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MicroRNAs expression signatures are associated with lineage and survival in acute leukemias

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Abstract

MicroRNAs (miRNAs) are small (~22 nucleotide) non-coding RNAs whose altered expression has been associated with various types of cancers, including leukemia. In the present study, we conducted a quantitative PCR (qPCR) analysis of expression of 23 human precursor miRNAs in bone marrow specimens of 85 Chinese primary leukemia patients, including 53 acute myeloid leukemia (AML) and 32 acute lymphoblastic leukemia (ALL) cases. We show that 16 miRNAs were differentially expressed between AMLs and ALLs; Of them, eight were previously reported (i.e., miR-23a, miR-27a/b, miR-128a, miR-128b, miR-221, miR-222, miR-223, and let-7b) and eight were newly identified (i.e., miR-17, miR-20a, miR-29a/c, miR-29b, miR-146a, miR-150, miR-155, and miR-196b). More importantly, through correlating miRNA expression signatures with outcome of patients, we further show that expression signatures of a group of miRNAs are associated with overall survival of patients. Of them, three (i.e., miR-146a, miR-181a/c, and miR-221), five (i.e., miR-25, miR-26a, miR-29b, miR-146a, and miR-196b), and three (i.e., miR-26a, miR-29b, and miR-146a) miRNAs are significantly associated with overall survival (P<0.05) of the 32 ALL, 53 AML, and 40 non-M3 AML patients, respectively. Particularly, the expression signature of miR-146a is significantly inversely correlated with overall survival of both ALL and AML patients. The prognostic significance of miR-146a in AML has been confirmed further in an independent study of 61 Chinese new AML patient samples. We also identified 622 putative target genes of miR-146a that are predicted by at least three out of the five major prediction programs (i.e., TragetScan, PicTar, miRanda, miRBase Targets, and PITA); Through gene ontology analysis, we found that these genes were particularly enriched (2–9 fold higher than expected by chance) in the GO categories of "negative regulation of biology processes", "negative regulation of cellular processes", "apoptosis", and "cell cycle", which may be related to the association of miR-146a with poor survival.

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Keywords

microRNA; acute leukemia; lineage; survival analysis; miR-146a

INTRODUCTION

MicroRNAs (miRNAs, miRs) are an abundant class of small (~22 nucleotides), non-coding RNAs that play important regulatory roles in diverse bioprocesses including development, cell proliferation, differentiation, and apoptosis [1–3]. It was predicted that over 50% or even 60% of the protein-coding genes in humans might be regulated by miRNAs [4,5]. Evidence is emerging that miRNAs can function as oncogenes and tumor suppressors, whose deregulation is clearly associated with the development and progress of various types of cancers [4,6–10]. Recent studies have unraveled complex regulatory networks where miRNAs frequently regulate hematopoietic differentiation and contribute to leukemogenesis [11–15].

Human acute leukemias, including acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), are genetically very diverse, arising from blood cell progenitors developing in the myeloid or lymphoid lineage or from primitive stem cells with multilineage potential [16,17]. AML is the most common acute leukemia in adults whereas ALL is the most common childhood cancer. We recently reported that miRNA expression signatures could accurately discriminate ALL from AML [18]. We identified 27 miRNAs that were differentially expressed between ALL and AML (miR-128a, miR-128b, miR-151*, j-miR-5, miR-130b, and miR-210 were highly expressed in ALL, whereas let-7b, miR-223, let-7e, miR-125a, miR-130a, miR-221, miR-222, miR-23a, miR-23b, miR-24, miR-27a, miR-27b, let-7a, let-7c, miR-199b, miR-26a, miR-335, miR-21, miR-22, miR-424, and miR-451 were highly expressed in AML). It was shown that expression signatures of any two of four miRNAs (i.e., miR-128a, miR-128b, let-7b and miR-223) could accurately discriminate ALL from AML samples [18].

Our Genome-wide, large-scale miRNA expression profiling assays [18,19] and those of others [20–27] have shown that some miRNAs are associated with specific leukemia subtypes and thereby may be able to serve as biomarkers for classification and diagnosis of these subtypes [11]. For example, miR-126 in CBF leukemia [19,20], miR-17-92 in *MLL*-associated leukemia [19,28], miR-196b in *MLL*-associated leukemia [19,29,30] as well as in AML with *NPM1* mutations [20], miR-224, miR-382 and miR-376 family in APL/t(15;17) leukemia [19–21], miR-10a and b in AML with *NPM1* mutations [20,22], and miR-155 in AML with *FLT3*-ITD [20,23] are likely to be such markers [11]. However, although the involvement of miRNAs in different subtypes of acute leukemias has been well-studied [18–26], only a few studies addressed the association of miRNA expression with outcome/survival of patients with AML [23,27,31]. The association of miRNA expression with outcome/survival of patients with ALL has not been reported. In particular, large-scale study of diagnostic and prognostic miRNAs expression in adolescent and adult acute leukemias in Asian populations is lacking.

In the present study, we employed quantitative real-time PCR (qPCR) to assess expression of precursors of 23 miRNAs in 85 Chinese adolescent and adult *de novo* acute leukemia bone marrow specimens including 53 AML and 32 ALL samples. These 23 miRNAs were reported by ourselves and others to be associated with hematopoiesis and/or leukemogenesis [12–15, 18,19,27]. We identified 16 miRNAs that were differentially expressed between the ALL and AML samples. More importantly, we also identified miRNAs whose expression was significantly associated with overall survival patients with ALL or AML. For miR-146a, which was associated with poor survival of both ALL and AML patients, we also validated its prognostic significance in an independent study of 61 Chinese new AML samples, and further

investigated the GO (gene ontology) distributions and significant enrichment of its predicted target genes.

MATERIALS AND METHODS

Patients

Bone marrow specimens were obtained from *de novo* acute leukemia patients at diagnosis during the period between 2002 and 2007 in the First Affiliated Hospital, Zhejiang University College of Medicine. See Table 1 for the characteristics of the patients. All cases were classified according to criteria of the WHO classification. Samples were obtained after informed consent and the study protocol was approved by the hospital Ethical Committee. APL patients were treated with all-trans retinoic acid (ATRA) 30mg/m^2 daily or Arsenic Trioxide 10mg daily when patients had high WBC count (>10×109/L) until patients obtained complete remission. After complete remission anthracycline-based chemotherapy for consolidation was used. Other AML patients were treated with DA regimen (daunorubicin 45mg/m^2 day 1–3, cytarabine 150 mg/m^2 at day 1–7). Patients with ALL were treated with VDCP (vincristine 1.4 mg/m^2 at day 1,8,15 and 22, daunorubicin 45 mg/m^2 at day 1–3, cyclophosphamide 0.75mg/m^2 at day 1 and 15, and prednisone 40 mg/m^2 at day 1–28) or VDLP (vincristine 1.4 mg/m^2 at day 1, 8, 15, and 22, daunorubicin 45 mg/m^2 at day 1–3, asparaginase 6000 units/m^2 at day 19–28, and prednisone 40 mg/m^2 at day 1–28) regimen.

RNA preparation and cDNA synthesis

Bone marrow samples were obtained after written informed consent had been obtained. Mononuclear cells were isolated through Ficoll-Hypaque gradient centrifugation (Invitrogen, Carlsbad, CA). Total RNA was extracted from the mononuclear cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA concentration was determined by measurement of the optical density at 260nm and the RNA was stored at −80° C until use. Total RNA was briefly exposed to RNAase-free DNAase I and 1 μg of total RNA was reversely transcribed to cDNA in a final volume of 20 μl, using random hexamer primers and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The final cDNA was diluted with 180 μl of H_2O.

Quantitative Real-time PCR (qPCR) and data analysis

The expression of the miRNA precursors was determined using real-time quantitative PCR. For real-time PCR, the 25 μl reaction contained 12.5 μl 2X SYBR GREEN PCR Master Mix (Applied Biosystems), 5 pmol each primer, and 2 μl of the diluted cDNA. The primer sequences were described previously[32]. Amplifications were carried out at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 PCR cycles at 95°C (15 seconds) and 60°C (1 minute). All reactions were carried out in MicroAmp optical 96-well reaction plates (Applied Biosystems) using a Bio-Rad IQ5 detection system. The threshold cycle CT is defined as the cycle number at which the ΔRn crosses a software-generated threshold defined as 10 standard deviations above baseline (during cycles 3–15). The CT is linearly proportional to the logarithm of the input copy number. U6 RNA was used as endogenous control. ΔC_T values were used for the analysis [32]. Briefly, in each sample, we calculated a $\Delta Ct_{(miRNA\text{-}U6)}$, which is equal to the difference in threshold cycles for a miRNA (i.e., target) and U6 RNA (i.e., reference) [i.e., $\Delta Ct_{(miRNA\text{-}U6)} = Ct_{miRNA} - Ct_{U6}$].

TIGR Mutiple Array Viewer software package (TMeV version 4.0) [33] was used to perform data analysis and to visualize the results. For two-class supervised analyses, we used the significance analysis of microarrays (SAM) method [34], which uses a modified t-test statistic (or F-test statistic for multiclass analysis), with sample-label permutations to evaluate statistical

significance [35]. In SAM analysis, *p*-values were obtained from permutation tests (10,000 permutations in each analysis if available).

Survival analysis

Using the normalized expression data of patient samples, we performed Cox regression analysis to evaluate the impact of expression values of each probe on overall survival. The set of probes significantly associated (P<0.05) with overall survival constituted a prognostic signature to be applied to the patients. In this set, a gene compound covariate predictor (referred to hereafter as an outcome summary value) was computed for each patient sample and the outcome summary value was assessed for association with overall survival. The outcome summary value was a linear combination of the expression values of the probes that defined the prognostic signature. The outcome summary value for patient i was $ci = \Sigma\, wj\, xij$, where xij was the log-transformed expression value for probe j in patient i and wj was the weight assigned to probe j. The sum was over all j probes included in the prognostic signature. The Cox regression coefficient from the training set analysis for each probe included in the signature was used as its weight in the outcome summary value. The outcome summary value in a given set of patient samples was dichotomized on the basis of the median value to separate patients into two groups. “Kaplan–Meier” curves were made to estimate actuarial probabilities of overall survival and “log-rank test” was used to evaluate the significance of the difference of overall survival between the two groups. Partek Genomics Suite (Partek Inc., St. Louis, Missouri, USA) was used for the Cox regression analysis. WinSTAT (R. Fitch Software, Chicago, IL, USA) was used for the Kaplan–Meier curves and the log-rank test.

Target gene analysis

Through searching five major miRNA-target prediction programs/databases including TragetScan (http://www.targetscan.org/vert_50/) [36], PicTar (http://pictar.mdc-berlin.de/) [37], miRanda (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl) [38], miRBase Targets (http://microrna.sanger.ac.uk/targets/v5/), and PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), we collected a total of 7,200 putative targets of miR-146a. We further used GSEA (Gene Set Enrichment Analysis; http://www.broad.mit.edu/gsea/) [39] to analyze the GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway distribution and significant enrichment of the putative miRNA targets. Chi-square test was used to detect the significance (*p* value).

RESULTS AND DISCUSSION

MiRNAs differentially expressed between ALL and AML

We performed qPCR to analyze expression profiles of 23 human precursor miRNAs in 85 acute leukemia samples including 53 AML and 32 ALL bone marrow samples (Materials and Methods; see Table 1A for the characteristics). The 23 miRNAs include let-7b, miR-17, miR-20a, miR-23a, miR-23b, miR-25, miR-26a, miR-27a/b, miR-29a/c, miR-29b, miR-126, miR-128a, miR-128b, miR-146a, miR-150, miR-155, miR-181a/c, miR-181b, miR-191, miR-196b, miR-221, miR-222, and miR-223, which were selected based on previous reports from ourselves and those of others [12–15,18,19,27].

To identify miRNAs differentially expressed between ALL and AML samples, we employed significance analysis of microarray (SAM) [34] and permutation tests (10,000 permutations) in a comparison between ALLs and AMLs. We identified 16 miRNAs differentially expressed between ALL and AML samples. Nine (i.e., miR-128a, miR-128b, miR-155, miR-150, miR-17, miR-29a/c, miR-29b, miR-146a, and miR-20a) were expressed at a significantly higher level in ALL than in AML; In contrast, the remaining seven (i.e., miR-223, miR-221, miR-222, miR-27a/b, miR-23a, miR-196b and let-7b) were expressed at a significantly higher

level in AML compared to ALL (see Fig. 1). Each differentially expressed miRNA has a *q* value < 0.05 and the overall false discovery rate (FDR) of the set of 16 differentially expressed miRNAs is less than 1%, which means that none of the 16 miRNAs is likely to be false positive.

Among the nine miRNAs that were expressed at a significantly higher level in ALL than in AML, only miR-128a and miR-128b were reported in our previous study [18]. Interestingly, the remaining miRNAs have been reported previously by others to play an important role in lymphoid cell development or be involved in ALL or chronic lymphocytic leukemias (CLL). For examples, miR-29a and miR-29c were overexpressed in CLL [25], whereas miR-146 was upregulated in CD4+ T cells [40]. miR-150 was reported to be highly expressed in mature and resting lymphocytes, but not in their progenitors [41,42]. It has also been reported to control B-cell differentiation [41], in particular, to block the transition from pro-B to pre-B cell stage [42]. miR-155 is also an important miRNA in lymphopoiesis and immune response [43–46]. Rodriguez et al. [44] developed a miR-155 knockout model and demonstrated a requirement of miR-155 for the function of B and T lymphocytes. Vigorito et al. [46] showed that B cells require miR-155 for normal production of isotype-switched, high-affinity antibodies and for a memory response, and that Pu.1 is a functionally important target of miR-155 in B cells. Ventura et al. [47] demonstrated that the miR-17-92 cluster is also essential for B cell development. Knockout of miR-17-92 leads to increased levels of Bim, a proapoptotic protein and a target of mir-17-92, and inhibits B cell development at the pro-B to pre-B transition [47]. Thus, the miR-17-92 cluster can promote the transition from proB to pre-B cell stage [47], whereas miR-150 blocks this transition [42]. Two independent loss- and gain-of function studies also showed that miR-17-92 is involved in lymphoid development [48,49]. Nonetheless, we have previously observed that miR-17-92 is also overexpressed in AML cases bearing *MLL* rearrangements [19,28], suggesting that a much larger number of patient samples covering various abnormalities are needed to identify really ALL- or AML-specific miRNAs.

Consistent with our previous study [18], miR-223, miR-128a and miR-128b are still the most discriminatory miRNAs. Let-7b was less discriminatory compared to the other miRNAs, which is consistent with our previous large-scale qPCR in analysis of expression of mature miRNAs [18]. Clearly, many discriminatory miRNAs such as miR-150, miR-155, and miR-223 are not ALL- or AML-related miRNAs; instead, they are lineage-related miRNAs. miR-223 is one of the most well-studied myeloid-specific miRNAs [50], whereas miR-150 and miR-155 are lymphoid-specific miRNAs [41–46]. Nevertheless, some miRNAs are ALL- or AML-related miRNAs. For example, as shown in our previous study, miR-128a, miR-128b are ALL-related miRNAs because they are particularly overexpressed in ALLs compared to not only AMLs but also normal controls (including CD19+ B cells and CD34+ hematopoietic stem/progenitor cells) [18]. Similarly, miR-221 and miR-222 appear to be AML-related miRNAs [18]. The function of miR-128 in hematopoiesis is unknown. miR-221 and miR-222 may play a role in erythropoiesis, and aberrant expression of miR-221 and miR-222 inhibits normal erythropoiesis and erythroleukemic cell growth probably through down-regulation of *KIT* [51].

MiRNAs associated with survival in patients with ALL

We then investigated whether some miRNAs were associated with survival of the ALL patients. We found that three miRNAs including miR-146a, miR-181a/c, and miR-221 were significantly associated with overall survival ($P<0.05$) of the 32 ALL patients (Table 2). Expression level of the first two precursor miRNAs was associated with a poor outcome (i.e., poor prognosis/short-term survival) whereas that of the latter was associated with a good outcome (i.e., good prognosis/long-term survival). We derived an outcome signature (see Materials and Methods) containing these three miRNAs. Expression level of this 3-miRNA outcome signature was inversely associated with overall survival ($r=-0.5933$; $P = 0.0039$). To

display the relation between the outcome summary value and the clinical outcome, the 32 ALL patient set was dichotomized at the median outcome summary value; the 16 patients with a higher level of outcome summary value exhibited a significantly shorter (p<0.03) overall survival than the other 16 patients with a lower value (Fig. 2).

MiRNAs associated with survival in patients with AML

We also investigated whether some miRNAs were associated with survival of the AML patients. We found that five miRNAs including miR-25, miR-26a, miR-29b, miR-146a, and miR-196b were significantly associated with overall survival (P<0.05) of the 53 AML patients (Table 3). Expression level of the first miRNA was positively whereas that of the latter four was negatively associated with a good outcome (i.e., good prognosis/long-term survival). Similarly, we also derived an outcome signature containing these five miRNAs (see Materials and Methods). Expression level of this 5-miRNA outcome signature was inversely associated with overall survival (r=−0.4606; P < 0.00024). To display the relation between the outcome summary value and the clinical outcome, the 53 AML patient set was dichotomized at the median outcome summary value (Fig. 3a). The 2-year overall survival rate was 42% (11/26) for patients with outcome summary values below the median and 11% (3/27) for those with values above the median. Because AML-M3 patients were treated very differently from other AML patients, we performed the above analyses with the 40 non-M3 AML patient samples. We found that only three miRNAs including miR-26a, miR-29b, and miR-146a were significantly associated with overall survival (P<0.05) of the 40 AML patients (Table 4). Expression level of all three miRNAs was negatively associated with a good outcome. Expression level of this 3-miRNA outcome signature was inversely associated with overall survival (r=−0.4256; P = 0.008). To display the relation between the outcome summary value and the clinical outcome, the 40 non-M3 AML patient set was dichotomized at the median outcome summary value (Fig. 3b). The 2-year overall survival rate was 35% (7/20) for patients with outcome summary values below the median and 0% (0/20) for those with values above the median. In analysis of the 13 AML-M3 patient samples, we could not find any miRNAs whose expression was significantly associated with overall survival of the patients, probably due to the small number of patients.

Thus, expression of miR-26a, miR-29b, and miR-146a was significantly associated with overall survival (P<0.05) of the entire set of 53 AML patients or the 40 non-M3 AML patients. Remarkably, expression of miR-146a was significantly associated with overall survival of both AML and ALL patients.

Signature of miR-146a is a potential prognostic marker

We further determined whether the signature of miR-146a alone could serve as a prognostic marker. As shown in Figure 4, the group of patients with a higher expression level of miR-146a exhibited a significant shorter (p<0.05) survival than that with a lower expression level of miR-146a in both ALL (Fig. 4a) and AML (Fig. 4b) cases. To validate the prognostic significance of miR-146a, we further collected 61 additional AML patient samples and conducted qPCR assays to assess expression of miR-146a. Then, we separated the 61 AML cases into two groups according to the expression level of miR-146a. As shown in Figure 4c, we confirmed that the expression signature of miR-146a was significantly (p<0.05) inversely associated with overall survival of AML patients. Thus, signature of miR-146a probably can serve as a prognostic marker for patients with acute leukemia.

Predicted targets of miR-146a

Because miR-146a is the only miRNA whose expression is significantly associated with overall survival of both AML (including or excluding AML-M3) and ALL patients, it is relevant to identify its potential targets. Through searching five common human miRNA-target prediction

programs/databases (i.e., TragetScan, PicTar, miRanda, miRBase Targets, and PITA; see Materials and Methods), we found that miR-146a has a total of 7,200 predicted targets. Of them, 7 (i.e., *IRAK1, DLGAP1*, *HMBOX1*, *KLF7*, *STRBP*, *CD79B*, and *IGSF1*), 102, 622, 1,809, and 4,660 putative targets were predicted by five, four, three, two, and one algorithm (s), respectively.

We focused on the 622 miR-146a targets that are predicted by at least 3 programs/databases for the GO and KEGG pathway analysis. In comparison with the enrichment in the 27,901 human genes that are putative miRNA targets as predicted by at least one of the above 5 programs, the enrichments of 98 GO BP (biological process), 35 GO MF (molecular function), and 17 KEGG pathway terms are significantly greater in the 622 putative targets of miR-146a that are predicted by at least 3 programs, with at least a 2-fold increase and a *P* value less than 0.05 (see Supplementary Table 1a, 1b, and 1c). Particularly, in GO BP (biological process) terms, genes related to “negative regulation of biology process”, “negative regulation of cellular process”, “apoptosis”, “cell cycle”, “cell cycle checkpoint”, “cell cycle arrest”, and “DNA damage checkpoint” were significantly more enriched in the 622 miR-146a putative target list than in the 27,901 total putative miRNA target gene list (see Fig. 4 and Supplementary Table 1a). This might contribute to the association of miR-146a with poor survival. In GO MF (molecular function) terms, genes related to “DNA binding”, “receptor activity”, “transcription factor activity”, and “kinase activity” are more enriched in the 622 miR-146a putative target set than in the 27,901 total putative miRNA target gene set (see Supplementary Table 1b). In KEGG pathways, genes related to “MAPK signaling pathway”, “WNT signaling pathway”, and “P53 signaling pathway” also are also more enriched in the 622 miR-146a putative target set than in the 27,901 total putative miRNA target gene set (see Supplementary Table 1c). In the future, when most of the direct targets of miR-146a are identified, further GO and KEGG analysis will provide more valuable insights into the pathway and function of miR-146a in the development and progression of AML.

In sum, our study shows that a group of microRNAs (e.g., miR-223, miR-128a and miR-128b) are discriminatory microRNAs between AML and ALL in Chinese patients, consistent with our previous finding in American patients. More importantly, our study also shows that expression of some microRNAs is associated with overall survival of patients with acute leukemias (e.g., miR-146a, miR-181a/c and miR-221 in ALL, and miR-25, miR-26a, miR-29b, miR-146a, and miR-196b in AML), which has not been reported previously. In particular, the prognostic significance of miR-146a has been further validated in an independent study of 61 additional AML patient samples. Furthermore, results from the GO and KEGG analysis of potential targets of miR-146a implicate some potential functions of miR-146a (e.g., negatively regulating genes that inhibit cell growth and promote apoptosis), which may be related to the association of miR-146a with poor survival. Nonetheless, further large-scale independent studies are needed to further verify our observations before we consider any potential clinical implication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
miRNA	microRNA
SAM	significance analysis of microarray
GSEA	Gene Set Enrichment Analysis
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes

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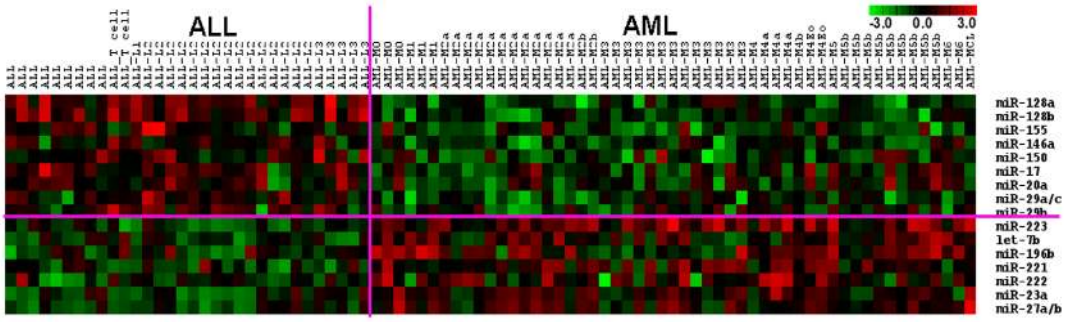

Figure 1. Expression profiles of 16 miRNAs that were differentially expressed between ALLs and AMLs as determined by qPCR in 32 ALL and 53 AML samples

Data are presented as ΔCt, which refers here as the difference in threshold cycles for a miRNA and U6 RNA. Expression data was mean centered and the relative value for each sample is represented by a color, with red representing a high expression and green representing a low expression (scale shown in the upper right). AML-MCL, mast cell leukemia.

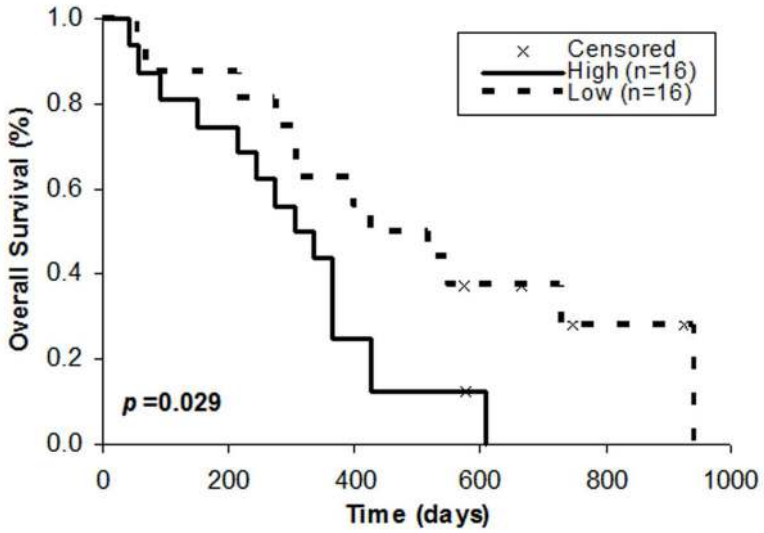

Figure 2. Overall survival curves for the 32 acute lymphoblastic leukemia (ALL) patients

For the purpose of display, the outcome summary value that reflects the expression levels of three miRNAs (i.e., miR-146a, miR-181a/c, and miR-221) in the 32 ALL patient samples was dichotomized on the basis of the median value to separate patients into two groups, and Kaplan–Meier curves were generated to depict outcomes. The *P* value was determined by log-rank test. Censored cases are those that were still alive when they were lost to follow-up or when the study ended.

Fig. 3a

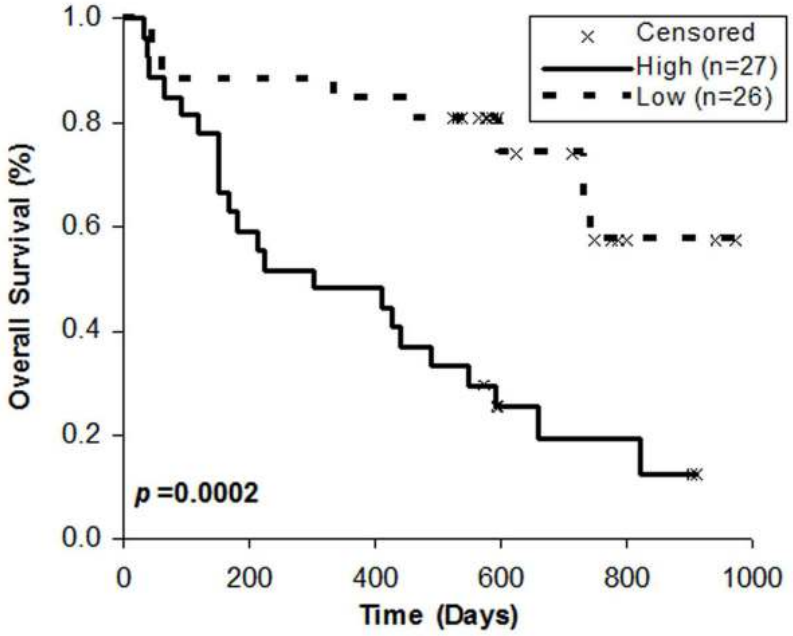

Fig. 3b

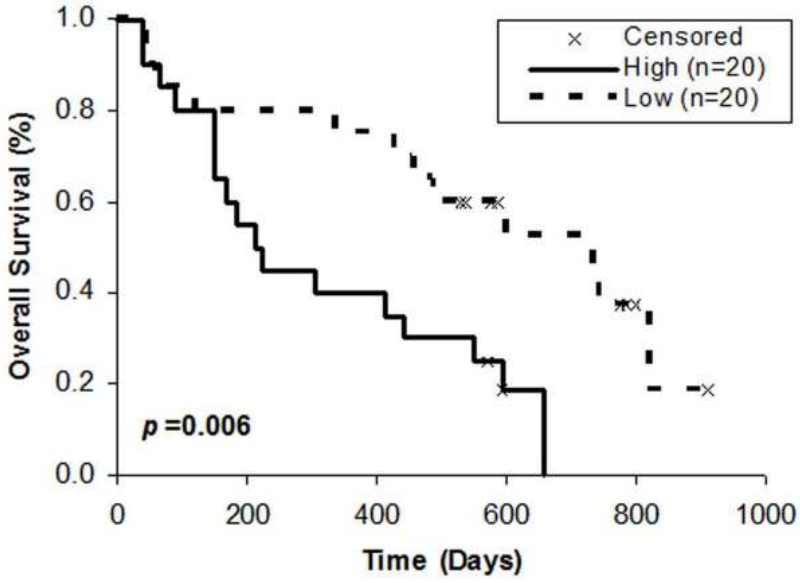

Figure 3. Overall survival curves for acute myeloid leukemia (AML) patients

(a) The outcome summary value that reflects the expression levels of five miRNAs (i.e., miR-25a, miR-26a, miR-29b, miR-146a, and miR-196b) in the 53 AML patient samples was dichotomized on the basis of the median value to separate patients into two groups; **(b)** The outcome summary value that reflects the expression levels of three miRNAs (i.e., miR-26a, miR-29b, and miR-146a) in the 40 non-M3 AML patient samples was dichotomized on the basis of the median value to separate patients into two groups, and Kaplan–Meier curves were generated to depict outcomes. The *P* value was determined by log-rank test.

Fig. 4a

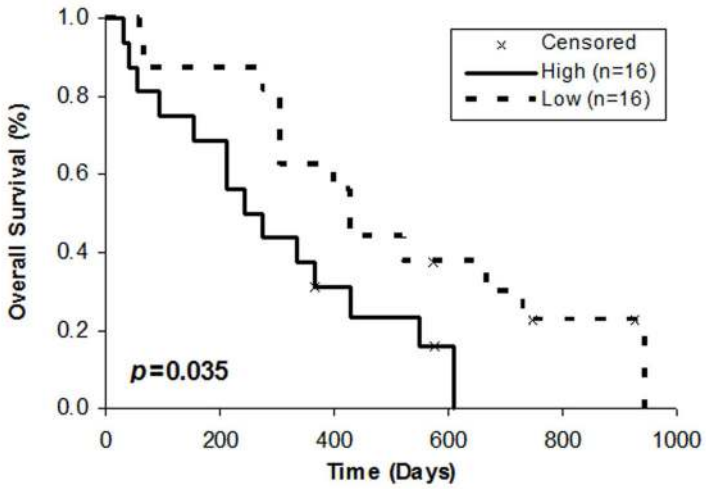

Fig. 4b

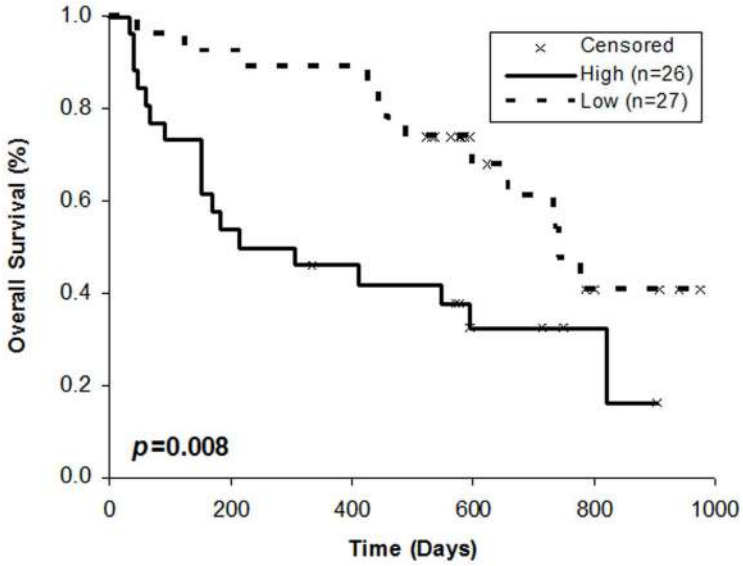

Fig. 4c

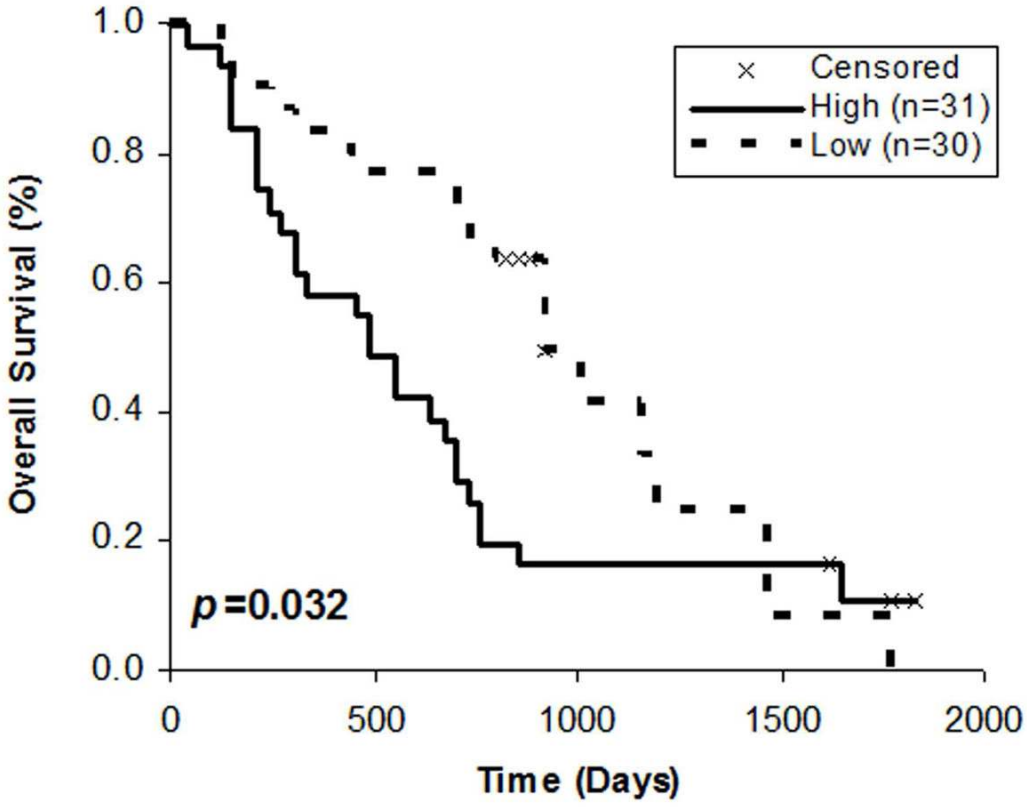

Figure 4. Expression signature of miR-146a probably is a potential prognostic marker

(a) The 32 ALL cases, **(b)** the 53 AML, and **(c)** the 61 additional AML patient cases were separated into two groups according on the basis of the median value of miR-146a expression, respectively. Kaplan–Meier curves were generated to depict outcomes. The *P* value was determined by log-rank test.

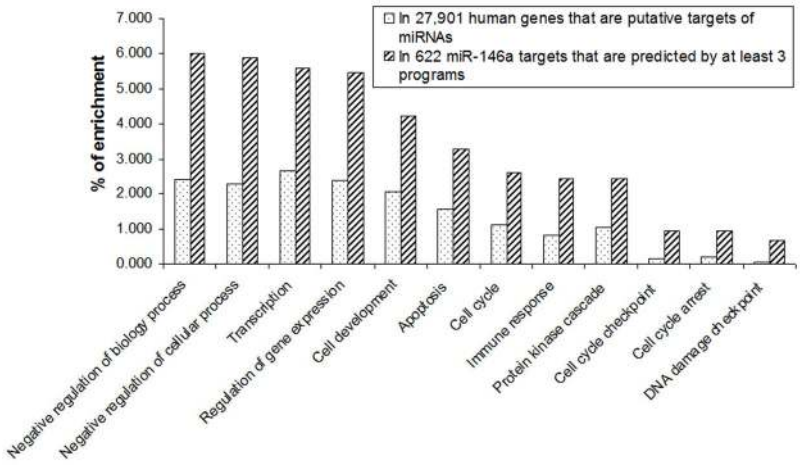

Figure 5. Example of 12 GO BP terms that have a significantly higher degree of enrichment of genes in the set of 622 miR-146a putative target genes than in the set of 27,901 whole putative target genes

See more details in Supplementary Table 1a.

Table 1

Characteristics of patients

A. 85 patients (32 ALLs and 53 AMLs) for qPCR	
Characteristic	**Number (n=85)**
Age	
Median	43
Range	14–82
Females (%)	43 (51)
Males (%)	42 (49)
FAB classification	
ALL	32
L1	1
L2	15
L3	5
T cell	2
Not classified	9
AML	53
M0	3
M1	3
M2	14
M3	13
M4	7
M5	10
M6	2
Mast cell	1

B. 61 AML patients for validation of prognostic significance of miR-146a	
Characteristic	**Number (n=61)**
Age	
Median	49
Range	13–71
Females (%)	29 (48)
Males (%)	32 (52)
FAB classification	
M0	4
M1	7
M2	18
M3	8
M4	7
M5	13

B. 61 AML patients for validation of prognostic significance of miR-146a	
Characteristic	**Number (n=61)**
M6	4

Table 2

Three miRNAs that are associated with overall survival of the 32 ALL patients

miRNAs	Cox Regression Coefficient[a]	Hazard Ratio	*p* Value[b]
Increased expression associated with a good outcome			
miR-221	−0.619053	0.538454	0.0379821
Increased expression associated with poor outcome			
miR-146a	0.525527	1.69135	0.0388068
miR-181a/c	0.52796	1.69547	0.0111003

[a] Cox regression models were used to assess the association between expression of individual miRNA precursors and overall survival. The 3 miRNAs with a significant association ($P<0.05$) constituted the outcome signature. A negative regression coefficient (which corresponds to a hazard ratio of <1) indicates that increased expression was associated with a good outcome, and a positive coefficient (which corresponds to a hazard ratio of >1) indicates that increased expression was associated with a poor outcome.

[b] *p* values show the significance of the Cox regression coefficient and were calculated.

Table 3

Five miRNAs that are associated with overall survival of the 53 AML patients

miRNAs	Cox Regression Coefficient	Hazard Ratio	*p* Value
Increased expression associated with a good outcome			
miR-25	−0.43302	0.648551	0.027855
Increased expression associated with poor outcome			
miR-26a	0.50379	1.65498	0.014152
miR-29b	0.422367	1.52557	0.0203
miR-146a	0.446804	1.56331	0.040721
miR-196b	0.47421	1.60674	0.004722

Table 4

Three miRNAs that are associated with overall survival of the 40 non-M3 AML patients

miRNAs	Cox Regression Coefficient	Hazard Ratio	*p* Value
Increased expression associated with poor outcome			
miR-26a	0.52378	1.6884	0.00677
miR-29b	0.331876	1.39358	0.049004
miR-146a	0.491423	1.63464	0.024642