MicroRNAs in Pediatric Acute Lymphoblastic Leukemia: Functions and Targets

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MicroRNAs in Pediatric Acute Lymphoblastic Leukemia: Functions and Targets

MicroRNAs in acute lymfatische leukemie bij kinderen:
functie en doelwit-genen

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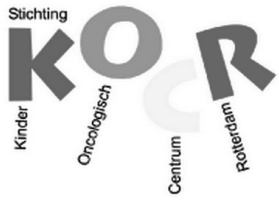

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"Human beings are members of a whole,
In creation of one essence and soul.

If one member is afflicted with pain,
Other members uneasy will remain.

If you've no sympathy for human pain,
The name of human you cannot retain!"

Saadi, persian poet, 1184-1283

To the children who are fighting leukemia and to their supporting parents

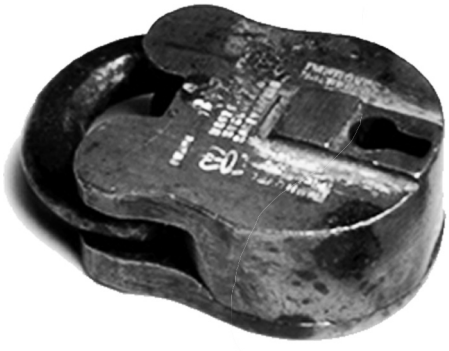
&

to the medical and research teams helping them.

CONTENTS

Chapter 1

Introduction



Normal and malignant Hematopoiesis

All different types of circulating blood cells originate from hematopoietic stem cells (HSCs). The pluripotential HSCs have the capacity of self-renewal, which provides the body with a secure backup of HSCs. HSCs can also differentiate into progenitor cells, which can further differentiate to different types of blood cells. This continuous process of blood cell production mainly happens in the bone marrow and is called “hematopoiesis”.

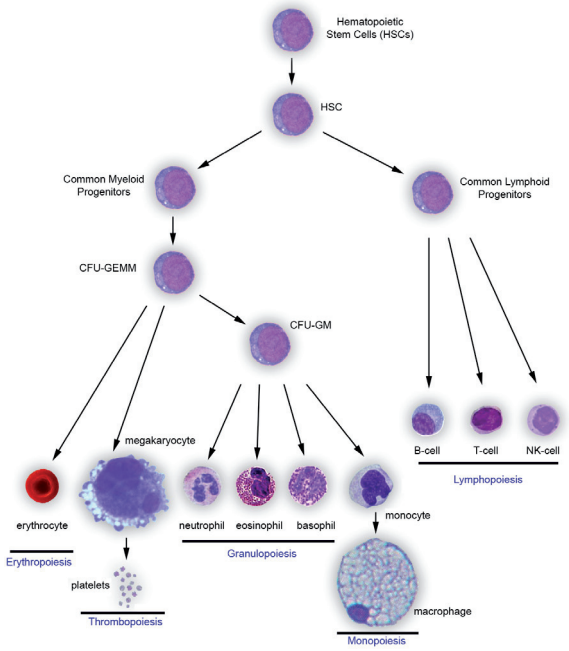
At the first step of hematopoiesis two distinct populations of cells are generated: common lymphoid progenitors and common myeloid progenitors. As shown in Figure 1, these common progenitors give rise to a variety of mature blood cells via several processes: thrombopoiesis (to produce thrombocytes), erythropoiesis (to produce erythrocytes), granulopoiesis (to produce basophils, neutrophils and eosinophils), monopoiesis (to produce monocytes and then macrophages and myeloid dendritic cells) and lymphopoiesis (to produce B-cells, T-cells and natural killer cells). During hematopoiesis, the differentiated cells lose their proliferative potential but gain more functionality. This is a crucial process, which keeps the number of circulating blood cells under control. Division and differentiation of hematopoietic cells are controlled by several regulators such as epigenetic modifiers, transcription factors, post-transcriptional modulators, cytokines and growth factors. Disruption in hematopoiesis may lead to a differentiation arrest and excessive growth of immature blood cells, which are called "blasts". Acute leukemia (leukos and haima means white and blood in Greek, respectively) is a disease of uncontrolled high proliferation of the blasts and classified according to the lineage origin of the blasts (lymphoid or myeloid). In addition, leukemia with slow onset and slow progression is called "chronic leukemia".

Pediatric Acute Lymphoblastic Leukemia

Leukemias constitute approximately one-third of cancers in children (age 0-18 years) during 1978-1997 in Europe [1]. Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer and represents almost one fourth of all childhood neoplasia [2]. ALL is also the most common type of childhood acute leukemias (~85%) and has a peak in incidence between the ages of 2 and 4 years [1]. In the Netherlands and other Northern and Western European countries, each year about 5 in 100,000 children are diagnosed with leukemia. In recent years, the average number of newly diagnosed cases with acute leukemia per year was approximately 140 in the Netherlands which includes 120 newly diagnosed ALL cases per year [3]. Pediatric ALL is a heterogeneous disease in which different genetic lesions result in the development of leukemia in T- and B-cell lineages [4]. As shown in Figure 2, ~15% of children with ALL are diagnosed with T-ALL which are divided into several subtypes based on cytogenetic abnormalities and type-A/type-B mutations [4, 5].

Around 85% of children with ALL are diagnosed with B-cell precursor ALL (BCP-ALL) [6]. The estimated frequency of specific genotypes in childhood ALL as well as frequency and risk assessment of cytogenetic subtypes of BCP-ALL is shown in Figure 2.

Figure 1: Normal hematopoiesis



Adapted from: http://daley.med.harvard.edu/assets/Willy/hematopoiesis.jpg, with the permission of Dr. M.W. Lensch PhD, Harvard Stem Cell Institute.

The major cytogenetic abnormalities that have been identified in BCP-ALL include t(12;21) resulting in a *ETV6-RUNX1* fusion, t(9;22) resulting in a *BCR-ABL1* fusion, several translocation involving either *TCF3* or *MLL* genes and global chromosomal copy number changes. These cytogenetic abnormalities are traditionally used to estimate the risk on treatment failure of patients and guide the choice of treatment to be given [7]. Due to improvements in medical care and treatment strategies in Western European countries, about 85% of children with ALL survive the first 5 years after diagnosis [3, 4]. In general, high hyperdiploidy (>50 chromosomes) and *ETV6–RUNX1*-positive ALL are associated with favorable outcome, whereas low hypodiploidy (<44 chromosomes) and *MLL* rearranged ALL (especially in infants and adults) are associated with poor prognosis (Figure 2). However, the highest absolute number of relapses still occurs in patients diagnosed as genetically unclassified BCP-ALL [8]. New molecular technologies such as high-throughput sequencing and high-resolution microarray profiling have resulted into the identification of new genetic lesions which affect regulator genes/molecules of lymphoid development, cell cycle, tumor suppressors or lymphoid signaling pathways: e.g. *PAX5*, *IKZF1*, *EBF1*, *CRLF2*, *CREBBP*, *JAK1* and *JAK2* [9]. Mutations and genetic aberrations of some of them (e.g. *IKZF1*-deletion and *CRLF2*-rearrangement) are associated with poor clinical outcome in BCP-ALL [9, 10]. Similarly, gene expression profiling provides the opportunity to detect genes which are differentially expressed between different types of leukemias. These genes can potentially be used as new candidate targets for treatment of drug resistant and/or relapsing leukemias. Moreover, gene expression profiling has the

potentials to be used for genome-wide classification of ALL and identify new sets of cases with similar clinical or biological behavior [11].

Figure 2: Frequency of genotypes in childhood ALL

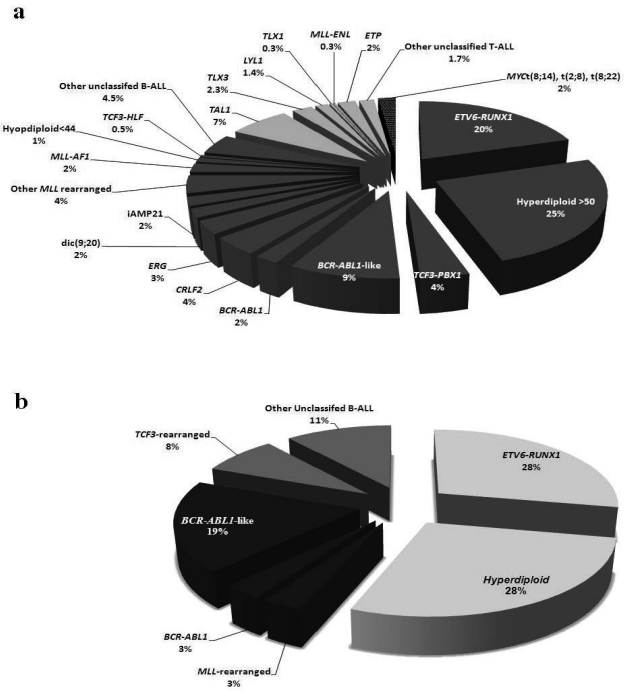


Figure 2: (a) The estimated frequencies of genotypes in childhood ALL [4], genetic lesions which are specific for BCP-ALL or mutually present in either BCP-ALL or T-cell ALL are shown in dark gray and T-cell ALL specific genetic lesions are shown in light gray. (b) The frequency of cytogenetic subtypes in BCP-ALL. Good prognostic subtypes are shown in light gray, poor prognostic subtypes in black and intermediate prognostic subtypes in gray.

Despite refinement in the diagnosis and treatment of pediatric ALL, there are still a considerable number of patients with poor clinical outcome due to resistance of leukemic cells to chemotherapy. This reflects the need to explore the disease from multiple aspects in order to better understand the pathobiology of pediatric ALL. Non-coding RNAs are recently discovered molecules that show a clear association with initiation and progression of leukemias. The tiny molecules of miRNAs were basically introduced 1-2 decades ago, have a high impact in driving normal and malignant hematopoiesis, and recently are introduced as early diagnostic molecules, predictive tools and therapeutic targets.

MicroRNAs in general

When in 1961, Jacob and Monod reported that some tiny RNA molecules can bind to messenger RNAs (mRNAs) through base-pairing and therefore inhibit mRNA expression [12], no one could imagine that in the following decades these small noncoding RNAs would open a new chapter in our understanding of how the expression of genes is being regulated.

The first functional clues appeared in 1993 when ~21-basepair-long RNAs were discovered in nematodes which were involved in post-transcriptional gene silencing [13]. Few years later, these newly called microRNAs (miRNAs) were found in *Drosophila melanogaster*, *Caenorhabditis elegans*, mammals and humans [14-16]. It was just the beginning of what we now know as one of the most conserved and abundant classes of gene regulatory molecules [17].

The highly conserved miRNAs-encoding genes are mapped all over the human genome and are categorized as inter-genic, intronic and exonic (Figure 3a) [18]. As shown in Figure 3b, primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II either in a single form or a cluster of multiple miRNAs. Each hairpin structure of pri-miRNA is composed of ~70-nucleotides and flanked by sequences which are necessary for further steps of maturation. Within the nucleus, pri-miRNAs are trimmed into premature miRNA (pre-miRNA) by a microprocessor complex consisting of DCR8 and RNase II enzyme, termed Drosha. The double-stranded hairpin structure of the pre-miRNA is characterized by a loop and a two-nucleotide overhang at 3' end. The pre-miRNAs are transferred to the cytoplasm by a multiprotein complex, which includes Exportin 5. In the cytoplasm, another protein complex comprising DICER and TRBP further processes pre-miRNAs to remove the hairpin-loop joining 3' and 5' arms, resulting into the imperfect duplex of miRNA:miRNA* which is about 22 nucleotides in length. At the final step, miRNAs are captured by the RNA induced silencing complex (RISC). RISC contains argonaute proteins (AGO), such as SLICER and the RNase H-like domain protein, PIWI. Proteins of RISC unwind the miRNA duplex and load the mature single strand miRNA to its partial complementary sequence in the 3' untranslated regions (UTRs) of the target protein-coding mRNA [19-21]. The passenger strand (miRNA*) is normally degraded. However, in some cases both strands of miRNA duplex may remain functional and target different mRNAs. Generally, miRNA-mRNA binding happens via a partial Watson-Crick complementarity [19, 22]. However, in most of the cases strict complementarity of the nucleotides 2-7 at the 5' end of miRNA – which is known as the "seed" sequence" – with the target sequence is mandatory [23]. As a consequence, miRNA binding to target mRNA may provoke mRNA degradation and/or translation inhibition (Figure 3b) via the following mechanisms: 1) Cap-40S initiation inhibition, 2) inhibition of 60S ribosomal unit joining, 3) elongation inhibition, 4) premature termination by ribosomal drop-off, 5) co-translational nascent protein degradation, 6) sequestration in P-bodies, 7) mRNA destabilization, 8) mRNA cleavage, and 9) translation inhibition through miRNA-mediated chromatin following by gene silencing [24].

MiRNAs play an important role in fine-tuning of the expression of more than 60% of human protein-coding genes [26]. MiRNAs regulate several general functions such as proliferation and cell cycle progression [27], apoptosis [28] and differentiation [29, 30]. In addition, they also were shown to regulate tissue-specific processes [31, 32]. One miRNA can target several hundred mRNAs; therefore, a change in expression levels of one miRNA can affect different transcripts. Aberrant expression levels of miRNAs correlate with development of multiple diseases, including cancer [33].

Figure 3: Biogenesis of miRNAs

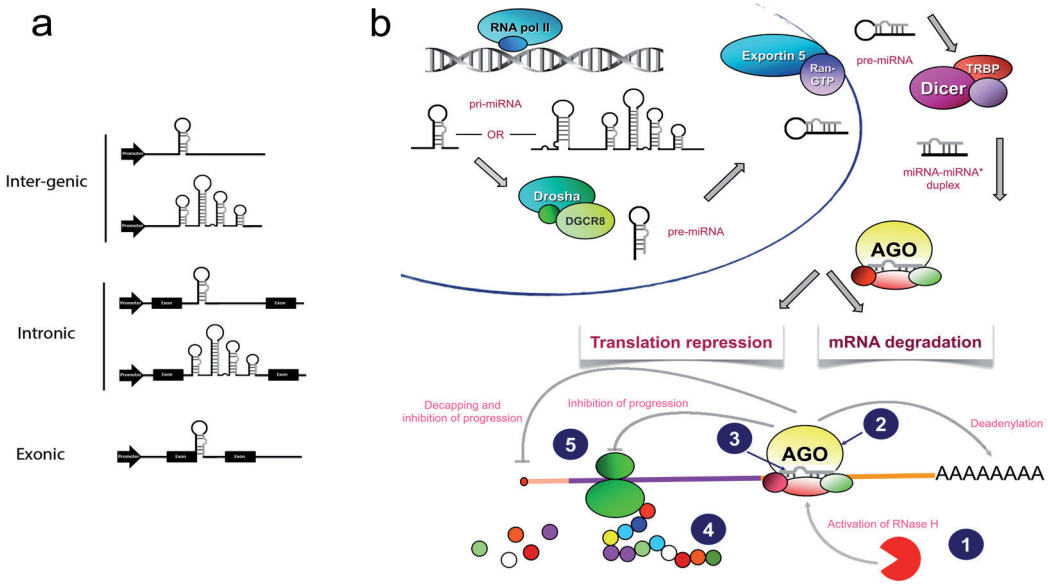


Figure 3: (a) The miRNAs originate from different locations within the human genome as: inter-genic, intronic or exonic miRNAs [25], (b) MiRNAs transcribed by RNA polymerase II, are processed in the nucleus by the microprocessor (Drosha) and exported to the cytoplasm by Exportin 5. Pre-miRNAs are further processed by Dicer and loaded to the RISC to bind to their partial complementary target mRNAs. As a result, this binding may degrade mRNA or inhibit translational progression.

MiRNAs in Leukemia

Expression levels of miRNAs are generally downregulated in malignant tissues [34]. For example: the let-7 family [35], miR-24 [36-38], miR-126 [39, 40] or miR-365 [41] are expressed at lower levels in various types of solid tumors. Since miRNAs are involved in normal hematopoiesis [42], it is not surprising that dysregulated expression of miRNAs can also be found in leukemias [43-45]. Several studies showed that the miRNA expression pattern is altered in ALL [45-48]. Aberrant expression levels of miRNAs such as miR-708, miR-365, miR-125b, miR-100, miR-99a and let7-a were observed in the ALL cells of children in comparison with normal $CD34^+$ cells [48]. Interestingly, we have previously shown that different cytogenetic subtypes of childhood ALL are characterized by distinct miRNA signatures [48-50]; Figure 4 shows the distinct miRNA expression pattern among different subtypes of childhood ALL using a selection of most differentially expressed miRNAs. In addition, we found also a subset of miRNAs to associate with clinical outcome in children with ALL [48].

However, our current knowledge about miRNAs in childhood ALL is mainly limited to the observed altered expression patterns of miRNAs in patients and its correlation with disease behavior including cellular resistance to chemotherapeutic drugs and clinical outcome. To further explore the clinical value of miRNAs more in depth studies are needed to identify the functional consequences of aberrant miRNA expression patterns. Till date, these type of studies have been limited in childhood ALL,

mainly due to technical reasons (see also chapter 2). Moreover, the currently known arsenal of miRNAs has been discovered in non-leukemic and often non-hematopoietic tissues which may result into a bias of knowledge about the role of miRNAs in childhood ALL. This knowledge is essential to determine the potential of miRNAs in diagnosis and treatment of children with ALL.

Figure 4: Discriminative miRNA signature among known cytogenetic subtypes of pediatric BCP-ALL

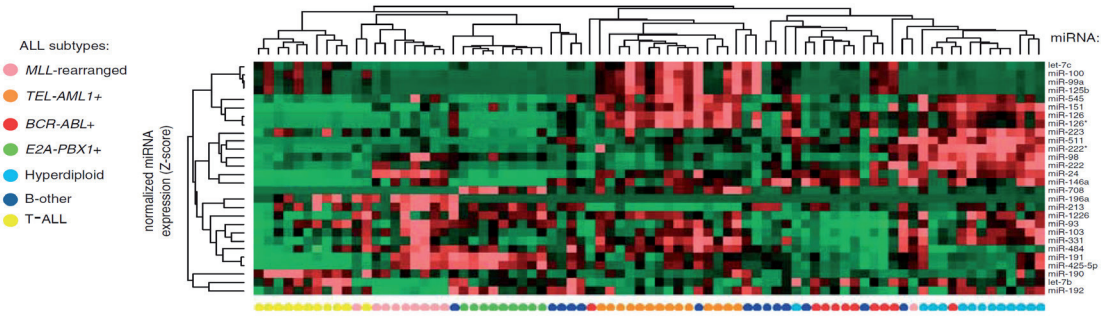


Figure 4: Subtypes of pediatric ALL cluster together based on similarities in miRNA expression patterns. The 28 miRNAs represent the top 5 most discriminative miRNAs per subtype of which 2 miRNAs being discriminative for ≥2 subtypes. The heatmap shows which miRNAs are overexpressed (in red) and which are underexpressed (in green) relative to snoRNA (adapted from Schotte *et al.* 2012 [50])

Outlines of this thesis

In this thesis we aimed to explore the function of aberrantly expressed miRNAs in pediatric BCP-ALL and to find biological target genes for these miRNAs. **Chapter 2** summarizes the currently available technologies to identify miRNAs target genes computationally and/or experimentally. The advantages and disadvantages of the most classical approaches as well as modern technologies for miRNA target identification are presented in this chapter. In **chapter 3**, we hypothesized that many miRNAs relevant to childhood ALL are yet unknown. This might be explained by tissue-specific biogenesis and function of miRNAs and the fact that the majority of currently known miRNAs were identified in non-hematologic tissues. Using high-throughput sequencing we identified known, novel and candidate novel miRNAs and miRNA*-sequences which were only expressed in ALL cells of children. **Chapter 4** contains the first report about miRNAs signature in poor prognostic *BCR-ABL1*-like ALL. This group of patients, which was recently identified among B-other cases negative for the known genetic lesions in BCP-ALL, is characterized by frequent genetic aberrations in B-cell development genes, similar to *BCR-ABL1*-positive ALL. We analyzed miRNAs in *BCR-ABL1*-like ALL in relation to clinical outcome of these patients. Moreover, we studied whether patients with *BCR-ABL1*-like and *BCR-ABL1*-positive ALL have similar miRNA signatures, which may imply an overlap in pathobiology of both diseases. The tissue-specific function of miR-24, miR-126 and miR-365, were presented in **chapter 5**. In this chapter, we investigated whether enforced expression of these three miRNAs alters the viability of *TCF3*-rearranged ALL cells. In **chapter 6**, we studied the contribution

of specific miRNAs in VCR-resistant ALL cells. We previously found that miR-125b, miR-100 and miR-99a are higher expressed in vincristine (VCR)-resistant leukemic cells of the children. Here we analyzed functionally whether individual or combined overexpression of these miRNAs affects the level of resistance to VCR. Moreover, we identified candidate target genes, which were downregulated upon combined expression of these miRNAs.

Finally, a summary of the results and the final conclusion are given in **chapter 7**. The detailed discussion and the perspectives for future studies are discussed further in **chapter 8**.

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Chapter 2

The hunting of targets: challenge in miRNA research

Akbari Moqadam F, Pieters R and den Boer ML

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs which control the expression of around 60% of the human protein-coding genes. In the past decade, deregulation of miRNAs (by expression and/or function) has been associated with the pathogenesis, progression and prognosis of different diseases, including leukemia. The number of discovered genes encoding miRNAs has risen exponentially in this period, but the numbers of miRNA-target genes discovered and validated lag far behind. Scientists have gained more in-depth knowledge of the basic mechanism of action of miRNAs, but the main challenge still remaining is the identification of direct targets of these important “micro-players”, in order to understand how they fine-tune so many biological processes in both healthy and diseased tissue. Many technologies have been developed in the past few years, some with more potential than others, but all with their own pros and cons. Here, we review the most common and most potent computational and experimental approaches for miRNA-target gene discovery and discuss how the hunting of targets is challenging but possible by taking the experimental limitations in consideration and choosing the correct cellular context for identifying relevant target genes.

Keywords

MicroRNA, cancer, computational target prediction, experimental target identification, target validation

Introduction

In 1993 two seminal reports (1, 2) described the discovery of post-transcriptional gene silencing by antisense RNA-RNA interaction in nematodes, introducing a new mechanism of gene expression regulation in metazoan. In a short time, several more small non-coding RNAs were identified in nematodes and insects (3, 4), and it was discovered that most of these molecules, termed "microRNAs" (miRNAs), were highly conserved in many other species (5). The miRNAs are involved in the translational regulation of around 60% of the human protein-coding genes (6), and it is now well established that miRNAs control different cellular processes such as apoptosis (7, 8), proliferation (9, 10) and differentiation (11). Altered expression of miRNAs correlates with initiation and progression of many diseases and malignancies (7, 8, 12-14) including different types of leukemia (13, 15-17); as such, miRNAs are associated with the prognosis of leukemic patients by controlling the level of sensitivity to cytotoxic drugs (18-20), growth rate (7) and differentiation (21, 22).

Global acts, local tasks

According to the latest version of the miRNA database (miRBase 18, released on November 2011, University of Manchester, UK), there are 18,226 miRNA hairpin precursor entries encoding 21,643 mature miRNA sequences in 168 species (23). The fine-tuning of basic cellular activities (e.g. growth, apoptosis and differentiation) by miRNAs implicates the contribution of these tiny molecules in biological activities all over the body (7, 11, 24). The miRNAs with proven contribution to general biological processes are summarized in Table-1. In addition, miRNA profiling studies reveal unique expression signatures discriminative for different types of healthy human tissues (13, 25). It has been shown that miRNA biogenesis, expression level and function are differentially controlled in tissues of different origin (26). As an example, both miR-221 and miR-222 inhibit proliferation in erythroblastic leukemia cells, while the same miRNAs act in an opposite way in many solid malignancies, promoting proliferation and survival (27). RNA-binding proteins (RBPs) which can either induce or suppress the expression of miRNAs and control their binding to their targets are putative important regulators of tissue-specific miRNA expression and function. (26, 28, 29).

Target discovery, the biggest challenge

Nowadays, generating miRNA expression signatures, the correlation of these expression patterns to disease stage and progression, and even discovery of new miRNAs is technically easily feasible (30, 31). Yet, the most challenging question is how to recognize direct target genes of miRNAs of interest. Several computational and experimental approaches toward miRNA target gene discovery have been developed and tested, which take into account the biological processing and the function of miRNAs, as well as the complex target gene binding parameters (32, 33).

Computational target prediction

Partial Watson-Crick complementarity of a miRNA to its target sequence is mandatory for binding (34, 35). Based on this simple but important fact, different computational algorithms have been developed to predict potential binding sites of a specific miRNA (35). The majority of these target prediction tools are based on the stringent pairing of the target sequence within the messenger RNA (mRNA) to the "seed" sequence, *i.e.* the nucleotides 2-7 of the 5' end of the ~22 nucleotide sequence encoding the mature miRNA (36). This area is the most conserved part of metazoan miRNAs; prediction based on complete seed base-paring significantly decreases the number of false positive results (34, 37). Most of these prediction algorithms are limited to browse the 3' untranslated regions (UTRs) of human mRNAs. However, this is not the only binding region for miRNAs, as there are also sites located in 5' UTR or even within the coding DNA sequence (CDS) of mRNAs (38-40). In addition, perfect seed base-pairing is not always an essential factor for miRNA-mRNA binding; in the presence of seed sequence mismatch, miRNA-mRNA binding may also happen through additional compensatory base-pairing of the 3' part of miRNA to its target sequence (40-43). The partial complementarity of miRNAs to their target genes together with the different forms of seed mismatch-pairing represent one of the major challenges in miRNA target recognition. Figure-1 represents major binding possibilities of miRNA-target mRNA.

The most common prediction algorithms and their characteristics are listed in Table-2. Tools using similar powerful prediction characteristics such as the mandatory stringent seed base-pairing used in TargetScan, PicTar and EIMMo3, have similar but not completely identical prediction results. Usage of different UTR databases as well as diverse internal criteria can be the reason of this dissimilarity. In addition, these prediction algorithms also vary in the individual ranking systems; e.g. the direct target of miR-196b, *HOXC8* (44), is ranked at position 1 in TargetScan and PicTar, at position 2 in EIMMo3 and at position 70 in PITA. This variation in prediction gives the scientists the ability to combine knowledge of disease biology and predicted targets to select target genes of interest. However, it can be difficult to select the best potential candidates from the long prediction lists. As an example, TargetScan, the most frequently used and referenced prediction algorithm, predicts 3 of the validated target genes of the let-7 family (*HMGA2*, *LIN28B* and *TRIM71*) among the top 5 predicted target genes, while other proven targets of human let-7 family, *NRAS* and *ITGB3*, are ranked at position 143 and 393, respectively. Moreover, TargetScan predicts only one of the 9 validated direct targets of miR-133a (*FSCN1*), albeit ranked at position 402. This poor prediction accuracy is observed for other computational prediction programs as well. Therefore, referring to only one target prediction tool is a risky and biased approach.

The majority of currently available miRNA target prediction algorithms (except EIMMO) are designed to predict possible targets of only one miRNA. However in some cases, several miRNAs simultaneously bind to and subsequently control the target mRNA. The combination of miRNAs then determines if the target gene is translated to protein (45). The miRNA multiplex phenomenon preludes also challenges in designing

functional experiments and requires the development of multicistronic expression vectors in order to over-express several miRNAs simultaneously (46).

Experimental target identification

Although development of computational prediction tools was a great step forward in miRNA target gene discovery, the percentage of false positive and negative findings is substantial. Therefore, these tools are mostly used to narrow down the list of potential targets, as well as to support correlation studies between miRNA and mRNA/ protein expression levels as an indication for miRNA targets. True targets of miRNAs can only be validated by different experimental procedures in the cell type of interest. A summary of the potential possibilities to discover miRNA target genes based on biogenesis and function of miRNAs are shown in Figure-2.

Gene expression correlation studies

An inverse correlation between expression level of miRNAs and mRNAs may indicate potential targets of miRNAs that are degraded upon miRNA-mRNA bindings. Therefore, changes in the mRNA levels after functional interference with miRNAs (e.g. ectopic induction or repression) may be indicative for targets regulated by miRNAs of interest (Figure-2a). Using this strategy it has been shown that over-expression of miR-1 and miR-124a in HeLa cells down-regulates the expression of more than 100 mRNAs (47). Subsequent analyses showed that the majority of these down-regulated mRNAs contain the seed complementary sequences in their 3' UTR regions associated with the over-expressed miRNAs (47). The analyses also revealed that over-expression of tissue specific miRNAs (miR-1, muscle specific, and miR-124a, brain specific) in a different context (HeLa cells, cervical cancer) partially turned the mRNA expression profile of the HeLa cells into that of the tissues that normally expressed those miRNAs (47). This confirms the important role of miRNAs in tissue development and maintenance. Rapidly, similar reports on miRNA target discovery were published by other groups (48, 49). Limited by the availability of tumor-representing cell line models, most of the over-expression studies are performed in a cell type different from the tissue aberrantly expressing the miRNA of interest. This can potentially result in missing tissue-specific target genes. Furthermore, most of the enforced expression studies will result in a supraphysiological increase in miRNA levels, augmenting the chance for off-target effects and false-positive read outs (49, 50). The disadvantages of miRNA over-expression strategies can be avoided by miRNA silencing methods; these are based on antisense oligonucleotides which scavenge the miRNA of interest by forming miRNA/anti-miR duplexes (51). However, the antisense (anti-miR) sequence may not be specific enough to distinguish between similar miRNAs like miR-196a and miR-196b which only differ in one nucleotide. A *caveat* is that these miRNA/anti-miR duplexes prevent the scavenging effect to be quantified since the duplex is not easily degraded and does not result into measurable reduction of miRNA level. Recent development in over-expressing RNA sequences complementary to miRNA seed sequence, known as

"miRNA sponges", might be the solution to create a proper and measurable silencing tool (52).

Biochemical approaches

The specificity of target discovery may be increased by analysis of miRNA-mRNA complexes isolated using co-immunoprecipitation of RNA-induced silencing complex (RISC) proteins like Argonaute (AGO) (53-56) (Figure-2b). This can be done by using epitope-tagged AGO (54, 57) or highly specific monoclonal antibodies against endogenous AGO proteins (53, 58). The precipitated fraction can then be analyzed for captured mRNA sequences by gene expression arrays or deep sequencing (53, 58). The precipitated mRNAs are 40-180 fold enriched for complementary seed sequences of over-expressed miRNAs which is significantly higher than expected by chance (55). In contrast to gene expression correlation analyses, purifying the active miRNA-mRNA complexes enables the discovery of target genes which can be regulated by both mRNA degradation and translational inhibition. However, this immunoprecipitation approach does not necessarily recapitulate the *in vivo* miRNA-mRNA interaction since different RNA-binding proteins, including AGO, may artificially and none-specifically associate with mRNAs upon cell lysis (58). In addition, the binding of miRNA-mRNA can be too weak for an efficient co-immunoprecipitation (59). To strengthen the interaction between miRNA-mRNA duplexes, RNA/protein complexes are crosslinked using ultraviolet irradiation prior to co-immunoprecipitation; this is subsequently followed by deep sequencing of the purified fractions (Figure-2b). This so-called high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) method was tested to identify miRNA targets in a mouse brain cell line (60) and *C. elegans* (61). HITS-CLIP may be a powerful strategy to identify biologically relevant targets of miRNAs *in vivo* as exemplified by the discovery of MMP2 as the target of miR-9 (62). However, the efficiency of ultraviolet irradiation to crosslink RNA-protein complexes is being controversially discussed (57). Recently, a new technique has been developed, called photoactivable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP), which aims at discovering the interaction between RBPs with cellular RNAs (63). PAR-CLIP is based on incorporating photoreactive ribonucleoside analogs like 4-thiouridine (4-SU) and/or 6-thioguanosine (6-SG) into newly synthesized RNAs of living cells. UV irradiation of the cells leads to efficient crosslinking which is followed by immunoprecipitation of the RBPs of interest. The bound mRNA sequences can be converted to cDNA and identified using high-throughput sequencing or gene expression profiling. 14 target genes of Epstein-Barr virus (EBV) miRNAs have been successfully identified and validated using PAR-CLIP (64). A major *caveat* for miRNA-mRNA and AGO/RISC co-immunoprecipitation studies is the fact that every miRNA-mRNA duplex has a similar chance to precipitate. Therefore, the share of miRNA-mRNA complexes of interest can only become discriminative upon over-expression or inhibition of desired miRNA prior to co-immunoprecipitation.

Applying tagged miRNAs can increase the specificity of immunoprecipitation (Figure-2c). E.g. transfection of cells with biotin-labeled miRNAs and subsequently

capturing the associated miRNA-mRNAs duplexes by streptavidin beads results in an enriched fraction for complexes of interest. This method has been first optimized in the fruit fly *Drosophila melanogaster* (65) and then applied in mouse E14 embryonic stem cells as well as HepG2 cells, confirming successful enrichment of previously discovered target genes of miR-10a and miR-122, respectively (40, 66). The streptavidin-mediated capture of biotinylated miRNA-mRNA complexes has been successfully used to discover a new target gene for miR-122 (*PRKRA*) and technical improvements in this methodology even yielded 14 new target genes for miR-34a (a.o. *ARAF*, *PIK3R2*, *MEK1*, *PLK1* and *SMAD4*) which were all confirmed as true targets of miR-34a (66, 67). This indicates the strength and value of this approach in the hunting of true miR-targets. In addition, a new regulating role of miR-10a was discovered showing that this miRNA targets the 5' UTR of ribosomal protein mRNAs resulting in an increase instead of the expected decrease of protein translation (40). Another tag successfully employed for miRNA target discovery in *C. elegans* and zebrafish is digoxigenin (68, 69). Albeit these tagged-miRNA approaches can yield informative data, one should keep in mind that over-expression of labeled miRNAs may potentially induce off-target effects and/or the 3' tag may affect the strength of miRNA-mRNA binding due to sterical hindrance.

No additional chemical modifications of miRNAs are needed for the parallel analysis of RNA ends (PARE) method. This novel method has been designed to identify the cleaved mRNAs that arise upon miRNA binding (Figure-2d). In principle, polyadenylated RNAs are extracted and an RNA adaptor is ligated to the 5' end of the cleaved mRNAs. Thereafter, the poly-A tail of mRNAs serve as primer for cDNA synthesis followed by PCR to amplify the cDNA. These products are further processed and used for high-throughput sequencing (70). The readout sequences are matched with genome browsers databases to identify the mRNAs which were cleaved upon over-expression of the miRNA of interest (70). PARE method is restricted to the identification of mRNA targets that are degraded. Alternatively, a strong detergent can be used to remove RISC proteins which will expose the bound miRNAs to serve as primers for cDNA synthesis upon incubation with reverse transcriptase (Figure-2e). The cDNA is then cloned and sequenced to identify target mRNAs that may be both targets of miRNA cleavage or translational inhibition (71). So far, this method was only successfully applied in *C. elegans* and showed K10C3.4 being the direct target of *let-7* (72). Recently, polysome profiling (Figure-2f) has been suggested for the analysis of translationally active mRNAs. In this method, elongating ribosomes are trapped by cyclohexamide and mRNAs bound to ribosomes are isolated by sucrose gradient centrifugation (73). Next, the ribosome-bound mRNAs are identified and quantified by gene expression microarrays and/or RNA-sequencing (RNA-seq).

Proteomic approaches

Proteomic technologies may provide the most relevant opportunity for miRNA target discovery since a change in protein expression level is the ultimate effect of miRNA function. Stable isotope labeling of amino acids in cell culture (SILAC) may be a tool to identify affected proteins (74, 75). Cells with a modulated level of a miRNA

and those with a mock control are kept in culture in two different media, one containing normal, the other containing heavy isotopes of essential amino acids (often lysine and arginine). The difference in *de novo* protein synthesis is quantified by comparing the ratio of peak intensities of heavy and light isotopes by mass spectrometry (Figure-2g). A ratio unequal to one indicates a gene which is either directly or indirectly controlled by the miRNA of interest (76, 77). Target genes of miR-143, miR-155 and miR-373 have been identified using the SILAC-based proteomic approach (78-80).

A second proteomic approach is two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) (Figure-2h). In this method, proteins extracted from cells under two different conditions (e.g. miRNA and mock-transfected cells) are labeled with distinct fluorescent dyes (e.g. Cy3 and Cy5) and separated by isoelectric focusing (1st dimension) and molecular size (2nd dimension) gel electrophoresis. Differentially expressed proteins are identified based on the Cy3/Cy5 ratio, followed by excision out of gel, trypsinization and subsequent analyses by mass spectrometry (81, 82). The excised spots still contain numerous differing proteins and subsequent analysis of 3' UTR sequences of the identified candidate proteins for presence of seed complement regions is required to help narrowing down the number of candidate targets.
Another method used is the analysis of protein expression levels by reverse-phase protein arrays (RPPA), for instance after exogenous manipulation of miRNA expression levels (83) (Figure-2i). However, this method is restricted to the availability of high-quality antibodies.

Target validation

After the identification of potential miRNA target genes, the physical binding of miRNA to candidate mRNAs and the subsequent translational modulation needs to be confirmed. The majority of target validation studies use a luciferase sequence as a reporter followed by 3' UTR sequence of the candidate target gene (84). A drop in luciferase activity upon over-expression of miRNA suggests a binding of the miRNA to its target 3' UTR mRNA sequence (Figure-2k). It is important to include proper controls to test specificity of the miRNA-mRNA interaction e.g. a mock miRNA-mimic sequence and a mutant form of the target 3' UTR mRNA sequence which has lost the predicted binding sites for miRNA (7-10, 24). For this approach, oftentimes, cell lines are chosen due to their transfectability (e.g. HEK293 cells); however, tissue-specific miRNA biogenesis and binding (26, 28, 29, 85) warrants to perform these studies in relevant cell line models resembling the tissue of origin (7, 10, 24, 86). For instance, *FBW7* was validated as target of miR-27a by a significant decrease in luciferase signal in MCF7 and U2OS cells, but not in the HEK293 cell line (10).

Summary and perspectives

Progress of our knowledge in miRNA biology provides a wide range of opportunities in computational and experimental miRNA target identification

approaches, albeit each of them has its limitations. Computational target prediction algorithms are provisionary tools to draw-up a putative landscape of miRNA function. Next, experimental approaches should determine which predicted gene may be a true target for the miRNA. The most important issue is to use cell line models resembling the original tissue in which the miRNA of interest was identified. Tissue-specific miRNA biogenesis and expression of RBPs illustrate the importance of cell type in miRNA target gene discovery. Experimental artifacts induced by manipulation of the expression level of miRNAs and the limitation in availability of suitable representative cell line models can be circumvented by the analysis of genome-wide patient datasets (19, 30, 87, 88). MiRNA, mRNA and/or protein expression data of different cohorts of patients can be integrated to discover biological and clinical relevant miRNA target genes (88). Recently, the importance to analyze primary patient samples was even further emphasized upon the discovery that the 3' UTR of genes can be shortened upon alternative splicing in cancer, yielding disrupted miRNA function compared to healthy tissue (89, 90).

Current knowledge of miRNAs biogenesis and function points to a clinical value of miRNAs. Several ways in reprogramming the altered expression of miRNAs into its original healthy signature have been tested in order to treat the pathological behavior of the affected cells. Tumor growth inhibition in breast cancer cell lines by miR-21 knockdown (91) and sensitization of drug-resistant glioblastoma cells by down-regulating miR-195 (92) are pivotal examples. Additionally, locked-nucleic-acid modified antisense sequences against highly expressed miR-122 was successfully applied in African green monkeys with hypercholesterolemia as one of the first *in vivo* experiments of using anti-miRs, albeit in non-human primates (93). The successful results of this experiment aiming at controlling the triglyceride and cholesterol level and disease manifestations with minimal side effects heralds a new field of research to develop more sensitive and powerful medications. Ongoing studies in developing more sensitive computational algorithms and in optimizing proteomic technologies for miRNA target recognition will further improve our understanding in miRNA function in healthy and diseased tissue. In the end, hunting of target genes of miRNAs will broaden our perspective of the role of miRNAs as a therapeutic tool.

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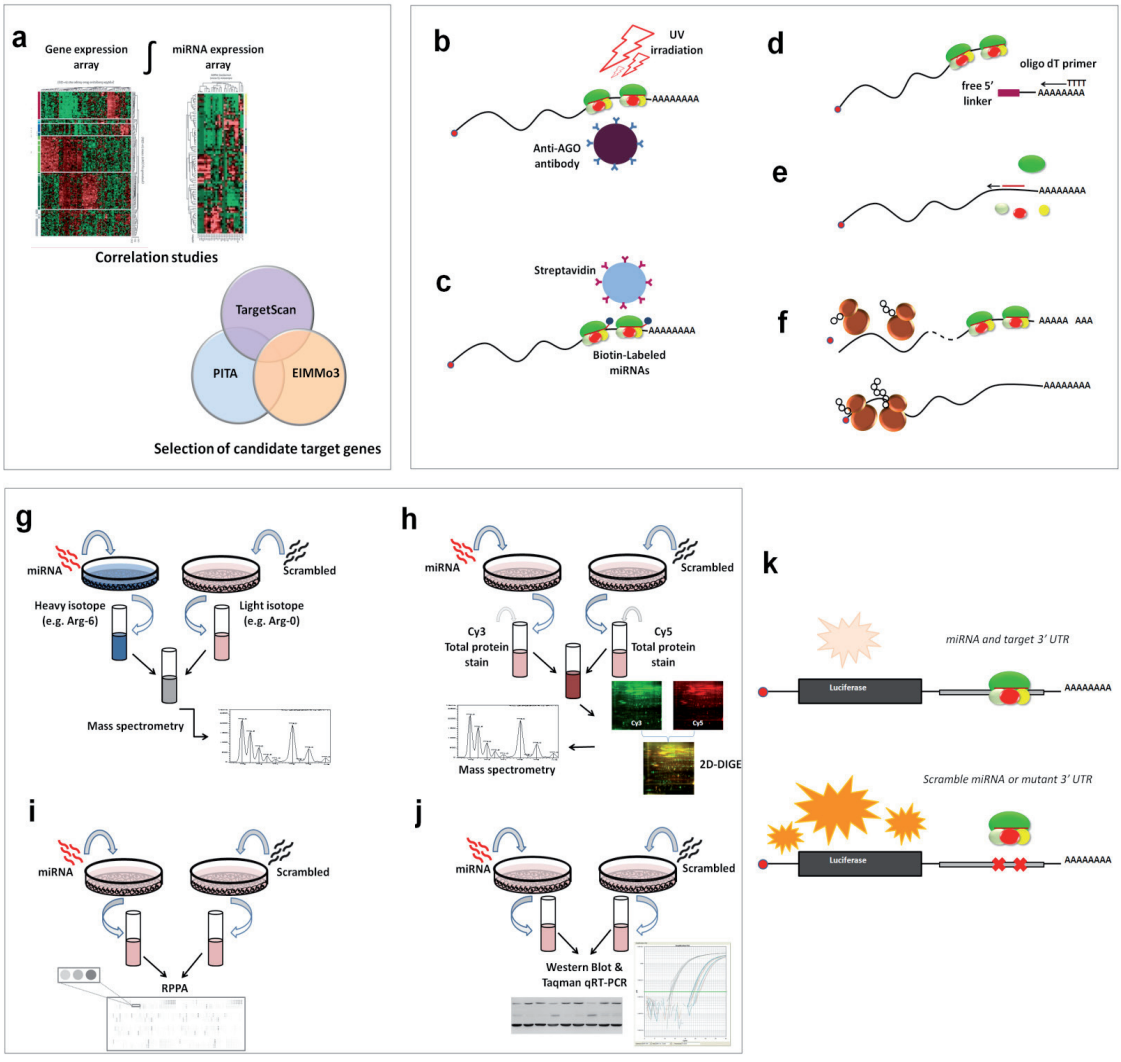
Conflict of interest

The authors declare no conflict of interest.

Figure1: Major possibilities in binding of miRNA-target mRNA

Seed sequence of mature miRNA is indicated in bold and red, solid lines indicate perfect Watson-Crick complementarity and circles demonstrate G:U wobble.

Figure 2: Common available approaches toward miRNA target identification:



Currently available approaches in miRNA target identification have been designed depending on biological biogenesis and function of miRNAs. **Integration and prediction studies**: (a) Finding significant (mainly inverse) correlations between miRNA and mRNA expression levels in combination with prediction of target genes by combining output of several target prediction algorithms. **Experimental interaction studies**: (b) co-immunoprecipitation of miRNA-mRNA complexes by RISC (AGO) precipitation with or without prior exposure to UV light (HITS-CLIP and PAR-CLIP); (c): selecting for mRNAs bound to tagged-miRNAs; (d): parallel analysis of RNA ends (PARE) possible after cleavage of target mRNA; (e): using the miRNAs as primers for cDNA synthesis after RISC and other proteins (e.g. RBPs) have been removed; (f): polysome profiling in which ribosome-bound mRNAs serve as input for gene expression arrays or sequencing. **Protein synthesis studies**: (g): aberrant de novo protein synthesis detected by using stable isotope labeled amino acids (SILAC); (h): aberrant expression levels detected by changes in Cy3/Cy5 labeling of proteins followed by separation using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE); (i): measuring expression levels of selected proteins using reverse-phase protein array (RPPA); (j): classical approach to analyze expression of one putative target gene both on protein (e.g. by western blot) and mRNA level (e.g. by Taqman qRT-PCR) after modulation specific miRNA expression levels. **Target validation studies:** (k): the specificity of the miRNA-mRNA interaction is mostly validated by 3' UTR binding assessment using luciferase reporter assays.

Table 1: A selection of miRNAs-target genes with associated biological functions

Function of miRNAs	miRNA	Proven target genes (Rank in TargetScan)	Reference
General function			
Cell cycle control and progression	miR-16 miR-24 miR-27a miR-34a	CCNE1(13) AURKB(-),CDK4(-), etc FBXW7(10) PLK1(-), SMAD4(393), etc	(94) (9) (10) (67)
Cellular movement	miR-124a miR-130a/b	CDK2(-), MCP1(-)	(95) (96)
Aging and senescence	miR-20a miR-146a/b	E2F1(688) IRAK1(2) FADD(-)	(97) (98) (99)
Cell survival and drug resistance	miR-130a miR-125b miR-155 miR-199a-3p	CSF1(496) BMF(281) FOXO3(216) CAV2(393)	(100) (101) (102) (103)
Tissue specific activities			
Cardiomyocytes/skeletal muscle differentiation and proliferation	miR-1 miR-133 miR-181a	HAND2(364) HDAC4(680) SRF(-), PTBP2(239) HOXA11(331)	(104) (105) (105, 106) (107)
Adipocytes differentiation	miR-143	ERK5(-)	(108)
Osteogenesis and chondrogenesis	miR-24 miR-199a/b		(96)
Neuronal morphogenesis and differentiation	miR-124 miR-132 miR-134 miR-24	Hes1(-) ARHGAP32(57) LIMK1(-) NOTCH1(-)	(109) (110) (111) (112)
Retinal development	miR-182 miR-96	ADCY6(400), MITF(2) ADCY6(17), MITF(101)	(113) (113)
Renal development	miR-192, 194, 204, 215, and 216		(114)
Hematopoietic differentiation	miR-15a miR-16 miR-150 miR-17-5p miR-223	BCL2(1232) BCL2(1232) MCL1(-) WIPF1(-) MYB(1) RUNX1(1139) NFIA(112), LMO2(64)	(115) (7) (115) (115) (116) (117) (118, 119)
Erythropoiesis and angiogenesis	miR-130a miR-20a miR-221 miR-223	MEOX2(126), HOXA5(671) VEGF(989) KIT(15) LMO2(64)	(120) (121) (122) (123)
Megakaryocytopoiesis	miR-10a miR-130a miR-146a miR-155	HOXA1(146) MAFB(490) CXCR4(-) ETS1(219), MEIS1(220)	(124) (124) (125) (126)

(-): Not predicted by TargetScanHuman, Release 6.2: June 2012

Table 2: Summery of most common miRNA target prediction algorithms

Considering 3' UTR only	
TargetScan	http://www.targetscan.org Stringent seed pairing, site number, site type, site context and score, likelihood of preferential conservation rather than site context
PicTar	http://www.pictar.org Stringent seed pairing for at least one of the sites for the miRNA, site number, overall stability of the bindings
EIMMo3	http://www.mirz.unibas.ch/EIMMo3/ Stringent seed pairing, site number, likelihood of preferential conservation, possibility of target prediction of several miRNA partners in combination
Diana-microT v3.0	http://diana.cslab.ece.ntua.gr/microT/ Conserved and poorly conserved binding regions, stringent seed pairing, thermodynamic stability of the bindings
PITA	http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html Moderately stringent seed pairing, no loops is allowed, only single G:U wobble is allowed in 7- or 8-mers
microRNA	http://www.microrna.org/microrna/home.do Moderately stringent seed pairing, site number
miRBase targets (microcosm)	http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/ Follows the previous algorithm of miRanda (microRna), moderately stringent seed pairing, site number, overall stability of the binding
miRDB	http://mirdb.org/miRDB Stringent seed pairing, based on support vector machines and high-throughput training datasets
Considering different parts of mRNA	
RNA22	http://cbcsrv.watson.ibm.com/rna22.html Moderately stringent seed pairing, matches to sequence patterns generated from miRNA set, overall stability of the binding, predicted pairing stability
miRWalk	http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/ Moderately stringent seed pairing, site numbers, both conserved and poorly conserved binding regions

Site numbers: The number of potential binding sites in the 3'UTR of the target mRNA, **Site type**: The complementarity of the miRNA, especially of its seed sequence, to the target mRNA (e.g. 8mer, etc. or 3' compensatory), **Site context and score**: Sum of the contribution of the following six features: site type, contribution of 3' pairing, local AU enrichment, site location, target site abundance and seed-pairing stability (127), Wobble: Non-Watson-Crick base-pairing between two nucleotides of RNA molecules which can be G:U, I (inosine):A, I:C and I:U.

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Chapter 3

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 ($-0.49 \leq$ Spearman's correlation coefficient (Rs) ≤ -0.27, $P \leq 0.05$); 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.

Keywords

Novel microRNAs, deep sequencing, pediatric 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].

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).

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.

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 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, $P<0.0001$).

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 x 10^{-4} up to 86 x 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).

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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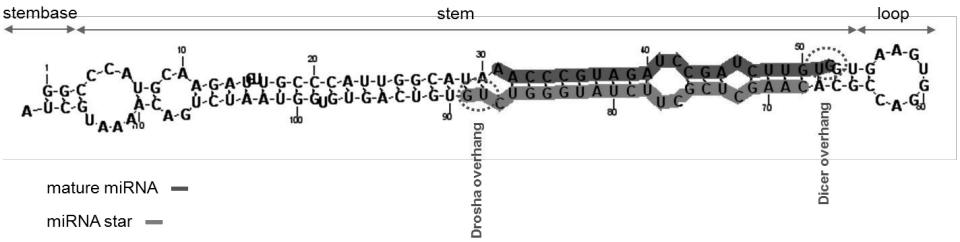
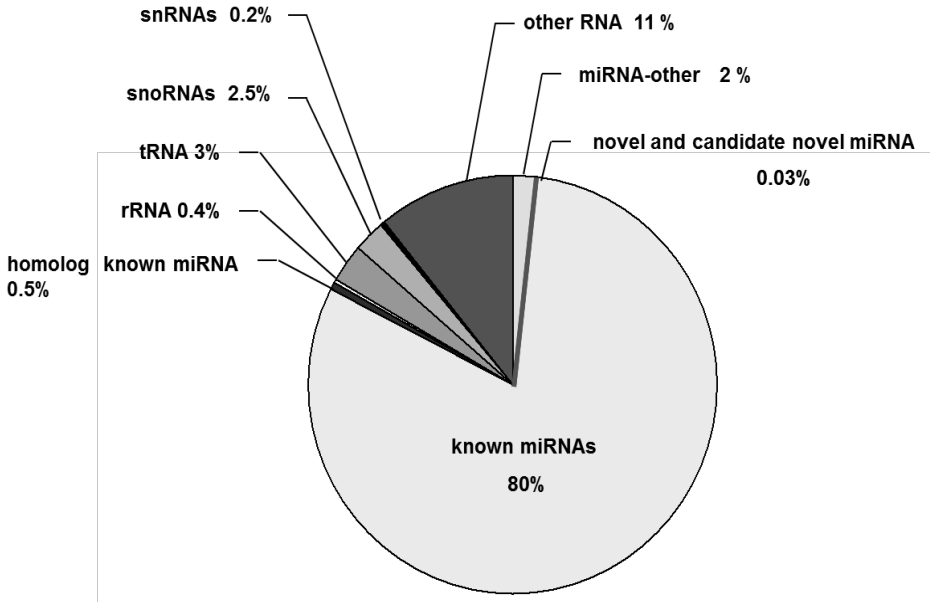
Figure 1: Parameters used to identify novel miRNAs



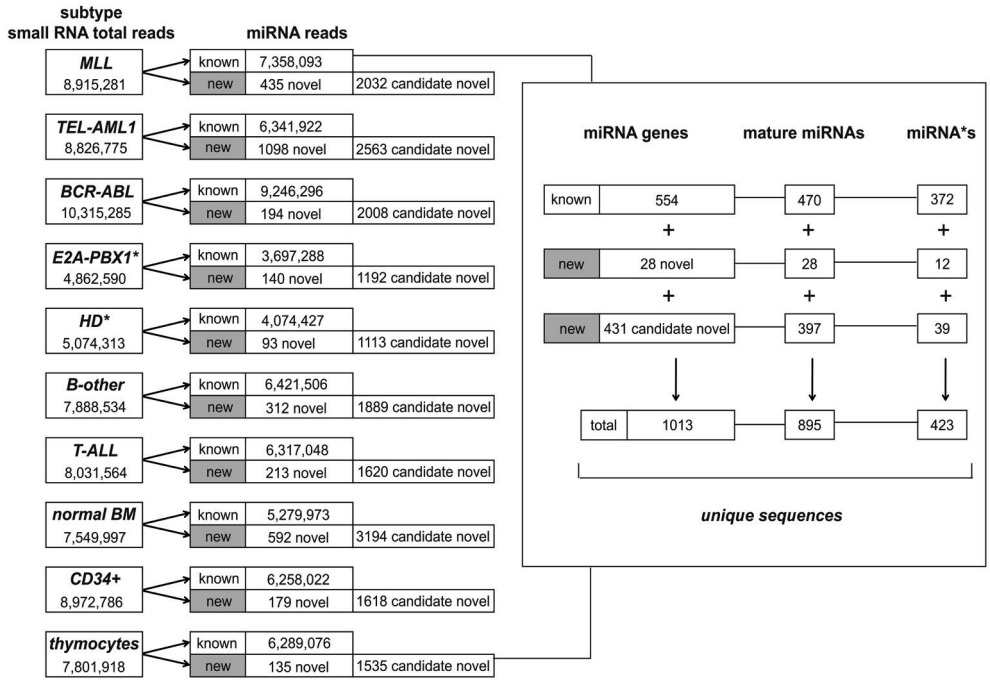
Small cloned RNA sequence reads are mapped to the human genome and the putative precursor is extracted by taking the miRNA sequence and the 100 nucleotides flanking sequence on either side of the miRNA. The precursor sequence is then computational folded into a hairpin structure, from which features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

Figure 2: composition of the small RNAome in leukemic cells of pediatric ALL patients



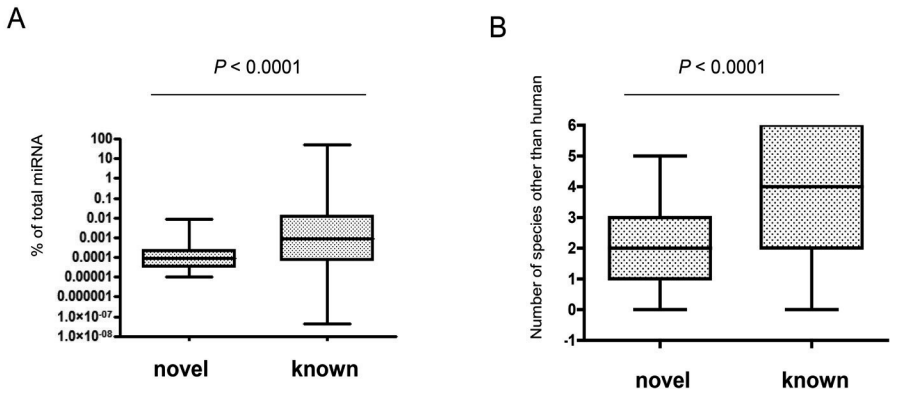
Frequency of non-coding small RNAs identified by high-throughput sequencing of ALL patient samples is shown. Frequencies of novel and candidate novel miRNAs are taken together. rRNA, ribosomal RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; tRNA, transfer RNA. 'Homolog to known' miRNAs refers to sequences that map to novel genomic loci of yet known miRNAs. The 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.

Figure 3: Overview of known and novel miRNAs in ALL types and normal hematopoietic cells



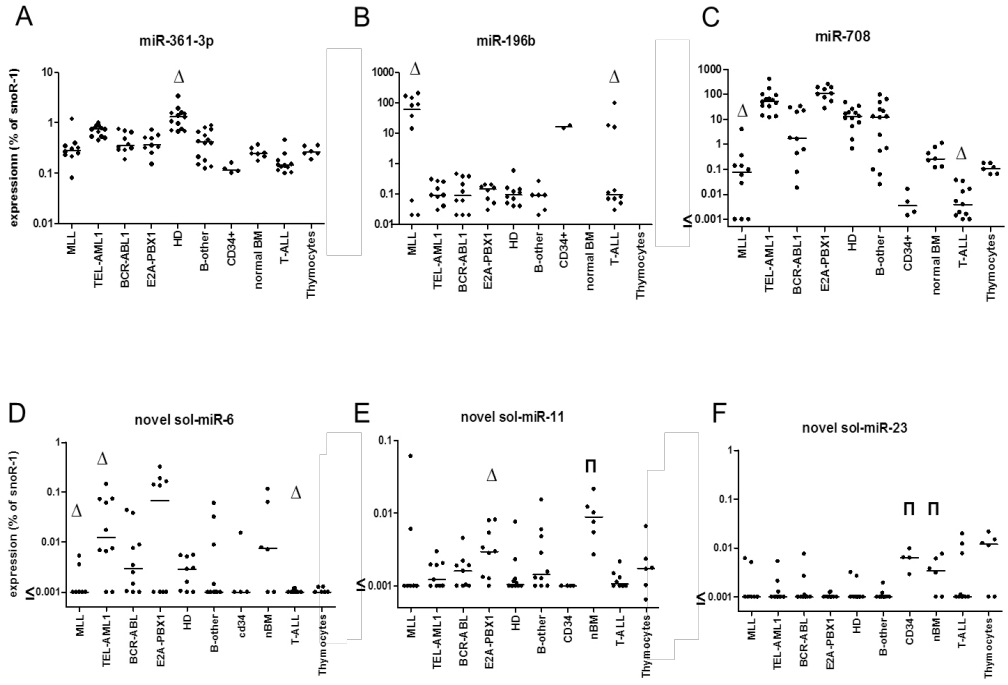
At the left, the total numbers of small RNA sequence reads are listed. At the right, the numbers of unique miR genes encoding mature miRNAs and miRNA-star forms (miRNA*s) are depicted. New miRNAs in both panels represent novel and candidate novel sequences (see Materials and methods section for explanation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

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 humans in which novel and known miRNAs are present. Mann–Whitney *U* test (MWU-test) was used to compare the abundance and number of non-human species for which the miRNA sequence can also be aligned.

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 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

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. 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. Abbreviations: miRNA, microRNA; rRNA, ribosomal RNA; tRNA, transfer RNA.

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
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).

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	*MLL*
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	
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	*TEL-AML1*
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	
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	*BCR-ABL*
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	
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	
1	novel sol-miR-6	hsa-mir-3150b	8,3	*E2A-PBX1*
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	
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	*hyperdiploid*
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	
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	*T-ALL*
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	
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.

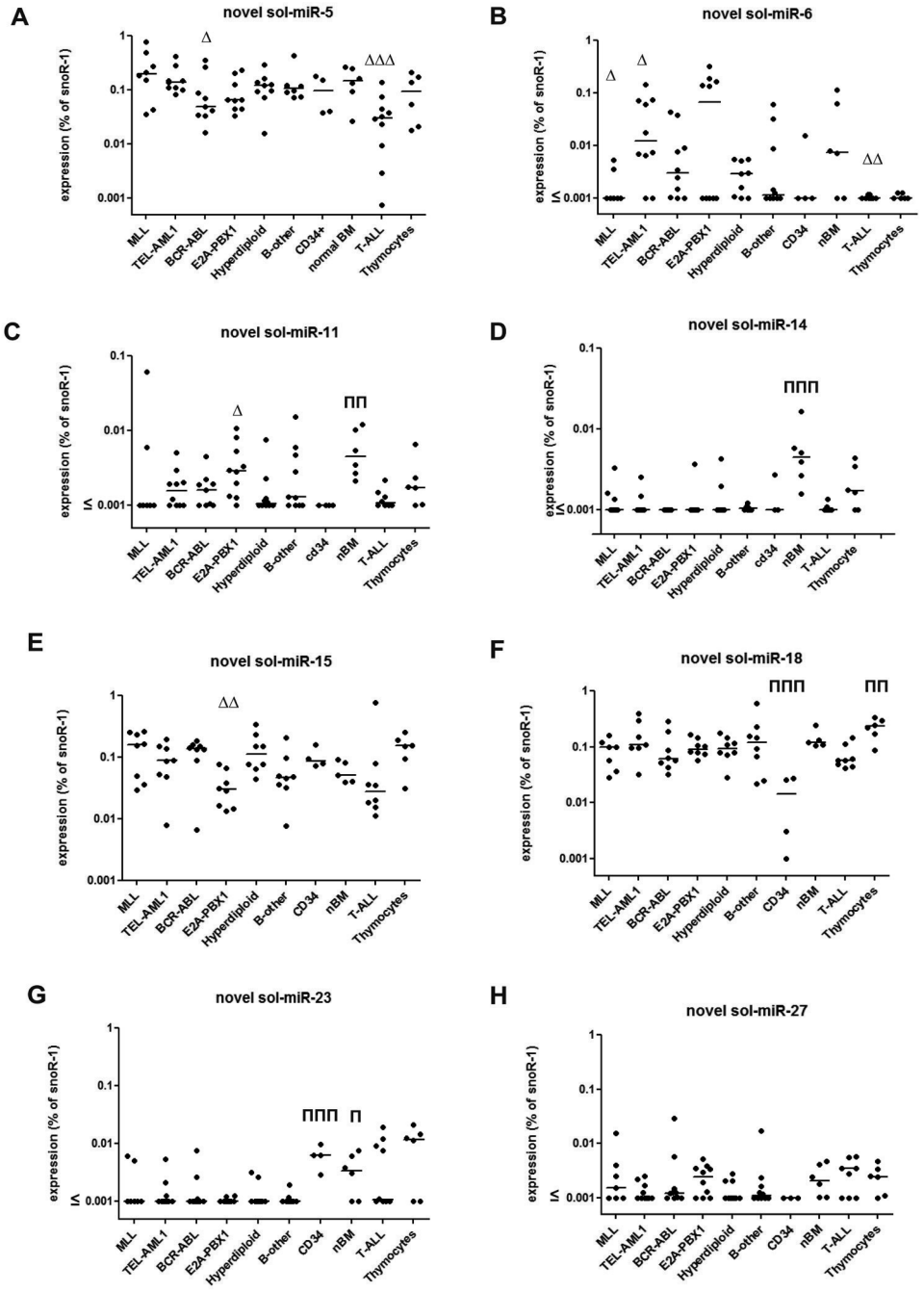
* Indicates the miRNA-star strand.

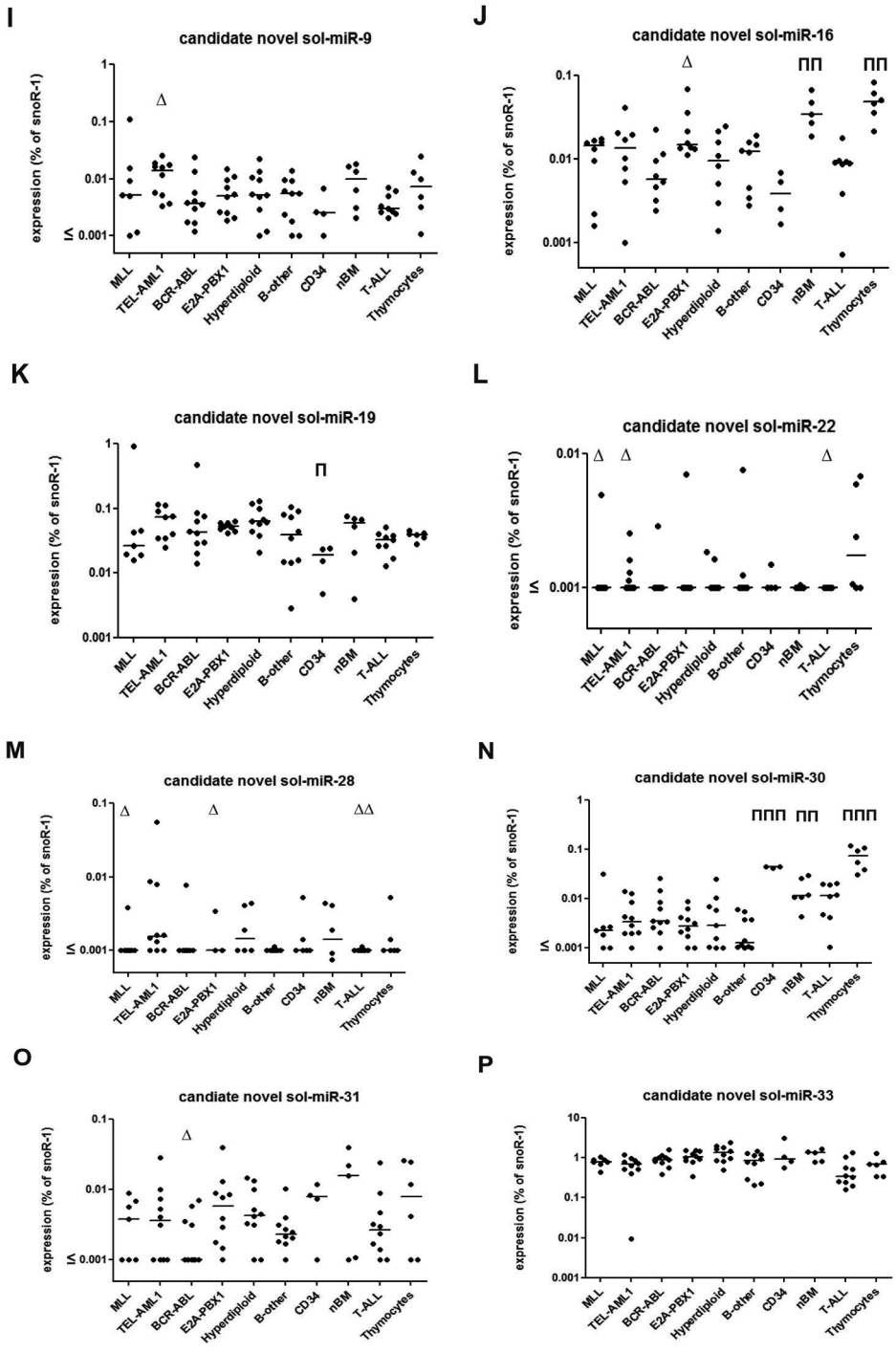
Table 4: Correlation between the expression level of the newly identified miRNAs and the expression level of their predicted targets

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 *

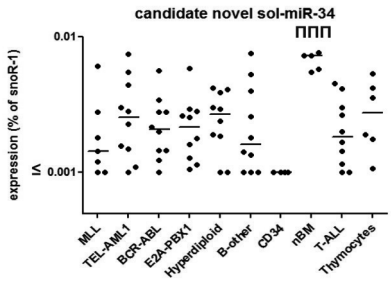
Target genes were predicted for novel and candidate novel miRNAs by Targetscan 5.1. MiRNA expression levels were determined by stem-loop RT-qPCR and compared with the expression of target genes as determined by Affymetrix U133 plus 2.0 GeneChips in the same patients (see Materials and methods section 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.

Supplementary Figure S1: Differential expression of novel and candidate novel miRNAs in genetic subtypes of ALL and normal hematopoietic cell populations



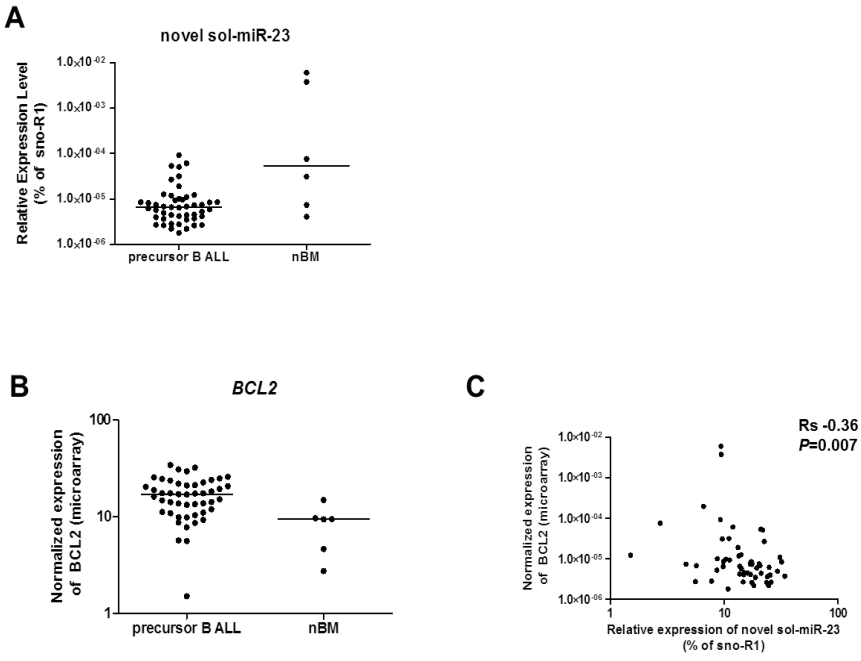

I
candidate novel sol-miR-9
expression (% of snoR-1)
MLL
TEL-AML1
BCR-ABL
E2A-PBX1
Hyperdiploid
B-other
CD34
nBM
T-ALL
Thymocytes
J
candidate novel sol-miR-16
K
candidate novel sol-miR-19
L
candidate novel sol-miR-22
M
candidate novel sol-miR-28
N
candidate novel sol-miR-30
O
candiate novel sol-miR-31
P
candidate novel sol-miR-33

Q



Expression levels of new miRNAs were measured by stem-loop RT-qPCR in different ALL types and normal hematopoietic cell samples. Dots represent expression levels as a percentage of the level of endogenous snoRNA-1 in individual patient samples. The median per type is depicted by a line. Levels below 0.001% were undetermined. nBM = normal bone marrow. Δ $P < 0.05$, $\Delta\Delta$ $P < 0.01$, $\Delta\Delta\Delta$ $P < 0.0001$; based on the comparison between expression levels of the indicated subtype and the remaining ALL cases, or Π $P < 0.05$, $\Pi\Pi$ $P < 0.01$, $\Pi\Pi\Pi$ $P < 0.0001$; based on comparison between normal bone marrow or CD34+ cells and precursor B-ALL or between thymocytes and T-ALL. HD = hyperdiploid.

Supplementary Figure S2: Expression of novel sol-miR-23 is inversely correlated with expression of its potential target gene *BCL2*



A) Expression levels of sol-miR-23 (in % of sno-R1 as reference) in 48 precursor B-ALL cases and 6 normal bone marrow samples (P=0.02; RT-qPCR data). B) Expression levels of *BCL2* detected by probeset 207005_s_at on Affymetrix U133 plus 2.0 GeneChips (P=0.006) and C) Correlation between expression of *BCL2* and sol-miR-23 in the same set of cases (Rs -0.36, P=0.007). Dots represent individual patients with the median per subtype represented by a horizontal line. nBM = normal bone marrow. Sol-miR-23 was recently annotated by miRBase as hsa-mir-4474 [14].

Supplementary Tables:

Supplementary tables are online available at:
http://www.nature.com/leu/journal/v25/n9/suppinfo/leu2011105s1.html?url=/leu/journal/v25/n9/full/leu2011105a.html

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Chapter 4

MiRNA signature in *BCR-ABL1*-like and *BCR-ABL1*-positive childhood acute lymphoblastic leukemia; similarities and dissimilarities

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LETTER TO THE EDITOR

Childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a heterogeneous disease in which the 5-years event-free survival rates are currently above 80% [1]. Yet, some genetic subtypes of this disease are characterized with poor clinical outcome: 5-years event-free survival in *BCR-ABL1*-positive [2] and *MLL*-rearranged leukemia [3] is reported ~50%. However, the highest absolute number of relapses still occurs in patients diagnosed as genetically unclassified BCP-ALL [4]. Using a genome-wide classification approach, we recently identified a poor-prognostic set of patients within this heterogeneous unclassified B-other group [5]. These patients have a high risk of relapse and a gene expression profile similar to *BCR-ABL1*-positive ALL cases. This so-called *BCR-ABL1*-like group of patients shows high frequencies of deletion in B-cell development genes such as *IKZF1*, *PAX5* and *VPREB1* [5]. Interestingly, similar genetic aberrations, most frequently *IKZF1* deletions, are found in children with *BCR-ABL1*-positive ALL [6].

Among different factors involved in clinical outcome of children with BCP-ALL, microRNAs (miRNAs) were also shown to have prognostic value [7]. These small non-coding RNAs regulate several genes post-transcriptionally and therefore, their aberrant expression is associated with the initiation and progression of multiple diseases and malignancies [8-10]. We reported earlier that known genetic subtypes of childhood BCP-ALL have subtype-specific miRNA signatures [7]. Besides, a subset of miRNAs in leukemia correlates with drug resistance and poor prognosis [7,11-12]. However, there is no available data about the expression pattern and possible role of miRNAs in the poor-prognostic *BCR-ABL1*-like leukemia.

Here, we investigate whether the miRNA signature in leukemic cells of children with *BCR-ABL1*-like ALL differs from those in other subtypes of childhood BCP-ALL. Further, we addressed whether altered expression levels of miRNAs measured at initial diagnosis has prognostic importance in *BCR-ABL1*-like ALL. MiRNA expression levels of leukemic cells of 32 newly identified children with BCP-ALL: i.e. 15 *BCR-ABL1*-like, 9 *BCR-ABL1*-positive, and 8 B-other patients (negative for *BCR-ABL1*-like ALL and known genetic lesions: hyperdiploidy, *BCR-ABL1*-positive, *ETV6-RUNX1*-positive, *TCF3*-rearranged, and *MLL*-rearranged ALL) were measured by Taqman® Array Human miRNA A and B cards V3.0 (TLDA cards, Applied Biosystems), using previously described instructions [7]. The institutional board of review approved the use of excess of diagnostic materials for research purposes and the written inform consent was obtained from patients, parents or legal guardians. This set of data was normalized and combined with our previously published set of miRNA expression data of 63 newly diagnosed patients (10 *MLL*-rearranged, 15 *ETV6-RUNX1*-positive, 9 *BCR-ABL1*-positive, 9 *TCF3*-rearranged, 14 hyperdiploid (>50 chromosomes), and 7 non- *BCR-ABL1*-like B-other patients) [7]. The miRNA expression data of two *BCR-ABL1*-positive patients, who overlapped between both data sets, was used to analyze the comparability of miRNA expression data of the two data sets. The expression levels of 257 miRNAs, which were measured in both data sets, showed significant correlation for these two

BCR-ABL1-positive patients (p<0.0001, Supplementary Figure S1a). Among these 257 miRNAs, 77 miRNAs with expression levels above detection threshold (>0.0001% relative to RNU44-48) were selected for further analyses. Similarly, the expression levels of these 77 miRNAs showed significant correlation for the two overlapping *BCR-ABL1*-positive patients between the old and the new data sets (p<0.0001, Supplementary Figure S1b). The average expression level of each miRNA within the B-others of each data set was used to normalize and combine both data sets. Briefly, the final values for relative expression level of each miRNA was calculated as $2^{-(\Delta CTmiRNA-\Delta CTaverageBOs)}x100\%$, where ΔCTmiRNA is equal to Ct-value of each miRNA minus Ct-value of reference non-coding RNA (RNU44-48), and ΔCTaverageBOs was the average of ΔCTs of the same miRNA among unclassified B-other cases. Thereafter, the two overlapping *BCR-ABL1*-positive patients as well as the B-others, which were used as a reference for normalization of the two data sets, were removed from the combined set before further analyses. Using the R statistics program (R-2.15, release June 2012), the Limma package was applied to compare the expression levels of miRNAs between the groups. Each genetic subtype was compared to the remaining cases to identify subtype-specific miRNAs. Top-10 most differentially expressed miRNAs, which were ranked based on p-values, are shown in Supplementary Table S1. Together, the subtype-discriminative miRNAs represent 44 unique miRNAs. These forty-four unique miRNAs were used for a supervised hierarchical clustering in GeneMaths XT software (upgraded version 2.0, Applied Maths, Belgium). The list of validated target genes for discriminative miRNAs of each subtypes and their predicted functions imply a possible role of these miRNAs in initiation and progression of leukemia (supplementary Table S2). As shown in Figure 1, children with *ETV6-RUNX1*-positive ALL cluster together and show a distinct miRNA signature. Similarly, children with hyperdiploid, *TCF3*-rearranged and *MLL*-rearranged ALL demonstrate a subtype-specific miRNA signature. In contrast, *BCR-ABL1*-positive and *BCR-ABL1*-like cases showed a more variable miRNA expression pattern. The heterogeneity in miRNA signature of *BCR-ABL1*-positive ALL in contrast to distinct miRNA signature of other known genetic subtypes of childhood BCP-ALL was reported earlier [7]. In addition, our current study revealed a partial overlap between the miRNA signature of *BCR-ABL1*-positive and *BCR-ABL1*-like ALL cases. As shown in Figure 1, seventy-three percent of *BCR-ABL1*-like patients (11 out of 15) had a miRNA expression pattern most similar to 43% of *BCR-ABL1*-positive patients (6 out of 14; cluster-I). These cluster-I patients displayed a miRNA signature discriminative from that found for other subtypes of BCP-ALL (Figure 1). The remaining 8 *BCR-ABL1*-positive and 4 *BCR-ABL1*-like cases showed a more heterogeneous expression pattern, which had a partial overlap with that of hyperdiploid ALL patients. These cases are assigned to cluster-II in Figure 1. The frequency of deletions in the *IKZF1* gene, a known prognostic factor in pediatric BCP-ALL [6], was 50% in *BCR-ABL1*-positive (7 out of 14) and 60% in *BCR-ABL1*-like cases (9 out of 15). This frequency did not significantly differ between the *BCR-ABL1*-like patients who co-clustered with *BCR-ABL1*-positive patients (cluster-I) and those in cluster-II (p>0.05, Fisher's exact test, Table 1). In addition, the frequencies of genetic aberrations for *PAX5*, *CDKN2A* and *CDKN2B* were higher but not significantly

different in the 12 *BCR-ABL1*-positive/*BCR-ABL1*-like patients of cluster-II compared to 17 cases of cluster-I (p>0.05, Fisher's exact test, Table 1). While alteration in *PAX5* and *CDKN2A*/*B* genes is common in ALL, there is no reported significant association between rearrangement of these genes and poor prognosis in childhood *BCR-ABL1*-positive ALL [13-15]. The expression levels of the 10 most discriminative miRNAs for *BCR-ABL1*-like did not differ between *BCR-ABL1*-positive and *BCR-ABL1*-like patients who relapsed and those who remained in continuous complete remission (mean time from diagnosis 5.5±2.8 years, p>0.05, Supplementary Figure S2). Although hampered by limited sample size and the fact that these cases were treated with different treatment protocols (supplementary Tables S3), none of these individual miRNAs nor the cluster signatures were predictive for an unfavorable outcome (pCIR-analysis, Fine and Gray test, p>0.05, Supplementary Figure S3 and S4). To explore the possible function of miRNAs in *BCR-ABL1*-positive and *BCR-ABL1*-like ALL, we integrated the miRNA and mRNA expression levels of 32 *BCR-ABL1*-positive, *BCR-ABL1*-like and unclassified B-other cases. Supplementary Table S4 shows the most significant correlation between expression levels of *BCR-ABL1*-positive and *BCR-ABL1*-like discriminative miRNAs and expression levels of their top-3 validated targets (available at www.mirtarbase.mbc.nctu.edu.tw/php/search.php) and top-5 predicted target genes (avauilable at www.targetscan.org). The heterogeneity in function of significantly correlated target genes does not suggest common pathways affected by miRNAs in *BCR-ABL1*-positive and *BCR-ABL1*-like childhood ALL.

In conclusion, the majority of children with *BCR-ABL1*-like leukemia have a miRNA expression pattern different from that of non-*BCR-ABL1*-positive genetic subtypes of pediatric BCP-ALL. Both *BCR-ABL1*-like and *BCR-ABL1*-positive groups display heterogeneity in miRNA expression pattern, yielding 2 clusters of patients. The prognosis of patients in both clusters, however, did not differ. There was no difference in *IKZF1* status between these two clusters whereas *IKZF1* deletions are a known predictive factor for relapse in children [6]. These data suggest that miRNAs are unlikely to explain the similarities and dissimilarities in pathobiology of childhood *BCR-ABL1*-positive and *BCR-ABL1*-like ALL.

Conflict of interest

The authors decline no conflict of interest.

Figure 1: Clustering of 77 BCP-ALL cases by expression of 44 miRNAs which are most differentially expressed between genetic subtypes. Overexpressed miRNAs are shown in red and underexpressed miRNAs are shown in green.

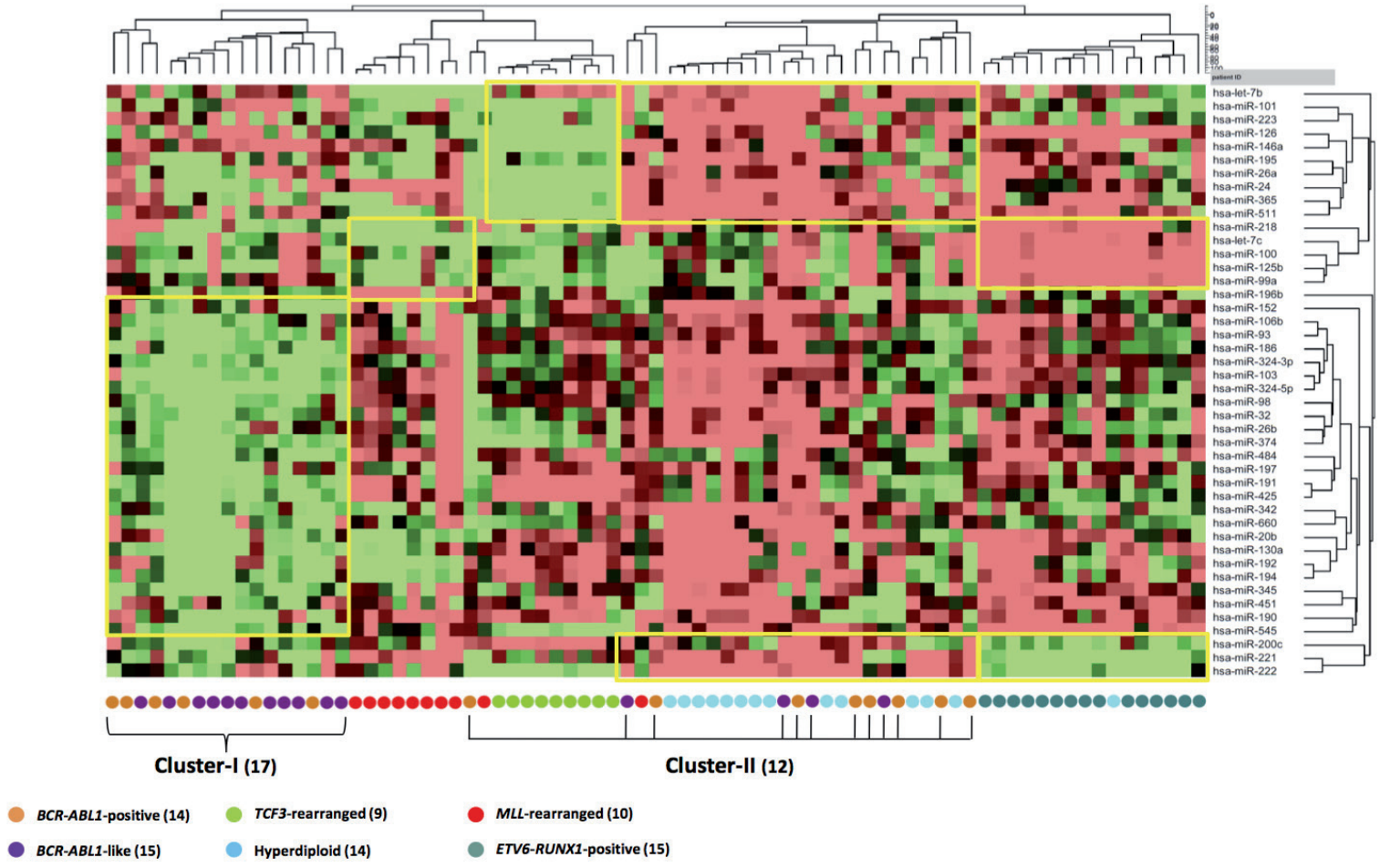
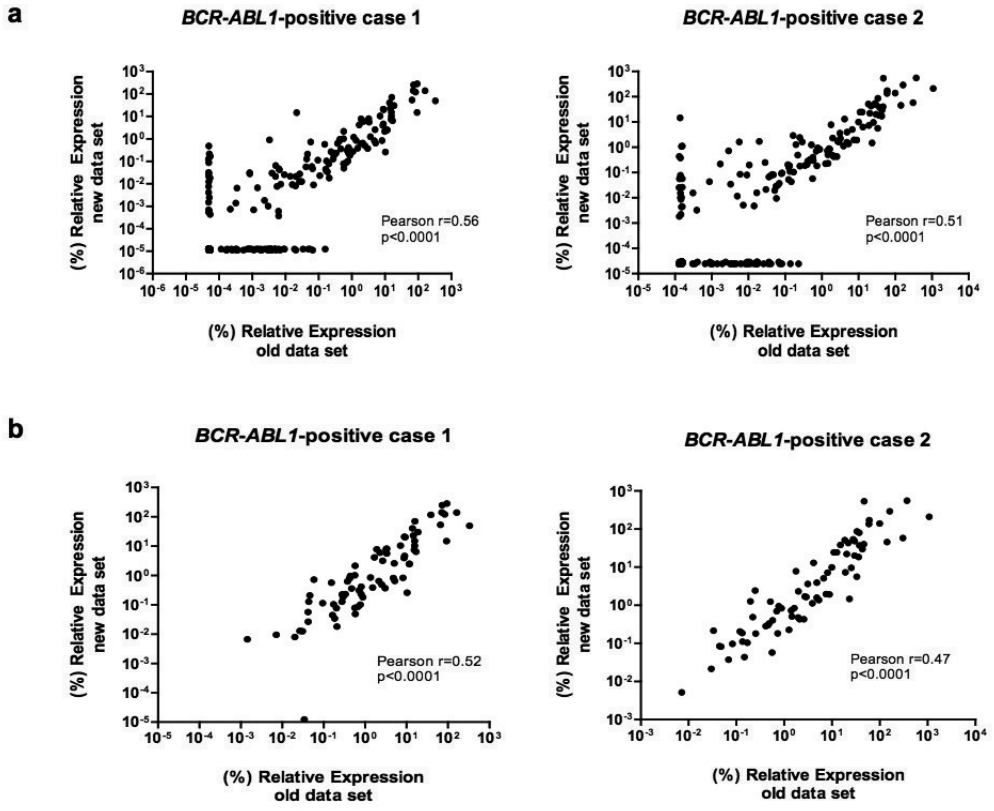


Table 1: The frequency of genetic lesions among the *BCR-ABL1*-positive and *BCR-ABL1*-like patients in cluster-I and cluster-II

		Cluster-I (17)			Cluster-II (12)		
		BCR-ABL1-positive (6)	*BCR-ABL1*-Like (11)	total in cluster-I (% excluding NTs)	*BCR-ABL1*-positive (8)	*BCR-ABL1*-Like (4)	total in cluster-II (% excluding NTs)
IKZF-1	Wild-type	1	3	4 (27%)	2	2	4 (44%)
	Aberration	4	7	11 (73%)	3	2	5 (56%)
	Not determined (NT)	1	1	2	3	0	3
PAX5	Wild-type	0	7	7 (58%)	3	0	3 (33%)
	Aberration	3	2	5 (42%)	2	4	6 (67%)
	Not determined	3	2	5	3	0	3
JAK2	Wild-type	5	11	16 (100%)	4	4	8 (100%)
	Mutation or translocation	0	0	0 (0%)	0	0	0 (0%)
	Not determined	1	0	1	4	0	4
CDKN2A	Wild-type	1	7	8 (67%)	3	0	3 (27%)
	Aberration	2	2	4 (33%)	4	4	8 (73%)
	Not determined	3	2	5	1	0	1
CDKN2B	Wild-type	1	6	7 (58%)	3	0	3 (27%)
	Aberration	2	3	5 (42%)	4	4	8 (73%)
	Not determined	3	2	5	1	0	1

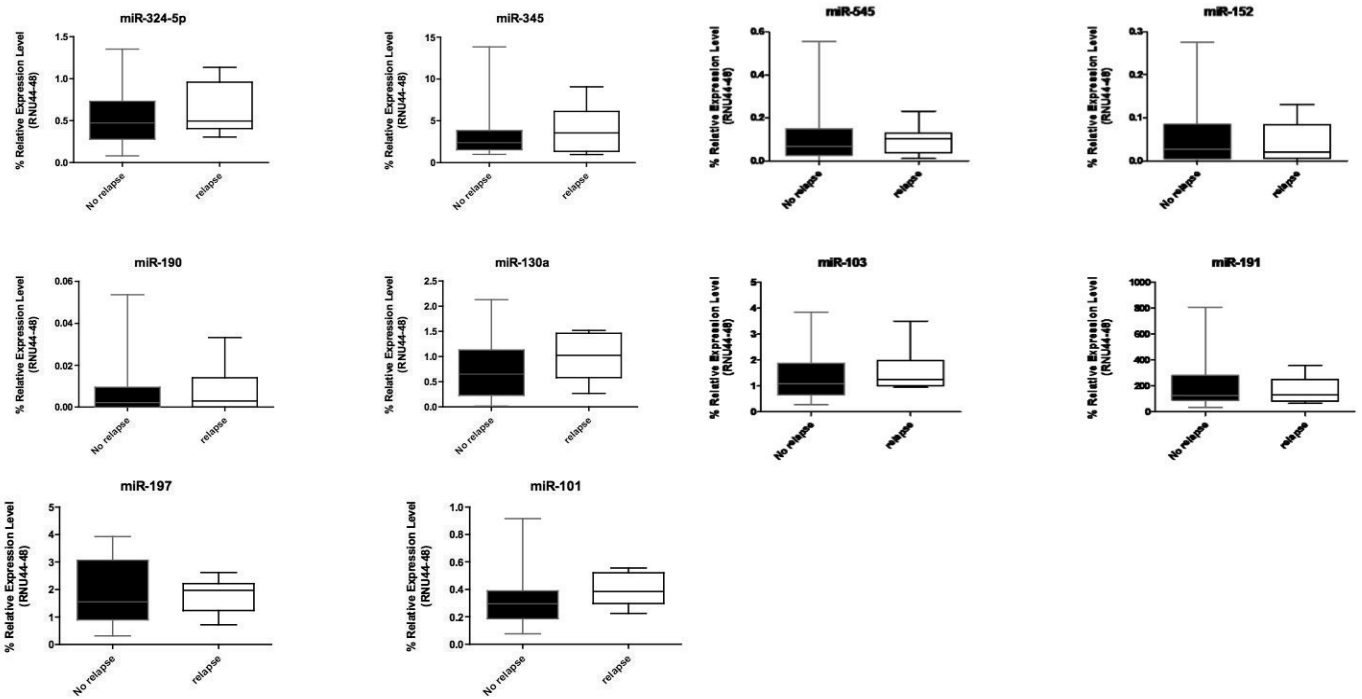
Number of aberrations in *IKZF1*, *PAX5*, *JAK2*, and *CDKN2A/B* genes comparing *BCR-ABL1*-positive and *BCR-ABL1*-like patients of Cluster-I and Cluster-II. Wild-type indicates no identified genetic lesion, Aberration shows all types of deletions of the gene and NT indicates not-determined status of genetic lesions. The frequency of genetic lesions of none of the studied genes differs significantly between patients of Cluster-I and Cluster-II ($p>0.05$, Fisher's exact test).

Supplementary Figure S1: Correlation of miRNA expression values of two *BCR-ABL1*-positive cases in two data sets.



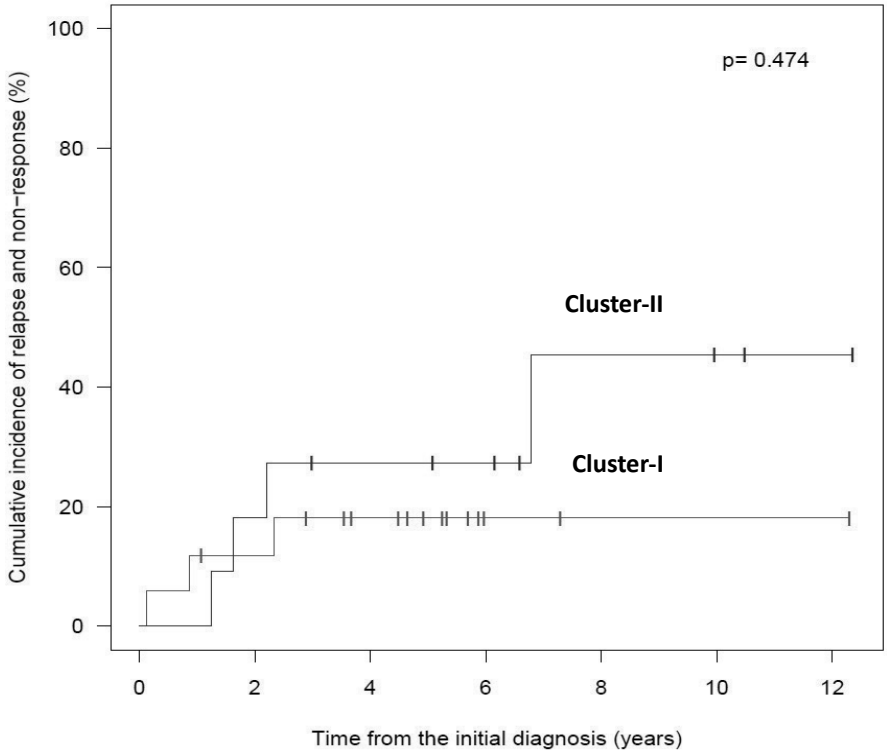
The expression levels of 257 miRNAs (a) for two *BCR-ABL1*-positive cases were significantly correlated between the old and the new data sets ($p<0.0001$, Pearson correlation coefficient test). Similarly, expression levels of 77 miRNAs that were higher than the detection threshold (>0.0001% relative to RNU44-48) were significantly correlated for two overlapping *BCR-ABL1*-positive cases between the two data sets (b, $p<0.0001$, Pearson correlation coefficient test).

Supplementary Figure S2: Discriminative miRNAs for newly diagnosed *BCR-ABL1*-like ALL and clinical outcome in childhood BCP-ALL.



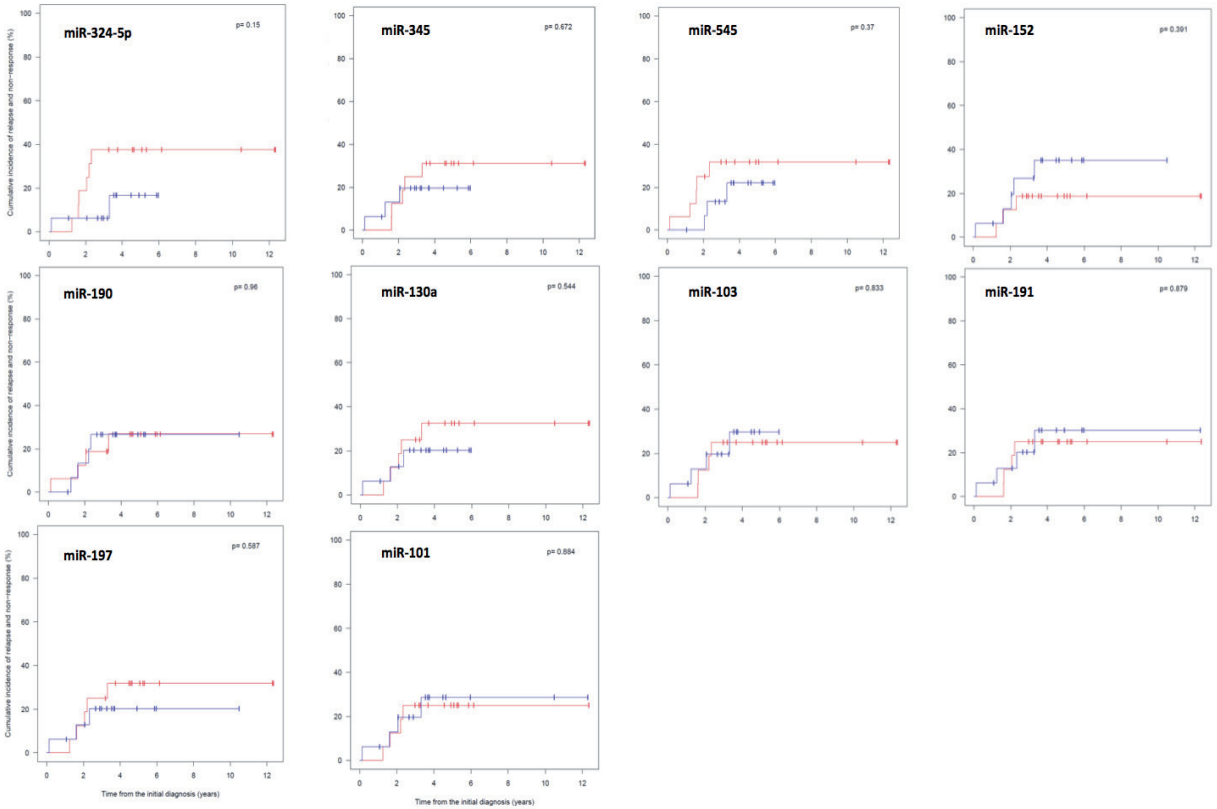
The expression levels of the top 10 most discriminative miRNAs for newly diagnosed *BCR-ABL1*-like ALL (see supplementary Table S1) were compared between patients who remained in continuous complete remission for 5.5±2.8 years (n=24) and those who relapsed (n=8). Each box shows the expression level of a miRNA in leukemic cells taken at initial diagnosis of 32 patients of the new data set with *BCR-ABL1*-like, *BCR-ABL1*-positive or B-other ALL. Non-parametric Mann-Whitney U test indicated no statistical difference in expression levels between patients who stayed in continuous complete remission (No relapse, filled box, n=24) and those who relapsed (open box, n=8, p>0.05).

Supplementary Figure S3: Cumulative incidence of relapse for *BCR-ABL1*-like and *BCR-ABL1*-positive patients according to the miRNA signature.



The probability of the cumulative incidence of relapse did not differ between *BCR-ABL1*-like and *BCR-ABL1*-positive cases who clustered together in cluster-I (red line, n=17) compared to those in cluster-II (blue line, n=12). Cluster-I and cluster-II were identified by the miRNA expression signature presented in Figure 1. The probability of the cumulative incidence of relapse (pCIR), with death as a competing event, was calculated using the method of Fine and Gray with the software packages mstate 0.2.6 and cmprsk 2.2-2 in R environment. Relapse and non-response to induction chemotherapy were considered to be events in the CIR model.

Supplementary Figure S4: Expression levels of top 10 most discriminative miRNAs for newly diagnosed *BCR-ABL1*-like ALL are not predictive for clinical outcome in childhood BCP-ALL.



The probability of cumulative incidence of relapse (pCIR) did not differ between patients with high (red) and low (blue) expression levels of the indicated miRNA in their leukemic cells. Thirty two patients with *BCR-ABL1*-like, *BCR-ABL1*-positive, and B-other ALL were divided into high or low miRNA expression category by the median expression level for each miRNA.

Supplementary Table S1: Most discriminative miRNAs per subtype of pediatric BCP-ALL

TOP#	miRNA	fold- change	multiple testing- corrected p value
***BCR-ABL1*-positive #1**	miR-29a	1.94	0.11
***BCR-ABL1*-positive #2**	miR-345	0.50	0.13
***BCR-ABL1*-positive #3**	miR-451	0.09	0.13
***BCR-ABL1*-positive #4**	miR-106b	0.70	0.18
***BCR-ABL1*-positive #5**	miR-93	0.69	0.23
***BCR-ABL1*-positive #6**	miR-186	0.72	0.23
***BCR-ABL1*-positive #7**	miR-103	0.66	0.23
***BCR-ABL1*-positive #8**	miR-324-3p	0.69	0.23
***BCR-ABL1*-positive #9**	miR-146a	1.92	0.26
***BCR-ABL1*-positive #10**	miR-32	0.74	0.26
***BCR-ABL1*-like #1**	miR-324-5p	0.30	<0.001
***BCR-ABL1*-like #2**	miR-345	0.33	<0.001
***BCR-ABL1*-like #3**	miR-190	0.04	<0.001
***BCR-ABL1*-like #4**	miR-130a	0.36	<0.001
***BCR-ABL1*-like #5**	miR-545	0.21	<0.001
***BCR-ABL1*-like #6**	miR-152	0.13	<0.001
***BCR-ABL1*-like #7**	miR-103	0.51	0.001
***BCR-ABL1*-like #8**	miR-191	0.54	0.003
***BCR-ABL1*-like #9**	miR-197	0.60	0.003
***BCR-ABL1*-like #10**	miR-101	1.73	0.003
***TCF3*-rearranged #1**	miR-146a	0.07	<0.001
***TCF3*-rearranged #2**	miR-126	0.03	<0.001
***TCF3*-rearranged #3**	miR-29a	0.25	<0.001
***TCF3*-rearranged #4**	miR-24	0.31	<0.001
***TCF3*-rearranged #5**	miR-511	0.01	<0.001
***TCF3*-rearranged #6**	miR-223	0.21	<0.001
***TCF3*-rearranged #7**	miR-26a	0.44	<0.001
***TCF3*-rearranged #8**	miR-365	0.17	<0.001
***TCF3*-rearranged #9**	miR-425	2.91	<0.001
***TCF3*-rearranged #10**	miR-191	2.40	<0.001
Hyperdiploid #1	miR-342	2.17	<0.001
Hyperdiploid #2	miR-660	2.46	<0.001
Hyperdiploid #3	miR-374	2.37	<0.001
Hyperdiploid #4	miR-222	3.14	<0.001
Hyperdiploid #5	miR-223	3.45	<0.001
Hyperdiploid #6	miR-195	2.10	<0.001
Hyperdiploid #7	miR-98	2.83	<0.001
Hyperdiploid #8	miR-511	22.11	0.001
Hyperdiploid #9	miR-345	2.38	0.003
Hyperdiploid #10	miR-324-3p	1.78	0.003
***MLL*-rearranged #1**	Let-7b	0.03	<0.001
***MLL*-rearranged #2**	miR-196b	342.95	<0.001
***MLL*-rearranged #3**	Let-7c	0.06	<0.001
***MLL*-rearranged #4**	miR-20b	0.20	<0.001
***MLL*-rearranged #5**	miR-192	0.43	0.002
***MLL*-rearranged #6**	miR-194	0.43	0.004
***MLL*-rearranged #7**	miR-425	2.17	0.012
***MLL*-rearranged #8**	miR-484	1.80	0.018
***MLL*-rearranged #9**	miR-186	1.65	0.018
***MLL*-rearranged #10**	miR-24	1.89	0.018

***ETV6-RUNX1*-positive #1**	miR-125b	29.33	<0.001
***ETV6-RUNX1*-positive #2**	miR-221	0.16	<0.001
***ETV6-RUNX1*-positive #3**	miR-99a	22.74	<0.001
***ETV6-RUNX1*-positive #4**	Let-7c	5.76	<0.001
***ETV6-RUNX1*-positive #5**	miR-100	32.07	<0.001
***ETV6-RUNX1*-positive #6**	miR-126	6.06	<0.001
***ETV6-RUNX1*-positive #7**	miR-222	0.34	<0.001
***ETV6-RUNX1*-positive #8**	miR-200c	0.34	0.001
***ETV6-RUNX1*-positive #9**	miR-218	20.84	0.002
***ETV6-RUNX1*-positive #10**	miR-345	2.33	0.003

The ten most differentially expressed miRNAs are listed for each subtype ranked by multiple testing-corrected p-values. Fold-changes and p-values were calculated based on comparison between expression levels of miRNAs of one specific subtype and remaining cases (e.g. *BCR-ABL1*-positive versus non-*BCR-ABL1*-positive cases).

Supplementary Table S2: Validated target genes for the most discriminative miRNAs per subtype of pediatric BCP-ALL and their possible function

		Validated targets	Predicted functions of the validated targets
***BCR-ABL1*-positive ALL**	**Upregulated miRNAs** miR-29a, miR-146a	NO	development of lymphohematopoietic cancer, function and differentiation of blood cells and leukocytes, etc.
	Downregulated miRNAs miR-103, miR-106b, miR-186, miR-32, miR-324-3p, miR-345, miR-451, miR-93	ABCF2, **AKAP11**, ANKFY1, AP1G1, **ATXN1**, BCL11B, BCL2, BTG3, C18orf32, C1orf21, CCND1, CD46, **CDC37L1**, CDKN1A, CNOT4, CREBBP, DUSP8, DVL2, DYRK2, E2F1, E2F3, EFCAB14, EXOC5, FBXL3, FBXL5, FEM1C, **FGF2**, FOXN2, INSIG1, KAT2B, **KATNAL1**, KMT2D, LHFPL2, **LUZP1**, MAPK9, MCL1, MLLT6, MTF1, MYO5A, NDEL1, NIN, NUAK1, **NUFIP2**, PHLPP2, PKD2, PLEKHA1, PON2, PPP1R12C, PRRG1, **PTEN**, **PURA**, RB1, RFK, RIMS3, RUNX1T1, SAMD8, SLC39A10, SMAD4, SOWAHC, SPRED1, STYX, SUV420H1, SYBU, **TAF6L**, TLE4, TMEM100, TMEM245, TNRC6B, TRIP10, TULP4, USP21, VEGFA, WDR33, WEE1, YTHDC1, **YWHAH**, ZBTB21, ZHX1, ZNF148, ZNF805	arrest in cell cycle progression, proliferation of tumor cell lines, *etc.*
***BCR-ABL1*-like ALL**	**Upregulated miRNAs** miR-101	NA	development of lymphohematopoietic cancer, control of proliferation of tumor cell lines, tumorigenesis of malignant tumors, *etc.*
	Downregulated miRNAs miR-103, miR-130a, miR-152, miR-190, miR-191, miR-197, miR-324-5p, miR-345, miR-545	ACVR1, AGR2, CDK6, DICER1, FBXW7, KLF4, PRKAR2A, RAD51	cell cycle progression, differentiation of cells, development of tumor, proliferation of tumor cell lines, *etc.*
***TCF3*-rearranged ALL**	**Upregulated miRNAs** miR-191, miR-425	NO	proliferation of tumor cells, development of hematological neoplasia, *etc.*
	Downregulated miRNAs miR-103, miR-130a, miR-152, miR-190, miR-191, miR-197, miR-324-5p, miR-345, miR-545	**BCL2**, BRCA1, CCNA2, CDK6, CPEB2, CPEB4, **E2F1**, FAF1, GLUL, **IL6**, IRS1, LIF, LMO2, MCM10, MYC, NASP, NFIX, PTEN, SMAD4, STRAD8, TNFAIP3	development of lymphohematopoietic cancer, lymphomagenesis, growth of tumor, *etc.*

Hyperdiploid ALL	**Upregulated miRNAs**	BCL2L11, CCND1, CHUK, DICER1, E2F1	quantity of hematopoietic progenitor cells, differentiation of cells, development of lymphohematopoietic cancer, *etc.*
	miR-195,miR-222, miR-223, miR-324-3p, miR-342, miR-345, miR-374, miR-511, miR-660, miR-98		
	Downregulated miRNAs	NA	NA
	NA		
***MLL*-rearranged ALL**	**Upregulated miRNAs**	BCL2, CMTM4, LBR, MED4, MIDN, NRIP1, PRPN9, RNF2, SLC11A2, WRB, YRDC, ZNF700, ZNF805	development of lymphohematopoietic cancer, proliferation of cells, lymphangiogenesis, *etc.*
	miR-186, miR-196b, miR-24, miR-425, miR-484		
	Downregulated miRNAs	ABCB10, ACSL1, ACVR2B, AGL, BCL2, CCNY, CDC25A, CDK6, CUL3, **DICER1**, E2F5, EFNB2, ELOVL1, HBEGF, HMGA2, IDI1, IFRD1, IGF1R, NAA50, NRAS, OSBPL10, PGM3, PPARG, PRKAR1A, RTCA, SCD, SEC23B, SLC1A4, TAF9B, TMTC3, UBE2D2, UGT8, ZADH2	cell cycle progression, cell viability of tumor cell lines, tumorigenesis of malignant tumors, *etc.*
	Let-7b, Let-7c, miR-192, miR-194, miR-20b		
***ETV6-RUNX1*-positive ALL**	**Upregulated miRNAs**	BCL2, **CDK6**, FGFR3, HMGA2, IGF1R, MMP13, MTOR, NTRK3, RPTOR, TRIM71	control the proliferation and apoptosis of tumor cell lines, development of hematological neoplasia, *etc.*
	Let-7c, miR-100, miR-125b, miR-126, miR-218, miR-345, miR-99a		
	Downregulated miRNAs	BBC3, BCL2L11, CDKN1B, CDKN1C, CERS2, CORO1A, DICER1, DIRAS3, ESR1, ETS1, FOS, FOXO3, HOXB5, ICAM1, KIT, KLF9, PTEN, SELE, TBK1, TCEAL1, TIMP3, TMED7, TNFSF10, TP53, TRPS1	control the proliferation and apoptosis of tumor cell lines, development of lymphoid cancer, *etc.*
	miR-200c, miR-221, miR-222		

The validated target genes for the most differentially expressed miRNAs are listed for each subtype using the data base available at http://mirtarbase.mbc.nctu.edu.tw/php/search.php. The genes which were validated for at least two of the top discriminative miRNAs are shown in this table and the validated target genes for ≥3 miRNAs are shown in **bold**. The possible functions of validated target genes for upregulated or downregulated miRNAs of each subtypes were predicted using Ingenuity systems (www.ingenuity.com). NA= not applicable, NO= no overlap.

Supplementary Table S3: Clinical information of *BCR-ABL1*-positive and *BCR-ABL1*-like ALL cases of cluster I and cluster II

Patient #	Cluster #	Genetic subtype	Disease free survival (year)	Relapse
1	Cluster I	*BCR-ABL1*-like ALL	5.87	NO
2	Cluster I	*BCR-ABL1*-like ALL	5.33	NO
3	Cluster I	*BCR-ABL1*-like ALL	4.64	NO
4	Cluster I	*BCR-ABL1*-like ALL	2.34	YES
5	Cluster I	*BCR-ABL1*-like ALL	3.54	NO
6	Cluster I	*BCR-ABL1*-like ALL	2.89	NO
7	Cluster I	*BCR-ABL1*-like ALL	4.58	NO
8	Cluster I	*BCR-ABL1*-like ALL	2.59	NO
9	Cluster I	*BCR-ABL1*-like ALL	2.01	NO
10	Cluster I	*BCR-ABL1*-like ALL	0.13	YES
11	Cluster I	*BCR-ABL1*-like ALL	5.24	NO
12	Cluster I	*BCR-ABL1*-positive ALL	NA	NA
13	Cluster I	*BCR-ABL1*-positive ALL	5.69	NO
14	Cluster I	*BCR-ABL1*-positive ALL	NA	YES
15	Cluster I	*BCR-ABL1*-positive ALL	1.07	NO
16	Cluster I	*BCR-ABL1*-positive ALL	4.92	NO
17	Cluster I	*BCR-ABL1*-positive ALL	12.29	NO
1	Cluster II	*BCR-ABL1*-like ALL	4.00	NO
2	Cluster II	*BCR-ABL1*-like ALL	6.15	NO
3	Cluster II	*BCR-ABL1*-like ALL	2.21	YES
4	Cluster II	*BCR-ABL1*-like ALL	12.35	NO
5	Cluster II	*BCR-ABL1*-positive ALL	NA	YES
6	Cluster II	*BCR-ABL1*-positive ALL	NA	NA
7	Cluster II	*BCR-ABL1*-positive ALL	NA	NA
8	Cluster II	*BCR-ABL1*-positive ALL	NA	NO
9	Cluster II	*BCR-ABL1*-positive ALL	5.07	NO
10	Cluster II	*BCR-ABL1*-positive ALL	1.25	YES
11	Cluster II	*BCR-ABL1*-positive ALL	10.48	NO
12	Cluster II	*BCR-ABL1*-positive ALL	1.48	YES

NA= data is not available

Supplementary Table S4: Correlation between expression levels of discriminative miRNAs and their validated/predicted target genes

		Validated target genes			Predicted target genes				
BCR-ABL1-positive ALL	miR-29a	***PIK3R1*** (RS -0.53) ***	***CDC42*** (RS -0.36) *	***DNMT3B*** (RS -0.32)	***ATAD2B*** (RS -0.23)	***COL3A1*** (RS 0.12)	***ELN*** (RS 0.22)	***HBP1*** (RS 0.11)	***COL4A4*** (RS -0.24)
	miR-345	***ABCC1*** (RS -0.39) *	***NTRK3*** (RS -0.19)	***CDKN1A*** (RS -0.19)	***HSPA12A*** (RS -0.08)	***CTTNBP2NL*** (RS 0.1)	***HABP4*** (RS -0.22)	***RFC1*** (RS -0.11)	***EPN3*** (RS -0.03)
	miR-451	***ABCB1*** (RS -0.28)	***MMP2*** (RS -0.23)	***MIF*** (RS -0.2)	***OSR1*** (RS -0.2)	***PSMB8*** (RS 0.05)	***TTN*** (RS -0.1)	***TSC1*** (RS 0.37) *	***AEBP2*** (RS 0.3)
	miR-106b	***ITCH*** (RS -0.5) ***	***VEGFA*** (RS -0.4) *	***RBL2*** (RS -0.32)	***FGO4*** (RS -0.2)	***PKD2*** (RS 0.03)	***MAP3K2*** (RS -0.25)	***ZNFX1*** (RS -0.02)	***PDCD1LG2*** (RS 0.26)
	miR-93	***LATS2*** (RS -0.38) *	***VEGFA*** (RS -0.19)	***PTEN*** (RS -0.18)	***FGO4*** (RS -0.38) *	***PKD2*** (RS 0.26)	***MAP3K2*** (RS -0.18)	***ZNFX1*** (RS -0.26)	***PDCD1LG2*** (RS 0.15)
	miR-186	***CSNK2A1*** (RS 0.1)	***P2RX7*** (RS 0.12)	***AKAP12*** (RS 0.13)	***GABRA4*** (RS 0.08)	***UBR2*** (RS 0.17)	***ANO6*** (RS 0.18)	***TMF1*** (RS 0.04)	***PID1*** (RS 0.08)
	miR-103	***DAPK1*** (RS -0.43) *	***CYP2C8*** (RS 0.36) *	***PTEN*** (RS -0.19)	***DICER1*** (RS -0.18)	***ANO3*** (RS -0.18)	***NF1*** (RS -0.04)	***EIF5*** (RS -0.18)	***ARIH2*** (RS -0.4) 0.03
	miR-324-3p	***CREBBP*** (RS -0.37) *	***WNT9B*** (RS 0.02)	***DVL2*** (RS 0.04)	***L1CAM*** (RS 0.45) *	***MDGA1*** (RS 0.33)	**FAM155B** (RS 0.3)	***MMP24*** (RS -0.06)	***ANKRD52*** (RS -0.12)
	miR-146a	***CXCR4*** (RS -0.39) *	***IL8*** (RS -0.3)	***ERBB4*** (RS -0.26)	***TRAF6*** (RS 0)	***IRAK1*** (RS 0.09)	***ZBTB2*** (RS 0.08)	***IGSF1*** (RS 0.07)	***LCOR*** (RS -0.23)
	miR-32	**CREBBP** (RS -046) *	***MSH3*** (RS -0.3)	***TAC1*** (RS -0.3)	***CD69*** (RS -0.05)	***FNIP1*** (RS -0.19)	***SLC12A5*** (RS 0.04)	***MAN2A1*** (RS -0.16)	***ACTC1*** (RS 0.367) *
BCR-ABL1-like ALL	miR-324-5p	***GLI1*** (RS 0.07)	***SMO*** (RS 0.48) **		***MGAT3*** (RS 0)	***PCYT1B*** (RS 0.08)	***SHPK*** (RS -0.37) *	***LYZL4*** (RS 021)	***LHFPL4*** (RS -0.05)
	miR-345	***ABCC1*** (RS -0.39) *	***NTRK3*** (RS -0.19)	***CDKN1A*** (RS -0.19)	***HSPA12A*** (RS -0.08)	***CTTNBP2NL*** (RS 0.1)	***HABP4*** (RS -0.22)	***RFC1*** (RS -0.11)	***EPN3*** (RS -0.03)
	miR-190	***VEGFA*** (RS -0.04)	***CDKN1B*** (RS -0.03)	***PHLPP1*** (RS 0.02)	***ERG*** (RS -0.35)	***BACH1*** (RS 0.14)	***CEBPA*** (RS -0.3)	***OSBPL6*** (RS 0.352)	***SMAD2*** (RS -0.23)
	miR-130a	***TAC1*** (RS -0.32)	***ESR1*** (RS -0.17)	***APP*** (RS -0.14)	***SLAIN1*** (RS 0.3)	***TSC1*** (RS 0.28)	***NDM4*** (RS 0.04)	***PAN3*** (RS -0.05)	***LCROL*** (RS 0.29)
	miR-545	***SNAI2*** (RS -0.16)	***LRP1*** (RS 0.02)		***ARGLU1*** (RS 0.06	***USP9X*** (RS -0.08)	***RPF1*** (RS 0.02)	***ZNF280C*** (RS 0.441) *	***ANKRD55*** (RS 0.03)
	miR-152	***IGF1R*** (RS -0.08)	***TGFA*** (RS -0.02)	***DNMT1*** (RS 0.01)	***CCKBR*** (RS 0.27)	***OSBPL11*** (RS 0.21)	***ATP6AP2*** (RS 0.1)	***CLCN6*** (RS -0.06)	***FXR1*** (RS -0.13)
	miR-103	***DAPK1*** (RS -0.43) *	***CYP2C8*** (RS 0.36) *	***PTEN*** (RS -0.19)	***DICER1*** (RS -0.18)	***ANO3*** (RS -0.18)	***NF1*** (RS -0.04)	***EIF5*** (RS -0.18)	***ARIH2*** (RS -0.4) *
	miR-191	***SATB1*** (RS -0.43) *	***IL1A*** (RS -0.21)	***NDST1*** (RS -0.17)	***TMOD2*** (RS 0.09)	***ZBTB34*** (RS 0.02)	***TJP1*** (RS 0.31)	***TMC7*** (RS -0.06)	***PLCD1*** (RS 0.29)
	miR-197	***ISYNA1*** (RS -0.36) *	***PEX13*** (RS -0.3)	***PIPOX*** (RS -0.26)	***TTPAL*** (RS -0.17)	***ADAT2*** (RS -0.17)	***SYNGR1*** (RS -0.03)	***NRF1*** (RS -0.05)	***ZIC3*** (RS -0.08)
	miR-101	***PTGS2*** (RS -0.53) ***	***ATXN1*** (RS -0.52) ***	***APP*** (RS -0.36) *	***FAM108C1*** (RS 0.17)	***GLTSCR1*** (RS -0.14)	***ZNF654*** (RS 0.08)	***EYA1*** (RS 0.6) ***	***FLRT3*** (RS 0.45) *

The expression levels of subtype-discriminative miRNAs were integrated with mRNA expression levels of their validated target genes (www.miRtarbase.mbcu.edu.tw) and top-5 predicted target genes (www.targetscan.org) for 32 *BCR-ABL1*-positive, *BCR-ABL1*-like and unclassified B-other cases. * indicates p<0.05, ** indicated p<0.01, and *** indicated p<0.005, RS= Spearman's correlation coefficient .

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Chapter 5

Altered expression of miR-24, miR-126 and miR-365 does not affect viability of childhood *TCF3*-rearranged leukemia cells

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Abstract

Among the microRNAs (miRNAs) that control different cellular processes, miR-24, miR-126 and miR-365 were shown to regulate cell cycle progression and apoptosis in various types of tumors. Interestingly, these three miRNAs were downregulated in pediatric *TCF3*-rearranged B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Here, we showed individual or combined overexpression of miR-24, miR-126 and miR-365 cannot alter cell cycle progression and amount of apoptosis in 697, KASUMI-2 or MHH-CALL-3 *TCF3*-rearranged leukemic cells. We further integrated the miRNA-mRNA expression data of 37 children with BCP-ALL to identify candidate target genes for the three miRNAs. However, the expression levels of selected candidate target genes (*ELL*, *EBF3* and *IRF4* for miR-24, *PITPNC1* for miR-126 and *ZAP-70* for miR-365) did not reduce upon miRNAs overexpression in MHH-CALL-3 *TCF3*-rearranged leukemic cells. While expression level of *AURKB* – a validated target for miR-24 – was reduced upon miR-24 overexpression in hepatocarcinoma HEP-G2 cells, overexpression of miR-24 cannot alter *AURKB* expression levels in MHH-CALL-3 *TCF3*-rearranged leukemic cells. Together, our data suggest that miRNAs function is highly tissue-dependent and that a defined biological target gene or function of a miRNA in a specific tissue cannot be extended as generalized target/function for that miRNA in all types of cells/tissues.

Keywords

miR-24, miR-126, miR-365, cell viability, *TCF3*-rearranged leukemia, miRNA-mRNA expression data integration

Introduction

MicroRNAs (miRNAs) are ~22 nucleotide-long non-coding RNAs that post-transcriptionally regulate the expression of more than 60% of human protein-coding genes (1). The impact of miRNAs in fine-tuning of different physiological processes has been well established (2, 3). Accordingly, altered expression of miRNAs is correlated with initiation and progression of different diseases and cancers (4, 5). Moreover, aberrant expression of miRNAs is associated with diagnosis, classification and/or clinical outcome of solid tumors (6, 7) as well as leukemias (8, 9). We previously showed that genetic subtypes of childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) demonstrate discriminative miRNA signatures (8). Similar to other reports, our results suggested prognostic values of a subset of miRNAs in ALL (8-10). Among genetic subtypes of childhood BCP-ALL, *TCF3*-rearranged leukemia displayed the most distinct miRNA signature (8). This group of patients is characterized by low expression of multiple miRNAs including miR-24, miR-126, and miR-365 (8).

Compared to lymphoid cells, high endogenous expression levels of miR-24 were observed in myeloid cells (11). Enforced expression of miR-24 (together with miR-23a and miR-27a) in hematopoietic progenitors resulted in decreased B lymphopoiesis, even if the cells were cultured in B-cell promoting condition (11). MiR-24 controls cell cycle distribution and amount of apoptosis (12, 13). Overexpression of miR-24 inhibited cellular proliferation and induced S/G2M arrest in human colon cancer and osteosarcoma cell line models (14). It was shown that the expression of genes that regulate cellular apoptosis – like *XIAP* (15) and *BCL2* (16) – as well as genes that control cell cycle progression – such as *E2F2*, *MYC* and *AURKB* (2) – are directly controlled by miR-24.

Similarly, enforced expression of miR-126 inhibited cell cycle progression and resulted in G1 arrest in solid tumors (17-20) as well as colony formation suppression and proliferation inhibition in colon cancer cells (20). MiR-126 overexpression in gastric cancer cells decreased cellular invasion and migration *in vitro*, and reduced tumorigenicity and metastasis of cancer cells *in vivo* (19). In hematopoietic stem cells (HSCs), upregulation of miR-126 induced quiescence and G0 arrest while downregulation of this miRNA resulted in cell cycle progression and HSCs expansion (21). High expression levels of miR-126 and miR-126* – which are both significantly lower expressed in *TCF3*-rearranged BCP-ALL – were shown to correlate with *in vitro* resistance to vincristine and daunorubicin in childhood ALL (8). MicroRNA-365 is also involved in control of cell cycle distribution in colon cancer cells (22), proliferation arrest of lung cancer cells (23), and apoptosis regulation in colon cancer (22), breast cancer (16), and human umbilical vein endothelial cells (24).

There is a growing body of evidence that multiple miRNAs collaborate to control distinct target genes/pathways and to modulate cellular functions (25, 26). In pediatric BCP-ALL, we recently showed that miR-125b synergizes with miR-100 and miR-99a in induction of vincristine resistance (27). Based on concomitant expression patterns of miR-24, miR-126 and miR-365 in childhood *TCF3*-rearranged ALL and their

similar reported functions, we investigated whether these three miRNAs, individually or in combination, may alter cell cycle distribution and amount of apoptosis in *TCF3*-rearranged leukemia. Next, we used a new miRNA target identification method (28) which together with published information on proven miRNA target genes as well as miRNA predicted target genes integrates the mRNA and miRNA expression profiles of 37 cases with childhood BCP-ALL. Following overexpression of miRNAs in *TCF3*-rearranged leukemic cells, changes in the expression levels of predicted candidate target genes in our miRNA-mRNA integration cohort were analyzed to discover functional relationships between these miRNAs and their predicted candidate target genes in BCP-ALL.

Methods and Materials

Cell lines

The *TCF3*-rearranged leukemic cell lines KASUMI-2 (#ACC-526), 697 (#ACC-42) and MHH-CALL-3 (#ACC-339) were purchased from DSMZ (Braunschweig, Germany). KASUMI-2 and 697 cells were kept in culture in RPMI 1640-GlutaMAX™ medium (Life technologies, Bleiswijk, The Netherlands) supplemented with 10% fetal calf serum (Bodinco BV, Zaandam, The Netherlands), 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.125 µg/mL fungizone (all from Life technologies, Bleiswijk, The Netherlands). MHH-CALL-3 cells were cultured in the similar medium mixture with 20% fetal calf serum. The hepatocellular carcinoma HEP-G2 cells were kindly provided by the department of Cell Biology, Erasmus MC, Rotterdam and were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. All cells were incubated at 37°C in humidified air containing 5% CO_2.

Transient induction of miRNAs

Precursor oligonucleotide sequences of miR-24, miR-126, miR-365 (Life technologies, Bleiswijk, The Netherlands) as single miRNAs (60 µM) or in combination (20 µM each) were added to $0.5x10^6$/mL KASUMI-2 and 697 leukemic cells and $0.8x10^6$/mL MHH-CALL-3 leukemic cells in the presence of Lipofectamine (Life technologies, Bleiswijk, The Netherlands), as explained earlier (27). Cells were cultured for another 48 hours. Similarly, 60 uM of scrambled control-miR (Life technologies, Bleiswijk, The Netherlands) was added to the cells following the same protocol. MiR-24 or scrambled control-miR was added to $0.2x10^6$/mL hepatocellular carcinoma HEP-G2 cells in triplicate and served as positive control for effective processing of precursor miRNAs and effective knock-down of *AURKB* expression levels, as reported earlier (2). recursor oligonucleotide sequences of miR-24, miR-126, miR-365 (Life technologies, Bleiswijk, The Netherlands) as single miRNAs (60 µM) or in combination (20 µM each) were added to $0.5x10^6$/mL KASUMI-2 and 697 leukemic cells and $0.8x10^6$/mL MHH-CALL-3 leukemic cells in the presence of Lipofectamine (Life technologies, Bleiswijk, The Netherlands), as explained earlier (27). Cells were cultured for another 48 hours. Similarly, 60 uM of scrambled control-miR (Life technologies, Bleiswijk, The Netherlands) was added to the cells following the same protocol. MiR-24 or scrambled control-miR was added to $0.2x10^6$/mL hepatocellular carcinoma HEP-G2 cells in

triplicate and served as positive control for effective processing of precursor miRNAs and effective knock-down of *AURKB* expression levels, as reported earlier (2).

RNA extraction and quantification of miRNA expression levels

Total RNA was isolated from 0.8 million cells using TRIzol isolation reagents as described in the manufacturer's protocol (Life technologies, Bleiswijk, The Netherlands). Concentration of the isolated RNA was quantified using a Nanodrop (ND-1000 spectrophotometer, Agilent, Amstelveen, The Netherlands) and RNA integrity was measured by RNA 6000 Nano Assay LabChips using Agilent Bio-analyzer 2100. All samples were checked to have an RNA integrity number of $\geq$7.0. The expression levels of each miRNA and scrambled control-miR were measured by real-time quantitative PCR (RT-qPCR) using mature-form specific miRNA stem-looped primers and miRNA-selective probes (TaqMan MicroRNA Assay, Applied Biosystems, Foster City, USA). RT-qPCR was performed using a Taqman 7900HT instrument (Life technologies, Bleiswijk, The Netherlands) and data were analyzed with SDS 2.3 software (Applied Biosystems, Foster City, USA). Endogenous small nucleolar RNA 1 (snoR-1, 5'-AUUUGCUACUGA GAGAUGGUGAUGACAUUUUAAACCACCAAGAUCGCUGAUGCA- 3') was used as a reference for input of RNA. Each assay was performed in duplicate and Ct value of miRNA and that of snoR-1 were measured. The expression of each miRNA was calculated as the percentage of snoR-1 expression level as $2^{-\Delta Ct}$x100% where the ΔCt is equal to the Ct-value for each miRNA minus the Ct-value of snoR-1.

Cell cycle distribution

Cell cycle distribution was determined as explained earlier (27). Briefly, 0.25 million of leukemic cells were fixed in 70% EtOH, resuspended in 20 µL PBS and then incubated for 5 minutes at room temperature in 45 µL of hypotonic buffer containing 3.5 mM sodium citrate, 0.1% Igepal® (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands), 1.5 mM spermin-tetrahydrochloride, 60 µg/mL Tris-hydroxymethylaminomethane and 3 mg/mL trypsin. Next, the nuclear suspension was incubated with 0.5 mg/mL trypsin inhibitor and 20 µg/mL ribonuclease A for 10 minutes at room temperature. Nuclei were incubated with 1.75 mM propidium iodide (all chemicals were provided by Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) for 15 minutes on ice and the amount of propidium iodide staining was measured using a MACSQuant device (Miltenyi Biotech, Bergisch Gladbach, Germany). The distribution patterns of G0/G1, S and G2M phases were analyzed by MACSQuant® software.

Apoptosis assay

The amount of apoptotic leukemic cells was quantified as described earlier (27). In summary, 0.25 million of cells were centrifuged and then resuspended in 200 µL of binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). Cells were incubated with 1:250 diluted Annexin V-Alexa Fluor® 647 conjugate (Life technologies, Bleiswijk, The Netherlands) and 4 µg/mL propidium iodide for 15 minutes at room temperature. Annexin-V/propidium iodide staining was quantified using a MACSQuant device (Miltenyi Biotech, Bergisch Gladbach, Germany). Data were analyzed using MACSQuant® analyzer software as follows: AnnexinV negative-PI negative population was used as a measure for live cells, and the remaining population of the cells was

considered as dead population (AnnexinV positive-PI negative cells as early apoptotic, AnnexinV positive-PI positive cells as late apoptotic, and AnnexinV negative-PI positive cells as necrotic population).

Performing and processing of mRNA and miRNA assays

Gene expression profiles of 37 cases with childhood BCP-ALL – 4 *BCR-ABL1*-positive, 3 *TCF3*-rearranged, 7 *ETV6-RUNX1*-positive, 13 hyperdiploid (>50 chromosomes) and 10 unclassified B-others negative for the above mentioned genetic aberrations – were obtained using the Affymetrix Human Genome U133 Plus 2.0 Arrays according to the manufacturer's guidelines (29). In summary, The RNA integrity was checked with Agilent's 2100 Bio-analyzer (Santa Clara, USA). cDNA and biotinylated cRNA were synthesized and hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, USA) according to the manufacturer's guidelines. Data were acquired using Bioconductor package 'affy', and probe-set intensities were normalized using the variance stabilization and normalization (VSN) (30) and batch corrected using ComBat (31) in the statistical data analysis environment R. We used the gene-based chip definition file U133plus2.gb.cdf from http://masker.nci.nih.gov/ev/. For the same 37 cases, the expression levels of 397 miRNAs were measured using a RT-qPCR reaction and analyzed as described earlier (8).

Data integration and candidate target identification

The above-mentioned mRNA microarray data were integrated with the miRNA expression profiles of the same patients (8) to identify the negatively associated miRNAs-mRNAs. We selected three commonly used tools for target prediction: TargetScan version 6.1 (32), microCosm version 5 (formerly miRBase Targets) (33) and PITA version from 2007 (1). In addition, we selected two databases containing validated targets: miRecords version November 25, 2010 (34), and miRTarBase release 3.5 (35). Candidate target genes were defined as genes that were predicted or validated target genes for a specific miRNA in at least one of these five databases. All candidate target genes together for a miRNA were called a gene set, and tested together for association of their expression with miRNA expression in the 37 patient samples. The integrated analysis was based on the globaltest (36) as described elsewhere in more details (28). MiRNAs which are significantly associated with mRNA expression levels (FDR<0.05) were selected. Based on the observed significant inverse association between expression levels of miRNAs and their candidate target genes and considering the possible function of candidate target genes in leukemogenesis, several candidate target genes were selected for further evaluation: *ELL*, *EBF3*, *IRF4* and *AURKB* for miR-24, *PITPNC1* for miR-126, and *ZAP-70* and *KAT2A* for miR-365.

Synthesis of cDNA, primers design and real-time quantitative PCR (RT-qPCR)

One µg of total RNA was incubated with 2.5 µL of a mixture of 25µM random hexamers and 200 nM oligodT in a total volume of 15 µL. This mixture was incubated at 70°C for 5 minutes. Thereafter, 0.5 µL of 10 mM dNTPs, 5 µL of 5x RT-buffer, 0.625 µL of 40U/µL RNasin (Life technologies, Bleiswijk, The Netherlands), 1 µL of 200U/µL mmLV reverse transcriptase enzyme (mouse moloney leukemia virus, Life

technologies, Bleiswijk, The Netherlands) and 2.88 µL H_2O was added. The reverse transcription step was preceded as follows: 37°C for 45 minutes, 42°C for 15 minutes and 94°C for 5 minutes followed by incubation at 4°C. Each cDNA mixture was diluted by addition of 100 µL H_2O.

Forward and reverse primers were designed using Oligo 6.22 software (Molecular Biology Insights, Cascade, USA) except for *AURKB* where previously described primers by Lal A, *et al* (2) were used. The list and the sequences of the primers are shown in Supplementary Table S1. Expression levels of candidate target genes were measured by RT-qPCR: 2.5 µL of cDNA was added to 12.5 µL of SYBR Green, 0.5 µL of ROX (#F410, Thermo Scientific, CO, USA), 0.75 µL of 10 pmol/µL forward primer, 0.75 µL of 10 pmol/µL reverse primer, 1.5 µL of 25mM $MgCl_2$ and 6.5 µL of H_2O (Applied Biosystems, Foster City, USA). RT-qPCR was performed using a Taqman 7900HT instrument (Life technologies, Bleiswijk, The Netherlands) and data were analyzed with SDS 2.3 software (Applied Biosystems, Foster City, USA). Expression level of each candidate gene was measured in duplicate and the endogenous expression of *GAPDH* was used as an internal reference for input RNA. The relative expression level of each gene was calculated as $2^{-\Delta Ct}$x100% where the ΔCt is equal to the Ct-value for each gene minus the Ct-value of the control reference *GAPDH*.

Statistical analysis and software

The non-parametric Mann-Whitney U test was used to compare the percent of apoptosis, cell cycle distribution, and expression levels of candidate genes in miR-24, miR-126 and miR-365 (individually and in combination) induced cells and scrambled control-miR transfected cells. We have used R version 2.2.0 (37). In addition, we used the following R Bioconductor packages: affy version 1.30.0, vsn version 3.20.0 and Biobase version 2.12.1. R code for combat procedure (31) was downloaded from the author's website at http://jlab.byu.edu/ComBat.

Results

Transient overexpression of miR-24, miR-126 and miR-365 does not affect cell cycle progression in BCP-ALL

Transfection of miR-24, miR-126 and miR-365 as single miRNA precursors in three different cell line models of *TCF3*-rearranged leukemia (697, KASUMI-2 and MHH-CALL-3) increased the expression levels of these specific miRNAs >100-fold compared to a scrambled control-miR transfected cells (Figure 1). Similarly, co-transfection of these miRNAs induced a >100-fold increase in expression levels of each specific miRNA compared to a scrambled miR-control (Figure 1). High expression levels of these three miRNAs in none of these three leukemic cell lines resulted in cell cycle arrest. As shown in Figure 2, overexpression of these miRNAs, either individually or in combination, did not result in G0/G1 arrest or reduced S/G2M phases compared to the effect of scrambled control-miR.

High expression level of miR-24, miR-126 and miR-365 did not affect apoptosis

level of *TCF3*-rearranged leukemic cells

Individual or co-transfection of miR-24, miR-126 and miR-365 did not change the amount of apoptosis in *TCF3*-rearranged 697 and KASUMI-2 leukemic cells (p>0.05, Figure 3a-b). The amount of apoptosis in KASUMI-2 leukemic cells upon enforced expression of miR-24 (26%), miR-126 (25%), miR-365 (28%) and their combination (23%) was not significantly higher than the cells after transfection of scrambled miR-control (27%). The percent of apoptotic 697 leukemic cells after transfection of miR-24 (9%), miR-126 (8%), miR-365 (8%) and their combination (14%) was comparable to that of the cells after transfection of scrambled miR-control (9%). Similarly, the amount of apoptosis in MHH-CALL-3 leukemic cells after overexpression of miR-24 (24±1%), miR-126 (25±1%), miR-365 (23±2%) and their combination (25±2%) did not significantly differ from that of the cells transduced with scrambled control-miR (28±4, p>0.05, Figure 3c).

Integration of miRNA and mRNA expression data predicts candidate target genes for miRNAs

The expression of miR-24, miR-126 and miR-365 varies across different cytogenetic subtypes of childhood BCP-ALL. As shown in Supplementary Figure S1a, low expression of miR-24 was found in *TCF3*-rearranged leukemia, while ~4-fold higher levels were found in hyperdiploid, *BCR-ABL1*-positive and *ETV6-RUNX1*-positive leukemia. Similar heterogeneity is observed in expression patterns of miR-126 and miR-365 between BCP-ALL subtypes which may reflect different functions of these miRNAs in those cytogenetic contexts (Supplementary Figure S1b-c). Integration of miRNA and mRNA expression levels of 37 newly diagnosed children with BCP-ALL revealed significant association between the expression of 29 miRNAs and their predicted target genes (FDR<0.05, p<0.005, Supplementary Table S2), including miR-24 (ranked at position #5), miR-126 (ranked at position #17) and miR-365 (ranked at position #20). Next, we looked at the individual miRNAs and their top inversely correlated target genes (Supplementary Figure S2). For miR-24, expression levels of *ELL* and *EBF3* were most strongly inversely associated with miR-24 and therefore, were chosen for further study (Pearson r -0.6, p<0.0001, Supplementary Figure S2a-b, Supplementary Table S3). In addition, *IRF4* was selected for further testing due to its important role in B-cell development and leukemogenesis (38) and significant inverse correlation between expression levels of this gene and miR-24 in our miRNA-mRNA expression data integration analysis (Pearson r -0.7, p<0.005, Supplementary Figure S2c). The distribution of expression levels of miR-24 and *IRF4* among different subtypes of BCP-ALL is shown in Supplementary Figure S1a and S3. Similarly, *PITPNC1*, which was inversely correlated with miR-126, and *ZAP-70*, which is involved in leukemogenesis (39, 40) and showed a significant inverse association with miR-365, were selected for further evaluation (Supplementary Figure S2e-f, Supplementary Tables S4 and S5). Moreover, *AURKB* a previously described validated target gene for miR-24 (2), and *KAT2A* as a gene which stabilize the oncoprotein *TCF3-PBX1* in ALL (41) and a predicted target for miR-365 (www.microRNA.org, Supplementary Figure S4) were

added to the study; however, there was no significant inverse association between these miRNAs/mRNAs in our miRNA-mRNA integration analysis (Supplementary Figure S2d and g).

Overexpression of miR-24, miR-126 and miR-365 in MHH-CALL-3 leukemic cells did not reduce the expression level of candidate target genes

The effect of high expression levels of miR-24 (>100-fold induced) on expression of candidate target genes *IRF4*, *ELL* and *EBF3* was measured in *TCF3*-rearranged MHH-CALL-3 leukemic cells. As shown in Figure 4, relative expression levels of *ELL*, *EBF3* and *IRF4* did not significantly differ between the cells overexpressing miR-24 compared to scrambled miRNA expressing cells. In addition, high expression levels of miR-24 in MHH-CALL-3 leukemic cells did not reduce mRNA expression levels of a previously reported target gene of miR-24, *AURKB* (2). The relative expression levels of *AURKB* upon enforced expression of miR-24 in MHH-CALL-3 leukemic cells did not differ from that observed in scrambled control-miR transduced cells (Figure 4, $p>0.05$). As positive control, transfection of miR-24 into hepatocellular carcinoma HEP-G2 cells reduced expression levels of *AURKB* by 4-fold, confirming the previously reported findings in this cell line (2) (Figure 5). In contrast to >100-fold increase in miR-24 expression levels in *TCF3*-rearranged leukemic cells (Figure 1), following the same protocol and using similar amount of miR-24 (60 nM) to transfect HEP-G2 cells induced >10,000-fold increase in miR-24 expression levels (Figure 5a).

Overexpression of miR-126 in MHH-CALL-3 leukemic cells did not reduce the expression levels of candidate target gene *PITPNC1* in miR-126 transduced cells in comparison to that of scrambled miR-control transfected cells (Figure 4). The expression levels of *ZAP-70* did not significantly decrease upon miR-365 overexpression compared with scrambled miR-control transduced MHH-CALL-3 cells (Figure 4). Similarly, the expression levels of *KAT2A*, which is predicted computationally as a target gene for miR-365, did not reduce after miR-365 overexpression in the cells (Figure 4). Combined overexpressing of miR-24, miR-126 and miR-365 in MHH-CALL-3 leukemic cells did not result in significant reduction of the expression levels of candidate target genes in comparison to that observed in scrambled control-miR transfected cells (Figure 4).

Discussion

Based on similar expression profiles of miR-24, miR-126 and miR-365 in *TCF3*-rearranged leukemia (8) and concomitant functions of these miRNAs in different types of tumors, we studied the possible effects of overexpression of these three miRNAs on cell cycle progression and amount of apoptosis in *TCF3*-rearranged leukemic cells. However, there are controversies in reported functions of these miRNAs in regulation of cellular viability; e.g. miR-24 which can inhibit the proliferation of K562 chronic myeloid leukemic cells (2), promotes cellular proliferation of primary keratinocytes, different solid cancer-derived cell lines (42) as well as hematopoietic cells (43). Our results, however, revealed that neither individual overexpression nor combined overexpression of these three miRNAs in 697, KASUMI-2 and MHH-CALL-3 *TCF3*-rearranged leukemic

cell lines significantly changed cell cycle distribution or apoptosis. In addition, ectopic expression of miR-24 in MHH-CALL-3 leukemic cells did not reduce the expression levels of candidate target genes for miR-24: *ELL*, *EBF3* or *IRF4*. Similarly, selected predicted candidate target genes for miR-126 and miR-365 were not affected by individual or combined overexpression of these miRNAs in MHH-CALL-3 cell line.

We showed earlier that *TCF3*-rearranged BCP-ALL is characterized by high mRNA and protein levels of *AURKB* (44). Knockdown of *AURKB* in *TCF3*-rearranged leukemic cells reduced the proliferation and increased the number of apoptotic cells (44). Therefore, *AURKB* which is earlier introduced as a target gene for miR-24 in a different tissue context (2) may be a likely candidate target gene in ALL. However, we did not observe an inverse correlation between expression levels of *AURKB* and miR-24 in our integration analysis. In addition, ectopic expression of miR-24 did not affect the expression levels of *AURKB* in the MHH-CALL-3 leukemic cell line whereas the control experiment showed that enforced expression of miR-24 significantly reduced the expression levels of *AURKB* in HEP-G2 hepatocarcinoma cells, confirming a previous study (2). We observed higher overexpression of miR-24 in HEP-G2 cells (>10,000-fold) compared with MHH-CALL-3 cells (>100-fold). However, this cannot explain the lack of any significant effect of miRNA overexpression in *TCF3*-rearranged leukemic cells because previous studies showed that only a 5 to 50-fold change in expression levels of miR-24 (2, 15, 16), miR-126 (19, 20, 45) or miR-365 (16, 24) was sufficient to regulate a target gene and induce a functional change. The ~100-fold change in expression level of miRNAs is often applied in functional studies since it is more comparable to that commonly happens in pathobiology of diseases. As shown in supplementary Figure S1, expression levels of miR-24 decreased by 4-fold in *TCF3*-rearranged ALL cases compared to healthy control $CD34^+$ cells. Similarly and compared to healthy control $CD34^+$ cells, expression levels of miR-126 decreased in 84-fold in *TCF3*-rearranged ALL cases whereas the expression levels of miR-365 did not differ from normal healthy control cells. In addition, stable overexpression of miRNAs using viral transduction often results in supraphysiological expression of miRNAs, which increase the chance of off-target effect (46) and also stimulates the in time escape mechanisms such as cellular adaptation. Moreover, successful overexpression of miRNAs as detected by mature miRNA-specific stem-loop RTq-PCR in our cell line models confirmed an effective transfection procedure and a functional miRNA-processing machinery in the current experiment.

Taken together, these data suggest that the function of miRNAs is highly tissue dependent. It is known that one specific miRNA might get involved in different – and even opposite – functions in different types of tissues. As an example, miR-221 and miR-222 are overexpressed in various types of solid tumors and promote cell proliferation while both miR-221 and miR-222 have inhibitory effects on cell proliferation in erythroblast and their expression levels are significantly downregulated in erythroblastic leukemia (47). In the current study, overexpression of miR-24 did not alter viability of *TCF3*-rearranged leukemic cells whereas cell proliferation and cell cycle progression in solid malignancies are regulated by miR-24 (12-16). Not only in completely different

types of tumors but also in malignancies with more related tissue context, miR-24 showed opposite expression pattern as this miRNA is upregulated in acute myeloid and non-*TCF3*-rearranged acute lymphoblastic leukemias but downregulated in acute promyelocytic leukemia (48). Interestingly, the expression patterns of miR-24, miR-126 and miR-365 vary between cytogenetic subtypes of BCP-ALL, possibly reflecting different functions of these miRNAs in those different cellular contexts.

We observed strong inverse correlation between some miRNAs and their candidate target genes in 37 BCP-ALL cases (e.g. miR24 with *ELL*, *EBF3* and *IRF4*, miR-126 with *PITPNC1* and miR-365 with *ZAP-70*); however, our functional study demonstrated no significant interaction between tested candidate target genes and miR-24, miR-126 and/or miR-365 in MHH-CALL-3 leukemic cells, a *TCF3*-rearranged cell line model. We should stress that there were only three cases of *TCF3*-rearranged ALL among the 37 cases of BCP-ALL included in our miRNA-mRNA data integration cohort. This suggests that the observed significant inverse correlation between some miRNA-mRNA pairs might mainly originate from other subtypes with higher number of cases in this cohort, such as hyperdiploid (13 cases) or unclassified B-others BCP-ALL (10 cases).

In conclusion, individual and combined overexpression of miR-24, miR-126 and/or miR-365 cannot alter the amount of apoptosis and cell cycle distribution in *TCF3*-rearranged leukemia. A series of extra changes in expression levels of other miRNAs and/or RNA-binding proteins might be needed in parallel to alter cell proliferation and viability of these leukemic cells. Our findings illustrate the complex and tissue-specific function of miRNAs and warns against generalizing data about miRNA-target gene interactions to all tissue types.

Acknowledgements

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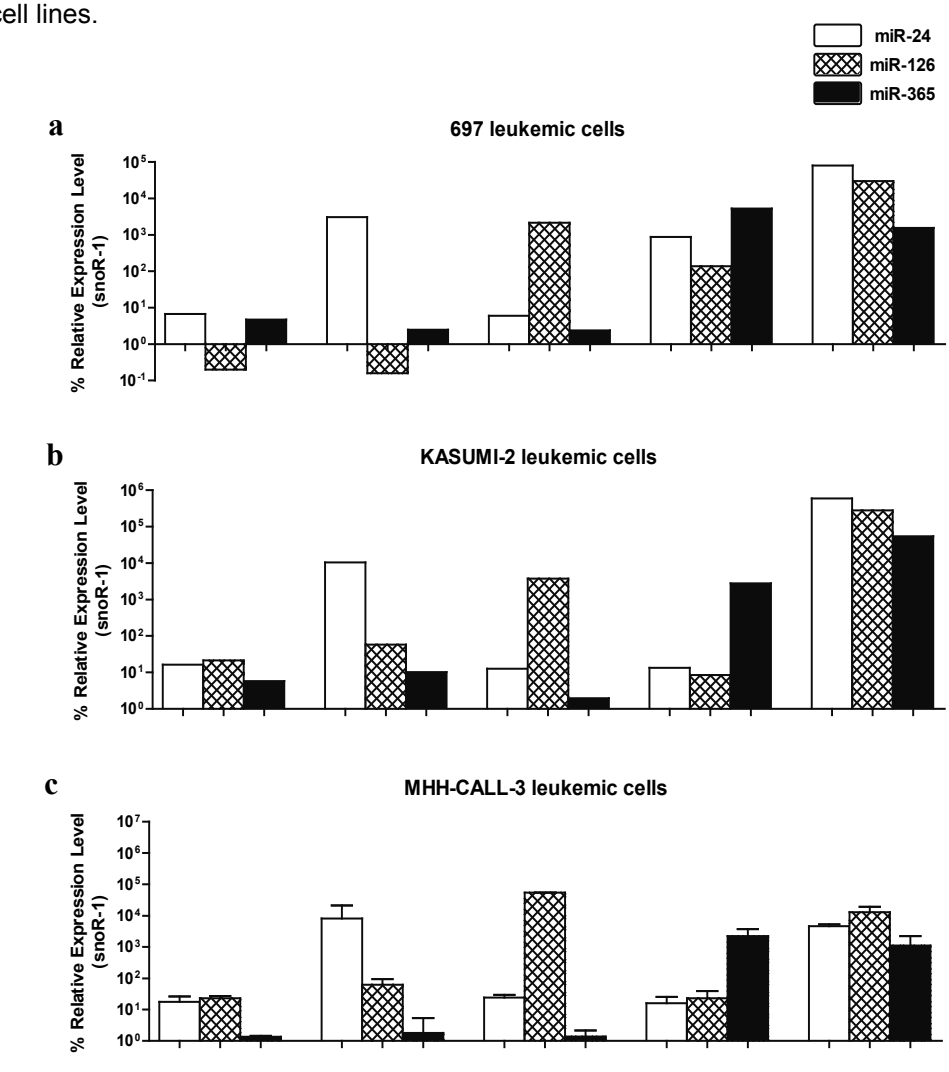
Conflict of interest

The authors declare no conflict of interest.

Author contributions

FAM performed research, analyzed data and wrote the manuscript; JMB preformed the miRNA-mRNA expression data integration, analyzed the data and revised the manuscript; EAML-T performed research, analyzed data and revised the manuscript; RP and MLdB supervised research, analyzed data, wrote and revised the manuscript. All authors approved the final version of the manuscript.

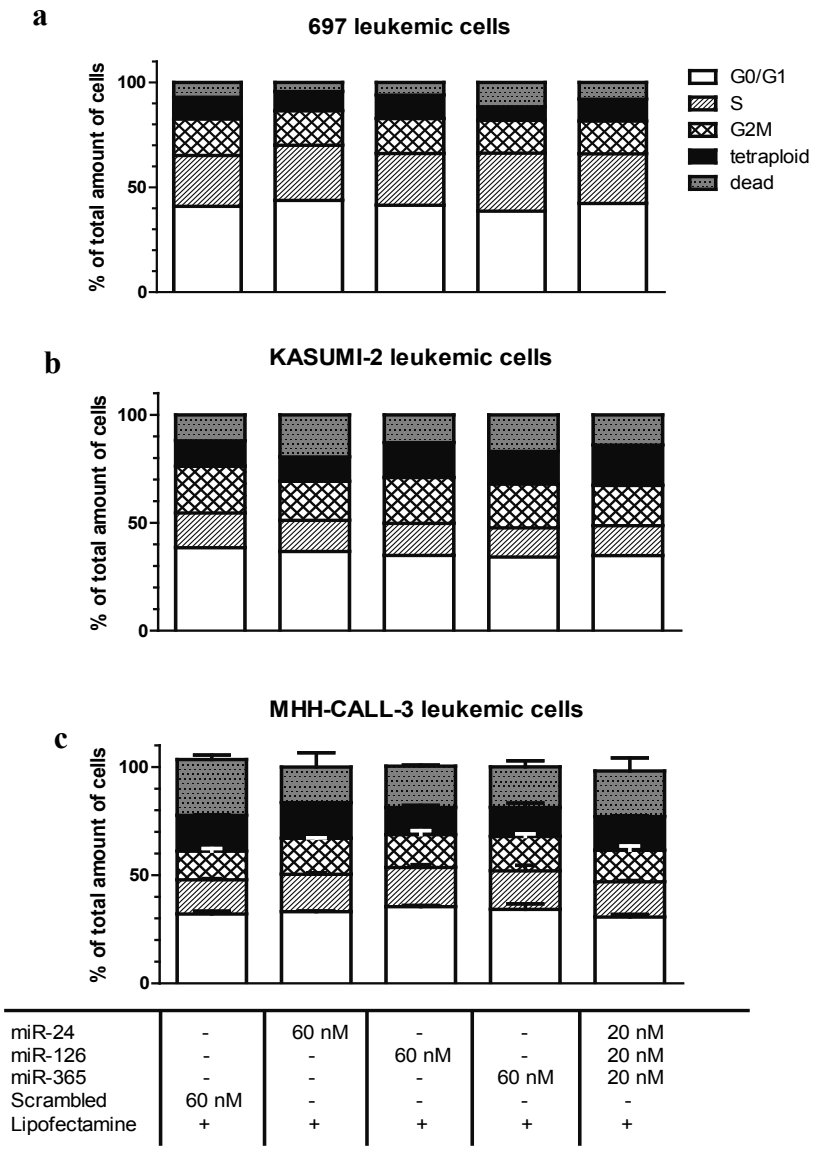
Figure 1: Successful overexpression of miRNAs in *TCF3*-rearranged leukemic cell lines.



miR-24	-	60 nM	-	-	20 nM
miR-126	-	-	60 nM	-	20 nM
miR-365	-	-	-	60 nM	20 nM
Scrambled	60 nM	-	-	-	-
Lipofectamine	+	+	+	+	+

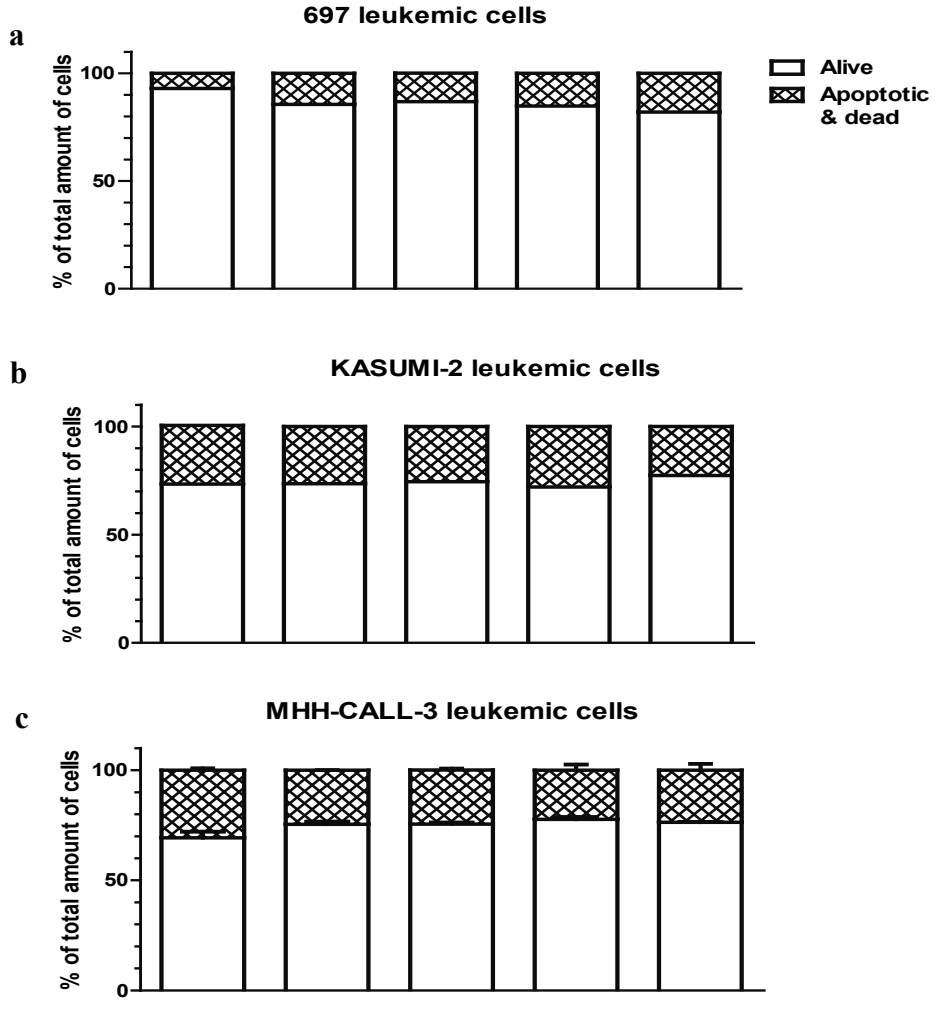
Expression levels of miRNAs (% relative to snoR-1) in *TCF3*-rearranged 697 (a), KASUMI-2 (b) and MHH-CALL-3 (c) leukemic cells after overexpression of miR-24, miR-126 and/or miR-365 compared to scrambled miR-control transduced cells. Open bar indicates expression levels of miR-24, crossed-line bar indicates expression levels of miR-126 and filled bar indicates expression levels of miR-365.

Figure 2: Cell cycle distribution in *TCF3*-rearranged leukemic cell lines upon miRNAs overexpression.



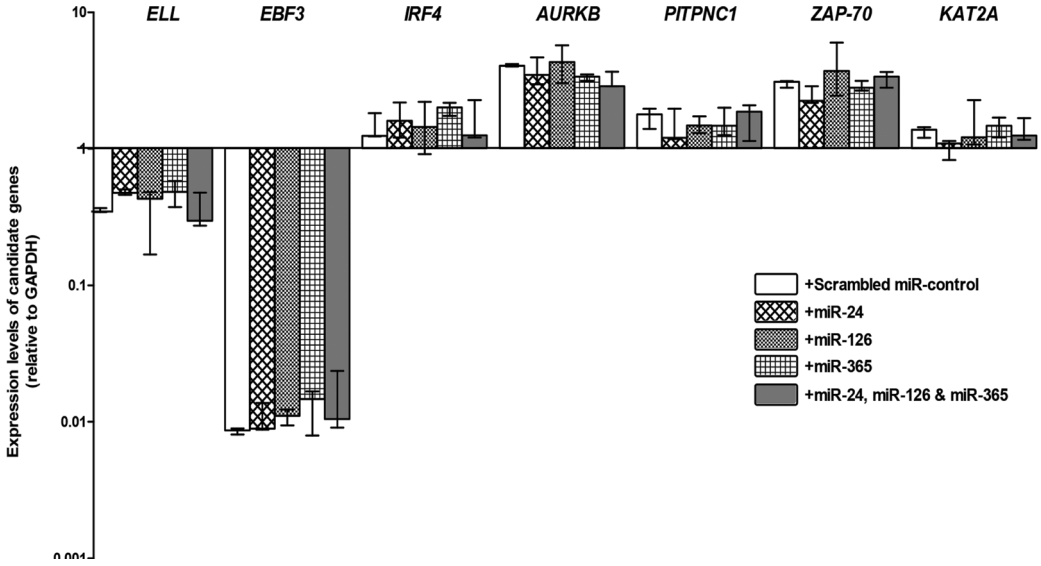
Individual or combined overexpression of miR-24, miR-126 or miR-365 in *TCF3*-rearranged 697 (a), KASUMI-2 (b) and MHH-CALL-3 (c) leukemic cell lines did not inhibit cell cycle progression compared to scrambled miR-control transduced cells (Mann-Whitney U test, $p>0.05$). The distribution of cell cycle was measured using hypotonic-PI method and the amount of the cells in G0/G1, S and G2M phase as well as dead and tetraploid population was measured using a MACSQuant device and shown as % of total amount of the cells.

Figure 3: Amount of apoptosis in *TCF3*-rearranged leukemic cell lines following miRNAs overexpression.



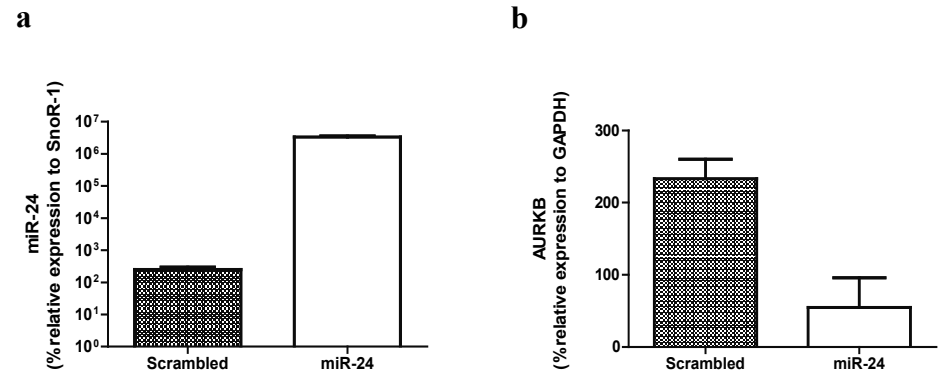
The number of apoptotic cells in *TCF3*-rearranged 697 (a), KASUMI-2 (b) and MHH-CALL-3 (c) leukemic cells did not significantly change upon individual or combined overexpression of miR-24, miR-126 or miR-365 compared to scrambled miR-control transduced cells (Mann-Whitney U test, $p>0.05$). Leukemic cells were stained using AnnexinV-PI method and the amount of stained-cells was measured using a MACSQuant device. AnnexinV negative-PI negative population was considered as alive cells and sum of AnnexinV positive-PI negative (early apoptotic cells), AnnexinV positive-PI positive (late apoptotic cells) and AnnexinV negative-PI positive (necrotic cells) populations was considered as apoptotic/dead fraction.

Figure 4: Modulation of expression levels of candidate target genes following miRNAs overexpression in *TCF3*-rearranged MHH-CALL-3 cells.



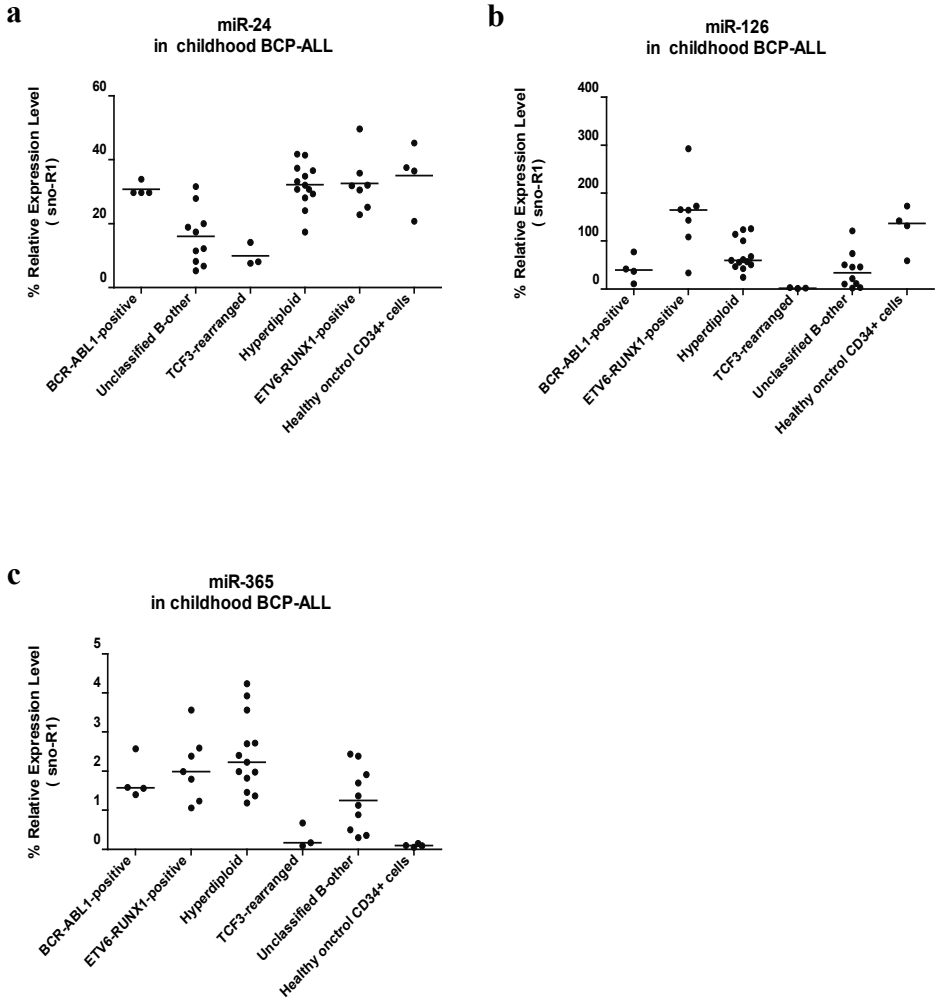
Following individual and/or combined overexpression of miR-24, miR-126 and miR-365 in *TCF3*-rearranged MHH-CALL-3 leukemic cells, the expression level of the candidate target genes for miR-24 (*ELL*, *EBF3*, *IRF4* and *AURKB*), for miR-126 (*PITPNC1*) and for miR-365 (*ZAP-70* and *KAT2A*) did not decrease significantly compared to scrambled miR-control transduced cells (three independent experiments, Mann-Whitney U test, P>0.05). The expression level of each gene was measured by RT-qPCR using specific primers and SYBR Green method. The median and ranges of expression level of each gene was shown relative to expression levels of the reference gene, *GAPDH*.

igure 5: MiR-24 overexpression reduces *AURKB* expression levels in hepatocarcinoma HEP-G2 cells.



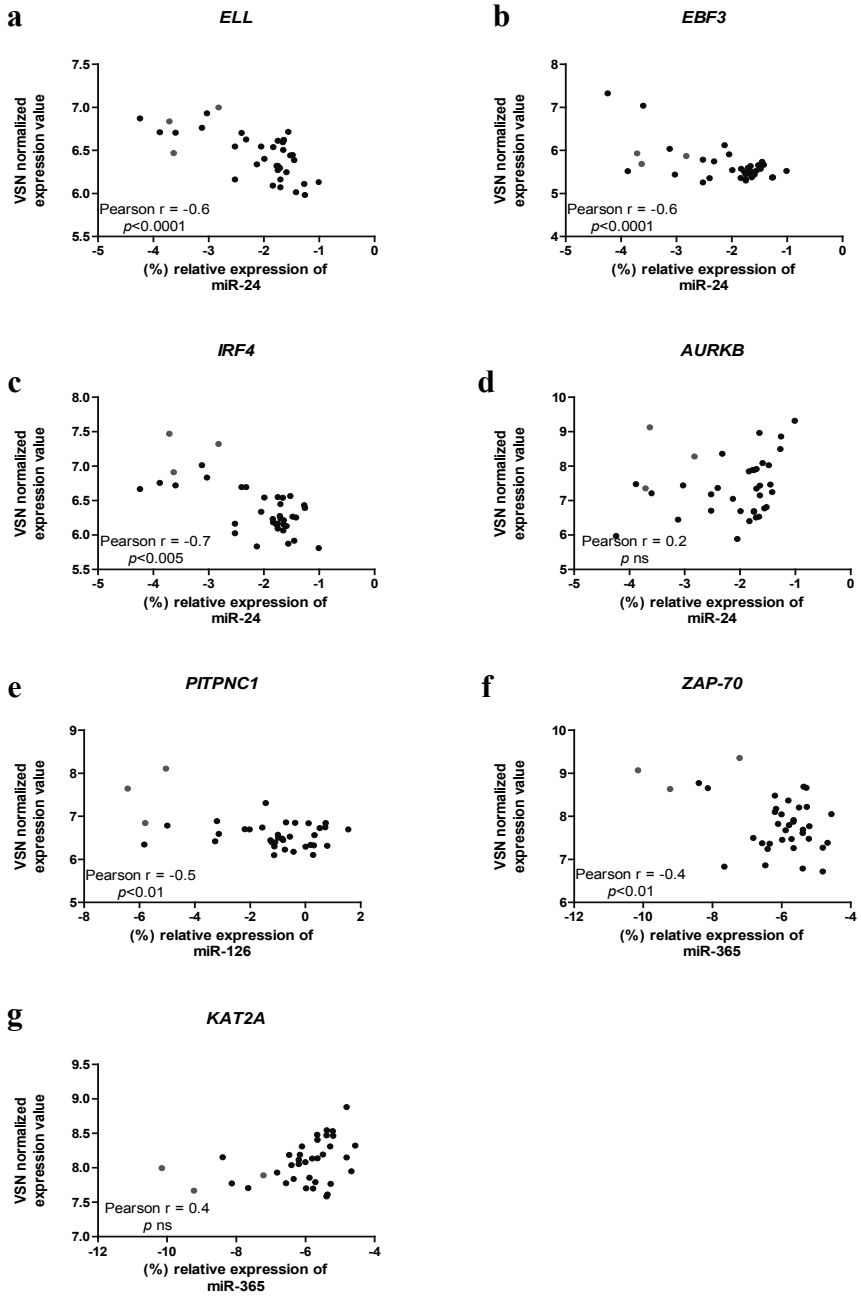
Two independent experiments of miR-24 transfection (60 nM) using Lipofectamine protocol resulted in >10,000-fold increase in expression levels of mature miR-24 in hepatocellular carcinoma HEP-G2 cells (a). Following overexpression of miR-24 in HEP-G2 cells, expression levels of *AURKB* decreased by 4-fold (b).

Supplementary Figure S1: Expression levels of miR-24, miR-126 and miR-365 in childhood BCP-ALL.



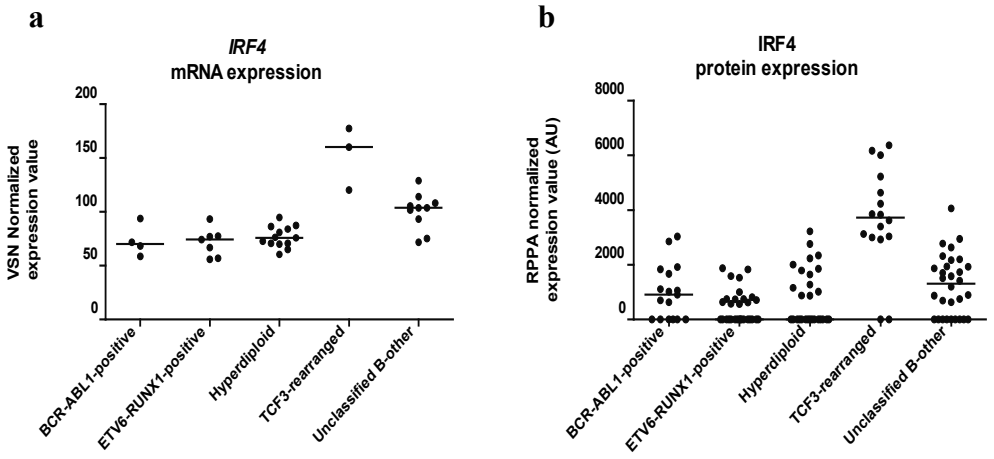
Heterogeneity in expression levels of miR-24 (a), miR-126 (b) and miR-365 (c) among different cytogenetic subtypes of childhood BCP-ALL. Expression of miR-24 is significantly lower in children with *TCF3*-rearranged leukemia compared to other BCP-ALL cases (Mann-Whitney U test, $p<0.02$). Compared to the leukemic cells of children with non-*TCF3*-rearranged leukemia, expression levels of miR-126 (by ~37-fold) and miR-365 (by ~6-fold) were lower in leukemic cells of children with *TCF3*-rearranged leukemia.

Supplementary Figure S2: Correlation between miRNA and mRNA expression levels of 37 children with BCP-ALL.



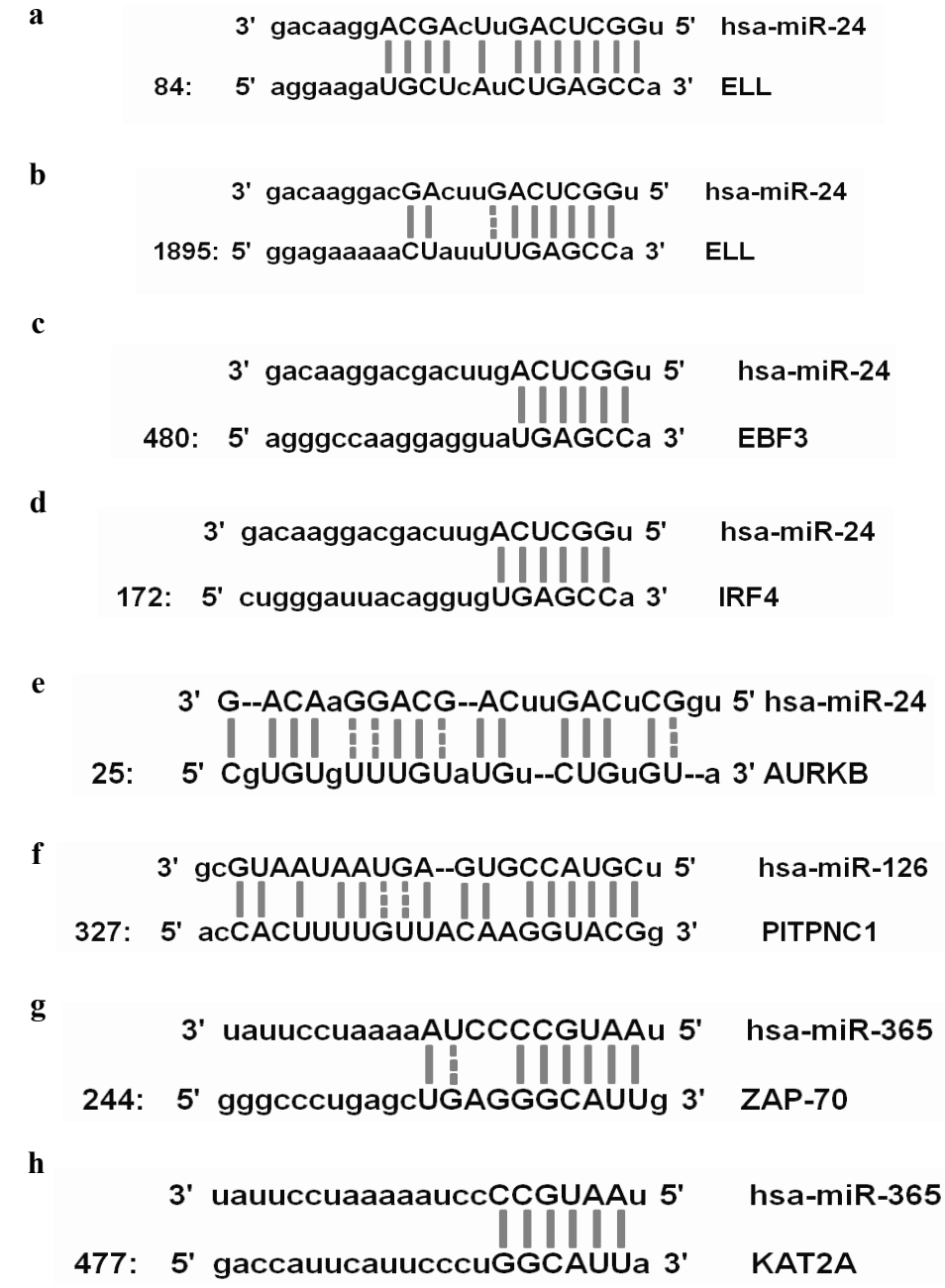
Using a cohort of 37 children with BCP-ALL (including three cases of *TCF3*-rearranged leukemia, shown in red dots) correlation between mRNA-miRNA expression levels was plotted. While *ELL*, *EBF3* and *IRF4* expression levels were inversely correlated with miR-24 expression (a, b and c, p<0.0001), *AURKB* showed no correlation with this miRNA (d, p>0.05, Pearson correlation coefficient test). Significant inverse correlation between expression levels of miR-126 and *PITPNC1* is shown in graph-e (p<0.01). Graph-f showed the significant inverse correlation between miR-365 and *ZAP-70* (p<0.01) while expression of this miRNA has no significant correlation with *KAT2A* expression (g, p>0.01).

Supplementary Figure S3: Expression levels of *IRF4*, candidate target gene of miR-24, in childhood BCP-ALL.



ontrast to expression levels of miR-24 in children with *TCF3*-rearranged leukemia (Supplementary Figure S1), expression levels of *IRF4* is significantly higher in these patients (a, Mann-Whitney U test, $p<0.006$). Among the cytogenetic subtypes of childhood BCP-ALL, protein expression levels of IRF4 is predominantly higher in children with *TCF3*-rearranged leukemia (b, Mann-Whitney U test, $p<0.0001$). The expression levels of *IRF4* (% relative to *GAPDH*) were measured using RT-qPCR in 37 cases with BCP-ALL (containing 3 *TCF3*-rearranged leukemia cases) and protein expression levels of IRF4 were measured using reverse phase protein array (RPPA) on a different cohort of 121 cases with BCP-ALL (containing 16 *TCF3*-rearranged leukemia cases). The protein expression values are shown after microarray data normalization and in an arbitrary unit.

Supplementary Figure S4: Possibility of miRNA-mRNA binding.



Predicted binding sites for miR-24 in the 3' UTR of *ELL* (a, b), *EBF3* (c), *IRF4* (d) and *AURKB* (e), possible binding site for miR-126 in the 3' UTR of *PITPNC1* (f) and binding sites for miR-365 in the 3' UTR of *ZAP*-70 (g) and *KAT2A* (h). The computational prediction to identify possible interaction between miRNAs and candidate target genes was done using miRanda algorithm (release August 2010, available at www.microrna.org), except for miR-24 and *AURKB* possible interaction which is visualized according to the suggested model of "Lal, *et al*" (e). The numbers at the 5' side of genes showed the position of starting base-pairs of the binding sites in the 3' UTR of the genes. Connected lines show the complementary base-pairs and dotted lines indicate G:U wobbles.

Supplementary Table S1: Sequences of the primers used for RT-qPCR measurement of expression levels of candidate target genes.

#	Gene name	Strand	Sequence
1	***ELL***	Forward	5' GGACGTTCTCCTTCTACCTC 3'
2		Reverse	5' ACCGTGATCTTGTCCTGTAT 3'
3	***EBF3***	Forward	5' CAGCAGTCCAATTACAACAC 3'
4		Reverse	5' GCAGGTGAGAATGAGAAAAT 3'
5	***IRF4***	Forward	5' CTTGGCGTTCTCAGACTG 3'
6		Reverse	5' TTGCAGGTCTGGTCTCTC 3'
7	***AURKB***	Forward	5' TCTGCTCTTAGGGCTCAAGG 3'
8		Reverse	5' TGCCACACATTGTCTTCCTC 3'
9	***PITPNC1***	Forward	5' GCCATGAACAGAGTGACC 3'
10		Reverse	5' ATTTCGGCAGAAAGGAAC 3'
11	***ZAP-70***	Forward	5'ATCCCTGTGAGCAATGTG 3'
12		Reverse	5' GACCCCATAGCTCCAGAC 3'
13	***KAT2A***	Forward	5' ACGCTGATGGAGTGTGAG 3'
14		Reverse	5' GCGGATGACCTCGTAGTA 3'
15	***GAPDH***	Forward	5' CTCCTGCGACTTCAACAG 3'
16		Reverse	5' TCTGGGATGGAAATTGTG 3'

Supplementary Table S2: The list of miRNAs for which the expression levels were significantly associated with the expression levels of their predicted/validated target genes in 37 children with BCP-ALL.

#	Name of miRNA	Global test P value	Number of candidate target genes	False discovery rate
1	**miR-222**	0.000	1033	0.00
2	**miR-511**	0.000	1103	0.00
3	**miR-660**	0.000	741	0.00
4	**miR-223**	0.000	917	0.00
5	**miR-24**	0.000	1225	0.00
6	**miR-505**	0.000	832	0.00
7	**miR-98**	0.000	1536	0.00
8	**miR-221**	0.000	1097	0.01
9	**miR-324-3p**	0.000	1009	0.01
10	**miR-545**	0.001	1447	0.01
11	**miR-30d**	0.001	1879	0.01
12	**miR-23a**	0.001	1713	0.01
13	**miR-30b**	0.001	1899	0.01
14	**miR-372**	0.001	1352	0.01
15	**miR-629**	0.001	867	0.01
16	**miR-103**	0.001	905	0.02
17	**miR-126**	0.001	616	0.02
18	**miR-101**	0.002	1427	0.03
19	**miR-130a**	0.002	1509	0.03
20	**miR-365**	0.002	948	0.03
21	**miR-26a**	0.002	1398	0.03
22	**miR-500**	0.002	687	0.03
23	**miR-195**	0.003	1777	0.03
24	**miR-26b**	0.003	1379	0.04
25	**miR-497**	0.003	1843	0.04
26	**miR-218**	0.004	1434	0.04
27	**miR-106b**	0.004	1857	0.04
28	**miR-324-5p**	0.004	847	0.04
29	**miR-27a**	0.004	1784	0.04

Supplementary Table S3: The top-20 genes for which expression levels were inversely correlated with miR-24 expression in 37 children with BCP-ALL and the identification of these genes by publicly available target prediction algorithms and/or databases of validated miRNA-mRNA interactions.

#	Gene symbol	miRNA-mRNA association P value	Microcosm	pita	TargetScan	miRecords	miRtarbase
1	*ELL*	0.000	FALSE	FALSE	TRUE	FALSE	FALSE
2	*EBF3*	0.000	FALSE	FALSE	TRUE	FALSE	FALSE
3	*MAD2L2*	0.000	TRUE	FALSE	FALSE	FALSE	FALSE
4	*TRIM62*	0.000	TRUE	FALSE	FALSE	FALSE	FALSE
5	*BCL2L11*	0.000	FALSE	FALSE	TRUE	FALSE	FALSE
6	*PCDH17*	0.000	FALSE	FALSE	TRUE	FALSE	FALSE
7	*ID4*	0.000	FALSE	TRUE	FALSE	FALSE	FALSE
8	*SNN*	0.000	FALSE	TRUE	TRUE	FALSE	FALSE
9	*CPNE7*	0.001	TRUE	FALSE	FALSE	FALSE	FALSE
10	*SPON2*	0.001	TRUE	FALSE	FALSE	FALSE	FALSE
11	*TFAP2C*	0.001	TRUE	FALSE	FALSE	FALSE	FALSE
12	*SERINC2*	0.001	TRUE	FALSE	FALSE	FALSE	FALSE
13	*NYNRIN*	0.001	TRUE	FALSE	FALSE	FALSE	FALSE
14	*ATAD3B*	0.001	TRUE	FALSE	FALSE	FALSE	FALSE
15	*MAPKBP1*	0.001	FALSE	FALSE	TRUE	FALSE	FALSE
16	*CORO7*	0.001	TRUE	FALSE	FALSE	FALSE	FALSE
17	*GAB2*	0.001	FALSE	FALSE	TRUE	FALSE	FALSE
18	*AFF2*	0.002	FALSE	TRUE	FALSE	FALSE	FALSE
19	*XYLT1*	0.002	FALSE	FALSE	TRUE	FALSE	FALSE
20	*MEGF11*	0.002	TRUE	FALSE	FALSE	FALSE	FALSE

The possibility of miRNA-mRNA binding was calculated using three commonly used tools for target prediction: TargetScan version 6.1 (www.targetscan.org), microCosm version 5 (formerly miRBase Targets, http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl), and PITA version from 2007 (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html). In addition, two databases containing validated targets were selected: miRecords version November 25, 2010 (http://mirecords.biolead.org/), and miRTarBase release 3.5 (http://mirtarbase.mbc.nctu.edu.tw/). TRUE indicates the possibility of miRNA binding to the candidate target gene and FALSE means there was no predicted binding site for the miRNA in 3' UTR of candidate target gene.

Supplementary Table S4: The top-20 genes for which expression levels were inversely correlated with miR-126 expression in 37 children with BCP-ALL and the identification of these genes by publicly available target prediction algorithms and/or databases of validated miRNA-mRNA interactions.

#	Gene symbol	miRNA-mRNA association P value	Microcosm	pita	TargetScan	miRecords	miRtarbase
1	*ANKS1B*	0.000	TRUE	FALSE	FALSE	FALSE	FALSE
2	*AEBP1*	0.001	TRUE	FALSE	FALSE	FALSE	FALSE
3	*SEMA6D*	0.001	TRUE	FALSE	FALSE	FALSE	FALSE
4	*FAM3B*	0.002	TRUE	FALSE	FALSE	FALSE	FALSE
5	*TWF2*	0.002	FALSE	FALSE	FALSE	FALSE	TRUE
6	*NYNRIN*	0.002	TRUE	FALSE	FALSE	FALSE	FALSE
7	*STAMBPL1*	0.003	TRUE	FALSE	FALSE	FALSE	FALSE
8	*HEATR1*	0.004	TRUE	FALSE	FALSE	FALSE	FALSE
9	*PITPNC1*	0.004	TRUE	FALSE	FALSE	FALSE	FALSE
10	*LARP6*	0.006	TRUE	FALSE	FALSE	FALSE	FALSE
11	*TESC*	0.011	TRUE	FALSE	FALSE	FALSE	FALSE
12	*ATAD3A*	0.012	TRUE	FALSE	FALSE	FALSE	FALSE
13	*ME3*	0.014	TRUE	FALSE	FALSE	FALSE	FALSE
14	*C20orf26*	0.016	TRUE	FALSE	FALSE	FALSE	FALSE
15	*TNFRSF11B*	0.017	TRUE	FALSE	FALSE	FALSE	FALSE
16	*PRKCD*	0.022	TRUE	FALSE	FALSE	FALSE	FALSE
17	*FGFR2*	0.023	TRUE	FALSE	FALSE	FALSE	FALSE
18	*CHD1L*	0.023	TRUE	FALSE	FALSE	FALSE	FALSE
19	*MATN4*	0.024	TRUE	FALSE	FALSE	FALSE	FALSE
20	*HERPUD1*	0.025	TRUE	FALSE	FALSE	FALSE	FALSE

The possibility of miRNA-mRNA binding was calculated using three commonly used tools for target prediction: TargetScan version 6.1 (www.targetscan.org), microCosm version 5 (formerly miRBase Targets, http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl), and PITA version from 2007 (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html). In addition, two databases containing validated targets were selected: miRecords version November 25, 2010 (http://mirecords.biolead.org/), and miRTarBase release 3.5 (http://mirtarbase.mbc.nctu.edu.tw/). TRUE indicates the possibility of miRNA binding to the candidate target gene and FALSE means there was no predicted binding site for the miRNA in 3' UTR of candidate target gene.

Supplementary Table S5: The top-20 genes for which expression levels were inversely correlated with miR-365 expression in 37 children with BCP-ALL and the identification of these genes by publicly available target prediction algorithms and/or of validated miRNA-mRNA interactions.

#	Gene symbol	miRNA-mRNA association P value	Microcosm	pita	TargetScan	miRecords	miRtarbase
1	*MYBL2*	0.000	TRUE	FALSE	FALSE	FALSE	FALSE
2	*CSMD1*	0.000	FALSE	FALSE	TRUE	FALSE	FALSE
3	*UGT8*	0.000	TRUE	FALSE	FALSE	FALSE	FALSE
4	*FGF9*	0.000	TRUE	FALSE	FALSE	FALSE	FALSE
5	*PITPNM2*	0.001	FALSE	FALSE	TRUE	FALSE	FALSE
6	*HDAC4*	0.001	FALSE	FALSE	TRUE	FALSE	FALSE
7	*SPON2*	0.002	TRUE	FALSE	FALSE	FALSE	FALSE
8	*PLEKHB2*	0.002	FALSE	FALSE	TRUE	FALSE	FALSE
9	*CHD4*	0.003	TRUE	FALSE	FALSE	FALSE	FALSE
10	*SLC15A1*	0.003	TRUE	FALSE	FALSE	FALSE	FALSE
11	*AFAP1L1*	0.004	TRUE	FALSE	TRUE	FALSE	FALSE
12	*MCOLN2*	0.005	TRUE	FALSE	FALSE	FALSE	FALSE
13	*DNAH12*	0.006	TRUE	FALSE	FALSE	FALSE	FALSE
14	*ZAP-70*	0.006	TRUE	FALSE	FALSE	FALSE	FALSE
15	*SLC6A15*	0.008	TRUE	FALSE	FALSE	FALSE	FALSE
16	*FAM60A*	0.008	TRUE	FALSE	TRUE	FALSE	FALSE
17	*EPAS1*	0.011	FALSE	TRUE	TRUE	FALSE	FALSE
18	*KCNA7*	0.012	FALSE	FALSE	TRUE	FALSE	FALSE
19	*SEMA6D*	0.014	FALSE	TRUE	FALSE	FALSE	FALSE
20	*SSTR3*	0.014	TRUE	FALSE	FALSE	FALSE	FALSE

The possibility of miRNA-mRNA binding was calculated using three commonly used tools for target prediction: TargetScan version 6.1 (www.targetscan.org), microCosm version 5 (formerly miRBase Targets, http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl), and PITA version from 2007 (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html). In addition, two databases containing validated targets were selected: miRecords version November 25, 2010 (http://mirecords.biolead.org/), and miRTarBase release 3.5 (http://mirtarbase.mbc.nctu.edu.tw/). TRUE indicates the possibility of miRNA binding to the candidate target gene and FALSE means there was no predicted binding site for the miRNA in 3' UTR of candidate target gene.

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Chapter 6

MiR-125b, miR-100 and miR-99a co-regulate vincristine resistance in childhood acute lymphoblastic leukemia

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Abstract

MicroRNA-125b (miR-125b), miR-99a and miR-100 are overexpressed in vincristine-resistant acute lymphoblastic leukemia (ALL). Cellular viability of *ETV6-RUNX1*-positive Reh cells significantly increased in presence of 9 ng/mL vincristine upon co-expression of miR-125b/miR-99a (91±4%), miR-125b/miR-100 (93±5%) or miR-125b/miR-99a/miR-100 (82±17%) compared with miR-125b-transduced cells (38±13%, P<0.05). Co-expression of these miRNAs resulted in downregulation of *DNTT*, *NUCKS1*, *MALAT1*, *SNRPE*, *PNO1*, *SET*, *KIF5B*, *PRPS2*, *RPS11*, *RPL38* and *RPL23A* (fold-change 1.3-1.9, p<0.05). Similarly, 7 out of these genes are lower expressed in vincristine-resistant ALL cells of children (p<0.05). The concerted function of miR-125b in combination with miR-99a and/or miR-100 illustrates the complexity of vincristine-resistant pediatric ALL.

Keywords

Leukemia, miRNA, vincristine resistance, miR-125b, miR-99a, miR-100

Introduction

MicroRNAs (miRNAs) are a group of small ~22 nucleotides-long non-coding RNAs which post-transcriptionally control the expression level of almost 60% of protein-coding genes (1). They suppress protein translation by binding perfectly or imperfectly to complementary sequences, which are mainly located in 3'-untranslated regions (UTRs) of target messenger RNA (mRNA) (2-3). MiRNAs regulate the expression level of genes involved in many cellular processes like cell cycle (4-5), differentiation (6-7) and apoptosis (8-9). Altered expression patterns of miRNAs are involved in leukemogenesis and clinical outcome of patients with leukemia (10-12). MiRNAs are also correlated with drug resistant leukemia (13-16). For example, overexpression of miR-125b induced proliferation (17-18) and decreased apoptosis upon downregulating proapoptotic proteins i.e. *Bmf* and *KLF13* (19).

MiR-125b is actively involved in normal and malignant hematopoiesis (20). In hematopoietic stem cells, it is highly expressed and increases self-renewal and survival (21). However, excessive expression of miR-125b resulted in B cell malignancies in transgenic mice (22). In non-hematological cells, miR-125b regulates the p53 network of cell survival after DNA damage (23). A known direct target gene of miR-125b, *BAK1*, modulates resistance to chemotherapeutic agents such as cisplatin resistance in ovarian cancer (24) and taxol resistance in breast cancer (25). In addition, high expression of miR-125b was correlated with drug resistance in leukemia (10, 26-27).

Previously, we reported a genetic subtype-specific miRNA signature in pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (10, 28). MiR-125b was expressed significantly higher in cases resistant to vincristine (VCR) or daunorubicin (DNR) and also in *ETV6-RUNX1*-positive ALL patients (supplementary Figure S-1) (10). Another study showed that silencing of miR-125b sensitizes the *ETV6-RUNX1*-positive leukemic cell line Reh to doxorubicin and staurosporine (26).

MiR-125b originates from two different loci of the human genome: miR-125b-1 at chromosome 11q24.1 and miR-125b-2 at chromosome 21q21.1 (Figure 1-a). Both loci encode identical mature forms of miR-125b. MiR-125b-1 is located downstream of miR-100 encoding gene and miR-125b-2 is located downstream of miR-99a encoding gene (Figure 1-a). The difference in nucleotide sequence of mature miR-99a and miR-100 is only one nucleotide (Figure 1-b). Interestingly, we found that both miR-99a and miR-100 are higher expressed in children with VCR and DNR resistant BCP-ALL, similar to miR-125b (supplementary Figure S-1) (10). Recent reports have shown that a combination of miRNAs can target one specific mRNA suggesting that the combination of miRNAs determines the fate of target gene(s) (29-30). Since miR-100, miR-99a and miR-125b are co-expressed and all have been linked to VCR resistance in ALL, we functionally studied whether these miRNAs individually or in combination regulate the resistance to VCR in childhood BCP-ALL.

Materials and Methods

More detailed description of the Materials and Methods and references are included as Supplementary information (Supplementary file 1).

Patient samples and Cell line model

Mononuclear cells were isolated from bone marrow and peripheral blood samples collected from children with newly diagnosed ALL. Immunophenotype was determined by flow cytometry and the cytogenetic abnormalities were determined by interphase fluorescence in-situ hybridization (FISH) and/or reverse transcriptase polymerase chain reaction (RT-PCR) techniques. The institutional review board approved the use of excess of diagnostic materials and written informed consent was obtained from patients, parents or legal guardians.

The *ETV6-RUNX1*-positive cell line Reh was chosen as the model to investigate the functional role of miR-125b, miR-99a and miR-100 in VCR resistant ALL. The human embryonic kidney (HEK293T) cells were used to produce lentiviral particles.

Lentiviral transduction and transient overexpression of miRNAs in leukemic cells

The plasmid pDual-miR-eGFP (Figure 1-c) is an in-house modified version of the double-promoter vector pHR SIN CS-F-W pUb-Em (31) to overexpress miR-125b or scrambled miR-control sequence. Lentiviral particles were produced in HEK293T cells and used to spin-infect the target Reh cells. Stably expressing miR-125b and scrambled miR-control GFP-positive cell lines were generated by culturing cells for at least two weeks. Parental Reh cells as well as stably miR-125b or scrambled miR-control expressing Reh cells were used for overexpression of additional miRNAs (individually or in combination) using pre-miR™ oligos and Lipofectamine reagent.

RNA extraction and quantification of miRNA expression levels

Total RNA was isolated using TRIzol isolation reagents. The expression levels of each miRNA and scrambled control miRNA were determined by real-time quantitative PCR (RT-qPCR). Endogenous small nucleolar RNA 1 (snoR-1) was used as a reference for input of RNA.

Cell cycle analysis and apoptosis assay

The distribution of the G0/G1, S and G2M phases of the cell cycle was determined by flow cytometric analysis of propidium iodide-stained nuclei. The amount of apoptosis was measured using the Annexin-V/propidium iodide staining method.

Drug resistance assay

The effect of miRNAs manipulation on cytotoxicity of vincristine was measured by incubating leukemic cell lines with 9 ng/mL VCR for 3 days followed by a methyl thiazol tetrazoliumbromide (MTT) assay. VCR resistance in primary patients' leukemic cells was determined after 4 days of exposure to a dilution series of VCR followed by an MTT assay. The concentration lethal to 50% of leukemic cells (LC50) was used for further analysis.

Gene expression profiling

The gene expression profile of Reh cells was determined using the Affymetrix Human Genome U133 Plus 2.0 Arrays according to the manufacturer's guidelines.

Statistical analyses

The non-parametric Mann-Whitney U test was used to compare viability of Reh cells with and without overexpression of miR-125b, miR-99a and/or miR-100. Spearman's rank correlation test was used to correlate the levels of expression of miR-125b, miR-99a and/or miR-100 and to study the connection between the expression of miR-125b and *in vitro* resistance to VCR in BCP-ALL samples. After data preprocessing using vsnrma, the Limma package was used within R environment (R version 2.14.1) to analyze the data raised from microarray experiments and identify differentially expressed genes after overexpression of miRNAs. The following R Biocoductor packages were used for this analysis: affy version 1.32.1, vsn version 3.22.0 and Biobase version 2.14.0).

Results

Individual overexpression of miR-125b, miR-100 or miR-99a does not induce VCR resistance

MiR-125b, miR-99a and miR-100 expression levels were highly correlated in leukemic cells of children with ALL in general and specifically in *ETV6-RUNX1*-positive primary ALL samples (supplementary Figure S1, Rs>0.9, p<0.0001). The median expression levels of these miRNAs in leukemic cells of children with ALL who were in vitro resistant to VCR were higher than those of cases sensitive to VCR: 25-fold for miR-125b, 21-fold for miR-99a and 14-fold for miR-100 (10). After 2 weeks of lentiviral-mediated transduction of Reh cells, miR-125b expression raised by >50-fold (Figure 2a, right panel). This high expression of miR-125b did not affect the in vitro response of Reh cells to VCR compared with that observed upon scrambled miR-control overexpression (Figure 2b, right panel). Lipofectamine-mediated transduction of Reh cells with pre-miR™ miR-125b precursors raised cellular miR-125b levels by >100-fold within 5 days of transfection (Figure 2a, left panel). Transient overexpression of miR-125b did not induce resistance to VCR compared to that observed in cells treated with the scrambled control miRNA (Figure 2b, left panel). This confirms the results obtained in stably transduced cells. However, compared with lentiviral-mediated infection, the procedure of transfection made Reh cells more resistant to VCR (Figure 2b, supplementary Figure S2; LC50 6 ng/mL versus 25 ng/mL, p<0.0001). Enforced expression of miR-125b did not change the cell cycle distribution or apoptosis rate compared to that observed in Reh cells expressing the scrambled control miRNA, in the absence of VCR (supplementary Figure S3).

Introduction of miR-125b, miR-99a and miR-100 precursors in Reh cells stably expressing the scrambled control miRNA resulted in a >100-fold increase of processed, mature levels of these miRNAs (Figure 3a, left bar graph). The high expression level of each miRNA had no effect or only a limited effect on the sensitivity to VCR (Figure 3b, left bar graph). The viability of Reh cells after 3 days of exposure to VCR was 20±5% for

miR-125b, 29±17% for miR-99a and 29±17% for miR-100, which was not significantly different from the 10±4% of viable cells seen in scrambled control-miR expressing cells ($p>0.05$). In addition, none of the miRNAs affected the cell cycle distribution or apoptosis rate in the absence of VCR (supplementary Figure S3).

Combination of miR-125b with miR-99a and/or miR-100 induces resistance to VCR

Similar to individual overexpression of miR-125b, miR-99a or miR-100, the combined overexpression of these three miRNAs did not change the cell cycle distribution and the apoptosis rate of Reh cells in the absence of VCR (supplementary Figure S3). Co-expression of miR-99a and miR-100 induced a slight resistance to VCR compared to cells expressing only the scrambled control-miR: the cell viability of miR-99a combined with miR-100 after exposure to VCR was 30±7% compared with 10±4% for cells expressing only the scrambled control-miR ($p<0.05$, left bar graph). Most remarkably, co-expression of miR-125b together with miR-99a and/or miR-100 induced a significant degree of resistance to VCR compared to the cell viability of cells expressing miR-125b only (Figure 3b, right bar graph; $p<0.05$) and compared to the viability of cells expressing each of these miRNAs separately (Figure 3b, left bar graph). The percentage of leukemic cells that survived three days of exposure to VCR increased to 91±4% for co-expression of miR-125b and miR-99a, 93±5% for co-expression of miR-125b and miR-100 and 88±17% for all three together. The viability significantly increased compared to the viability of cells expressing miR-125b as single miRNA (cell viability 38±13%; $p<0.05$, Figure 3b) or miR-99a and miR-100 without the co-expression of miR-125b (30±7%; $p<0.01$).

Potential target genes of miR-125b, miR-99a and miR-100

To identify potential target genes of the three miRNAs, a gene expression profiling study was performed on miR-125b expressing Reh cells induced with scrambled miR-control, miR-99a and/or miR-100. The most significant discriminative changes in gene expression levels were ranked by magnitude of fold-change and p-values below <0.05. The top 50 differentially expressed genes between cells with high levels of miR-125b, miR-99a and/or miR-100 and those only expressing miR-125b and scrambled miR-control are listed in supplementary Tables S1 (miR-125b and scrambled miR-control vs. miR-125b and miR-99a), S2 (miR-125b and scrambled miR-control vs. miR-125b and miR-100) and S3 (miR125b and scrambled miR-control vs. miR-125b, miR-99a and miR-100). Eleven downregulated genes were found to overlap in the top 10 of at least two of these comparisons. These genes were *DNTT*, *NUCKS1*, *MALAT1*, *SNRPE*, *PNO1*, *SET*, *KIF5B*, *PRPS2*, *RPS11*, *RPL38* and *RPL23A* (Figure 4). The median reduction in expression levels of these genes was 1.6-fold, with the largest reduction observed for *DNTT* (1.9-fold) and the smallest reduction for *PRPS2* (1.3-fold; Figure 4). Gene expression profiling of *ex vivo* VCR-resistant (n=75) and VCR-sensitive (n=90) leukemic cells of children with ALL revealed that the expression levels of these genes (with the exception of *NUCKS1*, *KIF5B*, *PNO1* and *SET*) were

significantly lower in resistant cases ($p<0.05$), as shown in supplementary Figure S4.

Discussion

Functional studies previously revealed that aberrant expression levels of miR-125b might contribute to leukemogenesis and the pathobiology of leukemia (17-18, 22, 27, 32). High expression levels of miR-125b were associated with *in vitro* resistance to VCR and daunorubicin in leukemic cells of patients with BCP-ALL and *in vitro* resistance of *ETV6-RUNX1*- positive Reh cell line to the daunorubicin – analogue doxorubicin (10, 26). In parallel, high expression levels of miR-125b made solid tumors more resistant to several drugs such as cisplatin and taxol (24-25, 33). These data imply that increased expression of miR-125b may induce resistance to drugs in cancer cells. In line with this hypothesis, we previously observed that resistance to VCR in pediatric ALL is associated with elevated levels of miR-125b, particularly in *ETV6-RUNX1*-positive ALL (10). We also observed that expression of miR-125b is correlated with that of miR-99a and miR-100 in pediatric ALL (10). These miRNAs are co-expressed not only in childhood ALL (10) but also in various types of solid cancers (34-35).

Here, we selected the *ETV6-RUNX1*-positive cell line Reh to explore the function of these 3 miRNAs in VCR resistance. The baseline expression levels of these miRNAs in Reh cells is in range with that observed in leukemic cells of children with *ETV6-RUNX1*-positive ALL (supplementary Figure S5a). *ETV6-RUNX1*-positive leukemia is relatively resistant to VCR (36), but large individual differences are found among the patients (supplementary Figure S5b). In contrast, *ETV6-RUNX1*-positive Reh cells are relatively sensitive to VCR (supplementary Figure S5b) and therefore, may serve as a suitable model to study the effect of miRNAs overexpression on inducing VCR resistance.

We showed that miR-125b in combination with miR-99a or miR-100 induced resistance to VCR in Reh cells whereas this effect was not observed for each of the miRNAs separately. The fold change in VCR-resistance after combined overexpression of miR-125b/miR-99a, miR-125b/miR-100 or miR-125b/miR-99a/miR-100 does not differ. This is not surprising since the sequence of mature miR-99a and miR-100 only differs in one nucleotide (Figure 1b) and hence there is also a considerable overlap in the lists of predicted target genes for these two miRNAs. Therefore, co-expression of only one of these two miRNAs with miR-125b is sufficient to induce resistance to VCR. Remarkably, in the absence of VCR, the apoptosis rate and cell cycle distribution did not differ between Reh cells expressing miR-125b together with miR-99a and/or miR-100 compared to those expressing miR-125b in combination with scrambled miRNA control. This suggests that the combined overexpression of miR-125b, miR-99a and/or miR-100 is a specific trigger for developing resistance to VCR in leukemic cells. Gene expression profiling revealed that 11 genes, including 4 genes encoded ribosomal proteins, were significantly downregulated in cells expressing high levels of miR-125b together with miR-100 and/or miR-99a compared to miR-125b and scrambled miR-control transduced cells. Enforced expression of these three miRNAs downregulated the expression levels of these target genes by median of 1.6-fold. Interestingly, 8 out of

these 11 genes were predicted by miRanda miRNA target prediction algorithm (www.microrna.org, August 2010 release) having a binding site in their 3' UTR for mature/star form of at least one of these three miRNAs (supplementary Table S4). Moreover, the expression levels of 7 out of these 11 genes (including all the ribosomal protein-coding genes) were reduced in primary leukemic cells of children who were *in vitro* resistant to VCR compared to the expression observed in the primary leukemic cells of *in vitro* sensitive children (supplementary Figure S4). The role of ribosomal proteins in VCR-resistant leukemia has not yet been functionally studied, but the present study as well as our previous report in a different set of pediatric ALL patients (37) underlines the importance to address the functional role of ribosomal proteins in VCR resistant leukemia. However, the expression levels of a candidate target gene with predicted binding sites for more than one miRNA (such as *MALAT1*) was not more decreased than a candidate target gene with one predicted binding site (such as *DNTT*), as shown in Figure 4. It must be taken into account that knockdown of one of these eleven candidate target genes might not be enough to induce VCR-resistance and similar to what we observed for miRNAs, combined downregulation of two or more of these genes might be necessary to induce VCR-resistance

The present study demonstrated the synergistic drug resistance-modifying effect of combined expression of miRNAs. The fact that miR-125b, miR-99a and/or miR-100 are often co-expressed suggests possible co-regulation of these miRNAs in leukemia. Remarkably, let-7c (21q21.1) expression levels also correlated with those of miR125b and miR-99a/miR-100, while let-7a-2 (11q24.1) showed no correlation (supplementary Figure S1b). This suggests that in childhood leukemia and especially in *ETV6-RUNX1*-positive ALL, it is the miR99a/miR-125b-2 locus on chromosome 21q21.1 which is actively transcribed

MiR-125b, miR-99a and/or miR-100 levels were linked to VCR resistance in *ETV6-RUNX1*-positive leukemic cells of children but let-7a-2 and let-7c levels were not. An imbalanced expression of individual members of a cluster has been seen before in the miR-17-92 cluster; inactivation or knockdown of p53 was shown to result in elevated levels of miR-92a and decreased levels of miR-17. This change in the expression pattern of these cluster members induced erythroleukemia in mice whereas a balanced expression of all 6 members of this cluster was shown to expand multipotent hematopoietic progenitors (38). The concerted action of miR-genes was also shown for miRNAs located on different chromosomes; the expression pattern of *ATXN1* in spinocerebellar ataxia type 1 is controlled by co-expression of miR-19, miR-101 and miR-130 (39). In brain tissue, both miR-7 and miR-153 were shown to co-regulate the expression of α-Synuclein thereby contributing to Parkinson disease (40). These examples illustrate the biological importance of miRNAs in combination.

In summary, we have demonstrated that concerted action between miR-125b, miR-99a and/or miR-100 induced resistance to VCR in *ETV6-RUNX1*-positive leukemia. The next step is to explore whether their directly regulated target genes (e.g. ribosomal proteins) and affected pathways may point to a way to modulate VCR resistance in *ETV6-RUNX1*-positive ALL.

Acknowledgements

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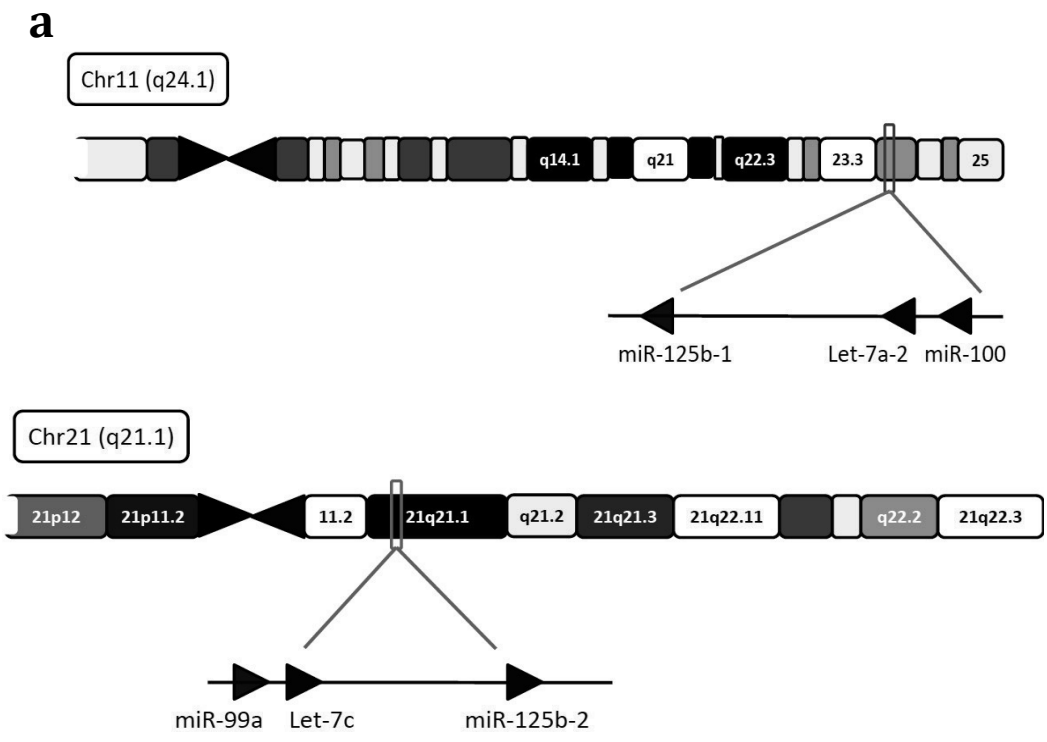
Conflict of interest

The authors declare no conflict of interest.

Author contributions

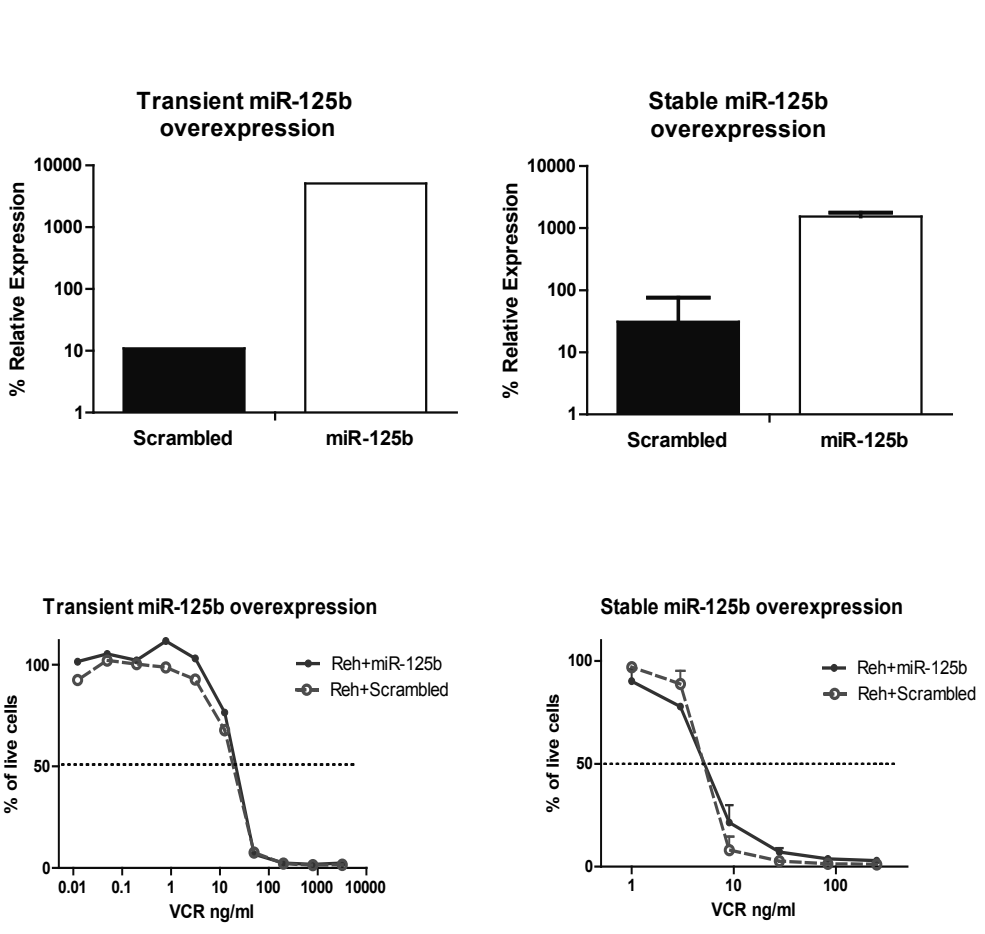
FAM performed research, analyzed data and wrote the manuscript; EAML-T performed research, analyzed data and revised the manuscript; IMA performed microarray of the patients and related data analyses; RP and MLdB supervised research, analyzed data, wrote and revised the manuscript. All authors approved the final version of the manuscript.

Figure 1: Chromosomal locations and sequences of miR-125b, miR-100 and miR-99a



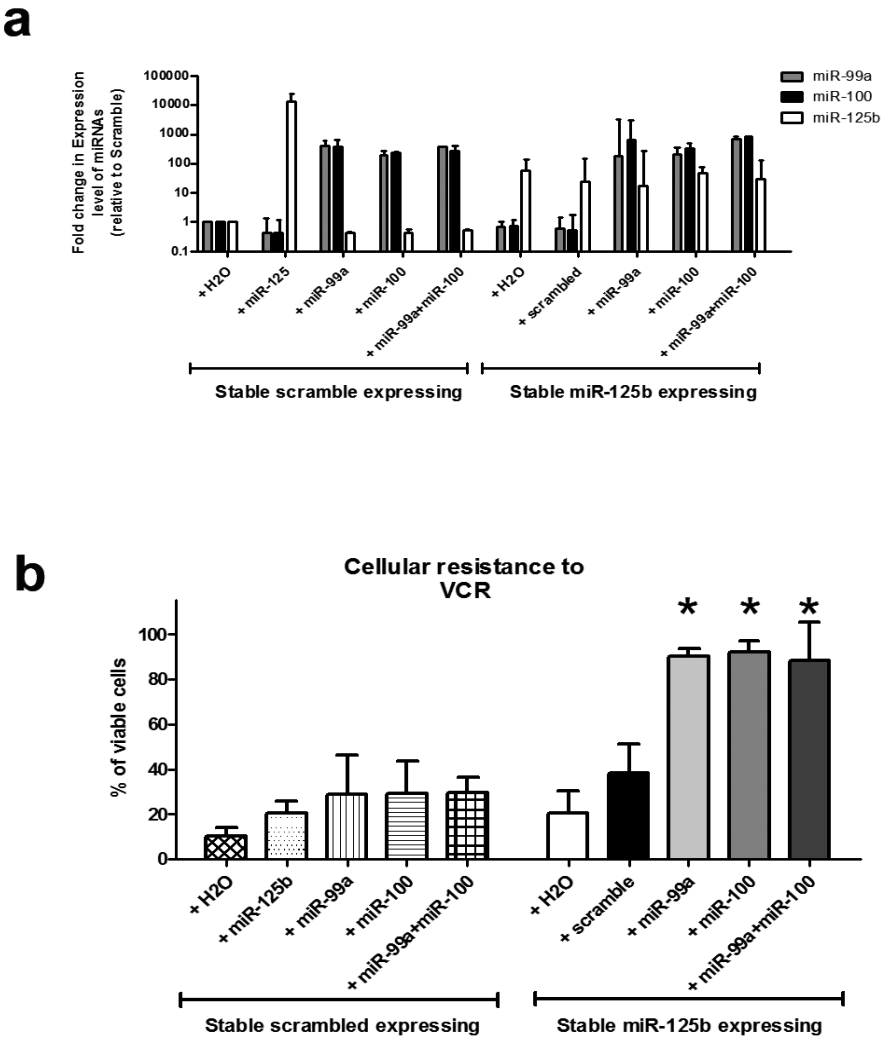
(a) Chromosomal location of miR-125b loci, (b) sequence similarities between miR-99a and miR-100 and between miR-125b-1 and miR-125b-2, and (c) schematic map of vector plasmid "pDual-miR-eGFP" which was used to induce the expression of mature miR-125b and a scrambled control miRNA by lentiviral-mediated transduction of *ETV6-RUNX1*-positive Reh cells.

Figure 2: Transient or stable overexpression of miR-125b did not alter resistance to VCR



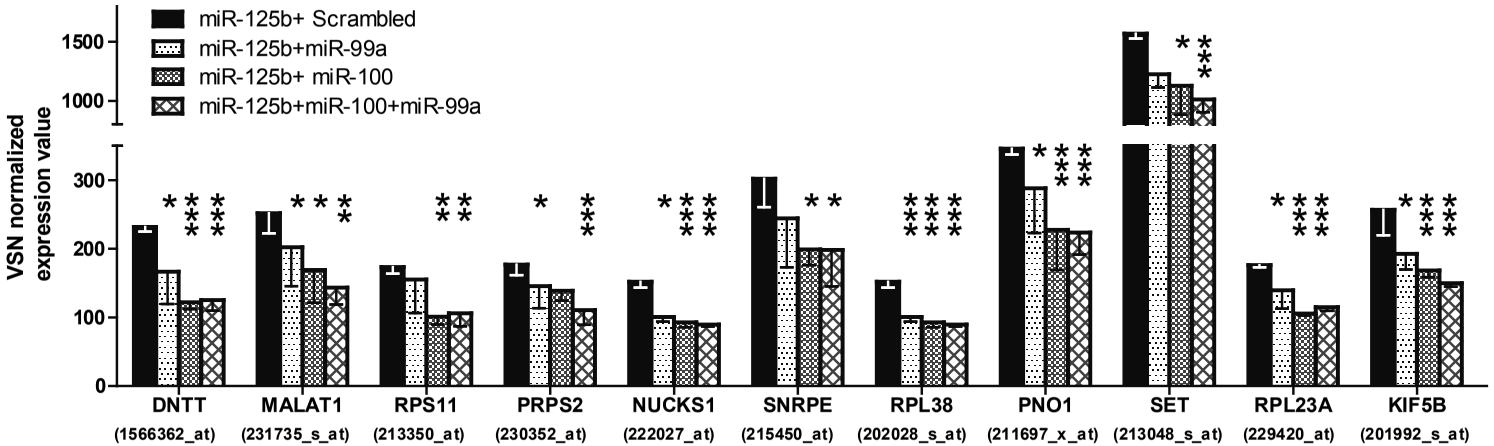
The effect of enforced expression of miR-125b in *ETV6-RUNX1*-positive Reh cells. (a) MiR-125b levels were raised by >100-fold compared to the levels observed in cells treated with a scrambled control-miR measured after 5 days of transfection with miR-125b precursor miRNAs (transient expression) and levels raised >50-fold after 2 weeks of lentiviral-mediated infection (stable expression, in duplicate), and (b) introduction of high expression levels of miR-125b did not alter the responsiveness of Reh cells to VCR compared to that observed in cells expressing high levels of the scrambled control miRNA.

Figure 3: Overexpression of miR-125b in combination with miR-100 and miR-99a induces significant VCR resistance in *ETV6-RUNX1*-positive Reh leukemic cells



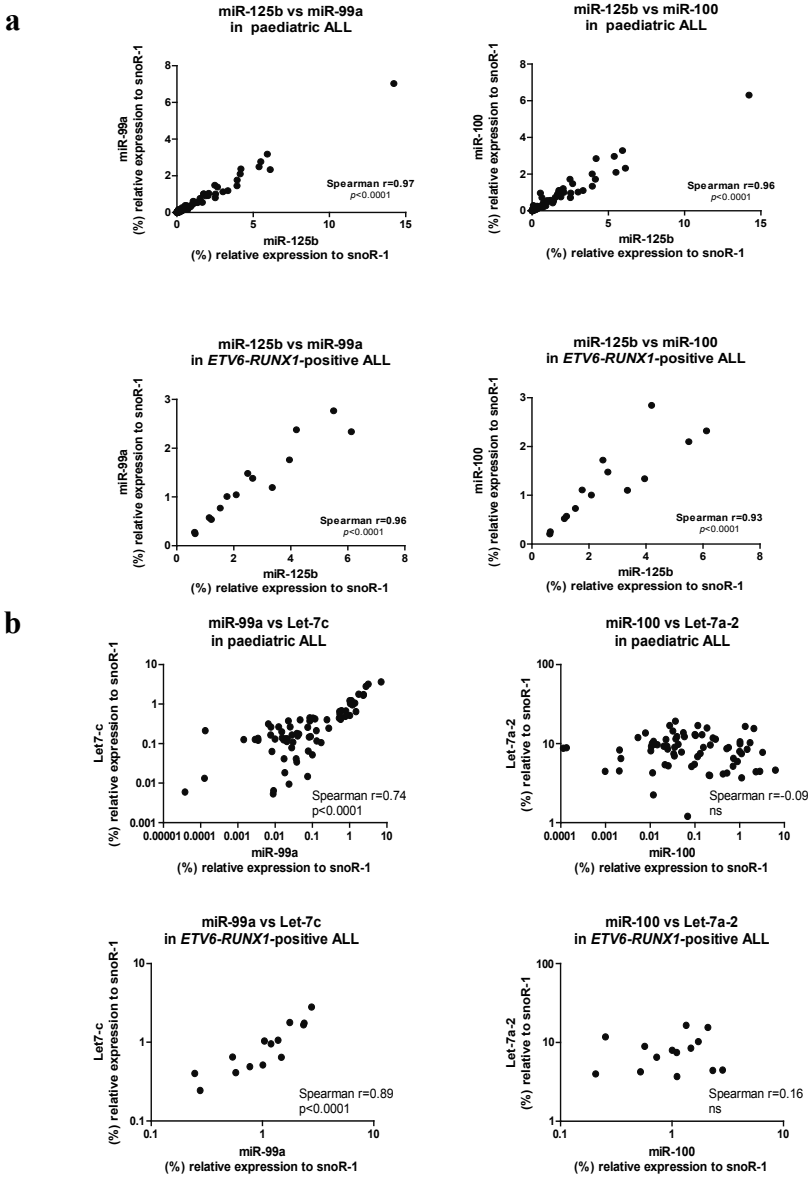
(a) Expression levels of miR-125b, miR-99a and/or miR-100 in *ETV6-RUNX1*-positive Reh cells were raised significantly compared to the intrinsic levels of these miRNAs in cells stably expressing the scrambled control miRNA (left bar graph) and compared to cells stably expressing miR-125b (right bar graph). Since the mature forms of miR-99a and miR-100 only differ in one nucleotide this hampers discrimination by qRT-PCR. In fact, we indeed observed that transduction of only miR-99a also resulted into an apparent raise of miR-100 levels and vice versa, (b) resistance to VCR is induced by co-expression of miR-125b, miR-99a and/or miR-100. Columns show the mean±SD percentage of viable cells remained after 3 days exposure to 9 ng/µL VCR (all experiments were done in triplicate). The *-symbol indicates statistical significance at $p<0.05$ level compared to scrambled control in stable miR-125b expressing cells.

Figure 4: Genes which are downregulated upon enforced expression of miR-125b in combination with miR-99a and/or miR-100



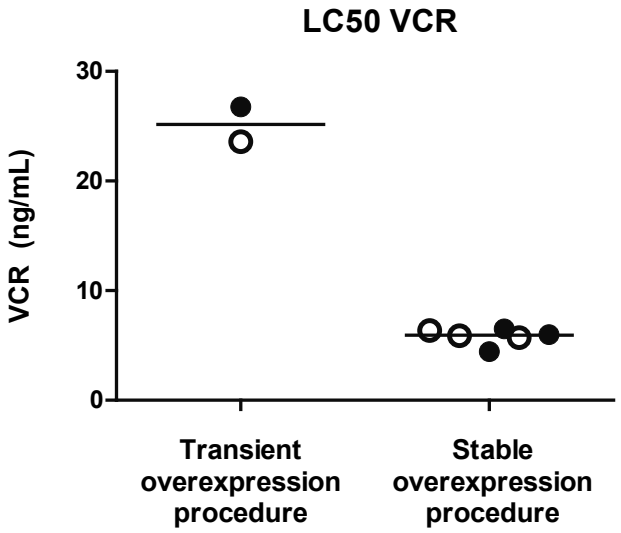
Genes with highest, statistically significant fold-reduction in expression in Reh cells overexpressing miR-125b, miR-99a and/or miR-100 compared to those expressing miR-125b and scrambled control-miR. The mean±SD of VSN normalized expression values of Affymetrix gene probes is shown (experiments were done in triplicate) *p<0.05, **p<0.01 and ***p<0.001 compared to miR-125b and scrambled control-miR.

Supplementary Figure S1: Significant correlation between expression levels of miR-125b, miR-100, miR-99a and Let-7c as well as LC50 VCR and expression level of miR-125b in pediatric ALL



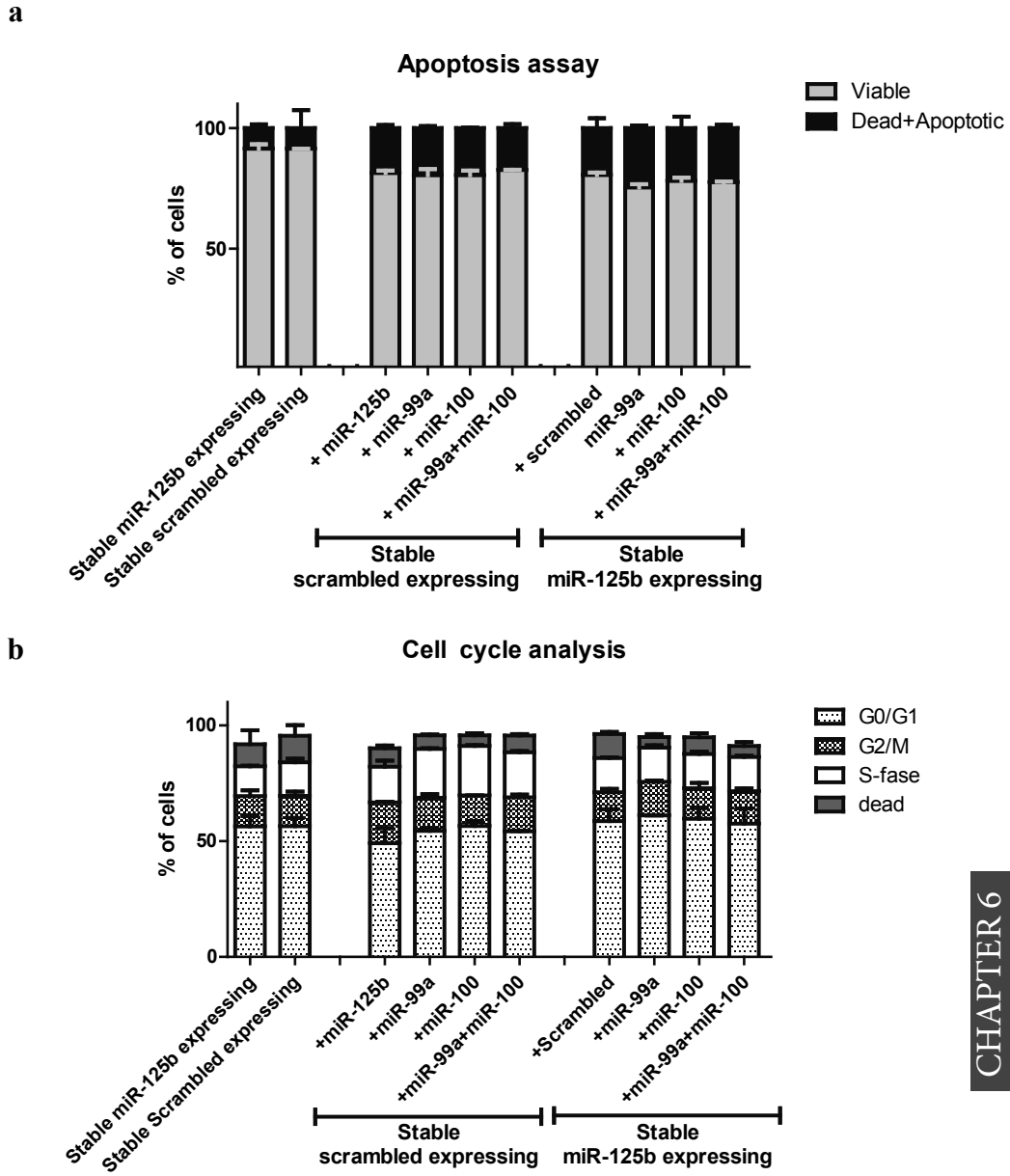
(a) Correlation between expression levels of miR-125b, miR-99a and miR-100 in pediatric ALL and, more specifically, in *ETV6-RUNX1*-positive pediatric BCP-ALL cases. (b) Correlation between expression levels of miR-99a and let-7c as well as miR-100 and let-7a in pediatric ALL and *ETV6-RUNX1*-positive pediatric BCP-ALL cases. (c) The correlation between the expression levels of miR-125b and LC50 values of VCR in children with ALL. The correlation between expression levels of miRNAs was calculated using Spearman's rank correlation test and $p<0.05$ was considered as significant correlation.

Supplementary Figure S2: Resistance to VCR in Reh leukemic cells increased after transient transfection procedures (lipofectamine-assisted) compared with stably transduction procedures (lentiviral-assisted)



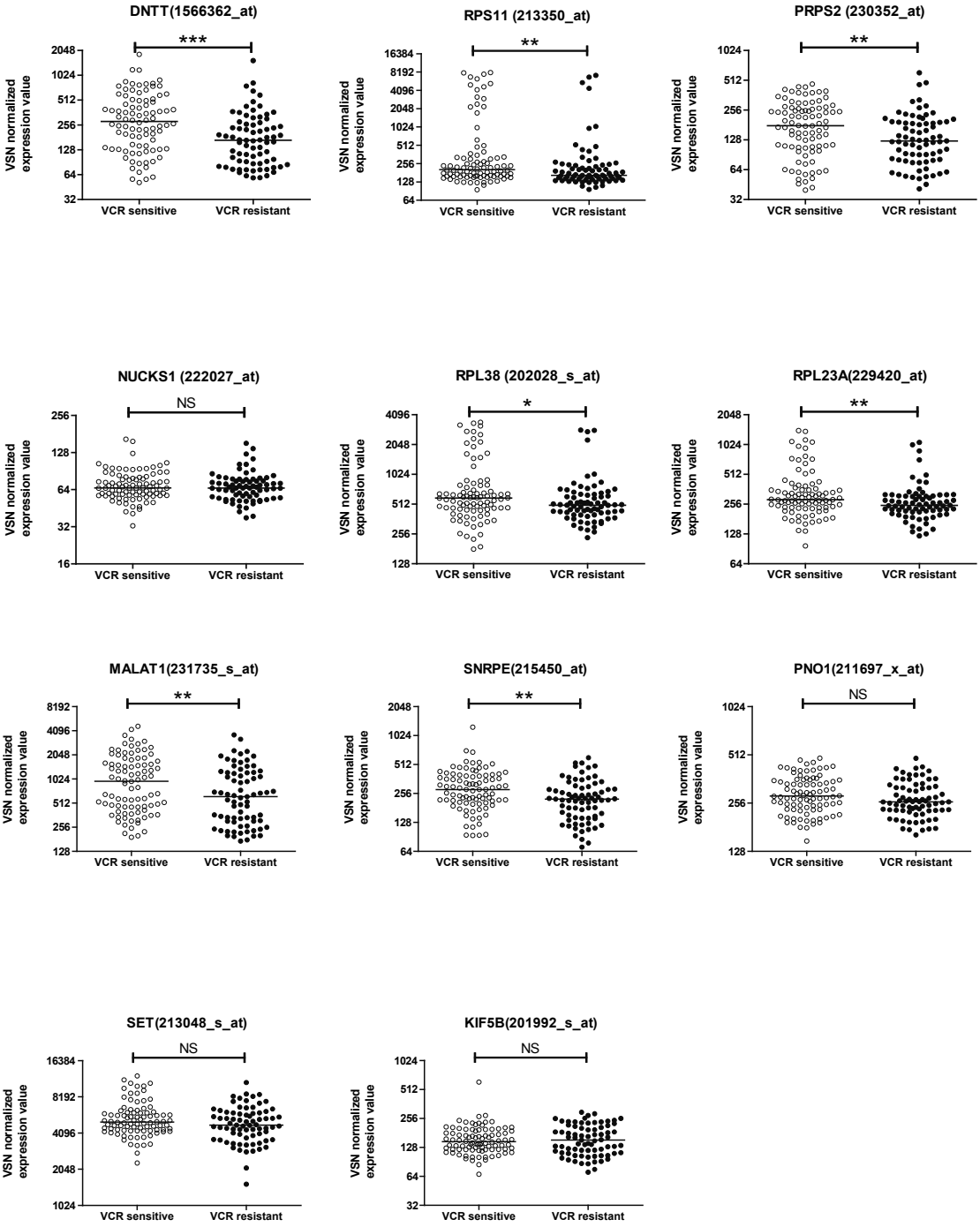
●-symbol indicates miR-125b overexpression and ○-symbol indicates scrambled control-miR overexpression (unpaired t test, $p<0.0001$).

Supplementary Figure S3: Overexpression of miR-125b per se or combined with miR-99a and/or miR-100 did not alter the amount of apoptosis and cell cycle distribution in Reh leukemic cells



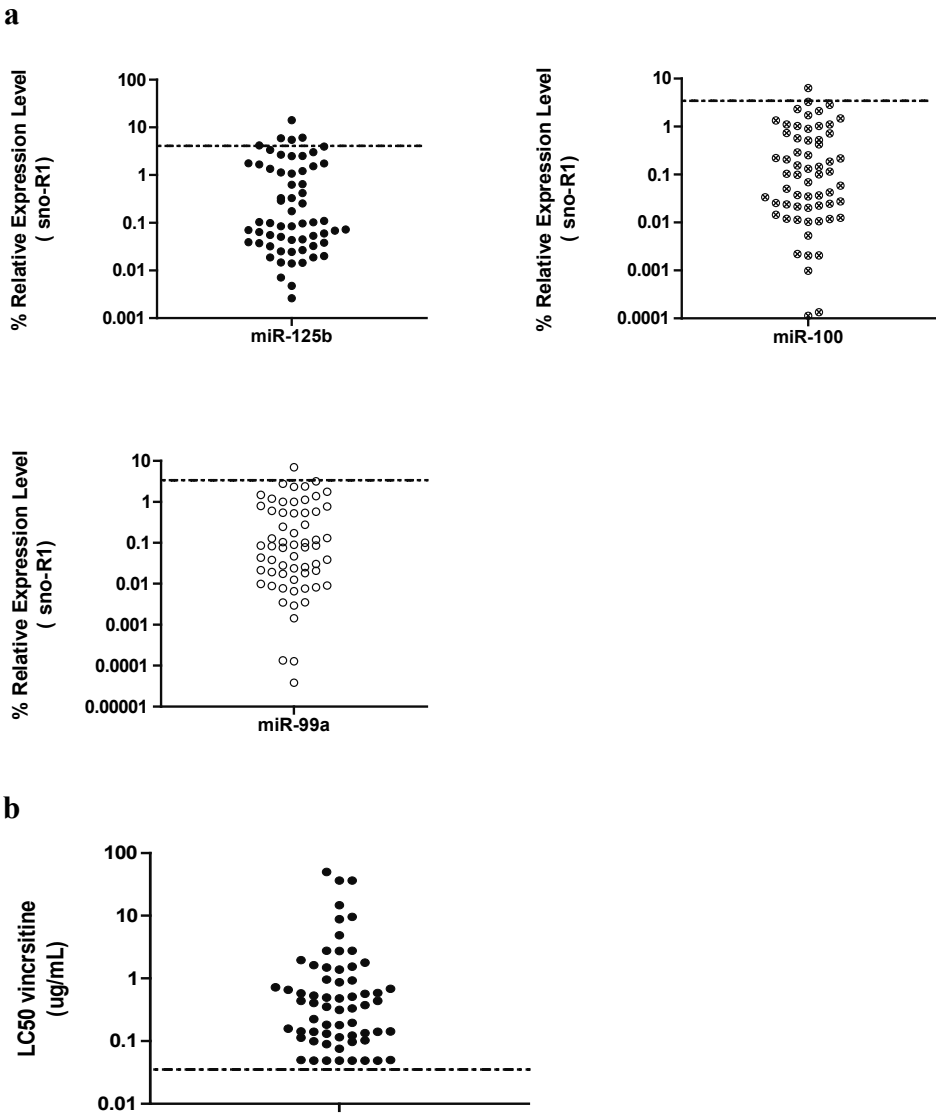
(a) Apoptosis rate after overexpression of miR-125b, miR-99a and/or miR-100 did not change compared to cells expressing the scrambled miRNA control or miR-125b in absence of VCR. The amount of apoptosis was measured using the Annexin-V/propidium iodide staining method. (b) Cell cycle distribution of VCR untreated *ETV6-RUNX1*-positive Reh cells did not change upon overexpression of scrambled control, miR-125b, miR-99a and/or miR-100. The cell cycle distribution was determined using flow cytometric analysis of propidium iodide-stained nuclei. Experiments were done in triplicate and the non-parametric Mann-Whitney U test was used to compare different conditions.

Supplementary Figure S4: Expression levels of 11 genes for which expression levels were downregulated upon introduction of miR-125b, miR-99a and/or miR-100 within *in vitro* VCR-resistant and VCR-sensitive pediatric ALL cases



VSN normalized expression values of patients are depicted by dots, the median value is indicated by a line. ○-symbol indicates VCR-sensitive and ●-symbol indicates VCR-resistant patients with ALL (FCR corrected p-value, *p<0.05, **p<0.01, ***p<0.001).

Supplementary Figure S5: Expression levels of miR-125b, miR-100 and miR-99a and LC50 VCR in children with *ETV6-RUNX1*-positive ALL compared with Reh leukemic cell line



(a) Relative expression of miR-125b, miR-100 and miR-99a in 61 children with *ETV6-RUNX1*-positive ALL (31 VCR sensitive and 30 VCR resistant cases). The expression level of each miRNA in the *ETV6-RUNX1*-positive cell line Reh is indicated by a dotted line, (b) VCR LC50 values of 61 children with *ETV6-RUNX1*-positive ALL (31 VCR sensitive and 30 VCR resistant cases). The VCR LC50 value of the *ETV6-RUNX1*-positive cell line Reh is indicated by a dotted line.

Supplementary Table S1: Top 50 differentially expressed genes upon co-expression of miR-99a and miR-125b compared to co-expression of scrambled control miRNA and miR-125b

#	Probe ID	Symbol	Gene name	Linear fold change	p-value
1	222027_at	NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	1.5	0.03
2	1566362_at	DNTT	deoxynucleotidyltransferase, terminal	1.4	0.02
3	202028_s_at	RPL38	ribosomal protein L38	1.3	0.00
4	229420_at	RPL23A	ribosomal protein L23a	1.3	0.05
5	201992_s_at	KIF5B	kinesin family member 5B	1.3	0.01
6	222576_s_at	EIF2C1	eukaryotic translation initiation factor 2C, 1	1.3	0.04
7	230352_at	PRPS2	phosphoribosyl pyrophosphate synthetase 2	1.3	0.04
8	211697_x_at	PNO1	partner of NOB1 homolog (S. cerevisiae)	1.3	0.05
9	228324_at	C9orf41	chromosome 9 open reading frame 41	1.3	0.00
10	235264_at	HCFC2	host cell factor C2	1.2	0.03
11	224321_at	TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2	1.2	0.04
12	234488_s_at	GMCL1L	germ cell-less homolog 1 (Drosophila)-like	1.2	0.03
13	243505_at	MAP3K3	mitogen-activated protein kinase kinase kinase 3	1.2	0.02
14	218006_s_at	ZNF22	zinc finger protein 22 (KOX 15)	1.2	0.02
15	1553519_at	C21orf94	chromosome 21 open reading frame 94	1.2	0.00
16	216652_s_at	DR1	down-regulator of transcription 1, TBP-binding (negative cofactor 2)	1.2	0.00
17	201404_x_at	PSMB2	proteasome (prosome, macropain) subunit, beta type, 2	1.2	0.03
18	229299_at	C5orf33	chromosome 5 open reading frame 33	1.2	0.03
19	229026_at	CDC42SE2	CDC42 small effector 2	1.2	0.00
20	235006_at	CDKN2AIPNL	CDKN2A interacting protein N-terminal like	1.2	0.01
21	226109_at	C21orf91	chromosome 21 open reading frame 91	1.2	0.02
22	209838_at	COPS2	COP9 constitutive photomorphogenic homolog subunit 2 (Arabidopsis)	1.2	0.00
23	244777_at	DCP2	DCP2 decapping enzyme homolog (S. cerevisiae)	1.2	0.01
24	203310_at	STXBP3	syntaxin binding protein 3	1.2	0.03
25	205632_s_at	PIP5K1B	phosphatidylinositol-4-phosphate 5-kinase, type I, beta	1.2	0.03
26	218202_x_at	MRPL44	mitochondrial ribosomal protein L44	1.2	0.04

27	1569025_s_at	FAM13A1	family with sequence similarity 13, member A1	1.2	0.02
28	241574_s_at	IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	1.2	0.02
29	220637_at	FAM124B	family with sequence similarity 124B	1.2	0.01
30	227416_s_at	ZCRB1	zinc finger CCHC-type and RNA binding motif 1	1.2	0.03
31	230679_at	WDR32	WD repeat domain 32	1.2	0.01
32	228201_at	ARL13B	ADP-ribosylation factor-like 13B	1.2	0.04
33	208482_at	SSTR1	somatostatin receptor 1	1.2	0.00
34	230903_s_at	C8orf42	chromosome 8 open reading frame 42	1.2	0.01
35	211967_at	TMEM123	transmembrane protein 123	1.2	0.04
36	218875_s_at	FBXO5	F-box protein 5	1.2	0.03
37	232557_at	LOC390595	similar to ubiquitin-associated protein 1 (predicted)	1.2	0.00
38	227904_at	AZI2	5-azacytidine induced 2	1.2	0.03
39	201382_at	CACYBP	calcyclin binding protein	1.2	0.01
40	208287_at	HCG9	HLA complex group 9	1.2	0.02
41	217956_s_at	ENOPH1	enolase-phosphatase 1	1.2	0.03
42	207092_at	LEP	leptin	1.2	0.01
43	216081_at	LAMA4	laminin, alpha 4	1.2	0.02
44	212297_at	ATP13A3	ATPase type 13A3	1.2	0.04
45	205140_at	FPGT	fucose-1-phosphate guanylyltransferase	1.2	0.05
46	223898_at	ZNF670	zinc finger protein 670	1.2	0.04
47	219592_at	MCPH1	microcephalin 1	1.2	0.01
49	221681_s_at	DSPP	dentin sialophosphoprotein	1.2	0.03
50	230569_at	KIAA1430	KIAA1430	1.2	0.02
50	220346_at	MTHFD2L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	1.2	0.04

Supplementary Table S2: Top 50 differentially expressed genes upon co-expression of miR-100 and miR-125b compared to co-expression of scrambled control miRNA and miR-125b

#	Probe ID	Symbol	Gene name	linear fold change	p-value
1	1566362_at	DNTT	deoxynucleotidyltransferase, terminal	1.9	0.00
2	202028_s_at	RPL38	ribosomal protein L38	1.6	0.00
3	229420_at	RPL23A	ribosomal protein L23a	1.6	0.00
4	213350_at	RPS11	ribosomal protein S11	1.6	0.02
5	231735_s_at	MALAT1	metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)	1.6	0.02
6	222027_at	NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	1.6	0.01
7	211697_x_at	PNO1	partner of NOB1 homolog (S. cerevisiae)	1.6	0.00
8	215450_at	SNRPE	small nuclear ribonucleoprotein polypeptide E	1.5	0.04
9	213048_s_at	SET	SET nuclear oncogene	1.5	0.01
10	201992_s_at	KIF5B	kinesin family member 5B	1.5	0.00
11	219558_at	ATP13A3	ATPase type 13A3	1.5	0.03
12	221943_x_at	RPL38	ribosomal protein L38	1.4	0.00
13	220725_x_at	DNAH3	dynein, axonemal, heavy chain 3	1.4	0.00
14	213826_s_at	H3F3A	H3 histone, family 3A	1.4	0.00
15	201602_s_at	PPP1R12A	protein phosphatase 1, regulatory (inhibitor) subunit 12A	1.4	0.02
16	219138_at	RPL14	ribosomal protein L14	1.4	0.01
17	1566990_x_at	ARID1B	AT rich interactive domain 1B (SWI1-like)	1.4	0.01
18	212044_s_at	RPL27A	ribosomal protein L27a	1.4	0.02
19	228204_at	PSMB4	proteasome (prosome, macropain) subunit, beta type, 4	1.3	0.03
20	223292_s_at	MRPS15	mitochondrial ribosomal protein S15	1.3	0.02
21	221681_s_at	DSPP	dentin sialophosphoprotein	1.3	0.00
22	1566989_at	ARID1B	AT rich interactive domain 1B (SWI1-like)	1.3	0.01
23	200908_s_at	RPLP2	ribosomal protein, large, P2	1.3	0.00
24	213642_at	RPL27	ribosomal protein L27	1.3	0.01
25	218006_s_at	ZNF22	zinc finger protein 22 (KOX 15)	1.3	0.00
26	224321_at	TMEFF2	transmembrane protein with EGF-like and two follistatin-like domains 2	1.3	0.02
27	202648_at	RPS19	ribosomal protein S19	1.3	0.01
28	222441_x_at	SLMO2	slowmo homolog 2 (Drosophila)	1.3	0.03
29	217813_s_at	SPIN1	spindlin 1	1.3	0.05
30	222556_at	ALG5	asparagine-linked glycosylation 5 homolog (S. cerevisiae, dolichyl-phosphate beta-glucosyltransferase)	1.3	0.03

31	1555272_at	RSPH10B2	radial spoke head 10 homolog B2 (Chlamydomonas)	1.3	0.03
32	200889_s_at	SSR1	signal sequence receptor, alpha	1.3	0.00
33	209750_at	NR1D2	nuclear receptor subfamily 1, group D, member 2	1.3	0.01
34	213494_s_at	YY1	YY1 transcription factor	1.3	0.00
35	238701_x_at	FLJ45803	FLJ45803 protein	1.3	0.05
36	202170_s_at	AASDHPPT	aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	1.3	0.05
37	234464_s_at	EME1	essential meiotic endonuclease 1 homolog 1 (S. pombe)	1.2	0.05
38	221449_s_at	ITFG1	integrin alpha FG-GAP repeat containing 1	1.2	0.00
39	213763_at	HIPK2	homeodomain interacting protein kinase 2	1.2	0.03
40	217494_s_at	PTENP1	phosphatase and tensin homolog pseudogene 1	1.2	0.05
41	200776_s_at	BZW1L1	basic leucine zipper and W2 domains 1 like 1	1.2	0.04
42	222156_x_at	CCPG1	cell cycle progression 1	1.2	0.04
43	223486_at	GTPBP8	GTP-binding protein 8 (putative)	1.2	0.02
44	1555772_a_at	CDC25A	cell division cycle 25 homolog A (S. pombe)	1.2	0.02
45	208541_x_at	TFAM	transcription factor A, mitochondrial	1.2	0.02
46	229322_at	PPP2R5E	protein phosphatase 2, regulatory subunit B', epsilon isoform	1.2	0.01
47	229174_at	C3orf38	chromosome 3 open reading frame 38	1.2	0.05
48	222565_s_at	PRKD3	protein kinase D3	1.2	0.05
49	235006_at	CDKN2AIPNL	CDKN2A interacting protein N-terminal like	1.2	0.00
50	203829_at	ELP4	elongation protein 4 homolog (S. cerevisiae)	1.2	0.01

Supplementary Table S3: Top 50 differentially expressed genes upon co-expression of miR-100, miR-99a and miR-125b compared to co-expression of scrambled control miRNA and miR-125b

#	Probe ID	Symbol	Gene name	linear fold change	p-value
1	1566362_at	DNTT	deoxynucleotidyltransferase, terminal	1.9	0.00
2	231735_s_at	MALAT1	metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)	1.7	0.01
3	213350_at	RPS11	ribosomal protein S11	1.7	0.01
4	230352_at	PRPS2	phosphoribosyl pyrophosphate synthetase 2	1.7	0.00
5	222027_at	NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	1.7	0.00
6	215450_at	SNRPE	small nuclear ribonucleoprotein polypeptide E	1.7	0.02
7	202028_s_at	RPL38	ribosomal protein L38	1.6	0.00
8	211697_x_at	PNO1	partner of NOB1 homolog (S. cerevisiae)	1.6	0.00
9	213048_s_at	SET	SET nuclear oncogene	1.6	0.00
10	201992_s_at	KIF5B	kinesin family member 5B	1.6	0.00
11	229420_at	RPL23A	ribosomal protein L23a	1.5	0.00
12	219558_at	ATP13A3	ATPase type 13A3	1.5	0.03
13	231784_s_at	WDSOF1	WD repeats and SOF1 domain containing	1.4	0.01
14	1567213_at	PNN	pinin, desmosome associated protein	1.4	0.05
15	229841_at	EIF2C2	eukaryotic translation initiation factor 2C, 2	1.4	0.05
16	228248_at	RICTOR	rapamycin-insensitive companion of mTOR	1.4	0.02
17	228204_at	PSMB4	proteasome (prosome, macropain) subunit, beta type, 4	1.4	0.01
18	201324_at	EMP1	epithelial membrane protein 1	1.4	0.02
19	229174_at	C3orf38	chromosome 3 open reading frame 38	1.4	0.00
20	203735_x_at	PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	1.4	0.00
21	212044_s_at	RPL27A	ribosomal protein L27a	1.4	0.01
22	210948_s_at	LEF1	lymphoid enhancer-binding factor 1	1.4	0.04
23	218006_s_at	ZNF22	zinc finger protein 22 (KOX 15)	1.4	0.00
24	201143_s_at	EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	1.4	0.02
25	217813_s_at	SPIN1	spindlin 1	1.4	0.01
26	221943_x_at	RPL38	ribosomal protein L38	1.4	0.01
27	216450_x_at	HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	1.4	0.03
28	222556_at	ALG5	asparagine-linked glycosylation 5 homolog (S. cerevisiae, dolichyl-phosphate beta-glucosyltransferase)	1.4	0.01
29	220725_x_at	DNAH3	dynein, axonemal, heavy chain 3	1.4	0.00

30	216609_at	TXN	thioredoxin	1.4	0.04
31	200776_s_at	BZW1L1	basic leucine zipper and W2 domains 1 like 1	1.4	0.00
32	1566989_at	ARID1B	AT rich interactive domain 1B (SWI1-like)	1.4	0.00
33	201602_s_at	PPP1R12A	protein phosphatase 1, regulatory (inhibitor) subunit 12A	1.4	0.02
34	219031_s_at	NIP7	nuclear import 7 homolog (S. cerevisiae)	1.4	0.02
35	223898_at	ZNF670	zinc finger protein 670	1.4	0.00
36	1555772_a_at	CDC25A	cell division cycle 25 homolog A (S. pombe)	1.3	0.00
37	1567014_s_at	NFE2L2	nuclear factor (erythroid-derived 2)-like 2	1.3	0.02
38	212075_s_at	CSNK2A1	casein kinase 2, alpha 1 polypeptide	1.3	0.00
39	235006_at	CDKN2AIPNL	CDKN2A interacting protein N-terminal like	1.3	0.00
40	228050_at	UTP15	UTP15, U3 small nucleolar ribonucleoprotein, homolog (S. cerevisiae)	1.3	0.02
41	228693_at	CCDC50	coiled-coil domain containing 50	1.3	0.01
42	201745_at	TWF1	twinfilin, actin-binding protein, homolog 1 (Drosophila)	1.3	0.03
43	1553575_at	ND6	NADH dehydrogenase, subunit 6 (complex I)	1.3	0.03
44	201016_at	EIF1AX	eukaryotic translation initiation factor 1A, X-linked	1.3	0.03
45	205191_at	RP2	retinitis pigmentosa 2 (X-linked recessive)	1.3	0.01
46	222441_x_at	SLMO2	slowmo homolog 2 (Drosophila)	1.3	0.01
47	201151_s_at	MBNL1	muscleblind-like (Drosophila)	1.3	0.01
48	240834_at	FAM105B	family with sequence similarity 105, member B	1.3	0.00
49	225073_at	PPHLN1	periphilin 1	1.3	0.05
50	214155_s_at	LARP4	La ribonucleoprotein domain family, member 4	1.3	0.01

Supplementary Table S4: The probability of the binding of miRNAs to the 11 downregulated candidate target genes upon co-expression of miR-125b, miR-100 and/or miR-99a

Gene symbole	miR-125b	miR-125b*	miR-100	miR-100*	miR-99a	miR-99a*
DNTT	1	0	0	0	0	0
MALAT1	2	2	0	5	0	5
RPS11	0	0	0	0	0	0
PRPS2	0	0	0	0	0	0
NUCKS1	1	3	0	2	0	1
SNRPE	0	0	1	1	1	1
RPL38	0	0	0	0	0	0
PNO	0	0	0	1	0	0
SET	1	1	0	2	0	0
RPL23A	1	0	0	0	0	0
KIF5B	1	0	0	0	0	0

Number of binding sites for the indicated genes to which miRNAs-125b, -100 and -99a may bind according to the computational target prediction algorithm of miRanda (www.microrna.org).

Supplementary file 1

Patient samples

Mononuclear cells were isolated from bone marrow and peripheral blood samples collected from children with newly diagnosed ALL, as described previously (1, 2). Mononuclear cells were isolated by sucrose density-gradient centrifugation (density 1.077 g/mL; Lymphoprep, Nycomed Pharma, Oslo, Norway). Cells were resuspended in RPMI 1640 medium (Life technologies, Bleiswijk, The Netherlands) supplemented with 20% fetal calf serum (Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Life technologies, Bleiswijk, The Netherlands), 200 µg/mL gentamicin (Life technologies, Bleiswijk, The Netherlands), 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.125 µg/mL fungizone (Life technologies, Bleiswijk, The Netherlands), 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL sodium selenite (ITS media supplement, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). If necessary, the mononuclear samples were enriched to >90% blasts by depleting normal cells using anti-CD immunomagnetic beads (DynaBeads; Dynal, Oslo, Norway). The institutional review board approved the use of patient leukemic cells and written informed consent to use excess diagnostic material in our studies was obtained from patients, parents or legal guardians. Immunophenotype was determined by flow cytometry. The cytogenetic abnormalities *ETV6-RUNX1* (*TEL-AML1*), *TCF3* (*E2A*)-rearrangement, *MLL*-rearrangement and *BCR-ABL1* were determined by interphase fluorescence in-situ hybridization (FISH) and/or reverse transcriptase polymerase chain reaction (RT-PCR) techniques, as previously described (3). High hyperdiploidy was determined using karyotyping (>50 chromosomes) and DNA index
(≥1.16).

Cell lines

The *ETV6-RUNX1*-positive cell line Reh as well as human embryonic kidney (HEK293T) cell line was purchased from DSMZ (Braunschweig, Germany). Reh cells were cultured in RPMI 1640 medium and HEK293T cells were cultured in DMEM medium (Life technologies, Bleiswijk, The Netherlands), both supplemented with L-glutamine (Glutamax) 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.125 µg/mL fungizone (all from Life technologies, Bleiswijk, The Netherlands) and 10% fetal calf serum (Integro, Zaandam, The Netherlands). Cells were cultured at 37°C in humidified air containing 5% CO_2.

Lentiviral transduction of miRNAs in leukemic cells

The plasmid pHR SIN CS-F-W pUb-Em was kindly provided by Dr Marry K. Collins and Dr David Scors (London, UK) (4) and modified in-house to overexpress the miRNA of interest. This modified plasmid contains an SFFV-promoter-driven miRNA sequence (e.g. miR-125b or scrambled miR-control sequence) and an ubiquitin-promoter-driven emerald green fluorescent protein (eGFP) reporter sequence. HEK293T cells (70-80% confluency) were transfected with pDual-miR-eGFP and the helper plasmids psPAX2

(developed by Professor D. Trono, Geneva, Switzerland; Addgene plasmid 12260; Addgene, Cambridge, USA) and pMD2.G (Addgene plasmid 12259) using the calcium phosphate technique as described previously (5). Briefly, pDual-miR-eGFP (20 µg), pMD2.G (3 µg) and psPAX2 (7 µg) and 2M CaCl2 (122 µL) were mixed with Hepes-buffered saline while bubbling, and added drop-wise to HEK293T cells in the presence of 25 µM chloroquine (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). The culture medium of HEK293T cells was replaced after 16-18 hours of transfection and virus-containing supernatant was collected and filtered (0.44µm pore-size) one and two days thereafter. The virus particles were concentrated by ultracentrifugation (32,000 rpm, 1hr, 4°C) and resuspended in L-glutamine containing RPMI 1640 medium. Viral titres in pg/mL were estimated with a HIV-1 p24 Antigen ELISA kit (ZeptoMetrix, Buffalo, USA) according to the manufacturer's instructions and expressed as Transforming Units per mL (TU/mL).

The *ETV6-RUNX1*-positive leukemic cell line Reh ($0.5x10^6$ cells) was spin-infected by adding virus particles at a multiplicity of infection (MOI) of 2.5 TU/cell and 5 µg/mL polybrene (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). After 48 hours, the infection rate was determined by measuring eGFP positivity by flow cytometry. Stably expressing miR-125b and scrambled miR-control cell lines were generated by culturing cells for at least two weeks.

Transient overexpression of miRNAs in leukemic cells

Reh cells ($0.8x10^6$/mL) were incubated in triplicate at 37°C in humidified air with 5% CO_2 with 66 µM precursor oligonucleotide sequences for miR-125b, miR-99a, miR-100 or a FAM-labeled scrambled control miR #1 (pre-miR™ oligos, Applied Biosystems, Foster City, USA) in the presence of Lipofectamine (Life technologies, Bleiswijk, The Netherlands). After 72 hours, new precursor oligos were added to the cells. In addition to parental Reh cells, the same procedure was also applied to stably miR-125b or scrambled miR-control expressing Reh cells. While the combination of 2 miRNAs was used for transfection, we used half amount (33 µM) of each miRNA to keep different experimental conditions comparable. Cells were harvested 48 hours after the second transfection, for further analysis.

RNA extraction and quantification of miRNA expression levels

Total RNA was isolated from 0.5 million cells, using TRIzol isolation reagents (Life technologies, Bleiswijk, The Netherlands) in accordance with the manufacturer's protocol. RNA was quantified using a Nanodrop (ND-1000 spectrophotometer, Agilent, Amstelveen, The Netherlands). The RNA integrity was determined using an Agilent Bio-analyser 2100 and RNA 6000 Nano Assay LabChips (Agilent, Amstelveen, The Netherlands). Only samples with an RNA integrity number (RIN) of ≥7.0 were used. The expression levels of specific miRNAs and scrambled control miRNAs were determined by real-time quantitative PCR (RT-qPCR) using a miRNA specific stem-looped RT-primer for cDNA synthesis and miRNA-selective primers and probes for PCR amplification (TaqMan MicroRNA Assay, Applied Biosystems, Foster City, USA) as described before

(6). RT-qPCR was performed using a Taqman 7900HT instrument (Life technologies, Bleiswijk, The Netherlands) and data were analysed with SDS 2.3 software (Applied Biosystems, Foster City, USA). Endogenous small nucleolar RNA 1 (snoR-1, 5'-AUUUGCUAUCUGAGAGAUGGUGAUGACAUUUUAAACCACCAAGAUCGCUGA UGCA- 3') was used as a reference for input of RNA. Each assay was performed in duplicate and Ct value of miRNA and that of snoR-1 were measured. The expression of each miRNA was calculated as the percentage of snoR-1 as $2^{-\Delta Ct}$x100% where the ΔCt is equal to the Ct-value for each miRNA minus the Ct-value of the control reference snoRNA (snoR-1).

Cell cycle analysis

The distribution of the G0/G1, S and G2M phases of the cell cycle was determined by flow cytometric analysis of propidium iodide-stained nuclei. Leukemic cells (0.25 million) were fixed with 70% EtOH, resuspended in 20 µL PBS and incubated for 5 minutes at room temperature in 45 µL of hypotonic buffer containing 3.5 mM sodium citrate, 0.1% Igepal® (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands), 1.5 mM spermin-tetrahydrochloride, 60 µg/mL Tris-hydroxymethylaminomethane and 3 mg/mL trypsin. Next, the nuclear suspension was incubated with 0.5 mg/mL trypsin inhibitor and 20 µg/mL ribonuclease A for 10 minutes at room temperature. Nuclei were incubated with 1.75 mM propidium iodide for 15 minutes on ice and propidium iodide staining was quantified using a MACSQuant device (Miltenyi Biotech, Bergisch Gladbach, Germany). The cell cycle distribution was analyzed by MACSQuant® analyzer software.

Apoptosis assay

Leukemic cells ($0.25x10^6$) were centrifuged and then resuspended in 200 µL of binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). Thereafter, cells were incubated with 1:250 diluted Annexin V-Alexa Fluor® 647 conjugate and 4 µg/mL propidium iodide (both from Life technologies, Bleiswijk, The Netherlands) for 15 minutes at room temperature. Next, the Annexin-V/propidium iodide staining was measured with a MACSQuant device (Miltenyi Biotech, Bergisch Gladbach, Germany). The data was analyzed by MACSQuant® analyzer software as follows: AnnexinV positive-PI negative population was used as a measure for apoptotic cells, and AnnexinV positive-PI positive population was considered as dead cells.

MTT drug resistance assay

Cytotoxicity of vincristine (TEVA Pharma, Petah Tikva, Israel) was measured by incubating the cells with 9 ng/mL vincristine using 3 days in-vitro methyl thiazol tetrazoliumbromide (MTT) drug resistance assay as described earlier (2, 7). In summary, *ETV6-RUNX1*-positive Reh cells after modulation in miRNAs level were incubated with 9 ng/mL VCR in duplicate. Cells were kept in culture in a humidified 37ºC incubator with 5% CO_2 for 3 days, and then 0.45 mg/mL of MTT solution was added. After 3 hours incubation, crystals produced by viable cells were dissolved in

acidified isopropanol and quantified by spectrophotometry at 562 nm on a VersaMax microplate reader. The MTT drug resistance assay of the patients' samples was done with the same method, but cells were incubated for 4 days to a dilution series of 0.05-50 µg/mL VCR, as described earlier (2, 7). The concentration of VCR lethal to 50% of the cells (LC50) was used to distinguish between VCR-resistant and VCR-sensitive patients. Among children with ALL, 90 VCR-sensitive cases with median LC50 of 0.13 µg/mL (<0.05-0.38 µg/mL, SD=0.1) and 75 VCR-resistant cases with median LC50 of 4.9 µg/mL (1.9->50 µg/mL, SD=13.5) were selected for further comparison. The LC50 value in Reh cells was <0.05 µg/mL after 4 days of exposure to VCR.

Gene expression profiling

The RNA integrity was checked with Agilent's 2100 Bio-analyzer (Santa Clara, CA, USA). cDNA and biotinylated cRNA were synthesized and hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, USA) according to the manufacturer's guidelines. Data were acquired using 'expresso' (Bioconductor package 'Affy'), and probe-set intensities were normalized using the variance stabilization and normalization (VSN) in the statistical data analysis environment of R, version 2.2.0, as explained earlier (8). The expression data of Reh leukemic cells after enforced expression of miRNAs is deposited in GEO, reviewer link at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lpyljsewycoosdg&acc=GSE46362

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Chapter 7

Summary and conclusion

Summary

For several decades, the central dogma of DNA → RNA → Protein stayed untouched as a holy principle. Since proteins are the final products involved in all cellular activities, protein-coding regions of DNA were of highest interest to scientists. Therefore, until the early nineties it was believed that the majority of non-protein coding DNA was non-functional and a waste of nucleotides. However, this concept was shown to be wrong in the last decennium by the discovery of non-coding RNAs, e.g. miRNAs [1]. The partial complementary binding of these small pieces of RNAs to their target mRNAs was shown to regulate the translation of mRNAs to proteins. Soon it was discovered that miRNAs are highly conserved [2] and are involved in fine-tuning of several cellular processes: i.e. it is reported that the expression of more than 60% of human protein-coding genes is regulated by miRNAs [3]. In less than 10 years our knowledge about miRNAs has grown extensively in the field of biogenesis, processing and function of miRNAs in either physiological or pathological conditions. However, the biggest challenge in the miRNA research yet remains: the identification of miRNA targets.

The most recent technologies available to predict and/or experimentally discover miRNAs target genes are summarized in **chapter 2**. The advantages and disadvantages of the most commonly used target prediction algorithms as well as possible experimental approaches to functionally study and discover miRNAs target genes are presented in that chapter. Moreover, the chapter contains the methods to validate a predicted/identified miRNA target gene in detail. Many computational target prediction algorithms have been developed in the past few years, but many questions about the ways miRNAs select and bind to their target genes are unclear, which is a bottleneck for the development of highly predictive algorithms. The findings of new fundamental mechanisms of miRNA action might help to reduce these artifacts and narrow down the huge list of miRNAs candidate target genes. We suggested that combination of a reliable target prediction tool with the recent state of the art developments in proteomics might open up new horizons in miRNAs target discovery. Since the majority of the studies to identify and explore the function of miRNAs were done in a non-hematological context and considering the fact that the biogenesis and function of miRNAs are tissue-specific [4], we hypothesized that using the most recent and sensitive method of high-throughput sequencing (deep sequencing) to explore hematologic malignant samples might result in identification of new tissue-specific miRNAs. Our results, which are presented in **chapter 3**, revealed 28 novel and 432 candidate novel miR-genes in 98 hematological samples of seven subtypes of ALL and three types of normal hematopoietic cell samples. In addition, the expression of 470 known miRNAs was identified confirming the value of high-throughput sequencing to discover miRNAs expressed in leukemic cells of patients. Many of these known and newly identified miRNAs showed a discriminative expression pattern between subtypes of childhood ALL and normal hematopoietic cells. Furthermore, newly discovered miRNAs were less conserved and less abundantly expressed which suggest a tissue-

specific biogenesis and may explain why these miRNAs were not identified earlier in other cellular contexts. The aberrant expression levels of selected novel and candidate novel miRNAs were confirmed by stem-loop RTq-PCR. To further explore the expression pattern of miRNAs among different subtypes of ALL (in particular, newly identified *BCR-ABL1*-like ALL [5]), expression levels of >600 miRNAs of leukemic cells were measured in 32 samples taken from children with ALL by real-time based miRNA arrays. **Chapter 4** contains the results of this study. This chapter discusses the identification of a miRNA signature indicative of *BCR-ABL1*-like ALL. Although hampered by limited sample size and the fact that the patients were treated with different treatment protocols, none of these individual miRNAs nor the cluster signatures were predictive for an unfavorable outcome. Interestingly, leukemic cells of the patients with *BCR-ABL1*-like and *BCR-ABL1*-positive ALL had partially overlapping miRNA signatures which may imply an overlap in pathobiology of both disease types. In **Chapter 5,** we explore the function of miR-24, miR-126 and miR-365 in *TCF3*-rearranged ALL cells. These three miRNAs were previously found to be expressed at low levels in this type of childhood leukemia [6]. Moreover, there were controversies about the role of these miRNAs in regulation of cell cycle progression and apoptosis in different cellular contexts [7, 8]. We showed that individual or combined overexpression of miR-24, miR-126 and miR-365 in *TCF3*-rearranged leukemia did not affect the cell cycle distribution or amount of apoptosis. In addition, we applied a new method to integrate miRNA-mRNA expression data [9] of 37 newly diagnosed children with different subtypes of BCP-ALL to identify candidate target genes for these miRNAs. Enforced expression of miR-24, miR-126 or miR-365 (individually or in combination) did not reduce the expression levels of candidate target genes. We conclude that miRNAs function is highly tissue-specific, and therefore, a newly identified or defined biological target gene/function of a miRNA in a specific tissue cannot be generalized to other cellular contexts. Finally, **chapter 6** contains our results about the role of miRNAs in vincristine (VCR) resistance in childhood ALL. We earlier reported that miR-125b, miR-100 and miR-99a are highly expressed in VCR-resistant leukemic cells of children with ALL [6]. Here, we showed that while individual expression of these three miRNAs does not affect the resistance level to VCR in *ETV6-RUNX1*-positive ALL cells, combined expression of miR-125b with miR-100 and/or miR-99a significantly induced VCR-resistance. In addition, enforced combined expression of these miRNAs resulted in significant downregulation of eleven candidate target genes (including four ribosomal proteins) in VCR-resistant ALL cells. The concerted function of miR-125b in combination with miR-99a and/or miR-100 illustrates the complexity of vincristine-resistance in pediatric ALL. The next step is to explore whether their directly regulated target genes (e.g. ribosomal proteins) and affected pathways may point to a way to modulate VCR resistance in *ETV6-RUNX1*-positive ALL.

Final conclusion

This thesis demonstrates that aberrant miRNAs can be found in childhood ALL. We identified new novel (candidate) miRNAs in hematological context with an

altered expression compared to normal hematopoietic cells suggesting a potential function in leukemogenesis. Our functional studies revealed that a concerted action of three miRNAs induced VCR-resistance in childhood ALL, whereas single miRNAs cannot alter the resistance to VCR of leukemic cells. This finding may be beneficial for treatment of drug-resistant ALL. We also showed that the function of miRNAs is tissue-dependent and this complicates generalizing the validated functions/targets of a miRNA in one type of tissue to another context. MiRNA signatures were not predictive for the poor clinical outcome associated with *BCR-ABL1*-positive and *BCR-ABL1*-like ALL. However, the observed overlap in miRNA signatures of *BCR-ABL1*-positive and *BCR-ABL1*-like ALL may point to an overlap in pathobiology of these two types of ALL. Taken together, our findings suggest that the complex function of miRNAs in initiation and progression of leukemia needs further investment. In this respect, discovery of the biological genes/pathways which are deregulated through aberrant expression of miRNAs might be the key to further clarify the mystery of ALL.

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Chapter 8

Discussion

MiRNAs and challenges in target genes identification

For several years, the non-protein-coding regions of DNA were assumed as non-coding or "junk" regions. However, in some cases these non-coding DNAs were transcribed to RNA molecules. Since these RNAs were not transcribed to proteins, they were known as non-coding or non-functional RNAs. In 1993, two seminal manuscripts reported the first evidence of functionality of these types of RNAs: some small non-coding RNAs were shown to bind mRNAs via antisense RNA-RNA interaction and therefore, resulted in post-transcriptionally gene silencing [1, 2]. The knowledge about the biogenesis and mechanism of action of these ~22-nucleotides small RNAs developed rapidly and introduced a new class of highly conserved gene expression regulatory molecules, called microRNAs (miRNAs). Yet, identification of direct targets for miRNAs remains the major challenge in miRNA research [3]. According to our current knowledge, miRNAs bind to their target sequence by a partial base-pair complementarity. It is known that miRNAs mainly bind to the 3' untranslated regions (UTRs) of target mRNAs and a stringent complementarity between the miRNA "seed" sequence (nucleotide 2-7 at 5' end) and the target mRNA is mandatory. This binding will decrease the production of target protein via either degradation of mRNA or translation inhibition. However, recently it is found that miRNAs can also bind to other parts of the mRNA: 5' UTR and exonic regions [4-6]. Moreover, in some cases miRNA can efficiently bind to a target sequence via partial complementary seed-pairing (seed-mismatch), especially when additional complementarity between the 3' end of miRNA and its target sequence are present [6-9]. These new binding criteria for miRNAs introduce a new challenge for the majority of computational prediction algorithms which are mainly designed for target gene prediction based on stringent seed-pairing [10]. Therefore, functional experiments are needed to identify miRNAs target genes and their biological impact. One of the most straightforward attempts to have a better biological clue of miRNA-mRNA interaction is to modulate the expression level of one or a subset of miRNAs and then measure the effect on expression levels of genes by gene expression profiling. However, a considerable number of target mRNAs do not degrade upon miRNA binding and therefore, these target genes are not identified by gene expression profiling. Another caveat of miRNA target identification by enforced expression of a miRNA is the resulting supraphysiological increase in the expression level of miRNA, which enhances the chance of off-target effects [11, 12]. A practical solution can be the application of anti-miRs (small RNA sequences complementary to miRNAs) to scavenge the miRNA of interest. This approach, however, has the disadvantage that a miRNA-anti-miR duplex is not easily degraded and therefore, the effective reduction in miRNA levels cannot be quantified properly using the commonly referred technologies and hence the experiment cannot be controlled.

Several biochemical methods were suggested to identify direct targets of miRNAs, e.g. co-immunoprecipitating miRNA-target sequence by targeting proteins of RISC complex (e.g. AGO) [13], isolation of miRNA-target sequence by crosslinking immunoprecipitation (HITS-CLIP or PAR-CLIP) [14-17] and using biotin-tagged miRNAs to pull-down miRNA-target sequence [18]. However, sensitive and specific antibodies against AGO proteins

as well as bioinformatics pipelines are the main challenges of these approaches. Application of recent proteomics technologies, such as mass spectrometry following stable isotope labeling of amino acids in cell culture (SILAC), might be a tool to identify changes in protein levels upon modulation of miRNAs expression levels [19]. However, these inclusive approaches need time-consuming development and optimization as well as complex bioinformatics analyses. In general, molecular biological technologies considerably increased our knowledge about miRNAs and their function, but tissue-specific characteristics of miRNAs and lack of suitable cell line models on top of the specific pitfalls of each technology yet remain as major challenges.

MiRNAs as classifier tools

In 2007, Zanette et al showed that a miRNA signature obtained from pooled bone marrows of 7 adults with different types of ALL differs from that of pooled healthy $CD19^+$ precursor B-cells purified from peripheral blood of six healthy individuals [20]. Subsequently, Ju et al profiled the miRNA expression levels of 40 newly diagnosed BCP-ALL children and reported high expression levels of miR-142-3p, miR-222 and miR-339 as well as low expression levels of miR-373* and miR-451 [21]. We also showed earlier that miR-30e-5p, miR-34b, miR-128a, miR-142, miR-150, miR-181, miR-193, miR-365, miR-582 and miR-708 were upregulated while let7-a, miR-99a, miR-100, miR-125b, and miR-196b were downregulated in leukemic cells of children with ALL compared with normal $CD34^+$ cells [22]. Comparison of miRNA signature of B-lineage versus T-lineage leukemia revealed that low expression levels of miR-151 as well as high expression levels of miR-148a and miR-424 are characteristics of T-lineage leukemia [23]. Interestingly, we identified a unique miRNA signature for each genetic subtype of pediatric BCP-ALL except for *BCR-ABL1*-positive and unclassified B-other cases [24]. Using a sensitive technology of high-throughput sequencing, we identified 16 novel and 170 candidate novel miRNA-miRNA* strands which were only expressed in ALL as well as 2 novel and 82 candidate novel miRNA-miRNA* strands which were unique for normal hematopoietic cells (**chapter 3**) [25]. The expression level of these novel (candidate) miRNAs was lower than known miRNAs. Some of these newly identified miRNAs were differentially expressed among genetic subtypes of BCP-ALL such as sol-miR-15 (later-on annotated by miRBase as hsa-miR-3151), which was significantly downregulated in *TCF3*-rearranged ALL [25]. In addition, we identified that the novel sol-miR-23 (later-on annotated by miRBase as hsa-miR-4474) is 10-fold lower expressed in leukemic cells of children with BCP-ALL compared to normal $CD-34^+$-sorted cells. We found a significant inverse correlation between expression levels of this miRNA with anti-apoptotic *BCL2*. This gene is also predicted as a potential target for novel sol-miR-23 using TargetScan (www.targetscan.org). These newly discovered miRNAs were identified in fresh leukemic cells taken from patients and $CD34^+$ hematopoietic progenitor cells and their abnormal expression levels in leukemic cells may therefore point to their contribution in pathogenesis of leukemia. We extended our studies [24] to determine whether miRNAs are also discriminative poor prognostic subtype of *BCR-ABL1*-like ALL [26]. Our results revealed that the miRNA signature of *BCR-ABL1*-like

ALL showed a partial overlap with that of *BCR-ABL1*-positive ALL (**chapter 4**). Since the gene expression signature of *BCR-ABL1*-positive and *BCR-ABL1*-like ALL also overlap [26], similarities in miRNA expression patterns of these patients may point to an overlap in pathobiology of these two subtypes of childhood ALL.
The recent findings support the hypothesis that miRNA signatures have the strong potential to identify biological differences among the subtypes of tumors that develop in association with diverse genetic backgrounds. The subtype-specific miRNA signature would ultimately give insights into the fundamental biology of human malignancies, however, clinical applications of miRNAs as classifiers is still under further adjustment and needs more validation.

MiRNAs as tissue-specific players

MiRNAs transcription, biogenesis/maturation, and function are differently controlled in tissues from different origins [27]. As an example, MYC and MYCN both stimulate expression of the miR-17-92 oncogenic cluster in lymphoma cells [28] and miR-9 in neuroblastoma cells [29], but these oncogenes inhibit the expression of several tumor suppressor miRNAs, such as miR-15a [30]. It is known that accumulation of the Dicer protein depends on the expression levels of TRBP and downregulation of TRBP leads to destabilization of Dicer [31]. The mutations, which diminish the expression levels of TRBP in human neoplasia, impair Dicer function [32]. The functionality of miRNAs is also controlled in downstream levels of biogenesis as recent data suggested that the miRNAs might be regulated via the changes in balance between different types of AGO proteins [33]. Moreover, the function of miRNAs can be modulated at the level of binding to the target mRNA. For example, RNA-binding protein Dead-End1 (DND1) binds to specific mRNAs and therefore, protects mRNA against miRNA-binding [34]. Interestingly, DND1 is differentially expressed in some cancer cells compared to healthy tissues which indicate another level of regulation of miRNAs function [35].

One specific miRNA may behave differently in two types of tissues. It was previously shown that miR-221 and miR-222 suppress cellular proliferation in erythroblastic leukemia while the same miRNAs promote cell growth and survival in solid tumors [36]. We earlier discovered that miR-24, miR-126 and miR-365 are significantly lower expressed in leukemic cells of children with *TCF3*-rearranged ALL [24]. There are several reports about the regulatory function of these three miRNAs in cell cycle progression and apoptosis in solid tumors [37-42]. However, there are strong controversies about the function of these miRNAs in different cellular contexts: e.g. miR-24 which can inhibit the proliferation of K562 chronic myeloid leukemic cells [43], promotes cellular proliferation of primary keratinocytes as well as different cancer-derived cell lines (gastric carcinoma, thyroid carcinoma and lung cancer cell lines) [44]. We showed that individual and combined expression of miR-24, miR-126 and miR-365 in *TCF3*-rearranged leukemia did not alter the viability of three different cell line models (**chapter 5**). Further, integration of miRNA and mRNA expression levels of 37 newly diagnosed children with BCP-ALL (including 3 *TCF3*-rearranged ALL cases) revealed significant association between the expression of 29 miRNAs and their predicted

target genes including miR-24 (ranked at position #5), miR-126 (ranked at position #17) and miR-365 (ranked at position #20). However, enforced expression of miR-24, miR-126 or miR-365 (individually or in combination) did not reduce the expression level of candidate target genes in MHH-CALL-3 leukemic cells. This suggests that the observed significant inverse correlation between some miRNA-mRNA pairs might mainly originate from other subtypes with higher number of cases in this cohort, such as hyperdiploid (13 cases) or unclassified B-others BCP-ALL (10 cases). In addition, ectopic expression of miR-24 did not affect the expression levels of *AURKB* in the MHH-CALL-3 leukemic cell line whereas the control experiment showed that enforced expression of miR-24 significantly reduced the expression levels of *AURKB* in HEP-G2 hepatocarcinoma cells, confirming a previous study [43].

Taken together, our findings illustrate the complex and tissue-specific function of miRNAs and warn against generalizing data about miRNA-target gene interactions to all tissue types. Therefore, it is suggested to explore miRNAs function in one type of tissue to avoid tissue-specific differences in miRNAs expression, maturation, and target recognition. Reported controversies emphasize the application of proper cell line models in miRNAs research. This issue must be also taken into account in miRNA target gene identification and validation. As an example, we could validate *FBW7* as biological target of miR-27a by a significant reduction of luciferase signal in MCF7 human breast adenocarcinoma and U2OS human bone osteosarcoma epithelial cells but not in HEK293 human embryonic kidney cells [45].

MiRNAs as outcome predictive tools

As miRNA expression profile of malignant cells differs from healthy cells, the expression pattern of one miRNA or a subset of miRNAs may be used for outcome prediction. As an example, the expression profile of 5 miRNAs (Let-7a, miR-137, miR-182, miR-221 and miR-372) is predictive for prognosis of non-small cells long carcinoma [46]. Similarly, increased overexpression of miR-196a predicts poor survival of the patients with pancreatic ductal adenocarcinoma [47], and miR-21 expression level is predictive for tumor aggression in pancreatic cancers [48]. Not only in solid tumors but also in different types of hematological malignancies, miRNAs are associated with outcome of patients: increased expression levels of miR-221 is predictive for shorter overall survival in T-ALL [49], and expression patterns of four miRNAs (miR-22, miR-92a, miR-146a and miR-330-3p) are correlated with clinical outcome in plasma cell leukemia [50]. Similarly, a miRNA signature is associated with prognosis in acute myeloid leukemia [51]. We previously showed that in pediatric ALL a subset of 14 miRNAs (miR-10a, miR-33, miR-134, miR-214, miR-215, miR-369-5p, miR-484, miR-496, miR-518d, miR-572, miR-580, miR-599, miR-624 and miR-627) is subtype-independently predictive for the clinical outcome [24]. Another study showed that miR-146a, miR-181a/c and miR-221 are significantly associated with survival in ALL [52]. MicroRNA-3151, which was recently discovered by our group (**chapter 3**) [25], is also shown to have inverse correlation with disease-free survival and overall survival in patients with cytogenetically normal acute myeloid leukemia [51]. These findings

emphasize the impact of miRNAs and their expression levels on clinical outcome of children with leukemia.

Currently, 5 years event-free survival in children with BCP-ALL is above 80% [53], yet the highest absolute number of relapses still occurs in patients diagnosed as genetically unclassified BCP-ALL [54]. We recently identified a poor prognostic group of patients within the unclassified B-others, which is characterized with frequent aberrations in B-cell development genes (such as *PAX5* and *IKZF1*) and gene expression profile similar to *BCR-ABL1*-positive ALL [26]. MiRNA expression profiling showed a partial overlap between this so-called *BCR-ABL1*-like ALL and *BCR-ABL1*-positive ALL. However, none of the 10 most discriminative miRNAs for *BCR-ABL1*-like patients differed in expression between patients who relapsed and those who remained in continuous complete remission (**chapter 4**). Majority of the cases with *BCR-ABL1*-like ALL (11 out of 15) has the miRNA signature similar to 6 out of 14 cases with *BCR-ABL1*-positive ALL and different from other known genetic subtypes of childhood BCP-ALL. There was no significant different in the clinical outcome of these patients (cluster-I) with the remaining cases of *BCR-ABL1*-like and *BCR-ABL1*-positive ALL (cluster-II). This could be due to limited sample size and differences in treatment protocols among the patients. Moreover, the frequencies of aberration in prognostic genes such as *IKZF1*, *PAX5* and *CDKN2A*/*B* did not significantly differ between these two clusters. Although miRNA signature, in contrast to genetic and mRNA signature, cannot be used as predictive tools in these types of childhood ALL, outcome prediction impact of miRNAs signature of patients with different types of solid malignancies is currently under investigation. Several experiments are still needed to validate the clinical impact of observed associations between the expression pattern of oncogenic or tumor-suppressor miRNAs and patients' outcome.

MiRNAs as micro-players in drug resistance

Cancer drug resistance remains a major clinical obstacle. Moreover, serious side effects of currently available medications emerge the needs for finding new targets for treatment. Better understanding of the mechanisms of drug resistance in cancer and the molecules/pathways, which are involved in induction of drug resistance, can help scientists to design new therapeutic strategies. Recently, increasing number of reports indicated the possible role of miRNAs in drug resistance. As an example, miR-221 and miR-222 reduced expression levels of *CDKN1B* (previously known as *p27kip1*) which resulted in resistance to tamoxifen in breast cancer cells [55]. In addition, doxorubicin resistant breast cancer is associated with functional interaction between several miRNAs and their target genes such as miR-127/*bcl-6*, miR-34a/NOTCH1, miR-27b/CYP1B1, miR-28/BRCA1 and miR-451/MDR1 [56].

In pediatric ALL, low expression levels of miR-708 were found in leukemic cells of the prednisone poor responder patients and were associated with impaired disease-free survival [57]. High expression levels of miR-363 in acute myeloid leukemia resulted in downregulation of RGS17 and HIPK3 and were associated with chemoresistance [58]. Our previously reported results revealed that expression levels of several

miRNAs are associated with vincristine and daunorubicin resistance in pediatric ALL [24]. According to our results, high expression levels of miR-99a, miR-100, miR-125b, miR-126, and miR-126* were found in the leukemic cells of children with vincristine-resistant and daunorubicin-resistant ALL [24]. Enforced expression of miR-125b, miR-100 and miR-99a as single miRNAs in *ETV6-RUNX1*-positive Reh leukemic cells did not affect the level of VCR-resistance of these cells (**chapter 6**) [59]. In contrast, co-expression of miR-125b with miR-100 and/or miR-99a significantly induced resistance to VCR in *ETV6-RUNX1*-positive Reh cells; ≥80% of the cells remained alive upon VCR exposure for any combination compared to only <40% of miR-125b-transduced cells. Remarkably, in the absence of VCR, the apoptosis rate and cell cycle distribution did not differ between Reh cells expressing miR-125b together with miR-99a and/or miR-100 compared to those expressing miR-125b in combination with scrambled miRNA control. This suggests that the combined overexpression of miR-125b, miR-99a and/or miR-100 is a specific trigger for developing resistance to VCR in ALL. Gene expression profiling revealed that 11 genes, including 4 genes encoded ribosomal proteins, were significantly downregulated in cells expressing high levels of miR-125b together with miR-100 and/or miR-99a compared to miR-125b and scrambled miR-control transduced cells. Interestingly, the expression levels of 7 out of these 11 genes (including all the ribosomal protein-coding genes) were reduced in primary leukemic cells of children who were *in vitro* resistant to VCR compared to that of *in vitro* sensitive children. The role of ribosomal proteins in VCR-resistant leukemia has not yet been functionally studied, but the present study as well as our previous report in a different set of pediatric ALL patients [60] underlines the importance to address the functional role of ribosomal proteins in VCR resistant leukemia. The genes encoding miRNA-125b, miR-100 and miR-99a are located closely on the human genome and the expression levels of these three miRNAs showed a significant correlation. Therefore, their collaborative function is not surprising, however, the concerted action of miR-genes was also shown for miRNAs located on different chromosomes. For example, the expression pattern of *ATXN1* in spinocerebellar ataxia type 1 is controlled by co-expression of miR-19, miR-101 and miR-130 [61]. In brain tissue, miR-7 and miR-153 co-regulate the expression of α-Synuclein in Parkinson disease [62]. These examples illustrate the biological importance of miRNAs in combination. Considering this fact may help scientists to approach miRNAs function not only as a result of single/separate modulators but also as teamwork. This complicates the experimental design to mimic or inhibit miRNAs biological function. However, recent development of “miRNA sponges” provides the opportunity to inhibit the expression of multiple miRNAs efficiently [63]. MiRNA sponges are short-oligonucleotide sequences which contain repetitive miRNA binding sites and are capable of scavenging several miRNAs at once. On the other hand, concomitant overexpression of a miRNAs cluster or related miRNAs with different chromosomal origins may be achieved by lentiviral transduction technologies. These recent technical developments, together with our better understanding of the function of miRNAs in chemoresistant malignancies, may provide new opportunities to successfully reprogram disregulated miRNAs networks and alter drug-resistance profiles of cancer cells.

MiRNAs as potential diagnostic tools and therapeutic targets

Since aberrant expression levels of miRNAs were associated with initiation, progression and outcome of human malignancies, these tiny molecules may serve novel candidates for early diagnosis of tumors [64]. In addition to the tumor-specific miRNA expression signature of a tissue, the expression pattern of secreted miRNAs in various body fluids can be used as biomarkers for early diagnosis of malignancy [65]. As an example, expression levels of Let-7 in tissue specimens [66, 67] and plasma expression levels of miR-21, miR-155 and miR-145[68] can be used for early detection of lung cancer. Similarly, aberrant tissue expression levels of a subset of miRNAs including miR-99a, miR-100 and miR-126* [69] as well as upregulated expression levels of circulating miR-195 [70] were associated with early diagnosis of breast cancer. Recently, downregulation of circulating miR-30 was suggested as potential diagnostic biomarker for breast cancer [71]. Dysregulated expression level of miR-26b-5p in blood as well as miR-1255b-5p in urine can be indicative for invasive bladder tumors [72].

In hematological malignancies, new findings support the diagnostic value of Let-7b, miR-233, miR-181a and miR-128 in acute leukemias [73]. Similarly, plasma levels of miR-150 and miR-342 were introduced as novel potential biomarkers for acute myeloid leukemia [74]. Our previous results revealed that miRNA expression signature in pediatric BCP-ALL is significantly different from childhood T-cell ALL and normal $CD34^+$ bone marrow cells of healthy individuals [24]. This finding, together with distinct miRNA signature for genetic subtypes of pediatric BCP-ALL can potentially be applied as diagnostic tool in the suspected cases [24]. Similarly, other studies [75-77] showed that miRNAs have strong potentials to advance cancer diagnosis and stratification beyond currently available methods, especially for those malignancies for which there is yet no reliable biomarker available. However, integration of miRNA expression analyses for cancer diagnosis (in particular leukemias) in daily practice remains challenging and needs further validation.

The role of circulating miRNAs, which can transfer a message from one cell to another, is a new developing topic of miRNA research. Tumor-derived miRNAs that are detectable in various body fluids are remarkably stable. There is a growing body of evidence to support circulating miRNAs as non-invasive sensitive biomarkers for cancers, especially solid tumors such breast, lung, prostate, pancreas and ovarian malignancies. As an example, significant increase in circulating expression levels of miR-195 (15-fold) and let-7a (5-fold) was suggestive for breast cancer in suspected cases [70]. Recent studies are however more focused on the possible ways of cell-to-cell communications by means of miRNAs. It is believed that the majority of secreted miRNAs are packed in extracellular vesicles which are characterized with a bilayer lipid membrane [78], however, miRNAs coated with proteins (e.g. AGO proteins) or miRNAs associated with high-density lipoproteins are also detected in plasma [78]. These miRNAs have the potentials as biomarkers of disease [79]. The use of exosomes and other extracellular vesicles as therapeutic delivery vesicles is extensively under study. It is shown that intravenous injection of THP1-macrophages, which were transfected with a chemically modified miRNA, into tumor-implanted nude mice resulted in an

increase in serum exosomes containing the modified miRNA and therefore, detectable expression of modified miRNA in the serum, kidney and tumors of the hosted animals [80]. The increasing number of fundamental research in parallel with translational studies about miRNAs provides a more clear picture of how miRNAs are involved in pathobiology of cancer. However, we are still at the start of a long way to apply miRNA mimics or inhibitors as therapeutic targets against cancer.

As abnormal expression pattern of several miRNAs is correlated with development and progression of human disorders, multiple studies demonstrated that the modulation of one or a subset of specific miRNAs using miRNA replacement (i.e. miRNA mimics technologies) or miRNA removal (i.e. anti-miR technologies) can restore physiological function of the miRNAs and repair the defected pathways in cancer cells. For efficient and specific target delivery of miRNAs or anti-miRs, viral strategies, tissue-specific non-viral vectors and nano-particles have been developed. Some miRNAs regulate expression of genes related to drug-resistance and therefore, downregulation or re-expression of these miRNAs could modulate the sensitivity of cancer cells to anti-cancer drugs. For example, enforced expression of miR-15 and miR-16 maintain the sensitivity of gastric cancer cells to chemotherapy through inhibition of BCL2 [81], or knockdown of miR-21 resulted in inhibition of cellular proliferation and tumor growth in breast cancer cell lines *in vivo* [82]. One of the first successful applications of miRNA-knockdown in non-human primates was performed on African green monkeys with hypercholesterolemia using intravenous injection of locked nucleic acid-modified anti-miR-122 [83]. Significant downregulation of miR-122 without severe side effects resulted in re-expression and function of aldolase A and therefore, controlled high triglyceride and cholesterol level and other manifestations of the disease [83]. Recently, it is shown that systematic application of locked nucleic acid anti-miR-155 oligonucleotides in Waldenstrom macroglobulinemia-engrafted mice significantly reduced the level of miR-155 and inhibited tumor growth [84]. Systemic administration of antisense oligonucleotide against miR-122, a liver-enriched miRNA, in mice and primates was shown to control lipid metabolism and hepatitis C viral load, and reduce liver damage [85-88]. These results introduced a new field of research in developing more sensitive and powerful medications to overcome different human diseases and malignancies. A growing body of evidence revealed that similar to many regulator proteins, several miRNAs could also collaborate to target one gene or a series of genes and regulate a cellular function. Therefore and as we studied the collaboration of miR-125b with miR-100 and/or miR-99a in induction of vincristine resistance in childhood BCP-ALL (**chapter 6**) [59], manipulation of the expression levels of multiple miRNAs at once can be a new strategy to explore the possible therapeutic impact of miRNAs in drug-resistant malignancies. However, there is no report so far on clinical trial about using miRNAs to regulate drug-resistance in human cancer (www.clinicaltrial.gov).

Regulation of the regulators

Rapid developments in technologies provide new opportunities for better understanding of miRNAs biogenesis and function. It has been shown that expression

of miRNAs as post-transcriptional gene expression regulators is controlled via different mechanisms. Genomic lesions of miR-genes (amplifications, deletions, mutations and translocations) have been found in cancer [89, 90]. Inter-genic miRNAs have committed promoters with common features of transcription such as CpG islands, histone marks, transcription factor biding sites, *etc.* Stimulation of cells with bone morphogenic protein (BMP) or tumor growth factor beta (TGF-β) may activate Smad proteins and result in efficient cleavage of specific miRNAs by Drosha [91]. In contrast, estrogen receptor alpha (ERα) can associate with microprocessor complex and inhibit the cleavage of other miRNAs [92]. In addition, the stability of RISC complex may regulate the expression level of mature miRNAs. It has been shown that Lin-41 acts as specific ubiquitin ligase for Ago proteins in mice cells and therefore, results in global reduction in miRNA-mediated gene silencing [93]. Aberrations in other genes can also affect the expression of miRNAs. There are numerous conserved pseudogenes and many of them contain 3' UTRs with conserved miRNA binding sites. These RNA sequences can act as decoys or biological miRNA sponges scavenging mature miRNAs and therefore, inhibit binding of these miRNAs to their cognate target mRNAs. These natural scavenging RNAs have been termed competing endogenous RNAs (ceRNAs) [94]. In this light, mutations or copy number loss of pseudogene *PTEN1* may disrupt the miRNA scavenging function of this gene and resulted in an increase in bioavailability of the miRNAs which target *PTEN* itself [95]. Similar to protein-coding genes, epigenetic alterations can affect the expression of miRNAs as well [96]. Treating SEMK2 t(4;11)-positive BCP-ALL cells with demethylating agent zebularine restored the expression of miR-200b, miR-200a and miR-429 as well as miR-424 and miR-503 [96], while the upstream regions of these miR-genes were hypermethylated before treatment. These findings open a new window for clinical application of miRNAs as possible therapeutic targets. Protein expression level of TRBP [31, 32] and balance between different types of AGO proteins [33] were also shown to be involved in regulation of miRNAs function. In addition to genomic and epigenetic changes, expression of other types of non-coding RNAs such as long non-coding RNAs (300 nt-100 kb, lncRNAs) can interact with miRNAs function [97]. It has also been reported that the miRNAs function may alter upon expression of RNA-binding proteins such as DND1 [34, 35]. Another RNA-binding protein, KH-type splicing regulatory protein (KHSRP) binds with high affinity to the conserved terminal loop region of pri-let-7a and is necessary for microprocessor cleavage [98]. The stimulatory role of KHSRP is antagonized by hnRNPA1, which binds to the same region in pri-miRNAs and therefore, competes with KHSRP binding [99]. Recently, it has been shown that mature miRNAs can regulate the biogenesis of other miRNAs. The first evidence was obtained in mouse cells where miR-709 was shown to negatively regulate biogenesis of miR-15a/16-1 via direct interacting with pri-miR-15a/16-1 and inhibiting the Drosha processing step [100]. These findings sound exciting and need further exploration to understand the impact of non-coding RNAs for the pathogenesis of diseases.

Final conclusion

There is no doubt that miRNA research is one of the hot topics in current

fundamental and translational research world. Attention has been paid to miRNAs as an effective tool to regulate the expression of target genes, as well as a “magic bullet” to suppress tumor progression. However, our knowledge about miRNAs is still in its infancy. For instance, whether all target genes of a miRNA are equally important still remains unclear. In addition, it is important to understand whether and how multiple miRNAs collaborate in a biological process. Moreover, application of miRNAs knowledge in cancer diagnosis, prognosis and treatment hardly resulted in an improved survival rate which is partially due to our still limited understanding of miRNA-miRNA, miRNA-target genes and miRNA-other regulators interactions.

The knowledge available in literature combined with findings reported in this book indicates that the biogenesis and role of miRNAs in childhood ALL is complicated. Therefore, studying miRNAs in leukemia remains challenging. It is important to realize that miRNA research not only provides a powerful tool to study the fundamental mechanisms of gene expression regulation, but may also broaden our view how to develop new therapeutic agents affecting gene regulation. Given the fact that still one out of five children relapses from ALL and the fact that the late effects of combination chemotherapy are considerable, any approach to improve treatment outcome is worth trying!

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About the Author

Farhad Akbari Moqadam was born in 22 July 1978, in the historical city of Yazd in Iran. He received his diploma in “Biology and Natural Sciences” from the high school of National Organization for Development of Exceptional Talents (NODET) in 1996. He enrolled the medical education program at Shiraz University of Medical Sciences, Shiraz, Iran and was granted with the MD degree at September 2003. Farhad learned the principles of biomedical researched in the department of Immunology and preformed his MD thesis in this department entitled “CTLA-4 Gene Polymorphisms in Southern Iranian Patients with Behçet’s Syndrome”.

His ambitions brought him to join several research groups across the country and he participated in different fundamental, translational and clinical research projects. Meanwhile, Farhad extended his clinical experiences by working as a physician in some small local medical institutes as well as in famous specialized hospitals in Shiraz such as *Dena* and *Shafa*. Although there were plenty of opportunities to work and study in medicine, Farhad decided to leave the country in 2007 using the opportunity to attend the prestigious research group of Prof. Dr. Dan Grandér at Cancer Centrum Karolinska (CCK), Stockholm, Sweden. This program, which was focused on the discovery of the role of cis-antisense RNAs in development and progress of leukemias, significantly improved his practical experiences and basic knowledge of RNA-RNA interaction in leukemias.

Farhad left Grandér’s lab at May 2008 and joined the leading group of Dr. Monique den Boer at Sophia Children’s Hospital, Erasmus MC, Rotterdam, the Netherlands. His PhD project in den Boer’s lab was focused on discovery of the functions and targets of microRNAs in pediatric ALL and was under supervision of Prof. Dr. Rob Pieters. His research during 2008-2012 resulted in better understanding of the complex function of miRNAs in pediatric ALL. The findings were presented at several international congresses and were published in peer-reviewed journals. Farhad succeeded to develop and optimize an efficient and reproducible viral transduction procedure to achieve a high-yield overexpression method for miRNAs and other genes/RNAs in leukemic cells. He could also successfully develop a scrambled control negative sequence for his lentiviral transduced miRNA overexpression experiments. After finalizing the experimental works of his PhD project, Farhad got involved in a new research topic from September 2012 on exploring the function of extracellular vesicles (microvesicles and exosomes) in childhood ALL.

He is married to Azadeh Jamalian since 2009, who is a young scientist with interest in quantitative cancer proteomics and new biomarkers identification (using techniques such as nano-HPLC, Mass spectrometry, *etc.*) as has a postdoctoral fellow position in Thermo Fischer Scientific in collaboration with department of Neuro-oncology, Erasmus MC. Azadeh and Farhad are gifted a daughter named Mana, born at 23 May 2012. Farhad aims to extend his knowledge in translational research and combine it with his clinical experiences in future.

LIST OF PUBLICATIONS

- **MiRNA signature in *BCR-ABL1*-positive and *BCR-ABL1*-like childhood acute lymphoblastic leukemia: similarities and dissimilarities.**

  F. Akbari Moqadam, E.A.M. Lange-Turenhout, A. van der Veer, J.R.M. Marchante, J.M. Boer, R. Pieters, M.L. den Boer. *Accepted* in Leukemia & Lymphoma 2013;October (IF:2.3)

- **Altered expression of miR-24, miR-126, and miR-365 does not affect viability of *TCF3*-rearragned leukemic cells**

  F. Akbari Moqadam, J.M. Boer, E.A.M. Lange-Turenhout, R. Pieters, M.L. den Boer. *Accepted* in Leukemia 2013;October (IF:10.16)

- **MiR-125b, miR-100 and miR-99a co-regulate vincristine resistance in childhood acute lymphoblastic leukemia**

  F. Akbari Moqadam, E.A.M Lange-Turenhout, I.M. Ariës, R. Pieters, M.L. den Boer. Leuk Res 2013;37(10): 1315-21 (IF:2.76)

- **The hanting of targets: challenge in miRNA research**

  F. Akbari Moqadam, R. Pieters, M.L. den Boer. Leukemia 2013;27(1): 16-23 (IF:10.16)

- **Discovery of new miRNAs by small RNAome deep sequencing in childhood acute lymphoblastic leukemia**

  D. Schotte*, F. Akbari Moqadam*, E.A.M. Lange-Turenhout, C. Chen, W.F. van Ijcken, R. Pieters, M.L. den Boer. Leukemia 2011;25(9): 1389-99 (IF:10.16).
  Both first authors were equally contributed to this work.

- **MirR-27a controls FBW7/hCDC4-dependent cyclin E degradation and cell cycle progression**

  M. Lerner, J. Lundgren, S. Akhoondi, A. Jahn, H.F. Ng, F. Akbari Moqadam, J.A. Oude Vrielink, R. Agami, M.L. den Boer, D. Grandér, O Sangfelt. Cell Cycle 2011, 10 (13): 2172-83 (IF:5.24)

- **MicroRNA characterize genetic diversity and drug resistance in pediatric acute lymphoblastic leukemia**

  D. Schotte, De Menezes RX, F. Akbari Moqadam, L.M. Khankahdani, E.A.M Lange-Turenhout, C. Chen, R. Pieters, M.L. den Boer. Haematological 2011, 96 (5): 703-11 (IF:5.93)

- **Teracheobronchomalacia and air trapping after mustard gas inhalation**

  M. Ghanei, F. Akbari Moqadam, M.M. Mohammad, J. Aslani. Am J Respir Crit Care Med 2006. 173 (3): 304-9 (IF:11.04)

- **HLA class II gene polymorphism in Parsees and Zoroastrians of Iran**

  S. Farjadian, F. Akbari Moqadam, A. Ghaderi. Int J Immunogenet 2006, 33 (3): 185-91 (IF:1.35)

PHD PORTFOLIO

Farhad Akbari Moqadam MD, PhD student at Department of Pediatric Oncology-Hematology
Molecular Medicine Research School, PhD period: 9 June 2008 – 8 January 2014
Promotor: Prof. Dr. R. Pieters, Copromotor: Dr. M.L. den Boer

PhD training	Year	Work load (ECTS)
Courses and workshops		
Basic and Translational Oncology Course Biomedical Research Techniques VIII	2008 2009	1.5 1.5
Browsing Genes and Genome in Ensemble	2010	0.6
Annual PhD day	2010	0.3
Workshop for Wiring Successful Grant Proposals	2012	0.6
Biomedical English writing and communication	2012	4.0
Workshop for Photoshop and Illustrator C6	2013	0.6
Annual Molecular Medicine day (Erasmus MC)	2008-2013	1.0
Overview of PhD project; 6 Oral presentations each year at the weekly Pediatric Research Meetings and Pediatric Oncology research meetings	2008-2013	7.2
Poster Presentation		
Discovery of New microRNAs by small RNAome deep sequencing in childhood ALL (16th MolMed day, Erasmus MC)	2012	1.0
Collaborative function of miRNAs in vincristine-resistant childhood ALL (17th MolMed day, Erasmus MC)	2013	1.0
MiR-125b, miR-100 and miR-99a co-regulate vincristine resistance in childhood ALL (54th ASH annual meeting, Atlanta)	2012	1.0
MiRNA signature is not predictive for poor prognosis in *BCR-ABL1*-like childhood ALL (45th SIOP conference, Hong Kong)	2013	1.0
MiR-125b collaborates with miR-100 and miR-99a to induce vincristine resistance in pediatric ALL (45th SIOP conference, Hong Kong)	2013	1.0
MiRNA signature in *BCR-ABL1*-positive and *BCR-ABL1*-like childhood ALL, similarities and dissimilarities (55th ASH annual meeting, New Orleans)	2013	1.0
Enforced expression of miR-24, miR-126 and miR-365 does not alter cellular viability in *TCF3*-rearranged leukemia (55th ASH annual meeting, New Orleans)	2013	1.0
International conferences		
MicroRNA in Cancer conference (Keystone, USA)	2009	2.0
54th ASH annual meeting (Atlanta, USA)	2012	2.0
55th ASH annual meeting (New Orleans, USA)	2013	2.0
Training and supervising	**Year**	**Work load (ETCS)**
Supervising Larissa de Graaf, HLO students for 6 months	2009	10
Supervising Alex Neagu, master student in Applied Sciences for 2 months	2010	3
Supervising Koen Bezemer, HLO student for 6 months	2011	10
Total		**53.3**

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The mission has now been officially completed!

At the beginning of this long road when I started my PhD project in 2008 at a group famous for its high demands for graduation, it was but a dream to see this moment. Like every other PhD project, I faced several difficult moments during my work which could be crushing for such an emotional and impatient person like me, however, having good friends, nice colleagues and a supportive family made life much easier! Apart from my own efforts, the success of this project depends largely on the encouragement and contributions of many others. I would like to take this opportunity to express my gratitude to these people who have been instrumental in the successful completion of this work.

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Before visiting you in person, I did not believe it to be possible to be both a successful clinician and, at the same time, run great in-depth basic and translational research projects. Thank you for being such a great exceptional example. I cannot say thank you enough for your tremendous support and help. Every time I attended our regular meetings, I felt motivated and encouraged. Without your trust and guidance, it would not have been possible to end this challenging project with such quality.

Dear **Monique**;

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You are a super-genius talented scientist who has great ideas and big dreams, perfectly knows how to approach and hit the question and is granted with exceptional abilities in presenting the visions and explaining the most complex concepts in an easily understandable way. In addition, I found you a person who knows herself and believes in her potentials and most importantly benefits from a marvelous supporting loving family and an endless network of good friends and great scientists. I won’t be surprised if I hear that your name is announced among the top scientists of the world in couple of years. I am so proud of doing my PhD under your supervision and would be so grateful to remain a friend to you and your family. I would like to extend my gratitude to your husband, **Etienne**, who is a good friend and our special artist. I wish you two

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Farhad, January 2014

ای عاشقان،ای عاشقان، آنکس که بیند روی او
شوریده گردد عقل او، آشفته گردد خوی او

معشوق را جویان شود، دکان او ویران شود
بر رو و سر پویان شود، چون آب اندر جوی او

در عشق چون مجنون شود، سرگشته چون گردون شود
آن کو چنین رنجور شد، نایافت شد داروی او

مولانا