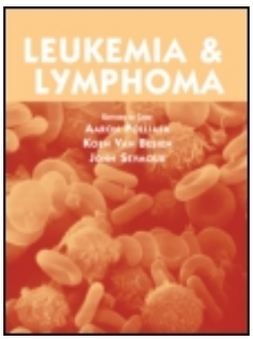

Leukemia & Lymphoma

ISSN: 1042-8194 (Print) 1029-2403 (Online) Journal homepage: http://www.tandfonline.com/loi/ilal20

MicroRNA profiling in pediatric acute lymphoblastic leukemia: novel prognostic tools

Neda Mosakhani, Virinder Kaur Sarhadi, Anu Usvasalo, Marja-Liisa Karjalainen-Lindsberg, Leo Lahti, Katja Tuononen, Ulla M. Saarinen-Pihkala & Sakari Knuutila

To cite this article: Neda Mosakhani, Virinder Kaur Sarhadi, Anu Usvasalo, Marja-Liisa Karjalainen-Lindsberg, Leo Lahti, Katja Tuononen, Ulla M. Saarinen-Pihkala & Sakari Knuutila (2012) MicroRNA profiling in pediatric acute lymphoblastic leukemia: novel prognostic tools, Leukemia & Lymphoma, 53:12, 2517-2520, DOI: 10.3109/10428194.2012.685731

To link to this article: http://dx.doi.org/10.3109/10428194.2012.685731

Leukemia & Lymphoma, December 2012; 53(12): 2517–2520

ISSN: 1042-8194 print / 1029-2403 online
DOI: 10.3109/10428194.2012.685731

informa
healthcare

LETTER TO THE EDITOR

MicroRNA profiling in pediatric acute lymphoblastic leukemia: novel prognostic tools

Neda Mosakhani[1]*, Virinder Kaur Sarhadi[1]*, Anu Usvasalo[2], Marja-Liisa Karjalainen-Lindsberg[1], Leo Lahti[3], Katja Tuononen[1], Ulla M. Saarinen-Pihkala[2] & Sakari Knuutila[1]

[1]*Department of Pathology, Haartman Institute and HUSLAB, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland,* [2]*Children's Hospital, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland and* [3]*Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands*

Childhood acute lymphoblastic leukemia (ALL) is a genetically and prognostically heterogeneous malignancy with a mortality rate of 20% [1]. The identification of novel prognostic biomarkers is, therefore, needed for treatment stratification. In many biological processes, microRNAs (miRNAs) are known to play a vital role, and their deregulation is associated with progression, metastasis, survival and treatment outcome in various types of cancer [2,3]. In pediatric ALL, miRNA expression in paired diagnosis–relapse pediatric samples and the association of miRNA expression with progression remain quite unexplored thus far.

To find prognostic biomarkers, we performed miRNA microarray profiling followed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) confirmation on 90 bone marrow core biopsies from 79 consecutive patients with pediatric ALL (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.685731), diagnosed and treated at Helsinki University Central Hospital, Finland, during 2000–2006. For the follow-up, patient data were, however, frozen on 31 December 2009. Eleven patients had samples available from core biopsies taken at the time of diagnosis and at relapse. The Nordic Society of Pediatric Hematology and Oncology (NOPHO)-ALL-2000 protocol for ALL [1] was used for all patients except for two infants, who were treated with the Interfant protocol. Of note, 15 patients were transplanted in first remission. The study was approved by the appropriate Institutional Review Boards and the National Authority for Medico-legal Affairs.

Total RNA, including miRNA, was extracted from core biopsies with a miRNeasy FFPE Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Quantification of RNA was determined with a NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). Integrity and quality of miRNA were assessed with RNA Nano 6000 and small RNA chips, analyzed using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Core biopsy material has an advantage, compared to bone marrow aspirate, in that tumor content can be determined by pathologists with high accuracy. In our core biopsies, the tumor content was similar among all of the samples ($> 80\%$). Also, our earlier study confirmed that core biopsy samples, which undergo a decalcification process in addition to formalin fixation, are technically and biologically reliable for use in miRNA profiling of cases of ALL [4]. For miRNA profiling, we used an Agilent miRNA microarray system (V3) (Agilent Technologies), containing 866 human and 89 human viral miRNAs. The raw data were processed with Agilent Feature Extraction Software and analyzed using GeneSpring Software v.11.0.2, preprocessed by log-2 transformation and normalized by the 75th percentile method. miRNAs not expressed in at least 100% of one group of samples and having less than a two-fold change (FC) were filtered out. The *t*-test was performed to find significantly differentially expressed miRNAs ($p < 0.05$ and false discovery rate or $q < 0.05$).

To evaluate the correlation of miRNA expression with overall survival (OS) and event-free survival (EFS), we performed unsupervised clustering to divide patients into two groups (K-means with two clusters and Euclidean distance) based on their miRNA expression profiles [Figure 1(a)], after which Kaplan–Meier survival analysis was performed and differences in OS and EFS between groups were compared by log-rank test. OS was calculated from the time of diagnosis until the date of death or last follow-up, and EFS was defined from the date of diagnosis until the event (relapse/death). OS and EFS analyses showed a significantly poorer prognosis in group 1 ($p = 0.02$ and $p = 0.05$, respectively) [Figures 1(b) and 1(c)]. Comparison of miRNA expression between these two patient groups showed 28 significantly up-regulated and two down-regulated miRNAs (*miR-423-5p* and *miR-*

*N. M. and V. K. S. contributed equally to this study.

Correspondence: Professor Sakari Knuutila, Haartman Institute, Department of Pathology, PO Box 21 (Haartmaninkatu 3), FI-00014 University of Helsinki, Finland. Tel: +358-9-191-26527. Fax: +358-9-191-26675. E-mail: sakari.knuutila@helsinki.fi

Received 2 February 2012; revised 4 April 2012; accepted 12 April 2012

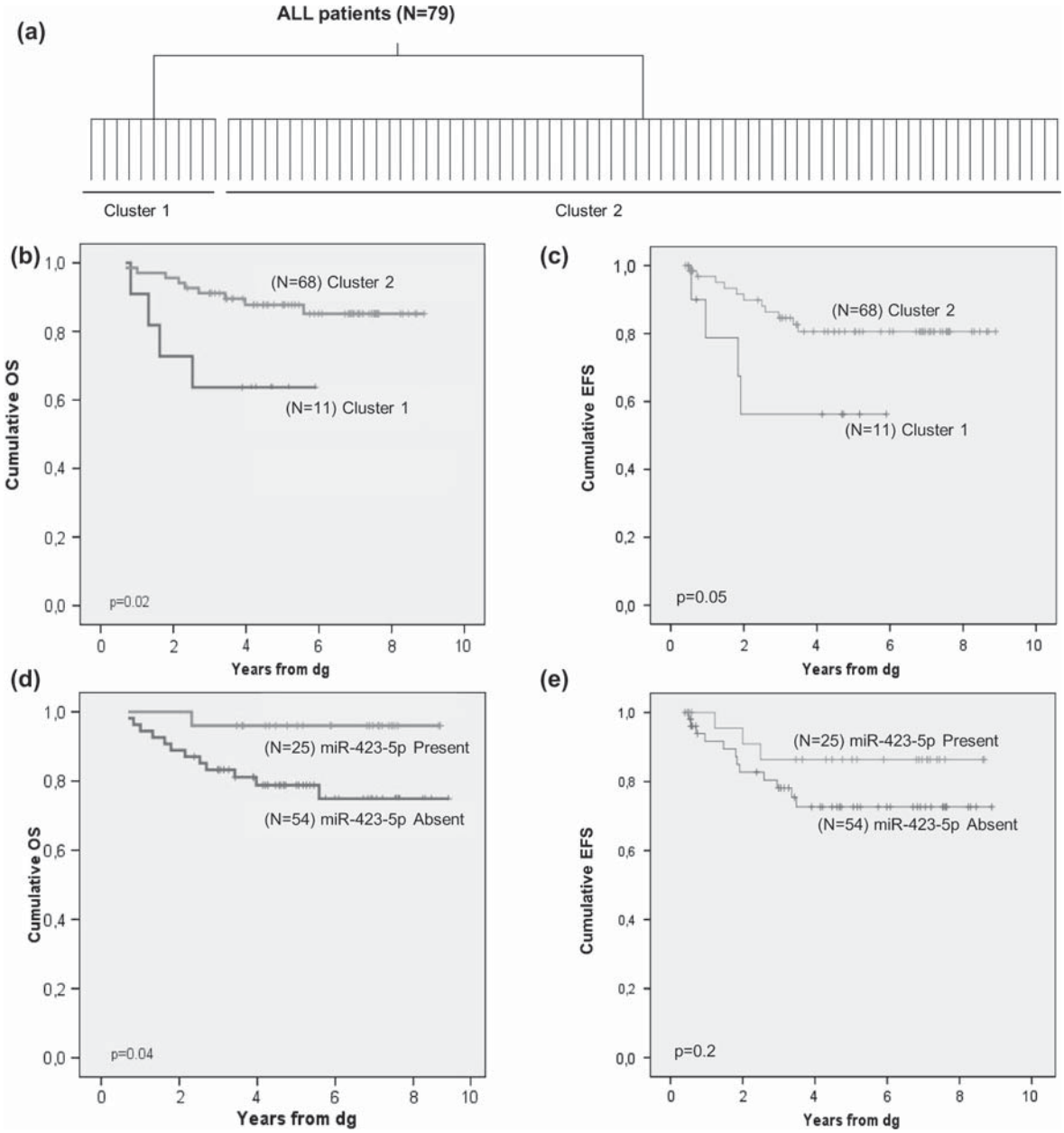

Figure 1. Clustering of patients with ALL and their survival analysis. (a) Division of patients into two clusters based on miRNA expression by 2K-mean clustering. Survival analysis by Kaplan–Meier shows that cluster 1 had a poorer prognosis and (b) shorter overall survival and (c) lower EFS than cluster 2. Absence of *miR-423-5p* expression, as studied by qRT-PCR, (d) correlates significantly with shorter survival in patients with ALL but (e) there is no significant correlation with EFS.

29c) in group 1 compared to group 2 (Table I). None of the other confounding variables of age, risk group, cytogenetic group and immunophenotype was significantly ($p < 0.05$) associated with the clusters, when quantifying by *t*-test for the continuous variable (age) and by hypergenomic enrichment analysis for the categorical variables. Expression of *miR-423-5p* was absent in all patients in group 1 (FC = 2.29, $q = 0.001$), and was, therefore, selected for validation by qRT-PCR performed using Light-Cycler software v.3.5 (Roche Applied Science, Mannheim, Germany) with a SYBR Green miScript PCR system (Qiagen). The snRNA U6 gene served as a control for normalization, and relative quantification of miRNA to U6 was calculated with the expression $2^{-\Delta\Delta Ct}$. Pooled RNA from bone marrow smears from healthy donors was used as the calibrator. Student's *t*-test was used to evaluate statistically significant differences in miRNA expression between the two groups. In accordance with the array results, qRT-PCR analysis showed a significant down-regulation of *miR-423-5p* ($p = 0.005$) in group 1. To determine whether *miR-423-5p* could independently predict survival, we performed Kaplan–Meier analysis on *miR-423-5p* expression data from qRT-PCR.

We studied OS and EFS in relation to absence or expression of *miR-423-5p*. Kaplan–Meier analysis clearly revealed that loss of *miR-423-5p* expression was associated with a poorer survival in patients with ALL ($p = 0.04$) [Figure 1(d)] but not significantly with EFS [Figure 1(e)]. A linear model indicated significant association of *miR-423-5p* expression with OS even after correcting for potential confounding factors such as immunophenotype, age and risk groups

Table I. Thirty differentially expressed miRNAs in group 1 with poorer prognosis versus group 2[§].

miRNA	Fold change
hsa-miR-1281	6.60
hsa-miR-1225-3p	5.60
*hsa-miR-877**	5.38
hsa-miR-1234	5.35
hsa-miR-1228	4.41
hsa-miR-766	3.73
hsa-miR-197	3.61
hsa-miR-940	3.46
hsa-miR-1249	3.41
hsa-miR-574-5p	3.14
hsa-miR-1280	3.09
hsa-miR-638	2.98
hsa-miR-923_v12.0	2.88
hsa-miR-572	2.83
hsa-miR-671-5p	2.75
hsa-miR-1915	2.66
hsa-miR-1268	2.66
hsa-miR-513a-5p	2.65
hsa-miR-1225-5p	2.58
hsa-miR-663	2.40
hsa-miR-939	2.28
hsa-miR-1207-5p	2.27
hsa-miR-365	2.24
hsa-miR-1202	2.14
hsa-miR-188-5p	2.08
hsa-miR-765	2.07
hsa-miR-1275	2.06
hsa-miR-483-5p	2.04
hsa-miR-423-5p[†]	2.29
hsa-miR-29c[†‡]	2.22

*indicates the mature miRNA is from the opposite arm of the precursor.
[§]For all except the final one, $q < 0.0001$, $p < 0.0001$.
[†]Down-regulated miRNAs.
[‡]$q = 0.001$, $p = 0.001$.

($p < 0.02$). *miR-423-5p* has not been associated with progression or OS in any cancer. To assess the association of *miR-423-5p* target genes with survival or prognosis and to detect pathways affected by this miRNA, we performed pathway analysis of putative target genes using Chipster Software Version 1.4.7 (http://chipster.csc.fi/). Only mRNA targets predicted by at least three of six algorithms, including TargetScan, miRanda, Sanger miRBase, mirTarget2, Tarbase and PICTAR, were screened for significant involvement in biological networks by the hyper-geometric test in ConsensusPathDB (CPDB) ($p < 0.05$). As a result we found, for *miR-423-5p*, 14 putative target genes (Supplementary Table II to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.685731) and six significantly affected pathways ($p < 0.05$) (Supplementary Table III to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.685731).

Some of the *miR-423-5p* target genes, such as *SRA1/SCARF1* and *FOXM1*, are related to prognosis and OS in various cancers, such as ovarian cancer, lymphoma and hepatocellular carcinoma [5–7]. In ovarian cancer, high expression of the *SRA1* gene is associated with disease aggressiveness, suggesting its involvement in the pathogenesis or progression of this disease [8], whereas low *SRA1* expression is related to a good prognosis and OS [5]. *FOXM1*, another *miR-423-5p* target gene, is a gene that predicts patient survival in multiple tumors, such as lymphoma and mesothelioma [7]. It has been identified as a target molecule for therapeutic regimens for patients with leukemia, since leukemia cell proliferation is inhibited by reducing its expression [9]. Moreover, amplification of the *FOXM1* oncogene locus at 12p13.33 in non-Hodgkin lymphoma is associated with an increased MYC oncogenic signaling signature [10]. Hence, negative regulation of these genes by *miR-423-5p* may demonstrate an important role for this miRNA and its target genes in survival of pediatric ALL.

Applying another approach, we also compared miRNA expression in patients with and without an event (relapse/death), and found significant down-regulation of *miR-150* in patients with an event (event = 17, no event = 62) (FC = 2.1, $q = 0.01$). In qRT-PCR, *miR-150* showed, however, no significant difference between patients with an event and those without an event, although the trend was similar to the microarray results. The expression level of *miR-150*, studied separately for precursor-B-ALL and T-ALL samples, also showed a decrease in expression in event versus no event samples. For *miR-150*, 16 putative target genes (Supplementary Table II) and 85 significant pathways ($p < 0.05$) (Supplementary Table IV to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.685731) were detected in our study.

Patients with ALL are often classified into risk groups based on clinical and cytogenetic evaluations (standard risk, intermediate risk and high risk) [1]. Among clinical risk groups, we could not find any differences in miRNA expression. We did not study miRNA profiling in different cytogenetic subtypes because the number of cases for most of the subtypes retained ultimately too small for statistical analysis. These analyses should, however, be performed in the future with larger series of ALL cases. Compared to samples at diagnosis in diagnosis–relapse pairs, miRNA profiling in relapse specimens revealed two significantly down-regulated miRNAs: *miR-431* (FC = 2.2, $q = 0.03$) and *miR-654-5p* (FC = 2.35, $q = 0.02$). For *miR-431*, screening for target genes in databases revealed 13 putative target genes (Supplementary Table II), while pathway analysis disclosed 35 significant pathways ($p < 0.05$) (Supplementary Table V to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2012.685731). Target genes of differentially expressed *miR-431* are related to epidermal growth factor receptor (EGFR) down-regulation, insulin-like growth factor 1 (IGF1) and Wnt signaling pathways. Our result revealing the differential expression of *miR-654-5p* and *miR-431* in matched diagnosis–relapse samples is, to our knowledge, the first result related to miRNA expression profiling studies in paired ALL samples. miRNAs identified in paired diagnosis–relapse comparison are not well known in ALL. However, *PKD1*, a target of *miR-431*, is a key player in mediating signal transduction related to angiogenesis by vascular endothelial growth factor (VEGF) in endothelial cells [11]. Since pathological angiogenesis is essential for tumor growth [12], molecular targeting of nuclear protein kinase D1 (PKD1) is considered to be one possibility for drug development – targeting angiogenesis in angiogenesis-related diseases [13]. However, the alteration of miRNAs in some relapsed ALL cases may result as likely from the treatment as from miRNA changes in the original tumor cells.

In conclusion, our study demonstrates that loss of *miR-423-5p* is related to a poorer prognosis, and it identifies, for the first time, miRNAs that may be important for relapse, progression and survival in pediatric ALL. Further studies are warranted to investigate the role of candidate miRNAs and target genes in survival.

Acknowledgements

We would like to acknowledge the Nona and Kullervo Väre Foundation for supporting this study.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

References

[1] Schmiegelow K, Forestier E, Hellebostad M, et al. Long-term results of NOPHO ALL-92 and ALL-2000 studies of childhood acute lymphoblastic leukemia. Leukemia 2010;24:345–354.
[2] Calin GA, Ferracin M, Cimmino A, et al. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 2005;353:1793–1801.
[3] Nymark P, Guled M, Borze I, et al. Integrative analysis of microRNA, mRNA and aCGH data reveals asbestos- and histology-related changes in lung cancer. Genes Chromosomes Cancer 2011;50:585–597.
[4] Borze I, Guled M, Musse S, et al. MicroRNA microarrays on archive bone marrow core biopsies of leukemias—method validation. Leuk Res 2011;35:188–195.
[5] Leoutsakou T, Talieri M, Scorilas A. Expression analysis and prognostic significance of the SRA1 gene, in ovarian cancer. Biochem Biophys Res Commun 2006;344:667–674.
[6] Ma HQ, Liang XT, Zhao JJ, et al. Decreased expression of Neurensin-2 correlates with poor prognosis in hepatocellular carcinoma. World J Gastroenterol 2009;15:4844–4848.
[7] Carter SL, Eklund AC, Kohane IS, et al. A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. Nat Genet 2006;38: 1043–1048.
[8] Scorilas A, Kyriakopoulou L, Katsaros D, et al. Cloning of a gene (SR-A1), encoding for a new member of the human Ser/Arg-rich family of pre-mRNA splicing factors: overexpression in aggressive ovarian cancer. Br J Cancer 2001;85:190–198.
[9] Nakamura S, Yamashita M, Yokota D, et al. Development and pharmacologic characterization of deoxybromophospha sugar derivatives with antileukemic activity. Invest New Drugs 2010;28: 381–391.
[10] Green MR, Aya-Bonilla C, Gandhi MK, et al. Integrative genomic profiling reveals conserved genetic mechanisms for tumorigenesis in common entities of non-Hodgkin's lymphoma. Genes Chromosomes Cancer 2011;50:313–326.
[11] Ha CH, Jin ZG. Protein kinase D1, a new molecular player in VEGF signaling and angiogenesis. Mol Cells 2009;28:1–5.
[12] Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997;18:4–25.
[13] Altschmied J, Haendeler J. A new kid on the block: PKD1: a promising target for antiangiogenic therapy? Arterioscler Thromb Vasc Biol 2008;28:1689–1690.

Supplementary material available online

Tables and Figure showing patient characteristics, target genes and results of pathway analysis