Citation: Blood Cancer Journal (2012) 2, e57; doi:10.1038/bcj.2012.1

www.nature.com/bcj

ORIGINAL ARTICLE

MicroRNA signatures in B-cell lymphomas

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Accurate lymphoma diagnosis, prognosis and therapy still require additional markers. We explore the potential relevance of microRNA (miRNA) expression in a large series that included all major B-cell non-Hodgkin lymphoma (NHL) types. The data generated were also used to identify miRNAs differentially expressed in Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL) samples. A series of 147 NHL samples and 15 controls were hybridized on a human miRNA one-color platform containing probes for 470 human miRNAs. Each lymphoma type was compared against the entire set of NHLs. BL was also directly compared with DLBCL, and 43 preselected miRNAs were analyzed in a new series of routinely processed samples of 28 BLs and 43 DLBCLs using quantitative reverse transcription-polymerase chain reaction. A signature of 128 miRNAs enabled the characterization of lymphoma neoplasms, reflecting the lymphoma type, cell of origin and/or discrete oncogene alterations. Comparative analysis of BL and DLBCL yielded 19 differentially expressed miRNAs, which were confirmed in a second confirmation series of 71 paraffin-embedded samples. The set of differentially expressed miRNAs found here expands the range of potential diagnostic markers for lymphoma diagnosis, especially when differential diagnosis of BL and DLBCL is required.

Blood Cancer Journal (2012) **2,** e57; doi:10.1038/bcj.2012.1; published online 17 February 2012

Keywords: microRNA; B-cell lymphoma; microarray; miRNA expression profile; lymphoma diagnosis

INTRODUCTION

B-cell non-Hodgkin lymphomas (NHLs) are a group of lymphoproliferative B-cell disorders that include Burkitt lymphoma (BL), chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), follicular lymphoma (FL), marginal zone lymphoma/mucosa-associated lymphoid tissue lymphoma (MZL/MALT), nodal marginal zone B-cell lymphoma (NMZL), splenic marginal zone lymphoma (SMZL) and various less frequent entities.[1] These definitions are based on a combination of clinical data and morphological, phenotypic and cytogenetic features. The use of surrogate markers for gene and chromosomal alterations specific to the different B-cell lymphoma types[1] is also useful at diagnosis.

Nevertheless, the distinctions between these disorders are somewhat blurred and most exhibit significant clinical and molecular heterogeneity.[1,2] New, consistent markers continue to be required to improve the accuracy of lymphoma diagnosis and therapy selection. An area of particular interest is the interface between BL and DLBCL,[3,4] two different lymphoma types that require different treatment. Previous studies have demonstrated the value of *C-MYC* translocations[3] and gene expression profiling data[4] for this purpose, but new markers are needed to delineate the boundaries between these two entities and to use this knowledge to identify diagnostic markers.

In recent years, the study of a new type of non-coding small RNA, microRNA (miRNA), has given renewed impetus to cell differentiation and cancer pathogenesis studies.[5] MiRNAs post-transcriptionally regulate the expression of thousands of genes, including key genes in cell differentiation and cancer pathogenesis.[6] Since evidence of the relationship between miRNAs and cancer first emerged, with the description of the loss of miR-15/16 in CLL cases,[7] an increasing number of specific miRNA changes have been identified in many tumor types.[5] The diagnostic potential of miRNAs is linked to their role in cellular differentiation, as demonstrated in hematopoietic cells and, for instance, by miR-150, in B-cell differentiation.[8] The potential of using miRNAs for differential diagnosis of tumors and hematopoietic malignancies has been recognized, for example, in acute lymphoblastic and myeloid leukemias, where the expression signatures of at least two of four miRNAs (miR-128a, miR-128b, miR-223 and let-7b) distinguished the two tumor types with about 95% accuracy.[9] Altered miRNA expression has a role in lymphoma development and potential diagnosis. MiRNA changes are associated with CLL (loss of miR-15a/16)[7] and SMZL (loss of miR-29a and miR-29b-1),[10] among others, and in both cases these miRNAs were located in frequently lost chromosomal regions: 13q14 and 7q32, respectively. There are differential signatures between the expression signature of patients with different lymphoma types, such as DLBCL and FL.[11] Furthermore, in DLBCL, miRNAs are differentially expressed in the germinal center (GC) and activated B-cell-like (ABC) subtypes; in particular, miR-21, miR-155 and miR-221 were found to be upregulated in the ABC subgroup.[12,13]

The purpose of this study is to test whether different B-cell lymphoma types have specific miRNA signatures, and to apply this knowledge to identify potential markers for distinguishing BL and DLBCL. In addition, the markers described here could be therapeutic targets in the treatment of B-cell lymphomas.

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Received 21 October 2011; revised 11 January 2012; accepted 13 January 2012

MATERIALS AND METHODS

Sample selection

A series of 147 fresh–frozen samples of NHL, including 12 BL, 29 DLBCL, 22 MCL, 17 SMZL, 18 CLL, 23 FL, 11 NMZL and 15 MZL/MALT, and 15 non-tumoral samples (7 reactive lymph nodes, 4 tonsils and 4 spleens) were collected. A formalin-fixed, paraffin-embedded (FFPE) series of 28 sporadic BL and 43 DLBCL samples were also investigated. Finally, an independent series of FFPE samples corresponding to 66 lymphomas (8 BL, 8 CLL, 12 DLBCL, 9 FL, 8 MZL/MALT, 8 MCL, 8 NMZL and 5 SMZL) and 8 controls (4 reactive lymph nodes, 3 tonsils and 1 spleen) was examined by quantitative real-time-polymerase chain reaction (qRT-PCR) for miRNA validation. CLL samples correspond to lymph node-involved CLL cases to facilitate comparison with the other lymphoma types. All cases were reviewed by a panel of three hematopathologists (SMRP, MAP, SMM) according to the current World Health Organization criteria.[1] All samples were diagnostic, taken before the patient received therapy. The project was approved by the ethics committee of the Instituto de Salud Carlos III (ISCIII). The majority of the BL cases (11/12 frozen cases; 20/28 FFPE cases) presented *C-MYC* translocation by FISH, whereas 31.7% (13/41) of DLBCL FFPE cases were positive for *C-MYC* translocation. On the other hand, 60% (15/25) and 40% (10/25) of DLBCL frozen samples were classified as ABC and GC type, respectively, according to the classifier of Wright *et al.*[14] based on gene expression profiling data, with four cases considered as not evaluable. FFPE samples of these cases permitted classification in 44% non-GC (11/25) and 56% GC (14/25)[15] based on Hans immunohistochemical classifier.[16] Finally, FFPE series of DLBCL used for qRT-PCR were classified as 54.76% non-GC (23/42) and 45.24% GC (19/42) (one case was not evaluable).

MiRNA detection

RNA from fresh–frozen tissues was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Quality was assessed using a bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA), and 100 ng of total RNA were hybridized on an Agilent 8 × 15K Human miRNA one-color platform (Agilent Technologies Inc.) containing probes for 470 human miRNAs according to the manufacturer's guidelines.[17] Data were extracted by Feature Extraction software (Agilent Technologies).

For FFPE samples, RNA was extracted by phenol–chloroform standard protocol after a deparaffinization step. RNA quality was assessed taking into account 260/280 ratio and 260/230 ratio. QRT-PCR was performed for 60 selected miRNAs according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA).

Statistical methods

Between-array median normalization was carried out and significantly deregulated miRNAs were computed using the Significant Analysis of Microarray (SAM; http://www-stat.stanford.edu/~tibs/SAM/) method.

Each lymphoma type was compared against the whole set of samples.[18] BL was also directly compared with DLBCL. MiRNAs with a false discovery rate (FDR) <0.01 and a >1.5-fold ($\log_2$) change were considered to be significantly up- or downregulated between lymphoma types.

MiRNA target prediction was performed using the software analysis tools included into the following websites: http://gencomp.bio.unipd.it/magia/start (Magia), http://www.targetscan.org (targetscan) and http://diana.cslab.ece.ntua.gr/pathways (Diana Lab).

The K-nearest neighbors method was also used for class recognition (http://tnasas.bioinfo.cnio.es) to test whether these lymphomas were classified correctly with the selected miRNAs.

Quantitative RT-PCR data were processed using the SDS 2.2 and Real-Time StatMiner (Integromics, Granada, Spain) programs, and normalized according to the most stable endogenous small RNA tested (RNU6B). $-\Delta$Ct values of BL and DLBCL were compared using a *t*-test (limma; available at http://pomelo2.bioinfo.cnio.es). MiRNAs with an FDR <0.05 were considered differentially expressed in the two groups. All clusters were computed using the web resource GEPAS 4.0 (www.gepas.es). The workflow is shown in Figure 1.

RESULTS

MiRNA signature of B-cell NHLs

MiRNA expression profiles of 147 NHL samples were studied. A general view of the B-cell lymphoma miRNA signature is shown in Supplementary Figure S1 comparing miRNA expression in tumor samples with non-tumoral controls (tonsils and lymph

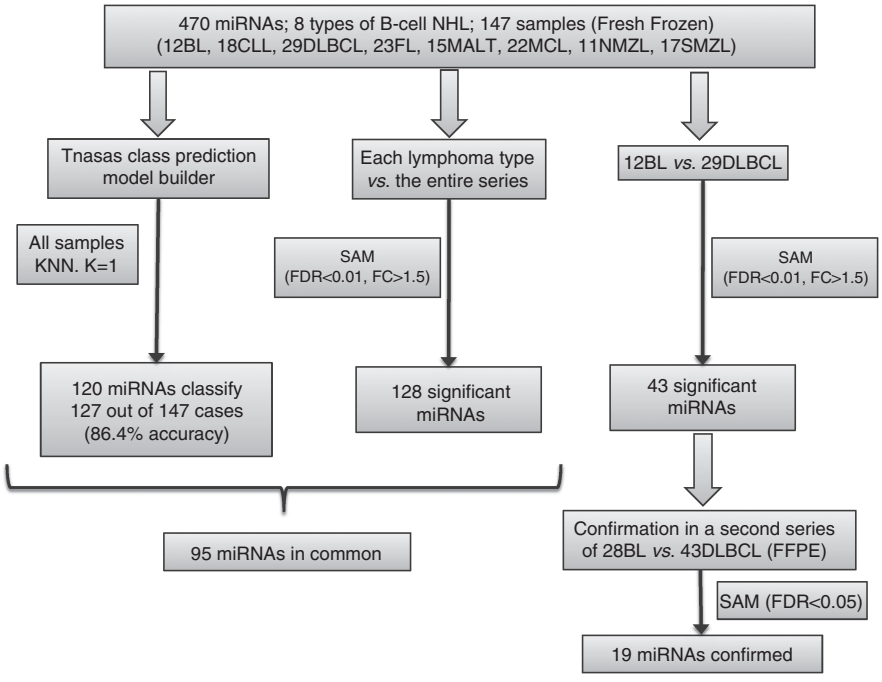

Figure 1. Workflow. The samples were analyzed first by SAM, comparing each lymphoma type against the whole series. In parallel, a class predictor model was investigated for the 147 samples. Also, BL and DLBCL samples were compared by SAM, and then significant miRNAs were analyzed in a second series of BL and DLBCL samples.

nodes or spleens). The heatmap reveals a fairly homogeneous expression pattern with a larger set of downregulated miRNAs in tumor cells. The upregulated miRNAs have a more heterogeneous pattern that varies with the lymphoma diagnosis.

The most strongly upregulated miRNAs compared with non-tumoral controls were miR-212, miR-487b, miR-513 and miR-770-5p, a set of miRNAs about which relatively little is known. On the other hand, downregulated miRNAs were more frequently observed. There was notable downregulation of let-7 family miRNAs, which are downregulated in various types of cancer, and key regulators of cell differentiation and apoptosis.[19] Interestingly, let-7a and let-7c loss participates in the genesis and maintenance of the lymphoma phenotype in BL cells through C-MYC regulation.[20] On the other hand, miR-23b was reported to be downregulated by C-MYC, playing a role in the MYC regulation of glutamine metabolism, and energy and reactive oxygen species homeostasis.[21] The miR-200 family is another large family downregulated in this B-cell lymphoma series. MiR-200 has been described, along with the let-7 family, as a key regulator of cell differentiation, whose loss is associated with increased stemness capacity.[19] MiR-10 (a and b) was also downregulated. This family is situated within the Hox cluster, and is also downregulated in myeloproliferative disorders.[22] MiR-15 and miR-16, already described to be downregulated in CLL,[7] were found to be downregulated in the CLL cases in this study. MiR-155, which is involved in the immune response and GC development, and is upregulated in Hodgkin lymphoma,[23] was found downregulated here in the BL cases.

A selection of 14 deregulated miRNAs (9 downregulated and 5 upregulated) was analyzed by qRT-PCR to validate the microarray data further. For this purpose, an independent series containing 66 lymphoma samples (8 CLL, 8BL, 12 DLBCL, 9 FL, 8 MZL/MALT, 8MCL, 8NMZL and 5 SMZL) and 8 controls (4 lymph nodes, 3 tonsils and 1 spleen) was used.

The miRNA expression observed by microarrays was confirmed by qRT-PCR (see Supplementary Figure S4). Nevertheless, miR-487b, miR-212 and miR-770-5p presented a slight variability among the different lymphoma types, especially SMZL and MZL/MALT. This variability can be explained by the different localization of SMZL lymphomas and NMZL, as SMZLs are localized in the spleen and MZL/MALT lymphomas are localized in a variety of different tissues (in this series: breast, eye, skin, salivary gland and intestine).

To select a lymphoma miRNA signature, data from all tumor samples, without previous normalization to controls, were studied. SAM analysis enabled comparison of each lymphoma type with the entire set of samples. A set of 128 miRNAs was considered to be significantly deregulated (FDR <0.01; >1.5-fold change in $\log_2$) in one or more lymphoma types (Table 1). The heatmap of the 128 significant miRNAs is shown in Figure 2.

MiRNA capacity for class recognition was tested with the K-nearest neighbors algorithm. Thus, 120 out of 470 miRNAs were identified that classified the eight subclasses of lymphomas (correct classification rate: 86.4%) (Supplementary Table S1). In all, 95 of these miRNAs coincide with those identified as significantly differentially expressed by SAM analysis (in blue in Table 1).

The miRNA signatures found by SAM analysis for each lymphoma type are described in more detail below.

Burkitt lymphoma. In all, 12 BL cases were analyzed. The majority of them (11/12) showed *C-MYC* translocation. A total of 35 (14 upregulated, 21 downregulated) miRNAs were deregulated in BL compared with the other NHLs. Interestingly, members of the miR-17–92 cluster (miR-17-3p, miR-18a, mir-19a, miR-19b and miR-92) were upregulated in BL. Among the downregulated miRNAs, we found the let-7 family miRNAs that are commonly lost in different neoplasias,[20] miR-155, miR-146a and others already described as lost in BL[24] and the miR-29 family (a, b and c), which regulates p53.[25]

Chronic lymphocytic leukemia. In all, 14 miRNAs were upregulated and 46 downregulated in CLL samples. MiR-197, which regulates the tumor suppressor gene FUS1,[26] was the most highly expressed miRNA in these samples. MiR-595 and miR-483 were also upregulated, as has been noted in Wilm's tumor.[27] Among the downregulated miRNAs, we found miR-15a and miR-16, already described in CLL, where the loss of the 13q14 minimally deleted region induces a clonal lymphoproliferative disorder that recapitulates the spectrum of CLL-associated phenotypes observed in humans.[7] Other downregulated miRNAs were miR-182, miR-199a*(5p), the let-7 family, miR-424, miR-10a, miR-7, mir-126 and miR-218, whose loss of expression is related with the activation of multiple survival pathways in various cancer models.[28]

Diffuse large B-cell lymphoma. No significant differential miRNA expression was found in this type of lymphoma by SAM analysis. This could be due to the intrinsic heterogeneity of DLBCL cases as an entity, which probably dilutes the miRNA expression differences with other types of lymphomas. This heterogeneity is reflected by the Tnasas web resource class predictor analysis results, where the DLBCL group has the highest error rate (27%; Supplementary Table S1). Indeed, gene expression profiling data in the series of DLBCL confirm this heterogeneity, revealing that 60 and 40% of the cases are ABC type and GC type, respectively, according to the classifier of Wright *et al*.[14] two DLBCL subtypes with specific miRNA signatures categories.[13,29]

Follicular lymphoma. Two significantly upregulated miRNAs, miR-138 and miR-9 (5p and 3p), were found. Increased expression of miR-9 was previously described in FL samples.[11] MiR-9, which is activated by MYC, has been shown to regulate nuclear factor-κB.[30] Overexpression of miR-9 or let-7a reduces PRDM1/BLIMP1 levels,[31] a finding of potential interest in FL cases, characterized by the tightly regulated expression of BCL6 and PRDM1/BLIMP1.

Marginal zone lymphoma/MALT. Three out of eight miRNAs significantly upregulated in MALT lymphoma cases studied belong to the miR-200 family (miR-200a, b and c). These miRNAs are components of two clusters, miR-200a/200b/429 on chromosome 1p36.33 and miR-200c/141 on chromosome 12p13.31. The other miRNAs located in these clusters, miR-429 and miR-141, were also upregulated. The miR-200 family inhibits the initiating step of metastasis, the epithelial–mesenchymal transition (EMT), by maintaining the epithelial phenotype through directly targeting the transcriptional repressors of E-cadherin, ZEB1 and ZEB2.[32] The only miRNA in MALT lymphoma cases downregulated relative to the other types was miR-126* (corresponding to miR-126-5p), an miRNA whose expression distinguishes acute myeloid leukemia cases with common translocations.[33]

Mantle cell lymphoma. Only miR-126* was downregulated, as in the case of MALT. Conversely, there were eight upregulated miRNAs, for example, miR-182 and miR-183, which are upregulated in colorectal cancer,[34] and miR-200c, which was also upregulated in MALT lymphoma cases. Downregulation of miR-126* and upregulation of miR-181c, miR-182, miR-363, miR-654 and miR-768-5p were also found in our previous study after comparing MCL cases with non-tumoral controls (tonsils and lymph nodes). In the same study, miR-182 was also upregulated in MCL cell lines compared with mantle cell controls.[17]

Nodal marginal zone lymphoma. Two miRNAs, miR-370 and miR-513, were downregulated in NMZL cases. MiR-370 has been shown to be downregulated in gastrointestinal stromal tumors with 14q loss[35] and upregulated in acute myeloid leukemia patients with t(15;17).[36] In all, 61 miRNAs were upregulated relative to the whole series. The most highly expressed miRNA in this series was miR-150, followed by miR-26b. MiR-150 regulates the expression of the transcription factor c-myb, and plays a key role in B-cell

Table 1. Differentially expressed miRNAs in each type of lymphoma compared with the rest of samples

	miRNA	BL	CLL	DLBCL	FL	MALT	MCL	NMZL	SMZL
1	hsa-let-7a	-1.54	-2.24	-	-	-	-	2.23	-
2	hsa-let-7c	-	-	-	-	-	-	1.86	-
3	hsa-let-7d	-1.53	-2.16	-	-	-	-	1.87	-
4	hsa-let-7e	-1.70	-2.07	-	-	-	-	1.84	-
5	hsa-let-7f	-1.67	-2.70	-	-	-	-	2.48	-
6	hsa-let-7g	-1.90	-2.01	-	-	-	-	2.55	-
7	hsa-let-7i	-	-	-	-	-	-	1.63	-
8	hsa-miR-1	-	-	-	-	-	-	1.96	-
9	hsa-miR-100	-	-2.02	-	-	-	-	2.07	-
10	hsa-miR-106a	-	-1.53	-	-	-	-	-	-
11	hsa-miR-107	-	-1.53	-	-	-	-	-	-
12	hsa-miR-10a	-1.62	-2.58	-	-	-	-	1.91	-
13	hsa-miR-10b	-1.67	-2.11	-	-	-	-	2.60	-
14	hsa-miR-125a	-	-1.68	-	-	-	-	1.69	-
15	hsa-miR-125b	-	-	-	-	-	-	1.82	-
16	hsa-miR-126	-	-2.27	-	-	-	-	2.33	-
17	hsa-miR-126*	-	-1.51	-	-	-1.58	-2.26	2.53	1.79
18	hsa-miR-127	-	-	-	-	-	-	-	1.56
19	hsa-miR-128b	-	-	-	-	-	-	1.52	-
20	hsa-miR-130b	1.61	-	-	-	-	-	-	-
21	hsa-miR-133a	-	-	-	-	-	-	1.59	-
22	hsa-miR-133b	-	-	-	-	-	-	1.55	-
23	hsa-miR-136	-	-	-	-	-	-	-	2.00
24	hsa-miR-138	-	-	-	2.07	-	-	-	-
25	hsa-miR-139	-	-	-	-	-	-	1.62	2.94
26	hsa-miR-141	-	-	-	-	1.54	-	-	-1.83
27	hsa-miR-143	-	-1.75	-	-	-	-	-	-
28	hsa-miR-144	-	-	-	-	-	-	-	3.22
29	hsa-miR-146a	-1.79	-1.67	-	-	-	-	-	-
30	hsa-miR-146b	-	-	-	-	-	-	1.74	-
31	hsa-miR-148a	-	-1.56	-	-	1.51	-	-	-
32	hsa-miR-148b	-	-	-	-	-	-	1.64	-
33	hsa-miR-150	-2.98	-	-	-	-	-	2.86	-
34	hsa-miR-152	-	-	-	-	-	-	1.57	-
35	hsa-miR-155	-2.12	-	-	-	-	-	1.59	-
36	hsa-miR-15b	-	-1.80	-	-	-	-	1.84	-
37	hsa-miR-16	-	-1.76	-	-	-	-	1.88	-
38	hsa-miR-17-3p	1.66	-	-	-	-	-	-	-
39	hsa-miR-17-5p	-	-1.55	-	-	-	-	-	-
40	hsa-miR-181c	-	-	-	-	-	1.60	-	-
41	hsa-miR-182	-	-2.90	-	-	-	1.71	-	-
42	hsa-miR-183	-	-	-	-	-	1.61	-	-
43	hsa-miR-18a	1.90	-	-	-	-	-	-	-
44	hsa-miR-18a*	1.66	-	-	-	-	-	-	-
45	hsa-miR-191	-	-	-	-	-	-	1.70	-
46	hsa-miR-192	-	-1.55	-	-	-	-	2.00	-
47	hsa-miR-193b	-	-1.96	-	-	-	-	-	-
48	hsa-miR-195	-1.69	-2.00	-	-	-	-	1.86	-
49	hsa-miR-197	1.81	2.93	-	-	-	-	-	-
50	hsa-miR-199a*	-	-2.81	-	-	-	-	2.19	-
51	hsa-miR-199b	-	-2.55	-	-	1.69	-	1.85	-2.12
52	hsa-miR-19a	2.05	-	-	-	-	-	-	-
53	hsa-miR-19b	1.69	-	-	-	-	-	-	-
54	hsa-miR-200a	-	-	-	-	2.66	-	1.55	-
55	hsa-miR-200b	-	-	-	-	2.68	-	1.91	-
56	hsa-miR-200c	-	-	-	-	2.02	1.50	-	-1.64
57	hsa-miR-203	-	-	-	-	1.75	-	-	-
58	hsa-miR-204	-	-	-	-	-	-	-	2.02
59	hsa-miR-206	-	1.81	-	-	-	-	-	-
60	hsa-miR-20a	-	-2.12	-	-	-	-	-	-
61	hsa-miR-20b	-	-2.08	-	-	-	-	-	-
62	hsa-miR-210	-	-	-	-	-	-	-	-1.90
63	hsa-miR-212	-	-	-	-	-	-	-	1.53
64	hsa-miR-215	-	-	-	-	-	-	1.85	-
65	hsa-miR-218	-	-2.63	-	-	-	-	1.64	-
66	hsa-miR-221	-	-	-	-	-	-	1.63	-
67	hsa-miR-223	-	-1.62	-	-	-	-	2.19	-
68	hsa-miR-224	-	-1.77	-	-	-	-	1.55	-
69	hsa-miR-26a	-1.54	-	-	-	-	-	2.08	-
70	hsa-miR-26b	-1.92	-2.11	-	-	-	-	2.81	-
71	hsa-miR-29a	-1.64	-	-	-	-	-	1.89	-
72	hsa-miR-29b	-1.74	-	-	-	-	-	1.63	-
73	hsa-miR-29c	-2.72	-	-	-	-	-	-	-
74	hsa-miR-30b	-	-	-	-	-	-	1.66	-
75	hsa-miR-30e-3p	-	-	-	-	-	-	1.95	-
76	hsa-miR-30e-5p	-1.82	-	-	-	-	-	1.69	-
77	hsa-miR-31	-	-	-	-	-	-	1.83	-
78	hsa-miR-328	-	1.51	-	-	-	-	-	-
79	hsa-miR-335	-	-	-	-	-	-	1.56	-
80	hsa-miR-338	-	-1.67	-	-	-	1.71	-	-
81	hsa-miR-340	-	-	-	-	-	-	1.54	-
82	hsa-miR-34a	-	-1.54	-	-	-	-	-	-
83	hsa-miR-34b	-	-	-	-	-	-	1.50	-
84	hsa-miR-363	-2.12	-2.59	-	-	-	3.26	-	-
85	hsa-miR-365	-	-1.79	-	-	-	-	2.11	-
86	hsa-miR-370	-	1.89	-	-	-	-	-1.83	-
87	hsa-miR-373*	1.73	-	-	-	-	-	-	-
88	hsa-miR-374	-	-2.14	-	-	-	-	2.18	-
89	hsa-miR-377	-	-1.62	-	-	-	-	-	-
90	hsa-miR-409-3p	-	-	-	-	-	-	-	2.11
91	hsa-miR-421	-	-	-	-	-	-	-	1.99
92	hsa-miR-424	-	-2.68	-	-	-	-	-	-
93	hsa-miR-429	-	-	-	-	2.16	-	-	-
94	hsa-miR-432	-	1.97	-	-	-	-	-	1.89
95	hsa-miR-451	-1.71	-2.18	-	-	-	-	2.06	4.06
96	hsa-miR-453	-	1.55	-	-	-	-	-	-
97	hsa-miR-454-3p	-	-1.54	-	-	-	-	1.65	-
98	hsa-miR-455	-	-	-	-	-	-	1.76	-
99	hsa-miR-483	-	2.29	-	-	-	-	-	-
100	hsa-miR-485-3p	-	2.39	-	-	-	-	-	-
101	hsa-miR-486	-	-	-	-	-	-	-	1.56
102	hsa-miR-487a	-	-	-	-	-	-	-	1.71
103	hsa-miR-487b	-	-	-	-	-	-	-	1.99
104	hsa-miR-513	-	-	-	-	-	-	-1.83	-
105	hsa-miR-520d	-	2.00	-	-	-	-	-	-
106	hsa-miR-520d*	-	-	-	-	-	-	-	1.77
107	hsa-miR-542-3p	-	-	-	-	-	-	-	1.89
108	hsa-miR-557	1.66	-	-	-	-	-	-	-
109	hsa-miR-560	2.02	-	-	-	-	-	-	-
110	hsa-miR-574	1.76	2.83	-	-	-	-	-	1.53
111	hsa-miR-595	-	2.59	-	-	-	-	-	1.95
112	hsa-miR-609	-	1.51	-	-	-	-	-	-
113	hsa-miR-625	-	-2.82	-	-	-	-	2.52	-
114	hsa-miR-629	2.51	-	-	-	-	-	-	-
115	hsa-miR-647	-	1.53	-	-	-	-	-	-
116	hsa-miR-650	-	-	-	-	-	-	-	2.05
117	hsa-miR-654	-	-	-	-	-	1.67	-	-
118	hsa-miR-663	3.32	-	-	-	-	-	-	-1.62
119	hsa-miR-7	-	-2.40	-	-	-	-	-	-
120	hsa-miR-766	-	1.58	-	-	-	-	-	-
121	hsa-miR-768-3p	-1.71	-	-	-	-	-	1.95	-
122	hsa-miR-768-5p	-1.77	-	-	-	-	2.00	-	-
123	hsa-miR-9	-	-2.00	-	1.67	-	-	-	-
124	hsa-miR-9*	-	-2.69	-	1.87	-	-	-	-
125	hsa-miR-92	1.52	-	-	-	-	-	-	-
126	hsa-miR-95	-	-2.05	-	-	-	-	2.22	2.64
127	hsa-miR-98	-	-2.24	-	-	-	-	2.09	-
128	hsa-miR-99a	-	-	-	-	-	-	2.56	-2.19

Abbreviations: BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FC, fold change; FDR, false discovery rate; FL, follicular lymphoma; KNN, K-nearest neighbors; MALT, mucosa-associated lymphoid tissue lymphoma; MCL, mantle cell lymphoma; miRNA, microRNA; NMZL, nodal marginal zone B-cell lymphoma; SMZL, splenic marginal zone lymphoma. MiRNAs differentially expressed in the different non-Hodgkin lymphoma types. FC in $\log_2$ is listed for all miRNAs with FDR <0.01 and FC >1.5. Numbers in red and green refer to upregulated and downregulated miRNAs, respectively. MiRNAs in blue are those also identified by KNN class prediction.

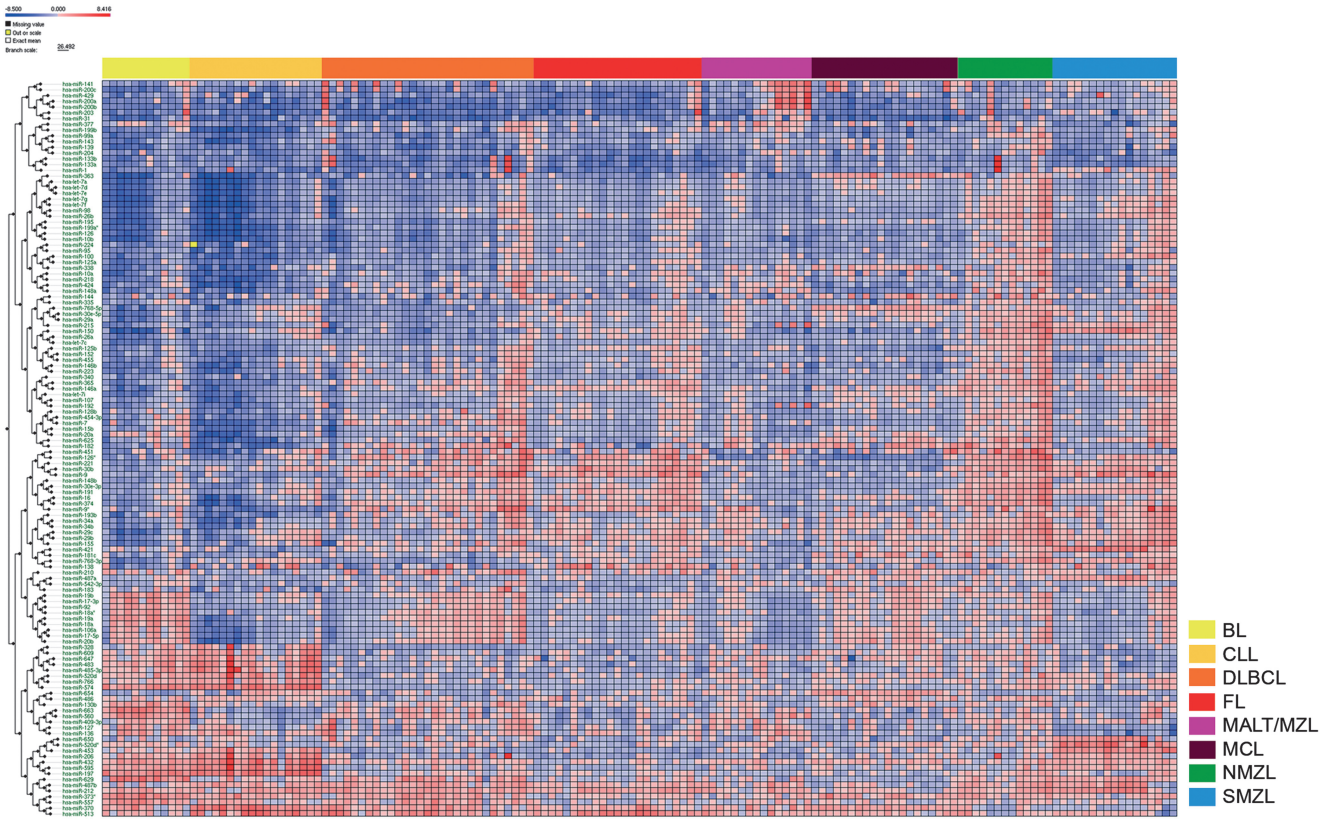

Figure 2. Differentially expressed miRNAs in B-cell lymphomas. In all, 128 miRNAs were differentially expressed in the series (FDR <0.01 and fold change (FC) >1.5) of 147 samples. For representation, data were normalized with non-tumoral controls (tonsils, reactive lymph nodes or spleens).

differentiation.[8] Interestingly, seven members of the let-7 family of miRNAs, which are commonly lost in tumors, were significantly overexpressed relative to the other lymphoma types.

Splenic marginal zone lymphoma. In all, 26 miRNAs (20 upregulated and 6 downregulated) were differentially expressed in SMZLs. The cluster miR-144/451 is highly overexpressed in SMZL. These two miRNAs are erythropoiesis regulators,[37] a finding that may be related to the splenic microenvironment. Among the downregulated miRNAs, we found the miR-200c/141 cluster, which was upregulated in other types of B-cell lymphomas in this series (MALT and MCL).

Identification of miRNAs differentially expressed in BL vs DLBCL

As a practical application of the data generated here, we compared the DLBCL and BL miRNA signatures. Microarray data from 12 frozen specimens of BL were compared with 29 DLBCL cases using SAM (details of the series, such as *C-MYC* translocation, ABC, GC, subgroups are described in Material and Methods section). In all, 43 miRNAs had a >1.5-fold in $\log_2$ differential expression and an FDR <0.01 (Figure 3). These miRNAs were investigated further by qRT-PCR in an additional series of 28 BL and 43 DLBCL FFPE samples.

Six of the miRNAs had low-efficiency qRT-PCR amplification, and were excluded from further analysis. Inefficient amplification could be due to the low quality of the RNA (extracted from FFPE samples), or to their generally low expression level.

Differential expression (FDR <0.05) was confirmed in 19 miRNAs (Table 2 and Figure 4). Thirteen additional miRNAs had the same tendency as observed in microarray analysis, but less significantly. Two miRNAs were significantly expressed, but oppositely with respect to the microarray. So, 32 of 37 miRNAs followed the array tendency (Supplementary Table S2).

MiR-155 was the most significantly lost miRNA in BL, confirming previous findings,[23] followed by miR-29b and miR-146a, whereas the most significantly lost miRNAs in DLBCL were miR-17-3p, miR-595 and miR-663.

MiR-29b, which is downregulated in BL cases, regulates TCL-1 expression,[38] a protein that is aberrantly expressed in this type of lymphoma and has been proposed as a diagnostic marker.[18,39] This miRNA is also negatively correlated with MCL-1 expression,[40] a key antiapoptotic protein of the BCL2 family, that is overexpressed in high-grade lymphoid neoplasms.

MiR-146a was already known to play an important role in inflammatory reaction and cancer.[41] MiR-34b that was downregulated in BL is targeted by p53, and is involved in maintaining self-renewal of pancreatic cancer stem cells, possibly by directly modulating the downstream targets BCL2 and NOTCH.[42]

The 19 significant miRNAs were submitted to the SOTA algorithm for samples unsupervised clustering. Only 5 of 71 cases were misplaced (7%): 4 DLBCL and 1 BL. As it is shown in Supplementary Figure S2, DLBCL cases that cluster with BL do not show any common feature in terms of GC/ABC type, *C-MYC* translocation or BCL2 immunohistochemical expression, although two of the cases carry on a *C-MYC* translocation. As some of the BL cases in this study do not carry *C-MYC* translocation, we analyzed C-MYC mRNA and protein expression levels. As shown in Supplementary Figure S3 and Supplementary Table S3 in Supplementary Information, C-MYC expression level does not significantly differ between BL cases with and without *C-MYC* translocation. On the other hand, the cluster shown in Supplementary Figure S2 demonstrates that these cases do not cluster depending on *C-MYC* translocation status or C-MYC expression level.

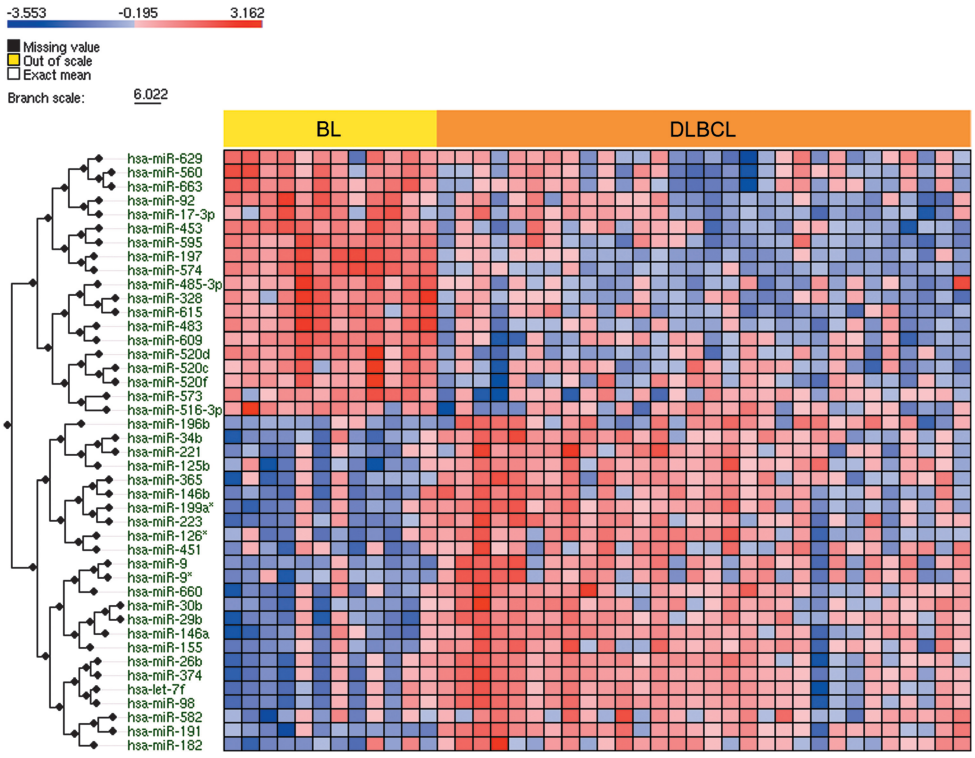

Figure 3. MiRNAs differentially expressed between BL and DLBCL. In all, 43 miRNAs were significantly (FDR <0.01 and fold change (FC) >1.5) differentially expressed in a SAM analysis comparing microarray data of 12 BL vs 29 DLBCL.

Table 2. Confirmation of miRNA expression by qRT-PCR

MiRNA	*FDR*	*Average FC*	*Upregulated in*
hsa-miR-155	<1.00E-07	2.67	DLBCL
hsa-miR-29b	3.00E-07	2.33	DLBCL
hsa-miR-146a	2.30E-06	1.97	DLBCL
hsa-miR-17-3p	2.30E-06	1.68	BL
hsa-miR-365	1.75E-05	1.59	DLBCL
hsa-miR-30b	2.92E-05	1.21	DLBCL
hsa-miR-595	3.74E-05	2.79	BL
hsa-miR-663	3.74E-05	1.17	BL
hsa-miR-573	0.0003	1.73	BL
hsa-miR-26b	0.0005	0.83	DLBCL
hsa-miR-374	0.0037	0.70	DLBCL
hsa-miR-520d	0.0037	1.57	BL
hsa-miR-92	0.0037	1.27	BL
hsa-let7f	0.0076	0.67	DLBCL
hsa-miR-516-3p	0.0096	0.83	BL
hsa-miR-9	0.0096	1.03	DLBCL
hsa-miR-629	0.0170	0.88	BL
hsa-miR-9*	0.0170	1.01	DLBCL
hsa-miR-34b	0.0502	0.80	DLBCL

Abbreviations: BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; FC, fold change; FDR, false discovery rate; miRNA, microRNA; qRT-PCR, quantitative real-time-polymerase chain reaction. List of 19 miRNAs differentially expressed between BL and DLBCL, confirmed by qRT-PCR: FDR <0.05. FC is expressed in $\log_2$.

DISCUSSION

Lymphoma diagnosis is based on the integration of clinical and histopathological data with chromosomal alterations and gene, or protein, expression data. In recent years, there have been many significant advances in lymphoma classification; nevertheless, additional molecular markers enabling a better distinction of specific lymphoma types and a more accurate prediction of response to therapy are still needed. The addition of miRNAs to the diagnostic algorithms is viable, given that qRT-PCR for miRNA expression is feasible in paraffin-embedded tissues, and it has been shown that the miRNA data generated in FFPE samples reproduce the data generated in frozen specimens.[13] In addition, the identification of miRNAs differentially expressed among lymphoma types could improve our understanding of lymphoma pathogenesis, ultimately enabling recognition of new therapeutic targets.

miRNA target prediction revealed interesting miRNA/gene interactions that could give some light on how lymphomagenesis takes place. For example, miR-133a and miR-23b, which are lost in our series of lymphomas, target *PAX5*, a gene involved in lymphocyte development and whose upregulation is related to the development of different B-cell lymphoma types reviewed by O'Brien *et al*.[43]

One of the most strongly lost miRNAs in our series, miR-31, is predicted to regulate the expression of the B-cell receptor pathway together with MAPK and JAK-STAT pathways.

Finally, target prediction tools showed some interesting genes that are commonly lost in different B-cell lymphoma types, and could be targeted by miRNAs upregulated in our series. Therefore, miR-9 and miR-513 are predicted to target *PRDM1*, and miR-770-5p and miR-212, whose predicted target gene is *TNFAIP3*.[44,45]

The expression of some of the miRNAs identified here reflects their role in B-cell differentiation, as has been shown for miR-17-5p, miR-20b, miR-223,[46] miR-150[(ref.8)] and miR-9.[46] In fact, the transcription factors LMO2 (GC marker) and PRDM1/BLIMP1 (plasma cell marker), which have a key role orchestrating B-cell differentiation, are found to be targets of some of these miRNAs,[46,47] which may suggest that the follicular lymphoma GC phenotype may be dependent on the concerted action of multiple miRNAs. Thus, miR-9, which is upregulated in FL cases, is involved in PRDM1/BLIMP1 downregulation,[46] which is consistent with the downregulation of PRDM1/BLIMP1 observed in reactive and

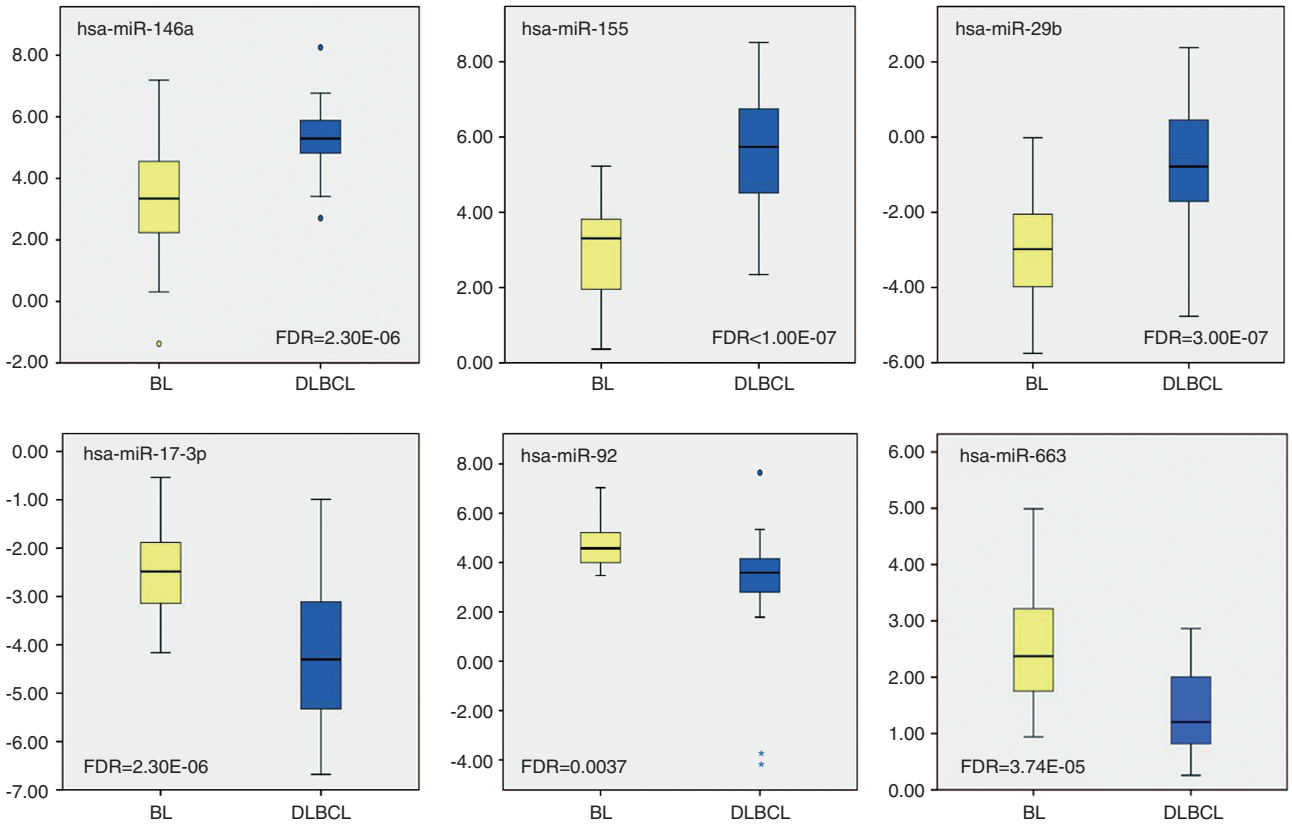

Figure 4. Box plots of qRT-PCR results. A selection of significant miRNAs (BL vs DLBCL) confirmed by qRT-PCR is illustrated. Mir-146a, miR-155 and mir-29b were upregulated in the DLBCL group, whereas miR-17-3p, miR-92 and miR-663 were upregulated in the BL group. $-\Delta Ct$ values are represented in $\log_2$ scale; *denotes outliers.

neoplastic GC B cells,[48] thus pointing to a possible cooperation of BCL6 and miR-9 in regulating terminal B-cell differentiation.[46] MiR-223 has been shown to inhibit LMO2 expression,[47] an observation that correlates with the strong expression of miR-223 in NMZL cases, a tumor that does not express LMO2 or other GC markers. Interestingly, miR-155 expression is downregulated in CLL and upregulated in NMZL cases, which probably reflects the observation that B cells lacking miR-155 generate reduced extrafollicular and GC responses and fail to produce high-affinity immunoglobulin G1 antibodies,[49] which is consistent with the data showing that MZL cells have a striking capacity for colonizing GCs and differentiating to become GC cells.

RAG1, BCL6 and *PRDM1/BLIMP1* are genes regulated by multiple miRNAs: at least eight miRNAs are predicted to target their 3′-UTRs, thereby emphasizing the need for the tight regulation of the expression of these transcription factors. MiR-127 is one of the miRNAs already known to regulate BCL6 expression.[50] Here, it was found to be upregulated in SMZL cases, a tumor that lacks BCL6 expression.[1] Normal B-cell differentiation and neoplastic B-cell phenotypes seem to be partially determined by the reciprocal antagonism between BCL6 and PRDM1/BLIMP1 expression,[51] which seems to be at least partially orchestrated through the interaction of multiple miRNAs.

MiR-182, overexpressed in MCL cases, was recently found to be overexpressed in MCL cell lines,[17] where a role as regulator of FOXO1, a putative tumor suppressor gene downregulated in breast cancer, has been proposed.[52]

Some of the changes detected here reflect the already established heterogeneity of several tumor types. Thus, DLBCL had the most heterogeneous miRNA signature of all the lymphoma types analyzed, extending previous observations.[47] In fact, heterogeneity of DLBCL has been characterized at the gene expression level, where the distinction between GC GC) type, ABC type and primary mediastinal large B-cell lymphoma has demonstrable biological, prognostic and therapeutic implications.[53] The series of DLBCL cases studied here reflects this heterogeneity with a 60% of ABC-type cases and 40% of GC-type cases in the series used for miRNA profiling. In fact, miRNA signatures related with the molecularly defined subgroups of DLBCL based on the cell of origin have been found by different groups.[13,29]

The heterogeneity is also reflected in C-MYC status; 32.5% (13/40) of FFPE series used for qRT-PCR were positive for *C-MYC* translocation. This uncommon high percentage of *C-MYC* translocated DLBCL is due to the fact that this series of DLBCL is a selection of DLBCL cases phenotypically similar to BL (44% non-GC (11/25) and 56% GC (14/25), as the source of the cases was a collection of DLBCL cases with features mimicking BL submitted for reference diagnosis.

The causes of these miRNA expression gains and losses are still to be investigated in many cases, although some can be attributed to changes in DNA copy numbers in the chromosomal regions where they are located. For instance, analysis of a deletion at 13q14.3 prompted the discovery of two physically linked miRNAs, miR-15a and miR-16-1, which were targets of these deletions,[7] and shown here to be lost in CLL. NMZL cases exhibited a gain of miR-191 located at chromosome 3p21.31, an area of known gains in NMZL.[54]

The data generated here could have a practical diagnostic value. We have explored whether miRNA data could contribute to the differential diagnosis of BL and DLBCL, a controversial issue that requires new approaches.[3,4,39] Despite the characteristic association between *C-MYC* translocation and the diagnosis of BL, a discrete number of DLBCL cases may carry the *C-MYC*

translocation.[1] Furthermore, a group of cases with typical histology of BL may lack the *C-MYC* translocation[55] and some association with the expression of selected miRNAs has been described.[56] Our results show that a few miRNAs could be of diagnostic value. For example, BL cases show loss of miR-155 and gain of multiple miRNAs belonging to clusters 17–92. Some of the miRNAs that characterize BL cases are probably C-MYC targets, as has been shown for the clusters 17–92 and other miRNAs.[24,57] In this study, miRNAs upregulated by C-MYC were upregulated in BL relative to other NHL cases (miR-17-3p, miR-18a, miR-19a, miR-92 and miR-130b) and specifically when compared with DLBCL (miR-17-3p). Other miRNAs downregulated by C-MYC were lost in BL compared with other NHL cases (let-7 family, miR-146a and miR-29 family), and some were confirmed when BL was compared with DLBCL (miR-29b and miR-146a). The expression of miR-155 in BL has been controversial, but recent reports agree that BL lacks miR-155 expression,[24] as confirmed here. In our study, it was the most significantly differentially expressed miRNA in BL compared with DLBCL, with a substantial 2.67-fold (log_2) change, meaning that expression in DLBCL was around six times stronger than in BL. This confirms miR-155 downregulation in BL, making it one of the most suitable markers for differential diagnosis. Nevertheless, a model including all 19 significant miRNAs or the most significant ones may increase the accuracy of the BL diagnosis as the unsupervised cluster failed to classify only 7% of the samples, and it can also form the basis of a better understanding of BL pathogenesis. These data facilitate a next level of analysis, where an independent series of BL, DLBCL and intermediate BL/DLBCL is currently being studied to obtain a signature with a lower number of miRNAs, which could potentially be used for diagnosis. Furthermore, a recently published paper by Lenze *et al*.[58] defines a signature of miRNAs that differentiates BL from DLBCL. Five of the miRNAs described in that paper, expressed in DLBCL, are confirmed by the work presented here, supporting the potential relevance of miRNAs in diagnosis.

Differences in miRNA signatures for different lymphoma types may have multiple causes. As B cells at different stages of maturation show specific miRNA signatures,[59] the differential expression of miRNAs between different lymphomas types may reflect distinct miRNA profiles of the cell of origin, the normal counterpart for each lymphoproliferative disorder. Alternatively, these differences could be due to the presence of some acquired recurrent genetic alteration of the specific lymphoma type, for example, miRNAs contained in frequently lost chromosomal regions, such as 13q14 deletion in CLL or 7q21 lost in SMZL. In addition, some of the changes observed here could depend on oncogenic changes in genes regulating the expression of multiple miRNAs, as demonstrated for C-MYC and BCL6.

In the initial series of 147 samples, K-nearest neighbors analysis correctly classified 86.4% of the samples. This rate might be even higher if more cases were introduced. Even though miRNA involvement in the pathogenesis of each specific lymphoma type still needs to be explored, and validation in an independent series of samples is recommended, the miRNA expression signatures described here could be a useful additional tool enabling a more accurate B-cell NHL diagnosis, in particular when BL vs DLBCL differential diagnosis is required, and a better understanding of B-cell lymphoma pathogenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the AECC, Fondo de Investigaciones Sanitarias (RETICS, PI051623, PI052800); (FI08/00038, LDL), (CP06/00002; NM); the Ministerio de Ciencia e Innovación (SAF2008-03871, SAF2007-65957-C02-02), Fundación la Caixa and the National Institute of Bioinformatics (GGL), Spain. We thank the Spanish National Tumour Bank Network and Socorro María Rodríguez Pinilla (CNIO), the Gregorio Marañon and La Paz Hospitals, Madrid, Spain, the Virgen de la Salud Hospital, Alberto Arribas, Toledo, Spain, Laura Cereceda and Carmen Almaraz, IFIMAV, Santander, Spain, for expert diagnosis, sample retrieval, tumor banking and technical support.

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Supplementary Information accompanies the paper on Blood Cancer Journal website (http://www.nature.com/bcj)