*<0.0001, Online Supplementary Figure S1-B-C). The mean of Ct-values for snoR-13 and -14 (TaqMan MicroRNA array) and snoR-1 (custom reactions) were used as reference to normalize the expression of miRNAs. These snoRNAs were chosen since expression levels did not differ significantly between genetic subtypes of ALL as well as between ALL and hematopoietic control cells (Online Supplementary Figure S2) and expression levels of these three snoRNAs were strongly correlated with each other (Online Supplementary Figure S3). The expression was calculated as a percentage of snoRNA as $2^{-\Delta Ct}$ x100% where the ΔCt is equal to the Ct-value for each miRNA minus the Ct-value of the control snoRNA. Processed miRNA expression values have been deposited in the NCBI's Gene Expression Omnibus and are accessible through GEO series accession number GSE23024 at http://www.ncbi.nlm.nih.gov/geo.

Affymetrix U133 plus 2.0 GeneChips (Santa Clara, CA, USA) were used to study mRNA expression levels of potential miRNA-target genes in ALL patients as described earlier (Online Supplementary text)[20]. Reverse phase protein detection were used to determine the protein level of Ras and c-Myc as previously described[21-23] (Online Supplementary text).

Statistical analysis

The Wilcoxon rank-sum test was used to compare miRNA expression levels between two groups. Differences were considered statistically significant if Benjamini-Hochberg's false discovery rate -corrected p-values (P_{FDR}) were <0.05[24]. R version 2.8[25] and the R package multtest (which corrects the p-values for multiple testing)[26] were used to perform these analyses.

Hierarchical clustering of patients by miRNA expression levels was done using GeneMaths 2.0 software (Applied Maths, Sint-Martens-Latem, Belgium) after Pearson's correlation as distance measure. Since TaqMan MicroRNA Arrays and custom-made assays make use of

different control snoRNA to correct for small RNA input, we calculated Z-scores for each miRNA. Z-scores were used for hierarchical clustering analyses.

Cox proportional hazard analysis was used to identify miRNAs that correlated to disease-free survival of children with newly diagnosed ALL. Both univariate and multivariate (corrected for ALL subtype) analyses were performed using relapse as event and miRNA expression as continuous variable. Mulitvariate analysis indicated that the expression levels of 14 miRNAs were of significant prognostic value ($P<0.05$; see results section). To visualize the prognostic value of the expression signature of these 14 miRNAs, we first divided the cases in two groups based on the median expression level per miRNA (see Online Supplementary Table S2 for the median values of each of the 14 miRNAs). Patients with high expression (above the median) of a prognostic *favorable* miRNA (e.g. miR-10a) were assigned to score 1 whereas patients with low expression (below the median) were given a score of 2. In case of a prognostic *unfavorable* miRNA (e.g. miR-33), patients were assigned a score 2 in case of an expression level above the median and a score of 1 if this level was below the median. Next, the sum of individual scores for the 14 prognostically informative miRNAs was calculated: this resulted in a minimum cumulative score of 14 and a maximum cumulative score of 28. The median of the cumulative scores for 78 patients was used to assign patients to a favorable (cumulative score ≤21, n=41) or unfavorable (cumulative score >21, n=37) group in order to study the prognostic value of a combined miRNA expression signature.

RESULTS

Distinct miRNA profiles in genetic subtypes of ALL

The expression of 397 miRNAs was measured in 81 pediatric ALL cases representing seven different subtypes of ALL and 17 control cases. This analysis includes miR-1972, miR-1974, miR-1975, miR-1976, miR-1977, miR-1978 and miR-1979 that we recently discovered as reported elsewhere[16]. Online Supplementary Table S2 shows the relative expression of all miRNAs in the different subtypes of ALL and normal hematopoietic cells. Seventy-two (18%) miRNAs were not expressed in at least 97 out of 98 cases and were, therefore, excluded from further analyses. Online Supplementary Figure S4 shows the comparison of the median expression levels of the remaining 325 miRNAs between ALL subtypes and normal hematopoietic cells. Hierarchical clustering using these 325 expressed miRNAs distinguished 12 clusters of ALL patients and control cases as shown in Figure 1. Online Supplementary Table S3 shows the genetic subtype, immunophenotype and karyotype of these patients. The majority of patients except for *BCR-ABL*-positive ALL and B-other ALL, clustered per subtype based on similarities in miRNA expression pattern. For example, all nine *E2A-PBX1* patients and 9 out of 11 T-ALL patients clustered in groups based upon similarities in their miRNA profiles. Figure 1 also shows that precursor B-ALL patients clustered differently from

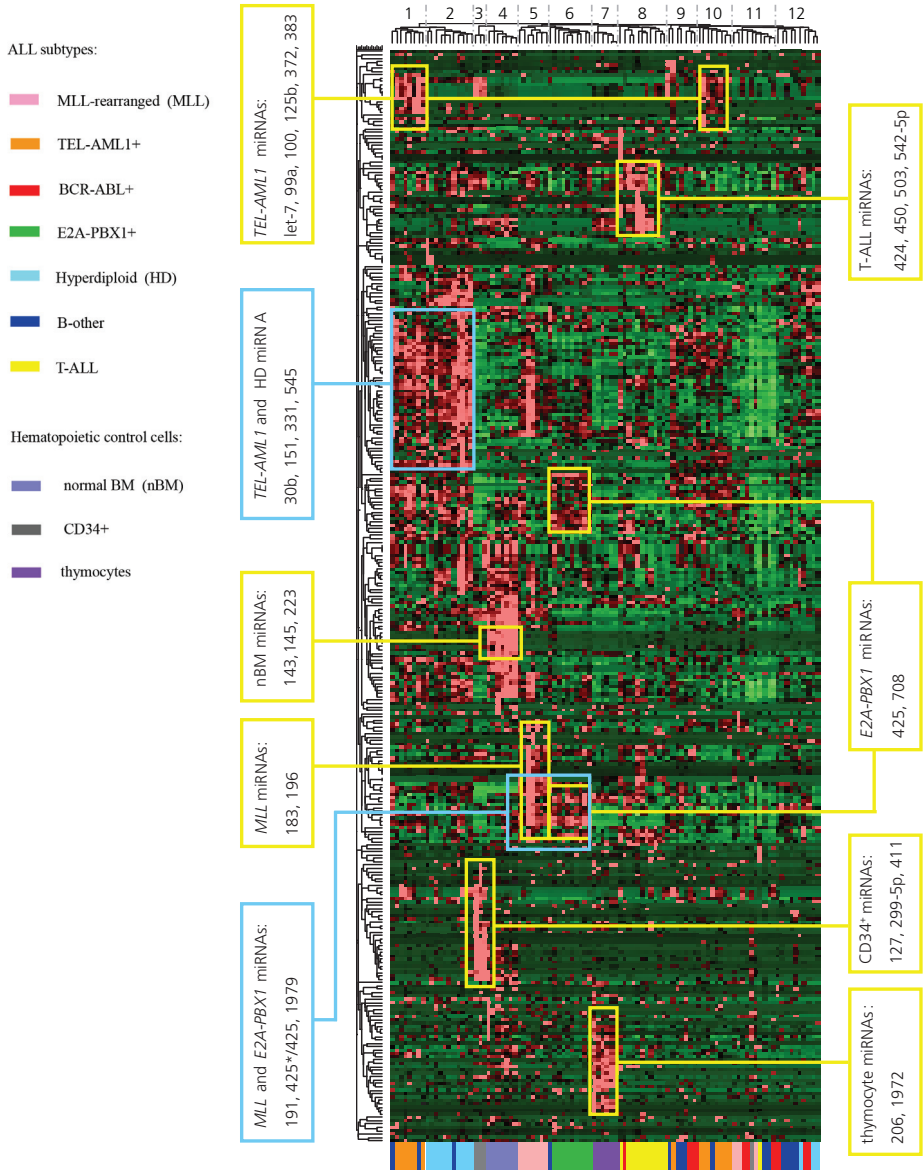

Figure 1. Clustering of ALL subtypes and normal hematopoietic control cells by expression levels of 325 miRNAs.

Hierarchical clustering of ALL patients, normal BM samples, CD34+ selected cells and thymocytes by expression levels of 325 (unselected) miRNAs. Heatmap shows which miRNAs are overexpressed (in red) and which are underexpressed (in green) relative to snoRNA input control. Expression levels are plotted as standardized Z-scores per miRNA.

normal bone marrow and CD34+ blood cells. For example, miR-143 was expressed 70-fold (P_{FDR}=0.0007) lower in precursor B-ALL patients than in normal bone marrow and miR-127 was expressed 140-fold (P_{FDR}=0.001) lower in precursor B-ALL patients than in normal CD34+ blood cells. Likewise, the expression pattern in T-ALL differed from that in normal thymocytes e.g. 28 miRNAs were significantly differentially expressed in T-ALL samples (P_{FDR}≤0.04; see Online Supplementary Table S2 for relative expression levels).

BCR-ABL-positive and B-other precursor B-ALL patients were spread among other genetic subtypes, including the hyperdiploid, *TEL-AML1* and *E2A-PBX1*-positive subtype, suggesting heterogeneity among *BCR-ABL* and B-other samples (Figure 1). In line with this, only 16 miRNAs with P_{FDR}<0.05 were found for *BCR-ABL*-positive patients whereas 50 to 89 miRNAs with P_{FDR}<0.05 were found for other subtypes (Online Supplementary Table S4). Moreover, these 16 miRNAs were less discriminative for *BCR-ABL* (fold-change in expression ≤6-fold) than the 50-89 miRNAs for the other subtypes (fold-change in expression ≤3884-fold; Table 1).

The majority of hyperdiploid samples clustered together and were distinct from other (non-hyperdiploid) ALL samples based on relatively high expression levels of miR-223, miR-222/222*, miR-98, and miR-511 (Table 1). Strikingly, genes encoding these miRNAs were all located on chromosome 10 or X, two chromosomes of which extra copies can be found in hyperdiploid cases[27]. *TEL-AML1*-positive ALL patients were distinguished from those with other (non-*TEL-AML1*) genetic subtypes by ~5- to 1700-fold upregulation of various miRNAs including miR-99a, miR-100, miR-125b, miR-383 and let-7c (P_{FDR}<0.001, Figure 1 and Table 1). In contrast to *TEL-AML1* cases, *MLL*-rearranged ALL showed a 16-fold downregulation of let-7c and a 69-fold lower expression of let-7b compared with other precursor B-ALL patients negative for *MLL*-translocations (P_{FDR}<0.001, Table 1). MiRNA profiling revealed two different subpopulations of *TEL-AML1* patients (Figure 1). Cluster 1 (n=6) displayed similarities in miRNA pattern to hyperdiploid patients whereas cluster 10 (n=8) did not share these similarities. At present we do not have an indication that both *TEL-AML1* sub-clusters differ in clinical outcome. It should, however, be noted that this may be due to a limited number of relapses in the *TEL-AML1* cases (n=1). Both subtypes were characterized by a high expression of miR-30b, miR-151, miR-331 and miR-545 (Figure 1) and miR-126/126* (Online Supplementary Table S2). This may indicate a common biological mechanism underlying the *TEL-AML1* and hyperdiploid subtypes.

MLL-rearranged and *E2A-PBX1*-positive ALL shared a high expression level of 15 miRNAs including the recently discovered miR-1979 (Figure 1 and Online Supplementary Table S5). Forty percent of these 15 miRNAs are mapped within miRNA-clusters e.g. miR-191 and miR-425 on chromosome 3p21.31 and the cluster of miR-141 and miR-200c on chromosome 12p13.31. The expression of miR-191 and miR-425 (Rs=0.8) as well as of miR-141 and miR-200c (Rs=0.9) correlated well with each other (P<0.001), which may indicate co-regulation of these miRNAs since they are encoded less than 10 kilobases from each other. Correlation

Table 1. Most discriminative miRNAs per subtype of pediatric ALL.

top#	miRNA	chromosomal location	fold change	FDR-corrected p-value
MLL-1	let-7b	22q13.31	↓ 69	<0.001
MLL-2	let-7c	21q21.1	↓ 16	<0.001
MLL-3	miR-708	11q14.1	↓ 288	0.002
MLL-4	miR-192	11q13.1	↓ 3	0.002
MLL-5	miR-196a	17q21.32;12q13.13	↑ 2283	0.002
MLL-6	miR-194	1q41; 11q13.1	↓ 3	0.002
MLL-7	miR-497	17p13.1	↓ 24	0.003
MLL-8	miR-20b	Xq26.2	↓ 6	0.003
MLL-9	miR-133a	18q11.2; 20q13.33	↑ 47	0.009
MLL-10	miR-372	19q13.41	↓ 27	0.009
TEL-AML1-1	miR-213	1q31.3	↓ 5	<0.0001
TEL-AML1-2	miR-99a	21q21.1	↑ 38	<0.001
TEL-AML1-3	miR-100	11q24.1	↑ 30	<0.001
TEL-AML1-4	miR-125b	11q24.1; 21q21.1	↑ 35	<0.001
TEL-AML1-5	miR-126*	9q34.3	↑ 4	<0.001
TEL-AML1-6	miR-383	8p22	↑ 1671	<0.001
TEL-AML1-7	miR-221	Xp11.3	↓ 7	<0.001
TEL-AML1-8	miR-126	9q34.3	↑ 3	<0.001
TEL-AML1-9	miR-629	15q23	↑ 4	<0.001
TEL-AML1-10	miR-361-5p	Xq21.2	↓ 2	<0.001
BCR-ABL-1	miR-93	7q22.1	↓ 2	0.015
BCR-ABL-2	miR-484	16p13.11	↓ 2	0.015
BCR-ABL-3	miR-331	12q22	↓ 2	0.023
BCR-ABL-4	miR-103	5q35.1; 20p13	↓ 2	0.023
BCR-ABL-5	miR-1226	3p21.31	↓ 2	0.023
BCR-ABL-6	miR-345	14q32.2	↓ 3	0.024
BCR-ABL-7	miR-210	11p12	↓ 5	0.024
BCR-ABL-8	miR-301	17q22	↓ 3	0.024
BCR-ABL-9	miR-324-5p	17p13.1	↓ 2	0.024
BCR-ABL-10	miR-148b	12q13.13	↓ 2	0.024
E2A-PBX1-1	miR-126*	9q34.3	↓ 28	0.001
E2A-PBX1-2	miR-126	9q34.3	↓ 34	0.001
E2A-PBX1-3	miR-146a	5q33.3	↓ 14	0.001
E2A-PBX1-4	miR-545	Xq13.2	↓ 5	0.001
E2A-PBX1-5	miR-24	9q22.32; 19p13.13	↓ 4	0.001
E2A-PBX1-6	miR-29a	7q32.3	↓ 3	0.001
E2A-PBX1-7	miR-511	10p12.33	↓ 706	0.001

Continuation of Table 1. Most discriminative miRNAs per subtype of pediatric ALL.

top#	miRNA	chromosomal location	fold change	FDR-corrected p-value
E2A-PBX1-8	miR-365	16p13.12; 17q11.2	↓ 6	0.001
E2A-PBX1-9	miR-30d	8q24.22	↓ 2	0.001
E2A-PBX1-10	miR-193a	17q11.2	↓ 8	0.001
hyperdiploid-1	miR-223	Xq11.2	↑ 4	<0.0001
hyperdiploid-2	miR-222*	Xp11.3	↑ 5	<0.0001
hyperdiploid-3	miR-98	Xp11.22	↑ 3	<0.0001
hyperdiploid-4	miR-511	10p12.33	↑ 7	<0.0001
hyperdiploid-5	miR-222	Xp11.3	↑ 4	<0.001
hyperdiploid-6	miR-660	Xp11.23	↑ 3	<0.001
hyperdiploid-7	miR-361-3p	Xq21.2	↑ 3	<0.001
hyperdiploid-8	miR-374a	Xq13.2	↑ 3	<0.001
hyperdiploid-9	miR-532-5p	Xp11.23	↑ 3	<0.001
hyperdiploid-10	miR-501-5p	Xp11.23	↑ 3	<0.001
T-ALL-1	miR-191	3p21.31	↓ 3	<0.0001
T-ALL-2	miR-190	15q22.2	↑ 6	<0.0001
T-ALL-3	miR-151	8q24.3	↓ 48	<0.0001
T-ALL-4	miR-425-5p	3p21.31	↓ 4	<0.0001
T-ALL-5	miR-222*	Xp11.3	↓ 8	<0.0001
T-ALL-6	miR-542-5p	Xq26.3	↑ 234	<0.0001
T-ALL-7	miR-708	11q14.1	↓ 3884	<0.0001
T-ALL-8	miR-132	17p13.3	↓ 6	<0.001
T-ALL-9	miR-425-3p	3p21.31	↓ 3	<0.001
T-ALL-10	miR-342-3p	14q32.2	↑ 3	<0.001

The ten most differentially expressed miRNAs are listed for each ALL subtype on the basis of multiple testing (FDR)-corrected p-value. The fold-change and p-value are based on the comparison between expression levels of the specified precursor B-ALL subtype and remaining precursor B-ALL cases (e.g. TEL-AML1-positive versus non-TEL-AML1 precursor B-ALL) or between precursor B-ALL and T-lineage ALL cases. miRNA-targeted genes are listed in Online Supplementary Table S7. * refers to the star strand of the miRNA duplex that is (partly) complementary to the mature miRNA.

between expression levels of clustered miRNAs is not restricted to the *MLL* and *E2A-PBX1* subtypes, since in all ALL cases a strong association was also observed for miRNA genes within clusters miR-15a-16 (Rs=0.7; $P<0.001$), miR-17-92 ($0.3\leq Rs\leq 0.9$; $P<0.01$), miR-106b-25 ($0.7\leq Rs\leq 0.8$; $P<0.001$), miR-106a-363 ($0.3\leq Rs\leq 0.9$; $P<0.01$), miR-221-222 (Rs=0.8; $P<0.001$) and miR-371-373 cluster ($0.6\leq Rs\leq 0.8$; $P<0.001$, Online Supplementary Table S6).

In Online Supplementary Table S7 we have listed target genes that were biologically proven (known from literature) for the miRNAs that were found to be differentially expressed in different subtypes of ALL. Two proven targets of the let-7 family are *RAS* and *c-MYC*.

Interestingly, mRNA levels of *RAS* and *c-MYC* were 3- to 5-fold upregulated in *MLL*-rearranged compared to non-*MLL* precursor B-ALL (P<0.0001, Online Supplementary Figures S6 and S7)[28]. The elevated mRNA expression levels were associated with a 2-fold upregulation of c-Myc protein (P<0.05) in *MLL*-rearranged patients but not with an upregulation of Ras protein as measured by reverse phase protein detection (M.W.J. Luijendijk, manuscript in preparation, Online Supplementary Figures S6 and S7).

Distinct miRNA profiles in drug sensitive and resistant patients and their association with clinical outcome in pediatric ALL

For 61 out of the 70 patients with precursor B-ALL included in this study, the *in vitro* sensitivity to prednisolone, vincristine, L-asparaginase and daunorubicin was determined. Online Supplementary Table S8 summarizes the expression levels of 397 miRNAs for drug sensitive and resistant patients. Sixteen miRNAs were discriminative for resistance to one or more drugs (P_{FDR}<0.05). No miRNAs were associated with prednisolone resistance (P_{FDR}>0.05), only miR-454 was 1.9-fold lower expressed in L-asparaginase resistant cases (P_{FDR}=0.017) whereas 20 miRNAs were discriminative for vincristine and/or daunorubicin resistance (P_{FDR}<0.05). Table 2 and Online Supplementary Table S9 show the fold-change for discriminative miRNAs and their potential target genes, respectively. The 14 to 25-fold upregulation of miR-125b, miR-99a and miR-100 in patients resistant to vincristine (P_{FDR}≤0.002) and daunorubicin (P_{FDR}<0.05) is most striking (Figure 2). Remarkably, 12 out of 31 (39%) and 12 out of 29 (41%) patients resistant to vincristine and daunorubicin carried the *TEL-AML1* translocation. Cox proportional hazard analysis revealed that the expression level of 31 miRNAs significantly correlated with the probability of disease-free survival (P<0.05; Table 3). The prognostic value was independent of ALL subtype for 14 miRNAs: six miRNAs (i.e. miR-33, -215, -369-5p, -496, -518d, and -599) were associated with an unfavorable long-term clinical outcome (hazard ratio 1.30–1.52 with 95%CI 1.01–2.04; 0.003≤P≤0.046) whereas eight miRNAs (i.e. miR-10a, -134, -214, -484, -572, -580, -624 and -627) were linked to a favorable prognosis (hazard ratio: 0.59–0.82 with 95%CI 0.41–0.99; 0.004≤P≤0.045). Online Supplementary Figure S8 illustrates the disease-free survival curves for each of these 14 prognostic miRNAs in pediatric ALL. Next, ALL patients were divided in two groups based on the median cumulative score that combined the expression levels of these 14 miRNAs (Figure 3, see Design and Methods for details on the cumulative score). Kaplan-Meier analysis indicated that the profile obtained by combining these 14 miRNAs further disseminated the prognosis; patients with a favorable miRNA expression profile had a 5-year disease-free survival of 89.4%±7% (n=41) compared to 60.8±12% (n=37) for patients with a less favorable miRNA profile (P=0.001).

Table 2. MiRNAs that are differentially expressed between drug sensitive and resistant precursor B-ALL patients.

top#	miRNA	chromosomal location	fold change	FDR corrected p-value
VCR-1	miR-125b	11q24.1; 21q21.1	↑ 25	0.001
VCR-2	miR-99a	21q21.1	↑ 21	0.002
VCR-3	miR-100	11q24.1	↑ 14	0.002
VCR-4	miR-629	15q23	↑ 3	0.031
VCR-5	miR-126*	9q34.3	↑ 3	0.032
VCR-6	miR-126	9q34.3	↑ 3	0.046
VCR-7	miR-9	1q22; 5q14.3; 15q26.1	↑ 2	0.032
VCR-8	miR-625	14q23.3	↓ 2	0.006
VCR-9	miR-141	12p13.31	↓ 2	0.021
VCR-10	miR-200c	12p13.31	↓ 2	0.001
DNR-1	miR-383	8p22	↑ 250	0.037
DNR-2	miR-99a	21q21.1	↑ 21	0.033
DNR-3	miR-125b	11q24.1; 21q21.1	↑ 20	0.033
DNR-4	miR-100	11q24.1	↑ 19	0.041
DNR-5	miR-203	14q32.33	↑ 4	0.041
DNR-6	let-7c	21q21.1	↑ 4	0.033
DNR-7	miR-126	9q34.3	↑ 3	0.033
DNR-8	miR-126*	9q34.3	↑ 3	0.033
DNR-9	miR-335	7q32.2	↑ 3	0.033
DNR-10	miR-199b*	9q34.11	↑ 2	0.033
ASP-1	miR-454	17q22	↓ 2	0.017
ASP-2	–	–	–	–
ASP-3	–	–	–	–
ASP-4	–	–	–	–
ASP-5	–	–	–	–
ASP-6	–	–	–	–
ASP-7	–	–	–	–
ASP-8	–	–	–	–
ASP-9	–	–	–	–
ASP-10	–	–	–	–

miRNAs that are differentially expressed between patients sensitive and resistant for vincristine (VCR), daunorubicin (DNR) and L-asparaginase (ASP) are shown and ranked on the basis of the fold change in expression level. Fold-change is the ratio in expression level between resistant and sensitive patients. ↓ represents downregulation and ↑ refers to upregulation in resistant cells.
* points to the star strand of the miRNA duplex that is (partly) complementary to the mature miRNA. miRNA-targeted genes are listed in Online Supplementary Table S9.

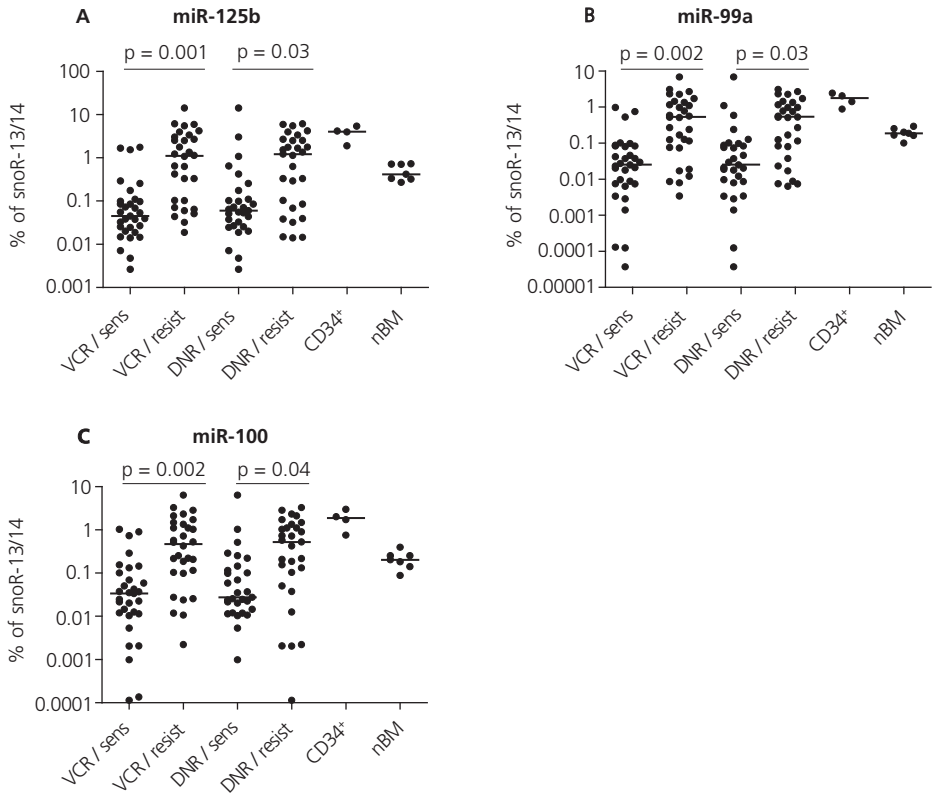

Figure 2. Discriminative expression of miR-125b, miR-99a and miR-100 between drug sensitive and resistant precursor B-ALL patients.

Expression levels relative to snoRNA are shown for miR-125b (**A**), miR-99a (**B**) and miR-100 (**C**). Dots represent individual samples of CD34+ selected cells (n=4), normal BM samples (nBM, n=7) and the following precursor B-ALL patients: vincristine sensitive (n=31; VCR/sens) and resistant (n=30; VCR/resist) or daunorubicin sensitive (n=29; DNR/sens) and resistant (n=29; DNR/resist). Lines indicate median expression level in each group. The indicated *P*-value is corrected for multiple-testing (FDR-corrected *P*-value).

Table 3. miRNAs associated with clinical outcome in pediatric ALL.

	uncorrected for ALL subtype		corrected for ALL subtype			
no.	**miRNA**	**p-value**	**miRNA**	**hazard ratio**	**confidence interval**	**p-value**
1	miR-10a	0.018	miR-10a	0.82	0.69-0.97	0.020
2	miR-23b	0.019	–	–	–	–
3	miR-27b	0.009	–	–	–	–
4	miR-33	0.025	miR-33	1.32	1.02-1.69	0.030
5	miR-99a	0.023	–	–	–	–
6	miR-107	0.028	–	–	–	
7	miR-125b	0.031	–	–	–	–
8	miR-126*	0.042	–	–	–	–
9	miR-134	0.025	miR-134	0.73	0.56-0.96	0.026
10	miR-193a	0.014	–	–	–	–
11	–	–	miR-214	0.73	0.59-0.90	0.004
12	–	–	miR-215	1.30	1.01-1.67	0.042
13	miR-219	0.033	–	–	–	–
14	miR-223	0.046	–	–	–	–
15	miR-335	0.034	–	–	–	–
16	miR-369-5p	0.049	miR-369-5p	1.30	1.01-1.67	0.046
17	miR-371	0.035	–	–	–	–
18	miR-372	0.040	–	–	–	–
19	miR-373	0.044	–	–	–	–
20	miR-449	0.043	–	–	–	–
21	miR-484	0.009	miR-484	0.81	0.69-0.94	0.008
22	–	–	miR-496	1.52	1.15-2.00	0.003
23	miR-518d	0.047	miR-518d	1.43	1.01-2.04	0.046
24	miR-572	0.010	miR-572	0.59	0.41-0.85	0.004
25	–	–	miR-580	0.81	0.65-0.99	0.045
26	–	–	miR-599	1.39	1.01-1.89	0.044
27	–	–	miR-624	0.79	0.67-0.93	0.006
28	miR-627	0.028	miR-627	0.68	0.49-0.93	0.019
29	let-7c	0.032	–	–	–	–
30	let-7d	0.048	–	–	–	–
31	let-7d*	0.014	–	–	–	–

Cox proportional hazard model (uncorrected and corrected for ALL subtypes) was applied to identify which miRNAs were related to the clinical outcome of children with ALL. MiRNA expression levels were considered as continuous variables and relapse as an event.
Hazard ratio represents the change in risk of relapse upon each 2-fold increase in miRNA expression level.

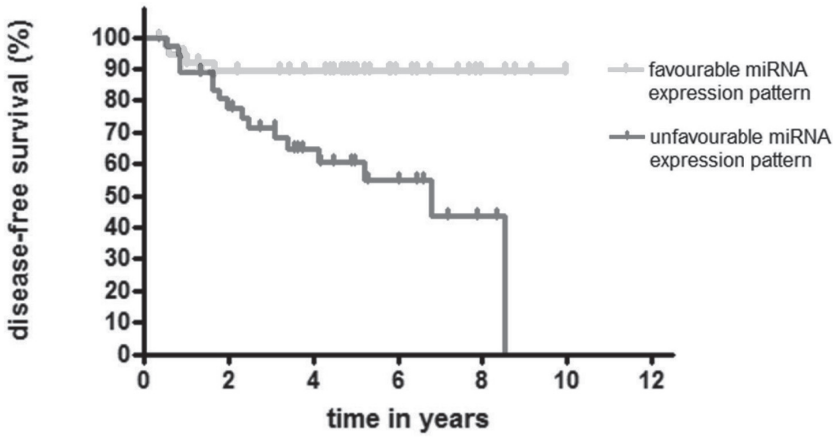

Figure 3. Prognostic value of a miRNA expression profile in newly diagnosed pediatric ALL. Kaplan-Meier estimates for the probability of disease-free survival of a miRNA expression profile are shown. This profile comprises the combined score of 14 miRNAs which were, independently of subtype, predictive for the clinical outcome of newly diagnosed pediatric ALL cases (see Table 3). See Materials and Methods section for details on how this profile was generated. Basically, patients were assigned a score 1 if the expression level of a given miRNA was associated with a good prognosis and patients were assigned a score 2 if this expression level was associated with a poor prognosis. Next, the sum of individual scores of 14 miRNAs was taken which resulted in a minimum score of 14 and a maximum score of 28. Patients were divided in two groups based on the median score (of 21) in 78 patients and Kaplan-Meier estimates were calculated. *P*=0.001, Cox proportional hazard analysis.

DISCUSSION

In this study we analyzed the expression levels of 397 miRNAs including recently cloned new miRNAs in pediatric ALL by highly specific, stem-looped RT-qPCR miRNA assays. The results demonstrated that different genetic subtypes of ALL and drug resistant cases have unique miRNA expression profiles and selected miRNAs were associated with clinical outcome of ALL patients.

Hierarchical clustering of 325 miRNAs that are expressed in ALL cells identified T-ALL, *MLL*-rearranged ALL, *TEL-AML1, E2A-PBX1* and hyperdiploid ALL-specific miRNA expression patterns (Figure 1). *BCR-ABL* and B-other ALL subtypes were not distinguishable as separate groups. This indicates that *BCR-ABL* and B-other cases are more heterogeneous than other subtypes and/or that miRNAs play a minor role in both subtypes. This is also reflected by the lower number of miRNAs that were significantly differentially expressed as well as the lower fold-change in expression levels found for miRNAs expressed in *BCR-ABL* and B-other subtypes compared to other (i,e. non-*BCR-ABL* and non-B-other) subtypes (Online Supplementary Table S4 and Table 1). Correspondingly, we and others found that the *BCR-*

ABL1 and B-other subtype are also difficult to classify based on mRNA expression levels[20,29,30]. Since samples used in both miRNA and mRNA studies contained >90% leukemic cells, the confounding effect of contaminating normal cells is kept to a minimum. This suggests that additional heterogeneity among the *BCR-ABL* and B-other samples prevents the finding of a characteristic miRNA-profile for each subtype. This explanation is strengthened by the fact that we recently identified a new subgroup of pediatric ALL among B-other cases which was characterized by abnormalities in B-cell differentiation genes[20].

MiR-196b, capable of promoting proliferation and survival of hematopoietic cells[31] was upregulated in *MLL*-rearranged ALL and T-ALL patients (Online Supplementary Table S2) which may be driven by activated *HOXA* genes in these patients[32]. In addition, DNA hypomethylation of the *miR-196b*-embedded *HOXA* area may contribute to the upregulation of the miRNA in *MLL*-rearranged precursor B-ALL patients[32]. As the upregulation is specific to the above mentioned ALL subtypes, these data imply that the expression and thus biological role of microRNAs depend on the type of cells (cellular context) in which they are expressed. The question we here addressed was to explore which miRNAs are discriminative for different genetic subtypes of pediatric ALL.

We observed two different groups of *TEL-AML1* patients based upon miRNA expression profiles (Figure 1). One group had a miRNA expression pattern which was most similar to that of hyperdiploid patients. *TEL-AML1* and hyperdiploid ALL are characterized by a high expression of several miRNAs including miR-126/126* (Table Online Supplementary S2), miR-151 and miR-545 (Figure 1). It was previously described that miR-126 and miR-126* inhibit apoptosis, stimulate cell viability in acute myeloid leukemia cells and enhances proliferation of mouse bone marrow progenitor cells when upregulated[33]. Also at mRNA level, overlapping gene expression profiles can be detected, supporting the hypothesis that *TEL-AML1* and hyperdiploid ALL have more in common than previously appreciated[20]. Albeit without prognostic difference, the second group of *TEL-AML1*-positive patients showed different miRNA expression patterns than the hyperdiploid patients. The level of miR-383, miR-125b, miR-100, miR-99a and let-7c expression was increased by 5- to 1670-fold in *TEL-AML1* patients whereas a 3- to 24-fold upregulation of miR-222/222*, miR-223, miR-511, and miR-660 was found in hyperdiploid cases only (Table 1). It should be noted that the expression level of miR-222/222*, -223, -511 and -660 in hyperdiploid cases can not simply be explained by a gene-dosage effect due to an extra copy of chromosome 10 and X characteristic for hyperdiploidy. MiRNAs specific for either hyperdiploid or *TEL-AML1*-positive ALL are of interest because they may point to mechanisms by which *TEL-AML1*-positive and hyperdiploid ALL diverge from the common underlying biology.

We only found a limited number of miRNAs associated with cellular resistance to vincristine, daunorubicin and L-asparaginase and none with resistance to prednisolone in pediatric ALL (Table 2). *In vitro* resistance to these drugs is associated with an unfavorable outcome in pediatric ALL[11-14]. Remarkably, 39 percent of the vincristine resistant patients

were of the *TEL-AML1* subtype, which supports earlier observations that TEL-AML1-positive patients are resistant to vincristine[34,35]. Interestingly, *TEL-AML1*-positive cases often show sensitivity to L-asparaginase in combination with resistance to vincristine[35]. We here observed that *TEL-AML1*-positive patients and vincristine resistant cases both expressed higher levels of miR-125b, which deserves further study since this may be linked to the vincristine-resistant/L-asparaginase sensitive discordant type of *TEL-AML1*-positive ALL. Overexpression of miR-125b reduced the amount of drug-induced apoptosis in preB-cells[36] and induced proliferation in CD34+ cells[37,38]. Therefore, interference with miR-125b function might point to a way to sensitize patients to these drugs.

The expression profile based on 14 miRNAs was predictive for the prognosis of ALL (Table 3 and Figure 3). None of these miRNAs were linked to the occurrence of central nervous relapses in pediatric ALL as previously reported[39]. We observed that, among others, miR-10a, miR-134 and miR-214 were linked to a favorable outcome in pediatric ALL, which may correspond to their described tumor suppressor activity by driving apoptosis (miR-10a)[40], inhibiting cell proliferation (miR-10a and miR-214)[41,42], and downregulating oncogene *SOX2* (miR-134)[43]. The expression of all three miRNAs was significantly lower (3- to 600-fold) in precursor B-ALL than in normal bone marrow or CD34+ cells (Online Supplementary Figure S4). Six miRNAs were associated with an unfavorable prognosis including miR-33, which was significantly upregulated in T-ALL compared to normal thymocytes (Online Supplementary Figure S4). Besides their prognostic value, these miRNAs may also serve as targets for new therapies. We recently discovered that downregulation of miR-10a in *MLL*-rearranged patients, due to DNA hypermethylation, could be reversed and brought back to normal levels by treatment with a demethylating agent[44]. In contrast, antagomirs could be chosen to downregulate the expression of aberrantly overexpressed miRNAs with unfavorable prognostic significance, such as miR-33.

In conclusion, we found that different genetic subtypes have unique miRNA expression profiles which point to several miRNAs with potential oncogenic and tumor suppressive activity in ALL. Moreover, our study indicates that specific miRNAs are associated with resistance to vincristine, daunorubicin and L-asparaginase, but not with resistance to prednisolone. Finally, expression levels of specific miRNAs correlate with outcome of pediatric ALL patients. Collectively, our data form a comprehensive repository of miRNAs being aberrantly expressed in genetic subtypes of ALL that can be used to explore the functional role of miRNAs in the corresponding types of pediatric ALL.

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Chapter 6

Expression of miR-196b is not exclusively *MLL*-driven but especially linked to activation of *HOXA* genes in pediatric acute lymphoblastic leukemia

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ABSTRACT

Background

Deregulation of microRNAs (miRNAs) may contribute to hematopoietic malignancies. MiR-196b is highly expressed in *MLL*-rearranged leukemia and has been shown to be activated by *MLL* and *MLL*-fusion genes.

Design and Methods

In order to test whether high expression of miR-196b is restricted to *MLL*-rearranged leukemia, we used quantitative stem-loop reverse transcriptase polymerase chain reaction (RT-qPCR) to measure the expression of this miRNA in 72 selected cases of pediatric acute lymphoblastic leukemia (ALL) i.e. *MLL*-rearranged and non-*MLL*-rearranged precursor B-ALL and T-ALL patients. We also determined the expression of *HOXA*-genes flanking *miR-196* by microarray and RT-qPCR. Furthermore, we used CpG island-arrays to explore the DNA methylation status of *miR-196b* and *HOXA*.

Results

We demonstrated that high expression of miR-196b is not unique to *MLL*-rearranged ALL but also occurs in T-ALL patients carrying *CALM-AF10*, *SET-NUP214* and inversion of chromosome 7. Like *MLL*-rearrangements, these abnormalities have been functionally linked with upregulation of *HOXA*. In correspondence, miR-196b expression in these patients correlated strongly with levels of *HOXA* family genes (Spearman's correlation coefficient (Rs) ≥ 0.7, $P \leq 0.005$). Since *miR-196b* is encoded on the *HOXA* cluster, these data suggest co-activation of *miR-196b* and *HOXA* genes in ALL. Upregulation of miR-196b coincides with reduced DNA methylation at CpG islands in the promoter regions of *miR-196b* and the entire *HOXA* cluster in *MLL*-rearranged cases compared with non-*MLL* precursor B-ALL and normal bone marrow cases ($P<0.05$), suggesting an epigenetic origin for miR-196b overexpression. Although *MLL*-rearranged ALL cases are highly resistant to prednisolone and L-asparaginase, this resistance was not attributed to miR-196b expression.

Conclusions

High expression of miR-196b is not exclusively *MLL*-driven but can also be found in other types of leukemia with aberrant activation of *HOXA* genes. Since miR-196b has been shown by others to exert oncogenic activity in bone marrow progenitor cells, our observations here imply a potential role for miR-196b in the underlying biology of all *HOXA*-activated leukemias.

INTRODUCTION

MicroRNA (miRNAs) were discovered to be small non-protein coding RNA molecules that post-transcriptionally regulate the expression of many protein-coding genes by complementary binding to their targeted mRNA[1]. Subsequently, the bound mRNA is cleaved or, like in the majority of cases, its translation into protein is repressed2. Despite their name, miRNAs play major roles in biological processes such as proliferation, differentiation and apoptosis and, consequently, aberrant activities of miRNAs have been found in a variety of malignancies. For example, let-7 has been identified as a tumor suppressor since let-7 downregulated the expression of oncoprotein Ras. Consequently, reduced expression of let-7 miRNA was associated with an increased expression of Ras in lung cancer patients, which may explain their unfavorable prognosis[3,4].

Recent studies also showed that aberrantly expressed miRNAs contribute to hematopoietic malignancies. For example, overexpression of the oncogenic miR-17-92 polycistron accelerated the formation of lymphomas in an *Eμ-Myc* transgenic mice model[5]. This miR-17-92 cluster is often amplified in human B-cell lymphomas, suggesting a role in lymphomagenesis[6]. Enforced expression of miR-155 in *Eμ-miR-155* transgenic mice resulted in preleukemic pre-B cell proliferation followed by mature B-cell leukemia, implying a leukemogenic contribution of miR-155[7]. Epigenetic silencing of miR-124a caused an upregulation of its target CDK6 in acute lymphoblastic leukemia (ALL) that may drive proliferation of leukemia cells and is associated with higher relapse and mortality rates among patients with ALL[8].

In general, the 5-year disease free survival (DFS) is 85% for children with ALL on contemporary treatment protocols. However, some subtypes such as cases with a rearrangement of the *Mixed Lineage Leukemia* (*MLL*) gene and *BCR-ABL1*-positive ALL have an unfavorable 5-year DFS of ~50%[9-11]. We previously observed aberrant miRNA expression patterns in different genetic subtypes of pediatric ALL[12]. One of the miRNAs that was most aberrantly expressed in *MLL*-rearranged cases was miR-196b. The expression level of miR-196b was 500-800 fold upregulated in the majority of *MLL*-rearranged precursor B-ALL cases and in about one-third of selected T-ALL cases compared with precursor B-ALL patients without *MLL* translocations. In addition, the expression in these leukemic cases was also elevated compared with normal bone marrow cells[12]. Interestingly, a recent report postulated that miR-196b may be involved in leukemogenesis and that its expression is induced by normal MLL and MLL fusion products such as MLL-AF4[13]. The MLL-AF4 fusion is most frequently found in infants with *MLL*-rearranged leukemia[14], which were included in our previous study[12]. MLL normally regulates the expression of the homeobox domain (*HOX*) gene family which plays an important role in regulating normal hematopoiesis[15]. The *HOXA* cluster genes, and especially *HOXA4*, *HOXA5,HOXA9* and *HOXA10,* are overexpressed in *MLL*-rearranged ALL[16-18]. However, aberrant expression of *HOXA* genes is not restricted to *MLL*-rearranged precursor B-ALL cases and has also been reported for T-ALL patients carrying *MLL*- or *HOXA*-

rearrangements e.g. inversion of chromosome 7, or fusion products including CALM-AF10 and SET-NUP214[19,20]. As *miR-196b* is mapped between the *HOXA9* and *HOXA10* genes on chromosome 7p15.2, the expression level of miR-196b may be linked to *HOXA* gene transcription, irrespective of the *HOXA* activating mechanism (either MLL fusions or other factors). To test this hypothesis, the expression levels of miR-196b and *HOX* gene-family members were measured in *MLL*-rearranged and non-*MLL*-rearranged precursor B-ALL and T-ALL cases carrying different genetic abnormalities leading to *HOXA* gene activation. In addition, expression levels of miRNAs may be regulated by gene methylation[21] and we therefore investigated the methylation status upstream of the *miR-196b* locus in *MLL*-rearranged cases. Since *MLL*-rearranged ALL cases are often highly resistant to prednisolone and L-asparaginase[22], two drugs that form major components of current ALL treatment, we also investigated whether miR-196b expression levels were linked to responsiveness to both drugs.

DESIGN AND METHODS

Patient samples

Leukemic cell samples of children with newly diagnosed ALL were obtained after informed consent of parents or guardians and approval by the institutional review board. The immunophenotype was determined by flow cytometry (T-ALL or precursor B-ALL), and the genetic subtype by fluorescent *in situ* hybridization (FISH) and/or reverse transcriptase PCR[11,20]. In total 12 *MLL*-rearranged precursor B-ALL i.e. five patients carrying t(4;11), six patients carrying t(11;19) and one was positive for t(9;11), 38 non-*MLL* precursor B-ALL and 22 T-ALL cases were included. The *HOXA*-linked T-ALL subgroup consisted of selected cases characterized by the fusion genes *MLL-AF6* (n=2), *CALM-AF10* (n=5) and *SET-NUP214 (*n=*3)* as well as one case with an inversion of chromosome 7 [inv(7)(p15q35)]. The *HOXA*-negative T-ALL group consisted of *TAL/LMO*-rearranged (n=4), *TLX3*-rearranged (n=2) and T-ALL cases negative for above-mentioned abnormalities (n=5). Mononuclear cells were isolated from bone marrow or peripheral blood samples using sucrose density centrifugation[23,24]. The percentage of leukemic cells was determined by May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. If the percentage was below 90%, samples were enriched by eliminating non-malignant cells with immunomagnetic beads[23,24].

Quantitative stem-loop real-time polymerase chain reaction analysis of miRNA and *HOXA* expression levels

Total RNA was extracted with TRIzol reagent (Invitrogen, Leek, the Netherlands) according to the manufacturer's guidelines with minor modifications as described before[25]. The 2100 bioanalyzer (Agilent, Amstelveen, the Netherlands) was used to determine the quality of

total RNA. All RNA samples had an RNA integrity number of ≥7.5. MiR-196b expression was measured by real-time quantitative PCR (RT-qPCR) using a specific stem-loop primer and probe combination designed by Applied Biosystems, USA[26]. Endogenous small nucleolar RNA 1 (RNU24) was used as reference for small RNA-input. Expression levels of *HOXA3*, *HOXA9* and *HOXA10* transcripts were quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using cDNA synthesized from total RNA as described previously[20]. Primer and probe sequences are depicted in Online Supplementary Table S1. All RT-qPCR reactions were performed on an Applied Biosystems 7900HT system. Details on sample preparation, primers, probes and real-time procedure are given in the Online Supplementary Design and Methods.

Gene expression microarray analysis

Affymetrix U133A and U133 plus 2.0 GeneChips (Santa Clara, CA, USA) were used to determine the expression of all *HOXA*, *HOXB* and *HOXC*-family genes in pediatric ALL cases, according to manufacturer's guidelines. Data extraction and normalization procedures of the 22,283 probe sets that both arrays have in common have been extensively described elsewhere[23]. The data collected are part of a larger data set which has been deposited in NCBI's Gene Expression Omnibus (GEO)[27] and are accessible via GEO numbers GSE13351 and GSE13425[23].

Assessment of methylation status

The methylation status of *miR-196b* and the *HOXA* cluster was assessed by the Differential Methylation Hybridization (DMH) procedure using 244K CpG island microarrays (Agilent Technologies, Santa Clara, USA). The microarray labeling and hybridization procedures were performed according to Yan et al[28] as described elsewhere[29]. The high-resolution microarrays contain 243,497 60-mer oligonucleotide probes, including numerous CpG island probes related to miRNAs. For the present study, the probes containing multiple CpG islands located at chromosome 7p15 in the 5' promoter region of the *miR-196b* and *HOXA* cluster genes were used. We used as common reference a genomic DNA pool derived from 10 healthy individual (five males and five females, Promega Benelux BV, Leiden, the Netherlands). Data extraction was performed using Agilent Feature Extraction 9.5.3 software. Subsequently, data normalization and assessment of differential methylation was performed in the R and Bioconductor Statistical environment as previously described[29]. Methylation data are presented as ratios of patient signal divided by the common reference signal. For nine *MLL*-rearranged cases, both miRNA methylation and matching miRNA expression levels were measured. Unprocessed genome-wide DNA methylation data was uploaded in the NCBI Gene Expression Omnibus under the GEO Series accession number GSE18400 as part of a previous study[29].

Drug resistance-assay

Responsiveness to the drugs prednisolone or L-asparaginase was determined by a 4-day *in vitro* methyl thiazolyl tetrazolium (MTT) drug resistance assay as described before[30,31]. The concentration ranges tested were 0.008-250 µg/ml for prednisolone and 0.003-10 IU/ml for L-asparaginase. The concentration of prednisolone or L-aspariginase that was lethal to 50% of the ALL cells (LC_{50}) was taken as a measure for the cellular drug resistance. These LC_{50} values are known to be predictive for clinical outcome[30] and are used to adapt treatment regimen[32,33].

Statistics

Differences in distribution of variables between groups of patients were analyzed by the Mann-Whitney *U* (MWU) test. Correlation between miRNA and mRNA levels was determined using the Spearman's correlation coefficient (Rs). *P*-values were two-tailed and considered statistically significant when less than 0.05.

RESULTS

The expression levels of miR-196b were measured in 72 pediatric ALL cases at diagnosis. Figure 1 shows that miR-196b is highly expressed in 9 out of 12 *MLL*-rearranged ALL cases and in 14 out of 22 T-ALL patients. In particular, all *CALM-AF10* (n=5), *MLL-AF6* (n=2), *SET-NUP214* (n=3) and inv(7) (n=1) positive T-ALL cases showed high expression levels of miR-196b comparable to the levels found in *MLL*-rearranged cases (Figure 1). Since these specific chromosomal abnormalities are linked to the activation of *HOXA* genes[19,20] and since *miR-196b* is mapped between *HOXA9* and *HOXA10*, we quantified the expression of the *HOXA9* and *HOXA10* transcripts by RT-qPCR. A strong correlation between the expression levels of miR-196b with *HOXA9* and *HOXA10* expression ($Rs \geq 0.7$, $P \leq 0.005$) was found in *MLL*-rearranged precursor B-ALL as well as in T-ALL patients (Figure 2). Figure 2 and Online Supplementary Figure S1 illustrate that patients with low expression levels of miR-196b (like non-*MLL* precursor B-ALL and the majority of T-ALL cases) also had low expression of *HOXA* genes. In those cases in which the levels of miR-196b were higher (such as the majority of *MLL*-rearranged precursor B-ALL and one third of T-ALL cases) *HOXA* levels were also elevated. The levels of *HOXA9* and *HOXA10* are also significantly correlated with each other as shown in Figure 2C and 2F ($Rs \geq 0.90$, $P < 0.0001$). These data suggest co-expression of miR-196b with *HOXA* cluster genes. To determine whether this was only restricted to the two adjacent *HOXA* genes, we also investigated the expression pattern of other *HOX*-family genes using a second technique. i.e. the Affymetrix human genome microarray platform. The results confirmed the strong correlation between the expression level of miR-196b and that of *HOXA9* ($Rs=0.8$, $P \leq 0.0001$) and *HOXA10* ($0.8 \leq Rs \leq 0.9$, $P \leq 0.0001$) and also revealed

that miR-196b levels correlated with the expression levels of nearly all other *HOXA* genes represented on the array platform (0.5<Rs<0.8 , $P < 0.05$, Figure 3A for all cases and Online Supplementary Figures S2 and S3 for *MLL*-rearranged ALL and T-ALL cases separately).

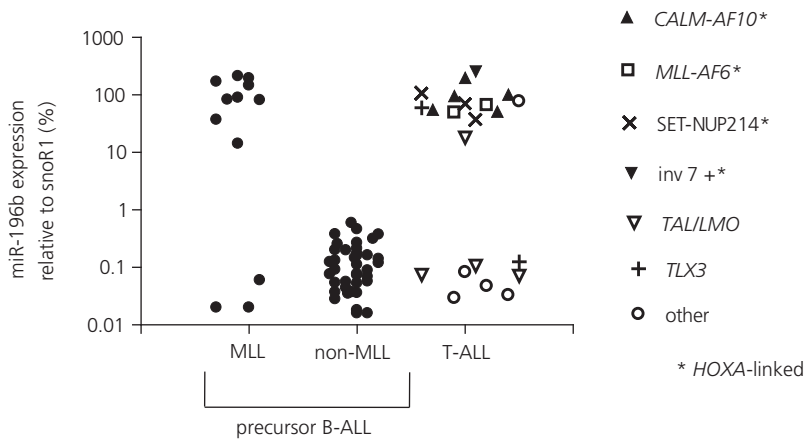

Figure 1. Expression of miR-196b in pediatric ALL.
The miR-196b level was measured in leukemic cells of 12 *MLL*-rearranged precursor B-ALL, 38 non-*MLL* precursor B-ALL and 22 T-ALL patients. * Refers to T-ALL cases that have genetic aberrations that are associated with activation of *HOXA* cluster genes. Dots represent the individual miR-196b levels as a percentage of the expression level of the endogenous reference, snoRNA-1. *MLL* versus non-*MLL* precursor B-ALL: *P*=0.003; T-ALL versus non-*MLL* precursor B-ALL: *P*=0.001).

Whereas miR-196b is encoded within the *HOXA* cluster, family member miR-196a that differs in only one nucleotide from miR-196b, is encoded by *miR-196a-1* located in the *HOXB* cluster (17q21.32) and *miR-196a-2* located in the *HOXC* cluster (12q13.13). The high homology between miR-196a and miR-196b may hamper the discriminative power of the stem-loop RT-qPCR procedure (and any other quantifying method) to determine solely miR-196b expression levels. However, no significant correlations were found between expression levels of miR-196b and those of members of the *HOXB* and *HOXC* cluster, except for *HOXB5*, *HOXB7*, *HOXB9*, and *HOXB13* (Figure 3B and 3C). Only one out of two and one out of three probe sets designed for *HOXB5* and *HOXB7* respectively, showed a significant correlation with miR-196b (0.4≤Rs≤0.6, *P*<0.05, Figure 3B). These correlations were less significant and less strong compared to the association observed between expression levels of miR-196b and family members of the *HOXA*-cluster. This suggests that miR-196b and the *HOXA* cluster are more likely to be co-transcribed than miR-196b and *HOXB* or *HOXC* family genes.

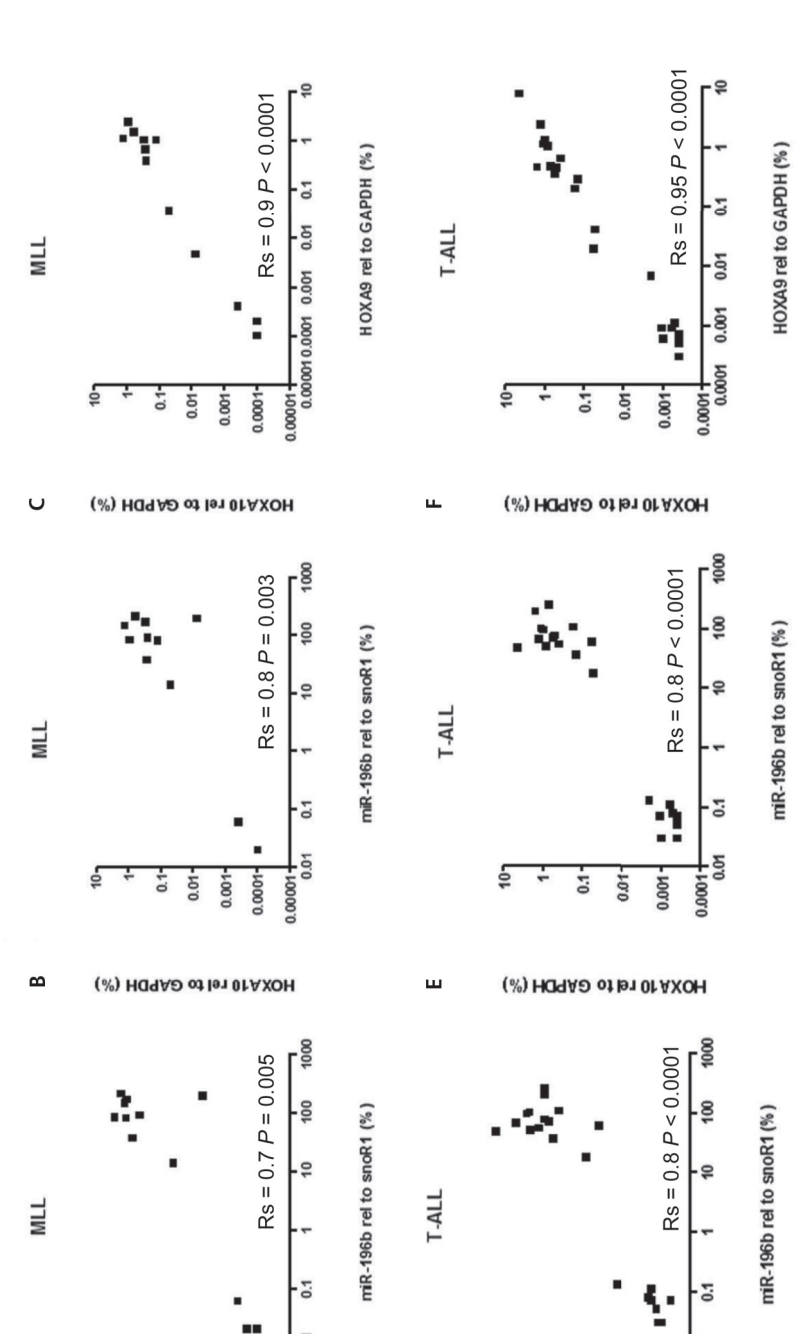

Figure 2. Correlation between expression levels of miR-196b, *HOXA9* and *HOXA10* in ALL patients.
The expression levels are compared between miR-196b and *HOXA9* (**A, D**), between miR-196b and *HOXA10* (**B, E**), as well as between *HOXA9* and *HOXA10* (**C, F**) in 12 *MLL*-rearranged precursor patients (upper panel, **A-C**) and 22 T-ALL patients (lower panel **D-F**). The expression level of miR-196b is normalized for the expression level of snoRNA-1 as measured by stem-loop RT-qPCR whereas the expression of *HOXA9* and *HOXA10* transcripts is normalized for GAPDH mRNA expression levels as measured by RT-qPCR. Rs and *P*-values are indicated in each panel.

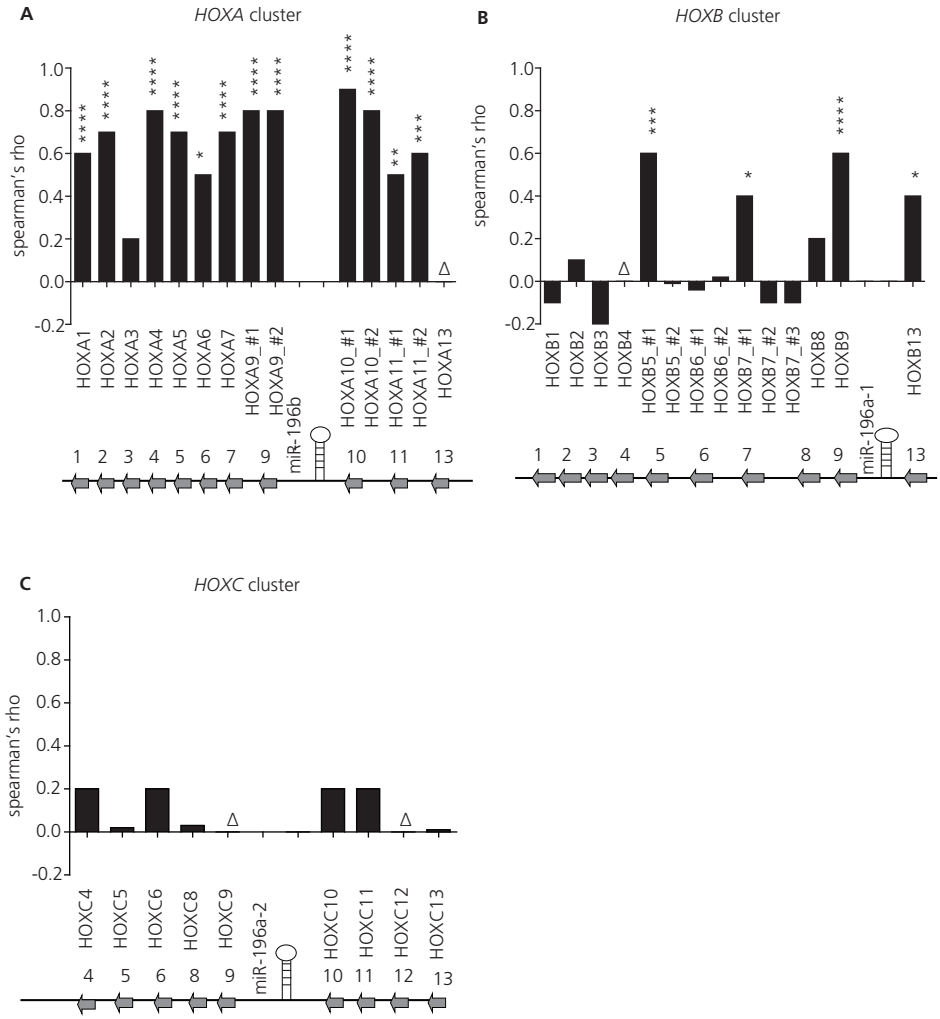

Figure 3. MiR-196b and *HOXA* cluster genes are co-transcribed in pediatric ALL.
The expression levels of miR-196b was compared with the expression of different members of the *HOXA* (**A**), *HOXB* (**B**) and *HOXC* (**C**) cluster in 12 *MLL*-rearranged B-ALL patients and 18 T-ALL patients. Spearman's correlation coefficient was calculated and plotted as bars. # 1, 2 and 3 refer to the different probe sets for the specified genes on the Affymetrix U133A platform (Online Supplementary Table S2). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Genomic location of miR-196b on 7p15.2 within *HOXA* cluster (**A**)*, miR-196a-1* on 17q21.32 within *HOXB* cluster (**B**) and *miR-196a- 2* on 12q13.13 within *HOXC* cluster (**C**) is indicated at the bottom of each graph. Δ indicates genes of which no probe sets were available on the U133A microarray.

Since the expression of miRNA genes and protein-coding genes can be affected by DNA methylation of promoter regions, we analyzed the methylation status of the 5' region upstream of *miR-196b* and the promoter region of all *HOXA* cluster genes. The methylation of the 5'region of *miR-196b* (Figure 4A) and of the entire *HOXA* cluster (Figure 4B) was reduced in *MLL*-rearranged cases compared with precursor B-ALL patients without *MLL*-translocation as well as normal bone marrow ($P \leq 0.01$ and $P < 0.05$, respectively), which may explain the increased expression levels of both miR-196b and the *HOXA* cluster in these cases.

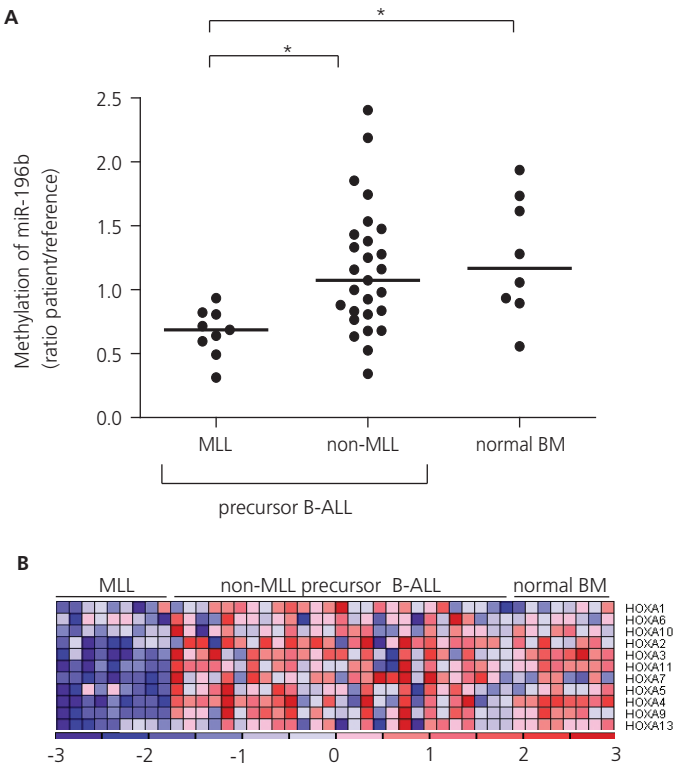

Figure 4. The promoters of *miR-196b* and *HOXA* genes have a lower level of methylation in *MLL*-rearranged precursor B-ALL patients than in non-*MLL* precursor B-ALL patients and normal bone marrow.
(**A**) Methylation status of the probe covering multiple CpG islands (y-axis) within the 5' region of *miR-196b* (27,175,990-27,176,034) was analyzed in nine *MLL*-rearranged precursor B-ALL patients, 27 non-*MLL* precursor B-ALL and eight normal bone marrow (BM) samples. Dots represent individual patients * $P \leq 0.01$. (**B**) Heatmap displaying methylation status of the *HOXA* cluster in the same patients as for Figure 4A. Columns represent patient samples and rows represent the different *HOXA* cluster genes. Relative DNA methylation levels are shown in red (high) and blue (low). Gene names are listed at the right. Heatmap was generated in GenePattern version 3.1.2[43]. *MLL* versus non-*MLL* precursor B-ALL $P < 0.0001$; *MLL*- precursor B-ALL versus normal BM $P < 0.0002$.

We also investigated whether the expression level of miR-196b was linked to sensitivity of the *MLL*-rearranged ALL and T-ALL patients to the drugs prednisolone and L-asparaginase, since resistance to these drugs is indicative for an unfavorable outcome[30,31]. Figure 5A and 5C show that the *in vitro* cytotoxicity values (LC_{50} values) for both drugs did not differ between patients with high and low expression levels of miR-196b. In correspondence, patients who were sensitive, intermediate or resistant to prednisolone or L-asparaginase did not have significantly different levels of miR-196b (Figure 5B and 5D, $P>0.05$).

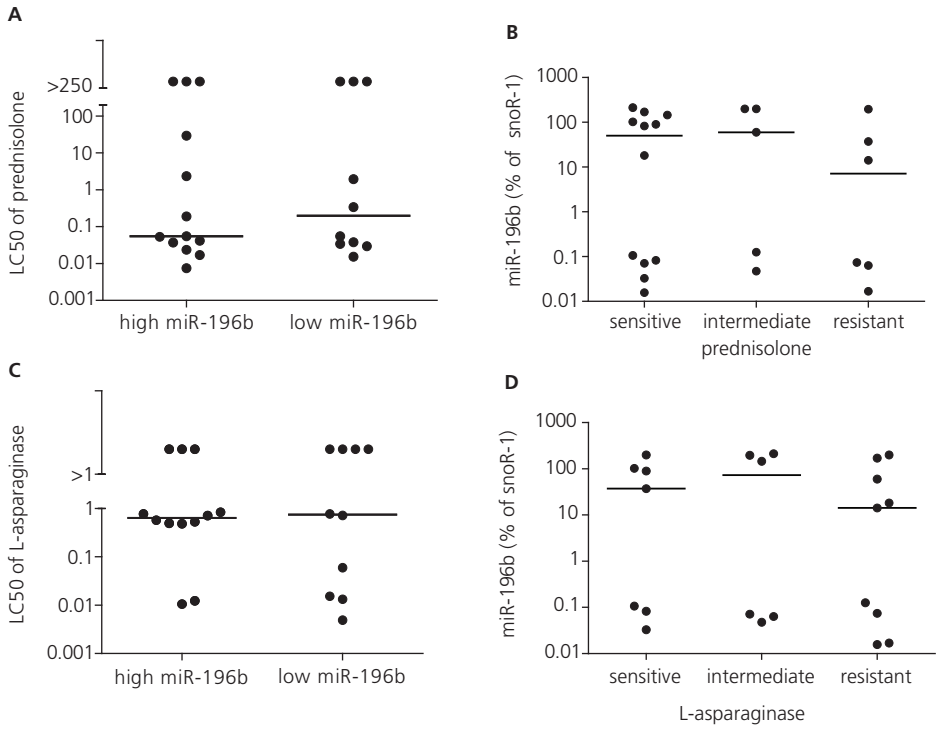

Figure 5. Expression levels of miR-196b are not associated with resistance to prednisolone and L-asparaginase in pediatric ALL cells.
In vitro cytotoxicity (represented by LC_{50} value) of prednisolone (**A**) and L-asparaginase (**C**) was measured in 11 *MLL*-rearranged precursor B-ALL and 11 T-ALL patients. Patients were separated according to their miR-196b expression level into two groups, i.e. low miR-196b (<1% of snoR-1) and high miR-196b (>1% of snoR-1). In **B** and **D** miR-196b expression is plotted against degree of resistance towards prednisolone (**B**) and L-asparaginase (**D**), i.e. sensitive, intermediate or resistant based upon previously established cut-off values[30,44]. $P>0.05$ for all comparisons.

DISCUSSION

In this study we demonstrated a strong association between the expression levels of miR-196b and genes belonging to the *HOXA* cluster in pediatric ALL. This co-transcription was not restricted to *MLL*-rearranged cases, but was also found for T-ALL cases characterized by activation of *HOXA* genes due to non-*MLL* mechanisms. Hypomethylation of CpG islands in the 5' upstream/promoter regions of miR-196b and *HOXA* cluster genes as demonstrated in *MLL*-rearranged cases may explain the high expression levels of this cluster and embedded miR-196b. *In vitro* resistance to prednisolone and L-asparaginase could not be explained by differential miR-196b expression levels.

Popovic et al reported that the expression of miR-196b is regulated by MLL and MLL fusion products[13]. In correspondence, we also observed high-level expression in *MLL*-rearranged cases. However, we also demonstrated that high expression of miR-196b is not restricted to *MLL*-rearranged cases but can also be found in patients having other cytogenetic abnormalities that are known to activate *HOXA* cluster genes, i.e. *CALM-AF10*, *SET-NUP214* and inv(7)(p15q35)-positive cases. The mechanism by which the *HOXA* cluster is transcriptionally activated may differ between these patients. It has been demonstrated that CALM-AF10, SET-NUP214 and MLL fusion recruit the DOT1L histone methyltransferase that facilitates gene transcription of the *HOXA* cluster by dimethylation of histone H3 lysine 79 residues (H3K79). The H3K79 dimethylation possibly allows further epigenetic modification that opens up the entire *HOXA* locus[20,34-36]. We here demonstrate CpG island hypomethylation of the *HOXA* cluster in *MLL*-rearranged patients suggesting that additional mechanisms that drive the *HOXA* expression may exist. Moreover, inv(7) cases have elevated *HOXA10* and *HOXA11* expression due to the rearrangement of the T-cell receptor beta locus into this region of the *HOXA* cluster[36]. Taken together these findings indicate that high expression levels of miR-196b and *HOXA* cluster genes are not exclusively *MLL*-driven but can also be due to other routes of *HOXA* locus activation. It should also be noted that not all *MLL*-rearranged cases have high expression levels of miR-196b and *HOXA* cluster genes (Figure 1 and 2). This corresponds with the fact that two distinct subgroups have been found in *MLL*-rearranged ALL that are separated based on the expression signature of *HOXA* cluster genes[17,37].

We demonstrated that the expression of miR-196b was highly correlated with most members of the *HOXA* cluster (Figure 3) whereas this correlation was less pronounced for the *HOXB* and *HOXC* cluster in pediatric ALL cells. For *HOXA3* microarray-based expression levels did not correlate with miR-196b expression levels. The lack of this correlation is due to a less optimal array-probe design for probe set 208604_s_at as exemplified by the fact that microarray and quantitative Taqman-based RT-qPCR data did not correlate for *HOXA3* whereas these data were highly correlated for other *HOXA* cluster genes such as *HOXA9* and *HOXA10* (Online Supplementary Figure S4). Since we can not rule out the possibility of such

non-optimal design for other array-probes, correlation between miR-196b and additional *HOX* genes may have been missed. However, since the miR-196b gene is positioned in between *HOXA9* and *HOXA10* and is being transcribed from the same DNA strand as the *HOXA* cluster, the high co-expression between the miRNA and *HOXA* genes suggests co-transcriptional activation. Correspondingly, the expression levels of both miR-196b and *HOXA9* are restored upon re-expression of *Mll* in *Mll*-deficient mouse embryonic fibroblasts[13]. A similar co-activation may explain the high association for miR-10a (positioned in between *HOXB4* and *HOXB5*) as well as miR-196a (encoded in between *HOXB9* and *HOXB13*) and the *HOXB* cluster as observed in acute myeloid leukemia (AML)[38,39]. Recent studies suggest that miRNAs, in general, are often expressed at lower levels in cancer cells than in their normal counterpart[40]. In case of ALL, this phenomenon may be caused by a high frequency of CpG island hypermethylation[21]. However, the fact that we observed that miR-196b and *HOXA*-genes are highly co-transcribed may indicate that this region has reduced DNA methylation. We demonstrated that *MLL*-rearranged cases display a lower level of methylation of the CpG islands in the 5' region of miR-196b and in the promoter region of the entire *HOXA* cluster than precursor B-ALL patients without *MLL*-rearrangements and healthy individuals do. Since *MLL*-rearranged ALL is characterized by hypermethylation of CpG islands across the genome[29], the hypomethylation of the *miR-196b/HOXA* region is remarkable. Whether this locus displays similar methylation status in *HOXA*-linked T-ALL cases needs to be explored.

Both *MLL*-rearranged and presumably *HOXA*-linked *CALM-AF10*-positive T-ALL patients have a poor clinical outcome[11,41]. It has been previously shown that both *MLL*-rearranged and T-ALL pediatric ALL cases are more resistant to prednisolone and L-asparaginase, as determined by an *in vitro* drug cytotoxicity assay[42]. These two drugs are extensively used in the treatment of pediatric ALL and resistance to these drugs is indicative for an unfavorable prognosis[30]. However, we did not find evidence that miR-196b contributes to resistance to these drugs since patients with high miR-196b expression were not more resistant to both drugs than patients with low miR-196b expression levels. In contrast to a role in drug responsiveness, miR-196b may have leukemogenic potential since ectopic expression of miR-196b resulted into increased proliferation and reduced differentiation of c-Kit+ bone marrow cells of mice[13].

In conclusion, we found that aberrant overexpression of miR-196b is not restricted to *MLL*-rearranged ALL cases (T-ALL or precursor B-ALL) but also occurs in T-ALL patients with other genetic abnormalities that activate the *HOXA* gene cluster. This observation is of great importance since miR-196b is known to have oncogenic activity[13] and may thus play a role in the biology underlying *HOXA*-activated precursor B-ALL and T-ALL. The high expression of miR-196b has no effect on the level of cellular responsiveness to prednisolone and L-asparaginase in pediatric ALL. The role of miR-196b in leukemogenesis and survival of *HOXA*-expressing ALL deserves further studies since targeting of miR-196b by 'antagomirs' reduced the proliferation capacity of *MLL*-rearranged bone marrow cells of mice[13].

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Chapter 7

Hypermethylation of specific microRNA genes in *MLL*-rearranged infant acute lymphoblastic leukemia: major matters at a micro scale

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ABSTRACT

MLL-rearranged acute lymphoblastic leukemia (ALL) in infants (<1 year) is the most aggressive type of childhood leukemia. To develop more suitable treatment strategies, a firm understanding of the biology underlying this disease is of utmost importance. *MLL*-rearranged ALL displays a unique gene expression profile, partly explained by erroneous histone modifications. We recently showed that t(4;11)-positive infant ALL is also characterized by pronounced promoter CpG hypermethylation. In this study, we investigated whether this widespread hypermethylation also affected microRNA (miRNA) expression. We identified 11 miRNAs that were down-regulated in t(4;11)-positive infant ALL as a consequence of CpG hypermethylation. Seven of these miRNAs were re-activated after exposure to the de-methylating agent Zebularine. Interestingly, five of these miRNAs are associated either with *MLL* or MLL fusions, and for miR-152 we found both *MLL* and DNA methyltransferase 1 (*DNMT1*) as potential targeted genes. Finally, a high degree of methylation of the miR-152 CpG island was strongly correlated with a poor clinical outcome. Our data suggests that inhibitors of methylation have a potential beyond re-expression of hypermethylated protein-coding genes in t(4;11)-positive infant ALL. In this study, we provide additional evidence that they should be tested for their efficacy in *MLL*-rearranged infant ALL in *in vivo* models.

INTRODUCTION

Hematological malignancies characterized by chromosomal translocations involving the *Mixed Lineage Leukemia (MLL)* gene are rare among children and typically affect infants (<1 year of age)[1]. Approximately 80% of infants diagnosed with acute lymphoblastic leukemia (ALL) carry such *MLL* rearrangements that independently contribute to disease aggressiveness[2]. The most frequent *MLL* translocation in infant ALL is t(4;11) fusing the N-terminus of the *MLL* gene to the C-terminus of transcription factor *AF4*, generating the oncogenic fusion protein MLL-AF4. Normally wild-type *MLL* functions as an important epigenetic regulator: a histone methyltransferase facilitating histone 3 tri-methylation at lysine 4 (H3K4me3)(3). As a result of *MLL* translocations, wild-type *MLL* becomes disrupted and its normal function is compromised by the loss of its methyltransferase domain. Instead, the *MLL* fusion proteins MLL-AF4, MLL-ENL, MLL-AF9 and MLL-AF10 recruit alternative histone methyltransferases like DOT1L leading to histone 3 di-methylation at lysine 79 (H3K79me2)[4,5]. In turn these illegitimate histone modifications lead to inappropriate activation of numerous genes, establishing a unique gene expression profile presumably favoring leukemia development[6,7].

Apart from gene activation guided by erroneous histone modifications, we recently showed that in *MLL*-rearranged infant ALL also numerous genes are inactivated or silenced as a consequence of DNA hypermethylation[8]. This is especially true for t(4;11)-positive infant ALL, in which the degree of genome-wide promoter hypermethylation appeared to be associated with an increased risk of relapse[8]. In the present study we asked whether the wide-spread hypermethylation patterns as observed in *MLL*-rearranged infant ALL could also have affected microRNA (miRNA) expression. Hypothetically, hypermethylation or silencing of miRNAs may successively lead to abnormally activated miRNA-targeted genes and hence, may further have contributed to the unique gene expression profile of *MLL*-rearranged infant ALL. To test this, we here studied the relation between two forces representing hallmark discoveries of contemporary biology: DNA methylation and miRNA expression[9].

MiRNAs are small (~22-nucleotides) RNA molecules that regulate the translation of over 60% of all human protein-coding genes[10] via transcript destabilization and/or translational repression. Increasing evidence has emerged that deregulated miRNA expression contributes to the development of various hematological malignancies[11-13]. Aberrant expression of certain miRNAs has also been observed in leukemias characterized by *MLL* translocations. For instance miR-196b was found to be overexpressed in *MLL*-rearranged infant ALL compared with other types of childhood precursor B-ALL[12], and appeared to participate in leukemogenesis by stimulating proliferation while blocking differentiation in hematopoietic progenitor cells[14]. More recently up-regulation of the entire miR-17-92 cluster was described in *MLL*-rearranged leukemias[15]. Strong binding of the *MLL* fusion protein to this miRNA cluster led to inhibition of apoptosis and promoted proliferation by regulation of the relevant target genes.

So far most studies in *MLL*-rearranged leukemia focused on miRNA overexpression[12,15,16]. However, in a recent genome-wide miRNA study we found putative tumor suppressing miRNAs, such as let-7b and miR-708, to be down-regulated in *MLL*-rearranged ALL, and to be associated with oncogene upregulation[12]. Therefore, the present study was designed to explore the silencing effects of genome-wide CpG island hypermethylation on miRNA expression, and the ability of the de-methylating agent zebularine to restore miRNA activity.

MATERIALS AND METHODS

Patient samples

We studied 22 newly diagnosed infants (<1 year) with t(4;11)-positive ALL enrolled in the international INTERFANT-99 treatment protocol(1). In order to make a valid comparison wild-type *MLL* infant ALL samples (n=10) and non-*MLL* childhood precursor B-cell ALL samples (n=10) from our cell bank were added. Mononuclear cells were isolated from bone marrow samples using sucrose density centrifugation[17]. The percentage of leukemic cells was determined by May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins, and in case blast percentages were below 90%, samples were enriched by elimination of non-malignant cells using immunomagnetic beads (Dynabeads, Dynal, ASA, Oslo, Norway). In the case of contaminating monocytes the CD14 marker was used, CD15 in the case of myeloid cells and E-1 antigen in the case of erythroid cells[18]. DNA methylation microarrays and gene expression microarrays were performed for 22 infant ALL patients. In addition CpG microarrays were performed for 10 wild-type *MLL* infant ALL samples and 10 non-*MLL* childhood precursor B-cell ALL samples. Due to restricted availability of patient material miRNA expression assays were performed for only 5 infant ALL patients. The miRNA expression assay specific for miR-152 could be performed for 13 infant ALL patients. Normal bone marrow (nBM) samples obtained from seven non-leukemic pediatric patients were included as controls. All samples were collected after approval of the institutional review board and informed consent from parents or legal guardians.

Cell line culture and zebularine treatment

SEMK2 is a subclone of the SEM cell line which is a t(4;11)-positive precursor B-ALL cell line derived from a 5-year old girl at relapse[19,20] (kindly provided by Dr. Scott Armstrong (Dana Farber Cancer Institute, Boston, MA, USA)). The cell line was maintained as a suspension culture in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen) supplemented with 10% FCS (Integro), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.125 µg/ml fungizone (Invitrogen) at 37°C in humidified air containing 5% CO_2. Cells were exposed to 100 µM of the de-methylating agent zebularine[21] for 3, 6 or 10 days whereas control samples were unexposed. Cell viability was assessed using the trypan blue exclusion method.

Isolation and purification of DNA and RNA

Genomic DNA and total cellular RNA were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The quality of the extracted DNA was assessed on 1.0% agarose gels, and the RNA integrity on a Agilent 2100 Bio-analyzer using RNA 6000 Nano Assay LabChips (Agilent, Amstelveen, the Netherlands). All RNA samples had an RNA Integrity Number (RIN) of ≥7.5.

Differential methylation hybridization for assessment of CpG island methylation

Extracted genomic DNA was used to assess the methylation status of CpG islands associated with miR-genes by the methylation-sensitive restriction enzyme-based Differential Methylation Hybridization (DMH) procedure using 244K CpG island microarrays (Agilent Technologies, Santa Clara, USA)[8]. These high-resolution CpG microarrays contain 243,497 60-mer oligonucleotide probes, including 1118 probes corresponding to 122 miR-genes encoding 113 mature miRNAs. Some of the probes represent CpG islands (embedded in between different miR-genes) associated with 2 or 3 mature miRNAs belonging to the same miRNA cluster. A genomic DNA pool derived from healthy males (n=5) and females (n=5) (Promega Benelux BV, Leiden, the Netherlands) was used as a common reference. Labeling and hybridization procedures were performed as reported previously[8]. Data extraction was performed using Agilent Feature Extraction 9.5.3 software and unprocessed genome-wide DNA methylation data was deposited in the NCBI Gene Expression Omnibus under the GEO Series accession number GSE18400 as part of our recent study on DNA methylation patterns in *MLL*-rearranged infant ALL[8]. CpG island methylation data are presented as normalized log-ratios of patient signal divided by the common reference signal.

miRNA expression analyses

Expression levels of 365 miRNAs were determined by using the Taqman MicroRNA array-platform based on stem-loop quantitative real-time PCR (stem-loop RT-qPCR, Applied Biosystems, Foster City, CA, USA)[22]. Reactions were performed according to the manufacturer's guidelines and the amplification of obtained cDNA was monitored on an ABI 7900HT Sequence Detection System (Applied Biosystems). MiRNA expression levels were determined in t(4;11)-positive infant ALL (n=5), and normal bone marrow (n=7) samples, as well as in zebularine- treated and untreated SEMK2 cells. An additional set of 13 t(4;11)-positive patients was examined for miR-152 expression by using a separate assay based on stem-loop RT-qPCR specifically for miR-152. This assay was performed in duplicate for all patients. The mean of Ct-values for snoR-13 and -14 (Taqman MicroRNA array), and snoR-1 (miR-152 assay) were used as a reference for input RNA. The expression was calculated as a percentage of snoRNA: $2^{-\Delta Ct}$ x 100, in which ΔCt is equal to "Ct^{miRNA} minus $Ct^{control\ snoRNA}$". Hypermethylated miRNAs were considered re-expressed by de-methylation when >2-fold

increases in expression were observed for at least two time points (3, 6 or 10 days) of zebularine treatment.

Gene expression analyses using Affymetrix GeneChips

Affymetrix HGU133 plus 2.0 GeneChips (Santa Clara, CA, USA) were used to study mRNA expression levels of the potential miRNA-target genes *DNMT1*, *MLL*, *ZEB2* and *HOXA3* in t(4;11)-positive infant ALL patients (n=22) and in normal bone marrow samples (n=7). Data were processed as described before[7] and raw infant ALL gene expression data was deposited in the NCBI Gene Expression Omnibus under the GEO Series accession number GSE 19475 as part of one of our recent studies[7].

Quantitative real-time PCR analysis

Total RNA was reverse transcribed as described before(17) and the obtained cDNA was used to quantify *MLL, DNMT1*, *ZEB2* and *HOXA3* mRNA expression in cell line SEMK2, using quantitative real-time PCR analysis. *B2M,* encoding human *beta-2-Microglobulin*, was used as a housekeeping reference gene. All oligonucleotides were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CA) and primer combinations are listed in Online Supplemental Table 1S. PCR products were amplified and stained using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) according to the manufacturer's recommendations, and detected on an ABI 7900HT Sequence Detection System (Applied Biosystems)[7]. Per experiment samples were analyzed in duplicate and all experiments were conducted twice. Highly expressed miRNA targeted genes were considered down-regulated by re-activation of the miRNA when mRNA expression was at least 2-fold decreased after zebularine treatment.

Statistical analyses

Normalization of the CpG island microarray data was performed using global locally weighted scatterplot smoothing (loess) normalization[23]. Differentially methylated CpG island loci and differentially expressed miRNAs were identified using linear models for microarray data (limma) as previously described[8,24]. Differential methylation and expression was regarded significant at *P*-values (adjusted for multiple testing according to the step-up procedure of Benjamini and Hochberg[25]) of <0.05 (i.e. false discovery rate (FDR) <0.05). All statistical analyses were performed in the statistical environment R using Bioconductor packages (R Development Core Team, 2007). Heatmaps were generated in GenePattern version 3.1.2 and principal component plots were produced using Genemath XT 1.6.1. software (Applied Maths, Inc., Austin TX, USA).

The risk of relapse and the overall survival were computed with the Kaplan-Meier estimator. Overall survival was defined as the time from diagnosis until death or the last follow-up. The log-rank test was used to compare outcomes between different patient groups. SPSS

16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for computation of survival statistics.

RESULTS

CpG island hypermethylation at miRNA loci in t(4;11)-positive infant ALL

We previously showed that *MLL*-rearranged infant ALL patients carrying translocation t(4;11) exhibit severely hypermethylated genomes[8]. In this study we investigated whether the genome-wide deregulation of DNA methylation also affected miRNA activity. For this, multiple datasets were successively analyzed following the flowchart depicted in Figure 1. First, an unsupervised principal component analysis using all the 1118 oligonucleotide probes related to 122 miR-genes (encoding 113 mature miRNAs) present on the 244K CpG island microarray (Agilent) readily separated the data into two groups, corresponding to t(4;11)-positive infant ALL (n=22) and normal bone marrow samples (n=7) (Online Supplementary Figure 1S). Fifty-nine out of these 122 miR-genes encoding 55 mature miRNAs were

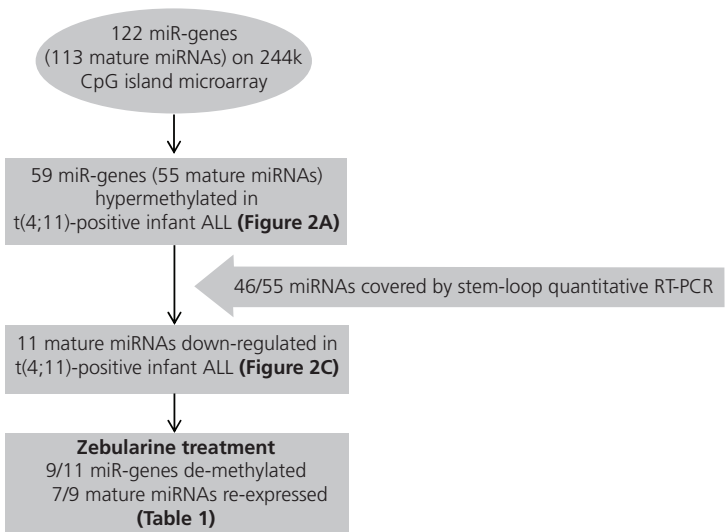

Figure 1. Flowchart showing the sequential analyses performed in this study.
Several sequential analyses were performed on different data-sets, and miRNAs were selected for further evaluation on the basis of the following criteria: hypermethylated miRNAs are transcriptionally silenced, and exposure to the DNA methyltransferase inhibitor zebularine results in de-methylation and re-expression. Seven miRNAs met these criteria.

significantly hypermethylated (FDR <0.05) in t(4;11)-positive infant ALL cases compared with normal bone marrows (Figure 2A). Agilent probe IDs, log-fold changes in methylation and *P*-values are listed in Online Supplemental Table 2S. As shown in Figure 2A, among the t(4;11)-positive infant ALL samples two subgroups could be identified that either displayed heavy (n=7) or light (n=15) miR-gene methylation. While we observed a similar phenomenon in our study of the methylation patterns of protein-coding gene promoters[8], the here presented separation between heavy and light miR-gene methylation is more pronounced (Figures 2A and 2B).

Downregulated expression of hypermethylated miR-genes

Subsequently, miRNA expression was assessed in t(4;11)-positive infant ALL patients (n=5) and in normal bone marrow samples (n=7) by stem-loop-based RT-qPCR. For 46 out of the 55 hypermethylated miRNAs the expression level could be evaluated, and 11 of these 46 mature miRNAs appeared to be consistently down-regulated in t(4;11)-positive infant ALL (FDR<0.05) (Figure 2C, Online Supplementary Table 2S), presumably as a consequence of CpG island hypermethylation[26]. For some miRNAs, such as miR-148a, miR-503, and miR-432, >100-fold down-regulated expression was observed in t(4;11)-positive infant ALL as compared with normal bone marrow.

Responsiveness of miRNA loci to de-methylation by zebularine

In t(4;11)-positive infant ALL, the expression of the majority of genes transcriptionally silenced by promoter hypermethylation could be re-activated by the DNA methyltransferase inhibitor zebularine[8]. We here examined the responsiveness of the silenced and hypermethylated miRNAs (Figure 2C) to de-methylation. For this, the t(4;11)-positive precursor B-ALL cell line SEMK2 (a subclone of the SEM cell line) was exposed to 100 μM of zebularine for 3, 6 or 10 days. At these consecutive time points both CpG island methylation and miRNA expression were determined. Nine miRNA loci could be de-methylated by zebularine (Online Supplementary Figure 2SA), and for 7 of these, i.e. miR-200b, miR200a, miR429, miR-152, miR-10a, miR-503 and miR-432, expression was increased (Table 1, Online Supplementary Figure 2SB). Interestingly, hypermethylation at these miR-gene loci turned out to be specific for t(4;11)-positive infant ALL when compared with wild-type *MLL* infant ALL samples (n=10) and non-*MLL* childhood ALL samples (n=10) (Figure 3).

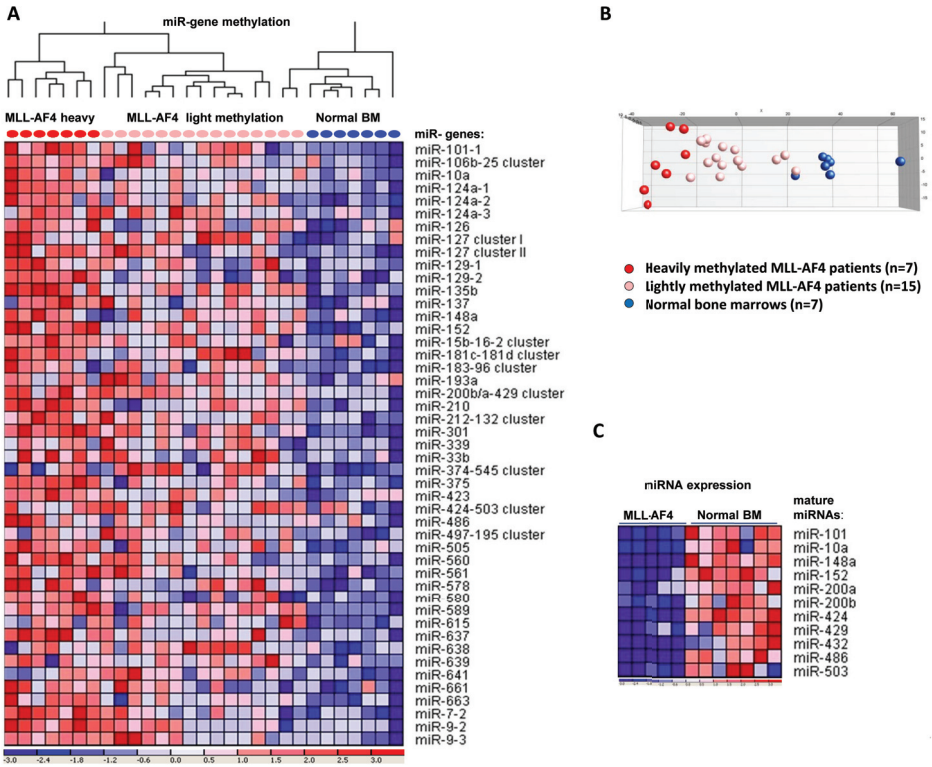

Figure 2. Hypermethylation and downregulated expression of miRNAs in t(4;11)-positive infant ALL.

(**A**) Heatmap showing the 59 miR-genes (corresponding to 55 mature miRNAs) hypermethylated in t(4;11)-positive infant ALL (n=22) compared with healthy bone marrow (BM, n=7) samples (FDR<0.05). Columns represent patient samples and rows represent miR-genes. Relative CpG methylation levels are shown in red (high) and blue (low). CpG probes located in close proximity to miRNA clusters are annotated as miRNA cluster. MiR-127 cluster I contains miR-433 and miR-127 and miR-127 cluster II relates to miR-432 and miR-136 (Figure 4C). Samples were ordered using hierarchical cluster analysis (Euclidean distance, complete linkage). (**B**) Principal component analyses (PCA) using the 59 hypermethylated miR-genes separating heavily (red, n=7) and lightly (pink, n=15) methylated t(4;11)-positive infant ALL, and normal bone marrow samples (blue, n=7). Based on the first three components of the PCA explaining 62.5% of the total variance, the samples were visualized. (**C**) Heatmap displaying the expression of miRNAs that were significantly downregulated in t(4;11)-positive infant ALL samples (n=5) as compared with normal bone marrows (n=7) (FDR<0.05). Relative expression levels are shown in red (high) and blue (low). MiRNA expression profiling could only be performed for 5 patients due to restricted availability of patient material. Among the 5 patients were 3 lightly methylated patients and 2 heavily methylated patients. Similarly as observed in our previous study, the separation between heavily and lightly methylated patients is not reflected at the expression level[8].

Table 1. Zebularine induced de-methylation and re-expression of silenced miRNAs.

miRNA	cluster	chromosomal location	t(4;11) vs nBM		re-expression Z0 Z3 Z6 Z10
			fold-change in expression	FDR	
miR-10a	–	17q21.32	-7	0.016	
miR-152	–	17q21.32	-13	0.001	
miR-200a			-3	0.042	
miR-200b	miR-200b/a-429	1p36.33	-4	0.016	
miR-429			-5	0.016	
miR-432	miR-127	14q32.2	-480	0.043	
miR-503	miR-424-503	Xq26.2	-218	0.036	

Table depicts hypermethylated miRNAs that were transcriptionally silenced, but de-methylated and re-expressed upon zebularine treatment. Expression levels were measured by RT-qPCR, and fold-changes between t(4;11)-positive ALL and normal bone marrow (nBM), as well as expression levels in the SEMK2 cell line before (Z0) and after treatment (for 3 (Z3), 6 (Z6) or 10 (Z10) days) are shown. MiRNAs were considered re-expressed by zebularine when the expression was increased more than 2-fold for at least two time points during treatment.

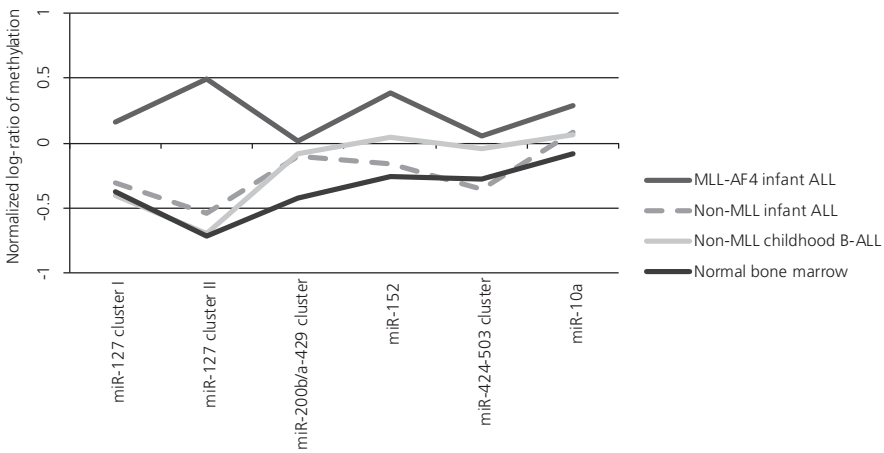

Figure 3. Methylation of selected miR-genes is specific for MLL-AF4-positive infant ALL.
Graphical representation of the methylation levels of the selected miR-genes in t(4;11)-positive infant ALL samples (n=15, dark grey line) along with the log-ratios of wild-type *MLL* infant ALL samples (n=10, interrupted line) and non-*MLL* childhood ALL samples (n=10, light grey line) relative to normal pediatric bone marrow samples (n=7, black line). CpG methylation data are presented as normalized log-ratios of patient signal divided by the common reference signal.

De-methylation and re-expression of miRNA clusters

About 40% of all miRNAs are found to be organized in clusters on human chromosomes[27]. By definition, miRNAs encoded less than 3 kb apart from one another are considered to be clustered and often are collectively expressed at comparable levels[9,27]. Five of the seven

miRNAs that were re-expressed upon de-methylation appeared to be part of such miRNA clusters. For instance, miR-200b is part of a miRNA cluster on chromosome 1p36.33 further including miR-200a and miR-429 (Figure 4A). Together with miR-424, miR-503 forms a miRNA cluster on chromosome Xq26.2 (Figure 4B), and miR-432 is located within a large miRNA cluster on chromosome 14.32.2 comprising miR-431, miR-432, miR-433, miR-127 and miR-136 (Figure 4C). Interestingly, neighboring miRNAs belonging to the same cluster also seem to be affected in a similar manner, albeit in some cases (e.g. miR-127 and miR-433) less significantly.

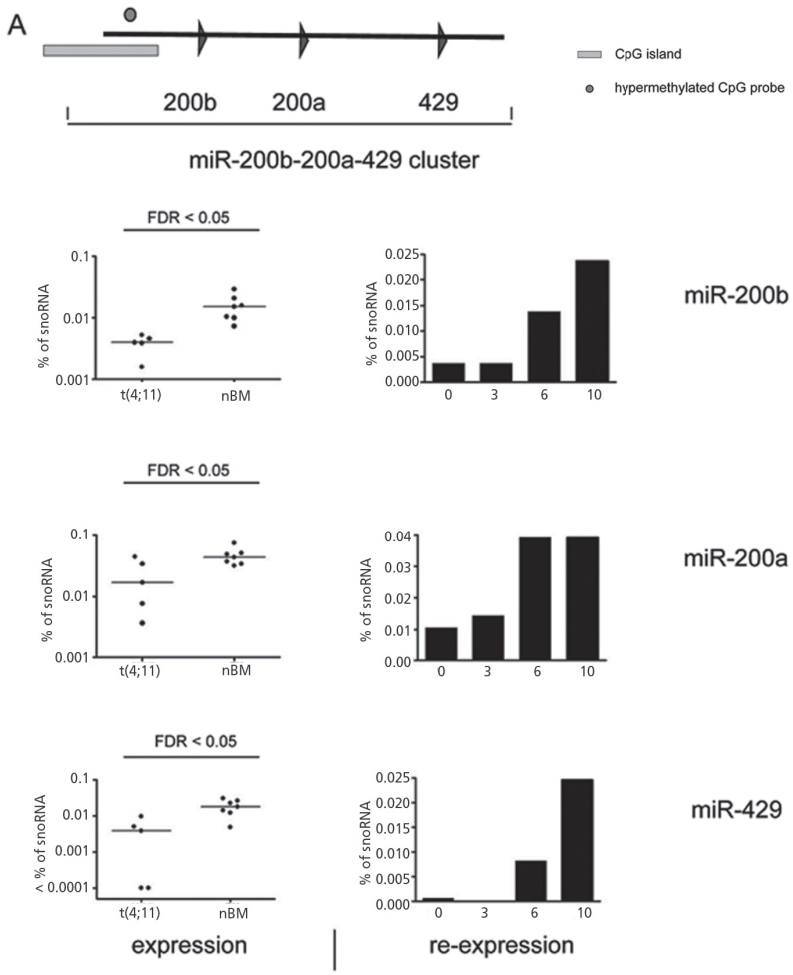

Figure 4. MiRNAs located in miRNA clusters.
(A) Schematic representation of the positions of the different miRNAs belonging to the miR-200b-200a-429 cluster on human chromosome 1p36.33.

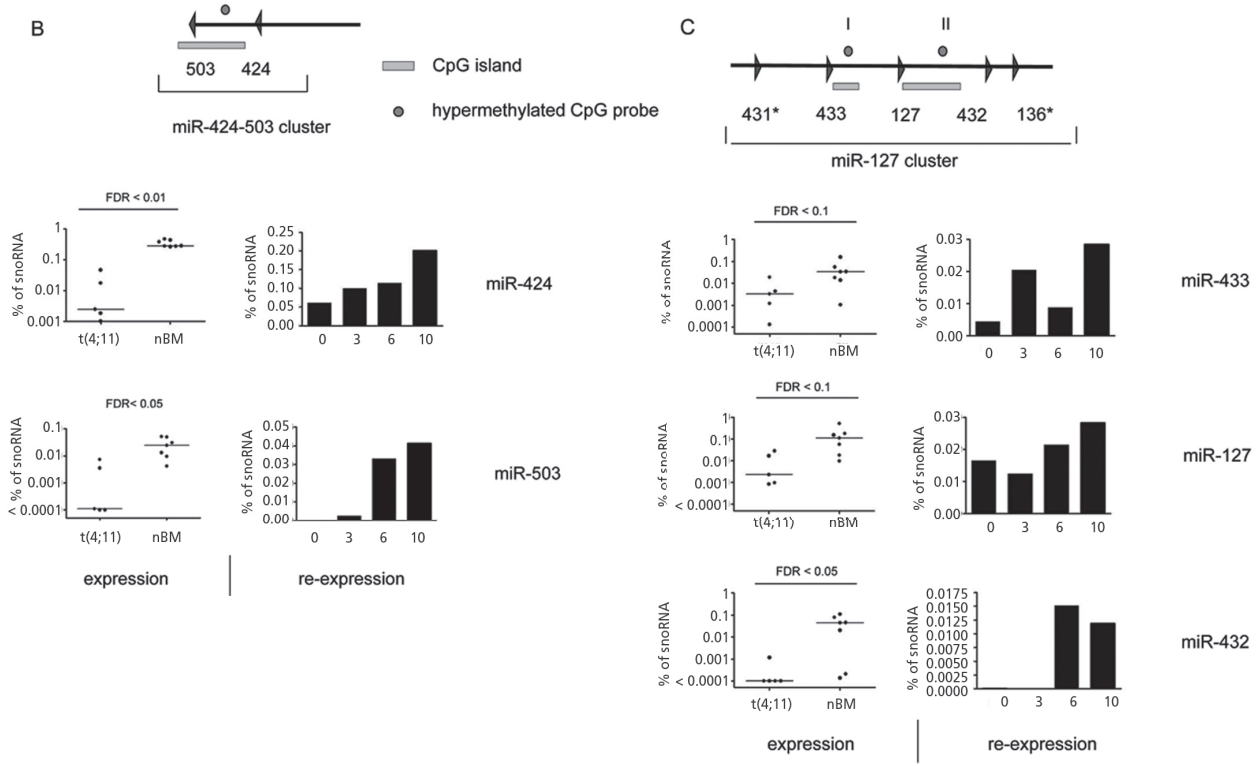

Figure 4. MiRNAs located in miRNA clusters.
(**B)** Schematic representation of the positions of the miRNAs from the miR-424-503 cluster on human chromosome Xq26.2. (**C)** Schematic representation of the positions of the different miRNAs belonging to the miR-127 cluster, miR-431, miR-433, miR-127, miR-432, and miR136, on human chromosome 14q32.2 MiRNAs indicated by an astrix (*) were not covered by RT-qPCR primers. "I" and "II" denote miR-127 cluster I and II respectively as used in Figure 2A. The locations of the miRNAs are indicated by triangles. The CpG island region is indicated as a grey bar, and the individual hypermethylated CpG island probes (Agilent microarrays) are depicted as circles. MiRNA expression levels were determined by RT-qPCR in t(4;11)-positive infant ALL patients (n=5) and in the SEMK2 cell line in the presence and absence of zebularine for 3, 6 or 10 days. *P*-values are from the limma model (Online Supplementary Table 2S).

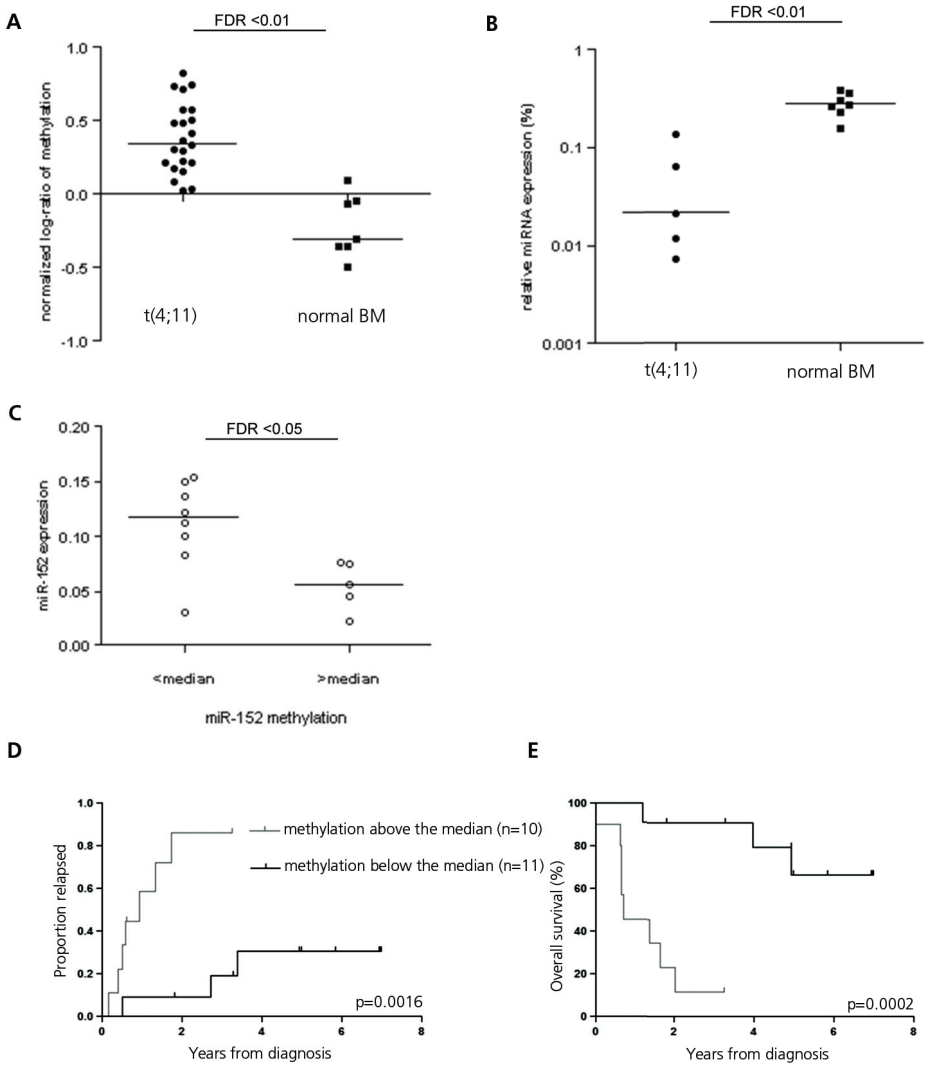

Figure 5. MiR-152 methylation, expression and patient survival.
(**A**) Methylation levels of the miR-152 CpG island as obtained by differential methylation hybridization (DMH) shown as normalized log-ratios of methylation in t(4;11)-positive infant ALL (n=22) and normal bone marrow (n=7) samples. P-value is from the limma model. (**B**) MiR-152 expression levels as determined by RT-qPCR in t(4;11)-positive infant ALL patients (n=5) and normal bone marrows (n=7). P-value is from the limma analyses. (**C**) MiR-152 expression in t(4;11)-positive infant ALL patients (n=13) as determined by using the miR-152 RT- qPCR assay in patients with methylation below or above the median value. (**D**) Risk of relapse as determined by the Kaplan Meier estimate and (**E**) overall survival (OS) in t(4;11)-positive patients (n=21) divided by the median degree of DNA methylation (Figure 5A). One patient died before start of treatment and was excluded from survival analyses (denoted early death). normal BM = normal bone marrow.

The two remaining miRNAs, miR-10a and miR-152, are both located on chromosome 17q21.32, however, the genomic distance between these miRNAs exceeds with >500kb the limit of 3 kb and they should therefore be considered as individual entities.

Clinical relevance of hypermethylation at miRNA loci

Epigenetically regulated miRNAs embedded within or near methylated CpG islands have been linked before to clinical outcome in precursor B-ALL[28]. In this study we present a similar finding in *MLL*-rearranged infant ALL involving miR-152. Although this miRNA locus is consistently methylated in all t(4;11)-positive infant ALL samples tested (Figure 1A), the magnitude of methylation and expression varied per patient (Figures 5A and 5B). Using the median log-ratio of miR-152 methylation as obtained from CpG microarray data (Figure 5A) as the cut-off value t(4;11)-positive patients, were divided into two groups displaying either high or low degrees of methylation. The median miRNA expression was 2-fold lower in the group with a high degree of methylation (FDR<0.05) (Figure 5C). Furthermore, heavy methylation of the miR-152 CpG island was associated with an increased risk of relapse (p=0.0016) (Figure 5D) and a reduced overall survival (p<0.001) (Figure 5E). The two known prognostic factors in infant ALL, such as age <6 months and white blood cell (WBC) count >300x10^9/L(1) did not differ significantly between the groups (Online Supplementary Table 3S). Although convincing, these results have to be interpreted with caution, given the low number of patients.

Potential target genes of miR-152

Encouraged by the clinical relevance of the methylation status of the miR-152 CpG island, we set out to identify possible miRNA-targeted genes by using the algorithms TargetScan and Pictar[29] which base their prediction on sequence homology between miRNA and potential mRNA target. Both algorithms showed that DNA methyltransferase 1 (*DNMT1)*, also known as *CXXC9*, on chromosome 19p13.2 represents a possible target for miR-152 (probability of conserved targeting (P_{CT}, a measure for assessing the biological relevance of predicted miRNA-target interactions)[10]: 0.77). Moreover, *DNMT1* has recently been verified as a target of miR-152 in hepatobiliary cancer[30]. Based on known sequence similarity we investigated whether the *MLL* gene on chromosome 11q23, alias *CXXC7,* could be another miR-152 targeted gene. Interestingly, the seed sequence of miR-152 appeared entirely present in the 3' untranslated regions (UTR) of both *DNMT1* and *MLL* (P_{CT}: 0.86) (Figure 6A).

Interestingly, miR-148, the other member of the highly conserved miR-148/152 family, which is also down-regulated due to CpG hypermethylation in t(4;11)-positive infant ALL (Figures 6B and 6C), has the same seed sequence as miR-152 and may therefore also target *DNMT1* and *MLL* (Figure 6A), which has already been proven for *DNMT1*[30]. *DNMT1* and *MLL* are highly expressed in t(4;11)-positive infant ALL patients (Figures 6D and 6E) and de-methylation of miR-152 by zebularine leading to up-regulation of miR-152 readily resulted in

down-regulation of mRNA expression of these genes (Figures 6F and 6G). In contrast to its family member miR-152, miR-148 could not be re-activated after exposure to zebularine for 10 days.

ZEB2 and HOXA3 as potential miRNA-target genes in t(4;11)-positive infant ALL

According to literature the *zinc finger E-box binding homeobox 2* (*ZEB2)* gene is the best-known validated target gene of the miR-200b/a-429 cluster[31], and the *homeobox A3* (*HOXA3)* gene has been described as a potential miR-10a targeted gene[32]. Both of these genes are known to be highly expressed in t(4;11)-positive infant ALL[5,7] which was validated on our t(4;11)-positive infant ALL cohort (Affymetrix GeneChips) (Online Supplementary Figures 3SA and B). After zebularine treatment the miR 200b/a-429 cluster was upregulated, as well as miR-10a (Table 1), and *ZEB2* expression was clearly down-regulated (Online Supplementary Figure 3SC) whereas *HOXA3* expression was only marginally decreased (Online Supplementary Figure 3SD).

DISCUSSION

MLL-rearranged ALL in infants represents a high-risk type of childhood leukemia characterized by a complex biology. Initiating transformation, the *MLL* fusion protein alters normal *MLL* histone methyltransferase activity guided by the fusion partner. In turn, these inappropriate histone modifications induce aberrant transcription of multiple genes, resulting in highly characteristic gene expression profiles[6,7]. Opposing widespread gene activation, we recently demonstrated that vast numbers of genes are epigenetically silenced by promoter hypermethylation[8], which was recently confirmed in an independent study by others[33]. Genome-wide hypermethylation is especially apparent in t(4;11)-positive infant ALL, and as shown in the present study extends its effects on miRNA expression.

Comparing t(4;11)-positive infant ALL with healthy bone marrow samples, a total of 59 out of 122 analyzed miR-genes appeared to be significantly hypermethylated. Seven of these miRNAs were selected for further evaluation because they were transcriptionally silenced, and exposure to the DNA methyltransferase inhibitor zebularine resulted in their de-methylation and re-expression: miR-200b, miR200a, miR429, miR-152, miR-10a, miR-503 and miR-432 (Table 1).

Interestingly, some of these miRNAs have been described to function as tumor suppressing miRNAs. For instance, miR-432 is located within the large miR-127 cluster (Figure 4C) that was previously shown to be silenced in various malignancies by CpG island hypermethylation and aberrant histone modifications[9]. In that study, re-expression of miR-127 resulted in down-regulation of the proto-oncogene B-cell CLL/lymphoma 6 (BCL6) at the protein level[9].

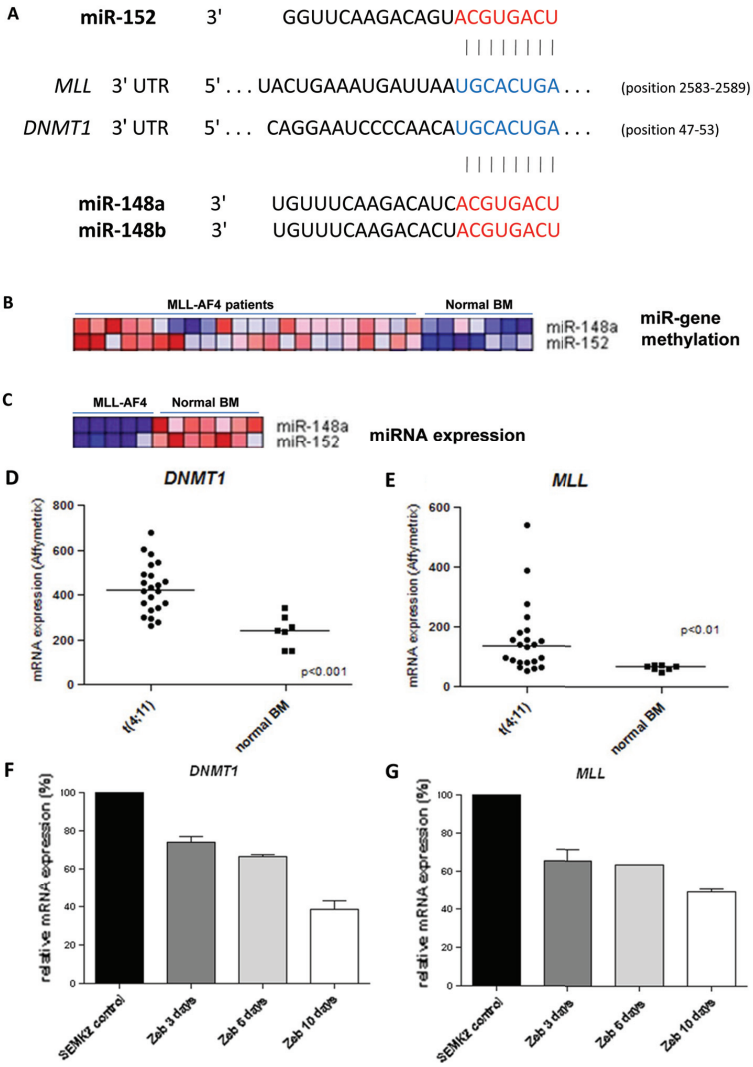

Figure 6. MLL and DNMT1 as potential miR-152 and miR-148 targeted genes.
(**A**) Putative miR-152 targeted genes were identified using TargetScan (*www.targetscan.org*) and Pictar[29]. MiRNA sequences were analyzed using miRbase[42] (*http://www.mirbase.org*) and detailed information on base pairing between miR-152 and its target sites in the 3'UTRs of the *DNMT1* and *MLL* genes was available through TargetScan. MiR-148, the other member of the broadly conserved miR-148/152 family, harbours a seed sequence identical to that of miR-152. (**B**) Methylation values for miR-148 (CpG island microarray data) in t(4;11)-positive infant ALL patients (n=22) and normal bone marrows (n=7), and (**C**) miR-148 expression data as obtained from RT-qPCR for t(4;11)-positive infant ALL patients (n=5) and normal bone marrows (n=7). (**D**) *DNMT1*, and (**E**) *MLL* normalized mRNA expression levels as obtained from gene expression profiling (Affymetrix GeneChips) in t(4;11)-positive infant ALL patients (n=22) and normal bone marrow samples (n=7). *P* values are from limma models. (**F**) *DNMT1*, and (**G**) *MLL* mRNA expression levels relative to the housekeeping reference gene *B2M* were determined by RT-PCR in the t(4;11)-positive cell line SEMK2 before and after exposure for 3, 6 and 10 consecutive days to 100 µM of zebularine. Expression levels in unexposed SEMK2 cells were set to 100%. Highly expressed miRNA-targeted genes were considered downregulated by re-activation of the miRNA when mRNA expression was at least 2-fold decreased after zebularine treatment.

In addition, down-regulation of miR-200b in human cancers leads to disease progression via elevated expression of the two E-cadherin transcriptional repressors: *ZEB1* and *ZEB2*[31]. Importantly, *ZEB2* is highly expressed in t(4;11)-positive ALL as a consequence of MLL-AF4 driven histone modifications[5]. Our data demonstrate both *ZEB2* down-regulation (Online Supplementary Figure 3SC) and re-activation of miR-200b (Online Supplementary Figure 2SB) by Zebularine, suggesting that *MLL* translocation- induced transcription involves the concerted regulation of both chromatin structure and miRNA activity.

Another example of MLL fusion-driven miRNA regulation involves the polycistronic miR-424-503 cluster. In t(9;11)-positive myeloid leukemias miR-424 expression was repressed by the MLL-AF9 fusion protein[34]. We here show that the miR-424-503 cluster is downregulated by CpG island hypermethylation in t(4;11)-positive ALL, possibly directed by the MLL-AF4 fusion. Likewise, miR-10a is downregulated in *MLL*-rearranged acute myeloid leukemia (AML)(35). In line with these observations, we found miR-10a to be down-regulated in t(4;11)-positive infant ALL. Although miR-10a is located within the *HOXB* cluster, one of its putative targets is *HOXA3*[32]. In concordance with miR-10a silencing, *HOXA3* expression is elevated in t(4;11)-positive infant ALL patients (Online Supplementary Figure 3SB). However, re-activation of miR-10a by de-methylation only marginally repressed *HOXA3* expression at the mRNA level, suggesting that miR-10a exerts its inhibition on putative target *HOXA3* largely on the protein level. Alternatively, additional forces retain the expression of this gene.

Of high interest it was that we identified the wild-type *MLL* gene as a potential target of miR-152. A recent report suggests that co-expression of wild-type *MLL* is required for MLL-AF9-induced leukemogenesis[36]. Whether wild-type *MLL* is also required for the development or maintenance of t(4;11)-positive leukemias remains uncertain. However, as we show in this study, epigenetic inactivation of miR-152 at least prevents the down-regulation of wild-type *MLL*. Treatment with the de-methylating agent zebularine induced expression of miR-152 and downregulation of wild-type *MLL* at the mRNA level. Moreover, the other miR-148/152 family member miR-148 utilizes the same "seed" sequence as miR-152 and therefore may also target the *MLL* gene (Figure 6A). MiR-148 is also downregulated as a consequence of CpG island hypermethylation in t(4;11)-positive infant ALL (Figures 6B and 6C), which may have further obstructed down-regulation of wild-type *MLL*. In addition, in case of an *MLL*-translocation, the N-terminal region of the *MLL* gene is usually retained in the fusion protein, whereas the C-terminus containing the 3' UTR is lost. Therefore, miR-148/152 would not be able to target the leukemic MLL-AF4 fusion. However, in the majority of t(4;11)-positive ALL samples, also the reciprocal *AF4-MLL* transcript is present[37], and from recently published mouse models it appears that the *AF4-MLL* fusion is essential for leukemic transformation(38). Hypothetically, besides targeting wild-type *MLL,* miR-152 may target the oncogenic reciprocal *AF4-MLL* fusion product. The fact that the *AF4-MLL* transcript is detected in 80% of t(4;11)-positive ALL samples indicates that this reciprocal transcript may contribute to the leukemogenic transformation into a full-blown leukemia, but may not

be essential for further maintenance of the leukemia. Epigenetic silencing of miR-152 and possibly miR-148 may therefore have contributed to this process by facilitating *AF4-MLL* driven induction of leukemic transformation. The selected miRNAs were not methylated in wild-type *MLL* infant ALL patients and non-*MLL* childhood ALL patients (Figure 3). In concordance with this none of the selected miRNAs in this study was found to be associated with CpG island hypermethylation in adult and pediatric precursor B-cell ALL patients in a previous study[28] which provides additional evidence that the here identified miRNAs are specific for t(4;11)-positive ALL. MiR-152 has been described before to be silenced due to CpG hypermethylation in cancer[39].

Apart from the associations of the selected miRNAs with *MLL* and MLL fusions, another notable feature stands out. The expression of at least three out of these miRNAs seems to be regulated by the genes executing DNA methylation: miR-200b, miR-200a and miR-10a were most significantly upregulated in a $Dnmt1^{-}/Dnmt3^{-}$ knock-out mouse model[32]. As DNMT1 and DNMT3 (DNA methyltransferase 1 and 3) facilitate maintenance and *de novo* DNA methylation respectively, expression of these miRNAs is evidently controlled by the epigenetic force of DNA methylation. MiR-152 appears to be an effector of the epigenetic machinery itself as one of its putative targets is *DNMT1*[30]. Therefore miR-152 can be added the growing list of so-called "epi-miRNAs", such as miR-29b and miR-290, which control the regulation of DNMTs[40]. MiR-148 has recently been added to this list of epigenetic effector miRNAs, because it targets DNMT3b[41]. As we have shown in the present study, the degree of miR-152 methylation varies among t(4;11)-positive infant ALL samples and is highly predictive for clinical outcome. Similarly, we recently found that the degree of genome-wide promoter methylation also varies among *MLL*-rearranged infant ALL and that patients with heavily methylated genomes are at extremely high risk of disease relapse[8]. Heavily methylated cases displayed a higher degree of methylation of the miR-152 CpG island (Online Supplementary Figure 4S). Thus, the varying levels of miR-152 methylation and *DNMT1* expression may determine the severity of genome-wide methylation. Zebularine is a cytidine analog that requires incorporation into the DNA where it covalently binds *DNMT1*, and protein levels of this protein will be reduced directly by this mechanism. Then, indirectly, through re-expression of miR-152, the conventional RNA silencing mechanism will downregulate the expression of *DNMT1* even further. Zebularine-induced re-activation of miR-152 that targets *DNMT1* may thus enhance the efficiency of the compound and explain its recently demonstrated potential in *MLL*-rearranged infant ALL[8]. Until recently, it was believed that the de-methylating actions of DNMT inhibitors, such as 5-azacytidine, decitabine (5-aza-deoxycytidine), and zebularine are limited to hypermethylated (tumor suppressor) genes. We here show that zebularine, through re-activation of important hypermethylated miRNAs, is able to downregulate proto-oncogenes in *MLL*-rearranged infant ALL. Obviously, the stability of zebularine was a key factor in allowing the re-expression of silenced miRNAs genes which peaks after 10 days. This

stresses the imperative need to test zebularine or comparable compounds in mouse models and subsequent clinical trials in infant leukemia.

Besides the previously published hypermethylation at protein-coding genes[8] we here showed that the aberrant DNA methylation in t(4;11)-positive infant ALL extended into methylation at miRNA loci. We identified miRNAs that were in-activated due to CpG island hypermethylation and the majority of these miRNAs have been found to be associated with the *MLL* gene or *MLL*-rearranged leukemia, implying an important role for the *MLL*-AF4 fusion in the deregulation of these miRNAs. We further show that the silenced status of miR-152 has impact on the survival of t(4;11)- positive infant ALL patients. This silenced status can be reversed by zebularine. All together, our study indicates that although miRNAs are small in size, they have major implications in t(4;11)-positive infant ALL. This emphasizes the need for further investigation of de-methylating agents, like zebularine, to improve the treatment of these young patients with leukemia.

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Chapter 8

Summary

Before the beginning of the new millenium it was thought that only protein-coding genes were of importance to the biology of cancer including leukemia. This believe changed with the discovery of microRNAs (miRNAs) in *C. elegans* followed by many studies dedicated to unravel the role of miRNAs in post-transcriptional silencing. These studies demonstrated that although miRNA molecules are small and non-protein coding their impact is major as they regulate the expression of many protein-coding genes. MiRNAs achieve this mainly via complementary basepairing within the 3' untranslated region (3'UTR) of target *messenger*RNAs (mRNA). Thereby miRNAs control crucial biological processes including proliferation, differentiation and apoptosis. As dysfunction of particularly these processes is a hallmark of cancer, our hypothesis was that miRNA dysregulation may -as well as protein-coding genes- contribute to the biology of leukemia.

Our current knowledge about the importance of these tiny biological players in acute leukemia is reviewed in **chapter 2**. In order to reveal which miRNAs are relevant to the most common form of childhood leukemia, i.e. acute lymphoblastic leukemia (ALL), we used a conventional small RNA concatemer-cloning procedure followed by Sanger cDNA-sequencing and thereby identified 105 known and 8 novel miRNA genes in leukemia cells of ALL patients (**chapter 3**). In **chapter 4** we applied high-throughput or deep sequencing to 98 patients covering seven subtypes of ALL and three types of normal hematopoietic cell samples. This approach was far more sensitive than the conventional Sanger cDNA-sequencing method and resulted in the discovery of another 28 novel and 431 candidate novel miR-genes. In addition to these new miRNAs, also the expression of 470 known miRNAs was demonstrated indicating the value of the high-throughput method. Many of the newly discovered as well as known miRNAs were uniquely expressed in subtypes of ALL and normal hematopoietic cells. Interestingly, newly cloned miRNAs were less evolutionary conserved and less abundantly expressed than known miRNAs which may point to cell-type specific miRNAs and therefore were not (yet) detected in other tissues. The dysregulated expression levels of selected novel and candidate novel miRNAs was confirmed by a second technique, i.e. stem-loop-based real-time quantitative polymerase chain reaction (RT-qPCR). To further explore which miRNAs may be potentially relevant to ALL, a large-scale RT-qPCR analysis was performed of 397 miRNAs (including novel miRNAs) in 81 patients covering seven subtypes of pediatric ALL and in normal hematopoietic cells (**chapter 5**). This analysis showed that T-ALL, *MLL*-rearranged, *TEL-AML1*-positive, hyperdiploid and *E2A-PBX1*-positive ALL but not *BCR-ABL*-positive and remaining precursor B-ALL (B-other) cases have distinct miRNA expression profiles. These leukemia-related expression profiles also differ from the miRNA expression patterns seen in normal hematopoietic cells. Examples are the upregulation of miR-196b characteristic for *MLL*-rearranged patients and a subset of T-ALL patients, and the upregulation of miR-708 in non-*MLL* precursor B-ALL patients **(chapter 3** and **5**). We also identified a signature of 14 miRNAs which predicted the clinical outcome of pediatric ALL patients independent from the specific ALL subtype (**chapter 5**). One of the determinants for a poor clinical outcome

relies on cellular resistance to drugs[1-4]. In **chapter 5** we discovered a set of miRNAs that were differentially expressed between drug sensitive and resistant leukemia cells. Among others higher expression levels of *miR-125b* and neighbouring *miR-99a* and *miR-100* genes were found in leukemic cells of patients being relative resistant to vincristine and daunorubicin. These drug-resistance associated miRNAs as well as subtype-discriminative and prognostic miRNAs warrant further biological studies.

In **chapter 6** we showed that high expression levels of miR-196b were predominantly found in *HOXA*-activated leukemias, including *MLL*-rearranged precursor B-ALL and T-ALL patients carrying *MLL*-rearrangements, *SET-NUP214*, *CALM-AF10* or an inversion of chromosome 7. As *miR-196b* is encoded by a gene located within the *HOXA* cluster, the correlated expression suggests co-activation of *miR-196b* and *HOXA* family members. The locus comprising *HOXA* cluster and *miR-196b* genes displayed less methylation of promoter CpG-islands in *MLL*-rearranged cases than in non-*MLL* cases and normal bone marrow cells. This points to DNA hypomethylation as one of the causes of miR-196b upregulation. Since miR-196b has been reported to have oncogenic activity in bone marrow progenitor cells[5], our findings point to a potential role for miR-196b in *HOXA*-activated leukemias.

MLL-rearranged ALL, in particular MLL-AF4/t(4;11)(p21;q23) positive cases, was characterized by downregulation of at least 11 miRNAs due to DNA hypermethylation (**chapter 7**). The epigenetic silencing of 7 miR-genes could be reversed by the demethylating agent zebularine, which is promising as downregulated miRNAs potentially exert tumor suppressive functions. One of the re-expressed miRNAs, i.e. miR-152, shared 3'UTR sequence complementarity with both germline *MLL,* the *AF4-MLL* fusion gene and the DNA methylating gene *DNMT1*. This is of interest because co-expression of germline *MLL* is essential to promote *MLL-AF9*-induced leukemogenesis whereas the AF4-MLL fusion protein is crucial for leukemic transformation of MLL-AF4-positive cells[6,7]. High DNMT1 expression may be responsible for the genome-wide hypermethylation of CpG islands in the promoter regions of t(4;11)-positive *MLL*-rearranged infant ALL which has previously been linked with an unfavorable outcome[8]. The re-expression of miR-152 induced by exposure to zebularine illustrates the potential of demethylating agents in the treatment of *MLL*-rearranged ALL especially since the degree of miR-152 methylation was shown to be inversely correlated with the clinical outcome of these patients.

In this thesis we identified many subtype-discriminative, drug resistance-associated, and prognostically relevant known and novel miRNAs in pediatric ALL. These miRNAs -in addition to relevant protein coding genes- now need further functional studies to address the biological role in leukemogenesis and cellular drug resistance. It is of special interest that the aberrant expression levels of some of the distinctive miRNAs could be restored by a demethylating agent. Although their functionality needs to be tested, this points to the potential of miRNAs as new targets to treat pediatric ALL.

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Chapter 9

General Discussion and Future Perspectives

"What we call little things are merely the causes of great things; they are the beginning, the embryo, and it is the point of departure which, generally speaking, decides the whole future of an existence. One single black speck may be the beginning of gangrene, of a storm, of a revolution."

by Henri Frederic Amiel, 1821-1881

DISCOVERY OF THE MICRORNAOME IN PEDIATRIC ALL

Previously scientists postulated that non-protein coding DNA was non-functional, in fact a waste of nucleotides or "junk" DNA. Research of the last decade proved this concept wrong as non-protein coding but nevertheless biologically relevant microRNAs (miRNAs) were discovered. Despite their name referring to their length of only 18-25 nucleotides, miRNAs play an important role in the physiology of cells since they fine-tune and control the balance of important cellular processes like proliferation, differentiation and apoptosis. The fine-tuning may be disrupted upon miRNA dysregulation resulting in the development of cancer. Many miRNAs that may be relevant to pediatric acute lymphoblastic leukemia (ALL) may not yet have been identified since their expression and function is cell-type dependent and the majority of miRNAs has been detected in non-hematopoietic/non-leukemic tissues.

In this thesis an old fashioned miRNA cloning technique (based on concatemer sequencing) and a new fashioned technique (followed by high-throughput sequencing) were used to identify 36 novel and 431 candidate novel miRNA genes (miR-genes, **chapter 3 and 4**). Many of these miR-genes were less abundant and less evolutionary conserved than known miRNAs. However the expression levels of selected novel miRNAs -as determined by stem-loop RT-qPCR- was similar to those of miRNAs yet known to play a role in hematopoiesis which indicates their potential importance. E.g. miR-708, discovered by us[1] and by others[2] was expressed at a level comparable to that of miR-196b known to affect proliferation of bone marrow cells[3]. Moreover, novel miRNAs were often unique to ALL or normal hematopoietic cells and were aberrantly expressed in ALL compared with normal hematopoietic tissue, which indicates a role for novel miRNAs in the underlying biology of ALL (**chapter 4**). As mentioned above, stem-loop RT-qPCR was used to measure the miRNA expression. This method makes use of an RT primer which shares complimentarity with a specific mature miRNA and has a stem-loop structure[4]. The loop structure of the RT-primer in combination with PCR primers specific for the mature miRNA prevents amplification of the miRNA precursor. Thereby this method is highly specific for the mature miRNA in contrast to many oligo-array detection systems which were used by others[5-7]. The number of miRNAs identified in this thesis are comparable to miRNA numbers identified by other high-throughput studies performed on pediatric ALL, AML and non-ALL tissues using similar stringent criteria to define a miRNA[8-12]. This indicates that the total number of miRNAs is not expected to dramatically change upon

new high-throughput sequencing attempts with similar samples unless new insights in the definition of a miRNA are obtained. The miRNAome covering more than 800 miRNAs identified in this thesis offer therefore a comprehensive basis for further functional studies.

LEUKEMIA-TYPE SPECIFIC EXPRESSION SIGNATURES OF MICRORNAS

Leukemic cells of adult patients with acute myeloid leukemia display aberrant miRNA expression signatures[13-15]. In line with these studies, this thesis shows that children with precursor B-ALL have different miRNA expression signatures than those observed in normal bone marrow and CD34$^+$-sorted cells (**chapter 5**)[16]. In addition, leukemia cells of children with T-ALL displayed different miRNA expression patterns than normal thymocytes[16]. While previous work often covered limited numbers of genetic subtypes[6,17,18], our study included pediatric patients with seven major subtypes of ALL. Except for B-other and *BCR-ABL*-positive cases the other five subtypes displayed unique expression signatures (**chapter 5**)[16]. For example *MLL*-rearranged precursor B-ALL cells were characterized by downregulation of let-7b and upregulation of miR-196b whereas *TEL-AML1*-positive ALL cells were characterized by downregulation of miR-221 and upregulation of miR-125b.

Such discriminative miRNAs may contribute to leukemogenesis, especially as studies with mice have identified several miRNAs with tumor suppressive and/or oncogenic capacities. For instance miR-29 was downregulated in *MLL*-rearranged AML and *NPM1 (nucleophosmin-1)*-wild type AML with normal karyotype and was able to reduce growth of leukemia cells by inhibiting *CDK6* (*cyclin dependent kinase 6)* and oncogene *TCL-1 (T-cell leukemia/lymphoma1)* in patient cells and transgenic mouse models[19-21]. MiR-155 and miR-21 were up-regulated in different types of leukemia and lymphoma and their introduction in mice gave rise to different hematologic malignancies including B-ALL by rescuing cells from apoptosis and boosting their proliferation [22-24]. Mice receiving liver cells that overexpressed miR-125b developed B-ALL and T-ALL, which suggests that the miR-125b upregulation in *TEL-AML1*-positive precursor B-ALL may play a role in the biology underlying this specific subtype[94].

Overexpression of miR-196b and downregulation of let-7 may contribute to the development of the *MLL*-rearranged ALL as others have shown that miR-196b exerts oncogenic activity in bone marrow progenitor cells[3] and that let-7 has tumor suppressive capacities by suppressing the oncogenes *RAS* and *c-MYC* in a variety of malignancies including lymphoma[25,26]. In fact, the aberrant downregulation of let-7 was associated with an upregulation of c-myc protein expression in *MLL*-rearranged precursor B-ALL cases (**chapter 5**)[16], which may suggest a mechanism responsible for the development of this leukemia-type.

MICRORNA EXPRESSION SIGNATURES MAY PREDICT PROGNOSIS OF LEUKEMIA PATIENTS

Previous studies of adult leukemia identified miRNAs that were associated with clinical outcome of patients. MiR-21[27] expression was associated with an unfavorable outcome whereas the expression of miR-29c[28], miR-223[28] and miR-181b[27] was linked to a favorable survival of chronic lymphocytic leukemia (CLL) patients. MiR-181b and family member miR-181a were associated with favorable prognosis of adult patients with high-risk cytogenetically normal AML (CN-AML) carrying unfavorable prognostic mutations including *FLT3-internal tandem duplication (FT3-ITD)*, wild type *NPM1* or both[29]. A recent study confirmed that high expression of miR-181a was predictive for a better outcome of patients with CN-AML[30]. Moreover, based on the expression of miR-181a in combination with that of miR-181b and 6 prognostic unfavorable miRNAs (i.e. miR-124, miR-128, miR-194, miR- 219-5p, miR-220a and miR-320) two subgroups of high risk CN-AML patients could be distinguished (probability of event-free survival of 11% versus 36%). Interestingly, this signature of eight different miRNAs could predict clinical outcome independent from other risk factors including mutational status (*FLT3-ITD*, wild type *NPM1* or *FLT3-ITD*/wild type) and white blood cell count[29]. In this thesis we identified 14 other miRNAs that each as single miRNA were predictive for relapse in pediatric ALL (**chapter 5**)[16]. Moreover, the combination of 14 miRNAs in one expression signature distinguished good and poor prognostic patients (5-year disease free survival of 90% versus 60%), which was independent of ALL subtype. Thereby our work and that of others demonstrate that next to established aberrant features such as the specific subtype (e.g. *MLL*-rearrangement) and specific mutations (e.g. *FLT3-ITD*), miRNA expression signatures comprised of a single or a combination of multiple miRNAs can have prognostic value.

Out of the 14 predictive miRNAs, eight miRNAs including miR-10a, miR-134 and miR-214 were linked to a favorable outcome in pediatric ALL which may correspond to their described tumor suppressor activity by driving apoptosis (miR-10a)[31], inhibiting cell proliferation (miR-10a and miR-214)[32,33] and downregulating oncogene *SOX2* (miR-134)[34]. High expression of six miRNAs i.e. miR-33, miR-215, miR-369-5p, miR-496, miR-518d and miR-599 was associated with an unfavorable prognosis (**chapter 5**)[16]. MiR-215 and miR-369-5p may contribute to a worse prognosis via induction of cell cycle arrest[35] and inhibition of differentiation[36], respectively. As the function for the here mentioned prognostic miRNAs is studied in non-ALL cells and miRNA function is often cell-type dependent, further studies are needed to confirm and/or reveal their function in ALL cells.

MiRNAs associated with survival were aberrantly expressed when compared to normal hematopoietic cells. Prognostically favorable miR-10a, miR-214 and miR-134 were downregulated in precursor B-ALL and prognostically unfavorable miR-33 was overexpressed in T-ALL[16]. If future studies confirm the functionality of the predictive miRNAs, the

normalization of their aberrant expression levels back to those levels found in normal cells may offer new therapeutic options in pediatric ALL.

MICRORNAS AND DRUG RESISTANCE

MiR-21 was upregulated in a chronic myelogenous leukemia (CML) cell line resistant to daunorubicin compared to its parental cell line sensitive to daunorubicin[37]. Sensitive CML cells became resistant to daunorubicin upon miR-21 overexpression via downregulation of tumor suppressor *PTEN* followed by PI3K/Akt activation [37]. Daunorubicin is one of the drugs used in the current therapy for pediatric ALL together with drugs such as vincristine, L-asparaginase and prednisolone. In this thesis we identified 10 other miRNAs that were associated with resistance to daunorubicin including the miRNA cluster comprising *miR-125b*, *miR-100* and *miR-99a* (**chapter 5**)[16]. Only miR-454 was associated with resistance to L-asparaginase. Although re-expression of silenced miR-128b and miR-221 cooperatively sensitized *MLL-AF4*-positive cell lines to glucocorticoids [38], none of these nor other miRNAs were associated with prednisolone resistance in pediatric ALL patients[16].

MiR-125b, miR-100 and miR-99a were highly expressed in leukemic cells of patients resistant to daunorubicin and/or vincristine[16,39]. Thirty-nine percent of the vincristine resistant patients in our study were *TEL-AML1-positive*, a subtype which is characterized by resistance to vincristine and sensitivity to L-asparaginase[40,41]. Gefen et al. found that treatment of *TEL-AML1*-positive precursor B-cells with an anti-miR-125b oligonucleotide sensitized for doxorubicin via stimulation of the apoptotic pathway. This finding is intriguing since doxorubicin displays cross-resistance with vincristine and daunorubicin[42,43]. MiR-125b may contribute to drug resistance via driving the proliferation of leukemia cells as this function of miR-125b was previously demonstrated in megakaryocytic progenitors[44]. At present the target gene for miR-125b is unknown. One candidate gene may be *IRF4* which has two possible binding sites for miR-125b in the 3' end of the untranslated region (3'UTR) of *IRF4* mRNA[45]. *IRF4* is involved in repressing proto-oncogene *B-cell CLL/lymphoma 6* (*BCL-6*) by binding to its promoter region. MiR-125b overexpression may contribute to drug resistance via *IRF4* inhibition resulting in overexpression of *BCL-6*. Although the underlying mechanism needs further research, this thesis and work of others suggest that miRNAs are involved in the response of leukemia cells to different drugs.

IDENTIFICATION OF TARGET GENES: A TRUE CHALLENGE

Our studies of miRNA profiling in acute leukemia were exploratory and the many discriminative, drug-resistance associated and prognostic important known and novel miRNAs identified in

ALL warrant new studies to demonstrate their function in the underlying biology of ALL. Since miRNAs have only been discovered in 2000, knowledge about their biological role is relatively limited. Initially miRNAs were postulated to inhibit the expression of target genes by complementary base pairing. Recent reports demonstrate that miRNAs can also enhance (instead of only repressing) the expression of their target genes by direct base pairing or through interaction with inhibitory regulators of protein translation. An example is miR-328 targeting hnRNPE2 thereby rescuing the translation of the *C/EBPα* gene[46-49]. This illustrates the emerging complexity of miRNA functioning. In addition, a single miRNA can affect the protein expression of multiple target genes.[50] However, the studies on multiple putative targets is limited by technical restrictions. Western blotting, Elisa's or protein arrays can be used for a direct candidate approach, but most of the target genes of miRNAs are yet unknown and/or specific antibodies are not (yet) available. Target predicting algorithms like TargetScan[45], PicTar[51] and EIMMo[52] often assign different genes with high likelihood to be targeted by a miRNA. However, genes for which functional studies have confirmed that they are targeted by specific miRNAs are not notified by these algorithms with the highest probability score[53]. For example *RAS* is a biologically proven target of let-7 but is ranked at position 283 by TargetScan[16]. This illustrates the limitation of the target prediction algorithms.

The fact that the biological role of miRNAs depends on cellular context in which they are expressed also complicates functional studies of miRNAs. Functional studies therefore need to be performed in the cell type in which the miRNA is aberrantly expressed.

MiRNAs may inhibit the translation of their target genes by mRNA degradation. In fact, the extent of mRNA breakdown may be more pronounced than initially appreciated as deep sequencing of ribosome-protected mRNA fragments demonstrated that three miRNAs i.e. miR-1, miR-155 and miR-223 downregulated their target genes mostly by degrading the mRNA instead of inhibiting the translational process itself[54]. Other studies suggest that mRNA decay triggers translational repression and that mRNA degradation and translational inhibition are thus coupled[55]. Therefore, correlation studies of miRNA and mRNA expression provide an alternative (large-scale) strategy to identify potential target genes. Unfortunately, it is yet not known to what extent mRNA decay is involved[55]. Moreover mechanistic studies such as the ribosome profiling study are limited to a small number of miRNAs and cell types. Therefore, we may not rule out the possibility that the proposed correlation studies exclude a considerable number of target genes regulated at the translational level as they only identify target genes that are affected by miRNAs at the mRNA level. All together, miRNA functioning is complex and not yet fully understood, which complicates the identification of the relevant target genes (among multiple candidates) for a specific miRNA.

DIFFERENT STRATEGIES TO REVEAL MICRORNA FUNCTIONING

Several strategies may be chosen to test miRNA-target gene interaction. Most commonly used is the 3'UTR luciferase binding assay[56]. Cells expressing the miRNA of interest are transfected with a construct containing a luciferase reporter sequence followed by the 3' UTR of its putative target gene. In case the miRNA binds to the 3' UTR binding sites, the transcription of the luciferase gene is prevented. The luciferase activity will thus be lower than the activity in cells that are transfected with a non-functional/mutated 3' UTR luciferase construct which prevents the miRNA from efficient 3' UTR binding[57]. In this way miR-320a and miR-494 were found to interact with anti-apoptotic *Survivin* resulting in apoptosis of leukemia cells[58]. This 3'UTR luciferase binding assay still tests miRNA-mRNA interactions in a non-physiological way driven by overexpression of reporter constructs and miRNAs. The same holds true for precipitation of Ago-2 bound miRNA/mRNA complexes[59,60]. The mRNA fraction will be either enriched (in case of silenced miRNA levels) or depleted (in case of an overexpressed miRNA) for targets of the miRNA under investigation. Although the precipitated targets are of putative biological relevance as they are bound by the miRNA/Ago-2 complex, they need to be validated by other techniques since this technique may yield many false positive targets.

Alternatively, gain- and loss-of-function studies may be more informative if studied in the correct cellular context. Overexpression of miRNAs in leukemia cells can be achieved in different ways. One may transfect cells with a synthetic precursor which is intracellularly processed into a mature miRNA or the miRNA precursor can be transduced into leukemic cells using lentiviruses. If lentiviruses are used as carrier the miRNA precursor encoding DNA is flanked by at least 150 nucleotide sequences required for the processing into mature miRNA[61]. This strategy results in cells which more stably express the miRNA of interest than in case synthetic precursors are transfected. The disadvantage of gain-of-function studies is an overload of the miRNA processing machinery, which may result in aspecific target binding by the introduced miRNA and/or may affect the processing of other miRNAs[56]. Therefore, the miRNA-target gene interaction should preferably be confirmed by loss-of-function studies.

Loss-of function of miRNAs may be studied by anti-miRNA molecules such as 2'-O-methyl-modified oligoribonucleotides, antagomirs and locked nucleic acid (LNA)-modified anti-miR oligonucleotides[56,62]. Antagomirs are very stable because of their phosphorothioate backbone[57]. Anti-miR-19 antagomir successfully inhibited miR-19 functioning which resulted in upregulation of proapoptotic *Bim*, tumor suppressor *PTEN*, AMP-activated kinase and phophatase *PP2A* in lymphocytes[63]. By means of the anti-miR-19 antagomir these genes -selected as putative target genes by 3' UTR luciferase binding assay- were confirmed as real target genes for miR-19 in *NOTCH-1*-induced T-ALL[63]. This example illustrates that a combination of different strategies to test miRNA-target gene interaction may result in the identification of real target genes.

REGULATION OF MICRORNA EXPRESSION

Dysregulation of miRNA expression can be caused by multiple factors. Lesions at the genomic level (deletions, amplifications, translocations and mutations) may comprise miR-genes and have been found in different types of cancer including leukemia[64,65]. A classical example is the deletion of *miR-15a* and *miR-16-1* on chromosome 13q14 of CLL patients. Both genes were shown to silence the expression of *BCL2* resulting in loss of apoptotic control of normal hematopoiesis[64,66]. Many other miRNAs are encoded on fragile sites relevant to pediatric ALL such as miR-34b (11q23.1), miR-100 (11q24.1) and miR-125b (11q24.1) near the *MLL*-breakpoint (11q23) and miR-181c (19p13.2) near the genomic region involved in the *E2A-PBX1*/t(1;19)(q23p13.3) translocation. However, none of these miR-genes were aberrantly expressed in either *MLL*-rearranged or *E2A-PBX1*-positive ALL patients. Apart from a mutation in the *miR-128b* gene resulting in the downregulation of miR-128b in a *MLL*-rearranged precursor B-ALL cell line[67], no other genetic aberration was found that could explain the aberrant expression of miRNAs identified in pediatric ALL. Genomic lesions affecting miR-genes are thus not the single explanation for dysregulation of miRNA expression in cancer.

Genomic aberrations may also disrupt miRNA expression by affecting other genes than miR-genes. The pseudogene *PTENP1* scavenges *PTEN*-targeting miRNAs by binding the miRNAs to the 3'UTR of *PTENP1* which is highly similar to the 3'UTR of *PTEN* itself[68]. Mutations and copy number losses comprising the 3'UTR of *PTENP1* resulted in upregulation of free *PTEN*-targeting miRNAs which may underlie the silencing of tumor suppressor *PTEN* in T-ALL cells[63,68].

MiRNAs may be co-transcribed when located within a cluster of genes that are transcribed as polycistronic transcripts[64]. An example is our finding that *miR-196b* is simultaneously transcribed with *HOXA* cluster genes, as *miR-196b* is located in between the *HOXA9* and *HOXA10* genes (**chapter 6**)[69]. Similar co-regulation may explain the correlation between miR-10a (encoded between *HOXB4* and *HOXB5*) and miR-196a (encoded between *HOXB9* and *HOXB13*) with the *HOXB* cluster genes in both adult and pediatric AML[70,71]. The high level of miR-196b expression is mainly seen in *HOXA*-activated *MLL*-rearranged leukemia and T-ALL cases with constitutively activated *HOXA* cluster genes caused by *CALM-AF10*/t(10;11)(p13;q14)-translocation, *MLL*-rearrangement or inv(7)(p15q35). These genetic subtypes of ALL have an epigenetic defect in common, i.e. the methylation of histones is facilitated due to recruitment of the histone H3K79 methyltransferase hDOT1L resulting in an opening of the chromatine structure. Together with activated *MLL* this results into selective activation of *HOXA* cluster genes[72-75]. In correspondence, we observed that high miR-196b expression was associated with hypomethylation of CpG islands in the promoter region of the *miR-196b/HOXA* locus in *MLL*-rearranged ALL patients (**chapter 6**)[69]. Aberrant epigenetic control of miR-genes in ALL has also been described for *miR-22* and *miR-124*[76,77].

Besides the fact that the expression of miR-genes can be epigenetically regulated, miRNAs themselves can affect genes involved in the epigenetic machinery. We observed that miR-152 is an effector of epigenetics ("epi-miRNA") as the seed of this miRNA shared sequence homology with the 3' UTR of the DNA methyltransferase *DNMT1* (**chapter 7**)[78]. Moreover, expression levels of miR-152 inversely correlated to those of *DNMT1* in *MLL*-rearranged ALL[78]. Other epi-miRNAs are miR-290 which was shown to control *de novo* DNA methylation by targeting *DNMT3*[79,80] and miR-29b which affects both *de novo* DNA methylation and its maintenance by negatively regulating *DNMT1* and *DNMT3*[81].

MICRORNAS AS TARGETS FOR THERAPY

Dysregulation of miRNAs by epigenetics may offer a therapeutic option. The demethylating agent zebularine reactivated 7 out of 11 miR-genes that were silenced in children with t(4;11)-positive *MLL*-rearranged ALL (**chapter 7**)[78]. These miR-genes (i.e. miR-10a, -152, -200a, -200b, -429, -432, and miR-503) were silenced through DNA hypermethylation suggesting that they may act as tumor suppressors. Accordingly, miR-432 is part of the large miR-127 cluster that downregulates oncogenic *BCL-6* at the protein level[82]. For miR-152 we identified germline *MLL*, *AF4-MLL* and *DNMT1* as putative targets based on sequence homology between the 3' UTR of the genes and the miRNA's seed sequence as predicted by the algorithms Targetscan[45] and Pictar[51,53]. Interestingly, co-expression of wild-type *MLL* facilitated leukemogenesis induced by the MLL-AF9 fusion protein[83] and also the AF4-MLL fusion product was a strong promoter of leukemic transformation[84]. Moreover, high levels of *DNMT1* may contribute to the genome-wide hypermethylation that characterizes t(4;11)/*MLL*-rearranged infant ALL which was associated with an increased risk of relapse in hypermethylated patients[85]. Interestingly, the methylation level of miR-152 inversely correlated with clinical outcome (**chapter 7**)[78], which indicates that demethylating agents like zebularine may be a new class of agents in the treatment of high-risk *MLL*-rearranged precursor B-ALL patients.

De-methylating agents provide an indirect way to manipulate miRNA expression since not only the miR-gene but also other (protein-coding) genes will become activated. A more direct strategy to increase miRNA expression levels comprise the use of synthetic precursor miRNAs and viral miRNA precursors. Such precursors are commonly applied in gain-of-function studies but may also serve a therapeutic function in case of precursors encoding favorable miRNAs (e.g. miR-10a and miR-152) or tumor suppressors (e.g. let-7). A viral vector with a tissue-specific promoter may carry the miRNA to the relevant tissue. Viral delivery of miR-26a resulted in an accumulation in the liver of mice where it effectively inhibited tumor formation[86]. A similar strategy using a viral vector with a promoter that is specific for the bone marrow may be used to introduce favorable prognostic miRNAs into leukemia cells.

A direct approach to inhibit miRNAs may involve antagomirs or other anti-miR-oligonucleotides. Treatment with an antagomir directed against miR-196b abrogated the growth advantage of MLL-AF9 transformed/miR-196b overexpressing bone marrow cells[3]. This suggests that a direct inhibition of even a single miRNA may be of clinical benefit. Besides this artificial model successful silencing with antagomirs has also been shown *in vivo*. Antagomirs directed against miR-16, miR-192, and miR-194 in mice resulted in an efficient and long-lasting inhibition of these miRNAs in multiple organs including bone marrow, liver and adrenal glands[57]. Anti-miR-122 was shown to effectively reduce expression levels of miR-122 and cholesterol in non-human primates[62]. Silencing of this miR-122 was also effective in treating similar primates being positive for chronic hepatitis C[87]. These findings suggest that interference with miRNA expression levels by anti-miRs may offer a new avenue for targeted therapy in humans. Alternative strategies make use of miR-masks[88] and miRNA sponges[89] to inhibit miRNA functioning. MiR-masks are oligoribonucleotides of similar length as that of miRNA and compete with miRNA for binding with the complementary 3'UTR site of the target mRNA[88]. The drawback of the miR-mask principle is that the miR-mask affects only one target gene of the targeted miRNA whereas other anti-miRNA molecules including antagomirs and miRNA sponges may affect multiple target genes at once. The miRNA sponges represent oligonucleotides containing multiple binding sites for miRNA and thereby the sponge literally absorbs its target miRNA and reduces the number of miRNA strands able to bind their target mRNA molecules[90, 91]. Whereas the cholesterol moiety at the 3' end of the antagomir supports the entrance into cells, the sponges need lentiviruses to be introduced. E.g. a lentiviral vector served as carrier for the miR-326 sponge, which effectively depleted miR-326 in T-cells of mice upon intravenous injection[92]. All together, these examples illustrate the therapeutic potential of miRNA-interference to treat acute leukemia. Potential targets for anti-miRNA therapy in pediatric ALL in the future may be those miRNAs that exert oncogenic action (e.g. miR-196b in *MLL*-rearranged leukemia), miR-genes associated with drug resistance (miR-99a, miR-100 and miR-125b) or those of unfavorable prognostic significance (e.g. miR-33)[16,69,78]. So far the toxicity of these miRNA-specific depletion strategies are low which encourages further exploration of their therapeutical benefit[93].

FINAL CONCLUSIONS

This thesis demonstrates that acute leukemia in childhood is not only a disease of dysregulated protein-coding genes, but also involves the regulators of the protein-coding genes. Although the biological value of miRNAs was unknown for a long time, present examples of aberrant miRNAs (e.g. miR-125b, miR-155 and miR-21) illustrate their potential importance in leukemia. Functional studies are currently ongoing to determine their role in leukemogenesis and drug resistance. Moreover, miRNAs warrant clinical attention especially

since we observed that aberrant expression and methylation levels are linked to clinical outcome and can be modulated. Therefore, we speculate that ALL patients may benefit from future miRNA-targeted therapeutic strategies. All together we conclude that miRNAs appear small, but may be of great value to understand and perhaps treat pediatric ALL.

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Chapter 10

Nederlandse Samenvatting

Het menselijk lichaam is opgebouwd uit heel veel verschillende soorten cellen. Er wordt geschat dat het aantal cellen oploopt tot 100 biljoen (10^{14}) afhankelijk van lengte, postuur en vele andere factoren. In deze cellen bevindt zich het DNA. In het DNA ligt de informatie opgeslagen van vele genen. De mens heeft ~25.000 genen die ieder coderen voor een ander eiwit. Deze eiwitten heeft een cel nodig om te kunnen functioneren. Als er een fout optreedt in een gen kan dit leiden tot een afwijkend eiwit. Dergelijke fouten in het DNA en afgeleide eiwitten kunnen er toe leiden dat een cel niet meer normaal kan functioneren. Abnormale deling en overleving van cellen kan op die manier tot kanker leiden.

Behalve eiwit-coderende genen bestaan er ook vele niet-eiwit coderende genen. Voor het jaar 2000 dacht men dat deze genen overbodig waren en dat alleen genen die voor eiwit codeerden belangrijk waren voor het functioneren van een cel. Dit besef veranderde echter door de ontdekking van microRNAs (miRNAs) in zoogdieren en de daarop volgende publicaties over de rol van miRNAs bij de remming van eiwitsynthese. Deze studies toonden aan dat hoewel miRNAs klein zijn en niet coderen voor eiwit, ze wel degelijk van groot belang zijn, omdat ze de eiwitproductie van vele eiwit-coderende genen kunnen remmen. MiRNAs doen dit vooral door het complementair binden met de zogenaamde 'untranslated region' aan het 3' uiteinde (3'UTR) van *messenger*RNA (mRNA) oftewel 'target mRNA'. Op deze wijze sturen miRNAs belangrijke biologische processen aan zoals proliferatie, differentiatie en apoptose. Dergelijke processen kunnen in het geval van leukemie, kanker van de witte bloedcellen, verstoord zijn en daarom luidde onze hypothese als volgt: afwijkende regulatie van miRNAs draagt, evenals die van eiwit-coderende genen, bij aan de onderliggende biologie van leukemie.

Een overzicht van de huidige kennis van miRNAs als kleine biologische spelers in acute leukemie is weergegeven in **hoofdstuk 2**. Om uit te zoeken welke miRNAs relevant zijn voor de meest voorkomende vorm van leukemie bij kinderen, acute lymfatische leukemie ofwel ALL, hebben we aanvankelijk een conventionele kloneringsmethode gebruikt. Hierbij werden zogenaamde concatemeren van small RNA moleculen gevormd, gevolgd door Sanger cDNA-sequencing. Middels deze methode werden 105 bekende en 8 nieuwe miRNA genen in de leukemiecellen van kinderen met ALL ontdekt (**hoofdstuk 3**). In **hoofdstuk 4** werd de high-throughput oftewel deep sequencing kloneringsmethode toegepast op 98 patiënten die zeven ALL subtypen en drie normale hematopoietische weefsels vertegenwoordigden. Deze methode bleek veel sensitiever dan de conventionele methode gebaseerd op Sanger cDNA-sequencing en resulteerde in de ontdekking van nog eens 28 nieuwe en 431 kandidaat nieuwe miRNA genen. Naast deze nieuwe genen werd de expressie van 470 bekende miRNAs aangetoond en daarmee ook de waarde van de high-throughput methode. Vele nieuwe, maar ook bekende miRNAs kwamen exclusief tot expressie in ALL subtypen en normale hematopoietische cellen. Opvallend was dat miRNAs die voor het eerste gekloneerd waren minder evolutionair geconserveerd en minder hoog tot expressie kwamen dan bekende miRNAs. Dit duidt mogelijk op het feit dat miRNAs specifiek kunnen zijn voor

leukemie en normale hematopoietische cellen en daarom niet eerder ontdekt zijn in andere weefsels. De ontregelde expressie van een selectie nieuwe en kandidaat nieuwe miRNAs werd bevestigd met een tweede techniek, namelijk de op stem-loop gebaseerde real-time kwantitatieve polymerase chain reaction (RT-qPCR). Om nader te bekijken welke miRNAs mogelijk relevant voor acute leukemie zijn werd er een grootschalige RT-qPCR analyse van 397 miRNAs (waaronder nieuwe miRNAs) verricht in 81 patiënten met zeven verschillende ALL subtypen en in normale hematopoietische cellen (**hoofdstuk 5**). Bij deze analyse werden miRNA expressieprofielen ontdekt die differentieerden tussen verschillende ALL subtypen waaronder T-ALL, *MLL*-gen herschikte, *TEL-AML1*- positieve, hyperdiploid en *E2A-PBX1*-positieve precursor B-ALL, maar niet onderscheidend waren voor *BCR-ABL*-positieve of andere precursor B-ALL (B-other, negatief voor de hier bovengenoemde genetische afwijkingen). De leukemie-gerelateerde expressieprofielen verschilden tevens van de profielen die werden gezien in normale hematopoietische cellen. Voorbeelden hiervan zijn de verhoogde expressie van miR-196b specifiek voor *MLL*-gen herschikte precursor B-ALL en een subgroep van T-ALL patiënten en de verhoging van miR-708 in patiënten met precursor B-ALL zonder *MLL*-gen herschikking (**hoofdstuk 3** en **5**). Tevens hebben we een set van 14 miRNAs geïdentificeerd die de prognose van kinderen met ALL kunnen voorspellen onafhankelijk van het desbetreffende subtype (**hoofdstuk 5**). Een slechte prognose wordt onder andere bepaald door cellulaire resistentie tegen medicijnen[1-4]. In **hoofdstuk 5** ontdekten we een set miRNAs die verschillend tot expressie kwamen in leukemie cellen die medicijn gevoelig waren in vergelijking met cellen die resistent waren. Zo werd er een hogere expressie van *miR-125b* en naastliggende genen *miR-99a* en *miR-100* gevonden in leukemie cellen van patiënten die relatief ongevoelig voor vincristine en daunorubicine waren. Deze resistentie-geassocieerde miRNAs verdienen samen met miRNAs die discrimineren tussen de verschillende ALL subtypen ofwel voorspellend zijn voor de prognose van ALL meer onderzoek naar de functionele rol in de hematopoiese.

In **hoofdstuk 6** laten we zien dat hoge expressie van miR-196b voornamelijk voorkomt in patiënten met *HOXA*-geactiveerde leukemie. Hiertoe behoren de patiënten met *MLL*-gen herschikte precursor B-ALL of T-ALL met een *MLL*-gen herschikking, *SET-NUP214*-translocatie, *CALM-AF10*-translocatie of een inversie van chromosoom 7. Aangezien miR-196b wordt gecodeerd door een gen wat binnen het *HOXA* genen cluster ligt, suggereert de gecorreleerde expressie van *miR-196b* en *HOXA* dat er een gezamenlijk mechanisme tot activatie bestaat. De promoter CpG eilanden binnen het DNA locus dat zowel het *HOXA* cluster als *miR-196b* omvat, bleken minder gemethyleerd te zijn in patiënten met *MLL*-gen herschikking in vergelijking met patiënten met een normaal *MLL*-gen en in vergelijking controles met normaal beenmerg. Dit wijst erop dat DNA hypomethylatie mogelijk (deels) de oorzaak is van de verhoogde expressie van miR-196b. Aangezien is beschreven dat miR-196b oncogenetische activiteit heeft in voorlopercellen van het beenmerg[5], duiden onze

bevindingen op een potentiële rol voor miR-196b in de pathologie van *HOXA*-geactiveerde leukemie.

MLL-gen herschikte ALL patiënten, vooral dragers van *MLL-AF4*/t(4;11)(p21;q23), werden gekarakteriseerd door verlaagde expressie van tenminste 11 miRNAs ten gevolge van DNA hypermethylatie (**hoofdstuk 7**). De epigenetische remming van 7 miRNA genen kon ongedaan gemaakt worden door toediening van het demethylerende medicijn zebularine. Aangezien miRNAs die verlaagd tot expressie komen een tumor remmende werking kunnen hebben, is het effekt van zebularine veelbelovend. Eén van de miRNAs die tot re-expressie werden gebracht was miR-152. Dit miRNA heeft een 3'UTR sequentie die complementair is aan zowel het normale *MLL*-gen, het *AF4-MLL* fusiegen en het DNA methylerende *DNMT1*-gen. Dit is opvallend aangezien co-expressie van het normale *MLL*-gen essentieel is voor *MLL-AF9*-geinduceerde leukemogenese en het AF4-MLL fusie eiwit cruciaal is voor de leukemische transformatie van *MLL-AF4*-positieve cellen[6,7]. De hoge expressie van *DNMT1* zou mogelijk de genoom brede hypermethylatie van promoter-CpG eilanden in t(4;11)-positieve ALL bij zuigelingen kunnen verklaren. Deze hypermethylatie bleek onlangs gekoppeld aan een ongunstige prognose[8]. De re-expressie van miR-152 ten gevolge van de blootstelling aan zebularine geeft aan dat de-methylerende medicijnen een toekomst kunnen hebben in de therapie van *MLL*-gen herschikte ALL, mede ook daar het methylatieniveau van miR-152 omgekeerd evenredig was aan de prognose van deze patiënten.

In dit proefschrift hebben we een aanzienlijk aantal bekende en nieuwe miRNAs geïdentificeerd die specifiek bleken te zijn voor diverse ALL subtypen en medicijn resistentie en die tevens de prognose van kinderen met ALL konden voorspellen. De afwijkende expressie van sommigen van deze onderscheidende miRNAs kon worden opgeheven middels behandeling met de-methylerende agentia. Ondanks hun bescheiden afmeting zouden miRNAs wel eens veel voor de behandeling van leukemie kunnen betekenen.

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About the Author

CURRICULUM VITAE

Diana Schotte (Rotterdam, 1976) behaalde in 1995 haar Gymnasium diploma aan het Emmauscollege te Rotterdam. In datzelfde jaar begon zij met de studie Medische Biologie aan de Universiteit van Utrecht alwaar zij vanaf 1998 ook Geneeskunde studeerde. In het kader van Medische Biologie werden verschillende wetenschappelijke stages doorlopen waaronder een van 9 maanden op de afdeling Immunologie van het UMC Utrecht (supervisoren: Drs. N.W. van de Donk, Dr. A.C.Bloem en Dr. H.M. Lokhorst) . Na het verkrijgen van beide doctoraal diploma's (2002) begon zij met haar coschappen welke onder andere aan de Flinder's University (Adelaide, Australië) werden doorlopen. In 2005 genoot zij een extra keuze-coschap op de afdeling Onco-Hematologie en Immunologie in het Wilhelmina Kinderziekenhuis te Utrecht (supervisor: Drs. A.T.H. van Dijk). Aansluitend startte Diana als promovendus op de afdeling Kinderoncologie/Hematologie aan het Erasmus MC-Sophia Kinderziekenhuis (promotor: Prof. R. Pieters) wat resulteerde in dit proefschrift. Bij aanvang van dit promotietrajekt heeft zij gedurende een jaar (2005-2006) in het laboratorium van Dr. Chang-Zheng Chen aan de Stanford University (Stanford, USA) gewerkt aan het kloneren van miRNAs in leukemie. In 2010 keerde Diana weer terug in de kliniek alwaar zij werkzaam was als arts-assistent niet opleiding in het Wilhelmina Kinderziekenhuis te Utrecht (hoofd: Prof.dr. E.E.S. Nieuwenhuis en Dr. J. Frenkel). In januari 2012 gaat zij van start met de opleiding tot kinderarts, welke begeleid zal worden door Prof.dr. H.J. Verkade en Mr.dr. A.A.E. Verhagen in het Beatrix Kinderziekenhuis te Groningen.

List of Publications

Schotte D, Pieters R, Den Boer ML; MicroRNAs in acute leukemia: from biological players to clinical contributors. *Leukemia 2011, Jun 24 [Epub ahead of print].*

Schotte D, Akbari Moqadam F, Lange-Turenhout EAM, Chen C, Pieters R, Den Boer ML; Discovery of new microRNAs by small RNAome deep sequencing in childhood acute lymphoblastic. *Leukemia 2011, May 24 [Epub ahead of print].*

Danen-van Oorschot A, Kuipers J, Arentsen-Peters S, **Schotte D**, De Haas V, Trka J, et al.; Differentially expressed microRNAs in cytogenetic and molecular subtypes of pediatric AML. *Pediatr Blood Cancer 2011, in press.*

Stumpel DJPM, **Schotte D**, Lange-Turenhout EAM, Schneider P, Seslija L, De Menezes RX, Marquez VE, Pieters R, Den Boer ML en Stam RW (**shared first** and last authorship); Hypermethylation of specific microRNA genes in MLL-rearranged infant Acute Lymphoblastic Leukemia: - Major matters at a micro scale - *Leukemia 2011; 25:429-39.*

Schotte D, De Menezes RX, Akbari Moqadam F, Lange-Turenhout E, Chen C, Pieters R, Den Boer ML; MiRNAs Characterize Genetic Diversity and Drug Sensitivity in Pediatric Acute Lymphoblastic Leukemia. *Haematologica 2011; 96:703-11.*

Schotte D, Lange-Turenhout E, Stumpel DJPM, Stam RW, Meijerink JPP, Pieters R, Den Boer ML; Expression of miR-196b is not exclusively MLL-driven but especially linked to activation of HOXA-genes in pediatric ALL. *Haematologica 2010; 95:1675-82.*

Kotani A, Ha D, **Schotte D**, Den Boer ML, Armstrong SA, Lodish HF; A novel mutation in the miR-128b gene reduces miRNA processing and leads to glucocorticoid resistance of MLL-AF4 acute lymphocytic leukemia cells. *Cell Cycle 2010; 9:1037-42.*

Kotani A, Ha D, Hsieh J, Rao PK, **Schotte D**, Den Boer ML, Armstrong SA, Lodish HF; miR-128b is a potent glucocorticoid sensitizer in MLL-AF4 Acute Lymphoblastic Leukemia cells and exerts synergistic effects with miR-221. *Blood 2009; 114:4169-78.*

Schotte D, Chau JCK, Sylvester G, Liu G, Chen C, Van der Velden, Pieters R, Den Boer ML; Identification of new miRNA genes and aberrant miRNA expression in childhood acute lymphoblastic leukemia. *Leukemia 2009; 23:313:22.*

Van de Donk NW, **Schotte D**, Kamphuis MM, Van Marion AM, Van Kessel B, Bloem AC, Lokhorst HM; Protein geranylgeranylation is critical for the regulation of survival and proliferation of lymphoma tumor cells. *Clin Cancer Res. 2003; 9:5735-48.*

Boven LA, Middel J, Breij ECW, **Schotte D**, Verhoef J, Nottet HSLM; Interactions between HIV-infected monocyte-derived macrophages and human brain microvascular endothelial cells result in increased expression of CC chemokines. *J Neurovirol. 2000; 6:382-9.*

PhD Portfolio

Summary of PhD training and teaching activities

Name PhD student: Diana Schotte
PhD period: 2005-2010
Erasmus MC department: Pediatric Oncology /Hematology
Promotor: Prof.dr. R. Pieters
Research School: Molecular Medicine
Co-promotor: Dr. M.L. den Boer

1. PhD training

	Year	Workload (ECTS)
General courses		
– English Biomedical Writing and Communication	2008	4
– Statistics: Classical Methods for Data-analysis	2007	5.7
Specific courses (e.g. Research school, Medical Training)		
– Molecular diagnostics, Rotterdam	2007	0.4
Seminars and workshops		
– Workshop on Basic Data Analysis on Gene Expression Arrays II, Rotterdam	2009	0.2
– Annual PhD day, Rotterdam	2008	0.2
– Workshop 'Browsing Genes and Genomes with Ensembl', Rotterdam	2007	0.4
– Annual Pediatric Oncology Symposium	2005, 2007-2009	0.8
– Annual Pediatric Research Day, Rotterdam	2006-2009	0.8
– Annual Molecular Medicine Day, Rotterdam	2007-2009	0.6
– Workshop on MiRNAs in Hematopoiesis and Cellular Development, Annapolis, USA	2007	1
– Stanford Microbiology and Immunology Departmental Retreat, Asilomar, USA	2005	0.4

	Year	Workload (ECTS)
Oral Presentations		
– MicroRNAs characterize Genetic Diversity and Drug Sensitivity in Pediatric Acute Lymphoblastic Leukemia; 51th ASH Annual Meeting	2009	1 1
– Expression of miR-196b is not exclusively MLL-driven but especially linked to Activation of HOXA genes in Children with Acute Lymphoblastic Leukemia; 51th ASH Annual Meeting	2009	1
– Differential Expression of MicroRNAs in Subtypes of Childhood Acute Lymphoblastic Leukemia; 40th SIOP Annual Meeting	2008	1
– Specific MicroRNA Profiles in Childhood Acute Lymphoblastic Leukemia; 6th Bi-Annual I-BFM Leukemia Symposium	2008	1
– MicroRNAs in Pediatric Acute Lymphoblastic Leukemia; 12th Annual Molecular Medicine Day	2007	1
– MicroRNAs in Acute Leukemia; Annual Pediatric Research Day, 2006; 1 ECTS	2006	
– Unique MicroRNA Profiles in Childhood Acute Lymphoblastic Leukemia; 48th ASH Annual Meeting	2006	1
– Overview of the PhD project; 6 presentations each year at the Weekly Pediatrics/Pediatric Oncology Research Meetings	2006-2009	3.6
Poster Presentations		
– Specific MicroRNA Profiles for Childhood Acute Lymphoblastic Leukemia; Keystone Symposium: RNAi, MicroRNA, and Non-Coding RNA	2008	1
– Identification of new miRNA genes in Childhood Acute Lymphoblastic Leukemia; Workshop on miRNAs in hematopoiesis and cellular development	2007	1
International conferences		
– 51th ASH Annual Meeting, New Orleans, USA	2009	1
– Keystone Symposium: RNAi, MicroRNA, and Non-Coding RNA, Whistler-Canada	2008	1
– 40th SIOP Annual Meeting, Berlin, Germany	2008	1
– 6th Bi-Annual I-BFM Leukemia Symposium, Glasgow, United Kingdom	2008	0.6
– 49th ASH Annual Meeting, Atlanta, USA	2007	1
– 48th ASH annual Meeting, Orlando, USA	2006	1

	Year	Workload (ECTS)
Other		
– **Visiting Researcher at Stanford University, USA** Department of Microbiology and Immunology under supervision of Dr. C.Z. Chen en Dr. M.L. den Boer	2005-2006	10
– **Awards and grants**		
– Travel grant for the 48th and 51th ASH Annual Meeting awarded by the ASH committee	2006, 2009	0
– Travel grant for Keystone Symposium: RNAi, MicroRNA, and Non-Coding RNA, awarded by Erasmus Trustfonds	2008	0.1
– Travel grant for the Workshop on MiRNAs in Hematopoiesis and Cellular Development awarded by the organising committee	2007	0
– Award for best oral presentation, 12th Annual Molecular Medicine Day	2007	0
– **Committee assignments** PhD committee, Erasmus MC, Rotterdam; representative of the Erasmus Postgraduate School Molecular Medicine	2007-2009	1

2. Teaching

	Year	Workload (ECTS)
– Supervising students participating in the Msc Molecular Medicine Program of the Erasmus University during their two-week traineeship of different laboratory techniques	2007	1
– Supervising Charlotte Verouden, student of Applied Science-Biomedical Science, Life Science, Fontys Hogeschool Toegepaste Natuurwetenschappen (Eindhoven) during her a 6-month traineeship on "The function of miR-708 in ALL"	2007-2008	10
Total		**55.8**

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Zonder startbewijs geen aanvang van de wielerkoers

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In geval van lekke band of gebroken ketting is de juiste technische ondersteuning onmisbaar

Ellen, ik durf te promoveren! Jouw ervaring en geroutineerdheid waren een luxe voor mij. Vol bewondering zie ik hoe je een gezin met 4 kids weet te combineren met je baan. Ik heb het je al eens gezegd, maar doe het nogmaals: ontzettend bedankt voor al je hulp.

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Voor het uitstippelen van de te nemen route

Waren mijn collega AIO's en post-docs uit de B-ALL groep onmisbaar:
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Zonder juist materiaal kun je niet koersen – without appropriate equipment you can not ride

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Om elkaar uit de wind te houden waren mijn fietsmaatjes onmisbaar

Om te delen in gezelligheid, de spannende en tegenvallende resultaten, een geaccepteerd abstract..!

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If you climb the hills abroad, you need some extra power

For that energy, the teaching of the lab techniques and fun during the lunch breaks and lab days, I thank my colleagues in the Chen lab:

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It's the little things that make big things possible

Rosalyn, strange things were happening around me. ... Thank you for doing all the paper work, your ideas of good places and certainly not to forget: the milk in the refrigerator and bread on the table in my new Stanford apartment, I immediately felt at home!

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