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

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Supplementary 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.[1,2] The percentage of leukemic cells was determined by May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins. If the percentage of leukemic cells was below 90%, samples were enriched by eliminating non-malignant cells with immunomagnetic beads (Dynabeads, M-450, Dynal, Norway) which were washed twice in culture medium before use.[1,2] In the case that non-T-ALL samples were contaminated by normal T cells, mononuclear cells were incubated for 30 min at room temperature with monoclonal antibody anti-CD2 directly coated on the immunomagnetic beads to eliminate the contaminating T-cells. In the case that non-T-ALL or T-ALL samples were contaminated by mononuclear cells other than T cells, mononuclear cells were incubated with one or more of the following mouse monoclonal antibodies: anti-CD14 (in the case of monocytes), anti-CD15 (in the case of myeloid cells, plus anti-CD13 if immature myeloid cells were also present), anti-E-1 (in the case of erythroid cells, plus anti-H1-antigen if immature erythroid cells were present as well). Anti-CD3 was used in the case that CD3-negative T-ALL samples were contaminated by normal T cells. The CD3-positive T-ALL samples in this study all contained more than 90% leukemic cells and did not need enrichment. After the incubation with the specific antibody for 30 min at room temperature, two washing runs were performed with protein-buffered saline and 0.1% bovine serum albumin to eliminate unbound antibody. This was followed by continuous mixing with beads coated with sheep anti-mouse immunoglobulin G. In all cases ten times more beads were used than contaminating target cells. Contaminating cells were extracted by placing the tubes containing cells and beads on a magnet for 2 min. The remaining cell suspension was extracted, assessed for purity of greater than 90% and used for further study.

$CD34^+$cells (>90% purity) were sorted from granulocyte colony-stimulating factor-stimulated blood cell samples from children with a brain tumor or Wilms' tumor by using the CD34 Progenitor Isolation Kit (Miltenyi Biotec, Utrecht, the Netherlands) according to the manufacturer's conditions for labeling of $CD34^+$ cells using the kit-included CD34 microbeads. $CD34^+$ cells were subsequently extracted using the autoMACS separator (Miltenyi Biotec).[3]

Thymocytes were isolated from thymic lobes that were resected from children during surgery for their congenital heart disease.[4] Thymic lobes were fragmented by removing the surrounding membrane and cutting. Fragments were then disrupted by gentle rubbing through a stainless steel filter and were washed with phosphate-buffered saline containing 5% fetal calf serum until only stroma remained. Thymocytes were then resuspended in the buffer containing 50 U/mL deoxyrubonuclease I.

All samples included 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 reverse transcriptase (RT) polymerase chain reaction (PCR) for genetic subtype and conventional karyotyping to determine the ploidy status of ALL cases. A total of 10 *MLL*, 14 *TEL-AML1*, 10 *BCR-ABL*, 9 *E2A-PBX1*, 13 hyperdiploid, 14 'B-other' (negative for the 5 previously listed genetic abnormalities) and 11 T-ALL cases were included. These cases were retrospectively selected on the basis of 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 (Bufa Pharmaceutical Products, Uitgeest, the Netherlands), vincristine (Oncovin; Eli Lilly, Amsterdam, the Netherlands), L-asparaginase (Medac, Hamburg, Germany) or daunorubicin (Sanofi Aventis, Gouda, the Netherlands) that were lethal to 50% of the ALL cells (LC_{50}) were measured by a methyl-thiazol-tetrazolium (MTT, Sigma, St Louis, MO, USA) drug resistance assay. As described previously, leukemia cells were incubated in duplicate with or without six different concentrations of drugs (with a range of 0.06-250 μg/mL for prednisolone; 0.05 to 50 μg/mL for vincristine, 0.003 to 10 IU/mL for L-asparaginase and 0.002-2 μg/μL for daunorubicin).[1,5] Cells were cultured in a humidified incubator in 5% carbon dioxide at 37 °C for 4 days. Then 10 μL of MTT (5 mg/mL MTT in saline) were added to the cell culture. After 6 h cells were gently mixed for 1 min and formazan crystals were dissolved in acidified isopropanol and quantified by spectrophotometry at 562 nm (Bio-Kinetics Reader; Bio-Tek Instruments, Winooski, VT, USA). Samples with more than 70% leukemic cells in the control wells and an optical density higher than 0.050 arbitrary

units (adjusted for blank values) were used to calculate the LC_{50}. 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 (RIN) 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).[6] 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.[3] 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.[6] 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 reactions contained 1 μL of diluted cDNA sample in 1x Universal TaqMan Master Mix and 1x specific primer/probe mix. PCR reactions were performed on an ABI 7900HT Sequence Detection System. Duplicate measurements of two independent experiments were stongly 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 snoRNAs 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 $2^{-\Delta Ct} \times 100$ where the ΔCt is equal to "Ct miRNA minus Ct control snoRNA". Processed miRNA expression data have been uploaded 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.[7] Data acquisition and data processing were exactly as previously described.[7] As part of this procedure, the dataset was e_{log} normalized by the variance-stabilizing normalization procedure (R version 2.3.1, package vsn). All Affymetrix data files are available through the website of the NCBI Gene Expression Omnibus (accession numbers GEO GSE13351 and GSE13425).

Reverse phase protein detection

Reverse phase protein detection was performed as previously described.[8-10] In brief, leukemic cells were lysed in Tissue Protein Extraction Reagent (TPER, Pierce Biotechnology, Rockford, IL, USA), supplemented with 300 mM NaCl, 1 mM orthovanadate and protease inhibitors. Lysates of patients' cells were spotted at 0.5 μg/μL in sextuplicate on glass-backed nitrocellulose coated array slides (FAST slides, Whatman plc, Kent, UK). Each spot was made by three hits performed by the Aushon Biosystems 2470 arrayer (Aushon Biosystems, Billerica, MA, USA). The first slide in every series of 15 slides was used to determine the total protein amount by Sypro Ruby Protein Blot Stain (Invitrogen) staining followed by visualization on a NovaRay CCD fluorescent scanner (Alpha Innotech, San Leandro, CA, USA). The remaining slides were stained with an antibody against Ras (Upstate, Temecula, CA, USA) or c-Myc (Cell Signaling, Danvers, MA, USA) followed by incubation with a biotinylated secondary antibody[10] using a DAKOcytomation autostainer. Slides were scanned using the NovaRay scanner. All slides were analyzed with the MicroVigene v2.8.1.0. software (VigeneTech, Carlisle, MA, USA). Finally, protein levels were calculated relative to the total amount of protein per sample.

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 (FDR)-corrected P values were less than 0.05.[11] R version 2.8[12] and the R package multtest (which corrects the *P* values for multiple testing)[13] 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 relapse-free survival of children with newly diagnosed ALL. Both univariate and multivariate (corrected for ALL subtype) analyses were performed using relapse as an event and miRNA expression as a continuous variable.

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 into two groups based on the median expression level per miRNA (see *Online Supplementary Table S2* for the median values 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 of 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 of 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.

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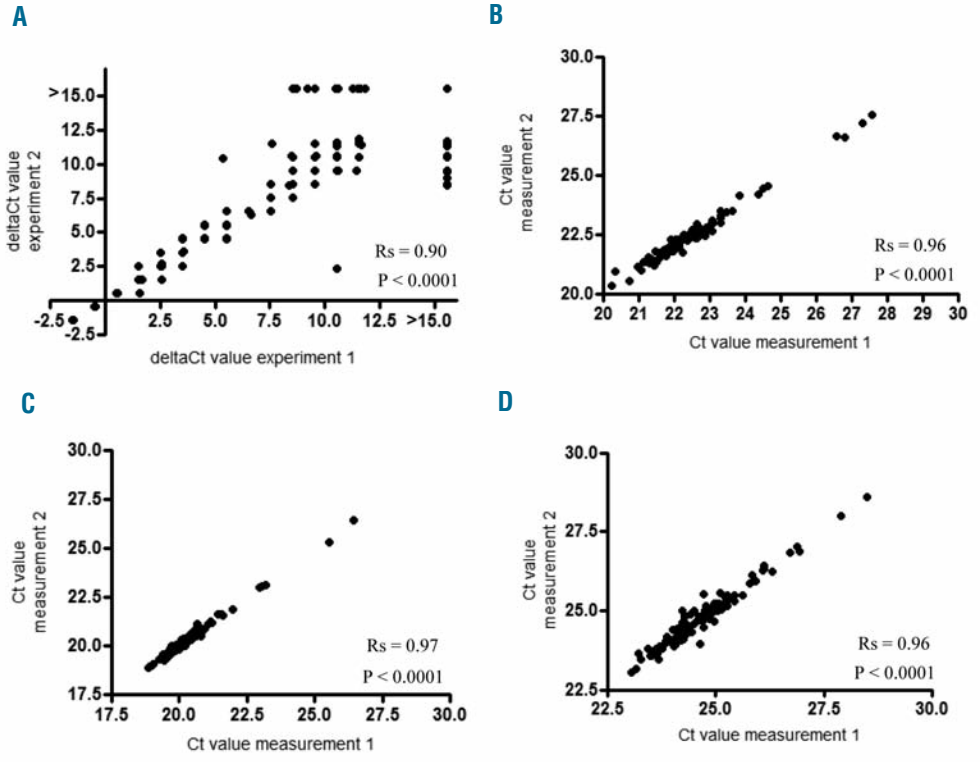
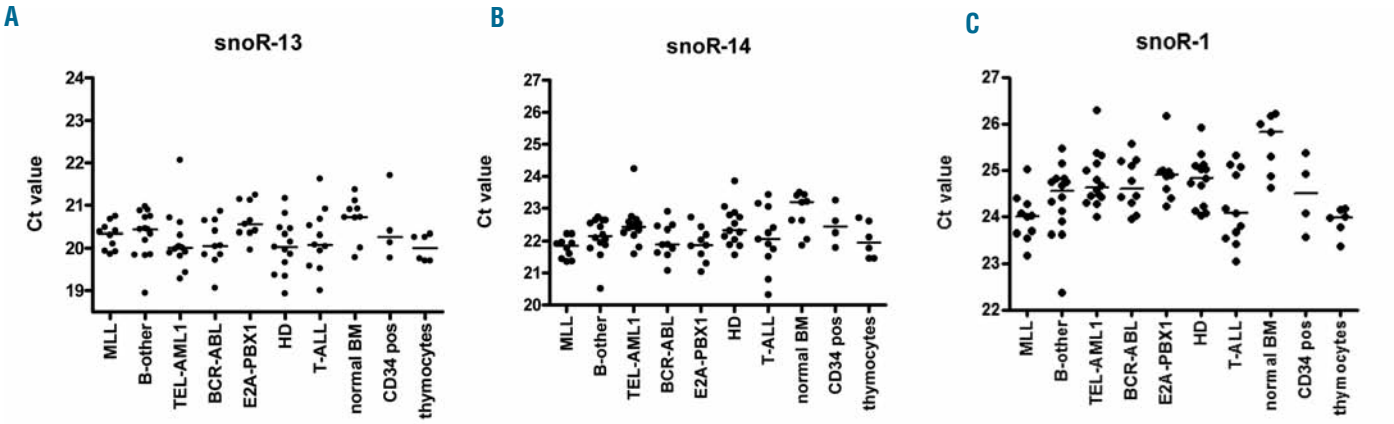
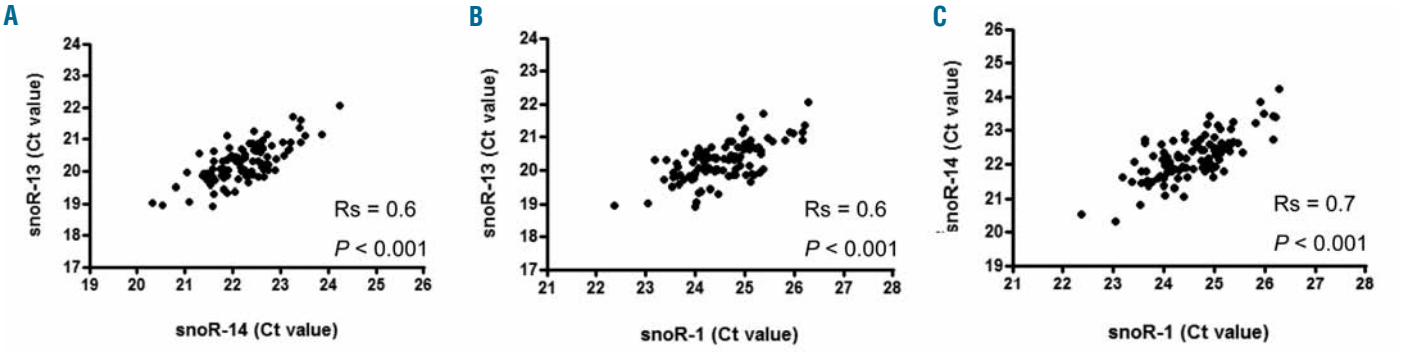


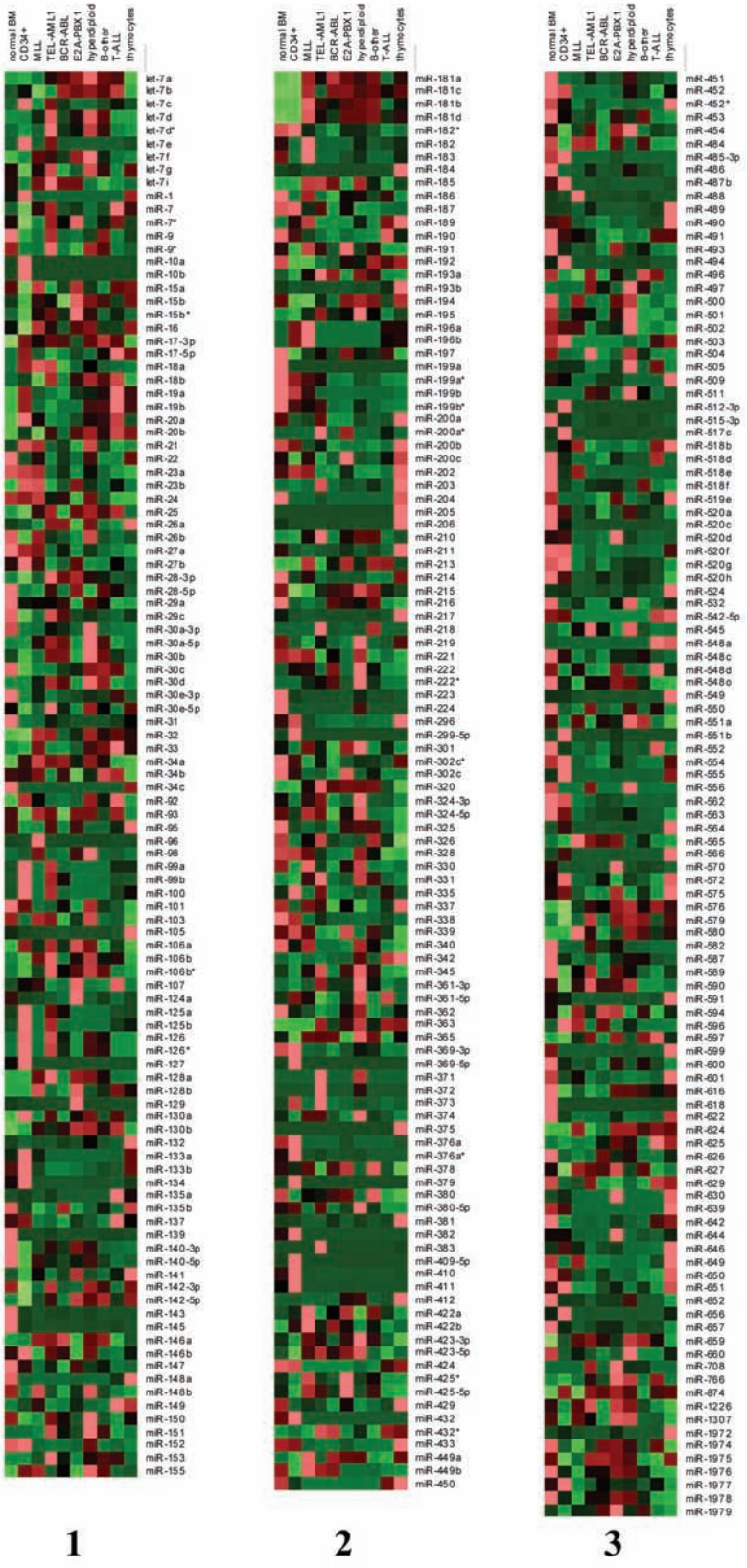
Online Supplementary Figure S1. Reproducibility of the RT-qPCR technique. Correlation between two independent RT-qPCR experiments (A), and between duplicate measurements for snoR-14 (B), snoR-13 (C) and snoR-1 (D) within a single experiment are shown with the corresponding Spearman's correlation coefficient (Rs) ≥ 0.90 and *P*< 0.0001. >15 ΔCt (A) corresponds to miRNA that were undetectable in 40 cycles of PCR reaction.



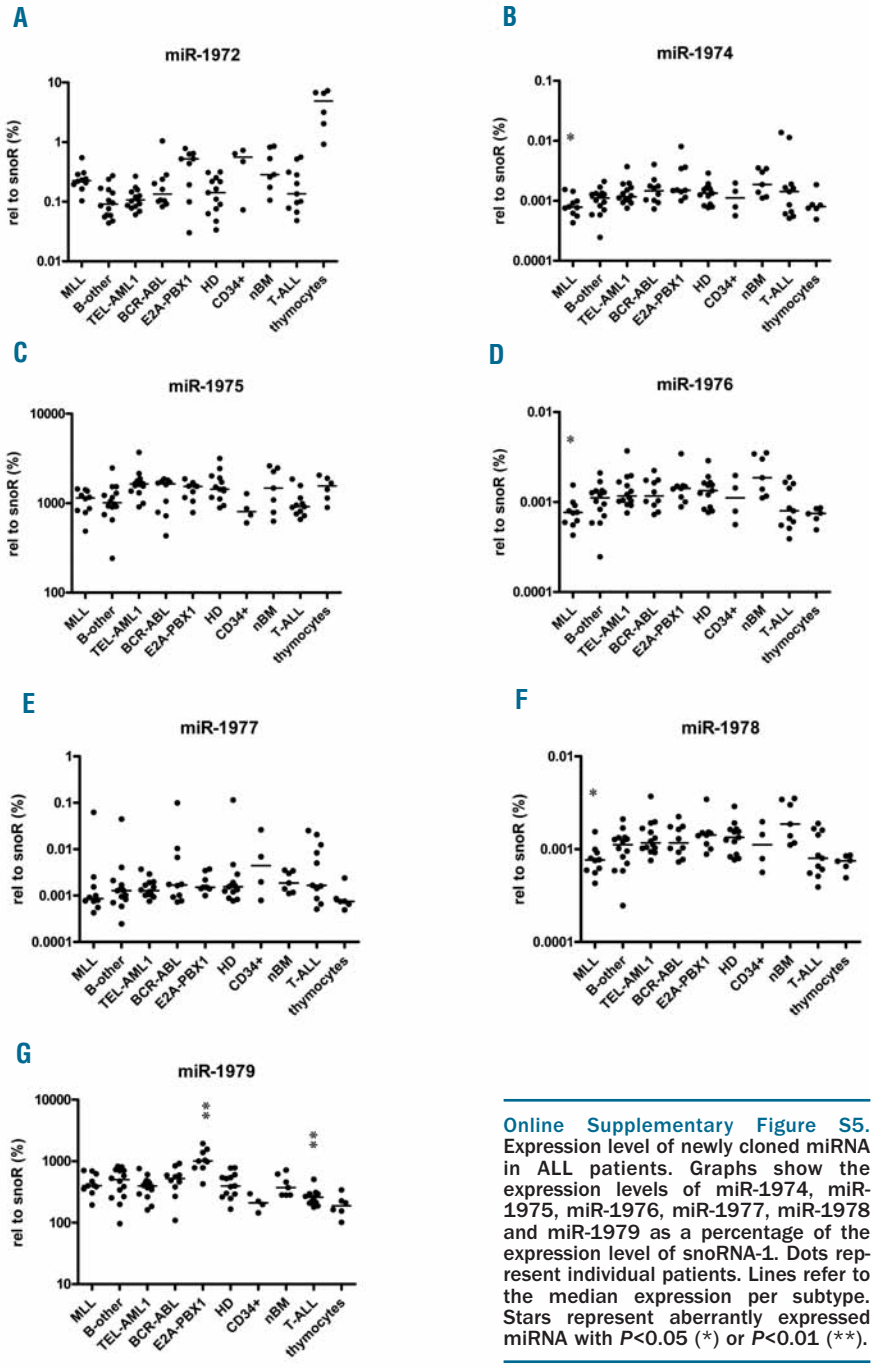
Online Supplementary Figure S2. Expression of snoRNA in ALL patients and their controls. Expression of snoR-13 (A), snoR-14 (B) and snoR-1 (C) was measured using multiplex RT-qPCR. Dots represent the mean Ct of octuple measurements for snoR-13 and snoR-14 or duplicate measurements for snoR-1 per sample. Lines represent the median value per group.



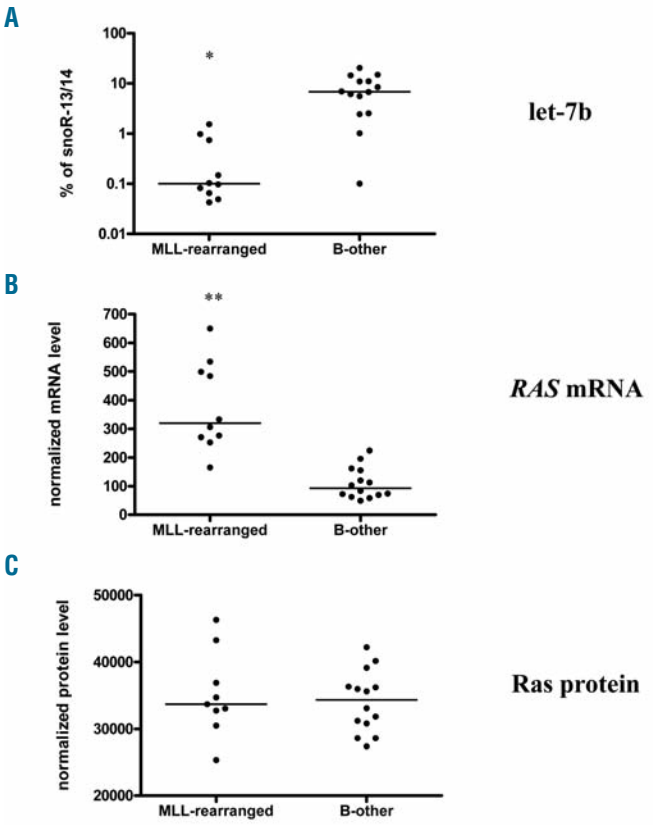
Online Supplementary Figure S3. Correlation between expression levels of different snoRNA. Expression levels of snoR-13, snoR-14 and snoR-1 were determined by multiplex RT-qPCR. Expression levels of the different snoRNA correlate with a Spearman's correlation coefficient of 0.6 (A), 0.6 (B) and 0.7 (C), all with $P<0.001$.



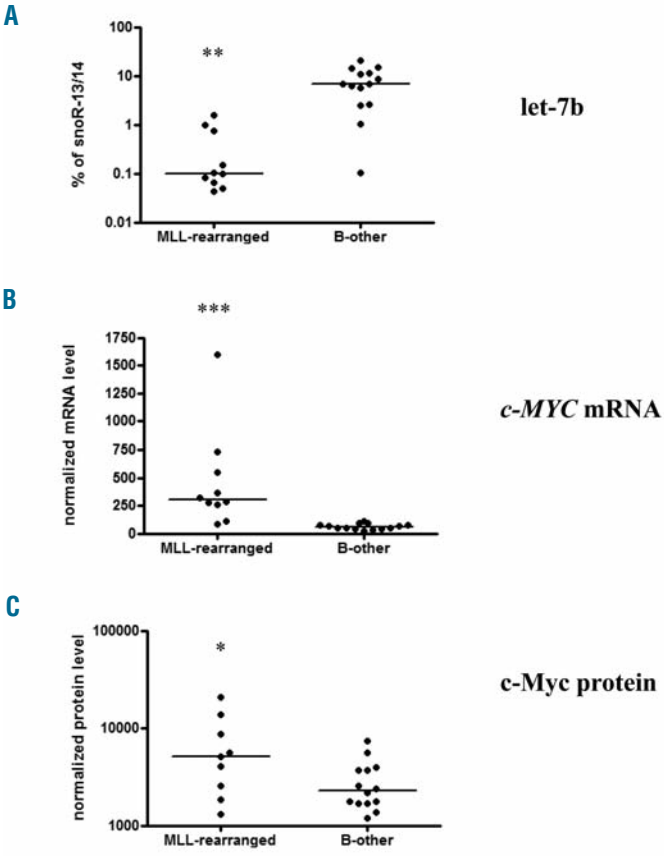
Online Supplementary Figure S4. Median expression of 325 miRNA in pediatric ALL subtypes and normal hematopoietic control cells. The heatmap shows which miRNA are over-expressed (in red) and which are under-expressed (in green) relative to snoRNA . Expression levels are plotted as standardized Z-scores per miRNA. * indicates the star form or complementary form of miRNA; Normal BM = normal bone marrow; MLL = *MLL*-rearranged ALL; $CD34^+$ = normal CD34-positive cells.



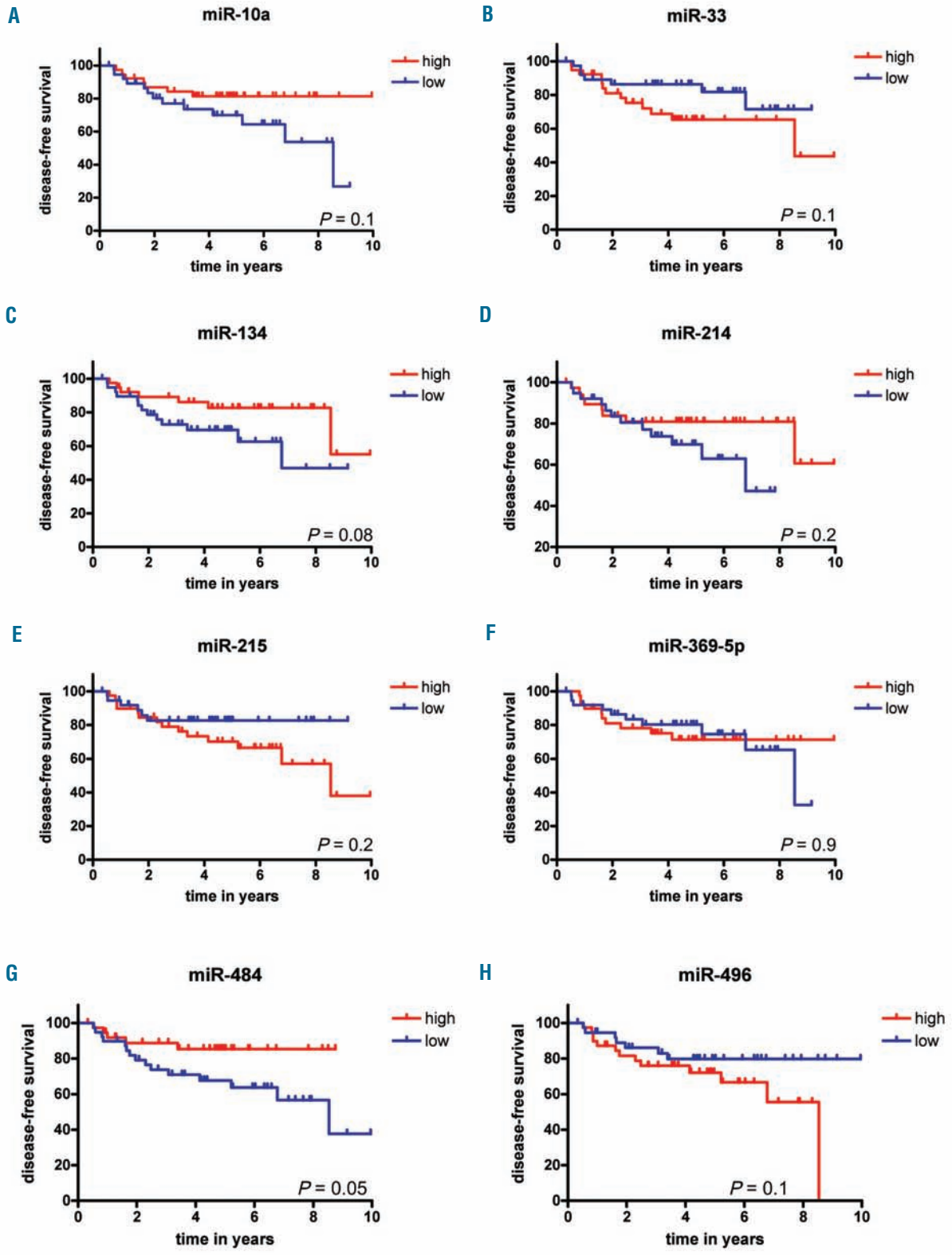
Online Supplementary Figure S5. Expression level of newly cloned miRNA in ALL patients. Graphs show the expression levels of miR-1974, miR-1975, miR-1976, miR-1977, miR-1978 and miR-1979 as a percentage of the expression level of snoRNA-1. Dots represent individual patients. Lines refer to the median expression per subtype. Stars represent aberrantly expressed miRNA with $P<0.05$ (*) or $P<0.01$ (**).



Online Supplementary Figure S6. Expression level of let-7 and KRAS in *MLL*-rearranged precursor B-ALL and B-other patients. The expression levels of let-7b as a percentage of snoR-13/14 determined by multiplex stem-loop RT-PCR (A), *RAS* mRNA as detected by Affymetrix GeneChips (B) and Ras protein measured by a reverse phase array-technique [M.W.J. Luijendijk, *unpublished data*, (C)] were measured in ten *MLL*-rearranged precursor B-ALL and 14 B-other patients without the *MLL* translocation. Due to limited sample size different patients are included in (A), (B) and (C). Dots represent individual patients with the median per subtype represented by a line. Stars refer to aberrant expression in *MLL*-rearranged cases *versus* 'B-other' cases with $P<0.001$ (*) or $P<0.0001$ (**). Ras protein levels are relative to the total amount of protein per sample.



Online Supplementary Figure S7. Expression level of let-7 and *c-MYC* in *MLL*-rearranged precursor B-ALL and 'B-other' patients. The expression levels of let-7b as a percentage of snoR-13/14 determined by multiplex stem-loop RT-PCR (A), *c-MYC* mRNA as detected by Affymetrix GeneChips (B) and c-Myc protein measured by a reverse phase array-technique [(C), M.W.J. Luijendijk, *unpublished data*] were measured in ten *MLL*-rearranged precursor B-ALL and 14 B-other patients without the *MLL*-translocation. Due to limited sample size different patients are included in (A), (B) and (C). Dots represent individual patients with the median per subtype represented by a line. Stars refer to aberrant expression in *MLL*-rearranged precursor B-ALL cases *versus* 'B-other' cases with $P<0.05$ (*), $P<0.001$ (**) or $P<0.0001$ (***). C-Myc protein levels are relative to the total amount of protein per sample.



Online Supplementary Figure S8. Kaplan-Meier estimates for the probability of disease-free survival.

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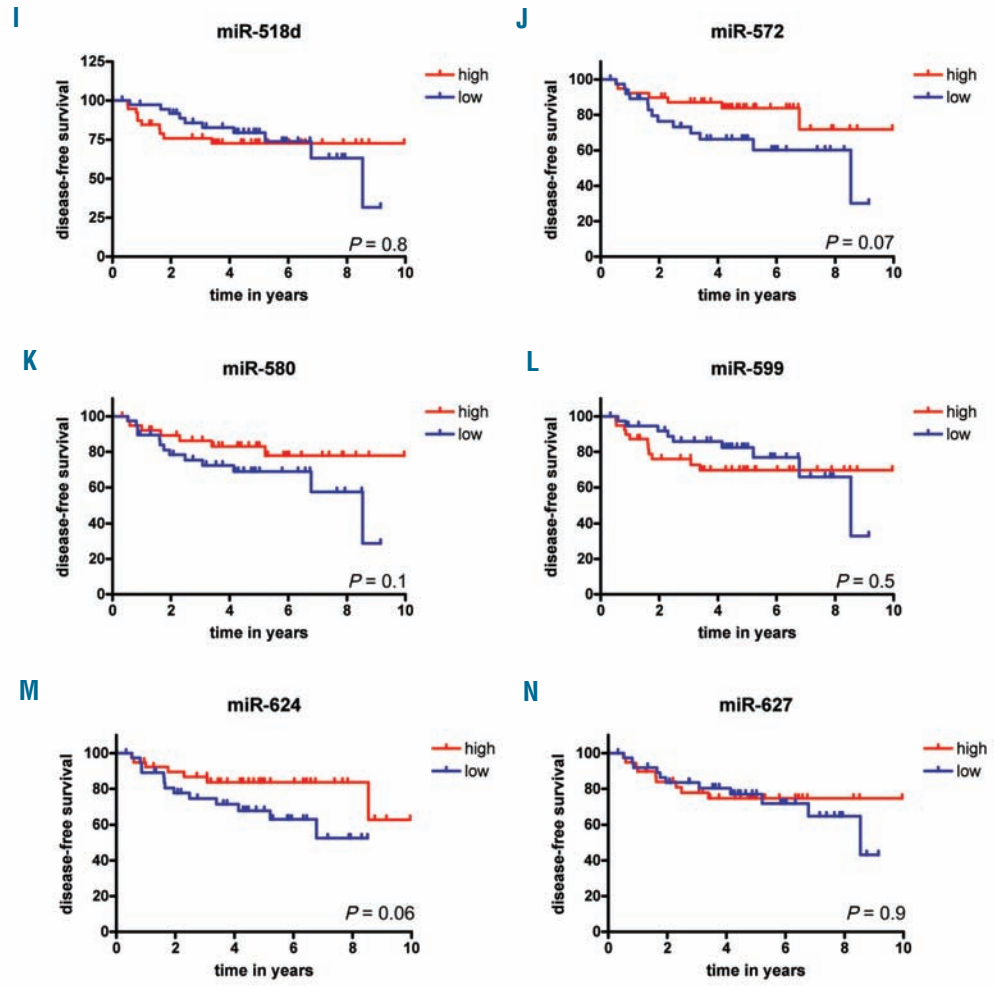

Online Supplementary Figure S8. Kaplan-Meier estimates for the disease-free survival in ALL patients with high and low expression of selected miRNAs. The following miRNAs are shown: miR-10a (A), miR-33 (B), miR-134 (C), miR-214 (D), miR-215 (E), miR-369-5p (F), miR-484 (G), miR-496 (H), miR-518d (I), miR-572 (J), miR-580 (K), miR-599 (L), miR-624 (M), miR-627 (N). To visualize the prognostic value of miRNA expression levels in pediatric ALL, we have divided cases by the median expression level in two groups; High and low refer to above or below the median expression level, respectively. The *P* values listed in panel A-O are obtained by Cox proportional hazard analyses of miRNA expression as a discrete variable (high or low expression). The median value is an abritrarily chosen cut-off for dividing the patients in two groups, moreover, these curves do not take into account that certain prognostic subtypes may be overrepresented in one of the expression categories and therefore may have confounded the analysis. For this reason we analyzed the prognostic value of miRNA expression as a continuous variable adjusted for ALL subtype. Data for this multivariate analysis are shown in Table 3.

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