MicroRNA profiling can classify acute leukemias of ambiguous lineage as either acute myeloid leukemia or acute lymphoid leukemia.

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Running Title: MicroRNA based classification of acute leukemias.

Word count: 4584

Disclosure: The authors have no conflicts of interest to disclose.

Number of figures: 3
Number of tables: 1
Number of supplemental tables: 4
Keywords: Leukemias of ambiguous lineage, Mixed Phenotype Acute leukemia, MicroRNA, Adult Acute Lymphoblastic Leukemia, Acute Myeloid Leukemia, Expression Profiling.

Statement of translational relevance (120-150)
Classification of acute leukemia (AL) is based on commitment of leukemic cells to the myeloid or lymphoid lineage. In some cases both lymphoid and myeloid features might be present and based on their immunophenotypic profile these cases are classified as AL of ambiguous lineage. It is unclear whether AL of ambiguous lineage benefit from an AML or ALL-based treatment protocol; this partly explains their unfavorable prognosis. Here we show that microRNA expression profiling clusters AL from ambiguous lineage with either myeloid or lymphoid leukemia control samples. Furthermore, based on five of the most lineage discriminative microRNAs we are able to define AL of ambiguous lineage as either AML or ALL. In conclusion, addition of microRNA evaluation to current diagnostic procedures helps deciphering the myeloid or lymphoid lineage predominance of an AL of ambiguous lineage and thus the treatment of choice.
Abstract (<250)

Purpose: Classification of acute leukemia (AL) is based on commitment of leukemic cells to the myeloid or the lymphoid lineage. However, a small percentage of AL cases lack straightforward immunophenotypical lineage commitment. These leukemias of ambiguous lineage represent a heterogeneous category of AL that cannot be classified as either myeloid AL (AML) or lymphoid AL (ALL). The lack of clear classification of acute leukemias of ambiguous lineage as either AML or ALL is a hurdle in treatment choice for these patients.

Experimental design: here, we compared the microRNA expression profiles of 17 cases with AL of ambiguous lineage and 16 cases of AML, B-ALL and T-ALL

Results: We show that leukemias of ambiguous lineage do not segregate as a separate entity but exhibit microRNA expression profiles similar to either AML, B-ALL or T-ALL. We show that by using only five of the most lineage discriminative microRNAs we are able to define AL of ambiguous lineage as either AML or ALL.

Conclusions: Our results indicate the presence of a myeloid or lymphoid lineage specific genotype, as reflected by microRNA expression, in these AL despite their ambiguous immunophenotype. MicroRNA-based classification of AL of ambiguous lineage might be of additional value in therapeutic decision making.

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Introduction

Acute leukemias (AL) are classified as myeloid or lymphoid by using immunological markers that determine their lineage commitment. Four percent of AL cases cannot be classified as acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL) due to co-expression of both lymphoid and myeloid lineage markers (1-6). The prognosis of leukemias of ambiguous lineage is worse compared to AML or ALL and no specific treatment programs exist for these leukemias. To date, it is unsettled whether patients benefit from AML, ALL or combination therapy which might cause their mistreatment and poor prognosis (7-12). The therapeutic strategies used for treatment of AML or ALL are considerably different, indicating the importance of classification of AL cases at diagnosis as either myeloid or lymphoid.

The diagnostic criteria for leukemias of ambiguous lineage were previously defined by the WHO 2001 classification and are based on the presence of immunological markers detected by flow cytometry (1). According to the WHO2001 scoring system many different combinations of immunophenotypic markers can define an AL as one with an ambiguous lineage and consequently this results in a heterogeneous group of biphenotypic acute leukemias (BAL). In 2008 these diagnostic criteria were refined: AML cases with recurrent chromosomal abnormalities and blast crises chronic myeloid leukemia (CML) cases were excluded from mixed phenotype AL (MPAL) (2). The major immunophenotypic markers used by the WHO2008 to determine the lineage for these AL cases are cytoplasmic myeloperoxidase (MPO), CD19, and cytoplasmic CD3. However, the current WHO2008 classification is difficult to interpret due to the implementation of expression intensities of B- and T-cell makers and the absence of clear cut-offs.

The discrimination between ALL and AML has been shown to be possible by morphologic, immunohistochemical, and immunological methods; however, also by gene expression profiling (13,14) and microRNA (miRNA) expression profiling (15,16). MiRNAs are small single stranded RNA molecules which regulate gene expression by promoting degradation of mRNAs or repressing their translation (17). Cancer, including leukemia, is characterized by globally aberrant miRNA expression patterns which are highly informative for tumor classification. Remarkably, in poorly differentiated tumors, miRNA expression profiles can...
successfully classify these tumors in contrast to mRNA expression profiles (18). In AML, genome-wide, large-scale miRNA expression profiles showed that miRNAs are associated with specific AML subtypes and may serve as biomarkers for classification and diagnosis of AML subtypes (19-23). Several of those miRNA expression profiling studies showed the association of miRNA expression profiles with the outcome of AML patients (22-25). Like in AML, miRNA expression signatures have been shown to be associated with prognosis of ALL patients (15). Moreover, miRNA expression patterns showed to reflect the lineage state of acute leukemia (15,16,18). Interestingly, from the 27 miRNAs differentially expressed between ALL and AML two were sufficient to accurately (97-99%) identify the myeloid or lymphoid identity of these AL (16). In another study, 16 miRNAs (mir-23a, mir-27a/b, mir-128a, mir-128b, mir-221, mir-222, mir-223, let-7b, mir-17, mir-20a, mir-29a/c, mir-29b, mir-146a, mir-150, mir-155, and mir-196b) were found to be differentially expressed between AML and ALL (15).

Because miRNA expression profiles can classify AL cases as either AML or ALL (15,16,18) we decided to investigate the miRNA expression profile of AL cases that do not have a clear lineage commitment based on immunophenotypical marker expression. We compared the miRNA expression profiles of leukemia cases of ambiguous lineage with those of ALL and AML cases and showed that the cases with an ambiguous lineage do not segregate as a separate entity but have miRNA expression profiles similar to either AML, B-ALL or T-ALL. Thus, AL without clear immunophenotypic lineage commitment can be classified as either AML or ALL based on their miRNA expression profile. This classification might help the diagnosis of AL of ambiguous lineage as either AML or ALL and therefore in treatment decision making.

Material and Methods

Patient samples and cell lines

Sixteen patients with a leukemia of ambiguous lineage and 12 patients with AML, B-ALL or T-ALL were selected by database review of all patients with acute leukemia treated at the VU University Medical Center, Amsterdam, The Netherlands from 2000 until 2012. The 12
AML, B-ALL and T-ALL samples were selected based on a clear immunophenotypic myeloid or lymphoid lineage commitment. Immunophenotypical analysis was performed on bone marrow (BM) samples or peripheral blood (PB) samples in case of no BM samples were available. The use of AML patient material for this study and the informed consent procedure has been approved by the medical ethical committee (MEC) of our institute. Bone marrow or peripheral blood samples from 9 patients diagnosed with a leukemia of ambiguous lineage and 11 patients with AML (n=5), B-ALL (n=4) or T-ALL (n=2) were used for miRNA expression analysis. Cell lines HL60 (CCL-240) and CCRF-CEM (CCL-119) were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA).

Immunophenotypic analysis

Analysis was carried out as part of regular diagnostic investigations. In short, red blood cells were lysed using Pharm Lyse lysing solution (BD Biosciences, San Jose, CA), washed with phosphate buffered saline (PBS) and incubated with antibodies defining or specific for the B cell, T cell and myeloid lineage. Samples were analyzed with four-color flow cytometry on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). The following FITC, Pe, PerCP or APC antibodies were used: CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD19, CD20, CD22, CD25, CD33, CD34, CD36, CD42b, CD45, CD56, CD61, CD64, CD65, CD71, CD90, CD117, CD133 and anti-HLA-DR. Cytoplasmic markers CD3, CD22, IgM, CD79a, TdT and MPO were evaluated after fixation with paraformaldehyde and subsequently permeabilization with Facs Lyse (Becton Dickenson, San Jose, CA). Antibodies were purchased from: BD Biosciences, Zebra biosciences (Enschede, The Netherlands), Dako (Glostrup, Denmark) or Sanquin (Amsterdam, The Netherlands). Data analysis was performed using CellQuest Pro software (BD Biosciences). Blasts were defined by low side scatter (SSC), diminished or low CD45 expression and CD34 expression. In case of CD34 negative AL, blasts were selected using other immature markers such as CD117, CD133, CD10 and cytIgM. For defining the B-lymphoid lineage of a MPAL according to the WHO2008 criteria, CD19 should be highly expressed on blasts. We considered blasts strongly positive for CD19 if at least 20% of the population was positive as compared with the appropriate isotype control combined with a mean fluorescence intensity as high as, or higher than normal B cells.
within the same sample. When normal B cells were absent, we compared expression levels with historical normal controls (1-3, 5).

Molecular diagnostics and cytogenetic analysis

Mononuclear cells were isolated using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) and subsequently snap frozen. DNA and/or total RNA was isolated from the cell pellets and used for determination of presence of translocations; t(9;22), t(8;21), t(15;17), MLL and FLT3-ITD by PCR following institutional standard operating procedures (www.modhem.nl). Chromosomal analysis was performed on pretreatment bone marrow at diagnosis. Specimens were processed using short term unstimulated cultures. Chromosome banding was performed by the use of standard techniques, and karyotypes were described according to the International System for Human Cytogenetic Nomenclature.

Cell purification

BM or PB cells derived from AL patients and stored in liquid nitrogen were thawed, washed and labeled with fluorochrome-conjugated antibodies. In general, blasts were stained with CD45, CD34 and various patient specific markers, mostly CD3, CD7, CD19, CD10, CD13 and CD33. The blast population from all AL cases were sorted by flow cytometry using FACS ARIA Cell Sorter (BD Biosciences, Franklin Lakes, NJ, USA), resulting in removal of normal B-lymphoid and myeloid progenitors.

RNA isolation and miRNA expression analysis

Total RNA was isolated with the NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany) according to manufacturer’s protocol. RNA was concentrated using a vacuum concentrator (SPD111V, Thermo Savant). Final concentration was measured with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific; Wilmington, DE). Human miRNA microarrays (Agilent Technologies; Palo Alto, CA), containing 60.000 probes representing 1205 human and 144 human viral miRNAs were used for miRNA expression profiling according to the manufacturers protocol. In short, 100ng of total RNA from each
sample was dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIP) and then
ligated with a Cyanine3-pCp molecule using Agilent miRNA Complete Labeling and Hyb
Kit (Agilent Technologies; Palo Alto, CA). Labeled RNA was hybridized to Human miRNA
microarray for 20 hours at 55°C. Microarray slides were scanned using a High-Resolution
C Scanner (Agilent Technologies) and images were analyzed with Feature Extraction TM
software, version 10.5.1.1 (Agilent). Microarray data are available on Array Express
(accession no. E-MTAB-1459). The expression of several miRNAs was confirmed by
Quantitative Real-Time Polymerase Chain reaction (Q-RT-PCR).

Quantitative Real-Time Polymerase Chain Reaction

Selection of the most differentiating miRNAs in our cohort was performed using
Significance Analysis of Microarrays (SAM) on the myeloid and lymphoid cluster as was
formed by unsupervised clustering of all samples (controls combined with AL of
ambiguous lineage). The five miRNAs with the highest observed relative difference
were selected. This signature included; miR-23a, miR-27a, miR-221, miR-223 and miR-
199b-5p (Supplemental Table 4). Specific primers (Applied Biosystems) for these five
lineage discriminating miRNAs were used for Q-RT-PCR validation. RNU48 and miR-
378, a miRNA which is highly expressed, has minimal variation in expression between
samples and is not discriminative between AML and ALL, were used as control. PCR
was performed on a 7300 Real-Time PCR System (Applied Biosystems). All
experiments were performed in duplicate and Ct-values were averaged. Expression was
calculated using $2^{\Delta\text{Ct}}$ method, log2-transformed and analyzed with BRB-ArrayTools.

Data analysis

Green median signal values from the microarray data were normalized using the quantile
method (26), and log2-transformed. As the arrays included probes mapping a set of 1347
miRNAs spotted multiple times, we averaged the probes representing the same miRNA.
The comparisons of the individual miRNAs between samples of the different lineage
groups were performed using a linear regression model. This model takes advantage of
the large number of miRNAs simultaneously studied to improve upon the individual estimates by means of empirical Bayes (27). P-value lists for each comparison were corrected by multiple testing using the step-up false discovery rate (FDR) procedure of Benjamini-Hochberg (28). Each miRNA was considered as differentially expressed between groups if its corresponding FDR was up to 0.05, unless otherwise stated. Hierarchical clustering was used to produce false-color heatmaps illustrating the data patterns. The clustering used average linkage and one-minus-correlation as distance measure.

Analyses were performed using the statistical package R (version 2.14.0) (29), the package limma (30) for empirical Bayes linear regression, and BRB-ArrayTools (version 4.2.0) for producing the heatmaps.

Results

MicroRNAs are differentially expressed between ALL and AML

To confirm the already observed differential expression of miRNAs in AML, B-ALL and T-ALL we analyzed the miRNA expression profiles of several AML, T-ALL and B-ALL cases. Blasts were purified by flow cytometry, RNA was isolated and hybridized with miRNA-arrays (Agilent). The microarray data was normalized and the miRNAs with the highest mean absolute deviation, highest variation in expression between samples, were selected for further analysis. The subsequent unsupervised clustering analysis of these AL control samples generated two separate clusters. One cluster that contained all AML samples while the other contained all the ALL samples. This lymphoid cluster could be further separated in a T-ALL and B-ALL cluster (Figure 1A).

Next, we performed a Linear Model for Microarray Analysis (LIMMA) on these AML and ALL samples to see whether we could identify a similar miRNA profile as previously described that discriminates between the myeloid and lymphoid lineage (15,16). The ten most significant miRNAs that are able to discriminate between AML and ALL are shown in Table 1. One of these miRNAs (mir-150) showed enhanced expression in ALL as
compared to AML, whereas the other nine miRNAs are higher expressed in AML as compared to ALL (miR-223, miR-199b-5p, miR-199a-3p, miR-27a, miR-27b, miR-23a, miR-340, miR-340* and miR-221). Seven of these 10 lineage discriminating miRNAs were described by others to discriminate between ALL and AML (15, 16).

Since previous reports have described lineage discriminating miRNA signatures we used these discriminative miRNAs on our AML and ALL samples. In the study by Mi et al. (16) twenty-seven miRNAs were found to be significantly differentially expressed between the two subtypes of AL. In the study by Wang et al. (15), 16 miRNAs were differentially expressed between AML and ALL. To generate a robust literature based miRNA signature we integrated the miRNA lists from these two studies which resulted in a miRNA signature containing 32 miRNAs (supplemental table 1). Using these 32 miRNAs in an unsupervised clustering analysis of our miRNA expression results we were able to define our control AL samples as either AML or ALL in the correct way (figure 1B). Moreover, using the expression of these 32 miRNAs, even the segregation of the lymphoid cluster in a B-ALL and T-ALL group was seen. Thus, our miRNA expression profiles can discriminate between AML and ALL in an unsupervised clustering approach by using either the most variable 10% expressed miRNAs or the 32 miRNA signature that was published before. These results confirm the potential of miRNA expression profiling in classification of AL as either belonging to the myeloid or lymphoid lineage.

Leukemias of ambiguous lineage are not a distinctive entity.

Because the immunophenotype of cells is a limited reflection of the genotype and, as shown before, miRNA expression profiling can identify AL cases as either AML or ALL we analyzed the miRNA expression profiles of 9 AL cases of ambiguous lineage and compared these profiles with the AML, B-ALL and T-ALL samples. Using LIMMA we analyzed the expression of all the miRNAs in the four morphological and immunophenotypical defined groups of AL cases (AML, B-ALL, T-ALL and AL of ambiguous lineage) (supplemental table 4A-C). First, this showed that there is no significant difference in overall expression pattern observed in AL cases of ambiguous lineage as compared to AML samples (lowest FDR >0.26), indicating that AL of ambiguous
lineage was not significantly different from AML. However, when comparing the overall miRNA expression of the AL of ambiguous lineage with B-ALL cases, the miR-320 family (miR-320a/b/c/d/e) was found to be differentially expressed. Furthermore, the ambiguous lineage samples showed differential expression of miR-29c and miR-513a-5p when compared to T-ALL. This differential expression of miR-29c, miR-513a-5p and the miR-320 family is also observed between B-ALL and T-ALL samples (supplemental table 4D), likely indicating that the differential expression of these miRNAs seen between AL of ambiguous lineage and B-ALL or T-ALL is a direct result of an intrinsic differences between B-ALL and T-ALL. Thus, we did not observe a distinctive pattern of overall miRNA expression between AL of ambiguous lineage and AML or ALL cases, suggesting that AL cases of ambiguous lineage are not a separate entity.

Leukemias of ambiguous lineage can be assigned to the lymphoid or myeloid lineage by miRNA expression profiling.

To study whether miRNA expression analysis is able to identify the myeloid or lymphoid origin of the leukemias of ambiguous lineage, we performed unsupervised clustering of the miRNA expression of the AL cases with ambiguous lineage as well as the AML and ALL cases (11 AL cases and 9 AL cases with ambiguous lineage). This resulted in the identification of three AL groups, a myeloid group containing all AML samples and a lymphoid group that could be further divided in B-ALL and T-ALL (Figure 2A). The miRNA expression signatures of the leukemias with ambiguous lineage showed great resemblance with the B-ALL, the T-ALL or the AML miRNA signature, resulting in grouping of these leukemias with either one of the AL groups (Figure 2A). Using the 32 miRNA literature based signature in an unsupervised way on all our samples (Figure 2B) showed as well grouping of leukemias with ambiguous lineage with either AML, T-ALL or B-ALL.

All together, our results show that leukemias without a clear immunophenotypic lineage commitment do not display a distinct miRNA expression profile but one that can classify these leukemias as either of the myeloid or the lymphoid lineage.
Acute leukemia of ambiguous lineage can be classified as ALL or AML by Q-RT-PCR expression analysis of a small selection of miRNAs.

AL patients are either treated with an ALL or an AML treatment protocol and the assignment of AL cases of ambiguous lineage as either one of those AL subtypes is therefore of most relevance. To investigate the feasibility of classification of AL cases of ambiguous lineage as either AML or ALL by the expression of only a limited set of miRNAs, we selected the top five significantly differentially expressed miRNAs that could distinguish between AML and ALL (Supplemental Table 3). These five miRNAs, miR-23a, miR-27a, miR-223, miR-221 and miR-199b, are as well present in the top list of differentially expressed miRNAs between AML and ALL in two published studies (15,16).

We analyzed the expression of these miRNAs by Q-RT-PCR in 19 AL cases that were used for the array hybridization (9 AL of ambiguous lineage, 5 ALL and 5 AML cases) as well as an additional 8 AL samples of ambiguous lineage, three ALL cases (two T-ALL, one B-ALL), two AML cases, the AML cell line HL60 and the T-ALL cell line CCRF-CEM. The expression profile of miR-23a, miR-27a, miR-221, miR-223 and miR-199b could discriminate two groups of AL cases, a myeloid and lymphoid group (Figure 3). The nine AL of ambiguous lineage cases (Universal patient number; UPN3, 5, 6, 7, 8, 12, 13, 18, 19) grouped in a similar fashion with either the AML or ALL cases by this Q-RT-PCR analysis as by array analysis. Moreover, the additional 8 AL of ambiguous lineage cases analyzed by Q-RT-PCR grouped either with the AML group or with the ALL group. Thus, the sole expression of five specific miRNAs using Q-RT-PCR can assign AL cases of ambiguous lineage as either from the lymphoid or myeloid lineage.

Correlation of immunophenotypic marker expression and miRNA classification.

The diagnosis of AL as either AML or ALL according to their miRNA expression profile might correlate with specific expression of one or more immunophenotypic markers already used to classify these leukemias. MiRNA profiling diagnosed 8 out of 17 leukemias of ambiguous lineage as AML and 9 cases as ALL. Overall the diagnosis according to the WHO2008 showed a more similar assignment to AML or ALL with the miRNA expression profiles than the WHO2001 criteria. This was most clear in samples that showed a myeloid
miRNA expression profile from which 7 out of 8 samples were diagnosed as AML by the WHO2008 criteria. In cases were miRNA profiling indicated a lymphoid origin the WHO2001 criteria were more likely to diagnose a lymphoid AL than the WHO2008 criteria. Three patients (UPN 13, 18, and 22) showed expression of several lymphoid markers (either B lymphoid or T lymphoid) in combination with MPO as single myeloid maker (supplemental Table 2). However, with miRNA expression profiling these cases showed a clear ALL profile (Figure 3). This might indicate that the importance of expression of MPO as a single myeloid marker in the classification as leukemia of ambiguous lineage is overestimated. Importantly, even in the absence of informative immunophenotypic markers, miRNA expression profiling can assign a lineage to these AL cases with undefined immunophenotypic lineage. For two of the AL of ambiguous lineage patients (UPN 12 and UPN 27) the diagnosis was unclear due to absence of cytoplasmic CD3, MPO and absence or low expression of CD19; miRNA profiling clustered these samples clearly within the AML group (UPN 12) and ALL group (UPN 27). Another two leukemias of ambiguous lineage expressed markers of all three lineages (UPN 5 and UPN 25) but miRNA analysis assigned these both to the lymphoid lineage. Both these cases and UPN 22 were the only cases expressing cytCD3 and all showed an underlying lymphoid lineage of origin possibly indicating the specificity of cytCD3 in lymphoid lineage commitment.

**Discussion**

AL of ambiguous lineage differs from ALL in atypical myeloid or combined B- and T-lymphoid antigen expression and from AML in atypical lymphoid antigen expression. However, the immunophenotype of AL, as detected by flow cytometry, is merely a limited reflection of the genotypic state. Therefore, we hypothesized that miRNA based classification might help to distinguish between a predominant myeloid or lymphoid origin of AL of ambiguous lineage. Our results indicate that AL of ambiguous lineage is not a separate entity but can be traced back to one of the hematopoietic lineages, lymphoid or myeloid. Reported data of gene expression profiling on thirteen pediatric MPAL cases showed that eight of the thirteen cases have a signature different from AML and B- or T-ALL. In this
study, the remaining five cases grouped with the AML cases (31). In contrast to our data, these data suggest that at least a part of the leukemias of ambiguous lineage can be assigned as a separate identity by gene expression profiling. However, the separate group clustered very close to the B-ALL group, suggesting it could as well be a subtype within B-ALL. Since miRNA expression profiling has shown to be more successful in classification of tumors than gene expression profiling(18) we hypothesized that miRNA expression profiling might be superior in classification of leukemias without clear immunophenotypic lineage commitment. Indeed, our results show a classification of AL cases with ambiguous lineage as either AML, T-ALL or B-ALL. The difference in classification of leukemias of ambiguous lineage by gene expression profiling (18) and miRNA expression profiling is possibly due to the fact that miRNAs target a large panel of genes influencing a diverse network of signaling pathways. As a consequence, miRNA expression profiling might be superior over gene expression profiling in classification of cancers belonging to the same subtype since subtype classification is based on common impaired signaling pathways. Moreover, using miRNA expression profiling to define subclasses of leukemia might be more promising than mRNA profiling since miRNAs are more stable and can be used in smaller numbers than genes.

We identified five miRNAs that could be used to assign AL cases of ambiguous lineage to either AML or ALL. All of these five miRNAs have previously been reported to be associated with AML, such as miR-199b and miR-221 (16). Furthermore, miR-223 has been show to function as a gene that plays a critical role in myeloid functions and differentiation (18, 32). MiR-23a and miR-27a are, together with miR-24-2, located in the same cluster which is regulated by PU-1. Each of these miRNAs are more abundantly expressed in myeloid as compared to lymphoid cells and both miR-23a and miR-27a are implicated in regulating the development of cells into the myeloid or lymphoid lineage (33). In contrast to Wang et al (15), we could not use miR-222 to discriminate between ALL and AML, due to high expression of miR-222 in several of the B-ALL samples. All of the five selected miRNAs have enhanced expression in AML as compared to ALL which is due to their enhanced discriminative character as compared with miRNAs higher expressed in ALL. This is probably due to the fact that most miRNAs have increased expression in either B- or T-ALL and not in lymphoid AL in general. The most significantly discriminative
miRNA with enhanced expression in ALL was miR-150. Since at diagnosis, the discrimination between myeloid or lymphoid is most relevant, no miRNAs associated with only B-ALL or T-ALL were selected.

MiRNA profiling diagnosed 8 out of 17 leukemias of ambiguous lineage as AML and 9 cases as ALL. Since it might be that the karyotype and molecular aberrancies have an effect on miRNA expression and thereby potentially influencing our clustering, we analyzed the distribution of the cytogenetic and molecular aberrations over the myeloid and lymphoid assigned AL of ambiguous leukemia groups. The most frequent cytogenetic abnormality was translocation t(9;22) which was positive in five leukemias of ambiguous lineage (UPN6, 8, 13, 27 and 33). Three of these cases had a miRNA profile that was similar to the ALL cases while the other two clustered with the AML cases. This indicates that despite an identical karyotype the microRNA expression profile is able to distinguish myeloid and lymphoid leukemia, suggesting that lineage specificity influences the microRNA expression profile more than the presence of cytogenetic and/or molecular aberrancies. Furthermore this suggests that translocation of BCR and ABL is not decisive in whether an AL is assigned to the myeloid or lymphoid lineage but that lineage commitment of AL with the BCR-ABL translocation is likely determined by the cell of origin receiving the mutation.

When we retrospectively classified the AL cases with ambiguous lineage containing an AML miRNA expression profile according to the WHO2001, all were diagnosed as AL with ambiguous lineage; while according to the WHO2008 seven out of eight cases were classified as an AML. The other AL case with ambiguous lineage with an AML miRNA expression profile could not be classified using the WHO2008 criteria due to absence of CD19, cytoplasmic CD3 and MPO. In the group AL with ambiguous lineage that had an ALL miRNA expression profiling, four out of nine cases were diagnosed as ALL by the WHO2001 criteria whereas only one out of nine cases was diagnosed as ALL by the WHO2008 criteria. Thus, classification by the criteria of the WHO2008 is less accurate in assigning ALL to the group of leukemias with a lymphoid miRNA expression profile than WHO2001, whereas it’s more accurate in assigning an AL case with a AML miRNA expression profile as an AML. The immunophenotypic classification of AL cases to the myeloid lineage is done when MPO is expressed as a single myeloid marker. This leads to
assignment of AL cases containing an ALL miRNA expression profile, but with MPO
expression as a single myeloid marker, to the group of leukemias of ambiguous lineage.
This might partly explain the better response to ALL treatment protocols of AL cases with
ambiguous lineage (12).

Acute leukemias with ambiguous lineage are associated with a poor prognosis as
compared to AML and ALL cases (7-11). The poor prognosis of these leukemias might be
due, at least partly, to their mistreatment. However, leukemias with ambiguous lineage
have been shown to be associated with several poor prognostic factors such as CD34+
phenotype, an unfavorable karyotype and PgP over-expression (7, 9). Our group was to
small to perform survival analysis. However, generally overall survival was poor (median
20 months, range 5-110 months) for AL of ambiguous lineage.

The 5-year overall survival rate of patients with AML is considerably lower than of
ALL patients and indeed, ALL patients have a better response to standard chemotherapy
than AML patients in almost every age group (34). This indicates that accurate diagnosis
of all AL cases as either AML or ALL is crucial for selection of appropriate therapy. To
base treatment decisions on miRNA expression profiles it is crucial that the results of a
microRNA expression test can be obtained within the timeframe of the start of appropriate
treatment. Our experience is that the turnaround time from isolation of the leukemic blasts
to miRNA expression results can easily be done within 1.5 day making it rapid and within
the time where a treatment decision has to be made. Whether treatment decisions based
on miRNA expression profiles improve outcome of patients with AL of ambiguous lineage
is difficult to demonstrate since these leukemias are very rare and effect of treatment can
only be measured in large randomized trials.

Our finding that expression analysis of five miRNAs could accurately classify AL cases
with ambiguous lineage as AML or ALL might add to the already used methods to classify
these AL cases of ambiguous lineage. In conclusion, our results indicate that leukemias of
ambiguous lineage are not an unique entity but can be classified as either AML or ALL
using miRNA expression profiling.
Acknowledgements

We thank Francois Rustenburg and Paul Eijck from the Microarray Facility of the VU University Medical Center assistance with the miRNA expression profiling. Furthermore, we are grateful to the people at the department of Hematology of the VU University Medical Center who generated a library of acute leukemia samples over the years.

Disclosure of Conflicts of Interest: none

Authors’ Contributions

Conception and design: D.C. de Leeuw, W. van den Ancker and L. Smit.
Acquisition of data: D.C. de Leeuw, W. van den Ancker and F. Denkers.
Writing, review and/or revision of the manuscript: D.C. de Leeuw and W. van den Ancker: T.M. Westers, R.X. de Menezes, G.J. Ossenkoppele, A.A. van de Loosdrecht and L. Smit.
Study supervision: G.J. Ossenkoppele, A.A. van de Loosdrecht and L. Smit
D.C. de Leeuw and W. van den Ancker contributed equally to this work

Legends

Title: Table 1
Caption: MicroRNAs are differentially expressed between AML and ALL.
Ten miRNAs were found to be differentially expressed between AML and ALL (B-ALL and T-ALL) by LIMMA. ‘Max. expression in dataset’ refers to the highest expression found in all samples for that particular miRNA. MiRNAs were considered expressed when median signal values after log2-transformation and normalization were higher than 6.0. ‘AML vs. ALL t’ represents moderated t-statistic values, ‘AML vs. ALL p’ represents p-values. ‘AML vs. ALL FDR’ represents False Discovery Rate.
**Title:** Figure 1

**Caption:** MicroRNA expression signatures discriminate between AML, B-ALL and T-ALL. (A) The top 10 percent of most variable miRNAs were selected (n=134) for unsupervised clustering analysis of the AL samples. AML samples cluster separately from ALL samples. Within the ALL cluster, B-ALL and T-ALL samples can be discriminated. (B) Unsupervised clustering of the AL samples using the 32 miRNA literature based signature. AML and ALL can be discriminated using previously reported lineage specific miRNA expression. In both A and B the columns represent patients; rows represent miRNAs; blue, AML samples; red, T-ALL samples; green, B-ALL samples; UPN, Unique patient number.

**Title:** Figure 2

**Caption:** Leukemias of ambiguous lineage show similar expression profiles as either AML, B-ALL or T-ALL. (A) Unsupervised hierarchical clustering was performed using all AL samples. Leukemias of ambiguous lineage show similar expression profiles as either AML, B-ALL or T-ALL. (B) Unsupervised clustering using the 32 miRNA literature based signature. Columns represent patients; rows represent miRNAs; blue, AML samples; red, T-ALL samples; green, B-ALL samples; purple, AL of ambiguous lineage; UPN, Unique patient number; Diagnose, diagnose based on immunophenotypical analysis; B/My, AL of ambiguous lineage expressing B-lymphoid and myeloid markers; T/My, AL of ambiguous lineage expressing T-lymphoid and myeloid markers; B/T/My, trilineage AL of ambiguous lineage.

**Title:** Figure 3

**Caption:** Expression analysis of miR-23a, miR-27a, miR-199b, miR-221 and miR-223 by QRT-PCR can diagnose leukemia of ambiguous lineage as myeloid or lymphoid. Unsupervised clustering of AL samples based on QRT-PCR expression analysis of miR-23a, miR-27a, miR-199b, miR-221 and miR-223 resulted in an AML and ALL group. UPN,
Unique patient number; Type, type of leukemia; WHO2001, diagnosis when using
WHO2001 criteria; WHO2008, diagnosis when using WHO2008 criteria; AML, acute
myeloid leukemia; B-ALL, B-cell acute lymphoid leukemia; T-ALL, T-cell acute lymphoid
leukemia; B/My, BAL/MPAL expressing B-lymphoid and myeloid markers; T/My,
BAL/MPAL expressing T-lymphoid and myeloid markers; B/T/My, trilineage BAL/MPAL;
UAL, unclassifiable acute leukemia; CL, cell line

Reference List


Table 1
MicroRNAs are differentially expressed between AML and ALL.

<table>
<thead>
<tr>
<th>Gene</th>
<th>AMLvsALL t</th>
<th>AMLvsALL p</th>
<th>AMLvsALL FDR</th>
<th>Max expression in dataset (selected &gt;6)</th>
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<td>-5.293199</td>
<td>0.000340</td>
<td>0.038177</td>
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</tr>
</tbody>
</table>
Figure 1

A

B
MicroRNA profiling can classify acute leukemias of ambiguous lineage as either acute myeloid leukemia or acute lymphoid leukemia.

David C de Leeuw, Willeijn van den Ancker, Fedor Denkers, et al.

Clin Cancer Res Published OnlineFirst February 26, 2013.