Splenic marginal zone lymphoma: comprehensive analysis of gene expression and miRNA profiling

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Splenic marginal zone lymphoma is a small B-cell neoplasm whose molecular pathogenesis is still essentially unknown and whose differentiation from other small B-cell lymphomas is hampered by the lack of specific markers. We have analyzed the gene expression and miRNA profiles of 31 splenic marginal zone lymphoma cases. For comparison, 7 spleens with reactive lymphoid hyperplasia, 10 spleens infiltrated by chronic lymphocytic leukemia, 12 spleens with follicular lymphoma, 6 spleens infiltrated by mantle cell lymphoma and 15 lymph nodes infiltrated by nodal marginal zone lymphoma were included. The results were validated by qRT-PCR in an independent series including 77 paraffin-embedded splenic marginal zone lymphomas. The splenic marginal zone lymphoma miRNA signature had deregulated expression of 51 miRNAs. The most highly overexpressed miRNAs were *miR-155*, *miR-21*, *miR-34a*, *miR-193b* and *miR-100*, while the most repressed miRNAs were *miR-377*, *miR-27b*, *miR-145*, *miR-376a* and *miR-424*. MiRNAs located in 14q32-31 were underexpressed in splenic marginal zone lymphoma compared with reactive lymphoid tissues and other B-cell lymphomas. Finally, the gene expression data were integrated with the miRNA profile to identify functional relationships between genes and deregulated miRNAs. Our study reveals miRNAs that are deregulated in splenic marginal zone lymphoma and identifies new candidate diagnostic molecules for splenic marginal zone lymphoma.

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Splenic marginal zone lymphoma has been recognized by the World Health Organization Lymphoma Classification as a regular entity.¹ Splenic marginal zone lymphoma is an uncommon form of small B-cell neoplasm infiltrating in the spleen, bone marrow and peripheral blood. It accounts for <1% of all lymphoid neoplasms, in which the relative rarity of splenic marginal zone lymphomas is a

significant obstacle to their molecular investigation and identification of specific diagnostic markers. The most common genomic alteration in splenic marginal zone lymphoma is the 7q31 deletion (26–45% of patients)^{2–4} involving the miRNA *miR-29b*^{4,5} and others. Translocations involving the 14q32 band, in which the immunoglobulin heavy chain variable region (IgV_H) locus is located, have been detected infrequently in splenic marginal zone lymphoma⁶ in comparison with other non-Hodgkin lymphomas.⁷ Around half of splenic marginal zone lymphoma patients carry an increased load of IgV_H somatic mutations, which some studies have found to be associated with improved prognosis.⁸ Analysis of gene expression profiles (GEPs) in

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splenic marginal zone lymphoma has revealed the deregulated expression of lymphoma oncogenes, such as *RHOH* and *TCL1A*, identifying clinical subgroups and new therapeutic targets.⁹ Very recently, genome sequencing studies have identified recurrent mutations in the *NOTCH2* gene that seem to be restricted to splenic marginal zone lymphoma among low-grade B-cell disorders.^{10,11} Nevertheless, the molecular pathogenesis of splenic marginal zone lymphoma is still poorly understood, and the data obtained so far suggest that miRNA could play a key role in the pathogenesis of the disease.

Although the diagnostic criteria for the recognition of splenic marginal zone lymphoma are clearly established in splenic tissue, this diagnosis often has to be performed in peripheral blood or bone marrow, where the diagnosis of splenic marginal zone lymphoma is often hindered by the lack of consistent markers differentiating it from chronic lymphocytic leukemia, follicular lymphoma and mantle cell lymphoma.

MiRNAs are 21- to 23-nt-long RNA molecules that regulate mRNA translation and, consequently, the expression of protein-coding genes. More than 1000 miRNAs have been identified in mammals, in which they regulate a range of biological functions, including cell differentiation and proliferation. B-cell differentiation is tightly regulated by miRNAs and the expression of characteristic sets of miRNAs distinguishes specific stages of B-cell differentiation and B-cell lymphoma main tumor types.^{12,13} The miRNA profile of splenic marginal zone lymphoma has been investigated by several groups, who have obtained interesting results related with 7q loss, hepatitis C virus presence or with normal spleen.^{4,5,14,15} Nevertheless, the miRNA changes in splenic marginal zone lymphoma are yet to be comprehensively described. We aimed to carry out an integrated genomics study to improve our understanding of the molecular mechanisms involved in the pathogenesis of splenic marginal zone lymphoma, and to suggest new targets, genes and miRNAs, with diagnostic and therapeutic potential.

Patients and methods

Patients and Tissue Samples

The series included 31 patients with splenic marginal zone lymphoma. Clinical information about these cases was retrieved from medical records, surgical pathology reports and referring clinicians (Table 1). For comparison purposes, we included a set of 35 spleen samples: 7 spleens with reactive lymphoid hyperplasia (reactive spleens), 10 spleens infiltrated by chronic lymphocytic leukemia, 12 spleens with follicular lymphoma and 6 spleens infiltrated by mantle cell lymphoma. To compare the miRNA

expression profile between splenic and nodal marginal zone lymphoma, we included 15 lymph nodes infiltrated by nodal marginal zone lymphoma that were included in a previous study by our group.¹⁶

The main criterion for inclusion was the availability of frozen tissue from the diagnostic specimen in each case. Morphological examination and CD20 immunostaining revealed the cases in which at least 75% of the cells were tumoral in this study.

To validate the levels of miRNA profiles found by microarray analysis, quantitative reverse transcription–polymerase chain reaction (qRT–PCR) studies were performed with formalin-fixed, paraffin-embedded tissues from an independent series including 141 cases: 77 splenic marginal zone lymphoma, 16 chronic lymphocytic leukemia, 20 follicular lymphoma, 22 mantle cell lymphoma and 6 reactive spleens.

All cases were provided by the Tumor Bank of the Hospital Virgen de la Salud (BioB-HVS, Toledo, Spain) and the CNIO Tumour Bank (Madrid, Spain). Cases were diagnosed on the basis of morphology, immunophenotypic and molecular findings according to the World Health Organization classification criteria.¹ Research was performed under the supervision of the Institutional Review Board of the HVS, Toledo, Spain.

Selection of B-cell Subsets

B-cell populations were obtained by magnetic cell separation from 5 tumoral patients and 4 healthy donors undergoing routine hematological analysis. The splenic marginal zone lymphoma and non-tumoral B cells were recognized by CD19⁺ expression.

RNA Isolation

For GEP and miRNA hybridization, total RNA was isolated from 59 frozen tumoral blocks and 7 control samples from reactive spleens by TRIzol Reagent (Invitrogen, Paisley, UK) following the manufacturer's recommendations. The quality of the RNA produced was checked by 1% agarose electrophoresis. Cases with poor-quality RNA ($A_{260/280}$ and $A_{260/230}$ ratios <1.8 measured in the NanoDrop; Thermo Scientific, Wilmington, USA) were discarded.

Microarray Procedures: GEP and miRNA Hybridization

RNA for GEP was hybridized on a Whole Human Genome Agilent 4 × 44K Oligonucleotide Microarray (Agilent Technologies, Santa Clara, CA, USA), as described elsewhere.¹⁷

The miRNA microarray experiments were done using the Agilent Human miRNA Microarray (V1),

Table 1 Clinical characteristics of the splenic marginal zone lymphoma series of patients

Case	Age/sex	IGHV	ID %	7q	Treatment	Progression	Status	OS
1	52/F	VH4-34	100	ND	S	NO	AWD	26
2	ND	ND	ND	ND	ND	ND	ND	ND
3	70/F	VH3-30	98	ND	S	NO	DOD	25
4	44/F	VH3-30	95.9	ND	S	YES	AWD	24
5	70/M	VH3-30	92.1	ND	S	YES	AWD	64
6	66/F	NR	NR	ND	S	NO	AWD	195
8	73/F	VH1-2/VH3-30	94.7/100	YES	S	NO	AWD	22
10	60/M	VH1-2	99.1	YES	S	NO	AWD	67
11	50/M	VH1-2	98.21	YES	S, CHT	NO	AWD	30
12	55/M	VH1-2	97.3	YES	ND	NO	AWD	81
13	67/M	NR	NR	ND	ND	ND	ND	ND
14	64/F	VH1-2/VH3-8	97.21/96.41	YES	ND	YES	DXT	61
15	66/M	VH4-39/VH3-74	100/100	YES	ND	YES	DXT	57
17	68/M	VH2-70/VH3-30	?/93.5	ND	ND	YES	DXT	78
18	63/M	VH1-18/VH3-3	99.5/100	YES	ND	YES	DXT	10
19	72/F	VH1-2	93.3	YES	ND	NO	AWD	24
20	70/F	VH1-2	96.4	YES	ND	YES	AWD	76
21	65/F	VH4-34	96.35	YES	ND	NO	AWD	53
22	61/M	VH1-2	93.4	NO	ND	YES	AWD	42
23	70/F	VH3-30	95.98	NO	ND	YES	DXT	18
24	63/F	VH6-1	94.5	ND	ND	NO	AWD	13
25	59/M	NR	NR	ND	ND	NO	AWD	34
26	72/F	NR	NR	ND	ND	NO	DOD	54
27	76/F	NR	NR	ND	ND	YES	DXT	92
28	67/F	VH1-2	99.55	YES	ND	YES	AWD	53
29	74/M	VH1-2	100	YES	ND	YES	DXT	3
30	66/F	VH1-2	99.1	YES	ND	YES	DXT	22
31	ND	NR	NR	ND	ND	ND	ND	ND

Abbreviations: F, female; M, male; ND, no data; NR, no rearrangement; ID, identity frequency; S, splenectomy; CHT, chemotherapy; D, dead; DOD, dead of disease; DXT, dead, non-lymphoma related; AWD, alive with disease.

SMZL patients had a median age of 64.7 years, ranging from 44 to 76 years; 54% of them were women.

8 × 15 K (Agilent Technologies). For each tissue sample, 100 ng total RNA were hybridized with the miRNA array and further processed as previously described.¹⁸

Microarray Data Analysis: GEP, miRNA, B-cell Subsets

The background subtraction of GEP microarray data was carried out using GEPAS 4.0 (<http://gepas.bioinfo.cipf.es/>). The data set was normalized by lowess within-array normalization and quantile between-array normalization, and then preprocessed. For miRNA profiling, microarray background subtraction was carried out using the normexp tool available in Bioconductor's limma package (<http://www.bioconductor.org>). To normalize this data set, loess within-array normalization and quartile between-array normalization were performed.¹⁹

We used *t*-tests (<http://pomelo2.bioinfo.cnio.es/>) to compare the expression of the splenic marginal zone lymphoma gene and miRNA signatures with that of the reactive spleens and with the other low-grade B-cell lymphoma types (chronic lymphocytic leukemia, follicular lymphoma and mantle cell lymphoma). In this comparison with other lymphomas, we used a *t*-test to compare splenic

marginal zone lymphoma cases with other lymphoma samples (all treated as a single class). In all these comparisons, the genes and miRNAs with FDR < 0.05 were considered significant.

For GEP signatures and miRNA signatures we used values of FDR < 0.01 and < 0.05, respectively, to indicate significance.

The *t*-statistic was calculated in the gene set enrichment analysis (GSEA) (<http://www.broad.mit.edu/gsea/>) to identify the pathways and functional groups enriched in the splenic marginal zone lymphoma signature. The GSEA gene sets used were selected from a curated version of the Biocarta, KEGG and CCG pathway databases, as previously described.²⁰ The gene sets with values of *P* < 0.05 or FDR < 0.25 were considered to be enriched and potentially relevant in each case.

MiRNA Target Prediction and miRNA Target Correlation

We followed the previously described procedure²¹ to identify associations between differentially expressed miRNAs (FDR < 0.05) and gene expression signatures (FDR < 0.05). The GSEA (Pearson correlation) was used to test the enrichment of gene sets related to each corresponding miRNA. Those with values of *P* < 0.05 and FDR < 0.25 were

considered to be enriched in each case. Gene target prediction for miRNAs of splenic marginal zone lymphoma signature were obtained using the TargetScan algorithm (Release 5.2) in conjunction with the mir2disease on-line database (<http://www.mir2disease.org/>).

Quantitative Real-Time RT-PCR Analysis

To validate miRNA microarray data we carried out a qRT-PCR assay. Total RNA was extracted from formalin-fixed, paraffin-embedded tissues sections of an independent patient group following the manufacturer's instructions and using a miRNeasy FFPE Kit (Qiagen, Valencia, CA, USA).

qRT-PCR experiments were performed on selected miRNAs using TaqMan probes (Applied Biosystems, Foster City, CA, USA), as previously described.^{9,21} The relative degree of change for each miRNA was calculated using the $RQ = 2^{-\Delta Ct}$ method, where $\Delta Ct = (Ct_{miRNA} - Ct_{U6})$, and with an RNU6B endogenous control. Ct values >32 were considered beyond the limit of detection.

Results were analyzed with *t*-tests and one-way ANOVAs (calculated in SPSS v.17.0). miRNAs with values of $P < 0.05$ were considered statistically significant.

Results

miRNA Signature in Splenic Marginal Zone Lymphoma

The miRNA signature in splenic marginal zone lymphoma, comprising the miRNAs differentially expressed in splenic marginal zone lymphoma relative to reactive spleen ($FDR < 0.05$; *t*-test), included 51 miRNAs, 9 of which were overexpressed and 42 were repressed. Within the overexpressed miRNAs we found miRNAs that are involved in B-cell development (eg, *miR-155*) and miRNAs that are deregulated in cancer (eg, *miR-21*, *miR-34a* and *miR-193b*). Among the underexpressed miRNAs we found a high number of miRNAs located in 14q32 (14 of 52 miRNAs, ~27% of the signature), such as *miR-377*, *miR-376a* and *miR-136*, and other relevant miRNAs, like *miR-24*, *miR-27b* (both located in the 9q22 band) and *miR-145* (Table 2; Figure 1).

These findings prompted us to compare the expression of these 14q32 miRNAs of the signature with those found in other lymphomas involving the spleen and nodal marginal zone lymphoma. Interestingly, we found that some of the miRNAs in 14q32 were also significantly repressed in splenic marginal zone lymphoma compared with nodal marginal zone lymphoma. Although the values were not significant, these miRNAs in 14q32 had a lower level of expression in splenic marginal zone lymphoma than chronic lymphocytic leukemia,

follicular lymphoma, and mantle cell lymphoma, with less than onefold change. Ultimately, miRNAs in 14q32 were also repressed in lymph nodes infiltrated by splenic marginal zone lymphoma. No miRNA in 7q appears in the signature, even though *miR-29a* and *miR-29b* repressed in splenic marginal zone lymphoma have been described^{4,5} (Table 2; Figure 2).

Comparison of Splenic Marginal Zone Lymphoma B Cells and Normal B Cells

Taking into account the essential role of the stromal microenvironment in low-grade lymphomas, we took another approach to compare CD19+ B cells isolated from peripheral blood of splenic marginal zone lymphoma patients with those from healthy donors. This enabled us to identify some of the most important miRNAs in the splenic marginal zone lymphoma miRNA signature, both upregulated (*miR-21* and *miR-193b*) and downregulated (*miR-483*, *miR-485-3p* and *miR-328*) (Table 2; Supplementary Table 4). It is of particular note that *miR-29b* and *miR-592*, located at 7q32, are repressed in splenic marginal zone lymphoma B cells compared with healthy donors (Supplementary Table 4).

Integration of miRNA and GEP Data

Although the GEP in splenic marginal zone lymphoma has been previously described,^{9,22,23} as we are now interested in identifying the association between miRNA signature and GEP, the same series used in the miRNAs was also hybridized in GEP. Therefore, we determined the GEP of splenic marginal zone lymphoma relative to reactive spleen using a *t*-test ($FDR < 0.01$) and GSEA software ($P < 0.05$, $FDR < 0.25$). The GEP signature determined in splenic marginal zone lymphoma comprised 753 genes, 194 of them upregulated and 559 repressed. Among the overexpressed genes we found integrins (*CD40*), BCR signaling genes (*SYK*, *BLNK*, *NFATC1*), proliferation-related genes (*MUM1*, *RHOH*, *AIM2*), interleukins (*IL2RA*, *IL7*, *IL6*, *IL21*), anti-apoptotic genes (*BCL2*, *BCL2L10*, *FOXP1*, *BIRC3*), TNF signaling (*CD70*, *TNFRSF9*) and normal marginal zone-related genes (*TACI*, *CD44*, *IL2RA*, *MMP12*). Among the repressed genes we found genes located in 7q (*MEST*, *MET*, *PTN*), others with important functions in B cells (*FCGR3A*, *FCGR3B*, *TNFAIP1*, *TNFAIP2*, *CXCL1*, *CXCL12*), and cell adhesion-related genes (*TGFB1* and *ICAM2*). GEP data in SMZL are shown in Supplementary Table 2.

The GSEA analysis revealed the enrichment of pathways related to cell survival, such as NF- κ B, BAFF signaling and cell cycle, and crucial B-cell pathways, such as memory B-cell-related genes ($IgM^+ IgD^+ CD27^+$), PRDM1 signaling and B-cell receptor genes (Supplementary Table 3).

Table 2 MiRNA expression profile in splenic marginal zone lymphoma

miRNA	Cytoband	SMZL vs. RLH		SMZL vs. other lymphomas			SMZL vs. NMZL		B cells		
		FDR	FC	FDR	FC (vs. FL)	FC (vs. CLL)	FC (vs. MCL)	FDR	FC	FDR	FC
hsa-miR-155	21q21.3	0.0001	1.3636	NS	-0.6234	-1.9369	-0.7545	0.0075	0.8876	NS	1.0121
hsa-miR-34a	1p36.22	0.0013	1.281	NS	-0.5482	-0.9769	-1.2861	0.0073	-0.8094	NS (0.0784)	1.392
hsa-miR-100	11q24.1	0.0028	1.1235	NS	-0.7022	-0.9698	-1.4994	0.0012	1.2583	NS	0.4678
hsa-miR-193b	16p13.12	0.0216	1.0595	NS	-0.7951	-0.4008	-1.8453	NS	-0.5294	0.035	1.6673
hsa-miR-330	19q13.32	0.001	0.8598	NS	-0.7498	-0.9477	-1.0091	0.0008	1.0633	NS (0.0539)	1.5244
hsa-miR-21	17q23.2	0.0474	0.8513	0.0049	-1.1313	-1.0129	-1.3443	0	-2.1378	0.0132	1.9599
hsa-miR-26b	2q35	NS	0.2715	0.041	-0.3368	-1.4802	-0.8624	0.0005	-1.0987	0.0329	-1.687
hsa-miR-19b	13q31.3	NS	0.1102	0.0499	-0.3911	-1.1701	-1.3954	0.0006	-0.8801	NS	-1.3312
hsa-miR-660	Xp11.22	NS	0.0263	0.0225	-0.7694	-1.0851	-1.0885	NS	-0.4423	NS	0.6627
hsa-miR-448	Xq23	NS	-0.1061	0.033	-0.762	-1.0343	-0.9973	0.0001	-0.9103	NS	-0.4552
hsa-miR-646	20q13.33	NS	-0.1896	0.033	-0.7637	-1.027	-1.0011	0	-1.3027	NS	-0.2437
hsa-miR-323	14q32.31	NS	-0.3022	0.0447	-0.784	-1.0144	-1.0373	NS	0.2477	NS	0.6538
hsa-miR-567	3q13.2	NS	-0.3514	0.041	-0.8072	-1.0267	-1.0057	0.0315	-0.4447	NS	-0.9589
hsa-miR-589	7p22.1	0.0334	-0.4116	0.0447	-0.7876	-1.0331	-1.0393	NS	-0.3662	NS	-0.1161
hsa-miR-381	14q32.31	0.0022	-0.8884	NS	-0.8363	-1.0544	-1.0361	0.0394	-1.0007	NS	-1.3213
hsa-miR-494	14q32.31	0.0102	-0.9253	NS	-0.1518	-0.3148	-0.4096	NS	0.5589	NS	-0.1063
hsa-miR-382	14q32.31	0.0024	-0.9511	NS	-0.7663	-1.1678	-1.1157	NS	-0.325	NS	0.3914
hsa-miR-154	14q32.31	0.0001	-1.0173	NS	-0.8407	-1.1529	-1.0722	NS	0.2216	NS	-0.6378
hsa-miR-376b	14q32.31	0.0081	-1.0242	NS	-0.8409	-1.1961	-1.0566	0.0047	-1.7553	NS	0.3983
hsa-miR-410	14q32.31	0.0004	-1.0356	NS	-0.8425	-1.071	-1.0584	NS	-0.0101	NS	0.9009
hsa-miR-127	14q32.31	0.004	-1.2176	NS	-0.7677	-1.2333	-1.3005	0.0001	-1.3158	NS	0.7175
hsa-miR-758	14q32.31	0	-1.2837	NS	-0.8111	-1.0247	-0.9909	0.0102	-0.5757	NS	-1.5249
hsa-miR-199a	19p13.2	0.0005	-1.2864	NS	-0.7517	-1.0731	-1.4343	0	-1.1909	NS	0.8821
hsa-miR-376a	14q32.31	0.0009	-1.3704	NS	-0.7113	-1.4976	-1.3556	0.0195	-1.2118	NS	0.846
hsa-miR-136	14q32.31	0.0009	-1.4854	NS	-0.7217	-1.1304	-1.0163	0.0309	0.6645	NS	0.5251
hsa-miR-379	14q32.31	0	-1.4966	NS	-0.8855	-1.0778	-1.0051	NS	0.1304	NS	0.8097
hsa-miR-145	5q32	0.0004	-1.5099	NS	-1.128	-0.8589	-1.3141	0.003	0.9025	NS	0.983
hsa-miR-338	17q25.3	0.0004	-1.6084	NS	-0.9872	-1.1076	-1.0284	0.0007	-1.5367	NS	1.4012
hsa-miR-107	10q23.31	0	-1.6811	NS	-0.5584	-0.8375	-1.1204	0.0157	-0.6025	NS	-0.0679
hsa-miR-27b	9q22.32	0	-1.6847	NS	-0.6594	-1.2939	-0.9624	0.0001	-1.6646	NS	0.7803
hsa-miR-24	9q22.32	0	-1.7407	NS	-0.681	-0.8085	-1.0673	0.0001	-1.2365	NS	0.9969
hsa-miR-377	14q32.31	0	-1.8081	NS	-0.8439	-1.3491	-1.132	0.0037	-1.8887	NS	0.5973
hsa-miR-328	16q22.1	0.0001	-0.9722	NS	-0.1321	-0.0142	0.0541	0.0062	-1.3057	0.0222	-1.809
hsa-miR-485-3p	14q32.31	0.0026	-1.0844	NS	0.2086	0.3138	0.3265	NS	NS	0.0198	-1.8431
hsa-miR-483	11p15.5	0.0059	-1.2378	NS	0.249	0.2958	0.3923	0.0276	-1.5169	0.0084	-2.0841
hsa-miR-339	7p22.3	NS	-0.659	NS	-0.1099	-0.4604	0.8266	NS	NS	0.0059	-2.1783

SMZL vs. RLH columns corresponding to a *t*-test between splenic marginal zone lymphoma and reactive spleen. The data from SMZL vs. Other Lymphomas columns correspond to a *t*-test comparing splenic marginal zone lymphoma with follicular lymphoma, chronic lymphocytic leukemia and mantle cell lymphoma, considered as a single class. SMZL vs. NMZL columns correspond to a *t*-test comparing the two marginal zone classes. The B-cell column corresponds to a *t*-test with B-cell data, obtained from isolated CD19+ B cells from peripheral blood of splenic marginal zone lymphoma patients and healthy donors. FDR is the false discovery rate. FC is the *n*-fold change ($\log_2$ scale) difference between the corresponding averages of classes. NS, not significant.

The GEP data were integrated with the miRNA profile using a TargetScan prediction algorithm and Pearson correlation (GSEA software) to identify functional relationships between genes and deregulated miRNAs. Among the upregulated splenic marginal zone lymphoma genes targeted by downregulated miRNAs, we found genes relevant in B-cell survival signaling, such as *SYK*, *CD40*, *BCL2*, *CD44*, *AIM2*, *FOXP1*, *MUM1* (*IRF4*) and *RHOH*. Downregulated miRNAs targeting these genes were *miR-145*, *miR-27b*, *miR-376a* and *miR-377* (Tables 2 and 3; Figure 3; Supplementary Table 2).

Conversely, among the downregulated splenic marginal zone lymphoma genes targeted by upregulated miRNAs, we found apoptosis regulators such as *TNFAIP1* and *TNFAIP2* that are predicted

to be regulated by overexpressed miRNAs such as *miR-34a* and *miR-193b*. The overexpressed *miR-155* could regulate the *PLEKHG5* gene, which is involved in the NF- κ B signaling pathway. Similarly, the high level of *miR-21* could contribute to the expression of *TSPYL5*, which is implicated in PTEN-AKT signaling (Tables 2 and 3; Figure 3; Supplementary Table 2).

GSEA was done to determine the enriched pathways related to the most relevant miRNAs in the study. It revealed significant gene sets and pathways with a crucial role in B-cell survival, such as NF- κ B signaling, CD40 signaling, interleukins and inflammatory pathways, cell cycle and memory B-cell genes, to be associated with the expression of *miR-155*, *miR-34a* and *miR-21* (Table 4; Figure 3).

Figure 1 Splenic marginal zone lymphoma miRNA signature. Hierarchical clustering of the complete splenic marginal zone lymphoma miRNA signature: 52 miRNAs with $FDR < 0.05$ and -0.85 - to 0.85 -fold change in a t -test comparison with RLH (<http://pomelo2.bioinfo.cnio.es/>). Red and green indicate high- and low-level expression, respectively. SMZL, splenic marginal zone lymphoma; RLH, reactive lymphoid hyperplasia in the spleen.

Figure 2 MiRNAs in 14q32. The splenic marginal zone lymphoma miRNA signature contains 14 repressed miRNAs located in 14q32 chromosomal band (27% of the signature). These had a lower level of expression in splenic marginal zone lymphoma than in other classes: lymph node infiltrated by splenic marginal zone lymphoma (LN-SMZL), spleens infiltrated by chronic lymphocytic leukemia (CLL) and by mantle cell lymphoma (MCL), nodal marginal zone lymphoma (NMZL) and non-tumoral spleens (RLH).

Comparison Between Splenic and Nodal Marginal Zone Lymphomas

We also studied the miRNA expression patterns of splenic marginal zone lymphoma relative to nodal marginal zone lymphoma obtaining 44 miRNAs differentially expressed between these subtypes of marginal lymphoma: These included 19 miRNAs

from the splenic marginal zone lymphoma signature, some of which were the most important miRNAs, such as *miR-21*, *miR-155*, *miR-100*, *miR-27b*, *miR-376a* and *miR-377*. Similarly, among the miRNAs with increased expression in nodal marginal zone lymphoma were several already known to be overexpressed in this lymphoma type, such as *miR-199a*¹⁶ (Table 2; Supplementary Table 5).

Table 3 Gene target prediction for the most significant miRNAs in the assay

miRNA Signature	miRNA	FDR	FC	Validated targets (published studies)	Prediction targets (TargetScan)	GEP signature
Upregulated miRNAs	hsa-miR-155	0.0001	1.3636	AGTR1, SAMD1, SMAD5, BCL2 SHIP1, HIF, ETS1, AID, RHOA	PLEKHG5, PTN, RHOBTB1, RAPGEF5, GDDP5	Downregulated genes
	hsa-miR-34a	0.0013	1.281	E2F3, CCND1, ARAF, AXL, MEK1, MET, PIK3R2	CCND1, TNFAIP1, CDH4, RAPGEF5	
	hsa-miR-193b	0.0216	1.0595	CCND3, MCM2, MCM5, PLK1, SMAD4, WNT1, JAG1	CCND1, TNFAIP1, PKD2L1, RAPGEF5, CD160	
	hsa-miR-21	0.0474	0.8513	MCL1, CCND1, 14-3-3, SHMT2, AKR1C2	TSPYL5, TGFBI, RAPGEF5, FCGR3B ITGA8, GDDP5, CD160	
Downregulated miRNAs	hsa-miR-376a	0.0009	-1.3704	PTEN, TPM1, PDCD4, CDC25A, LRRFIP1, WNT1, JAG1	CD44, DLEU1, MUM1, IL2RA , IL7, IRTA4, FOXP1	Upregulated genes
	hsa-miR-145	0.0004	-1.5099	unknown	CD44, NFATC1, DLEU1, MUM1 LIMK1, IL6, CD40, FOXP1	
	hsa-miR-27b	0	-1.6847	FLJ21308, C-MYC	CD44, NFATC1, SYK, PLA2G2D, BCL2L10, DLEU1	
	hsa-miR-377	0	-1.8081	CYP1B1, NOTCH1 NF- κ B(lipopolysaccharide-induced activation)	MUM1, LIMK1, TNFRSF9, IRTA1, RHOH, AIM2, FOXP1	
				HO-1 (in combination with hsa-miR-217)	SYK, BCL2L10, BCL2, DLEU1, FCGR2B, FOXP1	

The validated targets column refers to experimentally validated mRNA targets of the respective miRNAs selected from literature while the prediction targets are the predicted mRNAs according to the TargetScan algorithm (Release 5.2) in the mir2disease on-line database (<http://www.mir2disease.org/>). The miRNAs and predicted target genes are those of the splenic marginal zone lymphoma signature.

Figure 3 Representation of the most important miRNAs in the signature with connections to pathways and biological functions involved. The connections between upregulated and downregulated miRNAs with corresponding target genes and involved target pathways are shown. The links between miRNAs and genes corresponding to prediction targets by TargetScan algorithm; the junctions within genes and pathways and biological functions are based on literature and on-line databases, while those between miRNAs and pathways and functions reflect positive Pearson correlations from GSEA.

Supervised clustering of the splenic marginal zone lymphoma miRNA signature (SOTA algorithm, Pearson correlation) was used to differentiate between splenic marginal zone lymphoma cases in the spleen, lymph node infiltrated by splenic marginal

zone lymphoma and nodal marginal zone lymphoma. Interestingly, lymph nodes infiltrated by splenic marginal zone lymphoma clustered with the splenic samples and not with the nodal cases (Figure 4).

Splenic Marginal Zone Lymphomas vs. Other Low-Grade B-Cell Lymphomas in the Spleen

To determine those genes and miRNAs with differential expression between SMZL and other low-grade B-cell lymphomas in the spleen (chronic lymphocytic leukemia, follicular lymphoma and mantle cell lymphoma), we carried out a *t*-test, in which we found 357 genes and 9 miRNAs that were significantly deregulated ($FDR < 0.05$). This enabled us to predict targets *in silico*.

The GEP comparison confirmed the stronger expression of *CD200* and *TCL1A* in chronic lymphocytic leukemia, the expression of germinal center markers *BCL6*, *CD10* and *GCTE1* in follicular lymphoma, and of *HDGFRP3* in mantle cell lymphoma. The differentially expressed miRNAs included *miR-21*, *miR-19b* and *miR-660*, all of

which were repressed in splenic marginal zone lymphoma relative to other low-grade B-cell lymphomas and had been previously identified as being deregulated in lymphoma cells.

In splenic marginal zone lymphoma, we found upregulated genes related to normal marginal zone, such as *TACI* (*TNFRSF13B*), interleukins (*IL6*, *IL7R*), relevant kinases, such as *PIP5K2A* and *EIF2AK2*, and genes already described in the splenic marginal zone lymphoma GEP, such as *MMP9*. All significant miRNAs had a lower level of expression in splenic marginal zone lymphoma than in other B-cell lymphomas. Thus, *miR-21* expression in splenic marginal zone lymphoma was a half that in follicular lymphoma and mantle cell lymphoma (Table 2; Figure 5).

Table 4 GSEA Pearson analysis

GSEA gene sets	miR-155 FDR	miR-34a FDR	miR-21 FDR
Memory B cells IgMgDCD27	0.127	NS	0.174
B-cell-T-cell calcium signaling	0.067	0.024	0.023
NF- κ B target gene	0.039	0.016	0.027
NF- κ B total pathway	0.123	0.04	0.036
CD40 during GC development	0.065	0.078	NS
Integrin pathway	NS	NS	0.136
IL2 pathway	0.113	0.179	NS
IL2RB pathway	0.077	NS	0.184
IL3 pathway	NS	0.165	NS
IL6 pathway	0.108	0.154	0.094
IL12 pathway	0.129	0.175	0.029
Inflammatory cytokine pathway	NS	0.149	0.151
T-cell cytokine signaling	0.099	0.162	0.025
RHO pathway	0.162	NS	0.137
TNFR2 pathway	0.081	0.018	0.245
JAK-STAT2 pathway	0.122	NS	0.031
JAK-STAT2 receptors	0.111	0.146	0.023
G2 pathway	0.121	NS	0.056
Cell cycle	0.118	0.197	0.149
Death pathway	0.139	0.127	0.138

Significant ($FDR < 0.25$) Pearson correlations associating upregulation of the most relevant overexpressed miRNAs in the assay with the enrichment of crucial B cells pathways and tumorigenesis-involved signaling pathways.

Validation of Microarray Data in an Independent Formalin-Fixed, Paraffin-Embedded Series

Microarray results were validated by a quantitative RT-PCR assay in an independent formalin-fixed, paraffin-embedded series. The set of miRNAs selected for validation by quantitative RT-PCR was a combination of those found to be differentially expressed in the previous analysis, including *miR-21*, *miR-155*, *miR-193b*, *miR-29c*, *miR-27b*, *miR-377*, *miR-145* and *miR-660* (Table 2).

The quantitative RT-PCR analysis confirmed higher expression levels in splenic marginal zone lymphoma *versus* reactive spleen of *miR-21*, *miR-155* and *miR-193b*, and a lower level of *miR-27b*, *miR-377* and *miR-145*, with significant *P*-values for all miRNAs except for *miR-193b*. The differential expression between splenic marginal zone lymphoma and the other B-cell lymphomas in the spleen was also confirmed for *miR-21* and *miR-660*. Full results are shown in Figure 6.

Discussion

The lack of specific markers in splenic marginal zone lymphoma is an obstacle to its diagnosis,

Figure 4 Splenic marginal zone lymphoma miRNA signature. Supervised clustering of splenic marginal zone lymphoma miRNA signature was able to discriminate between splenic marginal zone lymphoma cases in the spleen, lymph node infiltrated by splenic marginal zone lymphoma (LN-SMZL) and nodal marginal zone lymphoma (NMZL). Splenic marginal zone lymphoma cases in the spleen or lymph node appear clustered in comparison with nodal marginal zone lymphoma. The cluster was derived in the GEPAS 4.0 analysis suite (<http://gepas.bioinfo.cipf.es/>) using the SOTA algorithm. Significance was that associated with the Pearson correlation coefficient.

Figure 5 Splenic marginal zone lymphoma expression patterns with respect to other low-grade B-cell lymphomas: miRNAs and GEP. Hierarchical clustering of genes with FDR < 0.05 and -1- to 1-fold change, and miRNAs with FDR < 0.05 and -0.85- to 0.85-fold change. Splenic marginal zone lymphoma cases were compared with other lymphoma samples (follicular lymphoma, chronic lymphocytic leukemia and mantle cell lymphoma, all treated as a single class) using a *t*-test (<http://pomelo2.bioinfo.cnio.es/>). Some relevant genes are indicated with arrows.

especially in those cases where the diagnosis is required to be made from bone marrow or peripheral blood, but without information about the splenic histology. In the present work, we have investigated the GEP and miRNA expression pattern in a splenic marginal zone lymphoma series selected using highly conservative diagnostic criteria. This approach reduced the number of cases that could be analyzed but improved the chance of identifying consistent and specific molecular markers. Cases with intermediate properties or insufficient clinical data were excluded from the analysis.

The close similarity of the gene expression and miRNA profiles in the splenic marginal zone lymphoma series suggests that this disorder is a unique entity and highlights the homogeneity of our data. The miRNA expression profile in splenic marginal zone lymphoma comprises miRNAs regulating important B-cell pathways such as NF- κ B signaling, CD40 signaling, interleukins (IL2, IL6, IL12), inflammatory pathways and memory B-cell-related pathways. These findings confirm the similarity of splenic marginal zone lymphoma with the marginal zone.^{12,24–26} As already described in other low-grade lymphomas,¹⁶ the inflammatory cytokines present in the microenvironment may be involved in the onset and progression of disease contributing to the survival of tumor cells, consistently with what is already known about the

role of the inflammatory microenvironment in this lymphoma type.

Comparison of the miRNA signatures of splenic and nodal marginal lymphomas show that both entities differ in the expression of a group of selected miRNAs. Interestingly, lymph nodes infiltrated by splenic marginal zone lymphoma seem to cluster with spleens infiltrated by splenic marginal zone lymphoma in comparison with nodal marginal zone lymphoma, thus suggesting that splenic marginal zone lymphoma can be distinguished from disseminated nodal marginal zone lymphoma cases. Therefore, although these data require a further validation in a larger series, the two diseases seem represent separate entities, not just different stages of the same disorder.

We found previously described miRNAs forming part of the splenic marginal zone lymphoma signature that were deregulated in this lymphoma type, such as the overexpressed *miR-21*, *miR-34a* and *miR-155*, the repressed *miR-139*,¹⁵ and miRNAs deregulated in other tumors, such as *miR-21*,^{27–30} *miR-34a*³¹ and *miR-193b*.³² The most significant miRNAs observed in the splenic marginal zone lymphoma signature were *miR-155*, *miR-34a*, *miR-193b*, *miR-21*, *miR-377*, *miR-376a*, *miR-27b* and *miR-145*. The expression pattern of these miRNAs has been previously reported in the different B-cell stages, so that *miR-155*, *miR-34a* and *miR-193b* were

Figure 6 Quantitative RT-PCR assay for miRNAs. Expression data (mean RQ) of the validated quantitative RT-PCR miRNAs included in the splenic marginal zone lymphoma signature compared with reactive spleen and other low-grade B-cell lymphomas (follicular lymphoma, chronic lymphocytic leukemia and mantle cell lymphoma). RQ values are shown below the corresponding graph, and the *P*-values associated with *t*-tests are shown on the bars for each comparison. The miRNAs provided valuable data in the quantitative RT-PCR assay with values of Ct < 32. Oblique hatching represents reactive spleen (RLH) cases; light gray, splenic marginal zone lymphoma (SMZL) cases; vertical hatching, follicular lymphoma (FL) cases; cross-hatching, chronic lymphocytic leukemia (CLL) cases; and black, mantle cell lymphoma (MCL) cases.

found overexpressed in memory B cells, the converse of what is found in naive B cells.¹² *Mir-21* has a higher level of expression in memory cells than in both naive and germinal center cells,^{12,13,25} *miR-145* is more weakly expressed in memory than in naive B cells,²⁵ and *miR-27b* has recently been described as being downregulated in memory B cells relative to both naive and GC B cells.^{12,25} In addition, *miR-21*, *miR-193b* and *miR-34a* were upregulated in splenic marginal zone lymphoma B cells relative to normal B cells in this series. Thus, the miRNome of splenic marginal zone lymphoma determined in the present study suggests that this tumor is a neoplastic expansion of memory B cells, similar to what our group has observed for nodal marginal zone lymphoma.¹⁶

Our findings confirm the increased expression of *miR-155* in splenic marginal zone lymphoma¹⁵ and other lymphoid neoplasias.^{27–29,33} *Mir-155* is involved in the development of different B-cell differentiation stages³⁴ and helps regulate essential

immune functions.³⁵ Also, *miR-155* targets the *PLEKHG5* gene, which is involved in the NF- κ B signaling pathway,³⁶ and thus *miR-155* could be involved in NF- κ B upregulation in splenic marginal zone lymphoma. The splenic marginal zone lymphoma signature contains others relevant overexpressed miRNAs, such as *miR-34a* and *miR-193b*, which are upregulated in cancer, *miR-34a* in chronic lymphocytic leukemia³¹ and *miR-193b* in malignant melanoma.³² These miRNAs have a predicted target in the *TNFAIP1* gene and could therefore regulate this gene. *TNFAIP1* was repressed in our splenic marginal zone lymphoma series and has been described as an apoptosis regulator,³⁷ so that overexpression of *miR-34a* and *miR-193b* could be involved in regulating apoptosis in splenic marginal zone lymphoma cells, via *TNFAIP1*.

Mir-21 is one of the miRNAs most closely related to lymphoma development, and is overexpressed in chronic lymphocytic leukemia,²⁷ diffuse large B-cell lymphoma,²⁸ acute myeloid leukemia,³⁰ Hodgkin

lymphoma²⁹ and other diseases. The *TSPYL5* gene, which is involved in PTEN-AKT signaling, is a target of *miR-21* and could be regulated by this miRNA. The overexpression of *miR-21* could be responsible for *TSPYL5* suppression, resulting in *PTEN* cellular deregulation, followed by changes in AKT activation. This *TSPYL5* suppression could also determine the expression level of *p21* and subsequently induce deregulation of cell growth.³⁸ Thus, the upregulation of this oncogenic miRNA could contribute to the onset and progression of this lymphoma type.

RHOH and *AIM2*, which have oncogenic activity and are upregulated in splenic marginal zone lymphoma,⁹ could be overexpressed as a consequence of the downregulation of *miR-27b*, as the loss of *miR-145* could allow the upregulation of the splenic marginal zone lymphoma-relevant integrin *CD40*. The relevance of genes regulated by these two miRNAs in splenic marginal zone lymphoma suggests that the loss of *miR-27b* and *miR-145* may contribute to this lymphoma subtype.

The enrichment of relevant pathways involved in B-cell survival, like NF- κ B, *CD40* and interleukins, and cancer-related pathways, such as *RHO*, *TNFR* and *JAK-STAT*, is correlated with the upregulation of miRNAs, such as *miR-155*, *miR-21* and *miR-34a* in this series of cases, suggesting that the concerted regulation of these miRNAs could help regulate these pathways and thereby contribute to the survival of lymphoma cells.

Although the miRNA signature of SMZL does not include miRNAs located in 7q32, when we compared the miRNA expression of B cells from splenic marginal zone lymphoma patients with those from healthy donors, we found two repressed miRNAs, *miR-29b* and *miR-592*, located in 7q32. This is consistent with the loss of this band in the splenic subtype of marginal zone lymphoma and with previous observations.^{4,5} Moreover, the expression of miRNAs may be subject to regulatory mechanisms independent of the loss in 7q, such as methylation and histone modification.

Comparison of the miRNA expression pattern in splenic marginal zone lymphoma and other low-grade B-cell lymphomas when the spleen is involved revealed a set of 9 miRNAs with statistically differential expression between the two groups. Of these, 3 were already known to be deregulated in lymphoma cells: *miR-21*, *miR-19b* and *miR-660*. In malignant lymphoma, an miRNA study found genomic upregulation of *miR-19b*,³⁹ while *miR-660* was found to be upregulated in leukemic cells⁴⁰ and *miR-21* has been described in cancer on several occasions.^{27,28,30} Similarly, GEPs from follicular lymphoma, chronic lymphocytic leukemia and mantle cell lymphoma involving the spleen compared with the splenic marginal zone lymphoma GEP showed overexpression of known markers and of previously described genes. The discovery of additional genes in the context of other

lymphomas is consistent with our results, eg, *CD200*⁴¹ and *TCL1A*⁴² in chronic lymphocytic leukemia, *HDGFRP3*⁴³ in mantle cell lymphoma, and germinal center markers such as *BCL6*, *CD10* and *GCTE1* in follicular lymphoma. The genes and miRNAs found in other lymphomas support our data in splenic marginal zone lymphoma, which include those from genes related to normal marginal zone (*TACI*), interleukins (*IL6*, *IL7R*) and several interesting kinases, such as *PIP5K2A* and *EIF2AK2*. *PIP5K2A* is a second messenger that regulates the levels of phosphatidylinositol by increasing *AKT* activity⁴⁴ and *EIF2AK2* is a kinase that takes part in regulating protein synthesis.⁴⁵ Lastly, we also found genes, such as *MMP9*, that have previously been described in the splenic marginal zone lymphoma GEP.⁹

Our study did not set out to identify the causes of the changes in the expression of the miRNAs identified. The miRNA splenic marginal zone lymphoma signature contains several downregulated miRNAs located in 14q32, including *miR-376a* and *miR-377* among others, which are lost in splenic marginal zone lymphoma compared with non-tumoral spleen and other low-grade B-cell lymphomas. The finding is consistent with the recently published results showing 14q alterations in splenic marginal zone lymphoma,⁶ which are also associated with poor prognosis.⁴⁶ Downregulation of these miRNAs could also be involved in the growth and progression of splenic marginal zone lymphoma through the regulation of oncogenic target genes such as *MUM1*, *FOXP1*, *SYK* and *BCL2*.

In summary, the present study shows that the splenic marginal zone lymphoma miRNA expression profile reproduces the normal marginal zone and memory B-cell program. The most relevant miRNAs of the signature have target genes involved in some of the most important pathways involved in the splenic marginal zone lymphoma pathogenesis, such as NF- κ B and *CD40* pathways, and thus could be deregulated through the concerted action of these miRNAs in splenic marginal zone lymphoma.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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