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Distinctive microRNA expression profiles in CD34+ bone marrow cells from patients with myelodysplastic syndrome

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Running title: MiRNA expression profiles in MDS

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KEYWORDS

myelodysplastic syndrome, miRNA expression, CD34+ cells, microarrays, 14q32 miRNA cluster

ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs functioning as regulators of hematopoiesis. Their differential expression patterns have been linked with various pathological processes originating from hematopoietic stem cells (HSCs). However, limited information is available regarding the role of miRNAs in myelodysplastic syndrome (MDS). Using miRNA arrays, we measured expression of 1,145 miRNAs in CD34+ bone marrow cells obtained from 39 MDS and acute myeloid leukemia (AML) evolved from MDS patients, and compared them to those of 6 healthy donors. Differential miRNA expression was analyzed and a panel of up-regulated (n=13) and down-regulated (n=9) miRNAs was found ($p<0.001$) in MDS/AML patients. An increased expression of a large miRNA cluster mapped within 14q32 locus was detected. Differences in miRNA expression of MDS subtypes showed distinction between early and advanced MDS; an apparent dissimilarity was observed between RAEB-1 and RAEB-2 subtypes. In early MDS, we monitored up-regulation of pro-apoptotic miR-34a which may contribute to the increased apoptosis of HSCs. Patients with 5q- deletion were characterized by decreased levels of miR-143* and miR-378 mapped within the commonly deleted region at 5q32. This is an early report describing differential expression in MDS CD34+ cells, likely reflecting their disease-specific regulation.

INTRODUCTION

Myelodysplastic syndrome (MDS) is a heterogeneous group of clonal preleukemic conditions with high risk of transformation into secondary acute myeloid leukemia with multilineage dysplasia (AML with MLD) occurring in approximately 30-40% of MDS cases. Individuals with MDS exhibit blood cytopenias, ineffective hematopoiesis and usually hypercellular or normocellular bone marrow (BM)[1]. Progression of the disease is assumed to be a multi-step process of acquisition of several successive genetic abnormalities in a normal hematopoietic stem cell (HSC) which ultimately leads to malignant transformation and expansion of the MDS clone[2]. According to the World Health Organization (WHO) classification system, MDS is divided into several subtypes with different characteristics and worsen prognoses: 5q-syndrome, refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD) and refractory anemia with excess blasts (RAEB-1 and RAEB-2)[3].

MicroRNAs (miRNAs) are small (~21nt) endogenous non-coding RNAs that negatively regulate gene expression of complementary mRNAs. Many crucial cellular processes are under control of miRNAs whose deregulation is implicated in the disease development and/or progression[4]. Only a limited number of reports investigating miRNA deregulation in MDS has been published to date[5-9]. Pons *et al.*[5] measured gene expression of 25 mature miRNAs in mononuclear cells (MNCs) isolated from bone marrow (BM) and peripheral blood of MDS patients. The authors reported an over-expression of miRNA cluster miR-17-92 in MDS and differential expression of miR-15a and miR-16 between low-risk and high-risk subgroups of patients. Hussein *et al.*[9] measured expression profiles of 365 miRNAs in total BM cells and demonstrated up-regulation of miR-1/miR-133a cluster. These studies examined miRNA expression in unsorted blood cells; however, gene expression profiles remarkably differ between various cell types[10]. Thus, increase of the blast count in high-risk MDS influences

miRNA levels when measured in whole blood samples. Therefore, separation of one cell type is essential for accurate gene expression profiling. Since MDS originates from HSC, we believe the testing of CD34+ fraction is of primary interest.

To understand impact of miRNA deregulation on MDS development and progression, we performed miRNA microarray analysis on purified CD34+ BM cells obtained from patients with MDS and AML evolved from MDS. Additionally, we profited from our previous gene expression profiling study[11] and associated miRNA levels with the appropriate gene expression data.

DESIGN AND METHODS

Patients

This study comprised 43 BM samples from Czech patients with primary MDS and AML evolved from MDS. According to the WHO 2001 classification criteria, patients' diagnoses were as follows: 5q- syndrome (n=6), RA (n=4), RARS (n=1), RCMD (n=6), RAEB-1 (n=7), RAEB-2 (n=10), and AML with MLD (n=9). Patients' age ranged between 31-79 years (average 63) and the female/male distribution was 16/27. The clinical characteristics of all patients are summarized in Table 1 and their karyotypes in supplementary Table 1. BM samples obtained from 9 healthy donors were used as controls. Their age ranged between 20-40 years (average 30) and the female/male distribution was 3/6. All tested subjects signed the informed consent and Institutional Review Board approved the study.

CD34+ cell separation

MNCs were separated from BM samples by Ficoll-Hipaque density gradient centrifugation (GE Health Care, Little Chalfont, United Kingdom). CD34+ cells were isolated from MNCs using magnetic cell separation according to the manufacturer's instructions (Direct CD34

Progenitor Cell Isolation MACS Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of isolated cells (>95%) was tested using FACSAria (Becton Dickinson, San Jose, CA, USA).

RNA extraction

Total RNA was isolated by the method of Chomczynsky and Sacchi[12] and subsequently treated with DNase I (Qiagen, Hamburg, Germany). Final RNA integrity was evaluated using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number (RIN) was found to be 9.1 on average and RNA quality was comparable within the different samples.

MiRNA microarray profiling

Human v2 MicroRNA Expression Profiling Kit (Illumina, San Diego, CA, USA) was used for measurement of gene expression of 1,145 human miRNAs (including 246 novel miRNA candidates denoted by HS codes; annotation file available at http://www.switchtoi.com/annotationfiles.ilmn). Only samples with sufficient RNA yield (39 samples of MDS/AML with MLD and 6 control samples, input 200ng of total RNA) were hybridized on Illumina 12-sample BeadArrays. Scanning was performed on the BeadStation 500 instrument (Illumina).

Microarray data analysis

Quality control, background subtraction and quantile normalization of the raw data were done by GenomeStudio software (Illumina). Only probes which reached detection P-value < 0.01 were included for further analyses. Logarithmic transformation of the data was done to obtain normal linear distribution. Subsequent analyses were performed using MeV v4.3.2 software[13].

Welch approximate t-test was applied to find out differentially expressed miRNAs between two groups of samples: (i) patients and controls, and (ii) patients with and without del(5q). Analysis of variance (ANOVA) was performed using a 1% false discovery rate threshold to identify differentially expressed miRNAs between different MDS subtypes. Hierarchical clustering of miRNA expression data was done using average linkage and Euclidean distance.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The microarray results were confirmed in the complete set of samples (43 samples of MDS/AML with MLD and 9 controls) by qRT-PCR using RotorGene 3000 apparatus (Corbett Research, Sydney, Australia). qRT-PCR was performed with following validated TaqMan MicroRNA Expression Assays (Applied Biosystems, Foster City, CA, USA): miR-10a (assay ID 000387), miR-34a (000426), miR-323-3p (002227) and miR-378 (000567). Resulting data were normalized to the endogenous control RNU48 and relative miRNA expression levels were calculated by the $\Delta\Delta C_T$ method. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA).

MiRNA target prediction

Putative miRNA targets were predicted by MicroCosm Targets v.5 (http://www.ebi.ac.uk/enright-srv/microcosm/), TargetScan 5.1 (http://www.targetscan.org/) and PicTar (http://pictar.mdc-berlin.de/) software.

RESULTS

MiRNAs differentially expressed between patients and healthy individuals

MiRNA expression profiles were measured in CD34+ cells isolated from BM of patients with MDS, AML with MLD and healthy donors (for normalized microarray data see

supplementary Table 2). Out of 1,145 tested miRNAs, 371 miRNAs were not detected and were excluded from further analyses. Subsequently, several statistical tests were applied on the filtered data set to identify differentially expressed miRNAs.

We defined a set of 22 miRNAs differentially expressed between patients and controls ($p<0.001$): 13 miRNAs were up-regulated (miR-299-3p, miR-299-5p, miR-323-3p, miR-329, miR-370, miR-409-3p, miR-431, miR-432, miR-494, miR-654-5p, miR-665, HS_40 and HS_206) and 9 miRNAs were down-regulated (miR-196a*, miR-423-5p, miR-525-5p, miR-507, miR-583, miR-940, miR-1284, miR-1305 and HS_122.1) in patients (hierarchical cluster in Figure 1). 9 up-regulated miRNAs (miR-299-3p, miR-299-5p, miR-323-3p, miR-370, miR-409-3p, miR-431, miR-432, miR-494 and miR-654-5p) mapped within 14q32 miRNA cluster. Also other miRNAs from this cluster displayed trend of increased expression in approx. 80% of MDS/AML patients (data in suppl. Table 2).

MiRNA expression profiles in MDS subtypes

To determine miRNA expression differences between MDS subtypes, we grouped the patients into five categories: 5q- syndrome, low risk MDS, RAEB-1, RAEB-2 and AML with MLD. Patients with RA (n=1), RARS (n=1) and RCMD (n=6) formed together the “low-risk MDS” group due to a small number of samples. Using one-way ANOVA test, we identified a set of 45 differentially expressed miRNAs with respect to the disease subtypes. Hierarchical clustering performed for the deregulated miRNAs resulted in the determination of five distinct clusters (Figure 2). MiRNAs grouped in red cluster showed slightly higher expression in low-risk MDS and RAEB-1 than in 5q- syndrome and high-risk patients. On the contrary, miRNAs in green cluster were down-regulated in low risk MDS and REAB-1 and displayed increased or normal levels in the other samples. MiRNAs in blue and brown cluster were down-regulated only in low-risk MDS category. Further, we detected 4 transcripts (miR-422a,

miR-617, HS_63 and HS_305_b; violet cluster) whose expression correlated with disease progression; their expression was low in normal samples and early MDS and high in advanced MDS and AML with MLD with elevation of their expression level between RAEB-1 and RAEB-2.

MiRNAs deregulated in patients with 5q- deletion

Our MDS subject cohort included 6 patients with 5q- syndrome and 6 patients with advanced MDS bearing 5q- deletion (RAEB-1_3, RAEB-2_3, RAEB-2_4, RAEB-2_9, RAEB-2_10, and AML with MLD_6). A non-parametric t-test was applied to identify miRNAs differentially transcribed in patients with and without deletion in the 5q32 region. Expression levels of 13 miRNAs were elevated and 7 of them were reduced in the patients with the deletion ($p<0.01$, Figure 3).

Figure 4 shows expression of miRNAs located in deleted region in 5q- syndrome (miR-143, miR-145, miR-146a and miR-378), demonstrating that only expression of miR-378 and miR-143* was significantly reduced in the patients with 5q- deletion.

Validation of microarray data

Using qRT-PCR, expression of 4 miRNAs was measured in the complete set of samples (43 patients and 9 controls): miR-10a, miR-34a, miR-323-3p, and miR-378 (Figure 5). We observed differences between miRNA levels in particular disease subtypes comparable to those obtained by microarrays. Moreover, level of miR-10a inversely associated with progression of the disease (miR-10a level was increased in 5q- syndrome and RA/RARS samples and reduced in RCMD, advanced MDS and secondary AML compared to healthy donors).

miRNA targets

To identify target genes of miR-10a, miR-34a, miR-378 and miR-127, we searched within previously published studies and also predicted new putative miRNA targets with potential implication in MDS (Table 2). We attempted to correlate miRNA expression levels with those of their targets measured in our previous study[11]. However, we confirmed inverse association of only a small number of target genes: increase of miR-34a corresponded to 70% reduction of *SPI1* transcript level in early MDS (p=0.04), reduction of miR-378 to 30% increase of *SUFU* level in 5q- samples (p=0.01) and miR-127 up-regulation to 100% reduction of *BCL6* level in all MDS (p=0.02).

DISCUSSION

Microarrays have been anticipated to bring new information to the current knowledge of molecular pathogenesis of MDS. Several studies focused on gene expression signatures of this disorder[11,14,15]; however, not many crucial questions have been answered. These studies identified many deregulated genes whose alteration may be critical to the MDS phenotype but no fundamental implications like new targets for therapeutic intervention or improvement of prognostic scoring system have been revealed. Recently, our laboratory performed another gene expression profiling of CD34+ progenitors in patients with MDS and secondary AML[11], and the miRNA profiling study presented here continues with further characterization of the same cohort of patients. We expected that the integration of mRNA and miRNA expression data might provide a more complex insight into the disease pathogenesis.

MiRNA expression analysis was done on CD34+ cells obtained from 39 patients (MDS and AML with MLD) and 6 controls. We identified a set of 22 miRNAs differentially expressed between patients and controls. Interestingly, 9 of the up-regulated miRNAs were located at 14q32. Up-regulation of several miRNAs from this region was observed also in AML bearing

t(15;17) translocation[16]. 14q32 comprises 40 miRNA genes with imprinted expression controlled by a distant differentially methylated region (DMR)[17]. In our study, we detected significant over-expression of the whole cluster in the majority of MDS/AML patients. We hypothesized that the cluster up-regulation might be caused by altered imprinting in DMR possibly due to uniparental disomy (UPD) and/or deregulated epigenetic mechanisms in 14q32. We read through recent publications whether any defect was detected in this region; however, no copy number variations or UPD found in 14q32[18,19] suggests rather a change in the methylation or acetylation status or aberrant characteristics of some transcriptional regulators. Limited information is available about the function of 14q32 miRNAs; however, several publications suggested their implication in cell development and oncogenesis. For example, miR-127, a member of 14q32 miRNA cluster, is involved in B-cell differentiation process through post-transcriptional regulation of *BLIMP1* and *XBP1*[20]. In MDS, we detected negative association of expression of another miR-127 target, proto-oncogene *BCL6*[21], functioning also as a transcriptional regulator required for B- and T-lymphocyte terminal differentiation[22]. Thus, over-expression of miR-127 may be linked with the down-regulation of genes involved in B-cell lineage differentiation previously seen in MDS[11, 23].

In CD34+ cells of all MDS subtypes, we confirmed up-regulation of miR-1/miR-133a cluster ($p < 0.05$, see supplementary Table 2), which was recently published by Hussein et al.[9] In contrast to increased expression in MDS/AML, this miRNA cluster was down-regulated in neutrophils from patients with polycythemia vera and essential trombocytosis[24], indicating its different roles in myeloid neoplasms.

5q- syndrome is a MDS subtype characterized by interstitial deletion of the long arm of chromosome 5. The commonly deleted region (CDR) at 5q32 contains 40 genes and several of them may be implicated in hematopoiesis but none of the genes seems to be responsible for all characteristics of 5q- syndrome[25-27]. Three miRNAs (miR-143, miR-145, and miR-378) are

mapped within the region and miR-146a is located in its proximity. Expression of these miRNAs was examined by Starczynowski *et al.*[28] who showed correlation between del(5q) and the loss of miR-145 and miR-146a in a murine model. However, in human CD34+ and unsorted BM cells any expression changes of miRNAs encoded in CDR were not detected[7,9]. Our data suggested down-regulation of miR-378 and miR-143* (complementary to miR-143 but less abundant) whereas the level of miR-145 and miR-146a remained at the control level. In addition, 50% decrease of miR-378 and miR-143* expression corresponded with the haploinsufficiency status. The discrepancy in the results is probably caused by small numbers of samples in the studies and should be clarified in future.

We hypothesize that an altered epigenetic mechanism at the remaining chromosome, perhaps as a compensational event, may explain why deletion at 5q32 does not lead to reduced expression of the presented miRNAs. Interestingly, we found specific down-regulation of miR-143* but not of miR-143 in 5q- syndrome. Expression of different miRNA variants is usually tissue-specific with lower expression of the complementary variants. It is generally thought that these minor variants are not so important for mRNA regulation. Therefore, 5q-deletion might be compensated only in the case of major variant of miR-143.

In 5q- patients, we observed significant reduction of miR-378 level. However, validation of the microarray data revealed rather its non-specific deregulation. miR-378 seems to be affected either directly (through chromosome 5 rearrangement as a part of complex karyotype) or indirectly (through deregulation of some upstream target) also in high-risk MDS. This miRNA promote cell survival, tumor growth and angiogenesis by targeting *SUFU* gene expression[29]. Our previously published gene expression data[11] showed an increase of *SUFU* transcript level in 5q- syndrome patients. Another putative target of miR-378 is transcription factor *ETV6*. This gene is involved in a large number of chromosomal rearrangements associated with leukemia, including MDS[30,31]. Since *ETV6* is required for

hematopoiesis, it may be candidate gene generating MDS phenotype. Thus, our findings suggest that the reduction of miR-378 expression may be an event contributing to the pathogenesis of MDS.

Our data provided evidence for deregulation of miRNAs in particular MDS subtypes. Interestingly, an apparent dissimilarity was observed between RAEB-1 and RAEB-2 expression patterns. The miRNA profile of RAEB-1 patients was more similar to controls and early MDS subtypes whereas RAEB-2 profiles rather resembled those of secondary AML, suggesting transformation of the disease as early as between RAEB-1 and RAEB-2.

Expression of miR-10a seems to negatively associate with progression status of MDS patients. Deregulation of miR-10a in various myeloid disorders has been previously reported (down-regulation in AML unsorted BM cells[16], down-regulation in chronic myeloid leukemia CD34+ cells[32] or up-regulation in BM MNCs of MDS patients[5]), indicating its different tissue-specific roles in myeloid neoplasms. MiR-10a regulates translation of *HOX* genes[33] that play important roles in regulation of early stages of hematopoiesis, including self-renewal of HSCs[34]. Thus, reduction of miR-10a level in advanced MDS may induce (through up-regulation of *HOX* genes) conversion of BM HSCs to leukemic cells, and its decrease might be a marker of MDS progression.

Further, we detected statistically significant over-expression of miR-34a in early MDS (approx. 10-fold, $p < 0.001$) and its level was also non-significantly increased in AML with MLD. MiR-34a is a direct pro-apoptotic transcriptional target of p53[35] and may play, by targeting *BCL2*[36], a role in the increased apoptosis of BM progenitors seen in low-risk MDS. In addition, we observed an inverse association between expressions of miR-34a and its putative target *SPI1* in early MDS. *SPI1* encodes transcription factor PU.1, mediating myeloid cell development[37], whose expression is down-regulated in particular subtypes of AML and MDS[38,39]. Taken together, our results suggest that the up-regulation of miR-34a in early MDS

possibly affects not only apoptosis of HSCs through *BCL2* but could also decrease PU.1 expression, which in consequence may impede myeloid signaling and contribute to ineffective hematopoiesis of early MDS.

In this study, we attempted to integrate miRNA expression data with the data from gene expression profiling previously performed in our laboratory[11]. However, we confirmed only a limited number of miRNA/mRNA interactions. It is known that miRNAs rather suppress target gene translation, not reflecting its effect on mRNA level. This regulatory impact of miRNAs at the post-transcriptional level may be reason why mRNA microarrays did not fully meet the expectations of finding new genes eligible for targeted therapy of MDS. We believe the mRNA expression profiling represents preliminary data for future proteomic approaches that will monitor effector molecules, and thus study the real situation in the cell.

This study identified miRNAs whose expression is deregulated in all MDS/AML with MLD patients and several miRNAs specific for particular disease subtypes. This is an early report describing complete miRNA expression profiles of MDS CD34+ cells measured by miRNA arrays. Deregulation of several miRNAs detected in this study may contribute to specific features of the disease; however, we are aware that their impact on the MDS characteristics has to be fully clarified by functional studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary information is available at European Journal of Human Genetics's website.

Supplementary Table 1. Patients' karyotypes.

Supplementary Table 2. Normalized signal intensities of all 774 detected miRNAs (detection P-value < 0.01) in CD34+ cells of individual MDS/AML with MLD patients and healthy donors. Background signal was subtracted.

REFERENCES

1. Aul C, Bowen DT, Yoshida Y. Pathogenesis, etiology and epidemiology of myelodysplastic syndromes. *Haematologica* 1998; **83**: 71-86.
2. Look AT. Molecular Pathogenesis of MDS. *Hematology Am Soc Hematol Educ Program* 2005; **2005**: 156-160.
3. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood* 2002; **100**: 2292-2302.
4. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281-297.
5. Pons A, Nomdedeu B, Navarro A et al. Hematopoiesis-related microRNA expression in myelodysplastic syndromes. *Leuk Lymphoma* 2009; **50**: 1854-1859.
6. Hussein K, Theophile K, Büsche G et al. Significant inverse correlation of microRNA-150/MYB and microRNA-222/p27 in myelodysplastic syndrome. *Leuk Res* 2010; **34**: 328-334.

7. Boultwood J, Pellagatti A, Cattan H et al. Gene expression profiling of CD34+ cells in patients with the 5q- syndrome. *Br J Haematol* 2007; **139**: 578-589.

8. Bousquet M, Quelen C, Rosati R, et al. Myeloid cell differentiation arrest by miR-125b-1 in myelodysplastic syndrome and acute myeloid leukemia with the t(2;11)(p21;q23) translocation. *J Exp Med* 2008; **205:** 2499-2506.

9. Hussein K, Theophile K, Busche G, et al. Aberrant microRNA expression pattern in myelodysplastic bone marrow cells. *Leuk Res* 2010; **34:** 1169-1174.

10. Merkerova M, Belickova M, Bruchova H. Differential expression of microRNAs in hematopoietic cell lineages. *Eur J Haematol* 2008; **81**: 304-310.

11. Vasikova A, Belickova M, Budinska E, Cermak J. A distinct expression of various gene subsets in CD34+ cells from patiens with early and advanced myelodysplastic syndrome. *Leukemia Research* 2010; e-pub ahead of print 17 March 2010; doi:10.1016/j.leukres.2010.02.021.

12. Chomczynsky P, Sacchi N. Single step Metod of RNA isolation by acid guanidinisothiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156-159.

13. Saeed AI, Bhagabati NK, Braisted JC et al. TM4 microarray software suite. *Methods in Enzymology* 2006; **411**: 134-193.

14. Pellagatti A, Cazzola M, Giagounidis A et al. Deregulated gene expression pathways in myelodysplastic syndrome hematopoietic stem cells. *Leukemia* 2010; **24**: 756-764.

15. Miyazato A, Ueno S, Ohmine K et al. Identification of myelodysplastic syndrome-specific genes by DNA microarray analysis with purified hematopoietic stem cell fraction. *Blood* 2001; **98**: 422-427.

16. Dixon-McIver A, East P, Mein CA et al. Distinctive patterns of microRNA expression associated with karyotype in acute myeloid leukaemia. *PLoS One* 2008; **3**: e2141.

17. Seitz H, Royo H, Bortolin ML, Lin SP, Ferguson-Smith AC, Cavaillé J. A large imprinted microRNA gene cluster at the mouse Dlk1-Gtl2 domain. *Genome Res* 2004; **14**: 1741-1748.

18. Gondek LP, Tiu R, O'Keefe CL, Sekeres MA, Theil KS, Maciejewski JP. Chromosomal lesions and uniparental disomy detected by SNP arrays in MDS, MDS/MPD, and MDS-derived AML. *Blood* 2008; **111**: 1534-1542.

19. Heinrichs S, Kulkarni RV, Bueso-Ramos CE et al. Accurate detection of uniparental disomy and microdeletions by SNP array analysis in myelodysplastic syndromes with normal cytogenetics. *Leukemia* 2009; **23**: 1605-1613.

20. Leucci E, Onnis A, Cocco M et al. B-cell differentiation in EBV-positive Burkitt lymphoma is impaired at posttranscriptional level by miRNA-altered expression. *Int J Cancer* 2010; **126**: 1316-1326.

21. Saito Y, Liang G, Egger G et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* 2006; **9**: 435-443.

22. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* 2010; **11**: 114-120.

23. Sternberg A, Killick S, Littlewood T et al. Evidence for reduced B-cell progenitors in early (low-risk) myelodysplastic syndrome. *Blood* 2005; **106**: 2982-2991.

24. Slezak S, Jin P, Caruccio L et al. Gene and microRNA analysis of neutrophils from patients with polycythemia vera and essential thrombocytosis: down-regulation of micro RNA-1 and -133a. *J Transl Med.* 2009; **7**: 39.

25. Boultwood J, Fidler C, Strickson AJ et al. Narrowing and genomic annotation of the commonly deleted region of the 5q- syndrome. *Blood* 2002; **99**: 4638-4641.

26. Lehmann S, O'Kelly J, Raynaud S, Funk SE, Sage EH, Koeffler HP. Common deleted genes in the 5q- syndrome: thrombocytopenia and reduced erythroid colony formation in SPARC null mice. *Leukemia* 2007; **21**: 1931-1936.

27. Ebert BL, Pretz J, Bosco J et al. Identification of RPS14 as a 5q- syndrome gene by RNA interference screen. *Nature* 2008; **451**: 335-339.

28. Starczynowski DT, Kuchenbauer F, Argiropoulos B et al. Identification of miR-145 and miR-146a as mediators of the 5q- syndrome phenotype. *Nat Med* 2010; 16: 49-58.

29. Lee DY, Deng Z, Wang CH, Yang BB. MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. *Proc Natl Acad Sci U S A* 2007; **104**: 20350-20355.

30. Mauvieux L, Helias C, Perrusson N et al. ETV6 (TEL) gene amplification in a myelodysplastic syndrome with excess of blasts. *Leukemia* 2004; **18**: 1436-1438.

31. Odero MD, Vizmanos JL, Román JP et al. A novel gene, MDS2, is fused to ETV6/TEL in a t(1;12)(p36.1;p13) in a patient with myelodysplastic syndrome. *Genes Chromosomes Cancer* 2002; **35**: 11-19.

32. Agirre X, Jiménez-Velasco A, San José-Enériz E et al. Down-regulation of hsa-miR-10a in chronic myeloid leukemia CD34+ cells increases USF2-mediated cell growth. *Mol Cancer Res*. 2008; **6**: 1830-1840.

33. Lund AH. miR-10 in development and cancer. *Cell Death Differ* 2010; **17**: 209-214.

34. Abramovich C, Humphries RK. Hox regulation of normal and leukemic hematopoietic stem cells. *Curr Opin Hematol* 2005; **12**: 210-216.

35. Hermeking H. p53 enters the microRNA world. *Cancer Cell* 2007; **12**: 414-418.

36. Wang X, Liu P, Zhu H et al. miR-34a, a microRNA up-regulated in a double transgenic mouse model of Alzheimer's disease, inhibits bcl2 translation. *Brain Res Bull* 2009; **80**: 268-273.

37. Nagamura-Inoue T, Tamura T, Ozato K. Transcription factors that regulate growth and differentiation of myeloid cells. *Int Rev Immunol* 2001; **20**: 83-105.

38. D'Alò F, Di Ruscio A, Guidi F et al. PU.1 and CEBPA expression in acute myeloid leukemia. *Leuk Res* 2008; **32**: 1448-1453.

39. Huh HJ, Chae SL, Lee M et al. CD34, RAB20, PU.1 and GFI1 mRNA expression in myelodysplastic syndrome. *Int J Lab Hematol* 2009; **31**: 344-351.

40. Ichimura A, Ruike Y, Terasawa K, Shimizu K, Tsujimoto G. miR-34a inhibits cell proliferation by repressing MEK1 during megakaryocytic differentiation of K562. *Mol Pharmacol.* 2010; **77**: 1016-1024.

41. Yan D, Zhou X, Chen X et al. MicroRNA-34a inhibits uveal melanoma cell proliferation and migration through downregulation of c-Met. *Invest Ophthalmol Vis Sci* 2009; **50**: 1559-1565.

Table 1. Patient characteristics.

Patients	N	Sex (F/M)	Age (average, range)	Blasts, % (average, range)	WBC, 10^9/l (average, range)	Karyotype (normal/abnormal /not available)
5q- syndrome	6	6/0	66 (57-75)	2.3 (1.4-5)	4.6 (2.4-6.0)	0/6/0
RA/RARS	4/1	0/5	62 (57-76)	1.1 (0.2-4.2)	6.6 (4.6-9.0)	5/0/0
RCMD	6	0/6	66 (59-79)	2.7 (1.4-4.2)	5.5 (2.2-10.6)	3/0/3
RAEB-1	7	3/4	65 (58-72)	7.2 (3.6-9.2)	2.2 (1.7-33.1)	2/5/0
RAEB-2	10	4/6	60 (31-79)	14.0 (10.0-18.6)	3.2 (1.1-4.7)	1/9/0
AML with MLD	9	3/6	63 (47-73)	31 (20.4-56.2)	22.9 (0.5-154.4)	2/7/0

Table 2: Previously published or putative miRNA targets with potential implications in MDS.

miRNA	miRNA deregulation in MDS	Previously published or putative targets
miR-10a	↑ early MDS ↓ advanced MDS	*HOX* genes (e.g. *HOXA1, HOXA3, HOXB3*)[33]
miR-34a	↑ early MDS	*BCL2*[36], *MEK1*[40], *MET*[41], *MYCN, SPI1**
miR-378	↓ in 5q- syndrome	*SUFU**[29], *ETV6, BCL2L2*
miR-127	↑ in all MDS	*BCL6**[21], *BLIMP1*[20], *XBP1*[20]

*Genes with inverse association of their transcript levels (compared with miRNA expression) in our previously published mRNA expression data[11].

TITLES AND LEGENDS TO FIGURES

Figure 1: Hierarchical clustering of 22 differentially expressed miRNAs between patients (MDS or AML with MLD) and controls ($p<0.001$). The binary logarithm of relative miRNA expression changes is expressed by a color gradient intensity scale, as shown at the top. Green color indicates down-regulation, red color indicates up-regulation of miRNA expression. Each column represents a separate sample and each row a single miRNA. N-control samples.

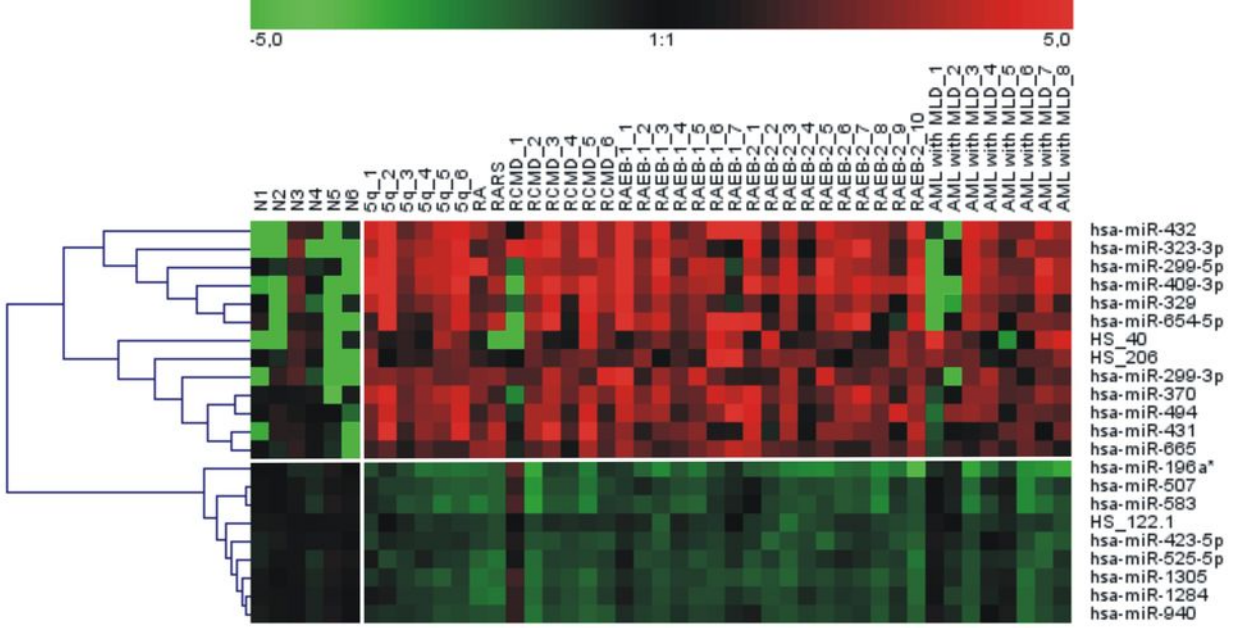
Figure 2: Hierarchical clustering of 45 differentially expressed miRNAs between different subtypes of the disease (5q- syndrome, low risk MDS (RA, RARS and RCMD), RAEB-1, RAEB-2 and AML with MLD) ($p < 0.001$). The binary logarithm of relative miRNA expression changes is expressed by a color gradient intensity scale, as shown at the top. Green color indicates down-regulation, red color indicates up-regulation of miRNA expression. Each column represents a separate sample and each row a single miRNA. Particular miRNA clusters are color-coded on the right side of heatmap. N-control samples.

Figure 3: Differentially expressed miRNAs in patients with and without the deletion at 5q32 region. The binary logarithm of relative miRNA expression changes is expressed by a color gradient intensity scale, as shown at the top. Green color indicates down-regulation, red color indicates up-regulation of miRNA expression. Each column represents a separate sample and each row a single miRNA. N-control samples.

Figure 4: Expression of miRNAs located within commonly deleted region at 5q32 (miR-143, miR-145, miR-378) or in its proximity (miR-146a) and their complementary variants (miR*) measured by Illumina microarrays. Values are expressed as binary logarithms of fold changes (logFC). Boxes represent means and 25-75 percentiles and bars indicate the minimum and

maximum values for particular patient category compared to the control group (NORM). The 5q- bar represents only patients with pure 5q- syndrome, whereas the bar denoted del(5q) illustrates miRNA expression in all patients with deletion at 5q32 region (all 5q- together with RAEB-1_3, RAEB-2_3, RAEB-2_4, RAEB-2_9, RAEB-2_10 and AML with MLD_6 patients). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; other differences between normal samples and patients with del(5q) were not statistically significant. Expression of miR-145* and miR-146a* was under the detection limit of our experiment.

Figure 5: Validation of microarray data of selected miRNAs using qRT-PCR. Relative fold changes (FC) of gene expression were measured in the complete set of samples and were calculated by ΔΔCT method. Values are expressed as binary logarithms of FC. Bars indicate the mean value of logFC plus standard error for particular patient category compared to the control group (NORM). The 5q- bar represents only patients with pure 5q- syndrome, whereas the bar denoted del(5q) illustrates miRNA expression in all patients with deletion at 5q32 region (all 5q- together with RAEB-1_3, RAEB-2_3, RAEB-2_4, RAEB-2_9, RAEB-2_10 and AML with MLD_6 patients).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RAEB-2_6
RAEB-2_7
RAEB-2_8
RAEB-2_9
RAEB-2_10
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AML with MLD_2
AML with MLD_3
AML with MLD_4
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AML with MLD_6
AML with MLD_7
AML with MLD_8
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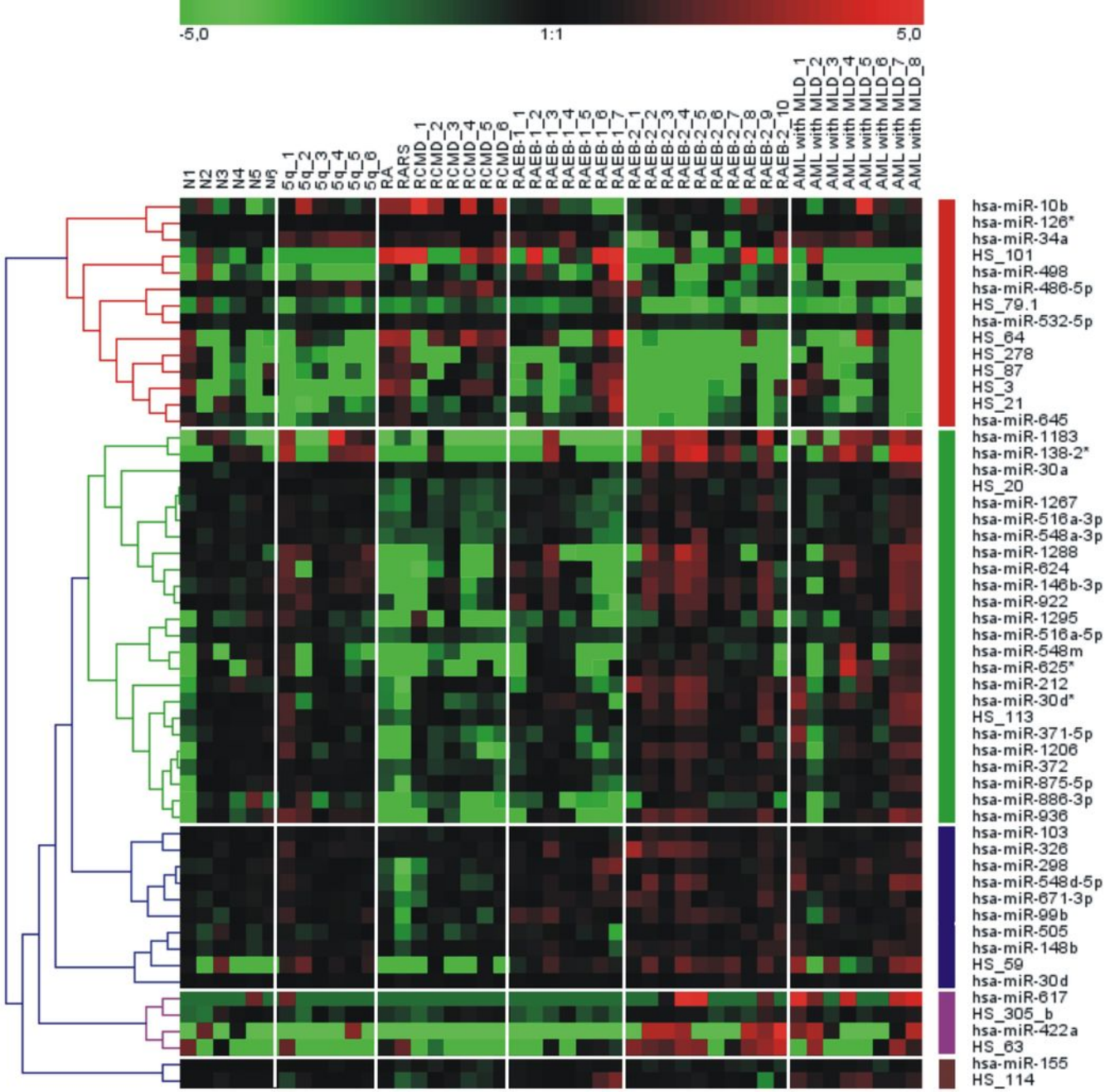
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RCMD_4
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RAEB-2_5
RAEB-2_6
RAEB-2_7
RAEB-2_8
RAEB-2_9
RAEB-2_10
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AML with MLD_2
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hsa-miR-126*
hsa-miR-34a
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hsa-miR-498
hsa-miR-486-5p
HS_79.1
hsa-miR-532-5p
HS_64
HS_278
HS_87
HS_3
HS_21
hsa-miR-645
hsa-miR-1183
hsa-miR-138-2*
hsa-miR-30a
HS_20
hsa-miR-1267
hsa-miR-516a-3p
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hsa-miR-1288
hsa-miR-624
hsa-miR-146b-3p
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hsa-miR-516a-5p
hsa-miR-548m
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hsa-miR-212
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hsa-miR-936
hsa-miR-103
hsa-miR-326
hsa-miR-298
hsa-miR-548d-5p
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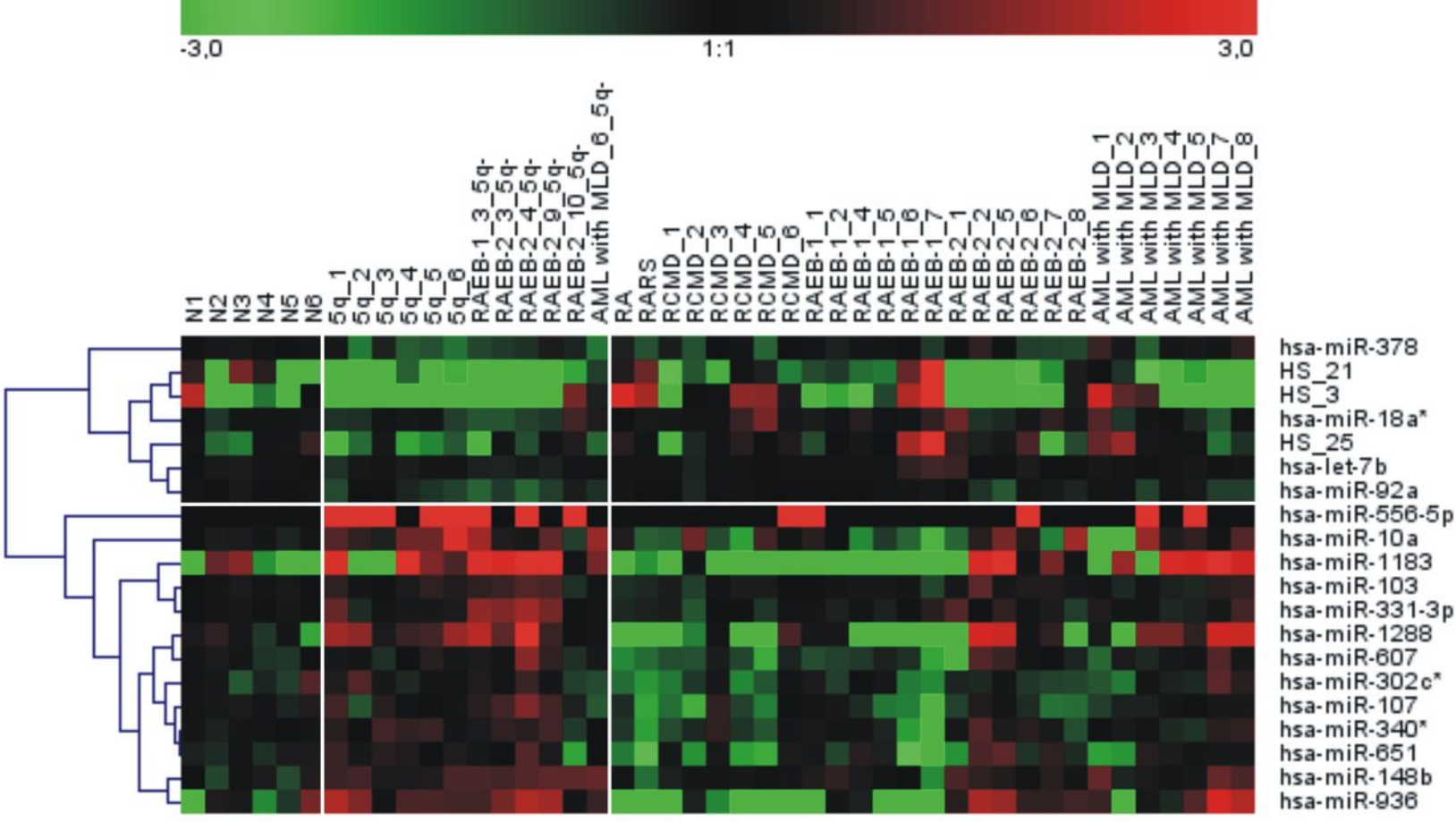
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N3
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N6
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5q_2
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5q_5
5q_6
RAEB-1_3_5q-
RAEB-2_3_5q-
RAEB-2_4_5q-
RAEB-2_9_5q-
RAEB-2_10_5q-
AML with MLD_6_5q-
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RARS
RCMD_1
RCMD_2
RCMD_3
RCMD_4
RCMD_5
RCMD_6
RAEB-1_1
RAEB-1_2
RAEB-1_4
RAEB-1_5
RAEB-1_6
RAEB-1_7
RAEB-2_1
RAEB-2_2
RAEB-2_5
RAEB-2_6
RAEB-2_7
RAEB-2_8
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AML with MLD_2
AML with MLD_3
AML with MLD_4
AML with MLD_5
AML with MLD_7
AML with MLD_8
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HS_3
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HS_25
hsa-let-7b
hsa-miR-92a
hsa-miR-556-5p
hsa-miR-10a
hsa-miR-1183
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hsa-miR-331-3p
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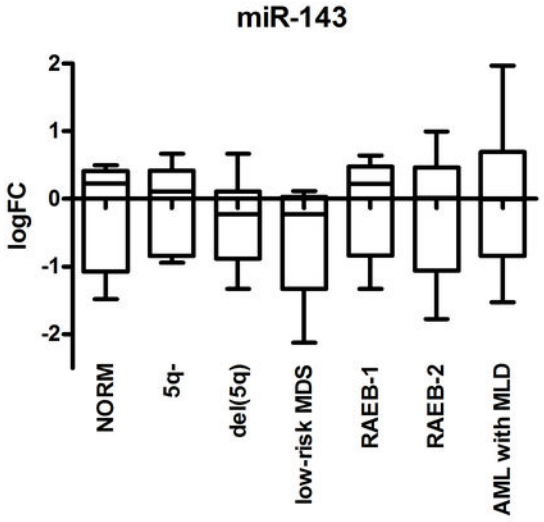
miR-143
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-1
-2
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5q-
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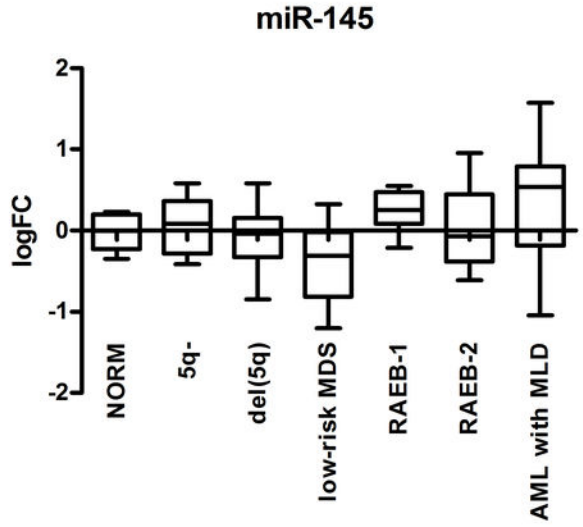
low-risk MDS
RAEB-1
RAEB-2
AML with MLD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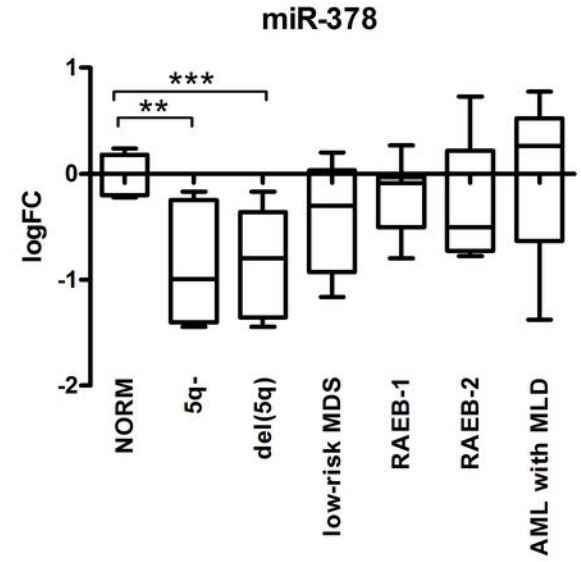
miR-378
***
**
logFC
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-1
-2
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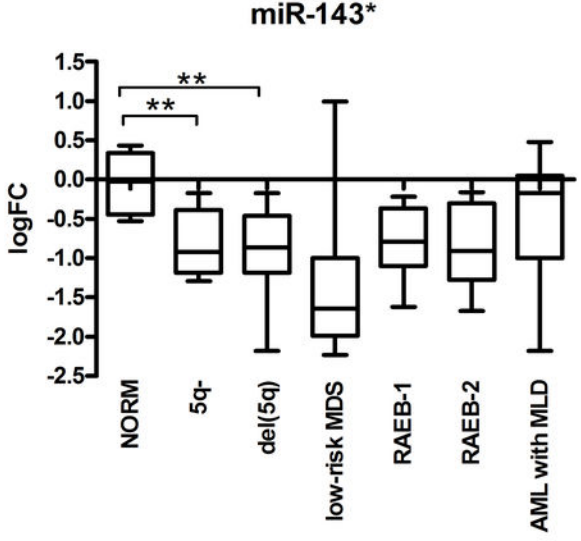
5q-
del(5q)
low-risk MDS
RAEB-1
RAEB-2
AML with MLD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**
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-2.5
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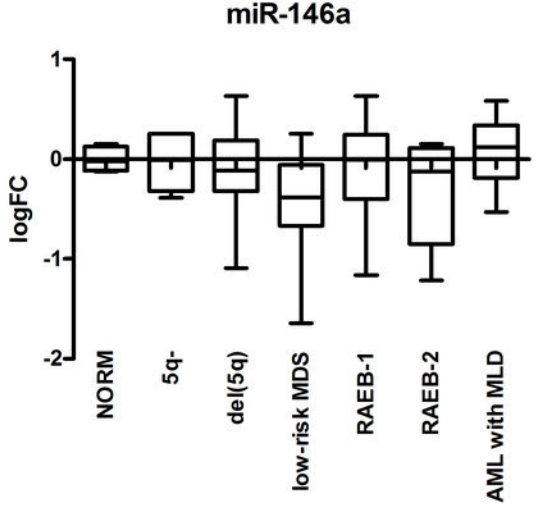
del(5q)
low-risk MDS
RAEB-1
RAEB-2
AML with MLD


miR-146a
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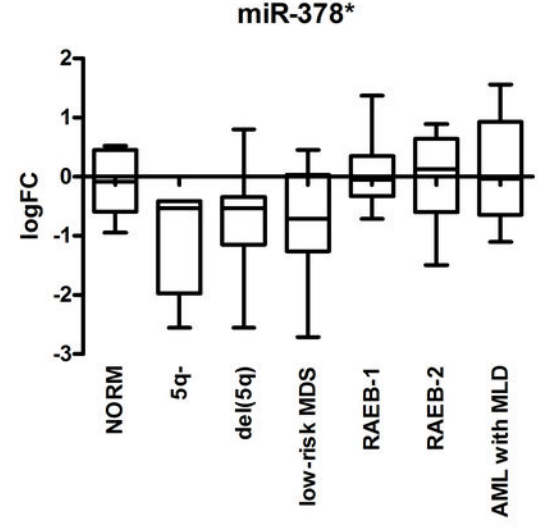
RAEB-2
AML with MLD


miR-378*
logFC
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1
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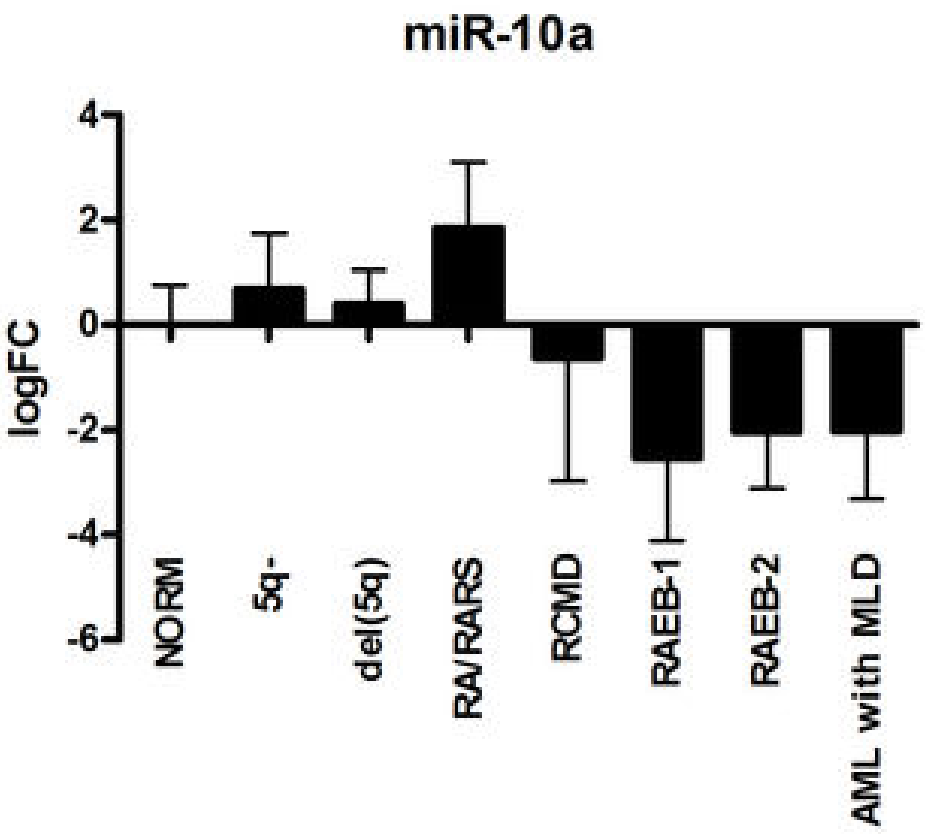
-1
-2
-3
NORM
5q-
del(5q)
low-risk MDS
RAEB-1
RAEB-2
AML with MLD


miR-10a
logFC
4
2
0
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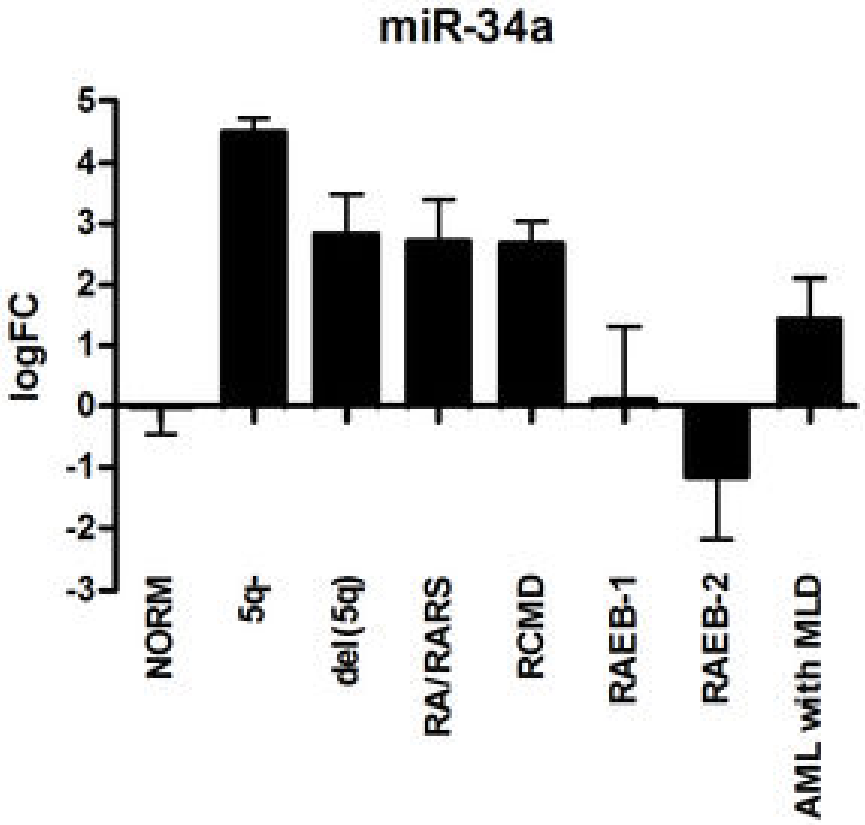
-6
NORM
5q-
del(5q)
RA/RARS
RCMD
RAEB-1
RAEB-2
AML with MLD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4
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RCMD
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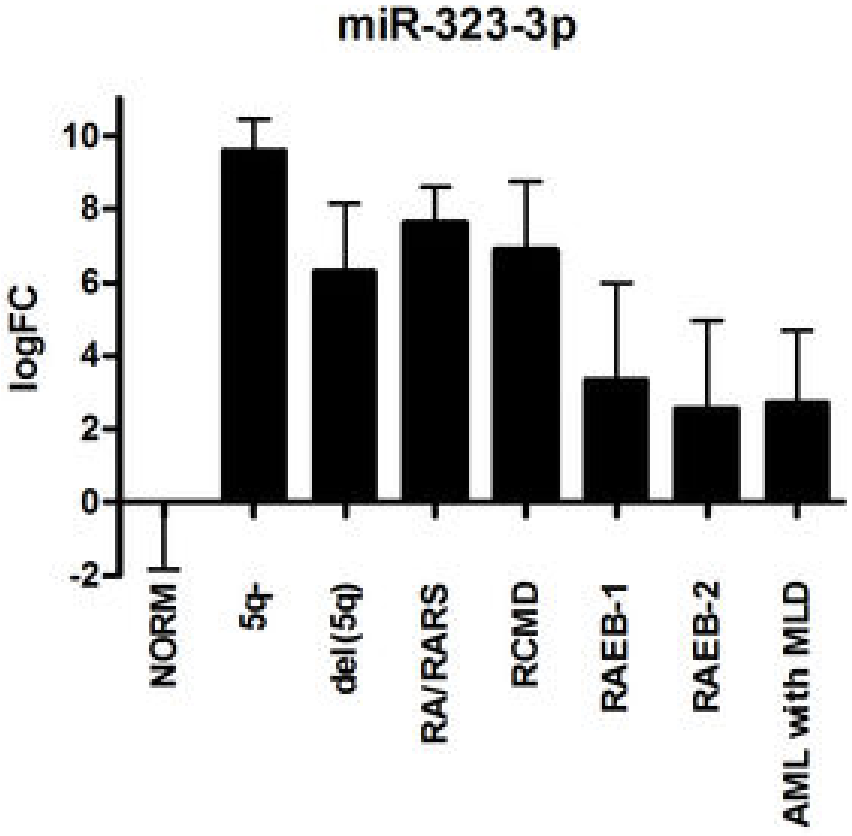
RAEB-2
AML with MLD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10
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4
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NORM
5q-
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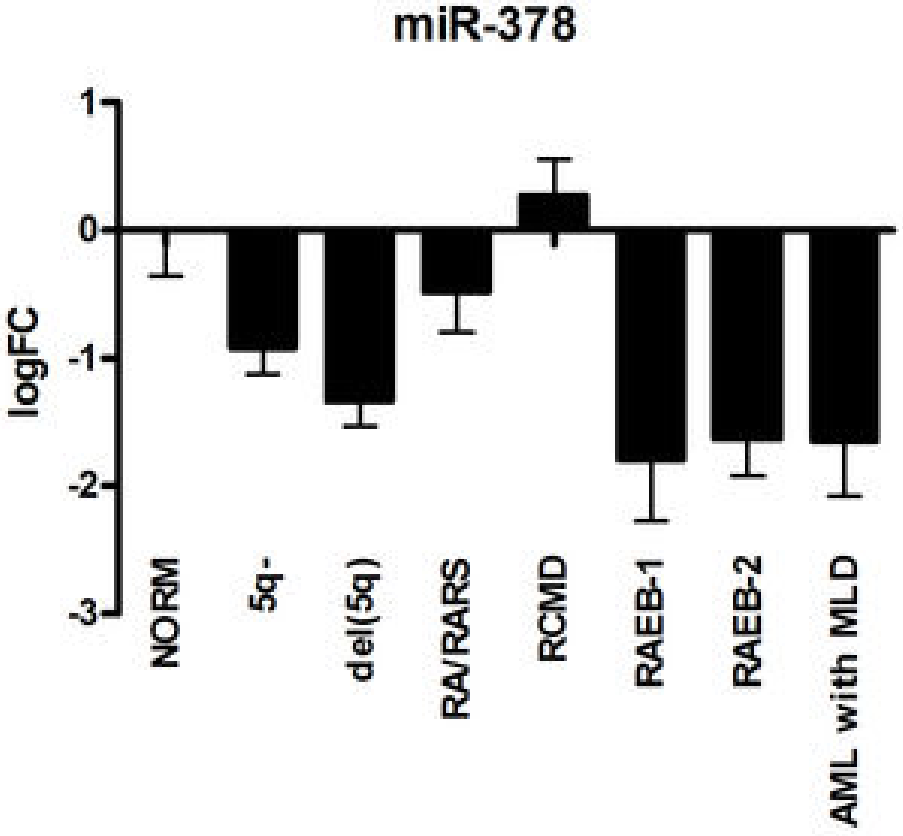
AML with MLD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NORM
5q-
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RA/RARS
RCMD
RAEB-1
RAEB-2
AML with MLD