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

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Acknowledgments: we thank Dr. F. J.T. Staal (Department of Immunology, Erasmus MC, Rotterdam, NL) for providing thymocyte samples. Dr. M.W.J. Luijendijk (Department of Pediatric Oncology, Erasmus MC, NL) and Prof. Dr. E.F. Petricoin (Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, VA, USA) are warmly acknowledged for their contribution to the reverse-phase protein detection studies. We thank Prof. dr. J.P.T.M. van Leeuwen (Department of Internal Medicine, Erasmus MC, NL) for sharing the Taqman Array block.

Funding: this research was financially supported by the Netherlands Organization for Scientific Research (NWO-Vidi Grant; MLdB), the Quality of Life Foundation and the Pediatric Oncology Foundation Rotterdam.

Manuscript received on April 8, 2010. Revised version arrived on January 10, 2011. Manuscript accepted on January 12, 2011.

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The online version of this article has a Supplementary Appendix.

ABSTRACT

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

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

Results
All major subtypes of acute lymphoblastic leukemia, i.e. T-cell, *MLL*-rearranged, *TEL-AML1*-positive, *E2A-PBX1*-positive and hyperdiploid acute lymphoblastic leukemia, with the exception of *BCR-ABL*-positive and 'B-other' acute lymphoblastic leukemias (defined as precursor B-cell acute lymphoblastic leukemia not carrying the foregoing cytogenetic aberrations), were found to have unique microRNA-signatures that differed from each other and from those of healthy hematopoietic cells. Strikingly, the microRNA signature of *TEL-AML1*-positive and hyperdiploid cases partly overlapped, which may suggest a common underlying biology. Moreover, aberrant down-regulation of let-7b (~70-fold) in *MLL*-rearranged acute lymphoblastic leukemia was linked to up-regulation of oncoprotein c-Myc (P_{FDR}<0.0001). Resistance to vincristine and daunorubicin was characterized by an approximately 20-fold up-regulation of miR-125b, miR-99a and miR-100 (P_{FDR}≤0.002). No discriminative microRNA were found for prednisolone response and only one microRNA was linked to resistance to L-asparaginase. A combined expression profile based on 14 microRNA that were individually associated with prognosis, was highly predictive of clinical outcome in pediatric acute lymphoblastic leukemia (5-year disease-free survival of 89.4%±7% *versus* 60.8±12%, P=0.001).

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

Key words: miRNA, genetic subtype, drug resistance, prognosis, pediatric leukemia.

Citation: Schotte D, De Menezes RX, Moqadam FA, Khankahdani LM, Lange-Turenhout E, Chen C, Pieters R, and Den Boer ML. MicroRNA characterize genetic diversity and drug resistance in pediatric acute lymphoblastic leukemia. Haematologica 2011;96(5):703-711. doi:10.3324/haematol.2010.026138

Introduction

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

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

Design and Methods

Patients' samples

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

Drug resistance

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

Expression analysis

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

Expression levels of 397 miRNA were analyzed by stem-loop RT-qPCR microRNA arrays (Applied Biosystems, Foster City, CA, USA).[19] Three hundred and sixty-five miRNA were assayed using TaqMan MicroRNA arrays with 100 ng of RNA as the input for each RT reaction according to the manufacturer's protocol. An additional 32 miRNA (*Online Supplementary Table S1*) were measured using miRNA assays that were custom designed by Applied Biosystems since these miRNA were not covered by the TaqMan MicroRNA array platform and/or were recently identified by our cloning study.[16] RT reactions for custom miRNA assays were performed in duplicate, in a total volume of 15 µL containing 0.5 mM dNTP, 10 U/µL RT, 1x RT buffer, 0.25 U/µL RNase inhibitor and 0.25x multiplex RT primer pool covering the 32 miRNA (Applied Biosystems). RT reactions were incubated as previously described.[19] Next, cDNA samples were diluted 10-fold in water. Duplicate PCR reactions of 15 µL were performed in a 96-well plate for each of the 32 miRNA. PCR reaction mixtures contained 1 µL of diluted cDNA sample in 1x Universal TaqMan Master Mix and 1x specific primer/probe mix. PCR reactions were performed on an ABI 7900HT Sequence Detection System. Duplicate measurements of two independent experiments were strongly correlated (Rs=0.9, $P<0.0001$, *Online Supplementary Figure S1A*). Moreover, multiple measurements for snoR-13 and snoR-14 were analyzed within one TaqMan Microarray-plate and were also strongly correlated (Rs≥0.96, $P<0.0001$, *Online Supplementary Figure S1B,C*). The means of the Ct values for snoR-13 and -14 (TaqMan

MicroRNA array) and snoR-1 (custom reactions) were used as references to normalize the expression of miRNA. These snoRNA were chosen since expression levels did not differ significantly between genetic subtypes of ALL or between ALL samples and hematopoietic control cells (*Online Supplementary Figure S2*) and expression levels of these three snoRNA were strongly correlated with each other *(Online Supplementary Figure S3*). The expression was calculated as a percentage of snoRNA as $2^{-\Delta Ct}$ x100% where the ΔCt is equal to the Ct-value for each miRNA minus the Ct-value of the control snoRNA. Processed miRNA expression values have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO series accession number GSE23024 at *http://www.ncbi.nlm.nih.gov/geo*.

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

Statistical analysis

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

Hierarchical clustering of patients by miRNA expression levels was done using GeneMaths 2.0 software (Applied Maths, Sint-Martens-Latem, Belgium) after Pearson's correlation as the distance measure. Since TaqMan MicroRNA Arrays and custom-made assays make use of different control snoRNA to correct for small RNA input, we calculated Z-scores for each miRNA. Z-scores were used for hierarchical clustering analyses.

Cox proportional hazard analysis was used to identify miRNA that correlated with the disease-free survival of children with newly diagnosed ALL. Both univariate and multivariate (corrected for ALL subtype) analyses were performed using relapse as the event and miRNA expression as a continuous variable.

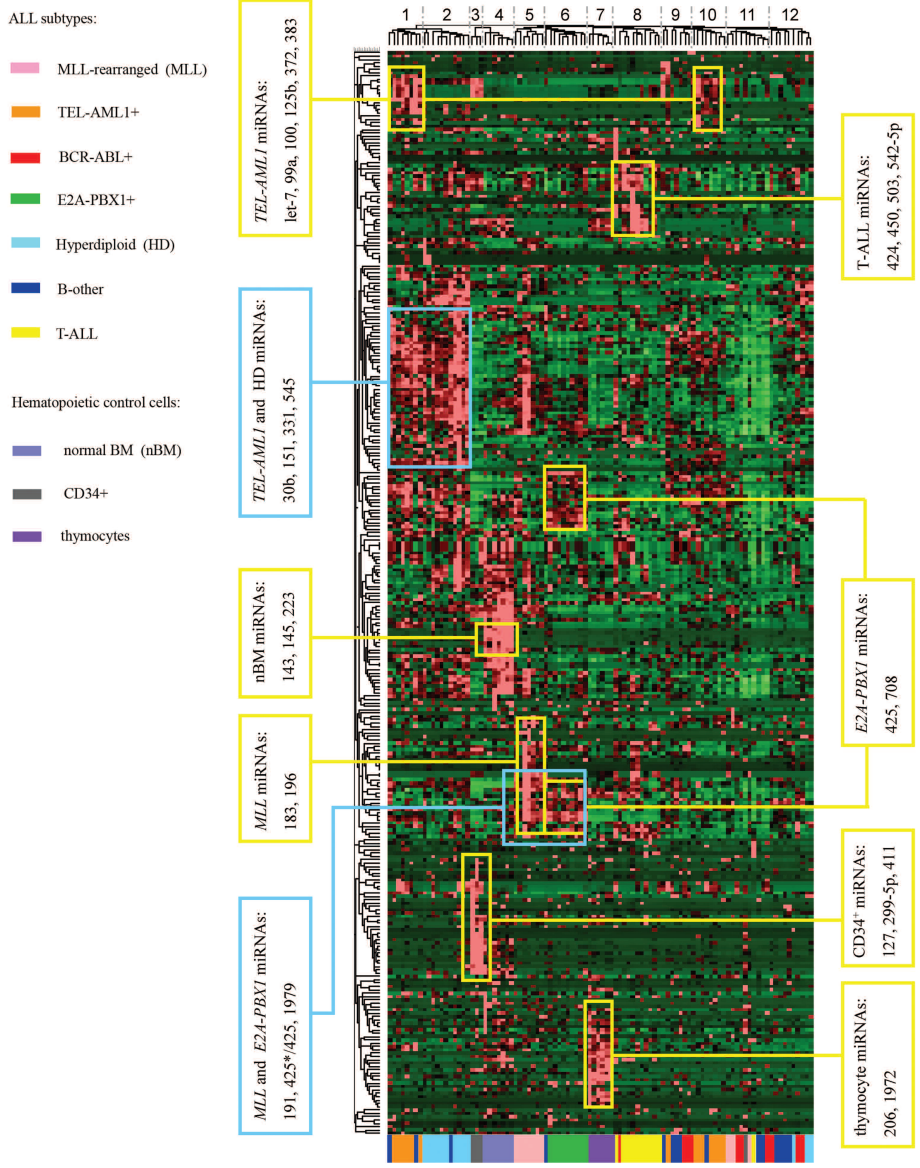

Figure 1. Clustering of ALL subtypes and normal hematopoietic control cells by expression levels of 325 miRNA. Hierarchical clustering of ALL patients, normal bone marrow samples, CD34+ selected cells and thymocytes by expression levels of 325 (unselected) miRNA. Heatmap shows which miRNA are overexpressed (in red) and which are underexpressed (in green) relative to snoRNA input control. Expression levels are plotted as standardized Z-scores per miRNA.

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

Top#	miRNA	Chromosomal location	Fold change	FDR-corrected *P* value
MLL-1	let-7b	22q13.31	↓ 69	<0.001
MLL-2	let-7c	21q21.1	↓ 16	<0.001
MLL-3	miR-708	11q14.1	↓ 288	0.002
MLL-4	miR-192	11q13.1	↓ 3	0.002
MLL-5	miR-196a	17q21.32;12q13.13	↑ 2283	0.002
MLL-6	miR-194	1q41; 11q13.1	↓ 3	0.002
MLL-7	miR-497	17p13.1	↓ 24	0.003
MLL-8	miR-20b	Xq26.2	↓ 6	0.003
MLL-9	miR-133a	18q11.2; 20q13.33	↑ 47	0.009
MLL-10	miR-372	19q13.41	↓ 27	0.009
TEL-AML1-1	miR-213	1q31.3	↓ 5	<0.0001
TEL-AML1-2	miR-99a	21q21.1	↑ 38	<0.001
TEL-AML1-3	miR-100	11q24.1	↑ 30	<0.001
TEL-AML1-4	miR-125b	11q24.1; 21q21.1	↑ 35	<0.001
TEL-AML1-5	miR-126*	9q34.3	↑ 4	<0.001
TEL-AML1-6	miR-383	8p22	↑ 1671	<0.001
TEL-AML1-7	miR-221	Xp11.3	↓ 7	<0.001
TEL-AML1-8	miR-126	9q34.3	↑ 3	<0.001
TEL-AML1-9	miR-629	15q23	↑ 4	<0.001
TEL-AML1-10	miR-361-5p	Xq21.2	↓ 2	<0.001
BCR-ABL-1	miR-93	7q22.1	↓ 2	0.015
BCR-ABL-2	miR-484	16p13.11	↓ 2	0.015
BCR-ABL-3	miR-331	12q22	↓ 2	0.023
BCR-ABL-4	miR-103	5q35.1; 20p13	↓ 2	0.023
BCR-ABL-5	miR-1226	3p21.31	↓ 2	0.023
BCR-ABL-6	miR-345	14q32.2	↓ 3	0.024
BCR-ABL-7	miR-210	11p12	↓ 5	0.024
BCR-ABL-8	miR-301	17q22	↓ 3	0.024
BCR-ABL-9	miR-324-5p	17p13.1	↓ 2	0.024
BCR-ABL-10	miR-148b	12q13.13	↓ 2	0.024
E2A-PBX1-1	miR-126*	9q34.3	↓ 28	0.001
E2A-PBX1-2	miR-126	9q34.3	↓ 34	0.001
E2A-PBX1-3	miR-146a	5q33.3	↓ 14	0.001
E2A-PBX1-4	miR-545	Xq13.2	↓ 5	0.001
E2A-PBX1-5	miR-24	9q22.32; 19p13.13	↓ 4	0.001
E2A-PBX1-6	miR-29a	7q32.3	↓ 3	0.001
E2A-PBX1-7	miR-511	10p12.33	↓ 706	0.001
E2A-PBX1-8	miR-365	16p13.12; 17q11.2	↓ 6	0.001
E2A-PBX1-9	miR-30d	8q24.22	↓ 2	0.001
E2A-PBX1-10	miR-193a	17q11.2	↓ 8	0.001
hyperdiploid-1	miR-223	Xq11.2	↑ 4	<0.0001
hyperdiploid-2	miR-222*	Xp11.3	↑ 5	<0.0001
hyperdiploid-3	miR-98	Xp11.22	↑ 3	<0.0001
hyperdiploid-4	miR-511	10p12.33	↑ 7	<0.0001
hyperdiploid-5	miR-222	Xp11.3	↑ 4	<0.001
hyperdiploid-6	miR-660	Xp11.23	↑ 3	<0.001
hyperdiploid-7	miR-361-3p	Xq21.2	↑ 3	<0.001
hyperdiploid-8	miR-374a	Xq13.2	↑ 3	<0.001
hyperdiploid-9	miR-532-5p	Xp11.23	↑ 3	<0.001
hyperdiploid-10	miR-501-5p	Xp11.23	↑ 3	<0.001
T-ALL-1	miR-191	3p21.31	↓ 3	<0.0001
T-ALL-2	miR-190	15q22.2	↑ 6	<0.0001
T-ALL-3	miR-151	8q24.3	↓ 48	<0.0001
T-ALL-4	miR-425-5p	3p21.31	↓ 4	<0.0001
T-ALL-5	miR-222*	Xp11.3	↓ 8	<0.0001
T-ALL-6	miR-542-5p	Xq26.3	↑ 234	<0.0001
T-ALL-7	miR-708	11q14.1	↓ 3884	<0.0001
T-ALL-8	miR-132	17p13.3	↓ 6	<0.001
T-ALL-9	miR-425-3p	3p21.31	↓ 3	<0.001
T-ALL-10	miR-342-3p	14q32.2	↑ 3	<0.001

continued in the next column

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

Multivariate analysis indicated that the expression levels of 14 miRNA were of significant prognostic value ($P<0.05$; see results section). To visualize the prognostic value of the expression signature of these 14 miRNA, we first divided the cases in two groups based on the median expression level per miRNA (see *Online Supplementary Table S2* for the median value of each of the 14 miRNA). Patients with high expression (above the median) of a prognostically *favorable* miRNA (e.g. miR-10a) were assigned a score of 1 whereas patients with low expression (below the median) were given a score of 2. In the case of a prognostically *unfavorable* miRNA (e.g. miR-33), patients were assigned a score 2 in the case of an expression level above the median and a score of 1 if this level was below the median. Next, the sum of the individual scores for the 14 prognostically informative miRNA was calculated: this resulted in a minimum cumulative score of 14 and a maximum cumulative score of 28. The median of the cumulative scores for 78 patients was used to assign patients to a favorable (cumulative score ≤21, n=41) or unfavorable (cumulative score >21, n=37) group in order to study the prognostic value of a combined miRNA expression signature.

Results

Distinct microRNA profiles in genetic subtypes acute lymphoblastic leukemia

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

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

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

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

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

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

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

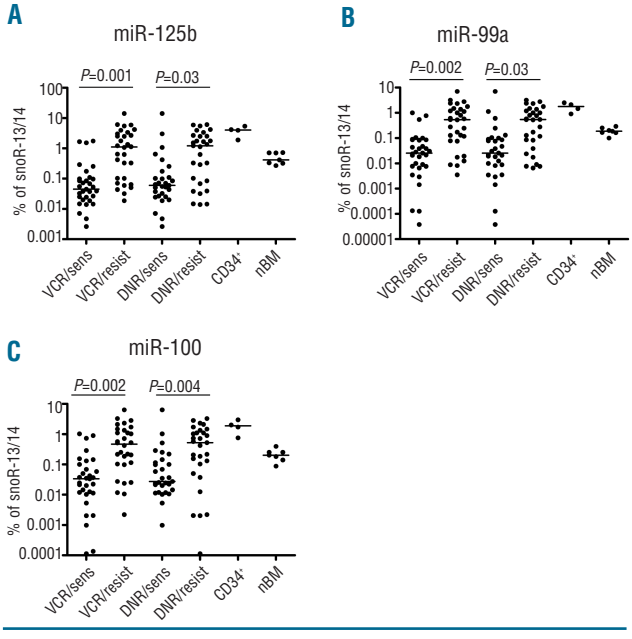

Figure 2. Discriminative expression of miR-125b, miR-99a and miR-100 between drug-sensitive and -resistant precursor B-ALL patients. Expression levels relative to snoRNA are shown for miR-125b (A), miR-99a (B) and miR-100 (C). Dots represent individual samples of CD34+ selected cells (n=4), normal bone marrow samples (=7) and the following precursor B-ALL patients: vincristine-sensitive (n=31; VCR/sens) and -resistant (n=30; VCR/resist) or daunorubicin-sensitive (n=29; DNR/sens) and -resistant (n=29; DNR/resist). Lines indicate median expression level in each group. The indicated *P* value is corrected for multiple-testing (FDR-corrected *P* value).

and miR-126/126* (*Online Supplementary Table S2*). This may indicate a common biological mechanism underlying the *TEL-AML1* and hyperdiploid subtypes.

MLL-rearranged and *E2A-PBX1*-positive ALL shared a high expression level of 15 miRNA including the recently discovered miR-1979 (Figure 1 and *Online Supplementary Table S5*). Forty percent of these 15 miRNA are mapped within miRNA-clusters e.g. miR-191 and miR-425 on chromosome 3p21.31 and the cluster of miR-141 and miR-200c on chromosome 12p13.31. The expression of miR-191 and miR-425 (Rs=0.8) as well as of miR-141 and miR-200c (Rs=0.9) correlated well with each other (P<0.001), which may indicate co-regulation of these miRNA since they are encoded less than 10 kilobases from each other. Correlation between expression levels of clustered miRNA is not restricted to the *MLL* and *E2A-PBX1* subtypes, since in all ALL cases a strong association was also observed for miRNA genes within clusters miR-15a-16 (Rs=0.7; P<0.001), miR-17-92 (0.3≤Rs≤0.9; P<0.01), miR-106b-25 (0.7≤Rs≤0.8; P<0.001), miR-106a-363 (0.3≤Rs≤0.9; P<0.01), miR-221-222 (Rs=0.8; P<0.001) and miR-371-373 cluster (0.6≤Rs≤0.8; P<0.001, *Online Supplementary Table S6*).

In Online Supplementary Table S7 we have listed target genes that were biologically proven (known from literature) for the miRNA that were found to be differentially expressed in different subtypes of ALL. Two proven targets of the let-7 family are *RAS* and *c-MYC*. Interestingly, mRNA levels of *RAS* and *c-MYC* were 3- to 5-fold up-regulated in *MLL*-rearranged ALL compared to non-*MLL* precursor B-ALL (P<0.0001, *Online Supplementary Figure S6* and *Figure S7*).[28] The elevated mRNA expression levels were associated with a 2-fold up-regulation of c-Myc protein (P<0.05) in *MLL*-rearranged patients but not with an up-regulation of Ras protein as measured by reverse phase protein detection (M.W.J. Luijendijk, *unpublished data*, *Online Supplementary Figures S6 and S7*).

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

	Uncorrected for ALL subtype		Corrected for ALL subtype			
N.	miRNA	*P* value	miRNA	hazard ratio	confidence interval	*P* value
1	miR-10a	0.018	miR-10a	0.82	0.69 - 0.97	0.020
2	miR-23b	0.019	-	-	-	-
3	miR-27b	0.009	-	-	-	-
4	miR-33	0.025	miR-33	1.32	1.02 - 1.69	0.030
5	miR-99a	0.023	-	-	-	-
6	miR-107	0.028	-	-	-	
7	miR-125b	0.031	-	-	-	-
8	miR-126*	0.042	-	-	-	-
9	miR-134	0.025	miR-134	0.73	0.56 - 0.96	0.026
10	miR-193a	0.014	-	-	-	
11	-		miR-214	0.73	0.59 - 0.90	0.004
12	-		miR-215	1.30	1.01 - 1.67	0.042
13	miR-219	0.033	-	-	-	
14	miR-223	0.046	-	-	-	
15	miR-335	0.034	-	-	-	
16	miR-369-5p	0.049	miR-369-5p	1.30	1.01 - 1.67	0.046
17	miR-371	0.035	-	-	-	
18	miR-372	0.040	-	-	-	
19	miR-373	0.044	-	-	-	
20	miR-449	0.043	-	-	-	
21	miR-484	0.009	miR-484	0.81	0.69 - 0.94	0.008
22	-		miR-496	1.52	1.15 - 2.00	0.003
23	miR-518d	0.047	miR-518d	1.43	1.01 - 2.04	0.046
24	miR-572	0.010	miR-572	0.59	0.41 - 0.85	0.004
25	-		miR-580	0.81	0.65 - 0.99	0.045
26	-		miR-599	1.39	1.01 - 1.89	0.044
27	-		miR-624	0.79	0.67 - 0.93	0.006
28	miR-627	0.028	miR-627	0.68	0.49 - 0.93	0.019
29	let-7c	0.032	-	-	-	-
30	let-7d	0.048	-	-	-	-
31	let-7d*	0.014	-	-	-	-

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

Distinct microRNA profiles in drug-sensitive and drug-resistant patients and their association with clinical outcome in pediatric acute lymphoblastic leukemia

For 61 out of the 70 patients with precursor B-ALL included in this study, the *in vitro* sensitivity to prednisolone, vincristine, L-asparaginase and daunorubicin was determined. *Online Supplementary Table S8* summarizes the expression levels of 397 miRNA for drug-sensitive and drug-resistant patients. Sixteen miRNA were discriminative for resistance to one or more drugs (P_{FDR} <0.05). No miRNA were associated with prednisolone resistance (P_{FDR} >0.05), only miR-454 was expressed at a 1.9-fold lower level in L-asparaginase-resistant cases (P_{FDR} =0.017) whereas 20 miRNA were discriminative for resistance to vincristine and/or daunorubicin (P_{FDR} <0.05). Table 2 and *Online Supplementary Table S9* show the fold-change for discriminative miRNA and their potential target genes, respectively. The 14- to 25-fold up-regulation of miR-125b, miR-99a and miR-100 in patients resistant to vincristine (P_{FDR} ≤0.002)

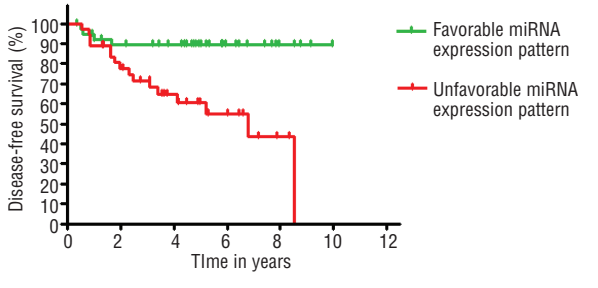

Figure 3. Prognostic value of a miRNA expression profile in newly diagnosed pediatric ALL. Kaplan-Meier estimates for the probability of disease-free survival of a miRNA expression profile are shown. This profile comprises the combined score of 14 miRNA which were, independently of subtype, predictive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

and daunorubicin (P_{FDR} <0.05) is most striking (Figure 2). Remarkably, 12 out of 31 (39%) and 12 out of 29 (41%) patients resistant to vincristine and daunorubicin carried the *TEL-AML1* translocation. Cox proportional hazard analysis revealed that the expression level of 31 miRNA significantly correlated with the probability of disease-free survival (P<0.05; Table 3). The prognostic value was independent of ALL subtype for 14 miRNA: six miRNA (i.e. miR-33, -215, -369-5p, -496, -518d, and -599) were associated with an unfavorable long-term clinical outcome (hazard ratio 1.30–1.52 with 95%CI 1.01–2.04; 0.003≤P≤0.046) whereas eight miRNA (i.e. miR-10a, -134, -214, -484, -572, -580, -624 and -627) were linked to a favorable prognosis (hazard ratio: 0.59–0.82 with 95%CI 0.41–0.99; 0.004≤P≤0.045). *Online Supplementary Figure S8* illustrates the disease-free survival curves associated with each of these 14 prognostic miRNA in pediatric ALL. Next, ALL patients were divided into two groups based on the median cumulative score that combined the expression levels of these 14 miRNA (Figure 3, see the *Design and Methods* section for details on the cumulative score). Kaplan-Meier analysis indicated that the profile obtained by combining information on these 14 miRNA further divided the prognosis; patients with a favorable miRNA expression profile had a 5-year disease-free survival of 89.4±7% (n=41) compared to 60.8±12% (n=37) for patients with the less favorable miRNA profile (P=0.001).

Discussion

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

Hierarchical clustering of 325 miRNA that are expressed in ALL cells identified T-ALL, *MLL*-rearranged ALL, *TEL-AML1*, *E2A-PBX1* and hyperdiploid ALL-specific miRNA expression patterns (Figure 1). *BCR-ABL* and 'B-other' ALL subtypes were not distinguishable as separate groups. This indicates that *BCR-ABL* and 'B-other' cases are more heterogeneous than other subtypes and/or that that miRNA play a minor role in both subtypes. This is also reflected by the lower number of miRNA that were significantly differentially expressed as well as the lower fold-change in expression levels found for miRNA expressed in *BCR-ABL* and 'B-other' subtypes compared to other (i,e. non-*BCR-ABL* and non-'B-other') subtypes (*Online Supplementary Table S4* and Table 1). Correspondingly, we and others found that the *BCR-ABL1* and 'B-other' subtypes are also difficult to classify based on mRNA expression levels.[20,29,30] Since samples used in both miRNA and mRNA studies contained more than 90% leukemic cells, the confounding effect of contaminating normal cells is minimum. This suggests that additional heterogeneity among the *BCR-ABL* and 'B-other' samples prevents the finding of a characteristic miRNA-profile for each subtype. This explanation is strengthened by the fact that we recently identified a new subgroup of pediatric ALL among 'B-other' cases which was characterized by abnormalities in B-cell differentiation genes.[20]

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

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

We only found a limited number of miRNA associated with cellular resistance to vincristine, daunorubicin and L-asparaginase and none with resistance to prednisolone in pediatric ALL (Table 2). *In vitro* resistance to these drugs is associated with an unfavorable outcome in pediatric ALL.[11-14] Remarkably, 39% of the vincristine-resistant patients had *TEL-AML1* subtype ALL, which supports earlier observations that *TEL-AML1*-positive patients are resistant to vincristine.[34,35] Interestingly, *TEL-AML1*-positive cases often show sensitivity to L-asparaginase in combination with resistance to vincristine.[35] We observed here that *TEL-AML1*-positive patients and vincristine-resistant cases both expressed higher levels of miR-125b, which deserves further study since this may be linked to the vincristine-resistant/L-asparaginase-sensitive discordant type of *TEL-AML1*-positive ALL. Over-expression of miR-125b reduced the amount of drug-induced apoptosis in pre-B cells[36] and induced proliferation in $CD34^+$ cells.[37,38] Interference with miR-125b function might, therefore, point to a way to sensitize patients to these drugs.

The expression profile based on 14 miRNA was predictive of the prognosis of ALL (Table 3 and Figure 3). None of

these miRNA was linked to the occurrence of central nervous relapses in pediatric ALL as previously reported.[39] We observed that, among others, miR-10a, miR-134 and miR-214 were linked to a favorable outcome in pediatric ALL, which may correspond to their described tumor suppressor activity by driving apoptosis (miR-10a),[40] inhibiting cell proliferation (miR-10a and miR-214),[41,42] and down-regulating oncogene *SOX2* (miR-134).[43] The expression of all three miRNA was significantly lower (3- to 600-fold) in precursor B-ALL than in normal bone marrow or CD34$^+$ cells (*Online Supplementary Figure S4*). Six miRNA were associated with an unfavorable prognosis including miR-33, which was significantly up-regulated in T-ALL compared to in normal thymocytes (*Online Supplementary Figure S4*). Besides their prognostic value, these miRNA may also serve as targets for new therapies. We recently discovered that down-regulation of miR-10a in *MLL*-rearranged patients, due to DNA hypermethylation, could be reversed and brought back to normal levels by treatment with a demethylating agent.[44] In contrast, antagomirs could be chosen to down-regulate the expression of aberrantly overexpressed miRNA with unfavorable prognostic significance, such as miR-33.

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

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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