MicroRNA Profiling Can Classify Acute Leukemias of Ambiguous Lineage as Either Acute Myeloid Leukemia or Acute Lymphoid Leukemia

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Abstract

Purpose: Classification of acute leukemia is based on the commitment of leukemic cells to the myeloid or the lymphoid lineage. However, a small percentage of acute leukemia cases lack straightforward immunophenotypical lineage commitment. These leukemias of ambiguous lineage represent a heterogeneous category of acute leukemia that cannot be classified as either acute myeloid leukemia (AML) or acute lymphoid leukemia (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 (miRNA) expression profiles of 17 cases with acute leukemia of ambiguous lineage and 16 cases of AML, B-cell acute lymphoid leukemia (B-ALL), and T-cell acute lymphoid leukemia (T-ALL).

Results: We show that leukemias of ambiguous lineage do not segregate as a separate entity but exhibit miRNA expression profiles similar to AML, B-ALL, or T-ALL. We show that by using only 5 of the most lineage-discriminative miRNAs, we are able to define acute leukemia of ambiguous lineage as either AML or ALL.

Conclusion: Our results indicate the presence of a myeloid or lymphoid lineage-specific genotype, as reflected by miRNA expression, in these acute leukemias despite their ambiguous immunophenotype. miRNA-based classification of acute leukemia of ambiguous lineage might be of additional value in therapeutic decision making. Clin Cancer Res; 19(8); 2187–96. ©2013 AACR.

Introduction

Acute leukemias are classified as myeloid or lymphoid by using immunologic markers that determine their lineage commitment. Four percent of acute leukemia cases cannot be classified as acute myeloid leukemia (AML) or acute lymphoid leukemia (ALL) due to coexpression of both lymphoid and myeloid lineage markers (1–6). The prognosis of leukemias of ambiguous lineage is worse compared with 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 acute leukemia cases at diagnosis as either myeloid or lymphoid.

The diagnostic criteria for leukemias of ambiguous lineage were previously defined by the World Health Organization (WHO) 2001 classification and are based on the presence of immunological markers detected by flow cytometry (1). According to the WHO 2001 scoring system, many different combinations of immunophenotypic markers can define an acute leukemia 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 crisis chronic myeloid leukemia cases were excluded from mixed phenotype acute leukemia (MPAL; ref. 2). The major immunophenotypic markers used by the WHO 2008 to determine the lineage for these acute leukemia cases are cytoplasmic myeloperoxidase (MPO), CD19, and cytoplasmic CD3. However, the current WHO 2008 classification is difficult to interpret due to the implementation of expression intensities for B-cell markers and the absence of clear cutoffs.

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-
Translational Relevance

Classification of acute leukemia is based on the 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 acute leukemia of ambiguous lineage. It is unclear whether acute leukemia of ambiguous lineage benefits from an acute myeloid leukemia (AML)- or acute lymphoid leukemia (ALL)-based treatment protocol; this partly explains their unfavorable prognosis. Here, we show that miRNA expression profiling clusters acute leukemia from ambiguous lineage with either myeloid or lymphoid leukemia control samples. Furthermore, based on 5 of the most lineage-discriminative miRNAs, we are able to define acute leukemia of ambiguous lineage as either AML or ALL. In conclusion, addition of miRNA evaluation to current diagnostic procedures helps deciphering the myeloid or lymphoid lineage predominance of an acute leukemia of ambiguous lineage and thus the treatment of choice.

stranded RNA molecules, which regulate gene expression by promoting degradation of miRNAs 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 patients with AML (22–25). Like in AML, miRNA expression signatures have been shown to be associated with prognosis of patients with ALL (15). Moreover, miRNA expression patterns showed to reflect the lineage state of acute leukemia (15, 16, 18). Interestingly, of the 27 miRNAs differentially expressed between ALL and AML, 2 were sufficient to accurately (97%–99%) identify the myeloid or lymphoid identity of these acute leukemias (16). In another study, 16 miRNAs (mir-23a, mir-27a/b, mir-128a, mir-128b, mir-221, mir-222, 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 acute leukemia cases as either AML or ALL (15, 16, 18), we decided to investigate the miRNA expression profile of acute leukemia 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-cell acute lymphoid leukemia (B-ALL), or T-cell acute lymphoid leukemia (T-ALL). Thus, acute leukemia 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 acute leukemia of ambiguous lineage as either AML or ALL and therefore in treatment decision making.

Materials and Methods

Patient samples and cell lines

Sixteen patients with 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 on the basis of a clear immunophenotypic myeloid or lymphoid lineage commitment. Immunophenotypical analysis was conducted on bone marrow samples or peripheral blood samples in case no bone marrow samples were available. The use of patient material for this study and the informed consent procedure has been approved by the Medical Ethical Committee of our institute. Bone marrow or peripheral blood samples from 16 patients diagnosed with leukemia of ambiguous lineage and 17 patients with AML (n = 7), B-ALL (n = 6), or T-ALL (n = 4) were used for miRNA expression analysis. Cell lines, HL60 (CCL-240) and CCRF-CEM (CCL-119), were obtained from American Type Culture Collection.

Immunophenotypical analysis

Analysis was carried out as a part of the regular diagnostic investigations. In short, red blood cells were lysed using Pharm Lyse lysing solution (BD Biosciences), washed with PBS, and incubated with antibodies defining or specific for the B cell, T cell, and myeloid lineage. Samples were analyzed with 4-color flow cytometry on a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences). The following fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), or allophycocyanin (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, immunoglobulin M, CD79a, Tdt, and MPO were evaluated after fixation with paraformaldehyde and subsequently permeabilization with Facs Lyse (Becton Dickenson). Antibodies were purchased from BD Biosciences, Zebra biosciences, Dako, or Sanquin. Data analysis was conducted using CellQuest Pro software (BD Biosciences). Blasts were defined by low-side scatter, diminished or low CD45 expression, and CD34 expression. In case of CD34-negative acute leukemia, blasts were selected using other immature markers such as CD117, CD133, CD10, and clygM. For defining the B-lymphoid lineage of a...
MPAL according to the WHO 2008 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 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) 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 conducted on pretreatment bone marrow at diagnosis. Specimens were processed using short-term unstimulated cultures. Chromosome banding was conducted by the use of standard techniques, and karyotypes were described according to the International System for Human Cytogenetic Nomenclature.

**Cell purification**

Bone marrow or peripheral blood cells derived from patients with acute leukemia 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 acute leukemia cases was sorted by flow cytometry using FACS ARIA Cell Sorter (BD Biosciences).

**RNA isolation and miRNA expression analysis**

Total RNA was isolated with the NucleoSpin miRNA Kit (Macherey-Nagel) according to manufacturer’s protocol. RNA was concentrated using a vacuum concentrator (SPD111V, Thermo Savant) and the concentration was measured with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Human miRNA microarrays (Agilent Technologies), containing 60,000 probes representing 1,205 human and 144 human viral miRNAs were measured with a NanoDrop 1000 Spectrophotometer (Macherey-Nagel) according to manufacturer’s protocol. Human miRNA microarrays were analyzed with Feature Extraction TM software, version 4.2.0) for producing the heatmaps.

**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 1,347 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 conducted 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 conducted using the statistical package R (version 2.14.0; ref. 29), the package limma (30) for empirical Bayes linear regression, and BRB-ArrayTools (version 2.14.0; ref. 29), the package limma (30) for empirical Bayes linear regression, and BRB-ArrayTools (version 4.2.0) for producing the heatmaps.

**Results**

**miRNAs 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 were 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 acute

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**Quantitative real-time PCR**

Selection of the most differentiating miRNAs in our cohort was conducted using significance analysis of microarrays (SAM) on the myeloid and lymphoid cluster as was formed by unsupervised clustering of all samples (controls combined with acute leukemia of ambiguous lineage). The 5 miRNAs with the highest observed relative difference were selected. This signature included miR-23a, miR-27a, miR-221, miR-223, and miR-199b-5p (Supplementary Table S4). Specific primers (Applied Biosystems) for these 5 lineage-discriminating miRNAs were used for qRT-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 conducted on a 7300 Real-Time PCR System (Applied Biosystems). All experiments were carried out in duplicate, and Ct values were averaged. Expression was calculated using 2^{-ΔΔCt} method, log2-transformed, and analyzed with BRB-ArrayTools.
leukemia control samples generated 2 separate clusters. One cluster that contained all AML samples, whereas the other contained all the ALL samples. This lymphoid cluster could be further separated in a T-ALL and B-ALL cluster (Fig 1A).

Next, we conducted 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 10 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 with AML, whereas the other 9 miRNAs are higher expressed in AML as compared with ALL (miR-223, miR-199b-5p,
miRNA-Based Classification of Acute Leukemias

Table 1. miRNAs differentially expressed between AML and ALL

<table>
<thead>
<tr>
<th>Gene</th>
<th>AML vs ALL t</th>
<th>AML vs ALL p</th>
<th>AML vs ALL FDR</th>
<th>Max expression in dataset (selected &gt;6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-199b-5p</td>
<td>-8.602706</td>
<td>0.000006</td>
<td>0.003259</td>
<td>7.61</td>
</tr>
<tr>
<td>hsa-miR-27b</td>
<td>-8.49291</td>
<td>0.000007</td>
<td>0.003259</td>
<td>6.94</td>
</tr>
<tr>
<td>hsa-miR-150</td>
<td>8.393688</td>
<td>0.000007</td>
<td>0.003259</td>
<td>10.94</td>
</tr>
<tr>
<td>hsa-miR-199a-3p</td>
<td>-7.008514</td>
<td>0.000035</td>
<td>0.011802</td>
<td>7.40</td>
</tr>
<tr>
<td>hsa-miR-223</td>
<td>-6.165671</td>
<td>0.000102</td>
<td>0.024114</td>
<td>13.21</td>
</tr>
<tr>
<td>hsa-miR-27a</td>
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<td>0.000107</td>
<td>0.024114</td>
<td>10.06</td>
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<tr>
<td>hsa-miR-340</td>
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<td>0.000154</td>
<td>0.028143</td>
<td>6.68</td>
</tr>
<tr>
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<td>-5.731613</td>
<td>0.000183</td>
<td>0.028143</td>
<td>6.68</td>
</tr>
<tr>
<td>hsa-miR-23a</td>
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<td>0.000260</td>
<td>0.033675</td>
<td>10.26</td>
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<tr>
<td>hsa-miR-221</td>
<td>-5.293199</td>
<td>0.000340</td>
<td>0.038177</td>
<td>8.26</td>
</tr>
</tbody>
</table>

NOTE: 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 versus ALL t" represents moderated t statistic values, "AML versus ALL P" represents P values. "AML versus ALL FDR" represents false discovery rate.

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 acute leukemia cases as either AML or ALL, we analyzed the miRNA expression profiles of 9 acute leukemia 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 4 morphologic and immunophenotypic defined groups of acute leukemia cases (AML, B-ALL, T-ALL, and acute leukemia of ambiguous lineage; Supplementary Table S4A–S4C). First, this showed that there is no significant difference in overall expression pattern observed in acute leukemia cases of ambiguous lineage as compared with AML samples (lowest FDR >0.26), indicating that acute leukemia of ambiguous lineage was not significantly different from AML. However, when comparing the overall miRNA expression of the acute leukemia 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 with 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 (Supplementary Table S4D), likely indicating that the differential expression of these miRNAs is a direct result of intrinsic differential expression between B-ALL and T-ALL. Thus, we did not observe a distinctive pattern of overall miRNA expression between acute leukemia of ambiguous lineage and AML or ALL cases, suggesting that acute leukemia 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 conducted unsupervised clustering of the miRNA expression of the acute leukemia cases with...
ambiguous lineage as well as the AML and ALL cases (11 acute leukemia cases and 9 acute leukemia cases with ambiguous lineage). This resulted in the identification of 3 acute leukemia groups, a myeloid group containing all AML samples and a lymphoid group that could be further divided in B-ALL and T-ALL (Fig. 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 acute leukemia groups (Fig. 2A). Using the 32 miRNA literature-based signature in an unsupervised way on all our samples (Fig. 2B) showed grouping of leukemias with ambiguous lineage with AML, T-ALL, or B-ALL as well.

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.

Leukemias of ambiguous lineage show similar expression profiles as AML, B-ALL or T-ALL. A, unsupervised hierarchical clustering was conducted using all acute leukemia samples. Leukemias of ambiguous lineage show similar expression profiles as 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, acute leukemia of ambiguous lineage.

Acute leukemia of ambiguous lineage can be classified as ALL or AML by qRT-PCR expression analysis of a small selection of miRNAs

Patients with acute leukemia are either treated with an ALL or an AML treatment protocol, and the assignment of acute leukemia cases of ambiguous lineage as either one of those acute leukemia subtypes is therefore of most relevance. To investigate the feasibility of classification of acute leukemia cases of ambiguous lineage as either AML or ALL by the expression of only a limited set of miRNAs, we selected the top 5 significantly differentially expressed miRNAs that could distinguish between AML and ALL (Supplementary Table S3). These 5 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 2 published studies (15, 16). We analyzed the expression of these miRNAs by qRT-PCR in 19 acute leukemia cases that were used for the array hybridization (9 acute leukemia of ambiguous lineage, 5 ALL, and 5 AML).
5 AML cases) as well as an additional 8 acute leukemia samples of ambiguous lineage. 3 ALL cases (2 T-ALL, 1 B-ALL), 2 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, and miR-199b could discriminate 2 groups of acute leukemia cases, a myeloid and lymphoid group (Fig. 3). The 9 acute leukemia of ambiguous lineage cases (unique patient number; UPN 3, 5, 6, 7, 8, 12, 13, 18, 19) were grouped in a similar fashion with either the AML or ALL cases by this qRT-PCR analysis as by array analysis. Moreover, the additional 8 acute leukemia of ambiguous lineage cases analyzed by qRT-PCR grouped either with the AML group or with the ALL group. Thus, the sole expression of 5 specific miRNAs using qRT-PCR can assign acute leukemia cases of ambiguous lineage as either from the lymphoid or myeloid lineage.

**Correlation of immunophenotypic marker expression and miRNA classification**

The diagnosis of acute leukemia 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 WHO 2008 showed a more similar assignment to AML or ALL with the miRNA expression profiles than the WHO 2001 criteria. This was most clear in samples that showed a myeloid miRNA expression profile from which 7 of 8 samples were diagnosed as AML by the WHO 2008 criteria. In cases where miRNA profiling indicated a lymphoid origin, the WHO 2001 criteria were more likely to diagnose a lymphoid acute leukemia than the WHO 2008 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 marker (Supplementary Table S2). However, with miRNA expression profiling, these cases showed a clear ALL profile (Fig. 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 acute leukemia cases with undefined immunophenotypic lineage. For 2 of the patients with acute leukemia of ambiguous lineage (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 2 leukemias of ambiguous lineage expressed markers of all 3 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

Acute leukemia 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 acute leukemia, 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 acute leukemia of ambiguous lineage. Our results indicate that acute leukemia 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 13 pediatric MPAL cases showed that 8 of the 13 cases have a signature different from AML and B- or T-ALL, whereas the remaining 5 cases were grouped with the myeloid leukemias (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. Because 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 acute leukemia cases with ambiguous lineage as AML, T-ALL, or B-ALL. The difference in classification of leukemias of ambiguous lineage by gene expression profiling (31) 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 because 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 because miRNAs are more stable and can be used in smaller numbers than genes.

We identified 5 miRNAs that could be used to assign acute leukemia cases of ambiguous lineage to either AML or ALL. All of these 5 miRNAs have previously been reported to be associated with AML, such as miR-199b and miR-221 (16). Furthermore, miR-223 has been shown 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 with 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 the study conducted by Wang and colleagues (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 5 selected miRNAs have enhanced expression in AML as compared with 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 acute leukemia in general. The most significantly discriminative miRNA with enhanced expression in ALL was miR-150. Because 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 of 17 leukemias of ambiguous lineage as AML and 9 cases as ALL. Because it might be that the karyotype and molecular aberrancies have an effect on mRNA expression and thereby potentially influence our clustering, we analyzed the distribution of the cytogenetic and molecular aberrations over the myeloid- and lymphoid-assigned acute leukemia of ambiguous leukemia groups. The most frequent cytogenetic abnormality was translocation t(9;22), which was positive in 5 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, whereas the other 2 clustered with the AML cases. This indicates that despite an identical karyotype, the miRNA expression profile is able to distinguish myeloid and lymphoid leukemia, suggesting that lineage specificity influences the miRNA expression profile more than the presence of cytogenetic and/or molecular aberrances. Furthermore, this suggests that the translocation of BCR and ABL is not decisive in whether an acute leukemia is assigned to the myeloid or lymphoid lineage but that lineage commitment of acute leukemia with the BCR-ABL translocation is likely determined by the cell of origin receiving the mutation.

When we retrospectively classified the acute leukemia cases with ambiguous lineage containing an AML miRNA expression profile according to the WHO 2001, all were diagnosed as acute leukemia with ambiguous lineage, whereas according to the WHO 2008, 7 of 8 cases were classified as an AML. The other acute leukemia case with ambiguous lineage with an AML miRNA expression profile could not be classified using the WHO 2008 criteria due to the absence of CD19, cytoplasmic CD3, and MPO. In the group acute leukemia with ambiguous lineage that had an ALL miRNA expression profile, 4 of 9 cases were diagnosed as ALL by the WHO 2001 criteria, whereas only 1 of 9 cases was diagnosed as ALL by the WHO 2008 criteria. Thus, classification by the criteria of the WHO 2008 is less accurate in assigning ALL to the group of leukemias with a lymphoid miRNA expression profile than WHO 2001, whereas it is more accurate in assigning an acute leukemia case with an AML miRNA expression profile as an AML. The immunophenotypic classification of acute leukemia cases to the myeloid lineage is done when MPO is expressed as a single...
myeloid marker. This leads to the assignment of acute leukemia 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 acute leukemia cases with ambiguous lineage (12).

Acute leukemias with ambiguous lineage are associated with a poor prognosis as compared with 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 overexpression (7, 9). Our patient group was too small to conduct survival analysis. However, generally overall survival was poor (median 20 months, range 5–110 months) for acute leukemia of ambiguous lineage.

The 5-year overall survival rate of patients with AML is considerably lower than that of the patient with ALL and indeed, patients with ALL have a better response to standard chemotherapy than patients with AML in almost every age group (34). This indicates that accurate diagnosis of all acute leukemia cases as either AML or ALL is crucial for the selection of appropriate therapy. To base treatment decisions on miRNA expression profiles, it is crucial that the results of a miRNA 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 obtaining 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 the outcome of patients with acute leukemia of ambiguous lineage is difficult to determine since these leukemias are very rare, and the effect of treatment can only be measured in large randomized trials.

Our finding that expression analysis of 5 miRNAs could accurately classify acute leukemia cases with ambiguous lineage as AML or ALL might add to the already used methods to classify these acute leukemia cases of ambiguous lineage. In conclusion, our results indicate that leukemias of ambiguous lineage are not a unique entity but can be classified as either AML or ALL using miRNA expression profiling.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D.C. de Leeuw, W. van den Ancker, G.J. Ossenkoppele, A.A. van de Loosdrecht, L. Smit
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.C. de Leeuw, W. van den Ancker, F. Denkers, A. A. van de Loosdrecht, L. Smit
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): D.C. de Leeuw, W. van den Ancker, F. Denkers, R.X. de Menezes, L. Smit
Writing, review, and/or revision of the manuscript: D.C. de Leeuw, W. van den Ancker, R.X. de Menezes, T.M. Westers, A.A. van de Loosdrecht, L. Smit
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.C. de Leeuw, W. van den Ancker, L. Smit
Study supervision: G.J. Ossenkoppele, A.A. van de Loosdrecht, L. Smit

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MicroRNA Profiling Can Classify Acute Leukemias of Ambiguous Lineage as Either Acute Myeloid Leukemia or Acute Lymphoid Leukemia

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