Predictive Biomarkers and Personalized Medicine

MicroRNAs Are Independent Predictors of Outcome in Diffuse Large B-Cell Lymphoma Patients Treated with R-CHOP

Alvaro J. Alencar¹, Raquel Malumbres¹, Goldi A. Kozloski¹, Ranjana Advani², Neha Talreja², Shideh Chinichian², Javier Briones³, Yasodha Natkunam³, Laurie H. Sehn⁶, Randy D. Gascoyne⁷, Rob Tibshirani⁴, and Izidore S. Lossos¹

Abstract

Purpose: Diffuse large B-cell lymphoma (DLBCL) heterogeneity has prompted investigations for new biomarkers that can accurately predict survival. A previously reported 6-gene model combined with the International Prognostic Index (IPI) could predict patients' outcome. However, even these predictors are not capable of unambiguously identifying outcome, suggesting that additional biomarkers might improve their predictive power.

Experimental Design: We studied expression of 11 microRNAs (miRNA) that had previously been reported to have variable expression in DLBCL tumors. We measured the expression of each miRNA by quantitative real-time PCR analyses in 176 samples from uniformly treated DLBCL patients and correlated the results to survival.

Results: In a univariate analysis, the expression of miR-18a correlated with overall survival (OS), whereas the expression of miR-181a and miR-222 correlated with progression-free survival (PFS). A multivariate Cox regression analysis including the IPI, the 6-gene model–derived mortality predictor score and expression of the miR-18a, miR-181a, and miR-222, revealed that all variables were independent predictors of survival except the expression of miR-222 for OS and the expression of miR-18a for PFS.

Conclusion: The expression of specific miRNAs may be useful for DLBCL survival prediction and their role in the pathogenesis of this disease should be examined further. Clin Cancer Res; 17(12); 4125–35. ©2011 AACR.

Introduction

Diffuse large B-cell lymphoma (DLBCL), the most common subtype of non-Hodgkin lymphoma, exhibits an aggressive and variable clinical course (1). An anthracyclin-based immunochemotherapy regimen [rituximab, cyclophosphamide, adriamycin, vincristine, and prednisone (R-CHOP)] is considered the current standard of therapy and leads to a complete remission in approximately 75% and a long-term failure-free survival rate of 50% in DLBCL patients (2). The International Prognostic Index (IPI), which incorporates 5 clinical parameters, is considered the gold standard for predicting prognosis in DLBCL patients; however, the variable outcomes of patients within specific IPI subgroups argue that clinical features alone cannot precisely predict response to therapy or clinical outcome. Similarly, routine histologic and immunophenotypic evaluation of diagnostic biopsies are insufficient to accurately predict clinical outcome in DLBCL patients (3, 4).

Gene expression profiling studies showed the presence of DLBCL subtypes associated with distinct cells of origin and clinical outcomes (5, 6). These pivotal studies suggested the existence of potential prognostic biomarkers, unique pathogenetic pathways, and different mechanisms of therapy resistance among the subtypes. To improve the accuracy of existing clinical prognostic methods, we previously established an IPI-independent 6-gene model that was capable of predicting progression-free (PFS) and overall survival (OS) in DLBCL patients (7, 8). This model was robust to predict outcome regardless of the type of tissue or platform used for analysis or the treatment regimen given to patients. Although this model was validated in 7 independent cohorts of patients (Supplemental Table S1) and incorporated into an ongoing multicenter prospective study, its inability to capture all of the variability in...
MicroRNAs (miRNA) have been reported to predict outcome in several malignancies, including chronic lymphocytic leukemia (CLL; ref. 9), acute myeloid leukemia (AML; refs. 10, 11), and solid tumors (12). Since miRNAs may be less susceptible to degradation than mRNAs (13), due to their shorter length, their expression is likely to be more robust for determination of cellular origin of multiple cancers (14). Initial reports proposed that specific miRNAs might be associated with outcome in DLBCL patients (15–17). However, these DLBCL studies were conducted on very small cohorts of patients and need validation in independent and larger cohorts of patients. We recently showed the stage-specific expression pattern of multiple miRNAs at distinct differentiation stages of peripheral B lymphocytes and identified a 9-miRNA signature that could separate germinal center B cell (GCB) from activated B-cell (ABC)–derived DLBCL cell lines (18). This 9-miRNA signature was derived from enriched tumor cells and may not be applicable to whole tumors that also contain nonmalignant cellular components that contribute to measured expression of miRNAs.

Therefore, in the current study, we set out to identify specific miRNAs whose expression in whole DLBCL tumors (without enrichment for malignant B cells) correlates with survival of DLBCL patients treated with R-CHOP. We also examined the relationship of these miRNAs to the 6-gene model and the IPI.

Materials and Methods

Patients

A total of 176 diagnostic specimens from DLBCL patients treated with curative intent by using R-CHOP chemotherapy at University of Miami, Miami, FL (n = 25), Stanford University, Stanford, CA (n = 49), Hospital Santa Creu i Sant Pau-Barcelona (n = 22), and British Columbia Cancer Agency, Vancouver, BC (n = 80) were used for analysis of expression of miRNAs and the genes comprising the 6-gene model (LMO2, BCL6, FN1, CCND2, SCYA3, and BCL2). The specimens were selected on the basis of the following criteria: (i) diagnosis of de novo DLBCL in HIV-negative patients; (ii) availability of tissue obtained at diagnosis before initiation of therapy; and (iii) availability of follow-up and outcome data at the treating institution. Criteria commonly used for prospective studies such as normal renal and liver functions, absence of comorbid conditions, and good performance status were not applied for case selection. Patients with primary mediastinal large B-cell lymphoma or involvement of central nervous system at presentation were not included. None of the patients in the current study were included in our prior study that led to the derivation of the 6-gene model (7), but some were included in the previous study analyzing the application of the 6-gene model in formalin-fixed paraffin-embedded (FFPE) specimens (8).

Institutional review board approval was obtained from all participating institutions for inclusion of anonymized data in this study in accordance with the declaration of Helsinki. The following information at the time of diagnosis was collected: age, sex, performance status, stage, number of extranodal sites involved, serum lactate dehydrogenase (LDH) level, presence or absence of systemic (B) symptoms, and IPI score. Staging was done in all patients according to the Ann Arbor system (19) on the basis of physical examination, bone marrow biopsy, and computed tomography of the chest, abdomen, and pelvis. OS and PFS were determined from the follow-up information retrieved from the patients’ medical records. Histological sections were reviewed to confirm the diagnoses of DLBCL according to the 2008 World Health Organization classification of hematopoietic tumors (20). The cellular context of the analyzed specimens consisted of at least 70% of large CD20+ lymphoma cells (range 70–95%), as determined by hematoxylin and eosin staining and immunohistochemistry.

RNA isolation and real-time PCR

Total RNA was extracted from two 5-μm-thick slices of FFPE sections as previously reported (21). RNA was successfully extracted from all 176 specimens. For measurement of expression of genes comprising the 6-gene model, RNA (2 μg) was reverse transcribed by the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol with a minor modification: addition of RNase inhibitor (Applied Biosystems) at a final concentration of 1 U/μL. The complete reaction mixes were incubated at 25°C for 10 minutes and 37°C for 120 minutes. For miRNA analysis, 5 μL of RNA at 2 ng/μL was mixed with 10 μL of TaqMan MicroRNA Reverse Transcription Kit reagent containing specific miRNA primers and reverse transcribed according to manufacturer’s instructions (Applied Biosystems). Complete reaction mixes were incubated at 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes.

Real-time PCR (RT-PCR) was carried out by the ABI PRISM 7900HT Sequence Detection System Instrument and software (Applied Biosystems), as previously reported (7, 21, 22). The following commercially available Assays-on-Demand were used for measurement of gene expression
Expression of miRNAs was normalized to the expression of endogenous RNU6B, whereas gene expression was normalized to phosphoglycerate kinase 1 (Human TaqMan Pre-Developed Assay Reagent; Applied Biosystems) that served as internal controls of RNA amount and integrity, as previously reported (21).

Identification of forkhead box protein P1 as mir-181a target

Three prediction algorithms, PicTar (ref. 23; http://picTar.mdc-berlin.de/), New York University, New York, and Max Delbruck Centrum, Berlin, Germany), miRanda (ref. 24, http://cbio.mskcc.org/mirnaviewer/, Memorial Sloan-Kettering Cancer Center, New York), and TargetScan (ref. 25; http://www.targetscan.org/, Whitehead Institute for Biomedical Research, Cambridge, MA), were used to find possible targets of hsa-miR-181a with potential role in DLBCL pathogenesis or prognosis. In addition, the Probability of Interaction by Target Accessibility (PITA) algorithm (ref. 26; http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html, Segal Lab of Computational Biology, Rehovot, Israel) was used to confirm the accessibility of the putative miRNA binding sites.

Forkhead box protein P1 (FOXP1) expressing DLBCL cell lines (HBL1 and VAL) were cultured in RPMI (Cellgro) with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin/L-glutamine (Cellgro), were transfected with each 3'UTR luciferase construct by using SiPort NeoFX (Ambion) according to the manufacturer’s instructions. Briefly, 45,000 cells per well were seeded over 50 μL of transfection mix in a final volume of 0.5 mL. The transfection mix was prepared by using 1 μL of SiPort, 0.4 μg of Luciferase pGL3 control-derived construct, 80 ng of pRL-TK (Promega), and 5 pmol of precursor hsa-miR-181a or precursor miR-negative control #1 (Ambion) per well. Cells were lysed 16 to 24 hours after transfection and Dual Luciferase Assay (Promega) was performed on a Sirius luminometer (Berthold). Measured luciferase expression values were normalized to nontargeted mutagenesis Kit (Stratagene).

For luciferase reporter experiments, HeLa cells, cultured in Dulbecco modified Eagle medium with high glucose (Invitrogen) and 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin/L-glutamine (Cellgro), were transfected in triplicate with each 3'UTR luciferase construct using 1 μL of SiPort, 0.4 μg of Luciferase pGL3 control-derived construct, 80 ng of pRL-TK (Promega), and 5 pmol of precursor hsa-miR-181a or precursor miR-negative control #1 (Ambion) per well. Cells were lysed 16 to 24 hours after transfection and Dual Luciferase Assay (Promega) was performed on a Sirius luminometer (Berthold). Measured luciferase expression values were normalized to nontargeting pre-miR-negative control #1, which measurement was given the value of 100%. Data are presented as average ±SEM. Each experiment was done in triplicates and repeated at least 3 times.

Similar experiments were done to examine the effect of miR-181a on expression of O6-methylguanine-DNA methyltransferase (MGMT) endogenously expressed in VAL and Jurkat cell lines.

Statistical analysis

The normalized gene expression values were log-transformed (on a base of 2) and the 6-gene mortality predictor score was calculated on the basis of the following equation: mortality predictor score = (−0.0273 × LMO2) + (−0.2103 × BCL6) + (0.0346 × CCND2) + (0.1800 × SCYA3) + (0.5527 × BCL2). miRNA expression and the 6-gene score were used as a continuous variable or categorically ranked the patients, by allowing their division into 2 groups: low and high molecular risk groups characterized by 6-gene mortality predictor score below or above the median, respectively, or low and high expression groups based on miRNA expression below or above the corresponding median.

Expression of miRNA and mortality predictor score were correlated to PFS and OS. OS was defined as the time interval from the date of diagnosis to the date of death or last follow-up. PFS was defined as the time interval between the date of initial diagnosis and the date of disease...
progression or death from any cause, whichever came first, or date of last follow-up evaluation. Survival curves were estimated by the product-limit method of Kaplan–Meier and were compared with the log-rank test. Univariate and multivariate analyzes were done. Multivariate regression analysis according to the Cox proportional hazards regression model (27) with OS or PFS as the dependent variables was used to adjust for the effects of the miRNA expression, mortality predictor score, and IPI. P value of less than 0.05 was considered significant.

To merge the predictive power of the IPI, the 6-gene model–derived mortality predictor score, and miRNAs, we constructed a combined model integrating these prognostic variables. The weights for each variable were obtained from their independent contributions, as inferred from a Cox proportional hazards fit to the data, and this same model was used to estimate the 5-year survival. The survival curves were generated by using the coxph function in the R software package (http://www.r-project.org/). This function estimates the baseline by using the Breslow method. In addition, the resulting confidence intervals for survival at each time were smoothed by cubic splines.

Results

Selection of a panel of miRNAs for quantitative RT-PCR

We selected a group of 11 miRNAs for this study. We previously reported that 8 of these miRNAs (miR-21, miR-146a, miR-146b-5p, miR-155, miR-222, miR363, miR-500, and miR-574-3p) were capable of distinguishing GCB- and ABC-like DLBCL cell lines (18). Three additional miRNAs (miR-18a, miR-140-3p, and miR-181a) were also selected because their expression has been suggested to be highly (refs. 16, 18, 28–31).

Expression of miRNAs and survival of DLBCL patients

We measured the expression of each of the 11 miRNAs and the internal control (RNU6B) for input mRNA by quantitative RT-PCR in 176 specimens of DLBCL patients treated with R-CHOP. The expression of the genes LMO2, BCL6, FN1, CCND2, SCYA3, and BCL2 comprising the 6-gene model was also measured and the mortality predictor score was calculated as previously reported (7). Patients’ median age was 59 years (range, 16–92) and their disease characteristics, including the 5 clinical parameters that comprise the IPI, are shown in Table 1. The follow-up period ranged from 15 days to 8.1 years (overall median 2.6 years; 25th and 75th percentiles of 1.4 and 4.1 years, respectively); 41 patients (23%) died, whereas 54 had documented disease progression or relapse. The median follow-up of patients who were alive was 2.84 years, whereas the median follow-up for patients who died was 0.86 years. Figure 1A shows the OS and PFS curves of all the 176 patients. Both the IPI, segregating the patients into low clinical risk (IPI 0–2) and high clinical risk (IPI 3–5) groups, and the 6-gene model–derived mortality predictor score segregating the patients into low molecular risk (lower than the median) and the high molecular risk (higher than the median) groups, predicted OS and PFS (Fig. 1B and C). The mortality predictor score also predicted OS and PFS as a continuous variable (data not shown). In a multivariate Cox regression analysis that included the IPI scores and the mortality predictor score also predicted OS and PFS (Fig. 1B and C).

Expression of 3 (miR-18a, miR-181a, and miR-222) of the 11 analyzed miRNAs was individually associated with survival of DLBCL patients in a univariate analysis. Expression of miR-18a analyzed as a continuous variable was statistically correlated with OS (P = 0.038), and increased expression of this miRNA was associated with a shorter OS (Fig. 2A). Expression of miR-18a was not associated with PFS. Expression of miR-181a, analyzed as a continuous variable, was statistically correlated with PFS (P = 0.026) but not with OS, and increased expression of this miRNA

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number (%)</th>
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<tr>
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<tr>
<td>≤60</td>
<td>92 (52%)</td>
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<tr>
<td>&gt;60</td>
<td>84 (48%)</td>
</tr>
<tr>
<td>Stage</td>
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<td>Stage I</td>
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<tr>
<td>Stage II</td>
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<tr>
<td>Stage III</td>
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<td>Stage IV</td>
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<td>4</td>
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</tr>
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<tr>
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<td>4</td>
<td>17 (10%)</td>
</tr>
<tr>
<td>5</td>
<td>2 (1%)</td>
</tr>
</tbody>
</table>
was associated with longer PFS (Fig. 2B). Expression of miR-222, analyzed as a dichotomous variable (above and below median expression), was statistically correlated with PFS \( (P = 0.004) \), but not with OS. Higher expression of miR-222 was associated with shorter PFS (Fig. 2C).

We next examined whether the prognostic significance of these 3 miRNAs was independent of the IPI score and the mortality predictor score derived from the 6-gene model. A multivariate Cox regression analysis that included the IPI score, the mortality predictor score, and the expression of miR-18a and miR-181a as continuous variables and miR-222 as a dichotomous variable, with OS or PFS as the dependent variables, was performed. The IPI, the mortality predictor score (either as a categorical or continuous variable), and expression of miR-222 were independent predictors of PFS (Table 2). Since 2 of the 3 miRNAs predict outcome as continuous variables, we elected to present their independence from the IPI and the mortality predictor score in Table 2 and not to display this data graphically in each relatively small IPI cohort, as the latter would require a selection of cutoff values that may be considered as a preoptimization step that would impose unnecessary bias into the data. No significant correlations between the expression of miR-18a, miR-181a, and miR-222 and age, stage, performance status, LDH, and extranodal involvement were observed (data not shown).

Given that the IPI, the mortality predictor score, and the expression of the 3 miRNAs independently correlated with the IPI, the mortality predictor score (either as a categorical or continuous variable), and expression of miR-181a and miR-222 were independent predictors of PFS (Table 2). Since 2 of the 3 miRNAs predict outcome as continuous variables, we elected to present their independence from the IPI and the mortality predictor score in Table 2 and not to display this data graphically in each relatively small IPI cohort, as the latter would require a selection of cutoff values that may be considered as a preoptimization step that would impose unnecessary bias into the data. No significant correlations between the expression of miR-18a, miR-181a, and miR-222 and age, stage, performance status, LDH, and extranodal involvement were observed (data not shown).
miR-181a regulates the expression of FOXP1 and MGMT

Given that the association between DLBCL outcome and miRNA expression most probably results from miRNA regulation of specific genes expression, and miR-181a is associated with outcome in other cancers (11), we explored novel miR-181a targets by searching for putative binding sites in the 3′-UTR of genes with a potential role in DLBCL pathogenesis or prognosis. By using 3 different prediction algorithms, we found that putative binding sites for miR-181a are harbored in the 3′-UTR of FOXP1 and MGMT. FOXP1 encodes a transcription factor whose expression was associated with outcome in DLBCL patients in some of the previous reports (32–35). To test whether this gene is regulated by miR-181a, we transfected the precursor hsa-miR-181a into HBL1 and VAL DLBCL cell lines, which expresses endogenous FOXP1. Western blotting of whole-cell lysates showed a decrease of native FOXP1 in both HBL1 and VAL cells transfected with hsa-miR-181a (Fig. 4A), compared with control miRNA transfectants. Examination of effects of hsa-miR-181a precursor on FOXP1 mRNA revealed a decrease in expression at 24 hours after transfection in the HBL1 cells, but an increase in the VAL cells with little effect at 48 hours after transfection (Fig. 4B), which suggests that the regulation is mainly occurring at the level of protein translation. Transfection efficacy in each experiment was confirmed by measurements of appropriate miRNAs by TaqMan MicroRNA Assays (Fig. 4C). To confirm direct effects, we fused the 3′-UTR sequence of FOXP1, containing 3 miR-181a putative binding sites, to a luciferase reporter gene. By cotransfecting the hsa-miR-181a precursor or a control precursor with the 3′-UTR FOXP1 luciferase reporter, we showed that miR-181a significantly repressed luciferase activity, compared with a nontargeting control (Fig. 4D). To show the specificity of the interaction, we generated a panel of four 3′-UTR FOXP1 luciferase reporter constructs containing individual mutations of each of the 3 putative binding sites (MUT1, MUT2, and MUT3) and a construct with combined mutations of sites 1 and 2 (MUT1+2; Supplemental Table S2). The specific binding sites chosen for mutagenesis were selected on the basis of analysis of accessibility of each putative miRNA binding site with the PITA algorithm. Mutagenesis of the seeds of sites 1 and 2 produced complete restoration of luciferase activity (not statistically different from the nontargeting control). Taken together, these data support a direct effect of miR-181a on FOXP1 in DLBCL cell lines.

MGMT encodes an enzyme that protects cells from the toxicity of alkylating agents that frequently target the O6 position of guanine and is one of the major mechanisms of resistance to alkylating drugs (36, 37). To test whether this gene is also regulated by miR-181a, we transfected the precursor of hsa-miR-181a into VAL and Jurkat cell lines, which expresses endogenous MGMT. Although miR-181a decreased protein levels of MGMT in both cell lines compared with control miRNA transfectants, the effect was indirect as shown by the absence of significant change in the 3′-UTR MGMT luciferase reporter assay (Supplemental Fig. S1).
Discussion

The ability to accurately predict response to therapy and survival may be crucial for initial treatment planning in patients with non-Hodgkin lymphomas. Robust prognostic tools may allow stratification of treatment modalities avoiding exposure to unnecessary treatment toxicity or suboptimal therapy. Besides serving as a tool for risk stratification and enabling comparisons among clinical trials, prognostic markers also assist in further understanding tumor pathogenesis and may facilitate the development of specific therapeutic agents.

miRNAs have recently been shown to play a key role in tumorigenesis and since their initial description, it has been shown that their expression is closely associated with outcome in hematologic neoplasms such as CLL (9) and AML (10). Here, we show that the expression of miR-18a, miR-181a, and miR-222 is correlated with survival of DLBCL patients treated with the current gold standard therapy—R-CHOP. Furthermore, we show for the first time that the expression levels of specific miRNAs in DLBCL tumors is associated with survival and is independent of the currently used clinical prognostic index IPI and the 6-gene model previously shown to be robust predictors of outcome in multiple cohorts of DLBCL patients.

Previous studies suggested the association between expression of specific miRNAs and outcome of DLBCL patients (15, 16). However, these findings were based on analysis of small cohorts of DLBCL patients mostly treated in the pre-rituximab era. In these studies, the expression cutoffs for increased and decreased expression of the analyzed miRNAs were most likely preselected and optimized for the reported cohorts. Furthermore, many of the analyzed miRNAs showed relatively limited variability across DLBCL tumors in our preliminary studies (data not shown), suggesting that they would have limited ability to separate patients with different outcome and thus were not selected for the current analysis. Indeed, our data did not confirm the predictive power of miR-21 and miR-155 which expression was previously reported to predict outcome of DLBCL patients (16, 17). This finding shows the need for adequate cohort size necessary for sufficient statistical power to identify robust prognostic biomarkers, which must be validated in independent studies. Our findings on prognostic value of miR-18a, miR-181a, and miR-222 in DLBCL patients are based on a multi-institutional DLBCL cohort of sufficient size necessary for statistical power to identify robust prognostic biomarkers. However, these findings still need to be validated in large independent cohorts of DLBCL patients. Although it is a

## Table 2. Multivariate Cox regression analyses for OS and PFS, including IPI score, mortality predictor scores, and expression of miR-18a and miR-181a (continuous variables) and miR-222 (dichotomous variable)

<table>
<thead>
<tr>
<th>Variable</th>
<th>OS</th>
<th>PFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z score</td>
<td>Coefficient</td>
</tr>
<tr>
<td>IPI score</td>
<td>4.12</td>
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<tr>
<td>6-gene</td>
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</tr>
<tr>
<td>miR-18a</td>
<td>2.53</td>
<td>0.752</td>
</tr>
<tr>
<td>miR-181a</td>
<td>-2.88</td>
<td>-3.603</td>
</tr>
<tr>
<td>miR-222</td>
<td>1.06</td>
<td>0.363</td>
</tr>
</tbody>
</table>

Figure 3. Probability of 5-year OS (A) and PFS (B) based on combined IPI, 6-gene mortality predictor score, and miRNA expression score. Curved lines represent 95% CIs.
common practice to validate prognostic factors on the basis of gene expression data sets available in public domains, currently, there are no publicly available miRNAs data sets in DLBCL patients with known outcome. Validation of this work will likely take much more time as patients are currently accrued into our multi-institutional prospective study (NCT00450385) in which we plan to validate the predictive power of these miRNAs before suggesting their application in clinical practice. Once data from these studies become available, recommendations about their application in clinical practice can be put forward. However, in the mean time, publishing of our data will offer the opportunity for other groups to repeat and/or validate and expand on our findings.

Currently, quantitative measurement of miRNA expression is most commonly accomplished by RT-PCR or microarrays. Since in the current study, only 3 miRNAs were shown to be associated with DLBCL prognosis and because RT-PCR allows more precise measurement of miRNA expression across a wider range and is more suitable for

**Figure 4.** Effect of miR-181a on the expression of FOXP1. A, effect of the overexpression of hsa-miR-181a on native FOXP1 protein levels in HBL1 and VAL cell lines at 24 hours after transfection, assessed by Western blot. GAPDH levels were used as loading control. Data were confirmed in triplicate experiments. B, effect of the overexpression of hsa-miR-181a on the mRNA levels of FOXP1 measured by RT-PCR by using TaqMan Gene Expression Assays (Applied Biosystems) at 24 and 48 hours after transfection. Values of triplicate wells are represented as fold expression with respect to the nontargeting control transfection. C, overexpression of hsa-miR-181a was confirmed by TaqMan MicroRNA Assays, expressed as fold increase about the control transfection. D, dual luciferase activity of reporter plasmids with the wild type or mutated 3'-UTR of FOXP1 fused to the luciferase gene following hsa-miR-181a precursor cotransfection in HeLa cells. □ represents cotransfections with the corresponding miRNA precursor, □ represents cotransfection of the same reporter vector with the nontargeting control. Values are normalized to the value of each control, which is noted as 100%. Mutation of putative binding sites is expressed as MUT1 for the most 5' site, MUT2 for the middle site, MUT3 for the most 3' site, and MUT1 + 2 for the combined mutation of sites 1 and 2. Statistical comparisons by 2-tailed t test with Bonferroni correction between different constructs are represented as -- Statistical comparisons between the cotransfected miRNA and the nontargeting control for the same reporter vector are noted over the black bars. Significant differences with associated P values less than 0.05 are expressed as * and nonsignificant difference as ns. Error bars correspond to SEM in all graphs.
continuous variables, we envision that in the future, RT-PCR will be adopted as the preferred methodology for clinical application. Although recent studies suggested that in situ analysis of miRNA expression can be done (38), this methodology may not be sufficiently quantitative for continuous variables and may not allow prediction of outcome by using the combined model integrating IPI, 6-gene mortality predictor score and the expression of the 3 miRNAs (Fig. 3).

In contrast to genes, some of which are specifically and exclusively expressed in GCB cells and tumors derived from them, the currently known miRNAs do not show exclusive expression patterns and are present in both malignant B-lymphocytes and nonmalignant cells comprising the tumor microenvironment. For example, miR-21, miR-155, miR-222, miR146a, and miR146b-5p are expressed at similar or higher levels in T cells in comparison with DLBCL cell lines (Supplemental Fig. S2). Consequently, the GCB- and ABC-like DLBCL subtypes defined by the previously reported miRNA expression signature (18) did not show differences in survival, although this cell of origin-based miRNA classification was derived from miRNA expression in tumor cells only and excluded nonmalignant cell types present in whole tumors that also contribute to the measured expression patterns of miRNAs. Future studies examining expression levels by in situ hybridization or in paired whole tumors and purified tumor B cells and nontumor infiltrating cells will help establish the cellular source of specific miRNAs.

We showed that increased expression of miR-18a was associated with inferior OS of DLBCL patients. miR-18a is a component of the miR-17-92 cluster, which is located at 13q31-q32 DNA region that is frequently amplified in human B-cell lymphomas (29). The components of this cluster are expressed at higher levels in normal GCB cells than naive and memory B cells (18). In addition, the region encoding this cluster is more commonly amplified in GCB-like than ABC-like DLBCL (28). He and colleagues initially suggested an association between increased expression of miRNAs comprising the miR-17-92 cluster and lymphoma (29). Accordingly, enforced expression of the miR-17-92 cluster, together with c-MYC in mice, accelerated the development of B-cell lymphomas and leukemias (29). Over-expression of the c-MYC mRNA, together with the miR-17-5p/miR-20a from this cluster, was associated with a more aggressive behavior in mantle cell lymphoma (39). Our findings of decreased OS in DLBCL patients, whose tumors express increased levels of miR-18a, are in agreement with these data.

Increased expression of miR-181a was associated with improved PFS in DLBCL patients. In cytogenetically normal AML, higher miR-181a expression was associated with a higher complete remission rate, longer OS, and longer disease-free survival (11). In contrast in CLL, high levels of miR-181a were associated with a shorter time from diagnosis to initial therapy (9). In this context, miR-181a may function as a tumor suppressor. miR-181 was implicated to have an antioncogenic role in CLL by downregulating TCL1, a known oncogene in T-CLL and B-CLL that is particularly overexpressed in the most aggressive subtypes of CLL (40). It was also reported that miR-181a can repress the expression of BCL-2 (30), a known prognostic factor in DLBCL and a component of the 6-gene model. However, in a multivariate analysis, both the miR-181a and the 6-gene model were independent predictors of outcome, suggesting that miR-181a regulates additional targets that influence patient outcome. Herein, we have shown that miR-181a directly downregulates the expression of FOXP1.

FOXP1 is a member of the FOXP subfamily (FOXP1–4) of transcription factors, characterized by a common DNA binding, winged-helix, or forkhead domain, together with N-terminal zinc finger and leucine zipper domains. The FOXP1 gene was mapped to chromosome 3p14.1, a locus that shows frequent loss of heterozygosity in solid tumors (41); both FOXP1 mRNA and protein expression are commonly affected in a range of solid tumors (42). FOXP1 was reported to be expressed in normal ABCs, mantle zone B cells, and some GCB cells (42, 43); however, the physiologic role of FOXP1 in normal lymphocytes is unclear. FOXP1 is recurrently targeted by chromosome translocations involving immunoglobulin heavy chain locus in marginal zone lymphomas and DLBCL, suggesting a potential role for FOXP1 in lymphomagenesis (44, 45). Previous studies showed more common expression of FOXP1 in a subset of non-GCB-like than GCB-like DLBCLs (32–35). Furthermore, some but not all previous studies showed association between FOXP1 expression and poor prognosis and survival (32, 33, 35). Downregulation of FOXP1 expression by miR-181a may at least partially explain the association between miR-181 and improved survival of DLBCL patients; however, like other miRNAs, miR-181a regulates multiple genes, some of which may also contribute to better prognosis associated with miR-181a expression. Evaluation of additional miR-181a targets with potential role in DLBCL pathogenesis is currently in progress in our laboratory.

Increased expression of miR-222 was associated with shorter PFS, thus confirming our previous observation in an extended cohort of patients (18). miR-222 is part of the miR-221/miR-222 cluster, which is highly expressed in ABC-like DLBCL cell lines (18) and ABC-like DLBCL tumors (16), but its specific role in DLBCL pathogenesis is unknown. miR-222 was reported to regulate the expression of the stem cell factor c-kit (46) and cyclin-dependent kinase inhibitors p27 and p57 (47, 48). The miR-222–induced downregulation of p27 and p57 may facilitate cell proliferation and survival (47, 48), particularly in pancreatic carcinomas (48) and melanomas (49) that were reported to express miR-222. Shorter PFS of DLBCL patients whose tumors express high levels of miR-222 may reflect the cellular origin of these tumors or specific biological effects of this miRNA. Further investigations are needed to address specific mechanisms associated with miR222 function.

The observation that miR-18a predicts OS while miR-181a and miR-222 are associated with PFS suggests different
biological effects on DLBCL cells. Association between miR-18a and shorter OS suggests that this miRNA predicts poor response to either upfront and salvage therapies, most likely by affecting biological aggressiveness of DLBCL tumors. In contrast, exclusive association between expression of miR-181a and miR-222 with PFS but not OS implies an effect on response to upfront R-CHOP but not salvage therapies, which can rescue these patients leading to similar OS. Indeed, our findings suggest that miR-181a indirectly decreases the expression of MGMT protein potentially contributing to better cyclophosphamide chemosensitivity and longer PFS. Further studies evaluating biological effects of these miRNAs are needed and are in progress.

The statistically significant association between the expression of specific miRNAs and either PFS or OS of DLBCL patients suggests that further studies to elucidate their role in lymphomagenesis are required. The observation that some miRNAs predict outcome as dichotomous variables while others as continuous variables may reflect the relatively small sample size or represent different biological effects of these miRNAs. The association of outcome with miRNA expression as a continuous variable suggests that even small changes in their expression may have profound effects on the expression of their targets, and implies a tightly regulated process. In contrast, dichotomous variables may imply an "all or nothing" modulation in which a certain threshold of miRNA expression must be reached for miRNA modulation. Since dichotomous division was based on median expression, it is possible that nonmedian classification could result in DLBCL subgroups with statistically significant outcome, but the latter approach would require independent confirmation in a separate cohort of patients, and thus was not done in the current study. Further studies are warranted to investigate these variations, to validate the prognostic impact of individual miRNAs in independent cohorts of DLBCL patients (as was done previously with the 6-gene model) and to elucidate their role in B-cell biology and lymphomagenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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