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MicroRNA expression in B-cell lymphomas

by

Lorena Di Lisio

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“Somewhere, something incredible is waiting to be known”

Cit. Carl Sagan

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Publications

L Di Lisio, M Sánchez-Beato, G Gómez-López, M E Rodríguez, S Montes-Moreno, M Mollejo, J Menárguez, M A Martínez, F J Alves, D G Pisano, M A Piris and N Martínez

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Summary/Resumen

Summary

Accurate lymphoma diagnosis, prognosis and therapy still require additional markers. Additionally, some aspects of lymphoma pathogenesis are still partially unexplained.

In this work the potential relevance of microRNA (miRNA) expression in a large series of cases that included all major B-cell lymphoma types has been investigated.

First, a series of 147 fresh frozen lymphomas and 15 controls were inspected for their miRNA expression profile. This analysis yielded a signature of 128 miRNAs that enabled the characterization of the different B-cell lymphoma types. Then a second series of cases was used to corroborate the differential expression of selected miRNAs *vs*. non-tumour controls.

The viability of using miRNAs as additional markers for differential diagnosis has been investigated by comparing Burkitt Lymphoma (BL) *vs.* Diffuse Large B-cell Lymphoma (DLBCL) and resulted in the identification of 19 significant miRNAs (False Discovery Rate < 0.05).

Not only: the correlation between miRNA expression profiles, gene expression profiles and pathway activation was examined in Mantle Cell Lymphoma (MCL), suggesting the implication of different miRNAs in pathway regulation. In particular, loss of miR-26a may contribute to NF-kB pathway activation.

Furthermore the clinical prognostic value of miRNAs has been examined in MCL cases, where miR-20b was identified as a good candidate for overall survival stratification of the patients.

Finally, selected miRNAs were sequenced to test the presence and the relevance of miRNA sequence variation in a series of 95 DLBCL cases.

In summary this work identifies good candidate miRNAs that might be used to better recognise the different B-cell lymphoma types (especially BL and DLBCL), and other miRNAs that could contribute to lymphoma pathogenesis elucidation.

Resumen

Introducción

El diagnóstico de los linfomas si bien está claramente establecido por la Organización mundial de la salud (WHO), está sujeto a continuas revisiones debidas a nuevos descubrimientos y a la mayor disponibilidad de técnicas que puedan ayudar a definir con precisión los distintos tipos tumorales.

Uno de los descubrimientos más valorados en los últimos años es la existencia de los microRNAs (miRNAs): secuencias cortas de RNA cuya actividad es la represión de la expresión de proteínas.

El papel de los miRNAs en el desarrollo de distintas enfermedades incluyendo el cáncer ya ha sido demostrado, y su expresión anómala ha sido relacionada con un número cada día mas alto de enfermedades complejas. Además, la expresión diferencial de los miRNAs ha sido demostrada en distintos tipos de tumores, pero mucho queda por investigar dado que los miRNAs son un grupo de moléculas que se han empezado a estudiar de forma sistemática hace solo una decena de años.

En este trabajo serán estudiados los principales tipos de linfomas de células B: linfoma de Burkitt (BL), leucemia linfática crónica (CLL), Linfoma B difuso de célula grande (DLBCL), linfoma folicular (FL), linfoma de células del manto (MCL), linfomas del tejido linfoide asociado a mucosas (MALT), linfoma ganglionar de zona marginal (NMZL) y linfoma esplénico de la zona marginal (SMZL). Los controles utilizados serán ganglios reactivos, amígdalas y bazos, además de células purificadas procedentes de la zona del manto en el análisis específico de los casos de linfoma de células del manto.

Objetivos

Este trabajo se propone:

- Analizar el perfil de expresión de miRNAs en una serie de linfomas B y controles
- Investigar la utilidad de los miRNAs como marcadores para diagnóstico diferencial y supervivencia global
- Evaluar el papel de los miRNAs en el desarrollo del linfoma de células del manto
- Buscar la existencia de variaciones en la secuencia nucleotídica de algunos miRNAs

Materiales y métodos

En primer lugar se han evaluado los niveles de expresión de los miRNAs de 147 linfomas B, 15 tejidos de control, 8 líneas celulares y 3 muestras no tumorales de células específicas de la zona del manto, mediante hibridación de arrays de expresión de miRNAs de un color, incluyendo 470 miRNA humanos.

Para validar la expresión de los miRNAs seleccionados se efectuó PCR cuantitativa en muestras de tejido congelado o parafinado.

En el caso de los linfomas de células del manto, se estudió también su perfil de expresión génica mediante arrays de expresión.

Los datos fueron normalizados mediante un script desarrollado por el grupo de bioinformática del CNIO con el programa R (http://www.r-project.org/).

La evaluación de los datos de arrays de miRNAs se efectuó mediante el programa *significance analysis of microarray* (SAM) y el algoritmo *K-nearest neighbour* (KNN) (http://tnasas.bioinfo.cnio.es/). Los datos de PCR cuantitativa se analizaron mediante t-test (http://pomelo2.bioinfo.cnio.es/) y comparando directamente los –ΔCT. El análisis de supervivencia se realizó con el programa SPSS.

Resultados

El análisis de los 147 casos procedentes de los distintos tipos de linfomas permitió detectar, mediante el programa SAM, 128 miRNAs significativos (FDR<0.01); mientras el algoritmo KNN identificó 120 miRNAs cuya expresión facilita la correcta clasificación del 86,4% de las muestras.

Las mismas 147 muestras fueron evaluadas también en conjunto frente a tejido no tumoral de ganglios reactivos y amígdalas (bazos para los SMZL). Una serie de miRNAs resultaron significativos y algunos de ellos se validaron en una segunda serie de 66 linfomas de células B (incluyendo todos los tipos de linfomas de la serie inicial) y 8 controles (4 ganglios reactivos, 3 amígdalas y un bazo). Los miRNAs que mejor se validaron fueron: miR-31 y miR-133a (perdidos en los tumores) y el miR-513 (ganado en los tumores).

Para evaluar la hipótesis de que los miRNAs pueden ser utilizados para discriminar entre dos tipos de linfomas se compararon también BL y DLBCL. Este análisis identificó 43 miRNAs

significativos, que fueron investigados en una segunda serie de 71 muestras. Diecinueve miRNAs fueron validados, entre ellos: miR-146a, miR-155, miR29b y miR-17-3p.

En la serie de casos de linfomas de células del manto analizada, se han identificado 117 miRNAs significativamente ganados o perdidos en los tumores (FDR<0.05) con respecto a los controles. La correlación de estos datos con datos de expresión de genes ayudó a identificar una serie de miRNAs potencialmente relevantes en la activación de rutas celulares relevantes. Entre ellos miR-26a, que también resultó perdido en las líneas celulares. Experimentos funcionales en las líneas celulares demostraron que la reintroducción de miR-26a obstaculiza la activación de la ruta de NF-kB, confirmando la hipótesis de que los miRNA pueden tener relevancia no solo sobre la expresión de proteínas, sino también en la activación de rutas biológicas.

La correlación de los miRNAs con la supervivencia fue inicialmente evaluada en 22 casos de los linfomas de células del manto utilizando los datos de los arrays de miRNAs. Los resultados fueron validados en una segunda serie de 54 casos procedentes de tejido parafinado. La expresión de miR-20b se encontró relacionada con la supervivencia global. La sobre-expresión de ese miRNA fue relacionada con un peor pronóstico.

Por último se secuenciaron 14 miRNAs en una serie de 95 casos de DLBCL. Este análisis identificó la existencia de diversos polimorfismos ya conocidos y también la existencia de variantes en miR-588 y miR-650 que en este momento aún no se encontraban descritas en las bases de datos del *National Center for Biotechnology Information* (NCBI) y Ensembl.

Discusión

El papel de los miRNAs en el desarrollo del cáncer ha sido demostrado numerosas veces. Este trabajo está dirigido por un lado a la identificación de miRNAs que puedan ayudar con el diagnóstico diferencial y con la evaluación del pronóstico, por otro lado a la identificación de miRNAs que tengan un papel en la patogénesis de los linfomas.

Algunos de los miRNAs encontrados diferencialmente expresados están probablemente relacionados con las características de cada tipo de linfoma, como por ejemplo la sobre-expresión de los miRNAs del *cluster* miR-17-92 y la sobrexpresión de MYC, especialmente en BL.

Algunos de los miRNAs relevantes en el diagnóstico diferencial entre BL y DLBCL han sido también encontrados en otra serie independiente, reforzando la hipótesis de que esta técnica es reproducible y que estos miRNAs son realmente útiles en el diagnóstico diferencial.

Una de las novedades introducidas en este trabajo es la correlación entre la expresión de los miRNAs con la activación de las rutas celulares. El miR-26a, cuya perdida en los casos de linfomas de células del manto se ha demostrado relacionada con la activación de NF-kB en líneas celulares, es uno de los pocos miRNAs que se estudiaron también *in vivo* y que quizás podría ser un buen candidato para desarrollar algún ensayo clínico.

Finalmente, la evaluación de la relevancia de variantes en la secuencia de los miRNAs es de difícil estimación debido a que aun hay pocos estudios sobre este tema y por la forma de actuar propia de los miRNAs.

ABBREVIATIONS

ABC Activated B-Cell

AID activation-induced cytidine deaminase

ATM ataxia telangiectasia mutated

BL Burkitt Lymphoma

BAX BCL2-associated X protein

BCL2 B-cell CLL/lymphoma 2

BCL6 B-cell CLL/lymphoma 6

BLIMP see PRDM1

BMI1 polycomb ring finger oncogene

BSA Bovine Serum Albumin

CCND1 cyclin D1

CCNE2 Cyclin E2

CD Cluster of Differentiation

CDKN1A cyclin-dependent kinase inhibitor 1A, also called p21

CDKN1B cyclin-dependent kinase inhibitor 1B, also called p27

CDKN2A cyclin-dependent kinase inhibitor 2A, also called p16 or ARF

CDKN2B cyclin-dependent kinase inhibitor 2B, also called p15,

CDK4 cyclin-dependent kinase 4

CDK6 cyclin-dependent kinase 6

cDNA complementary DNA

CHK2 checkpoint kinase 2

CHOP cyclophosphamide, doxorubicin, vincristine -also called Oncovin- and prednisone

Chr Chromosome

CGH Comparative genomic hybridization

CNV Copy Number Variantion

CLL Chronic Lymphocytic Leukaemia

Cluster 17-92 includes miR-17-18a-19a-19b-20a-92 (also known as oncomir-1)

Ct Threshold cycle

MYB v-myb myeloblastosis viral oncogene homolog

MYC is v-myc myelocytomatosis viral oncogene homolog

CNIO Centro Nacional de Investigaciones Oncológicas

Cy3 Cyanine 3-conjugated dUTP

Cy5 Cyanine 5-conjugated dUTP

DICER1 Dicer 1, ribonuclease type III

DLBCL Diffuse Large B Cell Lymphoma

DMEM Dulbecco's Modified Eagle Medium

DNA Desoxiribonucleic acid

dNTP 2'-Deoxyribonucleoside-5'-triphosphate

DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen

DTT Dithiothreitol

EBV Epstein Barr Virus

EZH2 enhancer of zeste homologue 2

FAM 6-carboxyfluorescein

FBS Fetal Bovine Serum

FC Fold change

FDR False discovery rate

FE Feature extraction

FF Fresh frozen

FFPE Formalin fixed paraffin embedded

FISH Fluorescent In Situ Hybridization

FL Follicular Lymphoma

FSCN1 fascin homolog 1, actin-bundling protein

FUS1 see TUSC2

GC Germinal Center

GCET2 germinal center expressed transcript 2

GEP Gene expression profile

GEPAS Gene expression profile analysis suite

GSEA Gene set enrichment analysis

h hours

H&E Haematoxylin and Eosin

HL Hodgkin lymphomas

HR Hazard Ratio

hsa-miR- Homo sapiens miRNA (Since most of the miRNA here described are of human origin "hsa" been generally omitted)

IARC International Agency for Research on Cancer

Ig Immunoglobulin

IgH Ig Immunoglobulin heavy chain

IgVH Ig Immunoglobulin variable heavy chain

IFIMAV Instituto de Formación e Investigación MArqués de Valdecilla

Intermediate BL/DLBCL B-cell lymphoma, unclassifiable, with features intermediate between BL and DLBCL

IPI International Prognostic Index

Ki-67 Antigen identified by monoclonal antibody

KNN K-nearest neighbour

KRAS v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

JAK Janus kinase

LN Lymph Node

LMO2 markers including LIM domain only 2

LPL Lymphoplasmacytic Lymphoma

MAPK mitogen-activated protein kinase

MAP3K2 mitogen-activated protein kinase kinase kinase 2, also called MEEK2

MAP3K14 mitogen-activated protein kinase kinase kinase 14, also called NIK

MALT Mucosa Associated Lymphoid Tissue

MCL Mantle Cell Lymphoma

MCL-1 myeloid cell leukemia sequence 1 (BCL2-related)

MEKK2 see MAP3K2

min minute

miRNA micro RNA/Micro ribonucleotic acid

mRNA Messenger RNA

ms milliseconds

MUM1 melanoma associated antigen (mutated) 1

MZL Marginal Zone Lymphoma

NA Not available

NCI National Cancer Institute

NCBI National Center for Biotechnology Information

ND No data

NF-kB Nuclear Factor Kappa B

NHL Non-Hodgkin lymphomas

NK Natural Killer

NMZL Nodal Marginal Zone Lymphoma

NSCLC non-small cell lung cancer

PAX5 target paired box 5

PBS Phosphate Buffered Saline

PcG DNA-binding Polycomb-group family

PCR Polymerase Chain Reaction

PRDM1 PR domain containing 1, with ZNF domain, also called BLIMP1

Pre-B-Cell Precursor B Cell

Pri-B-Cell Primitive B Cell

Pre-miR (or pre-miRNA) Precursor microRNA

Pri-miR (or pri-miRNA) Primitive microRNA

PTEN phosphatase and tensin homolog

PUMA P53 Up-regulated Modulator of Apoptosis

qPCR quantitative polymerase chain reaction

p65 see RELA

Oncomir-1 It includes miR-17-18a-19a-19b-20a-92 (also known as cluster 17-92)

OS Overall survival

RB1 retinoblastoma 1

RELA v-rel reticuloendotheliosis viral oncogene homolog A, also known as p65

R-CHOP Rituximab cyclophosphamide, doxorubicin, vincristine and prednisone

RISC RNA-induced gene silencing complex

RNA Ribonucleic acid

RNAi RNA interference

rmp Revolutions per minute

RPMI Roswell Park Memorial Institute medium

RRAS2 RAS viral oncogene homolog 2

RT Reverse Transcription

RT-qPCR Real time quantitative polymerase chain reaction

SAM Significance analysis of microarrays

sec second

SDS Sodium Dodecyl Sulfate

SEER Surveillance, Epidemiology and End Results

shRNA Short Hairpin RNA

SOCS1 suppressor of cytokine signalling 1

SOCS2 suppressor of cytokine signalling 2

SOTA Self-Organizing Tree Algorithm

SMZL Splenic Marginal Zone Lymphoma

SNP Single nucleotide polymorphism

STAT signal transducer and activator of transcription

T tonsil

TGF-β1Transforming Growth Factor-beta1

TCL1A T-cell leukemia/lymphoma 1A, also called TCL1

TNFAIP3 alpha-induced protein 3

TP53 tumour protein p53

TUSC2 tumour suppressor candidate 2 (also known as FUS1)

USA United States of America

UTR untranslated region

vs. versus

WB Western Blot

WHO World Health Organization

-3p miRNA derived from the 3' arm of the pre-miRNA

-5p miRNA derived from the 5' arm of the pre-miRNA

* less abundant miRNA mature form

1. Introduction

1.1. Lymphomas

1.1.1. Basic features of lymphomas distinction

Lymphomas constitute a large group of neoplasms derived from B, T or NK cells, or their progenitor cells. The first distinction to address among lymphomas is between Hodgkin Lymphomas (HL) and non-Hodgkin Lymphomas (NHL). The former type accounts for about 13% of lymphomas and its major recognizing feature is the presence of Reed Stemberg cells. The latter type, NHL, is a larger group of heterogeneous neoplasms accounting for the resting 87% of Lymphomas. Only a small fraction of NHL are derived from T and NK lymphocytes (about 10%), while the rest of NHL are derived from B-Cells (90%). B-cell lymphomas still represent a group of diseases that includes more than 20 neoplasms, some of them rare, but all of them sharing the common background of B-cell origin (Swerdlow et al., 2008).

1.1.2. Epidemiology

One of the most important organisms that take care about epidemiology of cancer in USA is the National Cancer Institute (http://seer.cancer.gov). According to their data, NHL represents about 4-5% of new diagnosed cancers. The incidence B-cell lymphomas is 16.5 out of 100.000 people per year. Survival rate is of about 70.4% at five years. The median age of diagnosis is 67 years. Lifetime risk is assessed at 2.12%, it means that about 1 person out of 47 will probably be diagnosed for a NHL during his (or her) lifetime.

In Europe there are about 88000 new cases per year of NHL and in Spain they are about 6300 as reported by the International Agency for Research on Cancer (AIRC) in collaboration with the World Health Organization (WHO) (http://globocan.iarc.fr). More information about cancer incidence in Spain is available at the National Statistical institute of Spain web site: INE at www.ine.es.

Lymphomas epidemiology data can be slightly different depending on the specific type of B-cell lymphoma, the age and geographic region of the group of study (Swerdlow et al., 2008). Figure 1 summarizes the frequencies of the most common B-cell lymphoma types (Armitage and Weisenburger, 1998). In general, Diffuse large B-cell lymphoma (DLBCL), followed by Follicular Lymphoma (FL) are the most frequently detected.

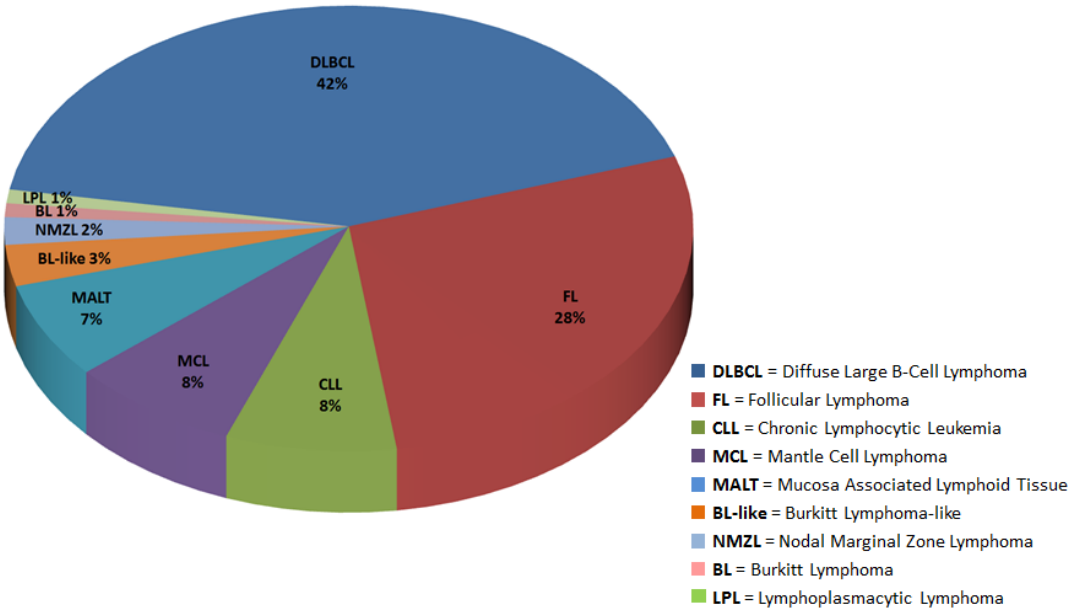


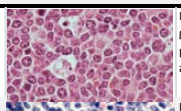
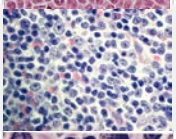
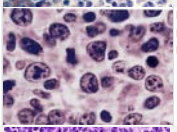
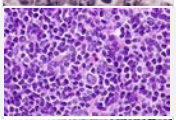
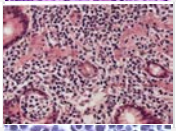
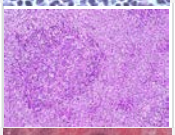
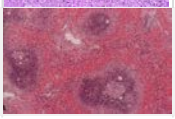
Figure 1. Relative frequencies of each B-cell lymphoma type (Armitage and Weisenburger, 1998).

1.2. B-cell lymphomas

The WHO establishes lymphoid tumour classification mainly according to the clinical history of the patient, the histopathological morphology of the tumour, the immunophenotype and chromosomal alterations. Their guidelines are collected in the book: "WHO classification of Tumours of Haematopoietic and Lymphoid Tissues" (Swerdlow et al., 2008). Moreover, B-cell lymphoma characteristics have been reviewed in many manuscripts (Jaffe, 2009; Kuppers, 2005; Lenz and Staudt, 2010). Principal details of the B-Cell lymphomas neoplasms included in this study are summarized in Table 1 according with WHO description (Swerdlow et al., 2008). Diagnosis, prognosis and relapse may vary according to the specific lymphoma type (Swerdlow et al., 2008).

Even if their classification and therapy protocols are already established, revision of these criteria is undertaken continuously.

Table 1. Summary of the main characteristics of the lymphoma types used in this work according whit WHO criteria (Swerdlow et al., 2008).

Lymphoma Type	Principal site of involvement	Principal alterations	Prognosis/OS	Differential diagnosis	Immunophenotype	Principal morphology features	
BL	Lymph node, Central nervous system possible involvement (sporadic form)	MYC translocation t(8:14)(q24;q32) or t(8;22)(q24 ;q11) or t(8;2)(q24;p12)	Aggressive, good response to treatment.		Positive for: CD19, CD20, CD22, CD10, BCL6, CD38, CD77, CD43, IgM (with light chain restriction), Ki67. Negative for: BCL2,TdT		Medium sized cells whit diffuse growth pattern and "starry sky" pattern. High proliferation and apoptosis fraction
CLL	Peripheral blood, bone marrow, lymph node, spleen, liver	13q14.3del in 50% of the cases, trisomy 12 is frequent. 11q22-23del (ATM) 17p13del (TP53), 6q21del	Sometimes indolent curse. There is an aggressive variant.	With MCL	Positive for: IgM, IgD, CD20, CD22, CD5, CD19, CD79a, CD23, CD43, CD11c (week). Negative for CD10 and CyclinD1.		Proliferation centres. Small lymphocyte, clumped chromatin, round nucleus, low mitotic activity. Some cases have plasmacytoid differentiation
DLBCL	Lymph node, gastro intestinal tract, bone marrow	ABC subtype: gain 3q, 18q21-q22, loss 6q21-q22. GCB subtype: gain 12q12 BCL2, BCL6 and MYC rearrangement	Heterogeneous. GCB subtype show better clinical outcome, 60% of treated patients survive more than 5 years	Wth BL and blastoid MCL	Positive for: CD19, CD 20+, CD22+, CD79a, Ki67, IgM>IgG>IgA. Note: CD10+ in 60%, BCL6+ in 80%, P53+ in 50%		Proliferation of large lymphoid cells. Partial nodal involvement. Different DLBCL subtypes.
FL	Lymph node, bone marrow, peripheral blood, spleen, gastro intestinal tract, skin	Up to 90% t(14;18)(q32;q21) BCL2 rearrangements. 5-15% 3q27 and or BCL6 rearrangement	Long term survival up to 8 years	With DLBCL. Progression to DLBCL is also possible	Positive for: Slg+, CD19, CD20, CD22, CD79a, BCL2, BCL6, CD10. Negative for: CD5, CD43, IRF4/MUM1. Note: (IgM+/-, IgD, IgG, or rarely IgA)		Neoplastic follicles are often poorly defined and usually have attenuated or absent mantle zones. Centroblasts and centrocytes lost their normal distribution (polarization)
MALT	Gastro intestinal tract, salivary gland, head and neck, ocular adnexa, skin, thyroid, breast	t(11;18)(q21;q21) (API2-MALT1) t(1;14)(p22;q32) (BCL10) t(14;18)(q32;q21) (MALT1) t(3;14)(p14.1;q32) (FOXP1)	Frequent indolent course and slow dissemination. Long disease free intervals	With H. pylori infection, FL, MCL, CLL, Progression to DLBCL	Positive for: IgM, (less frequently for IgA or IgG) CD20, CD79a, CD21 and CD35. Negative for: CD5, CD10, CD23. Note: Light chain restriction, CD43+/- (weak) and CD11c+/- (weak). No specific marker so far		Small tumoral lymphocytes infiltrates around the B-cell follicles, external to follicle mantle. Large cells resembling centroblast or immunoblast are usually present. Lymphoepithelial lesions.
MCL	Lymph node. Spleen, peripheral blood. gastro intestinal tract.	CyclinD1 alteration t(11;14)(q13;q32)	3-4 years. Aggressive forms whit worse course are identifed as blastic	MZL	Positive for: IgM/IgD, CD5, CD43, CyclinD1. Negative for: CD10, CD23, BCL6		Monomorphic lymphoid proliferation in the mantle zone area. Cells are from small to medium size. Nuclei can be irregular
NMZL	Lymph node	Trisomies 3,18 and 7	Generally low aggressivity: about 70% of patients survive longer that 5 years	Progression to DLBCL	Positive for: pan-B-cell markers, CD43 in 50% of the cases, BCL2. Negative for: CD5, CD23, CD10, BCL6, CIyclinD1. Note: IgD+ sometimes		Tumor cells surround reactive follicles and expand into the interfollicular areas. Tumor cells are composed of variable numbers of marginal zone (centrocyte-like and monocytoid) B-cells, plasma cells and scattered transformed B-cells
SMZL	Spleen	7q31-32del in 40% cases. 7q21 (CDK6) deregulation. Trisomy 3q	Slow progression. Sometimes indolent course, Aggressive only in some cases.	With CLL,MCL,FL, LPL	Positive for: IgM, IgD, CD20, CD79a. Negative for: CD5, CD10, CD23, CD43,annexinA1, CyclinD1, CD103 usually neg.		Lymphocytes replace splenic withe pulp or reactive germinal center with effacement of follicle mantle. Infiltration of red pulp. Plasma cells can be found in the center of white pulp nodules

1.2.1. B-cell lymphoma cell of origin

It is believed that the different lymphoma types have their origin at different stage of B-cell development. It is documented by somatic hypermutation status and surface marker expression of tumour cells (Alizadeh et al., 2000; Chapman et al., 1996; Chiorazzi et al., 2005; Hummel et al., 1994; Pascual et al., 1994; Swerdlow et al., 2008; Zhu et al., 1995).

The origin of the lymphoma types studied in this thesis is summarized in Figure 2 (Kuppers, 2005; Swerdlow et al., 2008).

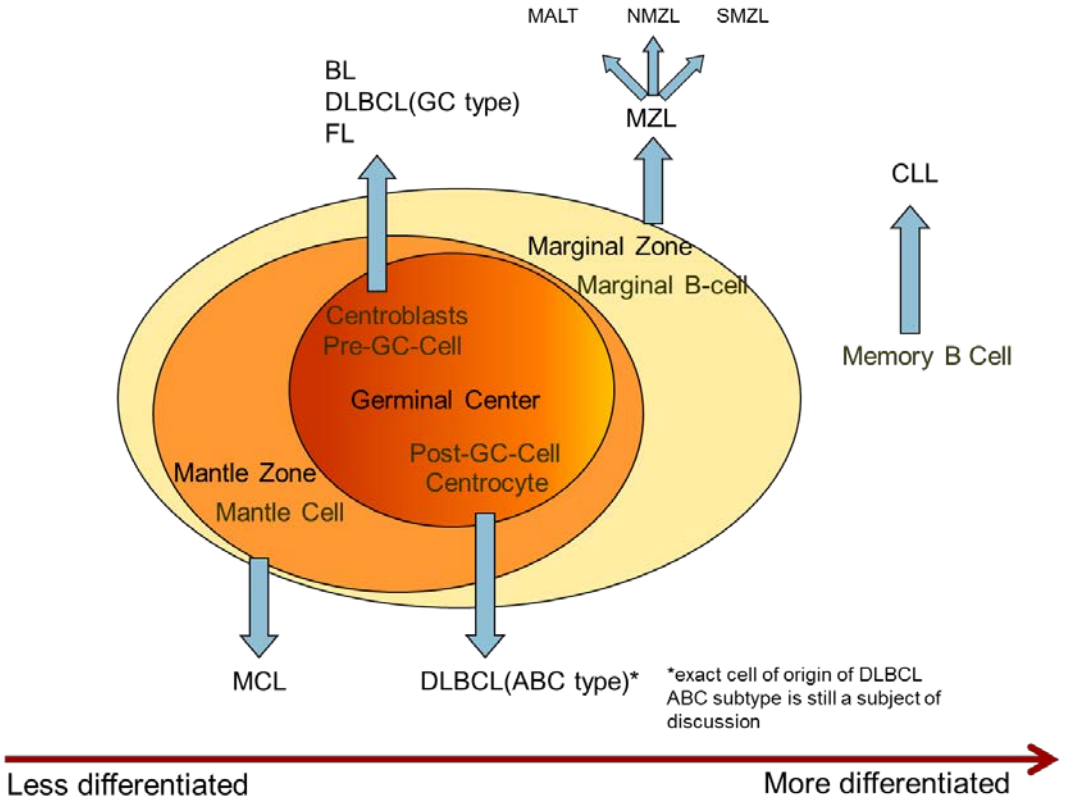


Figure 2. The different maturation steps of B-cell are here illustrated together with the hypothetical lymphomas which may derive from these cells. MCL=Mantle Cell Lymphoma, BL= Burkitt Lymphoma, FL=Follicular Lymphoma, CLL=Chronic Lymphocytic Leukemia, MZL=Marginal Zone Lymphoma, MALT=Mucosa Associated Lymphoid Tissue, NMZL=Nodal marginal zone lymphoma, SMZL=Splenic Marginal Zone Lymphoma, DLBCL=Diffuse large B-cell lymphoma, GC=Germinal Center, ABC=Activated B-Cell.

1.2.2. Lymphoma Treatment

Treatment scheme in NHL varies according to the lymphoma type, the extent of the tumour, the stage, the score of International Prognostic Index (IPI), the site of involvement and the expression of specific markers which account for their aggressiveness.

The web site of American Cancer Society (www.cancer.org) offers a general, and updated, view of different cancer treatments.

A description of the three B-cell lymphomas types investigated more in depth in this work (MCL, BL and DLBCL) is provided as follows.

1.2.3. Mantle Cell Lymphoma (MCL)

1.2.3.1. General features

Mantle cell lymphoma is a tumour accounting for 6-7% of non-Hodgkin lymphomas (Armitage and Weisenburger, 1998). The median age of presentation is about 63 years and high rate of male prevalence, unfortunately it is frequently diagnosed at stage III-IV (Armitage and Weisenburger, 1998).

The hallmark of this tumour is the overexpression of MCL cyclin D1 (CCND1) (de Boer et al., 1993), the other main characteristics of MCL are listed in Table 1.

Besides the classical high-grade, small B-cell form of MCL, other subtypes are described. Among them the blastoid variant is the most common, and is generally more aggressive (Raty et al., 2003).

Additional heterogeneity is documented inside this disease, and a further stratification has been proposed according to DNA abnormalities (Thelander and Rosenquist, 2008).

1.2.3.2. Molecular characteristics

MCL probably originates from follicular mantle zone cells (Hummel et al., 1994; Swerdlow et al., 2008), therefore the majority of MCL patients do not show hypersomatic mutations and expresses high levels of IgM and IgD (Jares et al., 2007). Nevertheless up to 40% of MCL cases may carry somatic hypermutation of IgVH genes, suggesting a different origin of this subset of tumour that is probably derived from cells that underwent germinal center reaction (Camacho et al., 2003; Kienle et al., 2003; Orchard et al., 2003; Welzel et al., 2001).

Overexpression of cyclin D1 can be detected in virtually all patient and is caused principally by the translocation t(11;14)(q13;q32) that brings the CCND1 gene under the control of the promoter of immunoglobulin heavy chain (IgH) (Jares et al., 2007; Raffeld and Jaffe, 1991).

The consequence of overexpression of Cyclin D1 is the deregulation of the cell cycle at the transition G_1/S. In fact Cyclin D1 participates in the control of the G1 phase by binding the cyclin-dependent kinase 4 (CDK4). These complexes phosphorylate and inactivate retinoblastoma 1 (RB1), abrogating its suppressor effect on cell cycle progression (Ewen et al., 1993).

There is a minor percentage of patients in which the classical genetic alteration of CCND1 is not detected. It has been proposed that in these subjects overexpression of Cyclin D2 (CCND2) and Cyclin D3 (CCND3) may drive similar effects of CCND1 alteration (Fu et al., 2005).

Nevertheless, MCL pathogenesis is yet to be fully explained, since genetic changes so far identified cannot account for all of the main features of the tumour cells. In fact, *in vivo* experiments, showed that cyclin D1 overexpression alone does not fully justify tumour development (Bodrug et al., 1994).

Indeed, there are still various MCL oncogenic features that are not explained by the alterations so far identified.

Other recurrent chromosomal alterations can be found in MCL samples.

These alterations affect the expression of several proteins involved in the regulation of cell cycle, such as the cyclin-dependent kinase 4 (CDK4) on Chr 12q13, the cyclin-dependent kinase inhibitor 2A (CDKN2A, also called p16 or ARF), which is on Chr 9p21 and MYC on Chr 8q21.

Other alterations affect the genes involved in apoptosis, for instance the B-cell CLL/lymphoma 2 (BCL2) on chr 18q21.3 and BCL2-like 11 (BCL2L11, also called BIM) on Chr 2q13.

DNA damage repair components are affected too, for instance the ataxia telangiectasia mutated (ATM) on chr 11q22, and the tumour protein p53 (TP53) on chr 17p13.3 (Bea et al., 1999; Bea et al., 2009; Camacho et al., 2002; Pinyol et al., 1997).

Additional deregulated pathways has been also described, among them NF-kB activation (Martinez et al., 2003). The most important deregulated pathways are showed in Figure 3 (Jares et al., 2007).

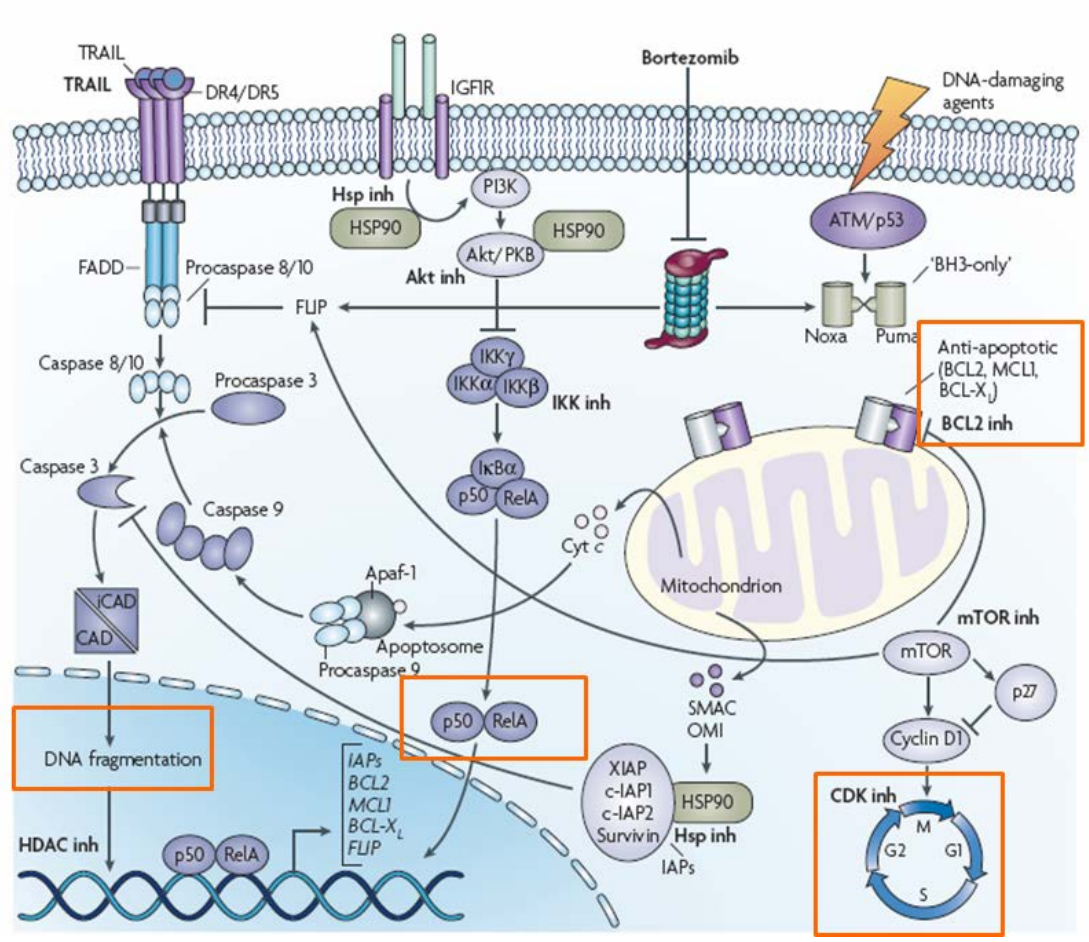


Figure 3. Chief deregulated pathways in MCL (Jares et al., 2007). The boxes outline deregulated pathways.

1.2.3.3. Prognosis, treatment and survival of MCL

The median survival of the patients is of about 4 years, even if a small percentage of cases (up to 15%) show longer survival period (Meusers et al., 1997; Weigert et al., 2009). In fact, clinical behaviour, as well as survival, can consistently vary from patient to patient (Fernandez et al., 2010; Jares et al., 2007; Meusers et al., 1997; Orchard et al., 2003; Rosenwald et al., 2003). Blastoid form is generally associated with more aggressive disease and poorer outcome (Raty et al., 2003), while IgVH somatic mutation is associated with good prognosis (Orchard et al., 2003).

MCL treatment is still heterogeneous, but generally consists of high dose chemotherapy, combined with rituximab (Dreyling et al., 2005; Romaguera et al., 2005).

Resistance to chemotherapy can occur and is associated with poor outcome (Alinari et al., 2012). Additionally, a subgroup of patients presents a rapid relapse (Rosenwald et al., 2003).

Efforts have been done to correlate the relapse to expression of specific markers and some results were found for CCND1, Sox11 and Ki-67 (Brizova et al., 2008; Klapper et al., 2009; Rosenwald et al., 2003; Wang et al., 2008) and IgVH somatic mutation (Kienle et al., 2003). High tumour cell proliferation is also a factor that has been associated to poor prognosis (Argatoff et al., 1997; Bosch et al., 1998).

Identification at time of diagnosis of non-responders at the first line therapy or of poor-outcome cases is crucial for deciding between alternative treatment schemes; thus, new markers are required for better stratification of patients.

1.2.4. Diffuse Large B-Cell Lymphomas (DLBCL)

1.2.4.1. General features

Diffuse large B cell lymphoma represent about 30-40% of non-Hodgkin lymphomas, being thus the most common B-cell lymphoma type in adults (see Figure 1). The median age of presentation is around 64 years (Armitage and Weisenburger, 1998): further details are shown in Table 1.

The existence of different subtypes of DLBCL has been broadly debated, and three main type of DLBCL lymphomas have been recognized: germinal center B-like DLBCL (GCB or GC DLBCL), activated B-like DLBCL (ABC DLBCL) and primary mediastinal B cell lymphoma (PMBL) (Alizadeh et al., 2000; Lenz et al., 2008b; Wright et al., 2003), which is the less frequent form.

The cases studied in this work belong mainly to GC or ABC subtype.

1.2.4.2. Molecular characteristics

Diffuse large B cell lymphoma is a heterogeneous disease (Gurbuxani et al., 2009). It is considered a high-grade large cell lymphoma, characterized by the proliferation of B cells that can have different origins and belonging to different developmental stage (see paragraph 1.2.4.3.).

Despite the heterogeneity typical of this disease, common chromosomal abnormalities can be identified. For instance translocation and consequent overexpression of B-cell CLL/lymphoma

6 (BCL6) (Chr 3q27) occurs in about 35% of the cases (Pasqualucci et al., 2003) and may lead to deregulation of B cell differentiation (Klein and Dalla-Favera, 2008). Another additional alteration is MYC translocation that can be found in about 10% of the cases (Hummel et al., 2006).

1.2.4.3. DLBCL sub-classification

In 2000 a study pointed out the existence of different subgroups of DLBCL (Alizadeh et al., 2000), the ABC and GC subtypes. These two sub-groups were proposed to derive from a different cell of origin; in particular GC subtype is thought to arise from germinal-center B cells whereas the ABC subtype may arise from post-germinal center B cells that are blocked during plasmacytic differentiation.

Some alterations have been found in association with the different DLBCL subtypes (see Table 1), for instance the gain in 3q, 6q21-22 and 18q21-q22 (that mediate BCL2 overexpression) in ABC subtype, and translocation of BCL2 in GC subtype (Lenz et al., 2008b; Rosenwald et al., 2002).

Due to these findings DLBCL is considered a heterogeneous group of tumours. This heterogeneity guides to a complex outcome prediction based on clinical and/or molecular parameters.

1.2.4.4. Prognosis, treatment and survival of DLBCL

Even if the International Prognostic Index (IPI) is broadly used for patient stratification, it has been reported an unusual heterogeneity and inadequate patient stratification in DLBCL disease. The addiction of further criteria of evaluation improved significantly the correct recognition of patients (Wright et al., 2003).

A variety of genetic abnormalities and clinical features, have been proposed to classify responses to treatment and prognosis prediction (Lossos and Morgensztern, 2006).

Anyway a significant contribution to adequate patient stratification has been achieved accordingly to the molecular subtype of DLBCL (Rosenwald et al., 2002). The ABC subtype is frequently associated with a shorter overall survival (Lenz et al., 2008a).

In general DLBCL is considered a potentially curable disease, the first line treatment is CHOP regimen (cyclophosphamide, doxorubicin, vincristine -also called Oncovin- and prednisone)

(Fisher et al., 1993). Furthermore, the addition of the monoclonal antibody, rituximab to CHOP regimen (R-CHOP) increases significantly the survival rate of patients and is now the standard treatment (Coiffier et al., 2002; Sehn et al., 2005). Nowadays, about 75% of patients achieve complete remission and half of the treated patients show 3- to 5-years of progression free survival (PFS) (Sehn et al., 2007).

1.2.5. Burkitt Lymphoma

1.2.5.1. General features

Burkitt Lymphoma is a germinal center originated lymphoma; highly aggressive, with fast proliferation. It was firstly described by Dennis Burkitt in 1958 (Burkitt, 1958) and recognized since then as a specific entity.

Three different types of BL are described: Endemic (frequent in Africa and in children), sporadic (more common in western countries and in the rest of the word) and immunodeficiency-associated (found in association mainly with HIV and EBV infections) (Swerdlow et al., 2008). To note also that BL can present differences depending on the age of occurrence, so that it is possible to refer to BL also as children or adult BL variant.

This study includes sporadic BL from both children and adults.

The incidence of BL in the sporadic form is about 1-2% of lymphomas found in adults where median age of presentation is 30 years. On the other hand sporadic BL has a really high incidence in children and young adults accounting for about 40% of children lymphomas (Swerdlow et al., 2008).

1.2.5.2. Molecular characteristics

The hallmark of BL is the Myc deregulation secondary to t(8;14). MYC is a transcription factor located at chromosome 8q24 and it is commonly translocated t(8;14)(q24;q32) on the IgH promoter in BL cases (more than 80% of BL cases) (Hecht and Aster, 2000; Magrath, 1990). Another 10-15% of the cases show translocations involving MYC on chromosome 2: t(2;8)(p12;q24) and 22: t(14;22)(q24;q11) (Gerbitz et al., 1999; Neri et al., 1988) (see also Table 1). There are a small percentage of cases where MYC translocation is not detected by Fluorescent In Situ Hybridization (FISH): for these cases the mechanism for MYC overexpression is not known. Additional mutation on MYC may enhance its tumorigenesis

(Yano et al., 1993). Other alterations at genetic level are also reported in CDKN2A, TP53, BCL2-associated X protein (BAX), BCL6 (Bellan et al., 2003; Klangby et al., 1998; Sanchez-Beato et al., 2001).

1.2.5.3. Prognosis, treatment and survival of BL

The tumour is highly aggressive and it grows very fast; thus, rapid decision and administration of specific therapy regimen is particularly important. Overall survival can have significant variations depending on the specific treatment. At present 5 years overall survival is achieved in about 70% of the cases (Perkins and Friedberg, 2008). An intensive combination chemotherapy regimen like CODOX-M/IVAC regimen (cyclophosphamide, vincristine, doxorubicin, and high-dose methotrexate alternating with ifosfamide, etoposide and high dose cytarabine, along with intrathecal methotrexate and cytarabine) results in cure rates up to 90% (Mead et al., 2002). Children generally respond better to therapy. New lines of treatment include rituximab administration (Meinhardt et al., 2010; Swerdlow et al., 2008).

Relapses are seen in a short period of time probably due to the high-growth fraction of BL that favours re-entry of remaining viable malignant cells into the cell cycle and rapid growth between chemotherapy cycles with subsequent development of resistance and fast growth in the remission phase. It occurs frequently within the first year and it is a very bad prognostic factor (Blum et al., 2004; Swerdlow et al., 2008). Regrettably at this point the optimal strategy is not well defined.

Unfortunately, reliability on clinical trials is limited to the fact that diagnostic criteria for BL are undergoing revision specially along the last years (Perkins and Friedberg, 2008) in which special attention has been given to MYC negative cases bringing additional genetic alterations. One of the most recent works is the definition of molecular BL based on gene expression data (Hummel et al., 2006). This explains why new markers are required for better BL classification and differential diagnosis.

1.2.6. An additional issue of differential diagnosis: intermediate BL/DLBCL cases

B-cell lymphoma, unclassifiable, with features intermediate between BL and DLBCL (Intermediate BL/DLBCL) is a relatively new described group of lymphoma. Even if it is not recognized as a separate entity so far, it is important to note that it includes cases that cannot be clearly assigned to neither BL nor DLBCL. It is defined as an aggressive lymphoma that has

morphological and genetic features of both BL and DLBCL (Swerdlow et al., 2008) as summarized in Table 2 (Thomas et al., 2011). This group also includes those BL previously known as Burkitt like lymphomas or the so called double hit BL.

This group of lymphoma shows low frequency, in part due to the underestimation derived from their difficult recognition. Immunoglobulin clonal rearrangement can be found, accounting thus for GC origin. About 50% of the cases present MYC translocation; BCL2 and BCL6 translocations are also frequent.

Some studies suggest the possibility that some of the cases defined as intermediate BL/DLBCL are effectively not well classified, and the creation of one algorithm based on gene expression profile data has been developed to facilitate their recognition (Dave et al., 2006).

Table 2. Summary of the characteristics of BL, BLBCL, and intermediate DLBCL/BL (Thomas et al., 2011). Features in common between the entities were underlined in the table. (a)Morphologically typical DLBCL with MYC rearrangement or otherwise typical BL without MYC rearrangement should not be classified in the intermediate category. (b)Can be associated with transformation of antecedent follicular center cell lymphoma EBV Epstein-Barr virus; GC germinal center.

Parameter	Burkitt Lymphoma (BL)	Intermediate BL/DLBCL[(a)]	Diffuse large B-cell lymphoma (DLBCL)
Age of presentation	Younger	Older	Older
Histology			
Cell size	Medium	Medium	Large
Nuclei	Round	Round to oval, irregular	Round to oval, irregular
Nucleoli	Prominent, multiple	Prominent, single	Prominent
Cytoplasm	Basophilic whit vacuoles	Basophilic whit vacuoles	Less basophulic
Mitotic activity	Very high	Very high	Lower
Starry sky pattern	Nearly all	Yes	Less common
Derivation	Early germinal center	Early germinal center	Germinal center
Ki-67 proliferative index	>95%	<95%	<90%
Immunophenotype			
B-cell	CD19+, CD20+, CD22+, CD79a+, sIgM+	CD19+, CD20+, CD22+, CD79a+	CD19+, CD20+
GC markers	CD10+, BCL6+, TCL1+, MUM1-, CD44-, CD138-	CD10±, BCL6±, MUM1-	CD10±, BCL6±, TCL1-, MUM1+, CD44+, CD 138±
Karotypes	Simple t(8;14)(q24;q32) t(2;8)(p11-12;q24) t(8;22)(q24;q11)	Complex t(14;18)(q32;q21)[(b)]	Complex t(14;18)(q32;q21)
Oncogenes	BCL2- *Ig-MYC* rearrangement	BCL2+ *Ig-MYC* rearrangement Non *Ig-MYC* rearrangement *MYC+BCL2* rearrangement	BCL2+ Nearly all *MYC*- negative *BCL6* rearrangement *BCL2* rearrangement
EBV-encoded RNAs (EBER)	LMP1- EBNA2- EBNA1+	Variable	LMP1+ LMP2+ EBNA2+

Unfortunately the most suitable approach for their therapy has not been yet identified (Swerdlow et al., 2008), and some heterogeneity in the response to treatment has been already described (Corazzelli et al., 2012; Li et al., 2012a; Macpherson et al., 1999).

1.2.6.1. Other B-cell lymphomas included in the series of cases

The other B-cells lymphomas here included, but not studied in depth are CLL, FL and the 3 types of Marginal zone Lymphomas: MALT, NMZL and SMZL. Their principal characteristics are included in Table 1.

1.3. MicroRNAs (miRNAs): new players in cell biology

miRNAs are short non-coding single strand RNA molecules of 19 to 25 nucleotides. They were firstly described in 1993 in C. Elegans (Lee et al., 1993; Wightman et al., 1993). Since their first appearance many efforts have been done to understand miRNAs biology and importance. At present, Sanger database contains data for more than 1500 human miRNAs (www.mirbase.org) (Basso et al., 2009; Bentwich et al., 2005; Berezikov et al., 2006). They are evolutionary conserved and their expression is temporarily regulated during development (Pasquinelli et al., 2000). More difficult and of big interest is understanding the role of these miRNAs (Krutzfeldt et al., 2006; Yousef et al., 2009). They have a big impact on protein expression (Baek et al., 2008) and it was possible to describe some role in cell biology for many of them, but much is left do discover. miRNAs have been shown to regulate quite different functions (Niwa and Slack, 2007) such as cell and tissue development (Ambros, 2004; Chen et al., 2004; Krichevsky et al., 2003), cell proliferation and death (Brennecke et al., 2003), and metabolism (Boehm and Slack, 2006). Many miRNAs are tissue specific, highlighting their role in the development and differentiation (Reynolds and Ruohola-Baker, 2008). Last but not least, miRNAs are described to be important players in many diseases: a web site that summarizes the existing information is available at www.mir2disease.org. miRNAs can be found in about any part of the DNA including introns (Ying and Lin, 2005), exons of coding genes, in repeated or imprinted sequences, in unstable genomic regions (Huppi et al., 2008) and regions frequently targeted by DNA breaks or Copy number alteration (Zhang et al., 2006).

Their mechanisms of transcription, maturation and action have been studied, and summarized in many publications (Bartel, 2004; Garzon et al., 2006; Miska, 2005) and here illustrated in

Figure 4. In brief: miRNAs are transcribed in the nucleus as long transcripts of variable length or cluster of miRNAs (pri-miRNAs) by RNA polymerase II (Lee et al., 2004), capped and polyadenilated (Cai et al., 2004). At this point they are individually cleaved by Drosha (Han et al., 2004), forming the so called pre-miRNAs structure, that have a length of 60 to 70 nucleotides and present a steam loop. Their export to the cytoplasm is operated by exportin 5 (Bohnsack et al., 2004). The dicer 1, ribonuclease type III (DICER) is then in charge of cutting the pre-miRNAs (Chendrimada et al., 2005) and the resulting double strand miRNAs undergo separation. In the majority of the cases only one strand is functional and it is the 5'UTR strand of the pre-miRNA, although this is not a fixed scheme. The RNA-induced gene silencing complex (RISC) binds the single strand miRNA to help it with finding its complementary sequence that is generally located at 3'UTR of a messenger RNA and brings them into physical interaction (Maroney et al., 2006).

It is believed that complementarity to mRNA is driven by a short sequence of 6 nucleotides generally in position 2 to 7 of the miRNA, the so called seed region, with a mechanism that is probably redundant (Lim et al., 2005). Interactions with regions other than 3' UTR of the messanger RNA are not excluded (Orom et al., 2008). Many prediction programs are available to predict the gene bounded by each miRNA, among them: miRanda (www.mirbase.org) (Griffiths-Jones et al., 2006), targetscan (www.targetscan.org) (Grimson et al., 2007) and microT available at DIANALAB web site (http://diana.cslab.ece.ntua.gr/) (Maragkakis et al., 2009) that also provides information about validated target and pathways potentially altered by miRNAs expression. Each of these programs gives as output a long list of potential targeted genes, but they have a high rate of false positive prediction (Rajewsky, 2006).

At the moment most of the predicted targets are not yet *in vitro* validated.

It is important to add some notes about miRNAs annotation. The initial three letters refer to the organism: for instance "hsa-miR-100" refers to human species (Homo sapiens) miR-100, while mmu-miR-100 refers to murine (Mus musculus) miR-100. Here, whenever omitted here the initial three letters, the miRNA has to be considered of human origin (for instance: miR-100 refers to hsa-miR-100). In many cases two mature miRNAs derive from a pre-miRNA (see Figure 4). To distinguish them, the less abundant miRNA product receives the same name of the primary product with the addition of the symbol "*": for instance miR-100*. When the data are not satisfactory to define which sequence is the predominant, the nomenclature

adopted is: miR-100-5p (from the 5' arm of the pri-miRNA) and miR-100-3p (from the 3' arm of the pri-miRNA). More details are available at www.mirbase.org/help/nomenclature.shtml.

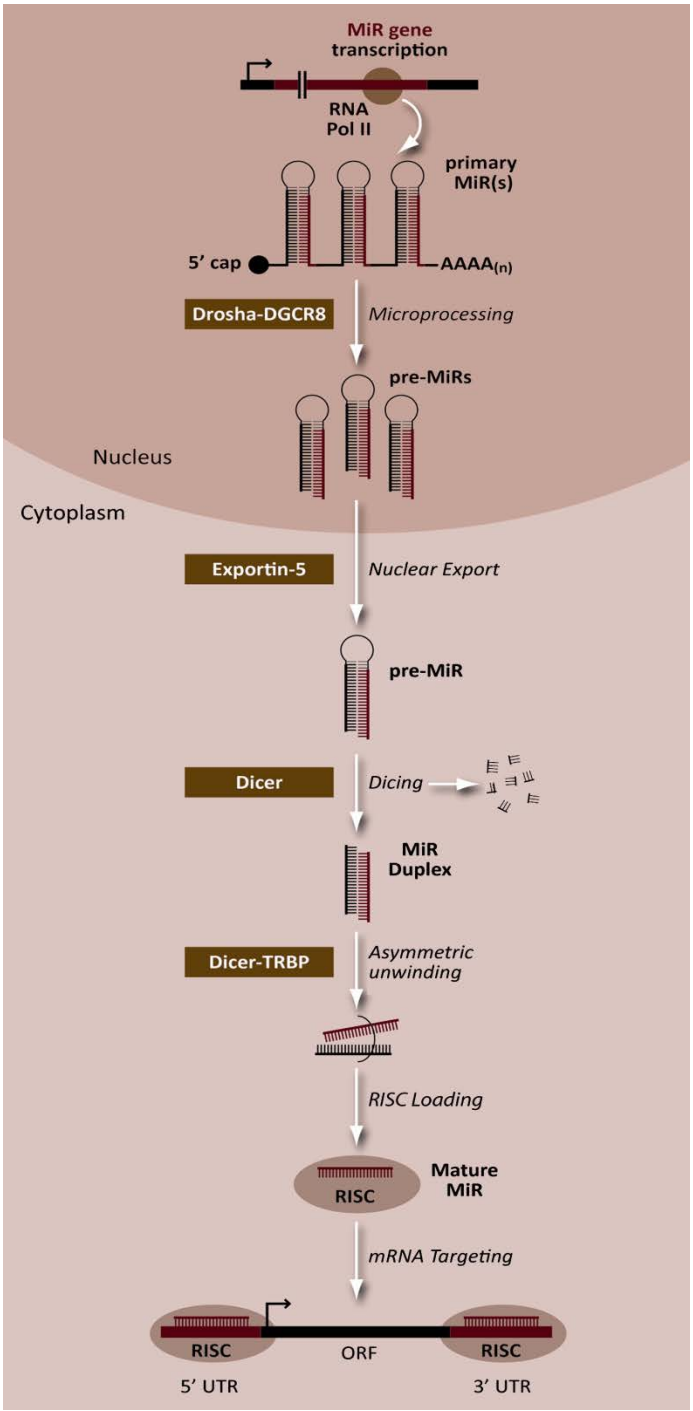


Figure 4. miRNA maturation process (Nana-Sinkam and Croce, 2010). Depending on the specific miRNA, after the step of unwinding, one strand or both of them can be functional.

miRNA-mRNA binding results in the modulation of expression of the correspondent protein through RNA degradation (Lim et al., 2005) (a mechanism that is probably similar to sh-si-RNA) or blocking/inhibition/degradation of protein translation (Eulalio et al., 2008; Pillai et al., 2007). The biologic significance of miRNAs resides here.

Moreover, in the complex miRNAs word it is necessary to consider that miRNAs may affect a protein only in a certain cell type, and may have a different role/target in another cell type. This is suggested by their fine regulation, in fact they are also regulated at post-transcriptional level (Thomson et al., 2006), and their expression is submitted to the same role of other genes, like transcription factor stimulation (O'Donnell et al., 2005) and methylation (Iorio et al., 2007; Lujambio and Esteller, 2007). They also may regulate their expression by themselves (Kai and Pasquinelli, 2010; Tuccoli et al., 2006). Finally it is interesting that miRNAs can affect pathways activation (Yamagishi et al., 2012).

1.3.1. miRNAs in lymphomas

In addition to the above-mentioned roles, miRNAs have been also implicated in several diseases including cancer.

When talking about cancer, a general classification according to miRNAs function and targets has been suggested: oncomirs for those miRNAs that target a tumour suppressor gene and stimulate cancer development, and tumour suppressor miRNAs for those miRNAs that target oncogenes thus blocking tumour development (Hammond, 2006; Hammond, 2007; Kent and Mendell, 2006). This definition is obviously strictly restricted to the disease and tissue we are talking about.

The first evidence of the role of miRNAs in cancer came from a study in lymphomas, on B-cell chronic lymphocytic leukemia where miR-15 and miR-16 were downregulated due to chromosomal deletion at 13q14 (Calin et al., 2002), which is found in about 50% of CLL cases.

More findings in B-cell lymphomas have been recently reviewed (Auer, 2011; Sandhu et al., 2011).

Another important evidence was the finding that miRNAs have specific expression pattern depending on tissue type and type of cancer (Lu et al., 2005).

The fact that several miRNA loci reside on chromosomal fragile sites is of particular interest in cancer and lymphomas also. For instance, the miR-15a/16 cluster mentioned for CLL is also deleted in pituitary adenomas (Bottoni et al., 2005). Another example is the miR-17-92 cluster at chromosome 13q31, composed by miR-17, miR-18a, mir-19a, miR-19b, miR-20a and miR-92, which is found amplified in some B-cell lymphomas (He et al., 2005; Ota et al., 2004) and lung cancers (Hayashita et al., 2005) and also miR143-145 that are located in the 5q33 deleted in lung cancer (Hosoe, 1996) and relevant in lymphomas (Akao et al., 2007).

Correlation of miRNA expression with DNA changes has been also investigated, for example in MCL and BL (Schiffman et al., 2011; Schraders et al., 2008).

Also oncogenic viruses can express miRNAs (Cullen, 2006; Pfeffer et al., 2005; Pfeffer et al., 2004), for instance EBV, whose infection is described in about 20% of BL and 10% of DLBCL, expresses about 25 miRNAs (www.mirbase.org). The role of the majority of viral miRNA and their impact on protein expression is still unknown, although some of these viral miRNAs has been demonstrated to promote survival of the host cell (Choy et al., 2008).

1.3.2. Expression profile investigation of miRNAs in lymphomas

During the last years several papers that describe miRNA expression profiles or their significance in lymphomas have been published, a nice review of the findings is from Sandhu and co-workers (Sandhu et al., 2011).

Table 3 tries to summarize major miRNA expression profile studies on B-cell lymphomas comparing different series of cases with non-tumour controls. To note that for BL studies were more prone to investigate differences between MYC positive and MYC negative cases (Onnis et al., 2010) rather than control lymph node.

Table 3. miRNAs found differentially expressed between B-cell lymphomas and controls. The table was originally taken from Sandhu and colleagues (Sandhu et al., 2011), changed and completed with additional lymphoma types. BL with MYC translocation have been also compared *vs*. BL without MYC translocation. LN = lymph node, T = tonsil, PBMC = peripheral-blood mononuclear cells.

Lymphoma	Number of cases/controls	Upregulated miRNAs	Downregulated miRNAs	Reference
BL	10 BL Myc pos vs 9 BL Myc neg	miR-17-5p, miR-20a	miR-9*, miR34b	Onnis et al., 2010
	25 BL vs 9 non-tumoral controls (LN, T, PBMC)		miR-155	Kluiver et al., 2006
CLL	50 CLL vs 14 normal B-cells	miR-34a, miR-141, miR-598, miR-451, miR-660, miR-155 miR-148a,	miR-449, miR-565, miR-139, miR-582, miR-107, miR-369-3p, miR-424, miR-143, miR-126, miR-368, miR-199a*, miR-130a, miR-326, miR-199a, miR-126*, miR-584, miR-125a, miR-181a, miR-181b	Pallasch et al., 2009
	56 CLL vs pool of CD19+ cells	miR-21, miR-150, miR-155	miR-92, miR-222, miR-29, miR-181	Fulci et al., 2007
DLBCL	98 DLBCL vs 12 normal lymphocyte population	miR-100, miR-10b, miR-125b, miR-143, miR-145, miR-155, miR-21, miR-34a, miR-451, miR-9	miR-150, miR-181a, miR-189, miR-223, miR-361, miR-363, miR-495, miR-584, miR-625, miR-768-5p	Lawrie et al., 2009
	58 DLBCL vs 7 LN	miR-210, miR-155, miR-106a, miR-17-5p	miR-150, miR-145, miR-328, miR-139, miR-99a, miR-10a, miR-95, miR-149, miR-320, miR-151, miR-let-7e	Roehle et al., 2008
FL	46 FL vs 7 LN	miR-9, miR-9*, miR-301, miR-213, miR-330, miR-106a, miR-338, miR-155, miR-210	miR-320, miR-149, miR-139	Roehle et al., 2008
MCL	30 MCL vs 5 CD19+ cells	miR-124a, miR-155, miR-328, miR-326, miR-302c, miR-345, miR-373*, miR-210	miR-29a/b/c miR-142-3p/5p, miR-150, miR-15a/b	Zaho et al., 2010
MALT	14 MALT vs adiacent non-tumour tissue	miR-150, miR-155	miR-184, miR-200a/b/c, miR-205	Cai et al., 2011
NMZL	15 NMZL vs 8 LN	miR-221, miR-223, let-7a	miR-494	Arribas et al., 2012
SMZL	20 SMZL vs 5 Spleens		miR-29a, miR29b-1	Ruiz-Ballesteros et al., 2007

1.3.3. miRNA targets in lymphomas

Once a miRNA expression profile is defined or one miRNA is found to be significantly gained or lost, one obvious question is: what is this miRNA doing?

Therefore, validation studies on miRNA targets and miRNAs significance have been performed by many groups. A list of miRNA validated targets can be found at DIANA labs web site (http://diana.cslab.ece.ntua.gr/tarbase/) (Sethupathy et al., 2006). According to this database about 1000 targets have been validated so far.

Among the more outstanding validated targets in lymphomas can be found:

miR-16/15a that targets BCL2 (Cimmino et al., 2005), miR-29 and miR-181 that regulates T-cell leukemia/lymphoma 1A (TCL1A, also called TCL1) expression (Pekarsky et al., 2006), miR-155 and miR-34a effect on the v-myb myeloblastosis viral oncogene homolog (MYB) and miR-155 modulate the suppressor of cytokine signaling 1 (SOCS1) (di Iasio et al., 2012; Zauli et al., 2011): all of them validated in CLL.

In FL miR-20a and miR-20b were found to be correlated with the expression of the cell cycle inhibitor CDKN1A and miR-194 was reversely correlated with the expression of the suppressor of cytokine signaling 2 (SOCS2) (Wang et al., 2011).

Interesting, it has been found that, in MCL, CCND1 is regulated by miR-16-1 (Chen et al., 2008) and the oncomiR-1 (Deshpande et al., 2009).

In MALT, it was demonstrated that miR-200a,b and c downregulate Cyclin E2 (CCNE2) alone or in combination (Cai et al., 2011).

miR-127 has been found to target BCL6 (Saito et al., 2006), a protein deregulated in about 30% of DLBCL cases.

The web site www.mir2disease.org stores most of the generated information and provides also information about the disease in which miRNAs has been investigated, thus it can be an additional useful tool to explore this issue more in depth.

1.3.4. miRNA lymphoma studies in mouse models

In order to study miRNAs role *in vivo,* a few mouse models accounting for the most commonly described and best known miRNAs, have been generated.

Models are available for miR-155 (Costinean et al., 2006) and oncomiR-1 (He et al., 2005; Ventura et al., 2008).

More recently a model of miR-21 has been used to investigate the correlation between this miRNA and lymphadenopathy and other lymphoid phenotypes (Medina et al., 2010).

Finally another model has been used to investigate the relevance of miR-15a/16 loss of in CLL (Klein et al., 2010; Raveche et al., 2007).

The availability of this mouse models has been also of great help because it was possible to study and show that the recovery of miR-15a/16 cluster increases drug sensitivity (Nutlin and Genistein): a really important finding for evaluation of personalized treatment therapy (Salerno et al., 2009).

1.3.5. miRNA correlation with patients outcome

The International Prognostic Index (IPI) is the primary clinical tool for predicting the outcome of patients with aggressive NHL (1993). Many adjustments and corrections have been done since 1993, especially in some specific lymphoma types.

miRNA expression revealed correlation with patients' outcome in different type of cancers including lymphomas.

While only a few findings are available in FL (Wang et al., 2011) and MCL (Jiang et al., 2010; Zhao et al., 2010), many studies have been done in DLBCL and CLL.

For instance, in DLBCL the first results point at miR-127 as one of the most significant miRNAs, whose low expression was associated with poor outcome (Roehle et al., 2008). In later studies low expression of miR-222 was found associated with better OS and better PFS (Malumbres et al., 2009). In another study the expression of miR-302, miR-608 and miR-637 were associated with poor prognosis, whereas high expression of a group of 9 miRNAs was associated with better outcome (Lawrie et al., 2009). miRNA expression profile has been also correlated with response to R-CHOP therapy and clinical outcome in DLBCL (Alencar et al., 2011; Montes-Moreno et al., 2011).

A series of miRNAs that are related to response to therapy has been distinguished also in CLL (Ferracin et al., 2010; Rossi et al., 2010; Zenz et al., 2009), while correlation with OS was

documented for: miR-29c and miR-223, miR-21, miR-650 (Mraz et al., 2012; Rossi et al., 2010; Stamatopoulos et al., 2010; Stamatopoulos et al., 2009).

miR-29c and miR-223 where proposed in association with IgVH unmutated status and ZAP70+ survival studies (Li et al., 2011), underlining the existence of some heterogeneity also inside CLLs.

Finally miR-181b was proposed as a biomarker of disease progression in CLL (Visone et al., 2011).

1.3.6. miRNAs and therapy

Despite the fact that potential utility or miRNAs in cancer therapy is generally recognized (Garzon et al., 2010; Kota and Balasubramanian, 2010), there are no trials involving miRNAs/anti-miRNAs delivery for cancer therapy at the web site http://clinicaltrials.gov/ of clinical trials. It demonstrates the difficulties that pass between an important discovery and use it in the practice.

Nevertheless, good results have been achieved in the preclinical stage. Principle advances are described in colon carcinoma with miR-145 and miR-33a (Ibrahim et al., 2011) in hepatocellular carcinoma with miR-26a (Kota et al., 2009). Interesting reports are also about metastasis inhibition with anti-miR-10b treatment (Ma et al., 2010a). Recently a couple of promising findings in lymphomas therapy have been reported: with miR-21 (Medina et al., 2010), miR-150 (Lachmann et al., 2011) and miR-15a/16-1 (Salerno et al., 2009) in mouse lymphoma models.

miR-122, which gave promising results in primates even if it was not a cancer model, (Elmen et al., 2008), is now one of the few anti-miRNA in clinical trial, but for hepatitis C treatment.

1.3.7. miRNA variants

The majority of miRNAs studies nowadays are more focalized on miRNAs expression levels. However the possibility that a miRNA-polymorphism could affect the expression of multiple genes involved in the same pathway activation, development, metabolism, cell cycle progression, drug resistance, patients outcome and risk to disease development came out recently (Mishra and Bertino, 2009), and thus the studies also dedicated their attention on miRNAs sequencing in order to describe miRNAs sequence alterations.

In fact, it is reasonable that miRNAs changes, especially in the seed region may affect miRNA-mRNA interaction and here in depth miRNAs can be affected into two ways: by losing their downregulation properties or by reaching a new target protein (Mishra and Bertino, 2009), another possibility is that or that miRNA variants may affect miRNAs maturation process (Duan et al., 2007; Han et al., 2006; Harnprasopwat et al., 2010).

The study of miRNAs polymorphisms are particularly complex, and probably the main difficulty so far is that variants in miRNAs are rarely found (Iwai and Naraba, 2005; Yazici et al., 2009). Right now there are only a few manuscripts that describe miRNAs carrying some variations correlated to some phenotype: some of them also in cancer (Zorc et al., 2012). For instance results are reported in breast/ovarian cancer and gastric cancer where the miRNAs involved are miR-146a (Shen et al., 2008; Xu et al., 2008) and miR-27a (Sun et al., 2010).

The fact that only few changes in miRNA sequence have been described (Zorc et al., 2012), may be consistent with the observation that mistaken miRNAs are depleted by the cell as also happens with mRNA that shows premature truncation or other types of defects, and it could mean that small changes in miRNAs are not acceptable and thus, somehow, eliminated.

Nevertheless, some miRNAs polymorphisms can be found with relatively high frequency in the population (Saunders et al., 2007). This observation offers the possibility that the presence of some variants in miRNAs sequence may not have the expected striking effects (Zeng and Cullen, 2003) afore-mentioned. It is possible that due to the fact that miRNAs protein regulation is a redundant mechanism and miRNAs belonging to the same family/cluster can target the same protein (Cai et al., 2011; Kojima et al., 2012; Ventura et al., 2008). In fact, the sequence of some miRNAs that belongs to the same family differs only in one nucleotide. This rationale can be further confirmed by searching in any target predictor website for two miRNAs that share all but a few nucleotides sequence.

Another interesting proposal that is giving nice results is that miRNAs polymorphisms may help patients stratification and may correlate to patients outcome or tumour developing risk, as found for miR-146a (Xu et al., 2010), miR-196a (Hu et al., 2008), miR-499 (George et al., 2011) and others (Hu et al., 2009b; Okubo et al., 2010).

2. Objectives

In the specific case of B-cell lymphoma, benefits derived from miRNAs investigation/knowledge can be accounted at different levels, such as:

-Molecular knowledge of lymphomas

-Use of miRNAs as diagnostic markers

-Use of miRNAs as survival markers

2.1. General purpose of the study

The main objective of this study is to perform a general description and characterization of miRNA profile in B-cell lymphomas.

For this purpose, 3 independent specific projects have been developed.

2.1.1. Project1: miRNA expression profile in B-cell lymphomas

The objective is to establish a miRNA expression profile for B-cell lymphomas that could be useful for differential diagnosis. The work was carried on a large series of B-cell lymphomas and comparing the different lymphoma types to each other and normal tissue. A practical application is offered comparing DLBCL and BL.

2.1.2. Project2: miRNAs deregulation and role in Mantle Cell Lymphoma

As an example of miRNAs importance in lymphomas, MCL has been investigated in depth to describe:

- miRNA expression profile in MCL

- Correlation between miRNA expression profile and Gene Expression Profile (GEP)

- Implication of miRNAs in pathways deregulation

- Correlation between miRNAs expression and Overall Survival

2.1.3. Project 3: Investigation of changes in miRNAs sequence

To investigate nucleotide changes in miRNAs from DLBCL patients and their possible role in lymphomagenesis.

3. Materials and Methods

3.1. Tissue Samples and cell lines

This work includes a big number of samples from lymphoma patients. Non-tumour controls and cell lines are also included.

3.1.1. Samples included in the study

All samples were collected at diagnostic step in collaboration with several Spanish and international Hospitals, and with the help of Spanish National Tumour Bank Network (CNIO), Madrid, Spain; or with the help of Addenbrooke's Hospital, Cambridge, UK. Diagnosis of all cases was reviewed according to the current World Health Organization criteria (Swerdlow et al., 2008). Table 4, Table 5 and Table 6 summarize the cases used in each project.

Table 4. Samples used in the project 1, collected at CNIO, Madrid. * Lymph node-involved CLL cases. FFPE=Formalin fixed paraffin embedded.

Lymphoma type	Fresh Frozen samples	FFPE samples
BL	12	28
CLL*	18	8
DLCBL	29	43
FL	23	9
MALT	15	8
MCL	22	8
NMZL	11	8
SMZL	17	5
Intermediate-BL-DLBCL	-	20
TOTAL tumour samples	147	137
Tonsils	4	4
Reactive lymph nodes	7	3
Spleens	4	1
TOTAL control samples	15	8

A fresh frozen cut of the samples verified that the blocks were representative tumours and that contained at least 80% of tumour cells.

All samples derived from lymph nodes. Only SMZL samples derived from spleen specimens, and MALT samples derived from skin, breast, stomach, lymph nodes, endometria, thyroid and lung specimens.

Table 5. Samples used in project 2, collected at CNIO, Madrid. FFPE=Formalin fixed paraffin embedded.

Lymphoma type	Fresh Frozen samples	FFPE samples
MCL	23	54
Tonsils	4	
Reactive lymph nodes	7	
Cell lines	8	
Sorted cells CD19+/IgD+/CD27-	3	

Twenty-two out of 23 MCL cases, all the reactive lymph nodes and all the tonsils included in the second project correspond to the samples included in the first project.

Table 6. Samples used in project 3, collected at Addenbrooke's Hospital, Cambridge, UK. Normal counterpart corresponds to bone marrow. FFPE=Formalin fixed paraffin embedded.

Lymphoma type	FFPE samples
DLBCL	95
Normal counterpart tissue	3

Human MCL Cell lines were grown at 37°C in a humidified atmosphere at 37°C and 5 % CO_2 using Roswell Park Memorial Institute medium (RPMI) 1640 medium (LONZA) supplemented with 10% of Fetal Bovine Serum (FBS) and 1% Pen/Strep antibiotics (both from Gibco). Only GRANTA cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) supported with 20% of FBS and 1% of Pen/Strep. REC-1, GRANTA and JEKO-1 were purchased from DSMZ (www.dsmz.de), UPN1, UPN2, MINO, Z138 and HBL2 were kindly provided by Dr. Martínez-Climent (Center for Applied Medical Research, University of Navarra, Pamplona, Spain).

3.1.2. Isolation of mantle B cells

Fresh tonsils derived from routine tonsillectomy were obtained with the collaboration of hospitals Gregorio Marañon, La Paz and Ramon y Cajal, Madrid. Cell suspensions were obtained by mincing and filtering the tissue in ice-cold culture medium (RPMI). Mononuclear cells were isolated by Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Suspensions with about $50x10^6$ tonsil single-cells were incubated with 10µl of CD19 (PE-Cy™7 Mouse Anti-Human CD19 antibody, BD biosciences, Franklin Lakes, NJ, USA), 25µl of IgD (FITC Mouse Anti-Human IgD antibody, BD) and 20µl of CD27 (APC Mouse anti-Human CD27 antibody, BD) antibodies in 300µl of PBS solution for 25 minutes at room temperature with shaking. $CD19^+/IgD^+/CD27^-$ cells were sorted, yielding a purity of at least 95%. Separation of

other cell subpopulation, like $CD19^{+}CD10^{+}$ (10µl of APC Mouse anti-Human CD10 antibody, BD) following the same protocol was also performed. All isolations were performed using the FACS Aria Sorter (BD), and analyzed with the Diva software (BD). An example of sorting selection is illustrated in Figure 5.

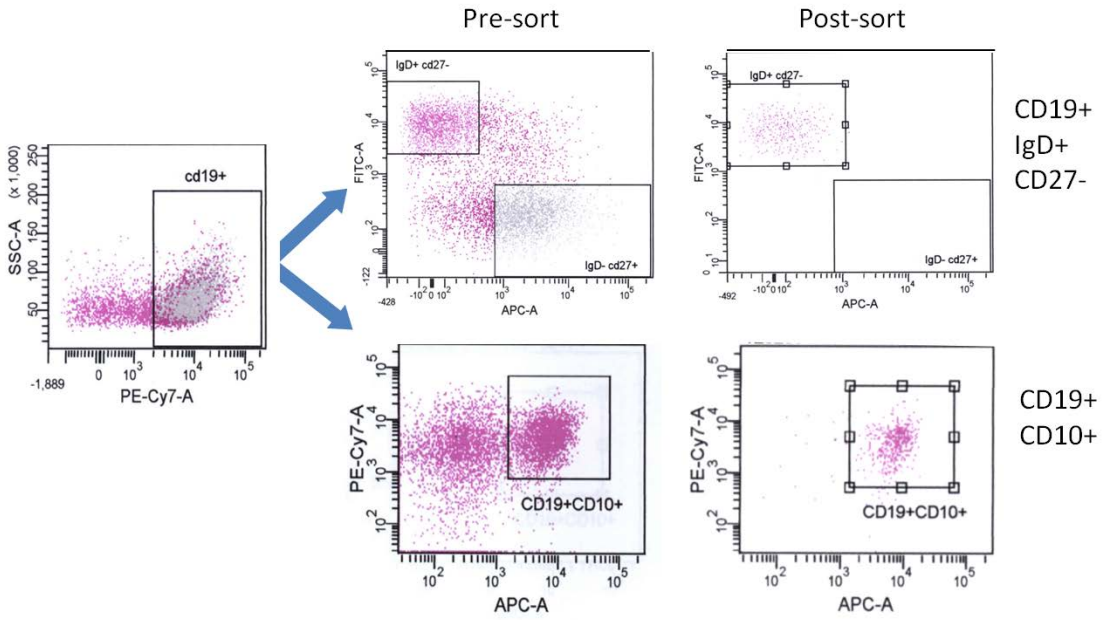


Figure 5. Different cell subpopulations sorted by FACS. $CD19^{+}/IgD^{+}/CD27^{-}$ (correspondent to B- cell mantle zone) and $CD19^{+}CD10^{+}$ (correspondent to GC B-cells).

3.2. RNA extraction

3.2.1. From fresh frozen samples

Twenty tissue sections of 20 µm thickness were disaggregated by Tri-reagent (1ml, SIGMA-ALDRICH, Steinheim, Germany) using a 1ml syringe and a needle (23G). The suspension was kept 10 minutes at room temperature and then, 200µl of chloroform were added. Samples were mixed thoroughly for 15 seconds and maintained 10 additional minutes at room temperature. After 15 minutes of centrifugation at 8000g (4°C), the aqueous phase was recovered in a fresh tube. The same volume of isopropanol was added and samples were kept at -20°C overnight. The day after, samples were centrifuged at 12000g, 15 minutes, at 4°C. Pellets were washed with 70% cold ethanol and resuspended in 30 µl of RNase free water.

RNA was quantified using the NanoDrop ND-1000 spectrophotometer device (Thermos scientific, Wilmington, DE, USA) and visualized on a 1% agarose gel.

For Gene Expression experiments, a higher purity of RNA was achieved by the RNeasy® Mini Kit from Qiagen (Hilden, Germany) following manufacturer's instructions, and including the DNase treatment (Qiagen). Finally, RNA was resuspended in 20-35µl.

The majority of RNAs also underwent bioanalyzer analysis (Agilent Technologies) to check RNA quality.

3.2.2. From formalin fixed paraffin embedded (FFPE) samples

Total RNA was extracted from 10 FFPE sections of 10µm. Deparaffinization was achieved by adding 1 ml of xylene and 10 minutes incubation at 65°C with shaking. Samples were centrifuged at 10000 rpm at room temperature for 15 minutes and then, the supernatant was removed. Ethanol wash was performed, afterwards tissue pellets were dried and incubated overnight at 65°C in 400 µl of tissue lysis buffer (Tissue & Cell Lysis Solution, Epicentre) + 4µl of proteinase K (50 µg/µl, epicentre). The day after, 480 µl of isoamilic-phenol-chloroform was added; samples were incubated 5 minutes and then centrifuged at 10000rpm for 5 minutes. Supernatant was then passed into a new tube, where, the same volume of isoamilic-chloroform was added and samples were incubated again for 5 minutes. After centrifugation (10000rmp for 5 minutes) the supernatant was recovered and precipitation was performed over night at –20°C, in an equal volume of isopropanol, supplemented with 1µl of linear acrylamide (Ambion, Austin, TX). On the following day a centrifugation at 4°C during 40 minutes was performed. Pellets were washed in 70% cold ethanol and resuspended in 30 µl of RNase-free water.

RNA concentration and purity (A260/A280 and A260/A230 ratios) were measured using a NanoDrop ND-1000 spectrophotometer device (Thermos scientific). RNA quality was considered good enough when 260/280>1,8 and 260/230 >1,5.

3.2.3. DNA extraction

DNA of FFPE tissues were collected at Addenbroke's Hospital using the QIAamp DNA Micro Kit (QIAGEN) following manufacturer instructions.

A general view of all the Methods used is summarized in Figure 6.

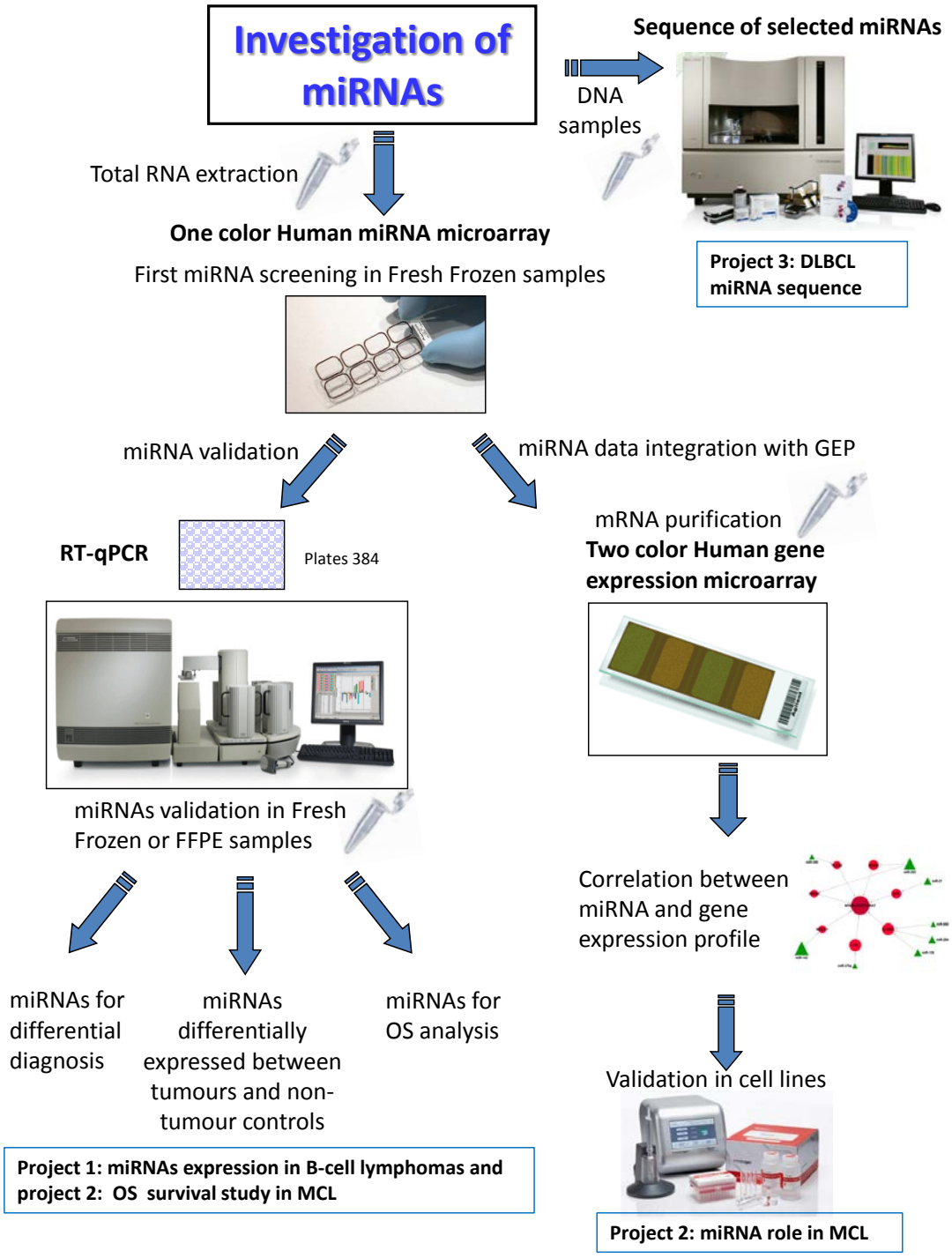


Figure 6. Summary of the methods used in this work. GEP=Gene Expression Profile, OS= Overall Survival. All images are available at www.appliedbiosystems.com, www.labplanet.com, www.agilent.com, www.invitrogen.com.

3.3. Hybridizations

3.3.1. miRNA Arrays

For miRNA profiling experiments, 100 ng of total RNA were hybridized on an Agilent 8x15K Human miRNA Microarray Kit (G4470A, one colour technique, Agilent Technologies, Inc., Santa Clara, CA) for the detection of 470 human and 64 viral miRNAs, following the manufacturer's instructions (Agilent Technologies) (Ach et al., 2008; Wang et al., 2007). Dephosphorylation step was performed for 30 min at 37°C by adding 4µl of RNA (concentration: 25ng/µl) to 3µl of Alkaline Phosphatase (CIP) master mix, prepared as follows: 0,7 µl of 10X Alkaline Phosphatase (CIP) Buffer (supplied with CIP enzyme) + 0,7µl Calf Intestine Alkaline Phosphatase (CIP, 16 U/µl) (GE- Healthcare, Amersham Place, Little Chalfont, UK) + 1.6µl of RNase free water. Denaturalization was accomplished by adding 5µl of DMSO to the samples, for 5 min at 100°C. All the following steps were undertaken in a minimum light environment. Samples were rapidly cooled on ice, and labelled with cyanine 3-CTP (Cy3) using the miRNA Labelling Reagent and Hybridization Kit (Agilent). For the labelling step, T4 ligase (GE Healthcare) working concentration (15 U/µl) was achieved by adding the correct amount of dilution buffer to the enzyme (1µl of 10X T4 ligase buffer, 1µl of BSA 0,1% and 8µl of RNase free water). The reaction mixture was composed by 2µl of 10X T4 ligase buffer, 2µl of 0.1% BSA, 3µl of Cy3 and 1µl of T4 ligase enzyme (15U/µl). Samples were incubated for 2 h at 16°C, purified with Bio-spin 6 columns (BioRad, Hercules, CA, USA) and dried in a speed vacuum at 45°C for 15 minutes.

Labelled RNA was resuspended in a total volume of 45µl using the hybridization mix containing 4,5µl of blocking agent, 22,5µl of hybridization buffer (Agilent) and 18µl RNase free water, incubated for 5 min at 100°C, quickly cooled on ice and placed in the 8x gasket (Agilent) previously positioned in a hybridization chamber (G2534A). Gaskets were covered with Agilent 8x15K human miRNA V1 slides (G4470A) and the chamber covers were secured. Incubation took place overnight in an Agilent oven at 55°C for 20h at 20rpm.

Washing steps were performed using the corresponding buffers provided from Agilent: Wash Buffer 1 at room temperature (Agilent) and Wash Buffer 2 at 37°C (Agilent) during 5 minutes in an ozone-free environment with shaking. Finally, the array was submerged in acetonitrile for 1 minute to help the drying process. Scanning was carried out immediately using the Microarray Scanner System (Agilent Technologies G2565AA).

3.3.2. Gene Expression Arrays

60-mer Agilent 4X 44K Human Whole Genome oligonucleotide microarrays (competitive two colour technique) and the correspondent hybridization kit were used (Agilent). 500ng of RNA were used for hybridization of each sample, and 500ng of a commercial pool of RNA from cell lines was used as a reference (Universal Human Reference RNA from Stratagene, La Jolla, CA).

Spike-in controls (Agilent) were added to the samples (1µl of the commercial already diluted mixture).

0.8µl of T7 promoter primer were added (total volume: 5.8µl). Template and primer were denatured for 10 minutes at 65°C and subsequently cooled on ice for 5 minutes.

The cDNA Master Mix was prepared (2µl of 5x First Strand Buffer + 1µl of 0,1M DTT + 0,5µl of 10mM dNTP mix + 0,6µl of MMLV-RT enzyme + 0,3µl of RNaseOUT) and then 4,4µl were added to each sample and incubated for 2 hours at 40°C. The enzyme was inactivated at 65°C for 15 minutes and the samples were immediately placed on ice for 5 minutes.

Dyes (0.5µl Cy3-CTP & 0.5µl Cy5-CTP) were added to Reference and Sample cDNA respectively and then 14.5 µl of the Transcription Master Mix, prepared as follows, were added to each sample: 3.83 µl of Nuclease-free water + 5 µl of 4x transcription Buffer + 1,5µl of 0,1 M DTT + 2µl NTP Mix + 1,6µl of 50% PEG + 0,12µl of RNaseOUT + 0,15µl of Inorganic Pyrophosphatase + 0,3µl of T7 RNA Polymerase. Samples were incubated for 2 h at 40°C.

The resulting cRNA was purified with Qiagen RNeasy kit columns and a total volume of 45µl (containing 1,1µg of labeled cRNA derived from patient and reference pool), was reached with Nuclease free water. For hybridization, 4µl of 10x control targets + 1,8µl of 25x fragmentation buffer were added. Incubation took place for 30 minutes at 60°C.

Finally, 45µl of 2x HRPM hybridization buffer were added to the mixture (90µl total) and quickly loaded into a 4X gasket slide positioned into a hybridization chamber, covered with a 60-mer Agilent 4X 44K Human Whole Genome oligonucleotide microarrays (G4112F, Agilent), locked and hybridized at 65°C for 17h at 10rpm.

The day after, washings were conducted as previously described for miRNA hybridizations with incubations of 1 minute (see paragraph 3.3.1.).

3.4. Real Time quantitative PCR (RT-qPCR)

3.4.1. Retrotranscription step

The expression of mature form of miRNA was validated by RT-qPCR with Applied Biosystems technology. Complementary DNA (cDNA) synthesis was carried out with a specific primer for each miRNA. These primers contain a specific loop that helps the binding only to the mature form of the miRNA and makes more difficult the annealing with immature forms and genomic DNA due to steric impairment.

The manufacturer's protocol was followed. Retrotranscription was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Approximately 2ng of RNA were used for each miRNA retrotranscription reaction. 0,5µl of Reverse Transciption Buffer + 0,33µl of MultiScribe Reverse Transciptase + 0,05µl of 100mM dNTPs mix + 0,05µl of of RNase inhibitor + 1µl of specific miRNA primer (or endogenous control, see Table 9, Table 10, Table 11 and Table 12 for details) + RNase free water until final reaction volume of 5 µl.

Whenever possible, after discussion with the technical department of Applied Biosystems and protocols optimization, pools containing a maximum of 15 primers were used in the retro transcription step.

In this case 200 ng of RNA was mixed with 0,2 µl of 100mM dNTPs, 1,33µl of 50U/µ MultiScribe Reverse Transciptase, 2µl of Reverse Transciption Buffer, 0,25µl of RNase inhibitor and 1µl of each one of the specific RT primers (mixed in a pool) in a final reaction volume of 20µl.

For retrotranscription step, samples were placed in a thermo cycler to undertake the program of Table 7.

Table 7. RT conditions used for cDNA synthesis.

Program	Temperature (°C)	Time (minutes)
Hold	16	30
Hold	42	30
Hold	85	5
Hold	4	Forever

3.4.2. Amplification step

Triplicates were performed in 10µl of total volume in 384-well plates (Applied Biosystems). Each well contained 5µl of Master Mix (Amperase free) + 0,5µl of Taqman probe. The amount of cDNA depended on the specific experiment and varied from 0,4µl (if the RT was done with the pool of primers) to 0,66µl (if a single RT primer was used in the RT step); volume adjustment up to 10µl were done with RNase free water.

To make the experiments and plates comparable to each other, at least two endogenous controls for relative quantification were always introduced in the plates.

The reactions were performed in the ABI PRISM HT 7900 Real-Time Sequence detection system (Applied Biosystems) using the conditions of Table 8.

Table 8. Program used for amplification reaction. Min= minutes, Sec= seconds.

Program	Temperature (°C)	Time
Hold	95	10 min
40 cycles	95	15 sec
	60	60 sec

Table 9 and Table 10 summarize the list of all miRNAs and endogenous controls used in the different series of cases and experiments performed.

Table 9. Primers used in the project 1 for the validation of BL *v.s* DLBCL miRNAs expression profile. NA*= Not Available; miR-560 is no longer considered a miRNA (www.mirbase.org/cgi-bin/mirna_entry.pl?acc=MI0003566).

	miRNA	Assy ID	Analysed as
1	hsa-let-7f	000382	Target
2	hsa-miR-125b	000449	Target
3	hsa-miR-126*	000451	Target
4	hsa-miR-146a	000468	Target
5	hsa-miR-146b	001097	Target
6	hsa-miR-155	000479	Target
7	hsa-miR-17-3p	000392	Target
8	hsa-miR-182	000597	Target
9	hsa-miR-191	000490	Target
10	hsa-miR-196b	000496	Target
11	hsa-miR-197	000497	Target
12	hsa-miR-199a*	000499	Target
13	hsa-miR-221	000524	Target
14	hsa-miR-223	000526	Target
15	hsa-miR-26b	000406	Target
16	hsa-miR-29b	000413	Target
17	hsa-miR-30b	000602	Target
18	hsa-miR-328	000543	Target
19	hsa-miR-34b	000427	Target
20	hsa-miR-365	001020	Target
21	hsa-miR-374	000563	Target
22	hsa-miR-451	001105	Target
23	hsa-miR-453	002318	Target
24	hsa-miR-483	001275	Target
25	hsa-miR-485-3p	001277	Target
26	hsa-miR-516-3p	001149	Target
27	hsa-miR-520c	001117	Target
28	hsa-miR-520d	001118	Target
29	hsa-miR-520f	001120	Target
30	hsa-miR-560	NA*	Target
31	hsa-miR-573	001615	Target
32	hsa-miR-574-3p	002349	Target
33	hsa-miR-582-3p	002399	Target
34	hsa-miR-595	001987	Target
35	hsa-miR-609	001573	Target
36	hsa-miR-615	001588	Target
37	hsa-miR-629	001562	Target
38	hsa-miR-660	001515	Target
39	hsa-miR-663	002857	Target
40	hsa-miR-9	000583	Target
41	hsa-miR-9*	002231	Target
42	hsa-miR-92	000430	Target
43	hsa-miR-98	000577	Target
44	RNU44	001094	Endogenous control
45	RNU6B	001093	Endogenous control

Table 10. Primers used in the project 1 for validation of B-cell lymphomas *vs*. non-tumour controls miRNAs expression profile.

	miRNA	Assy ID	Analysed as
1	hsa-let-7a	000377	Target
2	hsa-let-7c	000379	Target
3	hsa-let-7d	000380	Target
4	hsa-let-7e	000381	Target
5	hsa-miR-10b	000388	Target
6	hsa-miR-133a	000458	Target
7	hsa-miR-200a	000502	Target
8	hsa-miR-212	000515	Target
9	hsa-miR-23b	000400	Target
10	hsa-miR-31	001100	Target
11	hsa-miR-487b	001285	Target
12	hsa-miR-513	001146	Target
13	hsa-miR-770-5p	002002	Target
14	hsa-miR-9	000583	Target
15	RNU44	001094	Endogenous control
16	RNU6B	001093	Endogenous control

Table 11. Primers used in the project 2 for validation of MCL *vs*. non-tumour controls miRNAs expression profile of array paired fresh frozen samples.

	miRNA	Assy ID	Analysed as
1	hsa-miR-1	000385	Target
2	hsa-miR-106b	000442	Target
3	hsa-miR-126	000450	Target
4	hsa-miR-132	000457	Target
5	hsa-miR-150	000473	Target
6	hsa-miR-181c	000482	Target
7	hsa-miR-182	000597	Target
8	hsa-miR-198	000581	Target
9	hsa-miR-200b	001800	Target
10	hsa-miR-203	000507	Target
11	hsa-miR-24	000402	Target
12	hsa-miR-26a	000404	Target
13	hsa-miR-31	001100	Target
14	hsa-miR-320	000536	Target
15	hsa-miR-335	000546	Target
16	hsa-miR-363	001271	Target
17	hsa-miR-370	000558	Target
18	hsa-miR-497	001043	Target
19	hsa-miR-617	001591	Target
20	hsa-miR-7	000386	Target
21	hsa-let-7a	000377	Endogenous control
22	hsa-let-7d	000380	Endogenous control
23	RNU-44	001094	Endogenous control
24	RNU-48	001006	Endogenous control

Table 12. Primers used in the project 2 for validation of candidates miRNAs in Overall Survival study of MCL cases.

	miRNA	Assy ID	Analysed as
1	hsa-miR-130b	000456	Target
2	hsa-miR-181c	000482	Target
3	hsa-miR-198	000581	Target
4	hsa-miR-20b	001014	Target
5	hsa-miR-362	001273	Target
6	hsa-miR-363	001271	Target
7	hsa-miR-454	002323	Target
8	hsa-miR-532	001518	Target
9	hsa-miR-625	002431	Target
10	hsa-miR-660	001515	Target
11	hsa-miR-7	000386	Target
12	hsa-miR-99b	000436	Target
13	hsa-let-7a	000377	Endogenous control
14	hsa-let-7d	000380	Endogenous control
15	RNU-44	001094	Endogenous control
16	RNU-48	001006	Endogenous control

3.5. miRNA electroporation

miR-26a (pre-miR-26a, Applied Biosystems) and negative controls (NC) (pre-miR-negative control 1 which is a random sequence suggested by the manufacturer, Applied Biosystems) were electroporated at 60nM concentration using the Neon Transfection System with the corresponding buffer (Invitrogen, Carlsbad, CA, USA) in UPN-1, MINO and REC-1 cell lines.

Cells were grown without FBS 24h before electroporation. One million cells, were loaded in gold tips (100µl capacity), submerged in the microporation buffer (E2) and one pulse of 40ms and 1000V was applied. Electroporation efficiency using these conditions was about 90%.

Cells were then plated in 1ml of growth medium and recovered after 24, 48, 72 and 96 h of electroporation.

3.6. Protein expression

3.6.1. Protein extraction

About 10^6 UPN-1 cells were lysed by addition of 50µl of RIPA buffer (150mM NaCl, 50mM Tris, 1% IGEPAL CA-630, 0,5% sodium deoxycholate, 0,1% SDS) for 30 minutes on ice followed by 30 minutes centrifugation at 13200rmp at 4°C . Proteins were quantified using the BioRad protein

assay (BioRad) following the manufacturer's instructions and using bovine seroalbumin (BSA) to create a standard curve.

Protein concentration was measured at a wavelength of 750nm in the SynergyHT (Bio-TEK: BioTek Instruments, Inc. Winooski, VT, United States).

3.6.2. Western Blot

For Western blot, 30 µg of protein extracts in Laemmli buffer (5X composition: Tris-HCl 60mM, glycerol 25%, SDS 2%, 2-mercaptoethanol 14,4mM, bromophenol blue 0,1%) were subjected to electrophoresis on 12% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels using the Mini-Protean 3 system (BioRad) in the corresponding electrophoresis buffer (5X electrophoresis buffer composition: Tris-HCl 0,13M, glycine 0,95M, SDS 0,5%).

Afterwards, the proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham) using the Mini Trans-blot Cell system (BioRad). Transference conditions were 300 mA for 2 hours at 4°C in the appropriate transference buffer, supplemented with 20% of methanol (10X transference buffer composition: Tris-HCl 0,025M, glycine 0,2M).

Membranes were blocked in PBS-Tween (phosphate-buffered saline with 0.1% Tween-20) supported with 5% BSA for 1 hour with continuous shaking and then incubated over night at 4°C with MEKK2 rabbit-anti human primary antibody (EP626Y, Abcam Inc. Cambridge, MA, USA), at a dilution: 1:1000 (MEKK2 is also known as MAP3K2).

Primary antibody detection was carried out using fluorescent-labeled secondary antibodies (Alexa 680, Rockland, Gilbertsville, PA, USA) and images where taken by an Odyssey Infrared System Scanner (LI-COR Biosciences, Lincoln, NE, USA).

Tubulin mouse anti-human primary antibody, (Anti-α-Tubulin, T6074, Sigma-Aldrich Co., Saint Louis, Missouri, USA) was incubated for 30 minutes at room temperature (dilution: 1:10000) and protein expression was evaluated on the same membrane with a different secondary antibody (Alexa 800), as reference protein to evaluate differential expression of MEKK2.

3.7. Immunofluorescence

After recovering, electroporated cells were passed onto a slide by cytospin (Shandon Cytosin 4, Thermo electronic corporation) with the following conditions: 1000rpm for 1 minute; fixed and permeabilized with 100% acetone (10 minutes at room temperature). PBS supplemented with

10% FBS was applied for 30 minutes as blocking step. Incubations were performed in a humidified chamber to avoid sample drying.

RelA (p65) mouse anti-human antibody 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; SC-8008) in PBS solution supplemented with 5% of FBS for 1h was used for primary staining. Slides were washed 2 times with PBS. Secondary antibody staining was carried out with anti-mouse 1:200 (Invitrogen, Alexa Fluor 488) for 1h in PBS solution supplemented with 5% of FBS. Covers were then loaded using a loading solution (Vectashield mounting medium for fluorescence with DAPI, H-1200; Vectro Laboratories) containing 4,6-diamidino-2-phenylindole (DAPI) for nuclei visualization.

Images were obtained by a Leica TCS-SP2 (AOBS) confocal microscope (Leica Microsystems, Germany) with LCS v. 2.61 software (Leica Microsystems). Merged images were also collected.

3.8. Data analysis

Microarray images were processed using feature extraction software, 9.5 version (Agilent Technologies). The grid used in the feature extraction for miRNA microarrays (and thus, also the results described) is in accordance with Sanger database nomenclature version 9.1. It is to note that according to the specific version of miRBase release, miRNA description may slightly vary.

3.8.1. Microarray of miRNAs

Microarray background subtraction and data normalization was performed using an analysis script developed in collaboration with CNIO bioinformatics group. Between-array median normalization was carried out to render miRNA expression data sets comparable. Significantly deregulated miRNAs between two groups of samples were computed using Significance Analysis of Microarray analysis (SAM, which is a program that provides q-values directly) (Saeed et al., 2003; Tusher et al., 2001). The q-value corresponds to the false discovery rate (FDR) (Storey, 2002), which is an adjusted p-value corrected by multiple hypothesis testing. In terms of use, FDR=0.05 is more restrictive than p-value=0.05. The groups compared vary depending on the project and are described in the results section.

3.8.2. Microarrays of GEP

Microarray background subtraction and normalization of dataset were done also using a script developed in collaboration with CNIO bioinformatics group. Normalization was performed by loess within-array and quantile between-array normalizations. Differentially expressed genes were identified using the limma package (Ritchie et al., 2007). p-values were adjusted into a FDR values using the Benjamini & Hochberg correction. Those genes with FDR<0.05 were defined as being differentially expressed between controls and tumours.

3.8.3. Fold change

Fold change calculation refers to direct comparison of data when subtracting $\log_2$ values between the defined group of samples, controls or different subgroups of samples. It can be used both for array data (GEP and miRNA expression profile) and RT-qPCR data.

3.8.4. miRNA target searching

A variety of web resources and algorithms to investigate potential miRNA targets were used: miRanda (www.mirbase.org; Faculty of Life Sciences, University of Manchester), microRNA mirSVR score (www.microrna.org), Targetscan (www.targetscan.org; Whitehead Institute for Biomedical Research) and Diana-mir-Path (http://diana.cslab.ece.ntua.gr). Multiple searches can be achieved by the web page (http://gencomp.bio.unipd.it/magia/query/).

Usage of the specific algorithm or web page is detailed in the independent searches.

3.8.5. Association between miRNAs, GEP and biological pathways

In order to correlate miRNA expression profile to GEP, a contingency table connecting the miRNA and its predicted target genes whose probe was included in gene expression platform (Agilent) was constructed for each differentially expressed miRNA.

In this case the prediction softwares used were miRBase Targets Release v. 5.0 (www.mirbase.org; miRBase Targets Release uses an algorithm called miRanda) and TargetScan v. 5.1 including conserved and non-conserved target sites predictions (www.targetscan.org).

Fisher's exact test was used to evaluate statistical non-casual correlation between upregulated miRNAs with downregulated targets and vice versa: those miRNAs with a FDR<0.05 in the

Fisher's exact test result, were selected for further analysis on the basis of their non-random association with the gene expression signature of interest (Creighton et al., 2008).

For biological pathway correlation, targets were evaluated for their annotation into relevant biological pathways, especially those ones related with lymphomas, using Gene set enrichment analysis (GSEA, www.broad.mit.edu/gsea) (Subramanian et al., 2005). Pathways gene lists were taken from a curated version of the Biocarta (www.biocarta.com), and KEGG (www.genome.jp/kegg) with minor modifications (Aggarwal et al., 2009). Pathways were particularly curated for lymphomas neoplasm genes.

Significant associations between differentially expressed miRNAs (both Significant Analysis of Microarray and Fisher's exact test FDR<0.05) and enriched pathways resulted from GSEA, were investigated building a ranked list that included only the targets predicted of the significant miRNAs. The ranked list contained the genes and their correspondent moderated t-statistic values (that were included automatically in the GEP results output).

Downregulated miRNAs were tested for their association with upregulated genes, whereas upregulated miRNAs were tested for their association with downregulated genes. The ranked target list was subjected to gene set enrichment analysis (GSEA).

GSEA analysis was carried out independently using miRBase and TargetScan targets. Only miRNAs pathways with significant associations identified by both miRBase and TargetScan predictions were finally considered.

Figure 7 provides a flowchart of the entire data analytical approach for miRNA target identification.

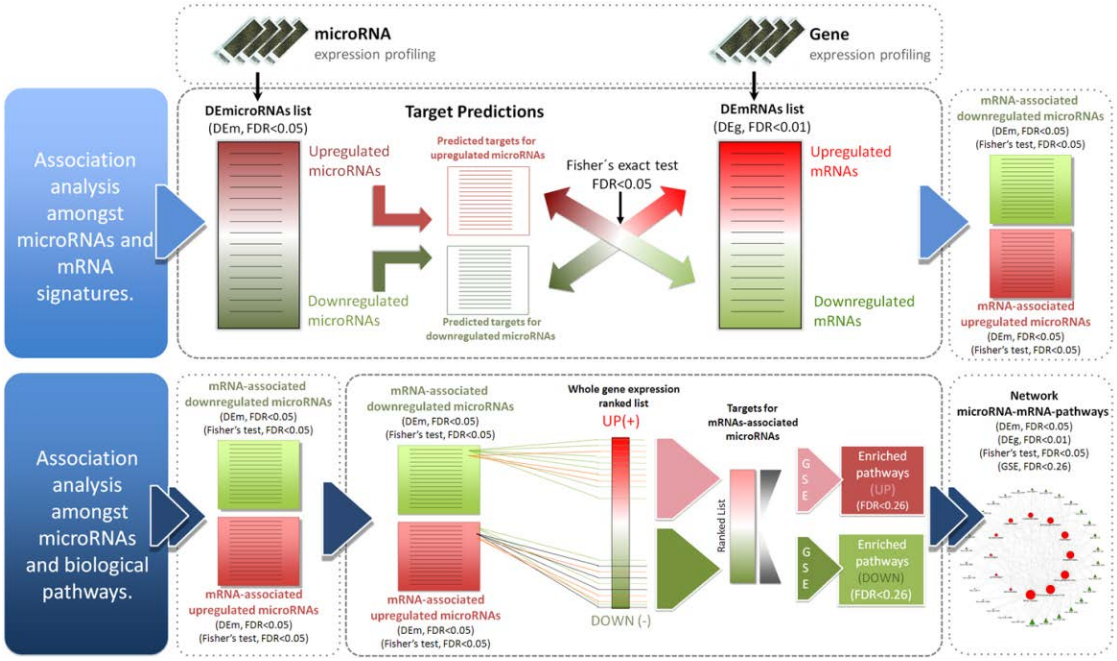


Figure 7. Work flow of data evaluation to compare miRNAs expression with pathway activation. DEm=differentially expressed miRNAs, DEg=differentially expressed mRNAs (genes), GSE= gene set enrichment method (GSEA) FDR=False Discovery rate. The association of each differentially expressed miRNA (DEm) with its differentially expressed predicted targets (DEg) was evaluated by Fisher's exact test (downregulated miRNAs were tested for upregulated predicted targets and upregulated miRNAs were tested for downregulated predicted targets). The resulting significant miRNAs were selected to investigate their statistically significant associations with enriched pathways: the gene set enrichment analysis (GSE) was applied using the list of predicted genes included in the gene expression array. Gene target predictions for human miRNAs were obtained by miRBase and TargetScan.

3.8.6. RT-qPCR analysis

Quantitative RT-PCR (RT-qPCR) data were processed and exported using the SDS software (SDS 2.2, Applied Biosystems). Data were analysed with Real-Time StatMiner program (INTEGROMICSTM; www.Integromics.com) using the ΔCT method. All assays were performed in triplicate. Reproducibility of triplicated curves was evaluated: inconsistent replicates were omitted.

Since the eligibility of the most stable endogenous gene depends on the samples analysed, and conventional endogenous genes (like 18S or GAPDH) are not recommended in small RNA RT-qPCR studies, different endogenous genes were investigated. Normalizations were done according to the most stable small RNA endogenous gene identified by StatMiner program. -ΔCT values were calculated with the following formula: -(Ct value of miRNA of interest – median Ct value for endogenous gene). -ΔCT were considered for further statistical analysis by

using a t-test corrected for multiple hypothesis testing, available at http://pomelo2.bioinfo.cnio.es, or by using SPSS program for survival studies.

3.8.7. Survival analysis

Correlation between miRNAs expression and overall survival was investigated in an initial series of 22 MCL patients. Gene Spring software v. 9.0 (Agilent Technologies Inc) was used to normalize miRNA intra-array data at the 75th percentile, as recommended by the manufacturer (Ach et al., 2008). Next, a Random Forests algorithm (Abba et al., 2007; Bienkowska et al., 2009; Hothorn et al., 2006), available from the SIGNS website http://signs.bioinfo.cnio.es, was used to select a set of 12 miRNAs related to patient survival (Table 12). These miRNAs were validated by RT-qPCR in a new set of 54 paraffin-embedded cases. Univariate Cox regression, available in SPSS v.15.0, (SPSS Inc, Chicago, IL, USA) was used to analyse the confirmation data set for the expression of the 12 miRNAs (Hoster et al., 2008). Overall survival curves were plotted with Kaplan–Meier method, stratifying the samples into low- and high-risk groups according to the median value of the miR-20b expression. Curves were compared by a log-rank test.

3.8.8. Class recognition analysis

miRNA espression data were tested for their reliability of grouping into different tumour types. The K-nearest neighbour (KNN) algorithm was used for class recognition using the Tnasas web tool that provides an internal cross-validation step (http://tnasas.bioinfo.cnio.es/).

3.8.9. Results visualization

miRNAs clusters were computed using the web resource: Gene Expression Profile Analysis Suite, GEPAS v.4.0 (www.gepas.org). The heatmap of GEP significant genes was created using Stanford Cluster software (complete linkage clustering for genes and arrays) and visualized with tree-view program (http://rana.lbl.gov/EisenSoftware.htm).

Interaction networks between pathways and miRNAs were depicted using Cytoscape bioinformatics software (http://www.cytoscape.org).

Kaplan-Meir curves were represented by overall survival was plotted by GraphPad Prism software v.5 (GraphPad Software Inc, v. 5, La Jolla, CA, USA).

3.9. miRNA sequencing

Fourteen miRNAs were selected based on literature findings (Table 13).

Table 13. Selected miRNAs for sequence evaluation.

	miRNA	Location	Why has been studied	Reference
1	hsa-miR-650	22q11.22	Lost in CGH	(Li et al., 2009)
2	hsa-miR-31	9p21.3	Lost in CGH	(Li et al., 2009)
3	hsa-miR-588	6q22.32	Lost in CGH	(Li et al., 2009)
4	hsa-miR-548a-1	6p22	Lost in CGH	(Li et al., 2009)
5	hsa-miR-570	3q29	Lost in CGH	(Li et al., 2009)
6	hsa-miR-16-1	13q14.2	Lost in CGH	(Li et al., 2009)
7	hsa-miR-15a	13q14.2	Lost in CGH	(Li et al., 2009)
8	hsa-miR-596	8p23.3	Lost in CGH	(Li et al., 2009)
9	hsa-miR-587	6q21	Lost in CGH	(Li et al., 2009)
10	hsa-miR-491	9p21.3	Lost in CGH	(Li et al., 2009)
11	hsa-miR-124-2	8q12.3	Lost in CGH	(Li et al., 2009)
12	hsa-miR-34b	11q23.1	Expression regulated by MYC	(Leucci et al., 2008)
13	hsa-miR-145	5q32	Expression regulated by MYC	(Chen et al., 2010)
14	hsa-let-7a	9q22.32	Expression regulated by MYC	(Sampson et al., 2007)

miRNAs sequences were downloaded from Ensembl database (www.ensembl.org/index.html). Primers used for amplification were selected using Primer3 program (available at the web site: http://frodo.wi.mit.edu/) and purchased from Thermo Electron (Thermo Electron Corporation, Ulm, Germany). Primers sequences are described in Table 14.

Table 14. Primers used for the PCR of the miRNAs.

	miRNA investigated	Primer name	Primer sequence	Fragment length
1	hsa-miR-650	miR_650_F1	AGGAGCTCAGGATGCAGATT	213
		miR_650_R1	ATCAGCCCAGAGGTCCCTGT	
2	hsa-miR-31	miR_31_F1	GGTGAAAGGAAAAATTTTGGAA	192
		miR_31_R1	CACAGCAATACACGAAGGACTG	
3	hsa-miR-588	miR_588_F1	AGTCTCTGCTGGTCGTCC	221
		miR_588_R1	CCAGGGCAGGTCTAGAAATTCC	
4	hsa-miR-570	miR_570_F1	GTGGGCCAAGTGTCCTGGG	222
		miR_570_R1	TGGCTTTTGCAAACATAGA	
5	hsa-miR-15a	miR_15a_F2	CGTGCTGCTAAGGCACTGCTG	197
		miR_15a_R2	TTTAGGCGCGAATGTGTGTTTAA	
6	hsa-miR-16	miR_16_F1	ATTGTGCTGCCTCAAAAATACA	245
		miR_16_R2	CTGAAAAGACTATCAATAAAACTG	
7	hsa-miR-596	miR_596_F2	AGTTCTGAGGAACGCATAGCAG	168
		miR_596_R1	AAGGACAGTGACCTAGACAGCA	
8	hsa-miR-587	miR_587_F2	CCTTGCTAAGAGGAGAGGT	310
		miR_587_R2	GACTCAGACCCCCTGAAGAGGC	
9	hsa-miR-491	miR_491_F2	AGCTAACAGACCAGCAGAAGC	224
		miR_491_R1	GCCTTCATCTTCTTCTTCATGG	
10	hsa-miR-124	miR_124_2_F1	CGCAGTGGGTCTTATACTTTCC	188
		miR_124_2_R1	TAAATTCCCTGCAATTGCTTTT	
11	hsa-miR-145	miR_145_F2	TGCTACAGATGGGGCTGGATGC	215
		miR_145_R1	CTGTGAAACCATGACCTCAAGA	
12	hsa-let-7a	Let_7a_F1	TGTGATTCCTTTTCACCATTCA	150
		Let_7a_R1	GCCTGGATGCAGACTTTTCTAT	
		Let_7a_F2	CAGCATAGATTATGCATGTAGC	348
13	hsa-miR-34b	miR_34b_F2	TTGCGCCCAGCCATGGTAG	344
		miR_34b_R1	AGAAACCGCGGGTTTCCTCG	
14	hsa-miR-548a	miR_548a_3p_F3	CCTACTGAATAAGTTATTGGAA	224
		miR_548a_3p_R2	GCTAACAGAAGGAAATTGCACC	

3.9.1. Polymerase chain reaction (PCR)

PCR was performed as follows: 2,5µl of DNA (that accounts for 12,5ng of DNA) + 5µl of AmpliTaq 360 Master mix (Applied Biosystems) + 0,5µl of Forward and Reverse primers (10mM) + water until 10µl of total volume. PCR reactions took place in a GeneAmp® PCR System 9700 (Applied Biosystems). The termocycler program is shown in Table 15.

Table 15. PCR program. Min= minutes, Sec= seconds.

PCR cycles	Temperature (°C)	Time
Hold	95	10 min
35 cycles	95	30 sec
	55	30 sec
	72	1 min
Hold	72	10 min
Hold	4	Forever

Presence, integrity and length of amplified products were checked on a 1,5 % agarose gel.

3.9.2. PCR product purification and sequencing reaction

PCR amplified products (2µl) were purified by adding 1 µl of exosap enzyme (GE-heatcare) in 10xPCR buffer (Applied Biosystems) plus water until a total volume of 5µl using the program shown in Table 16.

Table 16. Exosap reaction program.

PCR cycles	Temperature (°C)	Time (minutes)
Hold	37	20 min
Hold	80	20 min
Hold	4	Forever

Big Dye terminator sequence method was used as follows: 5µl of purified PCR product + 0,35µl BigDye terminator (Appied Biosystems) + 1,75µl of corresponding 5x buffer (supplied with BigDyes) + 2µl of Forward or Reverse primer (2mM) + water up to a total volume of 10µl were used with the conditions of Table 17.

Table 17. BigDyes terminators reaction program. Min= minutes, Sec= seconds.

PCR cycles	Temperature (°C)	Time
Hold	96	1 min
25 cycles	96	20 sec
	50	15 sec
	60	1 min
Hold	4	Forever

Forward primer was always used for sequencing. When necessary, also Reverse primer was used.

Products were purified by Dynabeads (Invitrogen) using DynaMag Sequencing Clean-up magnetic microbeads (Invitrogen); resuspended in 25µl of water and loaded into ABI3730 sequencer (Appied Biosystems).

3.9.3. Sequence evaluation

Fully annotated reference sequences were downloaded from the National Center for Biotechnology Information (NCBI) web site: www.ncbi.nlm.nih.gov.

Sequences were visualized by SeqScape program v2.5.0 (Appied Biosystems).

Previously reported variants were inspected at Ensembl genome browser (www.ensembl.org), NCBI for short genetic variation database (dbSNP, www.ncbi.nlm.nih.gov/projects/SNP/), and at a single nucleotide polymorphism (SNP) database specific for miRNA (www.bioguo.org/miRNASNP/) (Gong et al., 2012).

BibiServ web resource (Zuker and Stiegler, 1981) (http://bibiserv.techfak.uni-bielefeld.de/rnafold/submission.html) was used to evaluate miRNA secondary folding. Results were visualized by RNA StrAT web resource (Blin et al., 2010) (www-lbit.iro.umontreal.ca/rnastrat/?p=tools&p2=render).

4. Results

4.1. Project 1: miRNA expression in non-Hodgkin B-cell lymphomas

Three main parts of the project can be distinguished:

- Comparison of the different lymphoma types
- Comparison between lymphomas and non-tumour samples
- Differential diagnosis of BL *vs.* DLBCL

The work flow of this study is summarized in Figure 8.

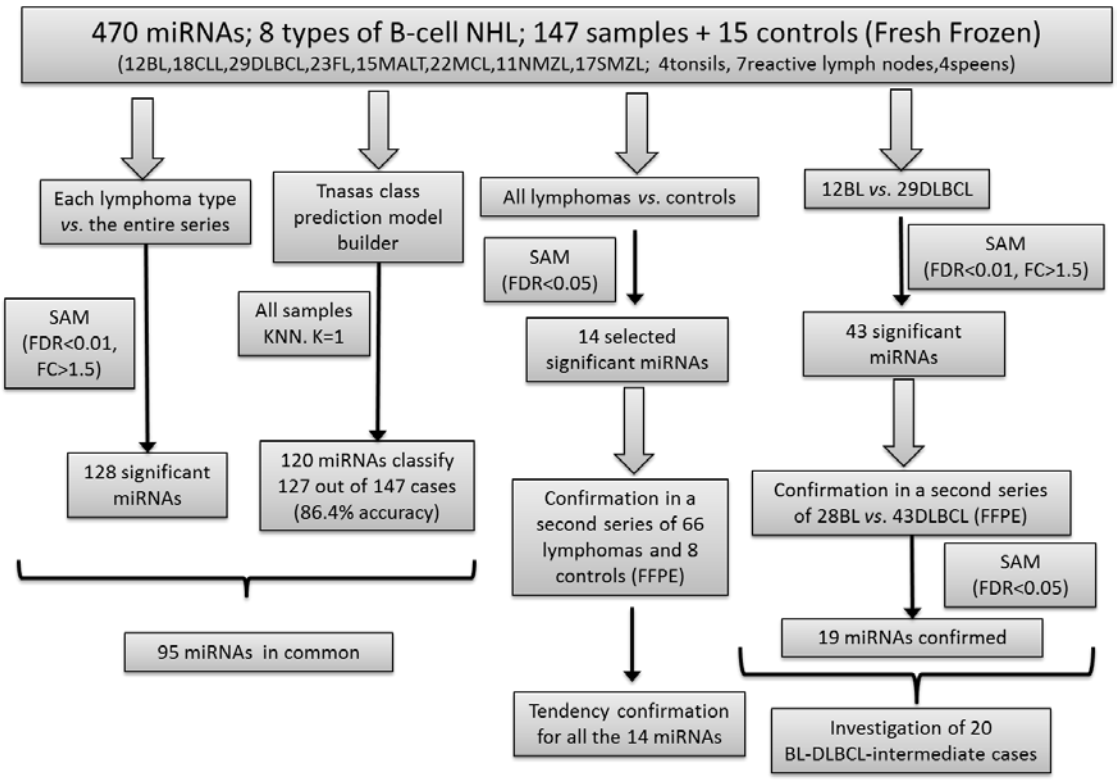


Figure 8. Summary of the samples and procedures used in the first project.

4.1.1. Comparison of the different lymphoma types

A series of 147 fresh–frozen samples of B-cell lymphomas including 12 BL, 29 DLBCL, 22 MCL, 17 SMZL, 18 CLL, 23 FL, 11 NMZL and 15 MZL/MALT (see Materials and Methods, Table 4) has been investigated.

To select a lymphoma miRNAs signature, data from all tumour samples, without prior normalization to non-tumour controls, were studied.

Each lymphoma type were compared towards the entire set of samples by SAM analysis; an approach that has been already used for meticulous description of lymphomas subtypes (Aggarwal et al., 2009).

miRNAs with a false discovery rate (FDR) <0.01 and a fold change >1.5 (log_2), were considered to be significantly up- or down-regulated between the different lymphoma types.

According to these criteria 128 miRNAs were considered to be significant in one or more lymphoma types. These miRNAs are listed in Table 18 and the heatmap of the 128 significant miRNAs is represented in Figure 9.

Table 18. Differentially expressed miRNAs in each type of lymphoma compared to the rest of the samples. Numbers in red and green correspond to significant miRNAs fold change (log_2). In red are shown gained miRNAs and in green loss miRNAs. miRNAs that are labeled in blue were also significant in KNN predictor for class recognition (see paragraph 4.1.1.1.).

	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	-

	miRNA	BL	CLL	DLBCL	FL	MALT	MCL	NMZL	SMZL
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

For heatmap visualization, data obtained from the tumours were normalized towards non-tumour controls: lymph nodes and tonsils (spleens were used only for SMZL cases normalization).

In the Figure 9 it is possible to appreciate the differences between miRNA expression levels found in the different B-cell lymphomas type and also whether these miRNAs are loss or gained in relationship with non-tumour controls (see also appendix figure).

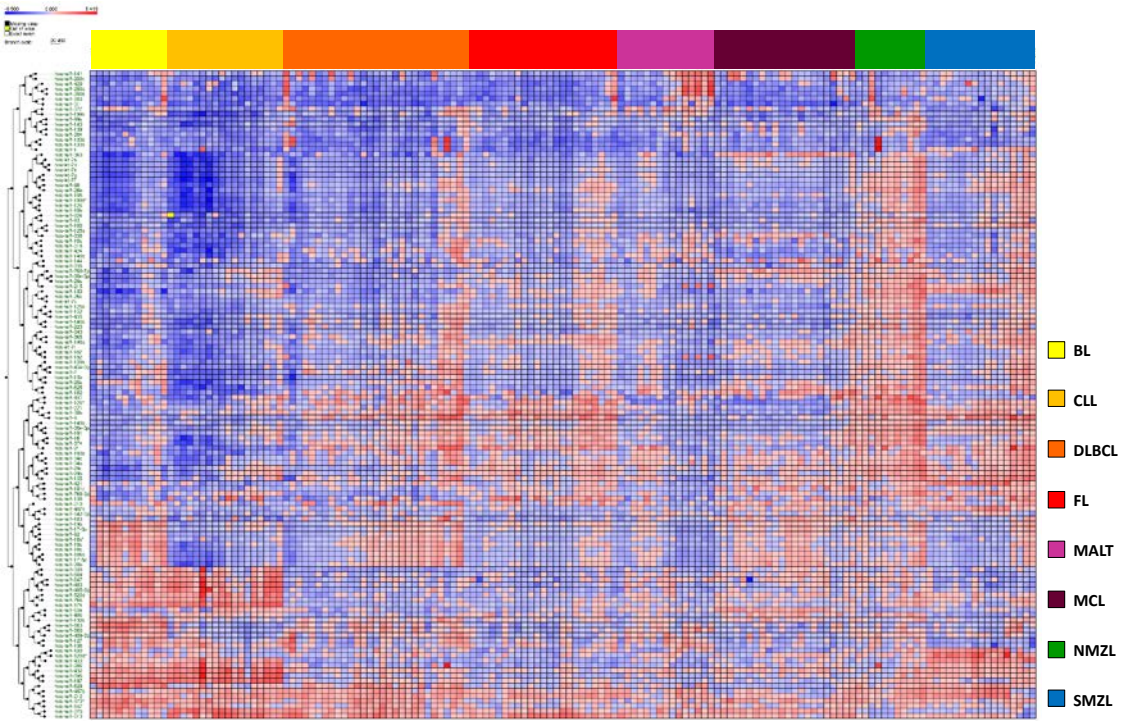


Figure 9. Heatmap of the significant miRNAs normalized with non-tumour controls. Red denotes high expression while blue denotes low expression. A higher resolution figure is provided in the appendix.

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

Burkitt lymphoma

Thirty-five (14 upregulated, 21 downregulated) miRNAs were deregulated in BL compared to the other B-cell lymphomas. The majority of BL (11 out of 12) showed MYC translocation.

Some of the members of the miR-17-92 cluster (miR-17-3p, miR-18a, mir-19a, miR-19b, and miR-92) were upregulated in BL as expected since these miRNAs are targeted by MYC transcription factor (Chang et al., 2008; O'Donnell et al., 2005; Robertus et al., 2010). The most upregulated miRNA was miR-663 and among the upregulated miRNAs, we can find also miR-130b as already described (Robertus et al., 2010). Inside the group of downregulated miRNAs, we found the let-7 family miRNAs and in particular let-7a downregulated in this series of BL, a data that is in line with the fact that let-7a is able to downregulate MYC expression (Sampson et al., 2007), so that let-7a loss might help with further MYC overexpression in BL cases. Other miRNAs were downregulated, for instance miR-150, miR-155 and miR-146a that were already described as lost in BL (Kluiver et al., 2006; Robertus et al., 2010).

Chronic lymphocytic leukemia

Sixty miRNA were deregulated: 14 miRNAs were upregulated and 46 downregulated in CLL samples. Several members of let-7 family were downregulated. Interestingly, miR-218, whose loss of expression might be related with the activation of NF-kB pathway (Gao et al., 2010) was downregulated. miR-106a and miR-363, which belongs to the same miRNA cluster in Xq26.2, where both lost.

Among the upregulated miRNAs found here, the most highly expressed miRNA was miR-197, which regulates the tumour suppressor candidate 2 (TUSC2, also known as FUS1: tumour suppressor gene) (Du et al., 2009a), and may somehow contribute to tumour phenotype.

Numerous works already focused their attention on loss of 13q14 region in CLL (which is found in about 50% of CLL cases) since this change may explain many features of this lymphoma. However it is still to consider that there is a group of cases that does not bring loss of 13q14. The findings here shown consider CLL as only one group and do not focus on the alterations that loss of 13q14 region may promote. Nevertheless, loss of miR-16, already described in CLL with 13q14del (Calin et al., 2002), can still be detected in this series of cases.

Diffuse large B-cell lymphoma

SAM analysis yielded no significant differential miRNA expression in this type of lymphoma. This result could be the consequence of the intrinsic heterogeneity of DLBCL cases as an entity, which probably dilutes the miRNA expression differences with other types of lymphomas. This

heterogeneity is also reflected by the Tnasas web resource class predictor analysis, where the DLBCL group presented the highest error rate (27%) (for more details, see paragraph 4.1.1.1. Table 19 and Table 20). Indeed, DLBCL lymphomas can be further divided into GC and ABC group with documented differences in gene expression profile (Alizadeh et al., 2000) and response to therapy (Dunleavy et al., 2009; Wright et al., 2003). The series of DLBCL cases here studied reflects this heterogeneity with a 60% of ABC type cases and 40% of GC type. Indeed, miRNA signatures related with the molecularly defined subgroups of DLBCL based on the cell of origin (GC or ABC) have been found by different groups (Culpin et al., 2010; Jima et al., 2010; Malumbres et al., 2009). Therefore, it is not surprising that DLBCL had the most heterogeneous miRNA signature of all the lymphoma types analysed, and that differences between this two DLBCL sub-groups may add some noise to the analysis.

Follicular lymphoma

This group is the one that showed the lowest number of significant miRNAs. Only three significantly upregulated miRNAs were found: miR-138, miR-9 and miR-9*; while no downregulated miRNAs were identifierd.

Increased expression of miR-9 was previously described in FL samples (Roehle et al., 2008). Overexpression of miR-9 reduces the PR domain containing 1, with ZNF domain (PRDM1, also called BLIMP1) levels (Nie et al., 2008), a finding of potential interest in FL cases, characterized by the tightly regulated expression of BCL6 and PRDM1. Most FL cases here studied carry on the translocation t(14;18) and gain of miR-138, consistently with their GC (Leich et al., 2011).

Mucosa Associated Lymphoid Tissue / Marginal zone lymphoma

The series of MALT here included are representative of different anatomic region localized MALT: skin, breast, stomach, lymph nodes, endometria, thyroid and lung. This choice was taken because the attention was focused more on the characteristic shared by all MALT tumors rather than to the features of the local tissue. Only one miRNA was found to be lost in MALT samples: miR-126* (corresponding to miR-126-5p), while eight miRNAs were significantly upregulated. Three of these upregulated miRNAs are components of miR-200 family: miR-200a, miR-200b and miR-200c. Interesting, these miRNAs are located into two clusters: miR-200a/200b/429 on chromosome 1p36.33, and miR-200c/141 on chromosome

12p13.31. It is to note that the others miRNAs located in these clusters, miR-429 and miR-141, were also upregulated.

Mantle cell lymphoma

Nine miRNAs were deregulated in MCL: only miR-126* was downregulated, while eight miRNAs were upregulated. The most upregulated miRNA was miR-363, that belongs to the cluster miR-106-363, which has similar miRNAs and functions of cluster miR-17-92 (Ventura et al., 2008), and whose overexpression has been associated with aggressive phenotypes (Rao et al., 2011). Upregulation of both miR-183 and miR-182 is not surprising since they belong to the same cluster located in 7q32.2.

Nodal marginal zone lymphoma

While only two miRNAs, miR-370 and miR-513, were downregulated in NMZL cases, a high number of upregulated miRNAs (sixty-one) relative to the whole series of samples has been identified. The most highly expressed miRNA in this series was miR-150, followed by miR-26b and miR-10b. miR-150 (here overexpressed in NMZL) regulates the expression of the transcription factor C-MYB, and plays a key role in B-cell differentiation (Xiao et al., 2007). miR-221, miR-223 and let-7f which were found upregulated, have been recently described as upregulated in NMZL in others studies too (Arribas et al., 2012).

The seven members of the let-7 family of miRNAs, which are commonly lost in tumours, were significantly overexpressed in NMZL cases compared with the other lymphoma types, which could be dependent of the controls here used.

Splenic marginal zone lymphoma

Twenty-six miRNAs (20 upregulated and 6 downregulated) were differentially expressed in SMZLs. The two most upregulated miRNAs belong to the cluster miR-144/451 on chromosome 17q11.2. These two miRNAs are described to be erythropoiesis regulators (Patrick et al., 2010; Rasmussen et al., 2010), which is a finding that may be related to the splenic microenvironment.

miR-141 and miR-200c (here downregulated), also belong to the same cluster located at chromosome 12q24.32. In this case their downregulation could be due to DNA methylation, as it was found in breast cancer cell lines (Neves et al., 2010). miR-127 which was upregulated is known to regulate BCL6 expression (Saito et al., 2006), a finding that is consistent with the observation that SMZL cases frequently lack BCL6 expression (Swerdlow et al., 2008).

4.1.1.1. Class recognition by miRNAs

In order to further evaluate whether the group of 128 miRNAs identified by the previous approach could be useful for class recognition, data were submitted to the KNN classifier algorithm to test whether B-cell lymphomas could be correctly classified by miRNAs expression level.

A group of 120 out of 470 miRNAs classified the eight sub-classes of lymphomas with a global correct classification rate of 86,4%.

The groups that were best classified were BL, CLL and SMZL. For these lymphomas subtypes only one case was incorrectly classified; while DLBCL was the group that showed the higher rate of error, and it is probably due to DLBCL heterogeneity (Gurbuxani et al., 2009), followed by NMZL.

In total 127 out of 147 tumour samples were properly classified. Results are shown in Table 19 and Table 20.

Table 19. The table shows correct and incorrect predictions of each group of lymphoma and the error rate associated with each class by using the 120 miRNAs identified by the KNN algorithm. Cases correctly classified are labelled in green, while wrong tumour samples predictions are labelled in dark red.

	Class	BL	CLL	DLBCL	FL	MALT	MCL	NMZL	SMZL	Error Per Class	% Error Per Class
Observed	BL	11	1	0	0	0	0	0	0	1/12	8.3%
Observed	CLL	0	17	0	1	0	0	0	0	1/18	5.6%
Observed	DLBCL	0	0	21	6	1	0	1	0	8/29	27.6%
Observed	FL	0	0	1	21	0	0	1	0	2/23	8.7%
Observed	MALT	0	0	0	1	13	1	0	0	2/15	13.3%
Observed	MCL	0	0	0	2	0	20	0	0	2/22	9.1%
Observed	NMZL	0	0	2	1	0	0	8	0	3/11	27.2%
Observed	SMZL	0	0	0	0	0	0	1	16	1/17	5.9%

Table 20. Estimated error found for each group of lymphoma by KNN algorithm.

Class	Total Error
BL	0.007
CLL	0.007
DLBCL	0.054
FL	0.014
MALT	0.014
MCL	0.014
NMZL	0.020
SMZL	0.007

Interestingly, 95 out of 120 miRNAs found in KNN analysis overlapped with the significant miRNAs identified by SAM analysis, these miRNAs labelled in blue in the previous Table 18.

4.1.2. Comparison between lymphomas and non-tumour samples

4.1.2.1. B-cell lymphoma miRNA expression compared vs. non-tumour controls

The same series of 147 fresh frozen samples of B-cell lymphoma (12 BL, 29 DLBCL, 22 MCL, 17 SMZL, 18 CLL, 23 FL, 11 NMZL and 15 MZL/MALT) was here compared with 15 non-tumour samples including reactive lymph nodes, tonsils and spleens (see table Table 4 in the Materials and Methods section), thus the expression profile of B-cell lymphomas considered as only one single group against non-tumour controls has been investigated.

As general consideration, B-cell lymphoma miRNA expression compared with non-tumour controls reveals a larger set of downregulated miRNAs, whereas the upregulated miRNAs have a more heterogeneous pattern that varies with the lymphoma type.

Downregulated miRNAs included miR-31, several members of let-7 family, miR-200 family (miR-200a and miR-200b), and miR-10 family (miR-10a and miR-10b) even though inside the same miRNAs family the significance of each different member may vary. Interestingly, both let-7 family and miR-200a/b are described as a key regulator of cell differentiation, whose loss is proposed to be associated with increased stemness capacity (Peter, 2009). miR-10 family (a and b) is situated within the Hox cluster that regulates development (Lund, 2010), and is downregulated in other myeloproliferative disorders (Agirre et al., 2008).

miR-31 was among the most downregulated miRNAs. One important finding on miR-31 has been recently published, where this miRNA seems to be connected to NF-kB pathway activation (Yamagishi et al., 2012), a feature frequently seen in lymphomas (Pham et al., 2011; Rosebeck et al., 2011).

miR-155, which is involved in the immune response and germinal center development, and is upregulated in HL (Kluiver et al., 2005), was highly downregulated in the BL cases as already described (Kluiver et al., 2006).

miR-15a and miR-16, were already known to be downregulated in 13q14 deleted CLL cases (Calin et al., 2002) and here they are also downregulated in the majority of CLL cases.

The most strongly upregulated miRNAs compared with non-tumour controls were miR-212, and miR-513, but little information are available about these miRNAs.

4.1.2.2. Validation of B-cell lymphoma miRNA expression compared to non-tumour controls

To confirm miRNAs differential expression of B-cell lymphomas compared with non-tumour controls and evaluate the reliability of microarray miRNA platform coupled with the methods used for analysis, a selection of 14 deregulated miRNAs (5 upregulated and 9 downregulated) was analysed by RT-qPCR in an independent series of 66 FFPE lymphoma samples (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). The endogenous gene that showed minor changes according to StatMiner program was RNU6B, which as a consequence was used as endogenous control for data evaluation.

These miRNAs were picked up due to different reasons: for their loss or gain in the majority of the samples independently on the lymphoma type (miR-513 and miR-133a), for their high differential expression as found in fold change (miR-212, miR-31 and miR-200b) and/or FDR values (miR-770-5p and miR-23b,) or because they cluterized together with the other chosen miRNAs (miR-487b, that clustered together with miR-212 and miR-770-5p) or for their relevance in the already published literature (let 7 family: let-7a,l et-7c, let-7d and let-7e) (Barh et al., 2010; Johnson et al., 2007; Johnson et al., 2005; Lee and Dutta, 2007; Leucci et al., 2008; Sampson et al., 2007; Tokumaru et al., 2008). Some of them were also chosen to test less

significant miRNAs (up: miR-9; down: miR-10b) to see whether even less significant results identified in this analysis are reliable.

Tendency of all miRNAs to be lost or gained compared to non-tumour controls was confirmed for all of them even when miR-487b, miR-212 and miR-770-5p presented a slight variability among the different lymphoma types: especially SMZL and MZL/MALT, as shown in Figure 10. This variability may be explained by the different localization of SMZL lymphomas and MZL/MALT, since SMZL are localized in the spleen and MZL/MALT lymphoma are localized in a variety of different tissues (in this series: breast, eye, skin, salivary gland and intestine).

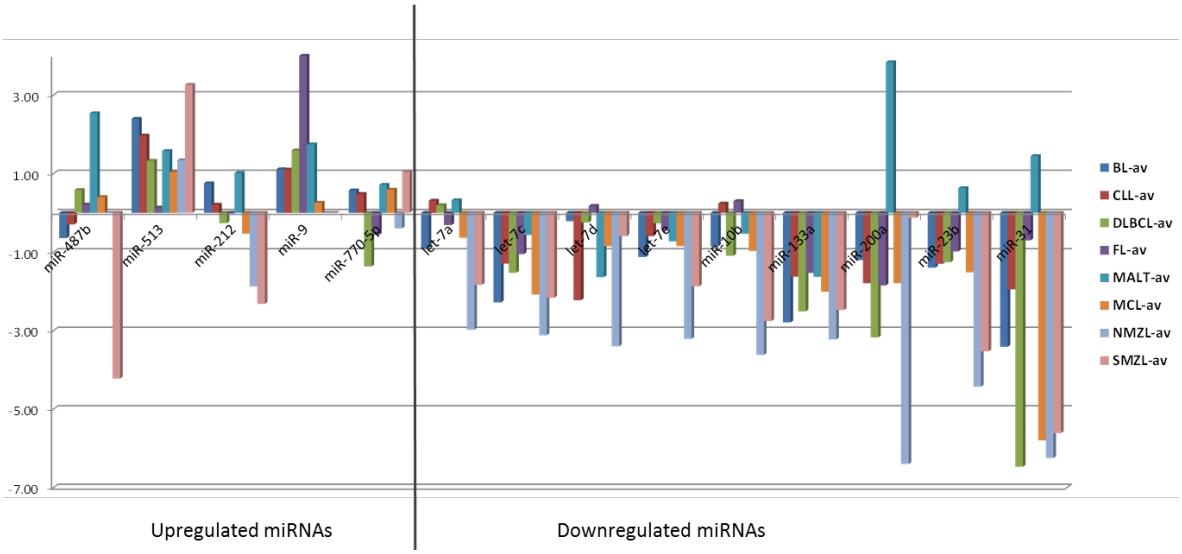


Figure 10. Expression level of 14 miRNAs deregulated in B-cell lymphomas measured by RT-qPCR. miRNA expression for each type of lymphoma is represented by the average expression of the cases (av). mRNA values (-ΔCt) are normalized with normal tissue (3 tonsils and 4 lymph nodes; spleen only for SMZL) and represented in $\log_2$ scale. av=average.

miR-31 is the miRNA that gave the highest rate of loss together with miR-133a, while miR-9 and miR-513 are the best confirmed up-regulated miRNAs.

Even miR-10b, which was less significant in the microarray results, confirms its tendency of being lost.

The potential targets of these miRNA were investigated by different algorithms. Predictions revealed interesting miRNA/gene interactions that could give some light on how lymphomagenesis takes place. For instance, according to miRanda algorithm and miRSVR scoring (downloaded at www.microrna.org/microrna/getDownloads.do), miR-133a and miR-23b, which are lost in this series of lymphomas, were predicted to target the paired box 5

(PAX5), a gene that is involved in lymphocyte development and whose upregulation is related to the development of different B-cell lymphoma types (O'Brien et al., 2011).

miR-31, which was the most strongly lost miRNAs in this series, was submitted to Diana Lab target prediction program (http://diana.cslab.ece.ntua.gr/pathways/) and it was predicted to regulate the expression of the B-cell receptor pathway together with MAPK pathway and JAK-STAT pathway. Not only recently a work in T cell lymphomas asses for miR-31 role in NF-kB pathway activation (Yamagishi et al., 2012), this miRNAs is among the most interesting candidate for further functional studies.

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, with the help of a multiple searcher of miRNAs target (http://gencomp.bio.unipd.it/magia/query/), miR-9 and miR-513 were found to have as potential target PRDM1, and for miR-9 experimental target validation is also available (Huang et al., 2011), while miR-770-5p and miR-212, where predicted to target the tumour necrosis factor, alpha-induced protein 3 (TNFAIP3, also known as A20) that has been described as a tumour suppressor gene frequently loss or inactivated in NHL (Honma et al., 2009; Kato et al., 2009).

4.1.3. Differential diagnosis in BL *vs.* DLBCL

4.1.3.1 miRNAs differentially expressed between BL and DLBCL

As mentioned in the introduction, differential diagnosis between BL and DLBCL is sometimes difficult, as recently reviewed by de Leval and Hasserjian (de Leval and Hasserjian, 2009).

In order to test whether this miRNAs microarrays data expression might be useful for diagnosis, the differential expression of miRNAs in BL and DLBCL was further investigated.

To this aim microarray data from fresh frozen specimens of 12 BL were directly compared with 29 DLBCL cases by SAM analysis. Forty-three miRNAs were considered significantly differential expressed (FDR<0.01 and fold change > 1.5($\log_2$)) between BL and DLBCL. These miRNAs (Figure 11) were further investigated by RT-qPCR in an additional series of 28 BL and 43 DLBCL FFPE specimens. In the second series of FFPE samples, FISH analysis confirmed MYC translocation in 20 out of 28 cases of BL, and in 31.7% (13 out of 41) cases of DLBCL.

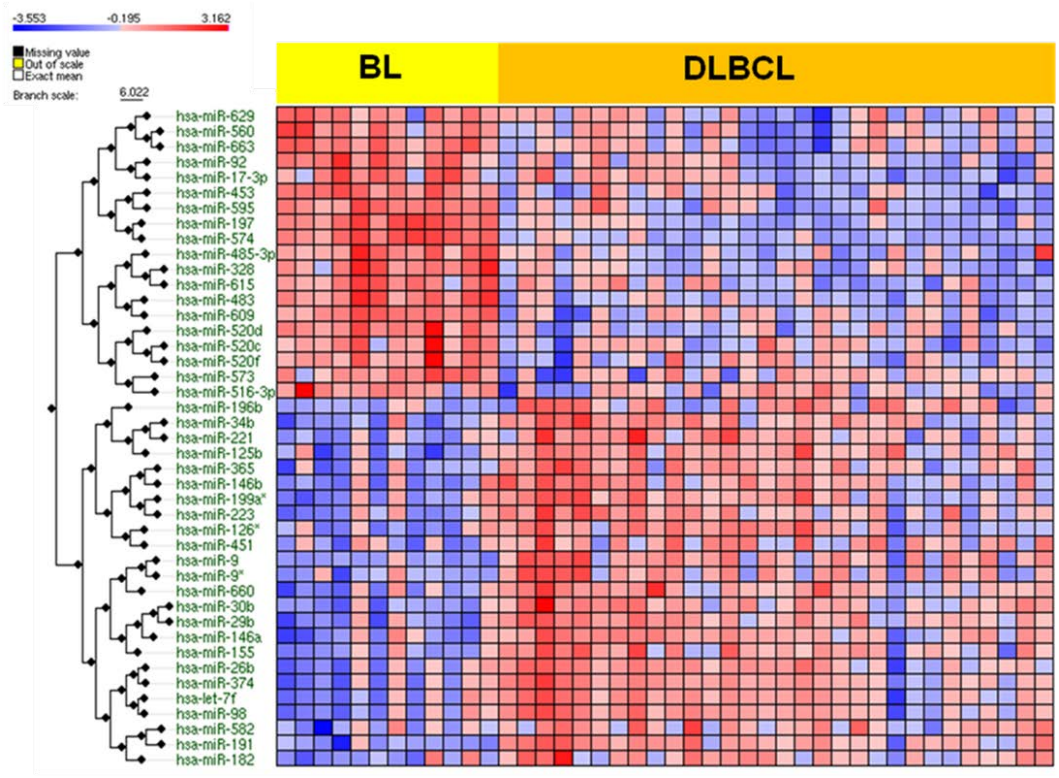


Figure 11. miRNAs differentially expressed in SAM analysis comparing microarray data of 12 BL *vs*. 29 DLBCL (FDR<0.01 and FC>1.5). Red denotes high expression while blue denotes low expression.

Differential expression was confirmed in 19 miRNAs (in blue in Table 21) by a t-test corrected for multivariant hypothesis (FDR<0.05). Thirteen additional miRNAs showed the same tendency as observed in microarray analysis, but with less significance. Two miRNAs were significantly expressed but oppositely with respect to the microarray. Six of the miRNAs had low-efficiency RT-qPCR 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 the miRNA low expression level. So in total, 32 out of 37 miRNAs followed the array tendency.

In terms of fold change miR-155 was the most significantly lost miRNA in BL, confirming previous findings (Kluiver et al., 2006; Kluiver et al., 2005), followed by miR-29b and miR-146a, whereas the most significantly lost miRNAs in DLBCL were miR-595, miR-573 and miR-17-3p. Only one miRNA belonging to these ones regulated by MYC was upregulated in BL: miR-17-3p.

Table 21. miRNAs used in qPCR to validate BL/DLBCL miRNA differential expression. miRNAs in blue have been confirmed and showed a significant FDR (FDR<0.05). ND = No data.

	miRNA	FC arrays	FC qPCR	Upregulated in
1	hsa-let-7f	2.030	0.668	DLBCL
2	hsa-miR-125b	1.909	0.146	DLBCL
3	hsa-miR-126*	2.198	0.218	DLBCL
4	hsa-miR-146a	2.486	1.968	DLBCL
5	hsa-miR-146b	2.011	0.477	DLBCL
6	hsa-miR-155	2.549	2.671	DLBCL
7	hsa-miR-17-3p	1.605	1.679	BL
8	hsa-miR-182	2.048	0.213	DLBCL
9	hsa-miR-191	1.999	0.332	DLBCL
10	hsa-miR-196b	1.741	0.943	DLBCL
11	hsa-miR-197	2.643	0.380	Not confirmed
12	hsa-miR-199a*	1.742	0.575	DLBCL
13	hsa-miR-221	1.798	0.398	Not confirmed
14	hsa-miR-223	1.692	0.458	DLBCL
15	hsa-miR-26b	2.054	0.835	DLBCL
16	hsa-miR-29b	1.979	2.331	DLBCL
17	hsa-miR-30b	1.898	1.209	DLBCL
18	hsa-miR-328	1.931	1.682	Not confirmed
19	hsa-miR-34b	1.889	0.804	DLBCL
20	hsa-miR-365	1.797	1.588	DLBCL
21	hsa-miR-374	1.897	0.696	DLBCL
22	hsa-miR-451	2.209	0.618	DLBCL
23	hsa-miR-453	1.846	ND	ND
24	hsa-miR-483	2.466	ND	ND
25	hsa-miR-485-3p	2.012	ND	ND
26	hsa-miR-516-3p	1.655	0.828	BL
27	hsa-miR-520c	1.809	ND	ND
28	hsa-miR-520d	1.814	1.566	BL
29	hsa-miR-520f	1.642	1.201	BL
30	hsa-miR-560	1.750	ND	ND
31	hsa-miR-573	1.638	1.728	BL
32	hsa-miR-574	3.069	0.815	Not confirmed
33	hsa-miR-582	2.014	0.436	Not confirmed
34	hsa-miR-595	1.940	2.785	BL
35	hsa-miR-609	2.120	ND	ND
36	hsa-miR-615	1.810	0.644	BL
37	hsa-miR-629	1.927	0.881	BL
38	hsa-miR-660	1.879	0.406	DLBCL
39	hsa-miR-663	2.504	1.173	BL
40	hsa-miR-9	2.577	1.028	DLBCL
41	hsa-miR-9*	2.060	1.011	DLBCL
42	hsa-miR-92	1.897	1.273	BL
43	hsa-miR-98	2.076	0.090	DLBCL

The 19 confirmed significant miRNAs were submitted to the Self-Organizing Tree Algorithm (SOTA) (Herrero et al., 2001; Wang et al., 1998) algorithm for samples unsupervised clustering (Figure 12). Only four DLBCL and one BL (five samples in total) out of 71 cases were misplaced (corresponding to 7%).

DLBCL cases that cluster with BL do not show any common feature in terms of GC/ABC type, MYC translocation or Bcl2 immunohistochemical expression, even though two of the cases carry on a MYC translocation. The BL case that clustered with DLBCL cases shows MYC translocation. Therefore, the cluster shown demonstrates that these cases do not cluster depending on MYC translocation status.

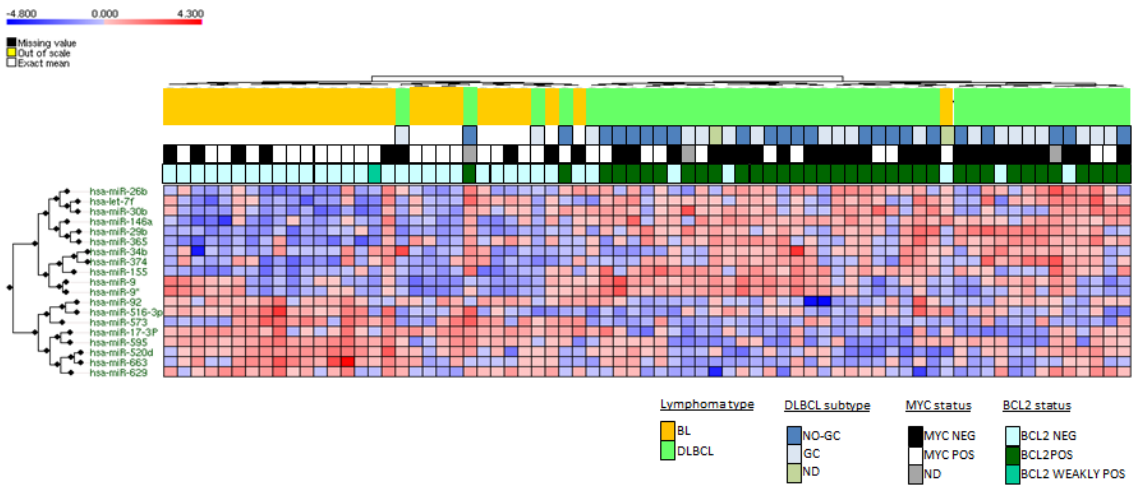


Figure 12. Unsupervised cluster of 28BL and 43DLBCL cases using –ΔCt values of the 19 miRNAs confirmed in RT-qPCR experiments. DLBCL subtype classification was done according to Hans' algorithm (Blood, 2004), and MYC and BCL2 status are annotated. Red denotes high expression while blue denotes low expression.

From t-test investigation, the most significant miRNAs lost in BL cases was miR-155 (FDR<10^{-7}), followed by miR-29b (FDR=$3x10^{-7}$) and miR-146a (FDR=$2.3x10^{-6}$), while the most significant miRNA lost in DLBCL cases was miR-17-3p (FDR=$2.3x10^{-6}$), as also showed in the box plot of Figure 13.

Among the most interesting downregulated miRNAs in BL cases we found miR-29b, which regulates TCL-1 expression (Pekarsky et al., 2006), a protein that is aberrantly expressed in this type of lymphoma and has been proposed as a diagnostic marker (Harris and Horning, 2006).

This miRNA is also negatively correlated with myeloid cell leukaemia sequence 1 (MCL-1) expression (Mott et al., 2007), an anti-apoptotic protein whose expression is commonly seen in lymphoid neoplasms with a high proliferation index (Soini et al., 1998) as BL.

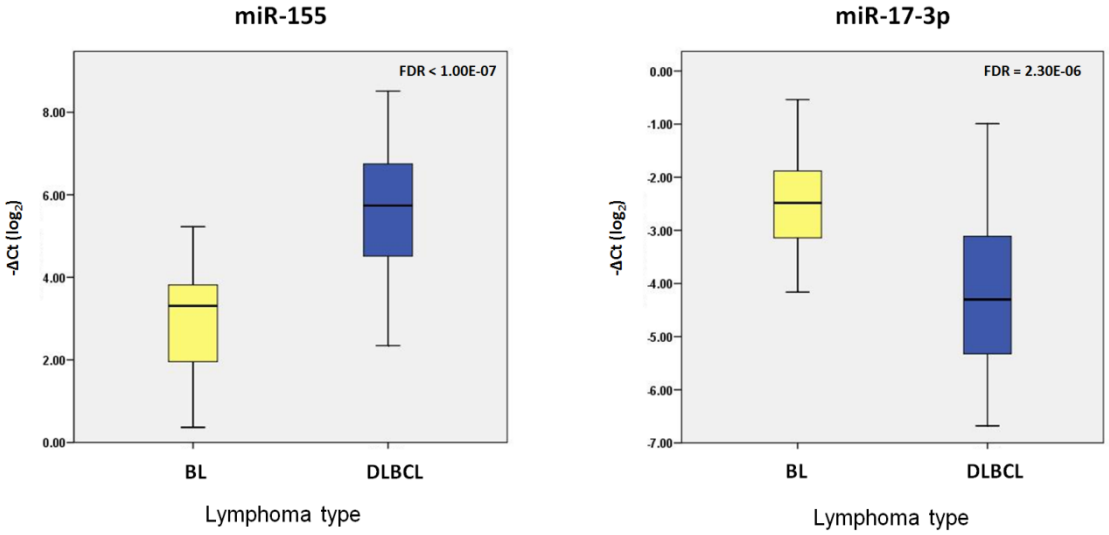


Figure 13. Results of RT-qPCR expression in BL and DLBCL cases of two of the most significant miRNAs: miR-155 and miR-17-3p. BL are represented by yellow boxes while DLBCL are represented by blue boxes.

miR-146a was already known to play an important role in inflammatory reactions and cancer (Li et al., 2010c). 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 BCL2 and NOTCH (Ji et al., 2009).

4.1.4. The issue of Intermediate BL/DLBCL cases

The scenario is even more complex if we consider the problem of unclassifiable BL, with features intermediate between BL and DLBCL cases (intermediate BL-DLBCL), an issue that demands deeper investigation because of the clinical implications of these diagnosis. For this reason a series of 20 B-cell lymphoma, Intermediate BL/DLBC, classified according to WHO criteria (Swerdlow et al., 2008), were added to BL and DLBCL cases.

Intermediate BL-DLBCL cases were investigated for the same miRNAs as DLBCL and BL.

Data were submitted to SOTA algorithm as before, and as expected in this investigation, samples clustering shows that the majority of the intermediate cases lay between BL and DLBCL, a finding that is in accordance with the existence of a distinguishable lymphoma entity, but still, a number of cases (eight) clearly segregates with DLBCL or BL.

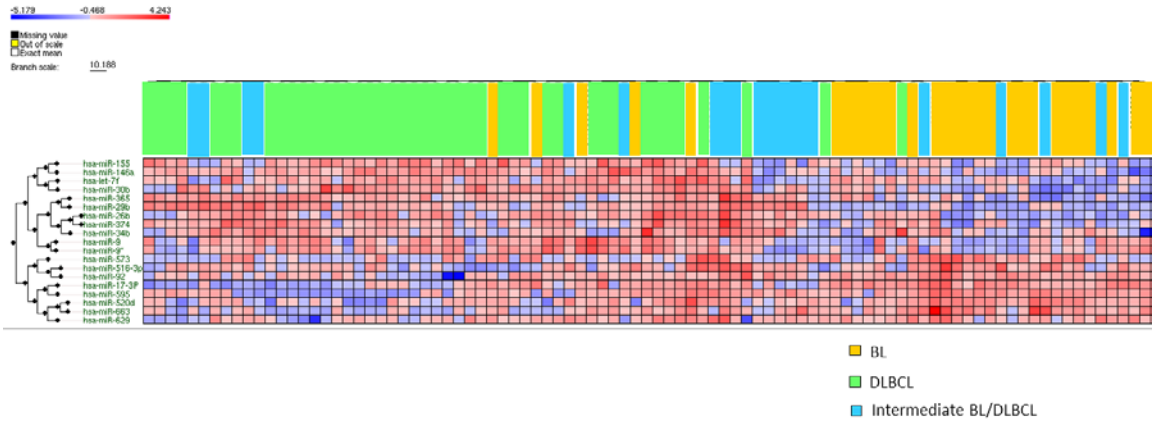


Figure 14. Unsupervised cluster of BL, DLBCL and intermediate BL/DLBCL cases. –ΔCt values were used. Red denotes high expression while blue denotes low expression.

These findings put the basis for a bigger project aimed to better characterize intermediate BL/DLBCL lymphoma subgroup.

4.2. Project 2: miRNA relevance in lymphomas: the example of MCL

MCL cases were studied more in depth and miRNAs expression profile was investigated in this type of tumour together with GEP. These data were integrated to explore miRNAs role in pathway deregulation and finally, since prognosis can be significantly different among MCL cases, the relevance of miRNAs expression was explored in relationship with patients OS.

4.2.1. miRNA microarray results in MCL cases *vs.* controls

Twenty-three MCL cases were compared to 11 non-tumour controls (7 reactive lymph nodes and 4 tonsils) by SAM analysis to define a miRNA expression signature that characterizes MCL tumour. All these samples proceeded from fresh frozen tissue. Cutoff for significant miRNAs was established at FDR<0.05 (Flavin et al., 2008).

117 miRNAs resulted significantly deregulated: 85 of them were downregulated and 32 were upregulated. FDR values are shown in Table 22 and Table 23.

The majority of significant miRNAs found by SAM analysis also showed a fold change higher than 1 ($\log_2$): it means that the loss of expression of these miRNAs was of at least the 50%, and for the upregulated miRNAs it means that miRNAs were expressed at least the double compared to controls. These 72 miRNAs with a fold change higher than 1($\log_2$) and FDR<0.05 were used to build the heatmap of Figure 15 with GEPAS web resource. This heatmap is a useful tool to describe the results, in fact it is easy to visualize miRNAs that are lost (in blue) or gained (in red) in MCL cases compared with controls. A yellow line in the figure helps with their distinction.

Interestingly, lost miRNAs were more numerous than these ones gained by the tumour. A bit of heterogeneity in the expression levels of non-tumour controls can be observed for lost miRNAs whereas gained miRNAs showed a more homogeneous pattern.

The heterogeneity observed between tonsils and reactive lymph node was expected due to a mayor epithelial component and probably periodic antigen stimulation of the tonsils. Both of them were used as controls for a better evaluation of the results.

Table 22. Results of SAM analysis between MCL cases and non-tumour controls: dowregulated miRNAs in MCL cases compared with non-tumour samples.

	Gene ID Agilent	FDR		Gene ID Agilent	FDR
1	hsa-miR-31	<0.001	44	hsa-miR-376a	<0.001
2	hsa-miR-148a	<0.001	45	hsa-let-7f	<0.001
3	hsa-miR-27b	<0.001	46	hsa-miR-151	<0.001
4	hsa-miR-199b	<0.001	47	hsa-miR-454-3p	<0.001
5	hsa-miR-224	<0.001	48	hsa-miR-199a	<0.001
6	hsa-miR-23a	<0.001	49	hsa-miR-650	<0.001
7	hsa-miR-27a	<0.001	50	hsa-miR-365	<0.001
8	hsa-miR-99a	<0.001	51	hsa-miR-204	<0.001
9	hsa-miR-424	<0.001	52	hsa-miR-335	<0.001
10	hsa-miR-7	<0.001	53	hsa-miR-223	<0.001
11	hsa-miR-23b	<0.001	54	hsa-miR-504	<0.001
12	hsa-miR-152	<0.001	55	hsa-miR-125a	<0.001
13	hsa-miR-200b	<0.001	56	hsa-miR-30a-3p	0.007
14	hsa-miR-199a*	<0.001	57	hsa-miR-196a	0.007
15	hsa-miR-181a*	<0.001	58	hsa-miR-629	0.007
16	hsa-miR-126	<0.001	59	hsa-miR-139	0.007
17	hsa-miR-200a	<0.001	60	hsa-let-7c	0.007
18	hsa-miR-1	<0.001	61	hsa-miR-143	0.007
19	hsa-miR-98	<0.001	62	hsa-miR-30e-3p	0.007
20	hsa-miR-133b	<0.001	63	hsa-miR-128a	0.007
21	hsa-miR-378	<0.001	64	hsa-miR-422b	0.007
22	hsa-miR-95	<0.001	65	hsa-miR-218	0.007
23	hsa-miR-146b	<0.001	66	hsa-miR-450	0.007
24	hsa-miR-125b	<0.001	67	hsa-miR-145	0.007
25	hsa-miR-150	<0.001	68	hsa-let-7g	0.007
26	hsa-miR-126*	<0.001	69	hsa-miR-323	0.012
27	hsa-miR-342	<0.001	70	hsa-miR-99b	0.012
28	hsa-miR-148b	<0.001	71	hsa-miR-582	0.012
29	hsa-miR-100	<0.001	72	hsa-miR-340	0.017
30	hsa-miR-149	<0.001	73	hsa-miR-30b	0.017
31	hsa-miR-10b	<0.001	74	hsa-miR-128b	0.017
32	hsa-miR-196b	<0.001	75	hsa-miR-136	0.026
33	hsa-miR-222	<0.001	76	hsa-miR-132	0.026
34	hsa-miR-203	<0.001	77	hsa-miR-26a	0.026
35	hsa-miR-502	<0.001	78	hsa-miR-22	0.026
36	hsa-miR-497	<0.001	79	hsa-miR-33	0.028
37	hsa-miR-21	<0.001	80	hsa-miR-532	0.028
38	hsa-miR-565	<0.001	81	hsa-miR-377	0.028
39	hsa-miR-133a	<0.001	82	hsa-miR-491	0.034
40	hsa-miR-221	<0.001	83	hsa-let-7e	0.034
41	hsa-miR-26b	<0.001	84	hsa-miR-660	0.034
42	hsa-miR-205	<0.001	85	hsa-miR-103	0.034
43	hsa-miR-24	<0.001			

Table 23. Results of SAM analysis between MCL cases and non-tumour controls: upregulated miRNAs in MCL cases compared with non-tumour samples.

	Gene ID Agilent	FDR		Gene ID Agilent	FDR
1	hsa-miR-617	<0.001	17	hsa-miR-372	0.006
2	hsa-miR-370	<0.001	18	hsa-miR-509	0.006
3	hsa-miR-654	<0.001	19	hsa-miR-124a	0.006
4	hsa-miR-494	<0.001	20	hsa-miR-202	0.006
5	hsa-miR-768-5p	<0.001	21	hsa-miR-182	0.006
6	hsa-miR-662	<0.001	22	hsa-miR-513	0.017
7	hsa-miR-765	<0.001	23	hsa-miR-525*	0.017
8	hsa-miR-610	<0.001	24	hsa-miR-563	0.017
9	hsa-miR-801	<0.001	25	hsa-miR-181c	0.017
10	hsa-miR-106b	<0.001	26	hsa-miR-606	0.026
11	hsa-miR-345	<0.001	27	hsa-miR-373	0.026
12	hsa-miR-636	<0.001	28	hsa-miR-627	0.026
13	hsa-miR-501	<0.001	29	hsa-miR-373*	0.026
14	hsa-miR-363	<0.001	30	hsa-miR-597	0.026
15	hsa-miR-198	<0.001	31	hsa-miR-134	0.026
16	hsa-miR-188	0.006	32	hsa-miR-216	0.026

The most significant miRNAs that were lost in all cases were miR-31, miR-148a and miR-27b, whereas the list of upregulated miRNAs was headed by miR-617, miR-370 and miR-654.

MCL cases displayed increased expression of the miRNAs miR-106b, miR-93 and miR-25 (these two with less significance) located at 7q22, which are functionally homologous to the 17-92 polycistron, known as *oncomir-1* (Tanzer and Stadler, 2004; Ventura et al., 2008) located on chromosome 13q31.3. Its amplification in lymphoma and other tumour types has been linked to an accelerated Myc-induced tumour development by suppression of the expression of the tumour suppressor PTEN (Poliseno et al., 2010; Xiao et al., 2008) and the proapoptotic protein BCL2L11 (also called Bim) (Kan et al., 2009).

The gain of function of the miR-106b cluster has been described to promote cell-cycle progression by silencing the cyclin-dependent kinase inhibitor (CDKN1A), a direct target of miR-106b overrides a doxorubicin-induced DNA damage checkpoint (Ivanovska et al., 2008).

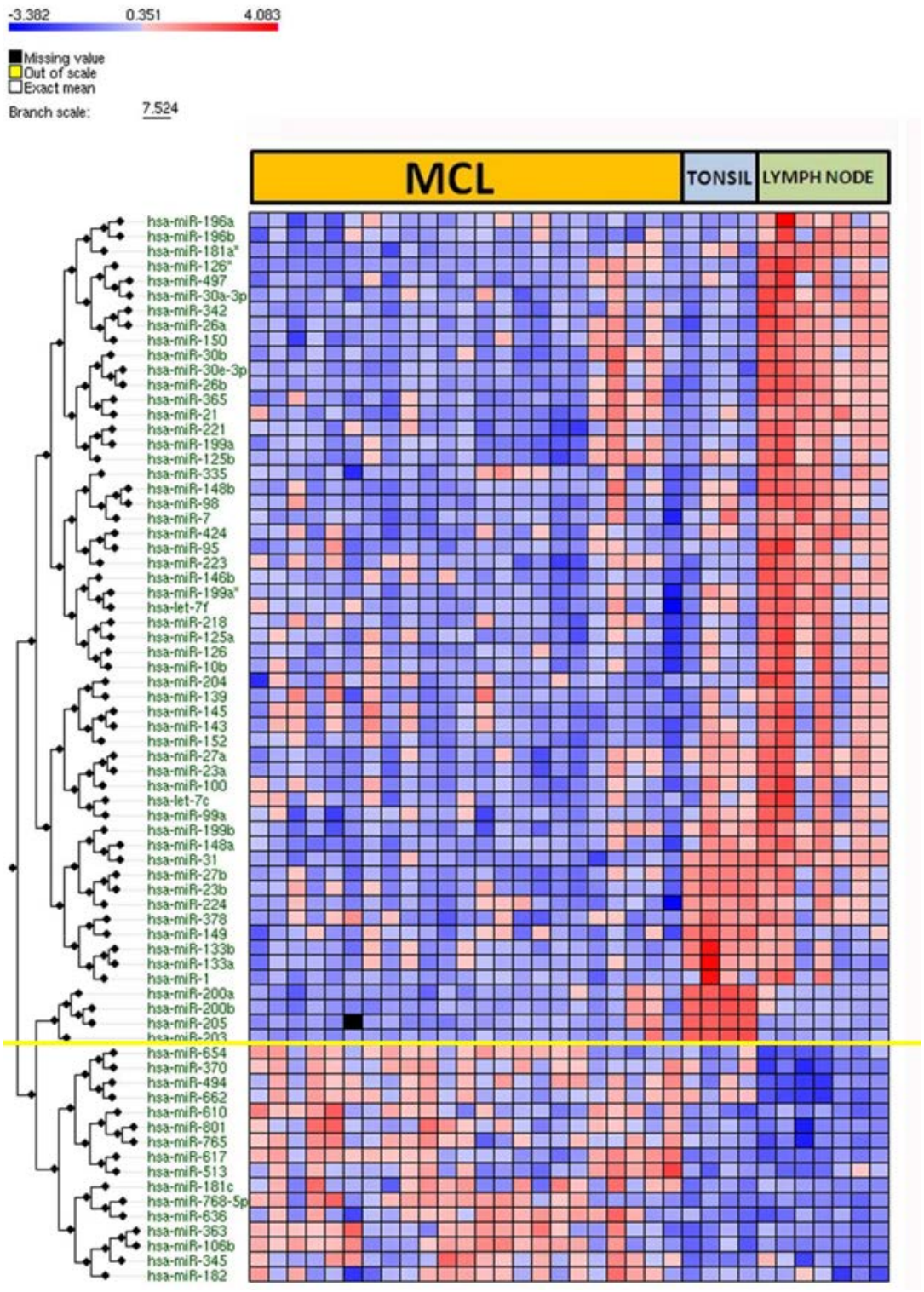


Figure 15. Heatmap of significant miRNAs between MCL and non-tumour controls (FDR<0.05 and FC>1). Red denotes high expression while Blue denotes low expression

4.2.1.1. RT-qPCR validation of array data

Nineteen miRNAs were selected to confirm their expression by RT-qPCR in all 23 MCL cases and in all the controls (7 reactive lymph nodes and 4 tonsils) on the basis of their statistical significance, and/or their potential role in MCL pathogenesis (Table 24).

Five miRNAs (RNU44, RNU48, let-7a, hsa-let7d and hsa-miR-320) were tested as endogenous controls. Let-7a, let-7d and miR-320, were selected because they presented minor variations in the arrays of the series studied (Davoren et al., 2008). Two additional small endogenous RNAs (RNU44 and RNU48) were used following the manufacturer's recommendations.

The most appropriate endogenous controls, according to StatMiner program were the combination of RNU44, RNU48 and let-7a, so the average of these three endogenous genes was selected for normalization.

Three out of the 19 miRNAs analysed (miR-198, miR-370 and miR-617) did not amplify efficiently, probably because of their low basal expression.

A t-test corrected for multivariant hypothesis was performed on -ΔCT values to compare MCL cases towards non-tumour controls. miRNAs with FDR<0.05 were considered significant (Flavin et al., 2008).

The significance of 13 miRNAs was confirmed (FDR<0.05). The remaining three miRNAs showed a similar tendency to that obtained in the microarray analysis, but their results were not statistically significant.

Thus, the majority of the results obtained from the array were confirmed by RT-qPCR (Table 24).

Some of these results overlap with other recently published findings in MCL, such as the loss of miR-150 together with the increased expression of miR-124a, miR-302c, miR-373* (Zhao et al., 2010) and miR-345 (Navarro et al., 2009).

miR-31 confirmed to be highly lost in MCL cases while miR-363 resulted the best validated up-regulated miRNA.

Table 24. miRNAs used for MCL expression profile validation in the same series of MCL cases. ND=No Data.

	De-regulated miRNAs	FDR qPCR	FDR array
	Down		
1	hsa-miR-31	<0.001	<0.001
2	hsa-miR-150	<0.001	<0.001
3	hsa-miR-24	0.004	<0.001
4	hsa-miR-26a	0.008	0.027
5	hsa-miR-200b	0.009	<0.001
6	hsa-miR-203	0.009	<0.001
7	hsa-miR-7	0.032	<0.001
8	hsa-miR-126	0.038	<0.001
9	hsa-miR-1	0.038	<0.001
10	hsa-miR-335	0.059	<0.001
11	hsa-miR-132	0.071	0.027
12	hsa-miR-497	0.118	<0.001
	UP		
13	hsa-miR-363	<0.001	<0.001
14	hsa-miR-106b	<0.001	<0.001
15	hsa-miR-182	0.002	0.007
16	hsa-miR-181c	0.032	0.017
17	hsa-miR-198	ND	<0.001
18	hsa-miR-370	ND	<0.001
19	hsa-miR-617	ND	<0.001

4.2.3. MCL gene expression profiling

Gene expression microarrays of the same 23 MCL cases were also performed to define the gene expression signature of paired MCL cases. A t-test corrected for multivariate hypothesis showed significance of about 5000 genes (FDR<0.05).

Results confirmed the pathological features of this lymphoma, with the distinctive signature including CCND1, SOX11, BMI1 and CDK4 overexpression, together with downregulation of a set of germinal center (GC) markers including LIM domain only 2 (LMO2), germinal center expressed transcript 2 (GCET2), membrane metallo-endopeptidase (MME, known also as CD10), BCL6 and others.

Results are shown in Figure 16, where for representation tumour samples have been previously normalized with controls.

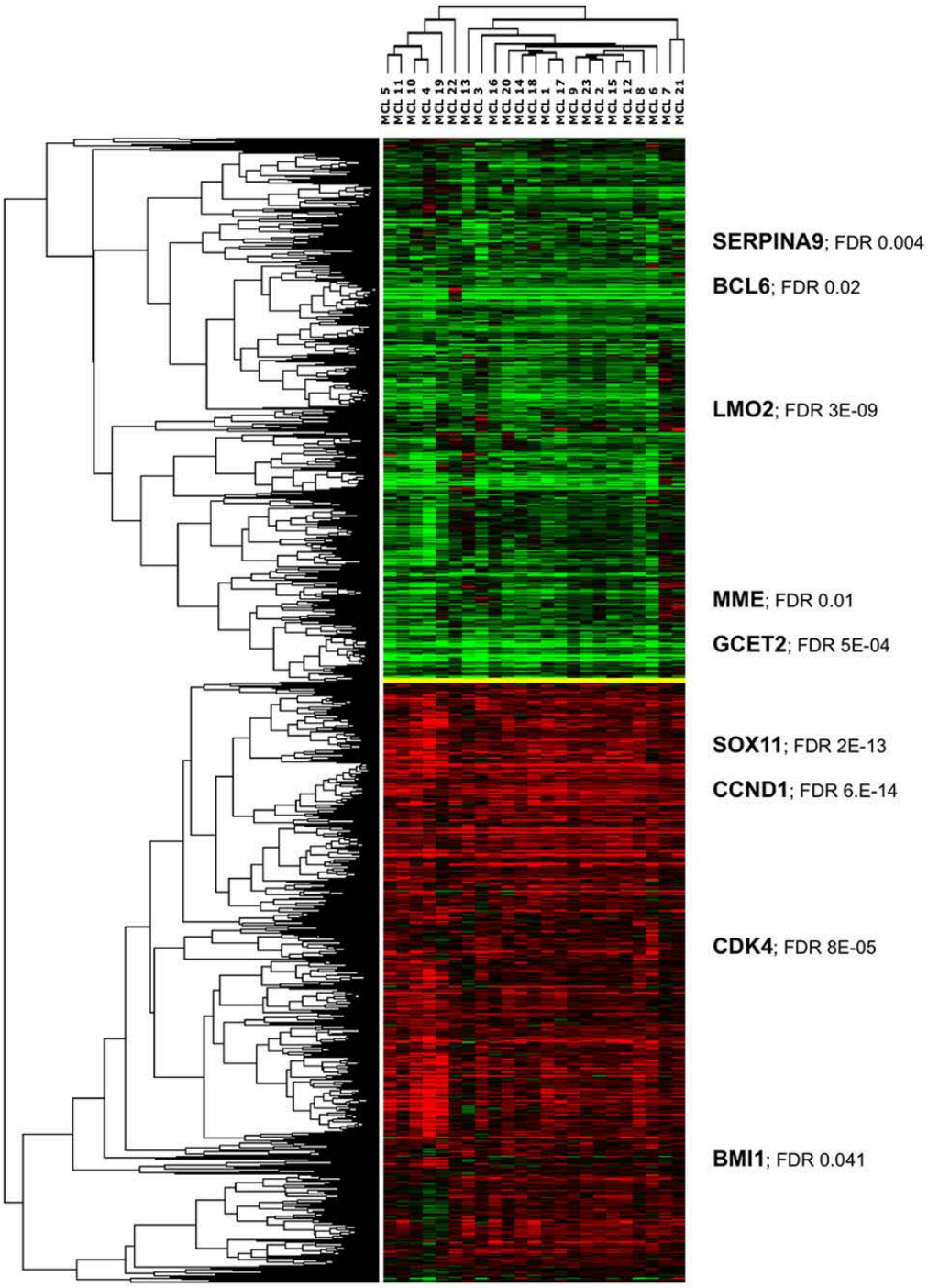


Figure 16. Gene expression profile of MCL cases. Heatmap of significant genes (FDR<0.05). Genes downregulated and upregulated with respect to reactive lymph nodes and tonsils are represented in green and red, respectively.

4.2.4. Association between miRNAs and mRNA signature genes and pathways

4.2.4.1. miRNA and mRNA association

The GEP profile of MCL itself does not confer any new piece of information, but the novelty in this study is that GEP has been correlated with miRNA signature. Data from 23 MCL samples and 11 controls (7 lymph nodes and 4 tonsils) were used.

All 117 significant miRNAs (FDR<0.05) were submitted to miRBase and TargetScan software to obtain a list of their predicted targets.

Then, significant genes resulted from GEP analysis (FDR<0.01) were selected for the following step.

As miRNAs function as gene repressors, a gain in miRNAs should be associated with the downregulation of the target mRNA or protein, whereas miRNAs loss should be associated with upregulation of the mRNA or protein target (Garzon et al., 2006).

Thus, a Fisher's exact test was applied to investigate the correlation between:

1) up-regulated miRNAs and their predicted targets (mRNA) that were down-regulated in GEP results (correlation investigated for miRBase and TargetScan predictions separately);

2) down-regulated miRNAs and their predicted targets (mRNA) that were up-regulated in GEP results (correlation investigated for miRBase and TargetScan predictions separately).

Twenty-one downregulated and four upregulated significant miRNAs were obtained (FDR<0.05) on the basis of a results of Fisher's exact tests for both Targetscan and miRBase.

4.2.1.2. miRNA and pathwas association

A further step to evaluate miRNA correlation to pathways activation has been done: connections between the miRNA signatures and the MCL-deregulated pathways were tested using GSEA.

For the analysis, two independent tables were built for the two predictors programs (miRBase and Targetscan) containing the statistic expression values (a so called ranked list for GSEA program) of significant mRNAs in Fisher's exact test.

Then, these two tables were further separated into down-regulated genes (mRNA) and up-regulated genes (mRNA). Each table contained the adjusted expression value of the listed genes.

A total of 4 tables were obtained:

-miRbase downregulated predicted targets (537 genes)
-miRbase upregulated predicted targets (3712 genes)
-Targetscan downregulated predicted targets (1951 genes)
-Targetscan upregulated predicted targets (3861 genes)

All these tables were separately submitted to GSEA together with a curated list of about 3000 genes involved in relevant pathways previously prepared that includes annotations from Biocarta, KEGG and gene ontology web resource.

The significant resulting pathways, significant miRNAs and significant predicted target genes, are listed in Table 25.

Table 25. The table shows the enrichment pathways linked to differentially expressed genes and differentially expressed miRNAs.

Gene included in the annoted pathways	miRNAs	GeneSet (Pathway)	FDR of GSEA analysis (miRanda prediction)	FDR of GSEA analysis (targetscan prediction)
CCDC50	hsa-miR-103	Bcells: IgMlgDCD27^{+}	0.15	0.16
CCDC50	hsa-miR-203	Bcells: IgMlgDCD27+	0.15	0.16
CD1D	hsa-miR-377	Bcells: IgMlgDCD27+	0.15	0.16
FCRL2	hsa-miR-27a	Bcells: IgMlgDCD27+	0.15	0.16
GPX7	hsa-miR-204	Bcells: IgMlgDCD27+	0.15	0.16
GPX7	hsa-miR-335	Bcells: IgMlgDCD27+	0.15	0.16
IGF2R	hsa-miR-143	Bcells: IgMlgDCD27+	0.15	0.16
IGF2R	hsa-miR-532-5p	Bcells: IgMlgDCD27+	0.15	0.16
BTK	hsa-miR-21	Blimp1 targets	0.12	0.21
CHKA	hsa-miR-143	Blimp1 targets	0.12	0.21
FTH1	hsa-miR-532-5p	Blimp1 targets	0.12	0.21
LYN	hsa-miR-376a	Blimp1 targets	0.12	0.21
MCM7	hsa-miR-103	Blimp1 targets	0.12	0.21
MYBL2	hsa-miR-143	Blimp1 targets	0.12	0.21
MYO1E	hsa-miR-143	Blimp1 targets	0.12	0.21
PHKG2	hsa-miR-103	Blimp1 targets	0.12	0.21
PRKDC	hsa-miR-203	Blimp1 targets	0.12	0.21
FCRL2	hsa-miR-27a	Blood Pan Bcell	0.08	0.19
OSBPL10	hsa-miR-21	Blood Pan Bcell	0.08	0.19
CHD1	hsa-miR-103	CD40 signalling during GC development	0.22	0.01
KCNN4	hsa-miR-103	CD40 signalling during GC development	0.22	0.01
LYN	hsa-miR-376a	CD40 signalling during GC development	0.22	0.01
NCKAP1L	hsa-miR-377	CD40 signalling during GC development	0.22	0.01
PHACTR1	hsa-miR-377	CD40 signalling during GC development	0.22	0.01
TCF3	hsa-miR-378	CD40 signalling during GC development	0.22	0.01
IKBKB	hsa-miR-203	MAPK PATHWAY	0.26	0.14
MAP2K3	hsa-miR-103	MAPK PATHWAY	0.26	0.14
MAP2K5	hsa-miR-103	MAPK PATHWAY	0.26	0.14
MAP3K2	hsa-miR-136	MAPK PATHWAY	0.26	0.14
MAP3K2	hsa-miR-26a	MAPK PATHWAY	0.26	0.14
ANXA6	hsa-miR-203	NFKB total PATHWAY	0.24	0.16
BTK	hsa-miR-21	NFKB total PATHWAY	0.24	0.16
IKBKB	hsa-miR-203	NFKB total PATHWAY	0.24	0.16
IL10RA	hsa-miR-136	NFKB total PATHWAY	0.24	0.16
IL10RA	hsa-miR-204	NFKB total PATHWAY	0.24	0.16
IL10RA	hsa-miR-660	NFKB total PATHWAY	0.24	0.16
LYN	hsa-miR-376a	NFKB total PATHWAY	0.24	0.16
NFATC1	hsa-miR-143	NFKB total PATHWAY	0.24	0.16
PLCG2	hsa-miR-340	NFKB total PATHWAY	0.24	0.16

The most remarkable up-regulated pathways associated with losses of miRNAs targeting the genes included in the corresponding pathway were those of the CD40, NF-kB and mitogen-activated protein kinase (MAPK) pathways. Pathways and miRNAs correlation are represented in Figure 17.

Significant relations were not found between upregulated miRNAs and downregulated pathways.

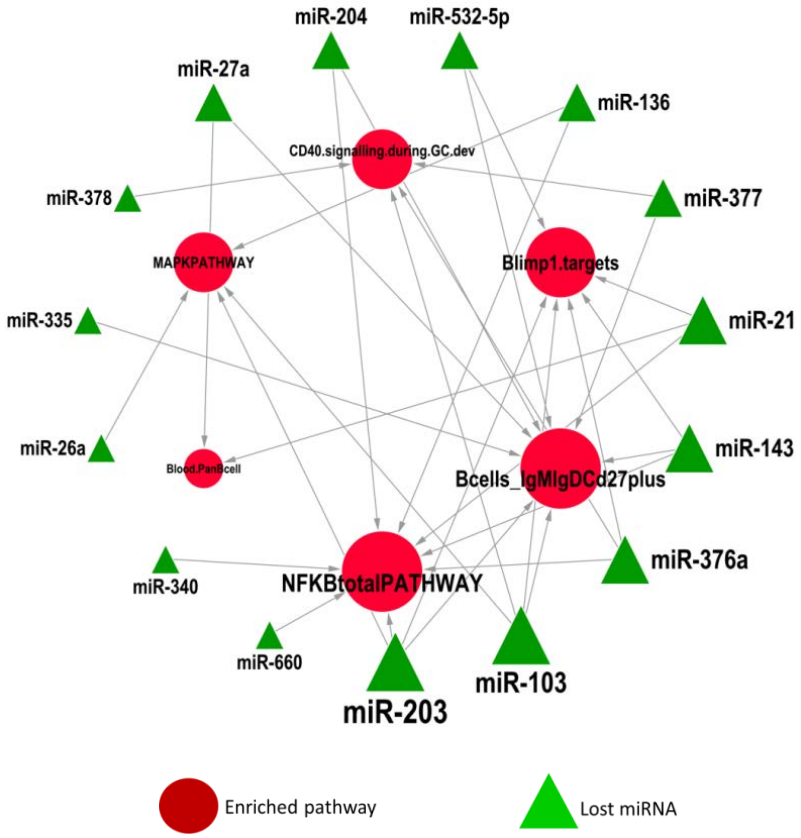


Figure 17. Downregulated miRNAs with connections to upregulated pathways. miRNAs are indicated by triangles, while pathways are represented by circles. Their size is proportional to their degree of connectivity. Red and green nodes represent, respectively, upregulated and downregulated elements. All the connections represent significant relations between the downregulated miRNAs and upregulated pathways targeted by the miRNAs

At this point validation of some targets/pathways was investigated in cell lines.

4.2.5. miRNAs profile in MCL-derived cell lines

miRNAs expression profile was evaluated also in 8 MCL cell lines and 3 samples of $CD19^{+}/IgD^{+}/CD27^{-}$ lymph node-sorted B cells, which corresponds to the cells derived from the mantle zone (Jares et al., 2007; Klein et al., 1998; Martinez et al., 2003).

SAM analysis revealed a miRNA signature (FDR<0.05) identified in MCL cell lines that included 15 miRNAs (Figure 18): all of them showing a fold change above 1 ($\log_2$). Only one miRNA was upregulated: miR-182, and was also significantly upregulated in MCL cases. Fourteen miRNAs were found downregulated in MCL cell lines, six of which were also lost in MCL cases: miR-26a, miR-151, miR-150, miR-223, miR-146b and miR-222.

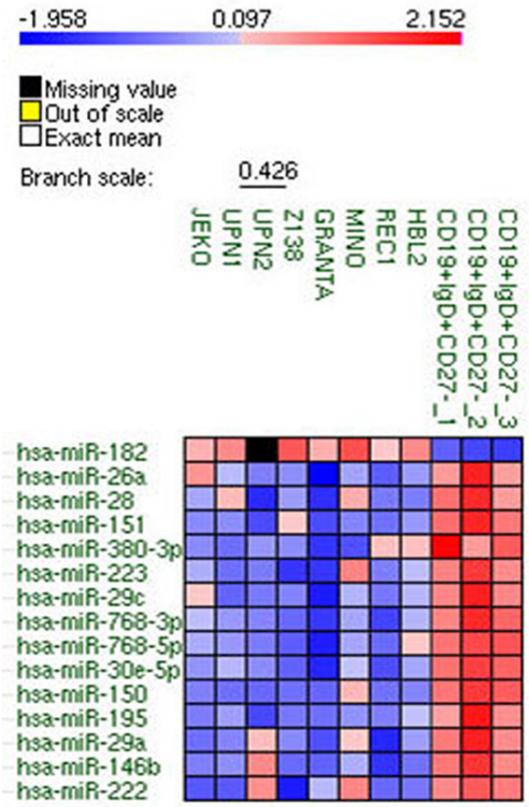


Figure 18. miRNA differentially expressed between MCL cell lines and mantle zone non-tumour cells. Red denotes high expression while blue denotes low expression.

Some of the MCL cell lines (for instance Jeko1) showed gain of *oncomir-1* (17-92 polycistron), confirming previous observations (Rinaldi et al., 2007; Tagawa and Seto, 2005), even though oncomir overexpression was not statistically significant.

Among the down regulated miRNAs, miR-26a was of particular interest because it was downregulated in MCL cases and in the cell lines, and it was significant in Fisher's exact test.

All these facts made miR-26a a good candidate for further studies of functional validation.

To further support the idea that miR-26a loss in MCL cases has some correlation with disease development/maintenance, miR-26a expression in normal mantle/naive cells has confirmed by other groups (Basso et al., 2009), and was already known to play a role in other types of cancer (Kota et al., 2009; Visone et al., 2007).

4.2.6. miR-26a target validation

One of the most interesting pathways activated in MCL is NF-kB, but its mechanism of activation is still essentially unknown.

Apparently, miR-26a, was not correlated in our analysis with NF-kB pathway, but a bibliography search brought to our attention a study where MAP3K2 (mitogen-activated protein kinase kinase kinase 2, also called MEKK2) was described as a NF-kB pathway-activating kinase, correlated to the delayed and persistent NF-kB activation (Schmidt et al., 2003; Winsauer et al., 2008).

Since MAP3K2 was upregulated in our analysis (FDR=0.0018), and is a predicted target of miR-26a both in miRanda and Targetscan predictors, miR-26a was chosen for functional studies on NF-kB patway activation in MCL cell lines.

First miR-26a was investigated for its capacity of modulate MAP3K2 expression.

MCL cells were electroporated with miR-26a and negative control (NC).

MAP3K2 regulation by miR-26a was evaluated by western blot 24h after electroporation in UPN1 cell line (Figure 19); its expression resulted lower than control, therefore MAP3K2 could be reasonably a potential target of miR-26a.

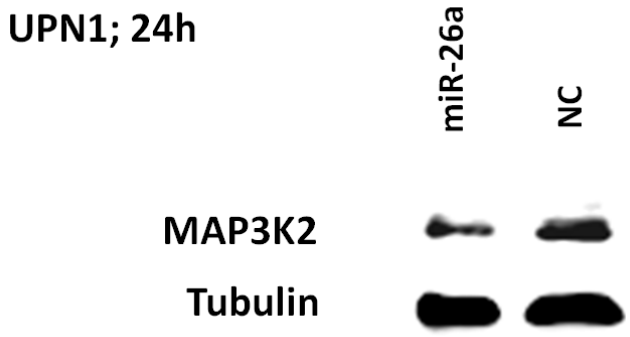


Figure 19. Evaluation of MAP3K2 expression after miR-26a reintroduction.

Correlation with NF-kB activation was then evaluated.

4.2.7. miR-26a relevance in NF-kB activation

Pathways activation is a complex end point to evaluate. Since one of the events directly correlated to NF-kB activation is the translocation of the v-rel reticuloendotheliosis viral oncogene homolog A (RELA, also known as p65) to the nucleus (see introduction Figure 3), sub-cellular location of RelA after electroporation of miR-26a was examined.

The MINO and REC-1 MCL cell lines resulted to be the best models for validation because they have low levels of miR-26a expression coupled with NF-kB activation, as demonstrated by RelA (p65) nuclear translocation.

Therefore, these cells where investigated for RelA sub-cellular location after miR-26a or negative control (NC) electroporation. Results are shown in Figure 20.

At 96h and 72h after miR-26a electroporation in MINO cell lines and REC-1 cell lines respectively, a smaller quantity of RelA compared with controls could be found in the nucleus. This means that the induced expression of miR-26a abrogated the nuclear translocation of RelA, and thus the activation of NF-kB pathway was impaired.

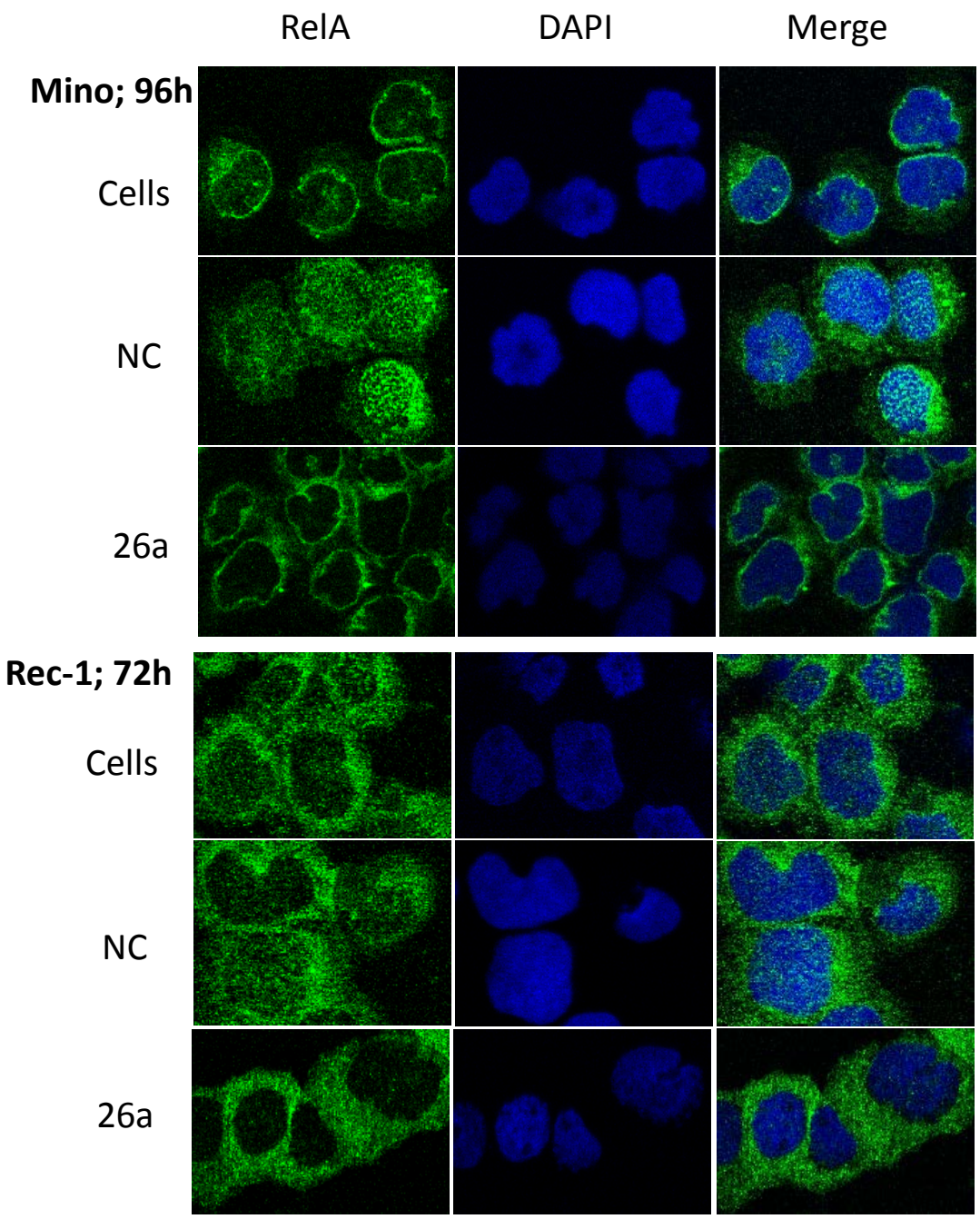


Figure 20. Immunofluorecence images of cell lines alone or after electroporation with negative control (NC) or miR-26a. RelA nuclear translocation is shown by Alexa Fluor 488 staining. Nuclei are stained with DAPI.

4.2.8. miRNA correlation with patients survival

Finally, in order to identify miRNAs of potential clinical prognostic value, miRNAs expression was investigated for their correlation with patient survival. Follow-up was obtained for 22 out of 23 MCL cases.

miRNA microarray data of 22 MCL cases were analysed with the Random Forest algorithm (Abba et al., 2007; Bienkowska et al., 2009; Hothorn et al., 2006) available at the SIGNS website http://signs.bioinfo.cnio.es/ (Diaz-Uriarte, 2008). This analysis yielded a set of miRNAs that described a Kaplan-Meier survival curve (log-rank p<0.001) in which 12 miRNAs were statistically significant (p<0.05). These miRNAs (see also Table 12 in materials and methods) were selected to confirm their expression in a second independent group of 54 FFPE cases by quantitative RT-qPCR. All the samples were recovered at the time of diagnosis. miR-198 was excluded from the analysis because it had a low efficiency of amplification by RT-qPCR.

After endogenous control normalization (RNU44 and RNU48), –ΔCt values were used for overall survival analysis by Cox regression analysis using SPSS programme. The significance of miR-20b as a prognostic marker was confirmed by univariate Cox regression analysis (p=0.013) (Table 26).

Table 26. Significance of the miRNAs investigated in OS studies. Univariate Cox regression analysis results. HR = hazard ratio.

miRNA	p-value	HR
miR-130b	0.222	1.150
miR-454	0.239	1.154
miR-99b	0.756	0.964
miR-7	0.63	1.057
miR-181c	0.828	0.979
miR-532	0.88	0.986
miR-362	0.999	1.000
miR-363	0.3	1.149
miR-625	0.3	1.112
miR-20b	0.013	1.388
miR-660	0.527	1.065

Samples were divided into two risk groups according to their median expression of miR-20b and results were plotted in a Kaplan-Meier survival curve (log-rank p=0.032) (Figure 21). At 60 months the cases expressing miR-20b below the median value had a survival probability of 56% whereas the patients expressing miR-20b levels above the median value only had a survival probability of 33%. So, the high-risk group of patients includes these ones that showed higher level of expression of miR-20b. This means that expression of miR-20b is an unfavorable marker.

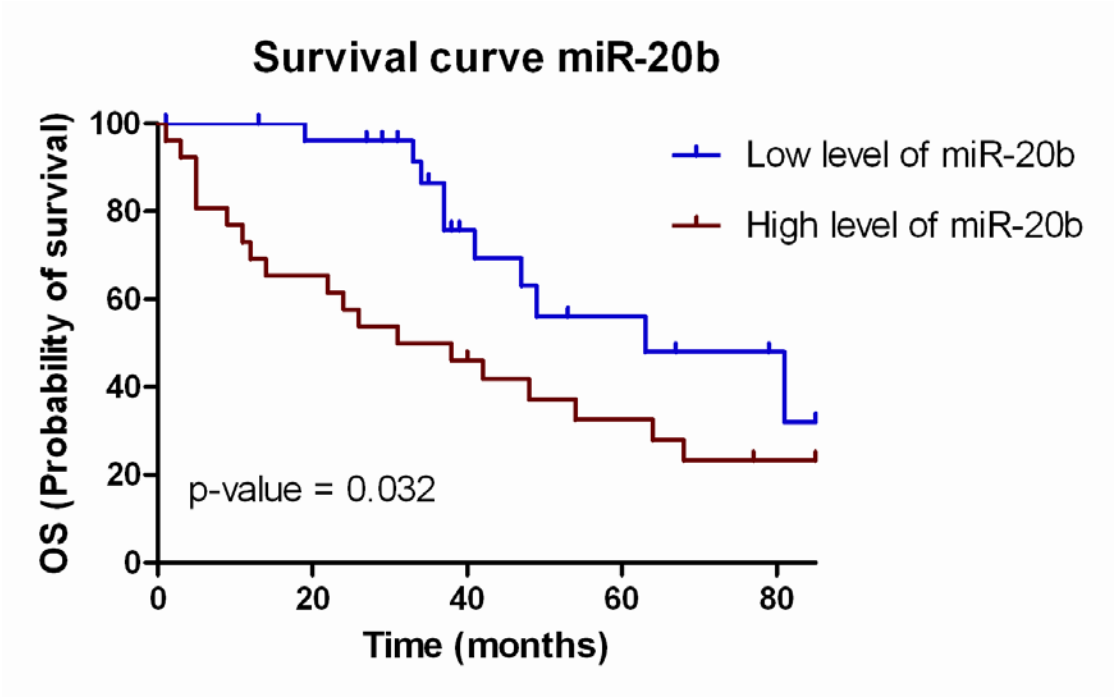


Figure 21. Kaplan-Maier distribution of survival results of MCL validation series of cases.

4.3. Project 3: miRNAs sequence

Fourteen miRNAs were sequenced in ninety-five cases of DLBCL. Samples were collected and selected at Addenbrooke's Hospital. Diagnoses were revised at Addenbrooke's Hospital too.

4.3.1. miRNAs selection and sequences achieved

The study was conducted to search for new miRNAs variants in DLBCL that may help with case stratification and/or pathogenesis elucidation.

In fact, some variability inside DLBCL has been already documented by GEP (Wright et al., 2003) and Comparative Genomic Hybridization (CGH) studies (Chen et al., 2006; Heyning et al., 2010; Robledo et al., 2009). Differential outcome and overall survival variability also have been documented (Lossos and Morgensztern, 2006). Thus, additional markers are required for better stratification of DLBCL patients and some variants in miRNAs may help with their recognition.

For miRNAs selection, the attention was focused on the genomic region lost in DLBCL previously identified for their relationship with miRNAs loss (Li et al., 2009). Not only: since about 10 % of DLBCL cases show MYC overexpression (Ladanyi et al., 1991; Swerdlow et al., 2008), also miRNAs that have been demonstrated to target MYC were chosen (Chen et al., 2010; Leucci et al., 2008; Sampson et al., 2007). According to the above mentioned criteria, a list of 14 miRNAs was identified for the analysis (see materials and methods table Table 13 or results Table 27).

After protocol optimization, PCR and sequence reaction of 95 DLBCL cases were achieved. All cases were sequenced at least for the Forward primer. Reverse primer sequence was performed for doubtful cases. In all cases the sequences included the mature miRNAs nucleotides, and also the pre-miR-sequence was evaluable in the majority of the cases.

Sequences were aligned to the reference sequences downloaded from the NCBI web-site and evaluated using SeqScape program. The quality of the sequences were generally good and only in a few cases the sequence was not readable.

Some of the variants found were discarded because of their uncertain reproducibility both in Forward and Reverse primer sequences, so that were not considered in the results. A summary of miRNAs and sequences read are in Table 27.

Nine miRNA variants were found in the analysis accounting for the sequence of six miRNAs: miR-650, miR-548a-1, miR-570, miR-596, miR-16-1 and miR-588. All the miRNAs showed the presence of only one variant, while miR-650 showed the presence of four variants.

Table 27. miRNA sequenced in DLBCL cases.

	miRNA	Sequences obtained out of 95 cases	Why has been studied	Variants found
1	hsa-miR-650	94	Lost in CGH	C>G, C>A, C>T, G>A
2	hsa-miR-31	94	Lost in CGH	No variant found
3	hsa-miR-588	92	Lost in CGH	T>G
4	hsa-miR-548a-1	95	Lost in CGH	T>G
5	hsa-miR-570	93	Lost in CGH	T>C
6	hsa-miR-16-1	95	Lost in CGH	T>C
7	hsa-miR-15a	95	Lost in CGH	No variant found
8	hsa-miR-596	94	Lost in CGH	T>C
9	hsa-miR-587	85	Lost in CGH	No variant found
10	hsa-miR-491	94	Lost in CGH	No variant found
11	hsa-miR-124-2	93	Lost in CGH	No variant found
12	hsa-miR-34b	85	Expression regulated by MYC	No variant found
13	hsa-miR-145	95	Expression regulated by MYC	No variant found
14	hsa-let-7a	95	Expression regulated by MYC	No variant found

4.3.2. Evaluation of already know variants

At this point Ensembl, dbSNP and a miRNA annotated variants database (miRNA-SNP) (Gong et al., 2012) were used to verify the existence of already known polymorphisms in the above mentioned miRNAs.

The variants found in miR-548a-1, miR-570, miR-596, miR-16-1 and one of the four variants found in miR-650 were already described (Table 28).

Table 28. Variants found that were already described.

Selected miRNA	Cases sequenced	Alredy known polimorfisms	Control tissue
hsa-miR-650	94	71C>G	Not investigated
hsa-miR-596	94	29T>C	Not investigated
hsa-miR-548a-1	95	42T>G	Not investigated
hsa-miR-570	93	34T>C	Not investigated
hsa-miR-16-1	95	55T>C	Not investigated

Some of the polymorphisms were pretty frequent, such as the polymorphisms in miR-570 and miR-650, while the variant found in miR-16-1 is really rare.

4.3.3. Evaluation of not yet described variants

Four variants found in this study were not included in Ensembl, dbSNP and miRNA-SNP databases (last check on April 20th 2012), so probably they have been identified in this study for the first time. Findings are listed in Table 29.

Table 29. Variants found not yet described. Codes refer to the different DLBCL patients.

Selected miRNA	Cases sequencied	New variant found	Control tissue
hsa-miR-650	94	L0027(C/A) L0032(C/T) L0032(G/A)L0037(C/A) L0078(C/A) L0121(C/A)	2 cases availables: L0027 and L0078
hsa-miR-588	92	L0050 (T/G)	available

All the variants found were confirmed by a fresh PCR and sub-sequent sequencing.

The not yet described variants here identified in miR-650 (C→A, G→A, and C→T) and miR-588 (T→G) were not included in the mature miRNA sequence. Their positions in relationship with the pre-miRNA bidimentional folding are illustrated in Figure 22 and Figure 23.

4.3.3.1. miR-588

The variant T→G (54T>G) found in the pre-miR-588 (which is codified in chromosome 6q22.32) is part of the complementary miRNAs sequence. It was found only in one patient.

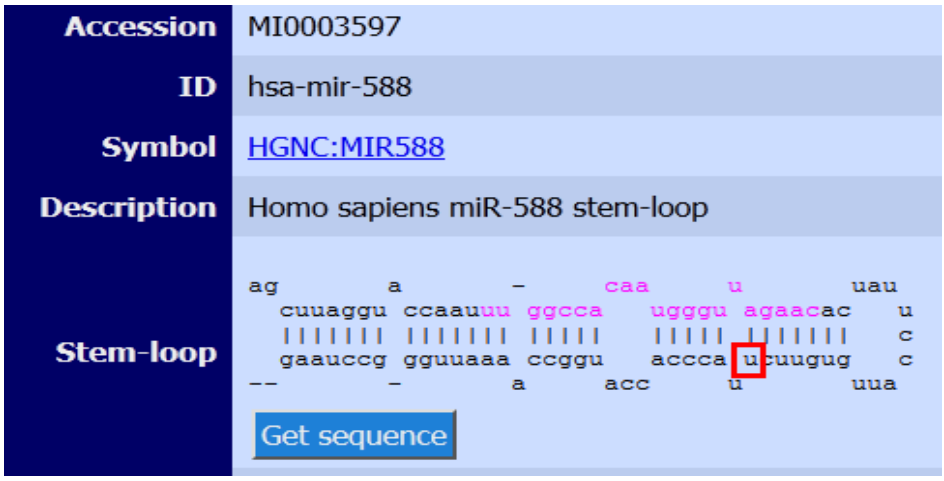


Figure 22. Location of the not yet described variant found in pre-miR-588.

4.3.3.2. miR-650

Three not yet described variants were identified on pre-miR-650 which is codified at chromosome 22q11.22.

The variant C→A is localized in the complementary miR-650 sequence (64C>A variant) while the other two: C→T and G→A were found at 3' and 5' end of the pre-miR-650 sequence respectively (6C>T and 86G>A variants) (Figure 23).

The 64C>A variant was described in 4 cases, while the other two (6C>T and 86G>A variants) were found in the same case.

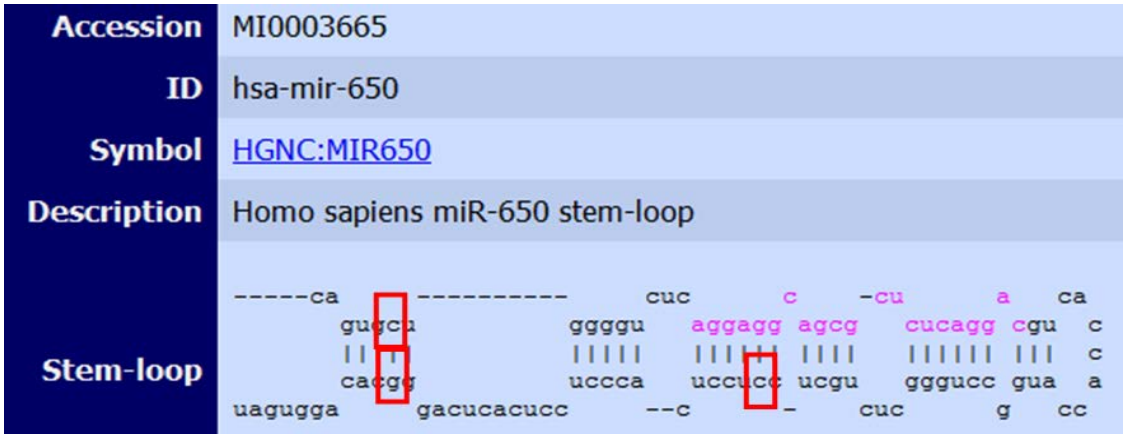


Figure 23. Location of the not yet described variants found in pre-miR-650.

It is particularly noteworthy that 6C>T and 86G>A variants rely exactly onto two nucleotides that are folded to form an overlapping structure and they are found in the same patient (Figure 23 and Figure 24). Whether it has a specific meaning cannot be said so far, but probably the variants G and C as well as the variants A and T may be found in the same allele; this way the complementarity would be conserved and the folding of the miRNA would not be impaired.

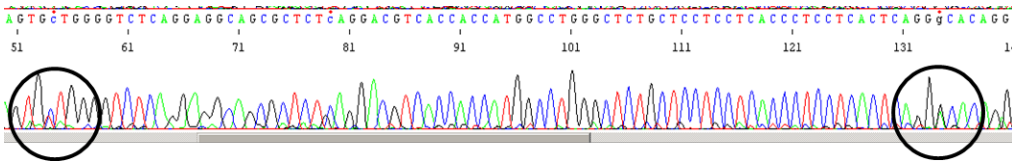


Figure 24. Sequence or miR-650: variants G→A and C→T.

4.3.4. Control tissues

For three out of six cases in which these variants were found, normal counterpart DNA proceeding from bone marrow tissue from paired patients was available in the laboratory, so that, PCRs and sequences were performed also in control tissue to investigate the presence of the variants.

Sequencing of the normal tissue counterpart of miR-588 showed the presence of only one peak at the site were the variant was described in the tumour and it was a T nucleotide (the wild type) (Figure 25). This finding accounts for the possibility that it could be a somatic alteration related to tumour development, even if its significance and validation is still to be fully demonstrated.

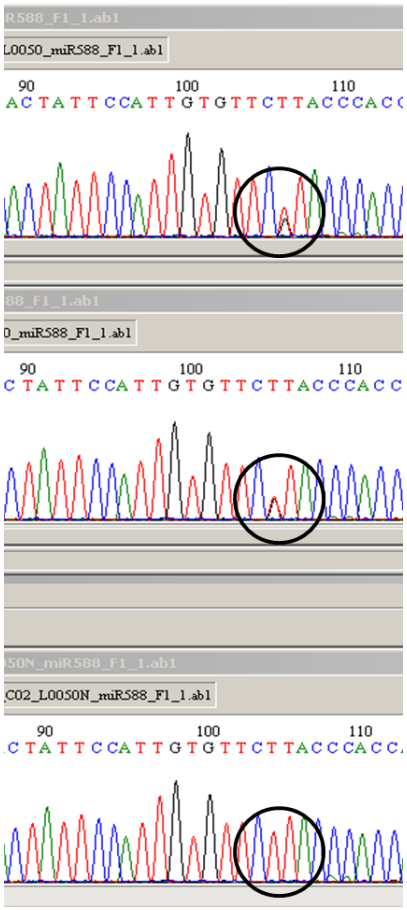


Figure 25. The first 2 sequences correspond to the case variant and the confirmation PCR respectively. The third sequence belongs to tissue control.

PCR and sequencing of the normal tissue counterpart DNA of miR-650 was possible only into two out of four cases in which the 64C>A variant was found (Figure 26).

In one patient only C variant (the wild type) was found in normal tissue DNA (Figure 26A, on the left), while both C and A nucleotides were shown in Sanger sequencing in normal tissue counterpart of the second case (Figure 26B, on the right).

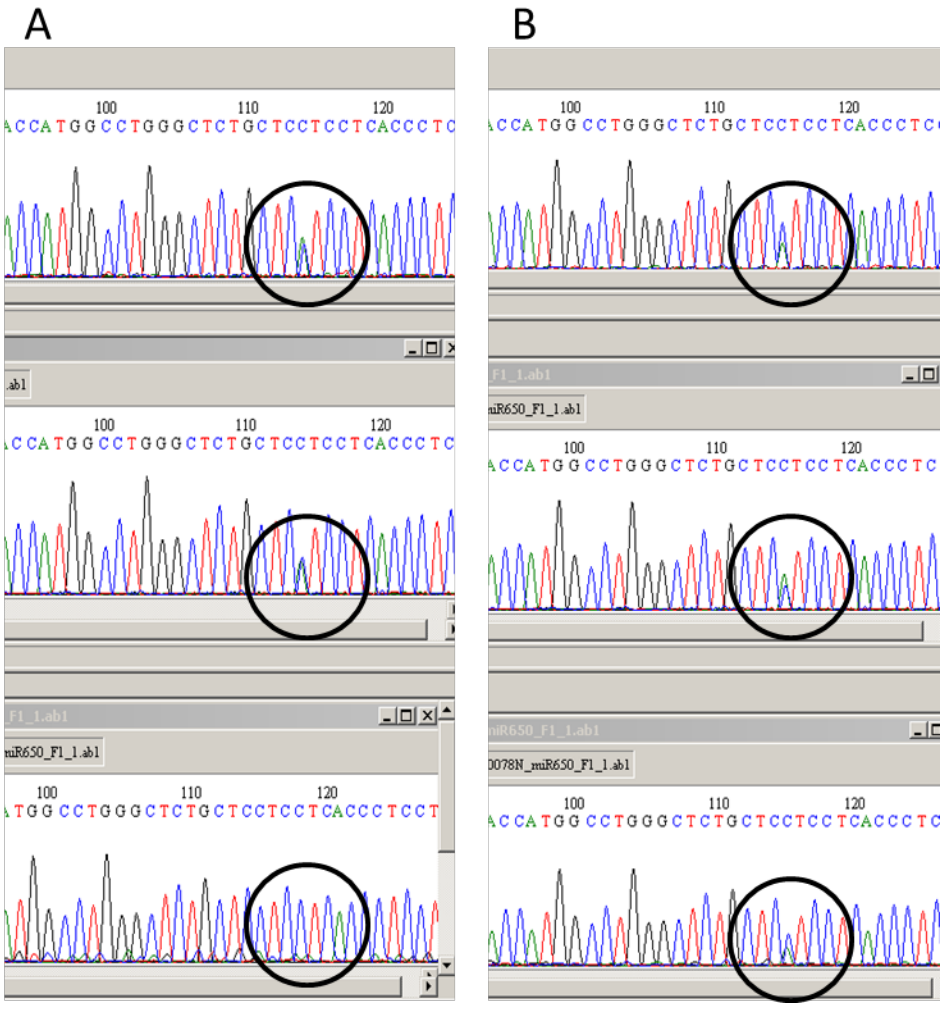


Figure 26. One case is shown on the left and another case is shown on the right. The first 2 sequences correspond to the case variant and to the confirmation PCR respectively. The third sequence belongs to tissue control.

The fact that in one case the normal counterpart tissue only presents the wild type sequence may account for a somatic alteration related to tumour development, but the finding that it can be found also in non-tumour control do not support this hypothesis. Data available so far are not sufficient to establish a reliable conclusion.

4.3.5. Investigation of the significance of the not yet described variants

It is interesting to note that two of the not yet described variants here identified (miR-650 64C>A, and miR-588 54T>G) are part of the complementary sequence of the miRNA. Frequently only one strand undergoes complete maturation (Hu et al., 2009a; Martinez et al., 2002), but there are many cases in which the complementary strand also undergoes

maturation and has a biological activity. The secondary mature strand is generally described in the miRBase with the same name as the miR- plus the symbol asterisk (*). In the case of miR-650 and miR-588, according to miRBase, the complementary strand of these two miRNAs is not reported so far. Nevertheless, it does not necessary mean that the complementary strand has no biological function. Moreover it has been proposed that the specific folding of pre-miRNA can influence the action Dicer during the maturation process and may alter whether the 3' or 5' arm of the pre-miRNA undergoes maturation (Schwarz et al., 2003).

Furthermore it is to note that new polymorphism data are continuously appearing. For instance, by the time of the work was done (July 2011) no data could be found about the polymorphism on miR-16-1.

To evaluate the importance that these nucleotides changes could have and their potential effects on pre-miRNA, the wild type sequences, and the other variant were submitted to BibiServ web resource that evaluates miRNA secondary folding changes.

In the case of pre-miR-588 only a minor change can be observed in the predicted folding (Figure 27).

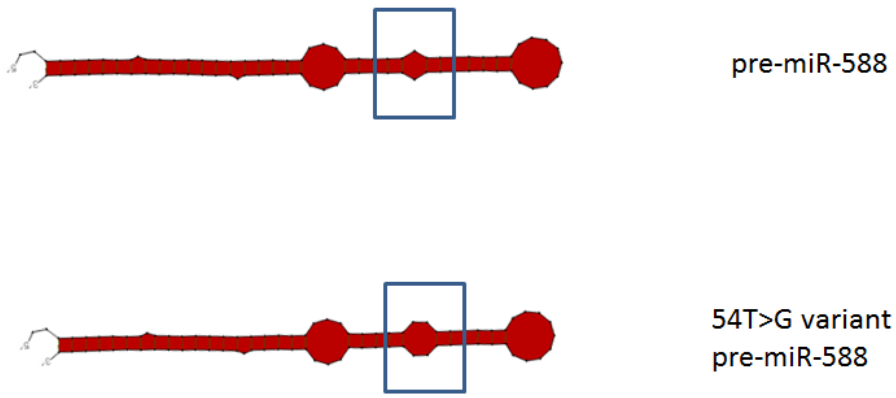


Figure 27. Change in the folder prediction in pre-miR-588 that results from T→G substitution. The blue box indicates the predicted change in the structure.

While in the case of pre-miR-650 the variant C→A predicts a large change in bidimentional pre-miRNA structure (Figure 28).

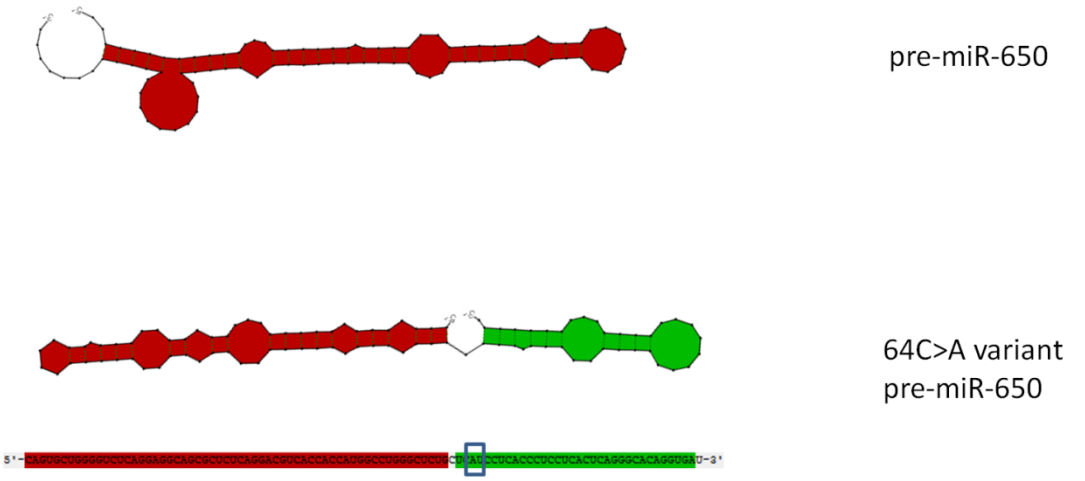


Figure 28. Change in the folder prediction in pre-miR-650 that results from C→A substitution. The blue box indicates the position of nucleotide substitution.

Even if an important change can occur in the pre-miR-650 folding, the value of the variants here found still need to be investigated, and so far a reliable significance cannot be assessed.

Data regarding the new variants and already known polymorphisms has been stored at Addenbrooke's Hospital database together with the other information concerning the series of DLBCL cases whit the aim of evaluate them in conjunction with the rest of the data for survival analysis, response to therapy and other features.

5. Discussion

As detailed in the guidelines of the WHO, lymphoma diagnosis is based on the integration of data proceeding from clinical history of the patient, histopathological aspect of the tumour, tumoural marker expression, chromosomal alterations and gene (or protein) expression data (Swerdlow et al., 2008).

Even if specific features for diagnosis are assigned to each lymphoma type, classification is continuously under review. Thus, additional markers for differential diagnosis and accurate prediction of response to therapy are still needed.

In this study the comparison of specific lymphoma type towards the entire series of lymphomas or non-tumour samples yielded a number of miRNAs whose expression was significantly altered suggesting new markers for differential diagnosis and patient stratification.

Additionally, some of the alterations found in lymphomas are not completely understood so far. For instance, pathway deregulation, as happens in MCL or in ABC subtype of DLBCL; variations in GEP and protein expression whose alterations are not yet elucidated, as happens in MYC translocation negative cases that however express high levels of MYC; and recurrent losses or gain in genomic regions that do not apparently codify for important genes.

miRNAs constitute promising candidates to enlighten these grey areas of lymphoma understanding, mainly due to their predicted impact on regulation of protein expression, indeed aberrant variations in miRNAs expression account for an undisputed role in alteration of cellular equilibrium and thus in cancer development.

Many of the miRNAs here identified have been already proven to be correlated to B-cell maturation and cancer development, while others are good candidates for clarification of the unexplained alterations in lymphomas.

Finally, efforts have been done to elucidate de origins of miRNA expression/function alteration by sequencing a selection of miRNAs and the role of copy number variantion (CNV) is also discussed.

5.1. Project 1: miRNA expression profile in lymphomas

Results yielded from the first project can be divided into three sections: the comparison of all lymphomas *vs*. non-tumour controls, the comparison of each lymphoma *vs.* the whole series of B-Cell lymphoma samples and the validation of a differential miRNA expression profile between BL and DLBCL: discussion is provided for all of them.

5.1.1. Comparison of all lymphomas *vs.* non-tumour controls

miRNA expression was investigated in the whole series of 147 B-cell lymphoma samples and compared with non-tumour controls (four tonsils, seven lymph nodes and four spleens), and a selection of these miRNAs was validated in a second series of 66 lymphoma cases. A number of miRNAs were broadly lost across the entire series of lymphomas: among them the ones that were more significant were miR-133a and miR-31. Upregulated miRNAs were less significant, but also some of them were upregulated in the whole series of cases; such as miR-513 and miR-9.

These data account for a potential important role of these miRNAs in B-cell lymphomas pathogenesis. Numerous works are available on miR-31. This miRNA has been found lost in gastric cancer (Zhang et al., 2010), its loss has been connected to metastasis development (Valastyan et al., 2009) and it has been also described as a tumour suppressor miRNA in malignant mesothelioma (Ivanov et al., 2010) due to its ability to inhibit proliferation, migration and invasion (further discussion about miR-31 can be found in section 5.4.1.). A role for miR-133a has been described in the regulation of lymph node metastasis of breast cancer (Wu et al., 2012) where, miR-133a, can suppress tumour cell invasion and migration probably by modulating the expression of fascin homolog 1, actin-bundling protein (FSCN1) (a regulator of cell mobility), which is a gene overexpressed in Reed-Sternberg Hodgkin's lymphoma cells (Kluiver et al., 2007), but not in non-Hodgkin lymphomas.

Another downregulated miRNA here identified, miR-23b, was found also to be lost in human colon cancer where *in vitro* experiments conferred to miR-23b impaired expression a role in attenuation of apoptosis and stimulation of cell proliferation, migration and invasion mediated by its action on different proteins involved in JNK, ERK and NF-kB pathway. Some of the targets identified for miR-23b are the mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase (MAP3K1), the p21 protein activated kinase 2 (PAK2) and the related RAS viral oncogene homolog 2 (RRAS2) (Zhang et al., 2011b).

Among the upregulated miRNAs, high expression of miR-9 was found correlated to metastasis development in breast cancer (Ma et al., 2010b) and in PDRM1 downregulation (Huang et al., 2011). Not only: miR-9 has been described as a DICER repressor and its inhibition in a HL xenograft model decreased tumour growth (Leucci et al., 2012).

Overexpression of miR-212 has been described in pancreatic cancer, where the retinoblastoma tumor suppressor (Rb1) was shown to be one of its targets (Park et al., 2011b), and in non-small cell lung cancer (NSCLC). In this last study the overexpression of miR-212 contributed to cell cycle progression (Li et al., 2012b).

Less published information is available for miR-513, since its overexpression was only described in retinoblastoma (Zhao et al., 2009).

The expression of miRNAs has been shown to be tissue-dependent (Lu et al., 2005), accordingly we have tried to select tissue-specific controls for each tumor type, thus avoiding a bias for the B-cell lymphomas subtypes typically localized outside the lymph nodes: MALT and SMZL lymphomas. This may explain some of the discrepancies of our findings with previous studies.

For instance, we have found upregulation of miR-200a compared with non-tumour controls (reactive lymph nodes and tonsils) both in the first and in the validation series of cases, while another group described the loss of miR-200a in conjunctival MALT compared with pared non-tumour conjunctival samples (Cai et al., 2011). Interestingly, only one MALT lymphoma in our validation series was derived from conjuntival tissue, and it was the case with the lowest expression level of miR-200a. This finding is in line with the evidence that miRNAs are specifically expressed in different tissue and that can have different role according to the tissues in which they are expressed. A similar approach in a study of gastric MALT has been done where matched adjacent normal gastric material from the same patients was used as non-tumour control (Craig et al., 2011). It is not surprising that MALT lymphomas express different miRNAs compared to the adjacent tissue since MALT lymphomas are derived from B-cells, while the adjacent tissue probably belongs to a completely different kind of tissue, mostly epithelial. Since the aim of this study was to compare the different tumours for lymphoma differential diagnosis, and a small amount of adjacent non-tumour tissue is always detectable in biopsies, MALT samples were selected from different anatomic localization to avoid that their expression profile could resemble the tumour location, thus making possible

the identification of miRNAs strictly correlated to MALT lymphoma independently of tumour anatomic localization, thus being a more useful approach for identifying markers for differential diagnosis.

The same problem had easier solution in SMZL cases, where the analysed samples were mostly infiltrated spleens. According to SMZL definition, these tumours are mainly localized in the spleens (Swerdlow et al., 2008), and in fact, we found that SMZL can be better normalized using spleens as non-tumour counterpart rather than lymph node and/or tonsil samples. For this reason, SMZL cases have been evaluated comparing them to normal spleens and not to lymph nodes, an approach shared also by others groups (Bouteloup et al., 2012; Ruiz-Ballesteros et al., 2007).

5.1.2. miRNAs differential expression among the different B-cell lymphoma types

Besides the finding that the expression profile of some miRNAs can differentiate all the B-cell lymphoma types here investigated from non-tumour controls; the potential value of the miRNAs expression has been investigated for differential diagnosis and pathogenesis elucidation of the different B-cell lymphoma types.

The initial analysis done by SAM software identified 128 miRNAs potentially useful for specific B-cell lymphoma type recognition. KNN analysis of the same 147 fresh frozen samples correctly classified 86,4% of the cases by using 120 miRNAs. Ninety-five miRNAs were significant both in SAM and KNN analysis (accounting for about the 3/4 of the significant miRNAs), confirming and enforcing thus the relevance of the miRNAs commonly identified by the two algorithms.

The group of cases poorer classified was DLBCL, a result somehow expected due to the already known DLBCL heterogeneity (Wright et al., 2003). Indeed, it is not a case that the error rate of DLBCL class recognition accounts alone for 5,4%, which represent more than 1/3 of the total error. It is interesting to note that 6 out of 8 of the misplaced DLBCL cases were classified as FL (see Table 19), confirming that differential diagnosis between these two lymphomas type is sometimes unclear. miRNA capacity of distinguish between FL and DLBCL has been investigated by other groups (Lawrie et al., 2009; Roehle et al., 2008) finding that miRNAs are able to distinguish between these two lymphomas with a confidence of 98%.

The rate of correct classification of this analysis could be even higher if more cases were introduced. In fact the more cases are used in predictor programs the more accurate the

results are, but unfortunately the availability of fresh frozen tissues is limited. Though this results can help with the selection of candidate miRNAs for further validation in a larger series of FFPE samples. It is also to consider that here eight different types of lymphomas are considered, so the difficulties of a correct classification are higher than classification just between two different tumour types.

Not only, the value of these results goes beyond lymphoma classification, since, as it has been shown in this work, some miRNAs identified by KNN algorithm and SAM analysis could be involved in the pathogenesis of each specific lymphoma type. Nevertheless, a role for many miRNAs has not been assigned so far and experimental validation of these markers still remains to be explored.

Moreover, a number of distinctive features, specific of each lymphoma type, may help to understand the implications and the rational of miRNAs expression in B-cell lymphomas pathogenesis and are discussed as follows.

One of the master regulators that may affect (and be affected by) miRNA expression is Myc protein. It is overexpressed in many B-cell lymphomas and particularly in BL. There is a vast group of miRNAs regulated by Myc (Robertus et al., 2010), and here, high expression of some of them: miR-17-3p, miR-18a, miR-19a, miR-92 and miR-130b, were found in BL after comparing to other B-cell lymphoma cases; while miRNAs that are supposed to be repressed by Myc were also downregulated in BL cases, such as miR-29 family (Mott et al., 2010) and miR-26a (Chang et al., 2008). Finally miRNAs that are demonstrated to downregulate MYC were lost in BL compared to other NHL cases (let-7 family) (Robertus et al., 2010; Sampson et al., 2007), a circumstance that may promote even higher expression of MYC.

Loss of miR-155 in BL, confirms the previous findings (Kluiver et al., 2006), and is consistent with the evidence that MYC translocation on IgH chain depends also on the expression of activation-induced cytidine deaminase (AID), an enzyme that promotes somatic hypermutation of immunoglobulin genes (Muramatsu et al., 2000), and whose expression is regulated by miR-155 (Dorsett et al., 2008).

In CLL cases, loss of miR-16 was found, as already described in this tumour (Calin et al., 2002; Fulci et al., 2007). Until date, the majority of the studies conducted on CLL cases examine CLL samples derived from peripheral blood, and compare them with peripheral blood or tonsil purified cell suspension controls (Calin et al., 2002; Fulci et al., 2007), while CLL samples and

controls of this study proceeded from fresh frozen tissues. Additional miRNAs were here identified to be significantly lost in CLL cells; for instance it is to note the loss of both the miR-126 and miR-126* (both deriving from the same pre-miRNA) as already documented (Pallasch et al., 2009). The role of loss of both these miRNAs in cancer has been already reviewed (Meister and Schmidt, 2010) and they have been shown to be able to inhibit metastasis formation in vivo using breast cancer cell lines (Tavazoie et al., 2008). miR-199a* (loss in CLL) has been described to be a pro-apoptotic miRNA (Kim et al., 2008). Finally it is remarkable that miR-143 (also lost in CLL cases) is a regulator of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) (Chen et al., 2009). KRAS overexpression (or activation) frequently occurs during cancer transformation events (Der and Cooper, 1983), and its overexpression has been described also in CLL (Gahrton et al., 1987).

MALT lymphoma cases overexpress all the members of miR-200 family: so thus, it could be possible that their redundancy have a specific role in this lymphoma type. Overexpression of miR-141 and miR-200a have been correlated with tumorigenesis in ovarian cancer (Mateescu et al., 2011), while upregulation of miR-200a and miR-200b has been connected to development and high serum levels in pancreatic cancer probably due to their hypomethylation (Li et al., 2010a).

In particular, miR-200c, was overexpressed in MALT and also in MCL cases. Upregulation of this miRNA has been correlated to chemoresistance and AKT pathway activation (Hamano et al., 2011): a feature commonly found in MCL cases (Rudelius et al., 2006), especially in MCL aggressive subtypes.

MCL cases also overexpress miR-183, an already described oncomiR (Sarver et al., 2010), whose upregulation regulates resistance to apoptosis, as shown in hepatocellular carcinoma, where this miRNA has been correlated to down regulation of the Programmed cell death 4 (PDCD4) and resistance to apoptosis induced by Transforming Growth Factor-beta1 (TGF-β1) (Li et al., 2010b). Additionally, miR-182 and miR-183, both upregulated in MCL, are located in the same cluster on chromosome 7q32.2. Upregulation of this cluster was correlated to the alteration of multiple pathways in medulloblatoma, driving cell survival and proliferation (Weeraratne et al., 2012).

SMZL cases showed loss of the cluster miR-141/200c on chromosome 12p13.31 which has been described also in renal carcinoma (Nakada et al., 2008) and in particular, loss of miR-141

has been connected to higher cell proliferation (Du et al., 2009b). Another miRNA loss in SMZL cases was miR-210: a miRNA that has been correlated with tumour proliferation and aggressiveness (Rothe et al., 2011), whose dowregulation in SMZL cases may be ascribable to lower aggressively of this type of lymphoma compared with the others included in the analysis.

A further interesting observation is that B-cells at different stages of maturation show specific miRNA signatures (Kuchen et al., 2010), thus the differential expression of miRNAs between different lymphoma types may reflect distinct miRNA profiles of the cell of origin as described in the introduction Table 1 and Figure 2.

In this study the eight B-cell lymphoma types that were included, derive from distinct B-cell of origin (see introduction Figure 2): MCL derive from mantle zone B-cells; BL, FL and DLBCL are mainly derived from GC cells (at least DLBCL CG subtype), while three of them are derived from the marginal zone: MALT, NMZL, SMZL, which accounts for post-GC cells origin and CLL derives from memory B-cells.

The analyses of the different lymphoma types reflect partially this cell of origin. This is the case of FL, a lymphoma derived from GC cells, where miR-138 and miR-9 are found to be upregulated as expected in GC cells (Basso et al., 2009; Jima et al., 2010; Zhang et al., 2009). miR-9 is also involved in PRDM1 down-regulation (Huang et al., 2011; Zhang et al., 2009): a finding observed in reactive and neoplastic Germinal center B -cells (Garcia et al., 2006).

Cells deriving from GC cells are described also to show upregulation of miR-130b (Basso et al., 2009; Jima et al., 2010) as can be observed in our BL series.

At the same time, miR-29c is downregulated in BL, again in accordance with the finding that this miRNA is lost in GC cells (Basso et al., 2009; Jima et al., 2010).

Another miRNA that may suggest a relationship with the expression pattern of the cell of origin is miR-223, which is strongly expressed in NMZL cases. This miRNA has been shown to inhibit LMO2 expression (Malumbres et al., 2009), a protein strongly expressed in the GC (Natkunam et al., 2007). Upregulation of this miRNA in NMZL cases is consistent with the observation that this tumour does not express LMO2 or other GC markers.

5.1.3. miRNAs differential expression between BL and DLBCL

Despite the characteristic association between MYC translocation and the diagnosis of BL, a discrete number of DLBCL cases may carry the MYC translocation (Ladanyi et al., 1991; Swerdlow et al., 2008), and the existence of a gray zone between these two entities has been already described (Bellan et al., 2009). Thus, the requirement of new approaches and markers for differential diagnosis has been a classical topic for research (Bellan et al., 2009; Dave et al., 2006; Harris and Horning, 2006; Hummel et al., 2006). miRNAs data were explored to test whether they could contribute to the differential diagnosis of BL and DLBCL and whether they could also set the basis of a more comprehensive understanding of the pathogenesis.

The analysis of 41 cases (12 BL and 29 DLBCL) by microarrays conducted on fresh frozen samples yielded to the selection of 43 candidate miRNAs that could significantly differentiate BL from DLBCL. Nineteen of these miRNAs were confirmed in a second series of 71 samples.

The results here found comparing BL *vs.* DLBCL show that a few miRNAs could help in their differential diagnosis. A model including all 19 significant miRNAs or only the most significant ones may increase the accuracy of the BL diagnosis since the unsupervised cluster correctly classified 93% of the samples.

The use of miRNAs for BL-DLBCL differential diagnosis was recently covered by another totally independent similar study conducted in parallel (Lenze et al., 2011) with the work here described. Even if not all significant miRNAs were coincident, a number of miRNAs were identified by both investigations. These miRNAs are: miR-146a, miR-155, miR-29b, and also others but less significantly like miR-26b miR-34b. A central finding in both studies is the loss of miR-155 that was the most significant miRNA in this work and one of the most important miRNA in the Lenze work.

Interestingly miR-34b was found to downregulate MYC expression (Leucci et al., 2008), while miR-26b was found to be downregulated by MYC expression (Chang et al., 2008; Koh et al., 2011): two findings that perfectly fits with their lost in BL cases.

miR-146a (here downregulated in BL compared with DLBCL) has been shown to be an apoptosis inhibitor and a suppressor of tumour growth in other types of cancers (Hou et al., 2011; Xu et al., 2011), an observation that is consistent with the observation that BL is the lymphoma with highest cell duplication rate (Swerdlow et al., 2008). miR-155 has been

described for its importance in B-cell maturation and its loss can be correlated with a blockade in immunoglobulin switching (Dorsett et al., 2008; Teng et al., 2008; Vigorito et al., 2007).

miR-155 is probably the most investigated miRNA among them, and here also probably the most promising miRNA for BL/DLBCL recognition. miR-155 high expression in DLBCL (Kluiver et al., 2005) and lack in BL have been two of the first discoveries in lymphomas (Kluiver et al., 2006), but its use for differential diagnosis was investigated until now.

Improvements in differential diagnosis in this case may have a clear clinical application in treatment decision, since standard doses of chemotherapy utilized for DLBCL such as CHOP are inadequate for treating Burkitt lymphoma and most of intermediate BL/DLBCL cases (Smeland et al., 2004).

5.1.3.1. miRNAs in the practice of differential diagnosis between BL and DLBCL

Usage of FFPE samples for RT-qPCR has been controversial in the last few years because results were considered less reliable than results obtained by fresh frozen samples. It is obvious that the RNA quality is lower if compared with fresh frozen tissue derived RNA, but still good enough for RT-qPCR studies as long as the investigated regions are not too extense. In the case of mature miRNAs the sequence to be amplified is only 20-30 nucleotides length. Here we have followed an approach based on using an initial relatively small series of fresh frozen samples with a comprehensive platform and select a reduced number of miRNAs to validate in a second group of FFPE samples. This approach has been used successfully for gene expression data (Sanchez-Espiridion et al., 2009) as well as for miRNAs investigation (Alencar et al., 2011; Laios et al., 2008; Montes-Moreno et al., 2011) where the addition of miRNAs to the diagnostic and prognostic algorithms have been demonstrated to be feasible. Thus, the evaluation of the expression levels of some miRNAs may provide additional criteria for a demanding differential diagnosis. Among the miRNAs that showed highest potential for differential diagnosis we found miR-155, miR-146a, miR-26b, miR-29b, miR-34b and some miRNAs included in the cluster 17-92, such as miR-17-3p and miR-92.

5.2. Project 2: miRNAs significance in pathway regulation and survival studies

Behind the description of a miRNA signature in MCL cases, the big novelty of the work done in MCL relies on the investigation of a correlation between miRNA expression profile, GEP and pathway activation. Gene expression profiling studies of MCL have revealed increased survival signalling (Jares et al., 2007; Martinez et al., 2003; Salaverria et al., 2007), but have not identified the mechanisms responsible for them. Thus the finding that miRNAs expression may be correlated to deregulation of MCL pathways such as CD40 activated signalling, MAPK and NF-kB (Basso et al., 2004; Homig-Holzel et al., 2008; Rosenwald et al., 2003) constitutes a stimulating step forward, because numerous works focused their attention on direct effects of miRNAs on their target protein, but did not investigated miRNAs role in pathways activation.

Since NF-kB pathway is of particular interest in MCL pathogenesis, miRNAs impact on this pathway was investigated. The finding that miR-26a restoration is able to obstacle NF-kB pathway activation, mainly through MAP3K2 protein modulation, consists in an additional elucidation of MCL pathogenesis.

It is important to remember that the approach followed here, looks at the association of miRNAs with mRNA expression and it accounts for only a part of the miRNAs' ability to modulate protein expression, since miRNA also regulates mRNA translation (Baek et al., 2008). We cannot exclude that additional findings could be found at investigating miRNA correlation with protein expression profile. However, mRNA destabilization still accounts for a big part of miRNA biological effects (Baek et al., 2008) and the same approach has been used to investigate others malignancies.

Later on other works confirmed the miRNAs importance in pathway activation (Bueno et al., 2011; Paik et al., 2011), for instance the role of miR-22 (here lost in MCL cases) in NF-kB activation was shown by a different group (Takata et al., 2011). Of great importance is the finding that miR-31 downregulation (a miRNA here lost in MCL cases) was associated to NF-kB pathway activation in T-cell lymphomas (Yamagishi et al., 2012).

Even if the majority of the significant changes have been detected in MCL cases and MCL cell lines, some minor variations can be observed among them. MCL cell lines are more representative of the blastoid form than of classic MCL, so we may hypothesize that the

aggressive transformation to blastoid form may include changes also in the expression of the miRNAs including the 17-92 polycistron (Rinaldi et al., 2007) that has been found upregultated in some cell line but not in classic MCL cases.

Some of our findings in MCL have been confirmed by a recent work, comparing $CD19^+$ purified tumoural lymph node cells with $CD19^+$ peripheral blood cells (Zhao et al., 2010). Some of the confirmed miRNAs in both series were miR-27a, miR-27b miR-21, miR-150, miR-30b, miR-26b and more interestingly, miR-26a (the miRNA that has been chosen here for functional studies), while among the significantly gained miRNA in both works there are: miR-345, miR-124a, miR-372 and miR-373*.

The study conducted on MCL series of cases also identifies a potential new prognostic marker. MCL response to therapy is not uniformly unfavourable, and some MCL cases follow a relatively indolent clinical course. Interestingly, weak expression of miR-20b can be useful for predicting clinical behaviour, enabling a group of MCL patients with higher survival probability to be distinguished. miR-20b expression has been found to have a role in other type of cancers, where its high level of expression was associated with worse prognosis (Katada et al., 2009; Landais et al., 2007; Sun et al., 2008), as we also found in MCL. It is of interest that miR-20b shares 21 out of 23 nucleotides with miR-20a, which is a member of oncomir-1 cluster. It is possible that miR-20b and miR-20a share also some common targets and it may have significance since as already discussed overexpression of the members of oncomir-1 cluster may have some correlation with additional aggressive features.

5.3. Project 3: variants in miRNAs sequence

miRNA sequence was evaluated in 95 cases of DLBCL. Nine variants were found in a total of six miRNAs. According with NCBI and Ensebl databases, five variants are already described, while the resting four variants, one on pre-miR-588 and three on pre-miR-650, are not described so far.

Even if a report in CLL affirms the possible existence of a germ line variant in tumour samples that may be relevant in the disease (Calin et al., 2005), miRNAs discoveries argument more for the relevance of alterations in miRNAs expression level, rather than miRNAs nucleotide changes.

Since the bidimentional conformation of pre-miRNAs is the result of the interaction between the entire sequence of pre-miRNA nucleotides, the potential effects of these changes on the pre-miRNA structure has been investigated by a predictor programme to evaluate wheatear these changes may disturb the pre-miRNAs structure. In the case of C→A variation on miR-650, the predicted structure of the pre-miRNA resulted deeply changed (Figure 28).

Pre-miRNA structure is considered of fundamental importance for miRNA maturation (Lund and Dahlberg, 2006; Starega-Roslan et al., 2011). For this reason a modification of the pre-miRNA conformation may alter the process of miRNA maturation as proposed by computational and biochemical analyses (Han et al., 2006), and in particular, it may perturb Dicer activity (Park et al., 2011a). Consequently, a lower-grade of mature miRNA expression would be expected (Duan et al., 2007; Harnprasopwat et al., 2010; Zhang and Zeng, 2010), as proposed for instance for miR-125a (Duan et al., 2007).

In this scenario a change in miRNAs sequence can be relevant and may have some effects on the regulation of the main cellular pathways. It might be reasonable to hypothesize that miRNAs variants may alter miRNA maturation, thus leading to a deregulation of the expression level of the miRNAs. These changes would be added to others caused by CNV in chromosome sites containing miRNAs.

Variants have been here identified for miR-588 and miR-650. Functional studies or validated targets are not available for miR-588, which makes the evaluation of these results even more difficult, while a recent publication arguments for miR-650 importance in CLL: lower expression of this miRNA was correlated to worse course of the disease, and higher rate of *in vitro* cell proliferation (Mraz et al., 2012).

So far the variants here described are not listed in Ensembl and dbSNP database. We cannot exclude the possibility that they could be found in the future in the healthy population, but still the importance of miRNAs polymorphism cannot be excluded as in all the rest of single nucleotide polymorphism (SNP) studies, since the importance of miRNAs variants may also rely on their correlation with patients' outcome, overall survival, response to therapy (Boni et al., 2011; Mishra et al., 2008; Mishra and Bertino, 2009) and assessment of cancer risk (Gao et al., 2011; Pastrello et al., 2010; Zeng et al., 2010).

5.4. Further developments and perspectives

5.4.1. Good candidates for further studies

It is interesting to note that some results on one single miRNA, miR-31, are recurrent in this work. It was downregulated when comparing MCL vs. normal controls and also after comparing the whole series of B-cell lymphomas *vs*. normal controls. miR-31 is predicted to be a regulator of the mitogen-activated protein kinase kinase kinase 14 (MAP3K14, also called NIK) expression, a gene that besides its obvious correlation to MAPK pathway, is also essential for the activation of the alternative NF-kB pathway (Sasaki et al., 2008) as demonstrated recently in T cell lymphomas (Yamagishi et al., 2012). Interestingly, NIK is a protein that is up-regulated in many lymphomas (Annunziata et al., 2007; Pham et al., 2011; Rosebeck et al., 2011; Saitoh et al., 2008). Moreover, the region in which miR-31 is located has been found lost in DLBCL, and correlation between this loss and miR-31 expression has been published (Li et al., 2009). All these observations make miR-31 a good candidate for further functional studies in MCL and the others B-cell lymphomas.

Another promising miRNA is miR-26a. It has been found able to regulate NF-kB activation: a pathway that is abnormal active in MCL and many other cancer types, including other lymphoma types. This miRNA has been found lost in many types of cancers, including hepatocellular carcinoma (Chen et al., 2011), nasopharyngeal carcinoma (Lu et al., 2011) and lung cancer (Dang et al., 2012). In these works restoration of miR-26a expression has been correlated with inhibition of cell proliferation, blockage in G1/S phase transition, induced apoptosis, inhibition of cell metastasis and *in vitro* inhibition of invasion (Dang et al., 2012), and suppression of cell proliferation and colony formation (Lu et al., 2011).

Additionally miR-26a downregulates a member of PcG complex: EZH2 (Lu et al., 2011; Zhang et al., 2011a), whose expression is important for histone methylation and regulation of different tumour suppressor gene expression.

Taken into account all these considerations, the restoration of miR-26a might confer a clinical advantage for the patients. A promising *in vivo* study describes the effects of reintroduction of miR-26a in a murine liver cancer model (Kota et al., 2009), where the restoration of miR-26a expression inhibited cancer cell proliferation and induced tumour cell specific apoptosis, cell cycle arrest, and protection from disease progression without toxicity. These findings make for miR-26a as a good candidate for future drug developments studies.

5.4.2. Causes of miRNA losses and gains: an issue that requires further investigation

The biological importance of miRNAs losses and gains is an element broadly recognised, and the effects of miRNAs deregulation have been largely investigated in normal and disease conditions.

Less information is available about the causes of miRNAs deregulation, in fact in most of the cases it is not known why changes in miRNAs expression level occur.

Efforts have been done also to correlate CGH data to miRNA expression profile. Good results are reported in CLL (Visone et al., 2009), DLBCL (Li et al., 2009) and other type of cancers (Nymark et al., 2011; Selcuklu et al., 2009; Tatarano et al., 2011). Among the miRNAs here found deregulated the miR-17-92 cluster that was found significantly related to gain at 13q31 region in lymphomas, particularly in MCL (Navarro et al., 2009; Rinaldi et al., 2007) In this series of MCL cases loss of the 9p21 and 17p13.1 cytobands, were miR-31 and miR-497 are located respectively was found (not published), but still CGH alone does not seem to explain the majority of miRNAs deregulation.

The possibility of epigenetic alterations has been also investigated in many studies and some of the miRNAs here deregulated have been found epigenetically controlled, for instance with miR-203 (Chim et al., 2011). Interestingly miR-141/200c cluster was found downregulated in our series of SMZL, and DNA methylation has been documented as one reason of its loss (Neves et al., 2010; Vrba et al., 2010).

Correlation between miRNAs overexpression and DNA hypomethylation has been also described (Li et al., 2010a), in specific for miR-200a and miR-200b: both upregulated in MALT and NMZL cases.

Not only, since miRNAs have a promoter and regulatory region like every other gene, conventional transcription factors are also able to induce miRNA expression. The most interesting case in B-cell lymphoma that has been documented is the regulation of oncomiR-1 by Myc (Chang et al., 2008; Robertus et al., 2010).

Other possibilities have been recently explored: for instance the alternative routes of miRNAs maturation as a mechanism that could determine the mature amount of miRNAs in the cells has been taken under consideration (Cheloufi et al., 2010; Yang et al., 2012).

Finally, changes in miRNAs sequence may impair the maturation of miRNAs, and confer another mechanism that may bring to miRNAs expression level reduction, as proposed for miR-125a (Duan et al., 2007) and miR-126 (Harnprasopwat et al., 2010), two miRNAs that here are lost in CLL cases compared with the rest of the lymphomas and in MCL cases compared *vs*. non-tumour controls. Due to the possibility that the maturation process could be affected by the changes in the sequence of miRNAs (Sun et al., 2010), the variant here reported on pre-miR-650 could be a good candidate to examine more in depth their effects on mature miRNA expression level.

6. Conclusions / Conclusiones

1. B cell lymphomas have a specific miRNA signature common to all types of lymphomas here studied, and different to non-tumour controls.
2. At the same time, the different types of lymphoma show distinct miRNA signatures, which confirm the importance of miRNAs in tumour development, and remark their potential use as diagnostic markers.
3. A signature of 19 miRNAs differentially expressed between BL and DLBCL could be used for differential diagnosis. Among them the most reliable are: miR-155, miR-29b, miR-146a and miR-17-3p.
4. The analysis of miRNA and gene expression profiles in MCL permitted to correlate miRNAs deregulation with gene pathways alterations. In particular, the loss of miR-26a was correlated to NF-kB pathway activation as shown in cell lines.
5. miRNAs relevance as potential prognostic markers was demonstrated in MCL, where high expression of miR-20b was found associated with a shorter overall survival
6. Sequence variations in miRNAs are a rare event and the significance of them needs to be fully investigated

1. Los linfomas de células B poseen un perfil de expresión de miRNAs común y diferente de los controles no tumorales.
2. Al mismo tiempo, los distintos tipos de linfoma B poseen un perfil de expresión de miRNAs específico, que confirma la importancia de los mismos en el desarrollo de cada linfoma y sugiere su uso para el diagnóstico diferencial.
3. Un perfil de expresión de 19 miRNAs diferencialmente expresados entre BL y DLBCL podría ser usado para el diagnóstico diferencial. Entre ellos, los miRNA más prometedores son miR-155, miR-29b, miR-146a y miR-17-3p.
4. El análisis de la expresión de los miRNAs y de los genes en linfoma de manto permitió relacionar la expresión de los miRNAs con las rutas biológicas alteradas. La perdida del miR-26a fue relacionada con la activación ruta de Nf-kB.
5. La relevancia de la expresión de los miRNAs en el pronóstico de los linfomas de manto ha sido demostrada tras la identificación de miR-20b, cuya expresión se encontró asociada a una peor expectativa de supervivencia.
6. Las alteraciones de las secuencias parecen ser un evento raro en los miRNAs, y sus relevancia en la célula requiere una mayor investigación

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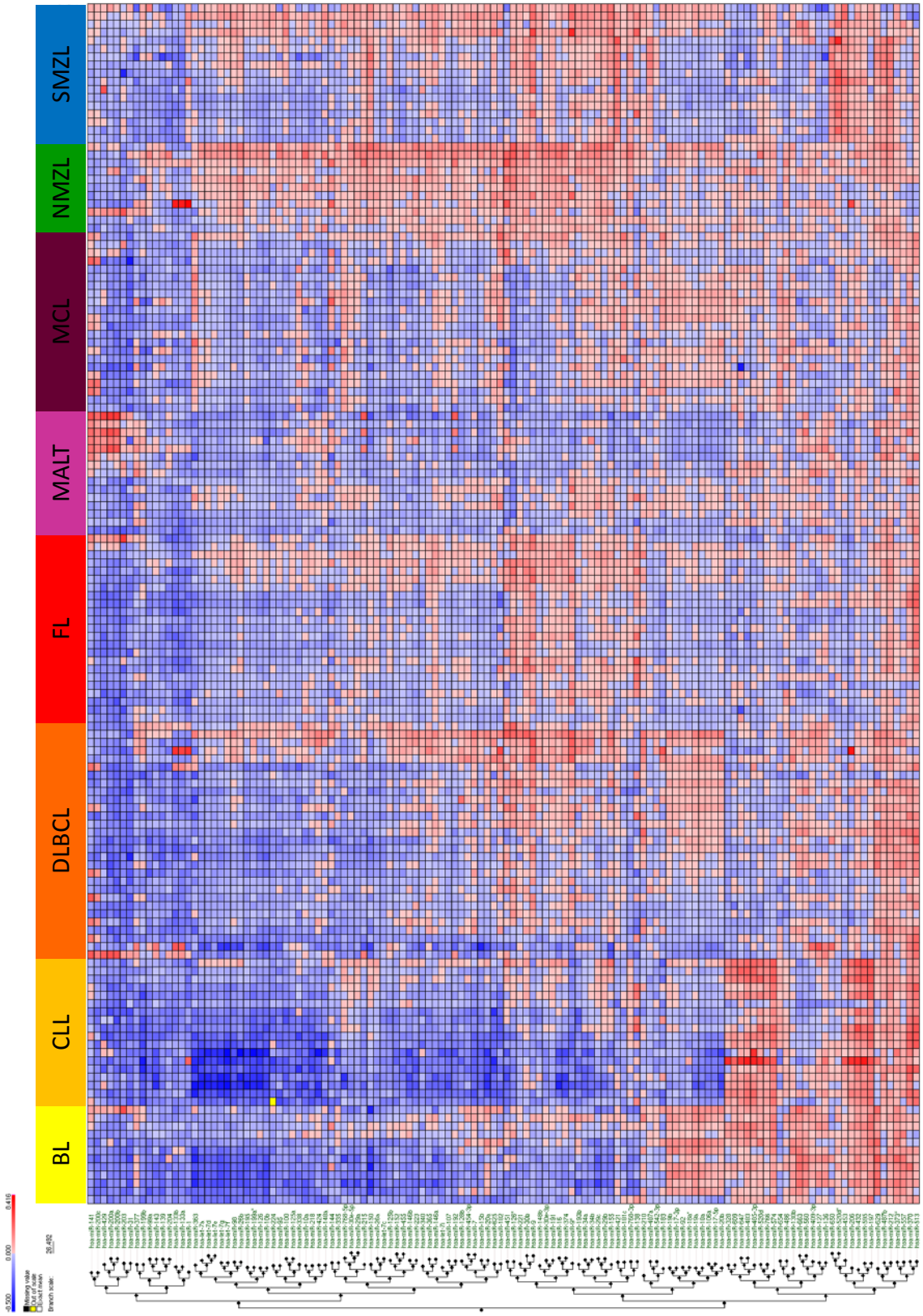
8. Appendix

Figure 9. Heatmap of the significant miRNAs normalized with non-tumour controls.



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ORIGINAL ARTICLE

MicroRNA signatures in B-cell lymphomas

L Di Lisio[1,2], M Sánchez-Beato[2], G Gómez-López[3], ME Rodríguez[1,2], S Montes-Moreno[1,2], M Mollejo[4], J Menárguez[5], MA Martínez[6], FJ Alves[7], DG Pisano[3], MA Piris[1,2,8] and N Martínez[1,2,8]

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.

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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.

[1]Genomica del Cancer, Instituto de Investigación y Formación Marqués de Valdecilla (IFIMAV), Santander, Spain; [2]Molecular Pathology Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain; [3]Structural Biology and Biocomputing Programme, Bioinformatics Unit, Spanish National Cancer Research Centre (CNIO), Madrid, Spain; [4]Department of Pathology Hospital Virgen de la Salud, Toledo, Spain; [5]Department of Pathology, HGUGM, Madrid, Spain; [6]Department of Pathology, Hospital Universitario 12 de Octubre, Madrid, Spain and [7]Department of Pathology, Hospital Universitario La Paz, Madrid, Spain. Correspondence: Dr N Martínez, Genomica del Cancer, Instituto de Investigación y Formación Marqués de Valdecilla (IFIMAV), Avda de Cardenal Herrera Oria S/N, 39011 Santander, Spain.
E-mail: ifimav.nmartinez@fmdv.org
[8]Senior authors.
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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

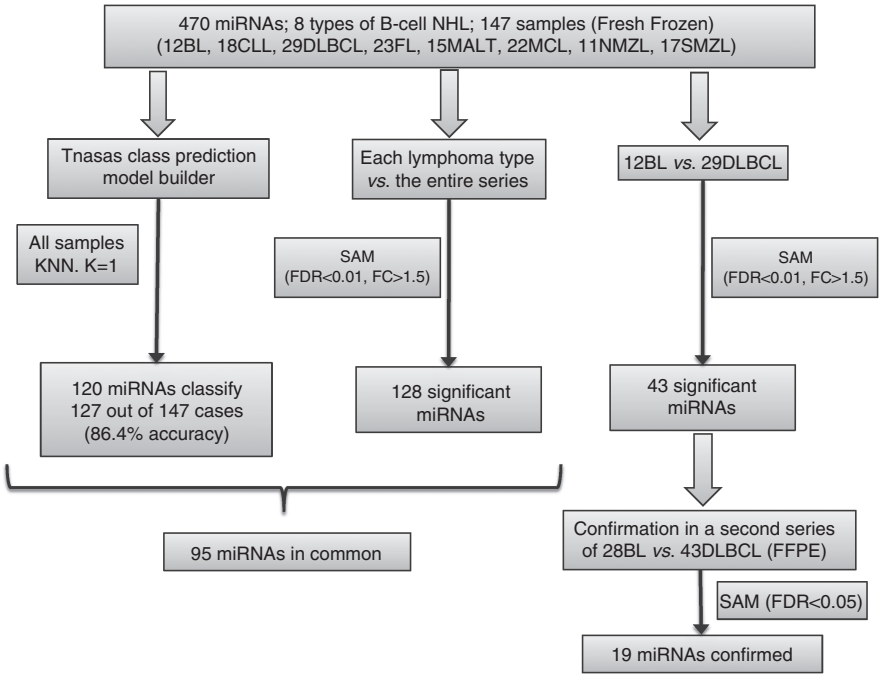

Figure 1. Workflow. The samples were analyzed first by SAM, comparing each lymphoma type against the whole series. In parallel, a class predictor model was investigated for the 147 samples. Also, BL and DLBCL samples were compared by SAM, and then significant miRNAs were analyzed in a second series of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

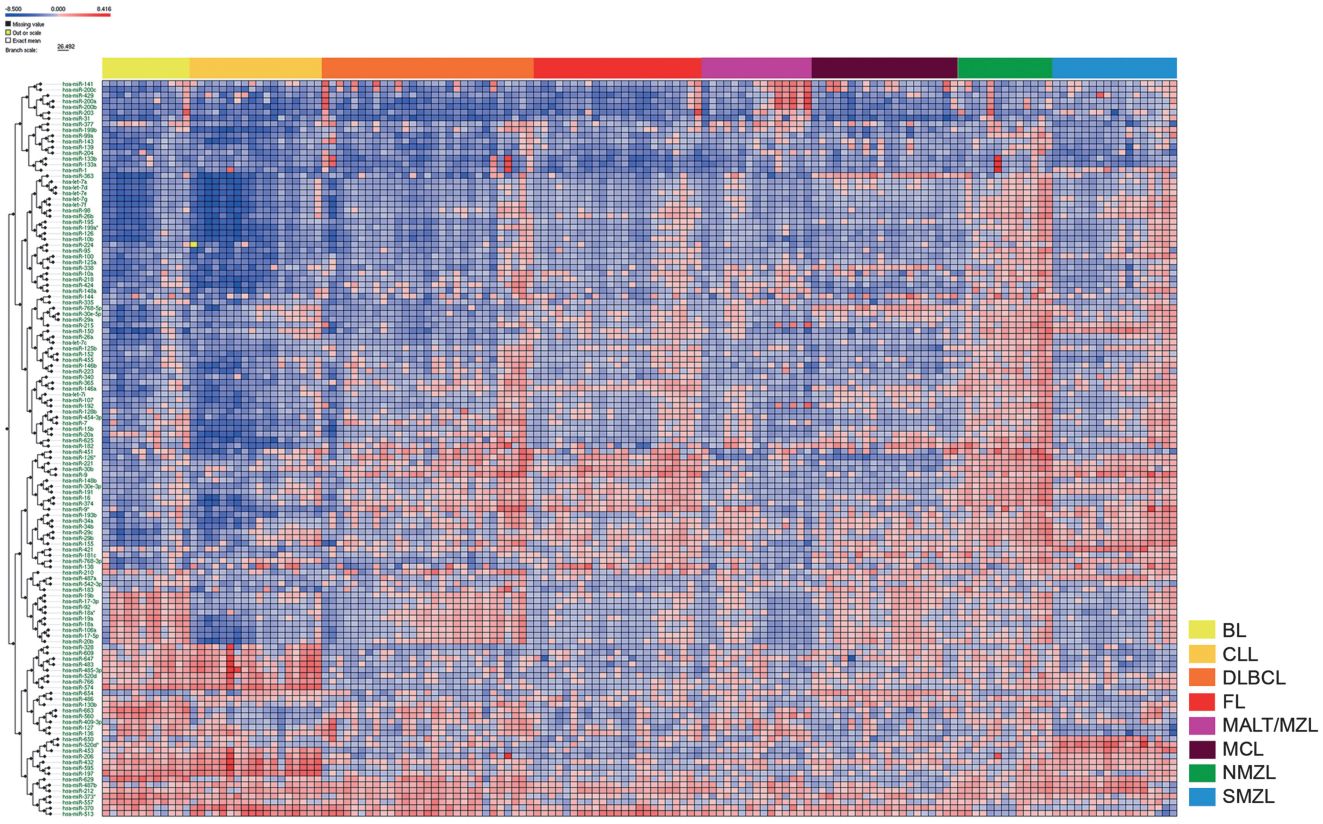

Figure 2. Differentially expressed miRNAs in B-cell lymphomas. In all, 128 miRNAs were differentially expressed in the series (FDR <0.01 and fold change (FC) >1.5) of 147 samples. For representation, data were normalized with non-tumoral controls (tonsils, reactive lymph nodes or spleens).

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.

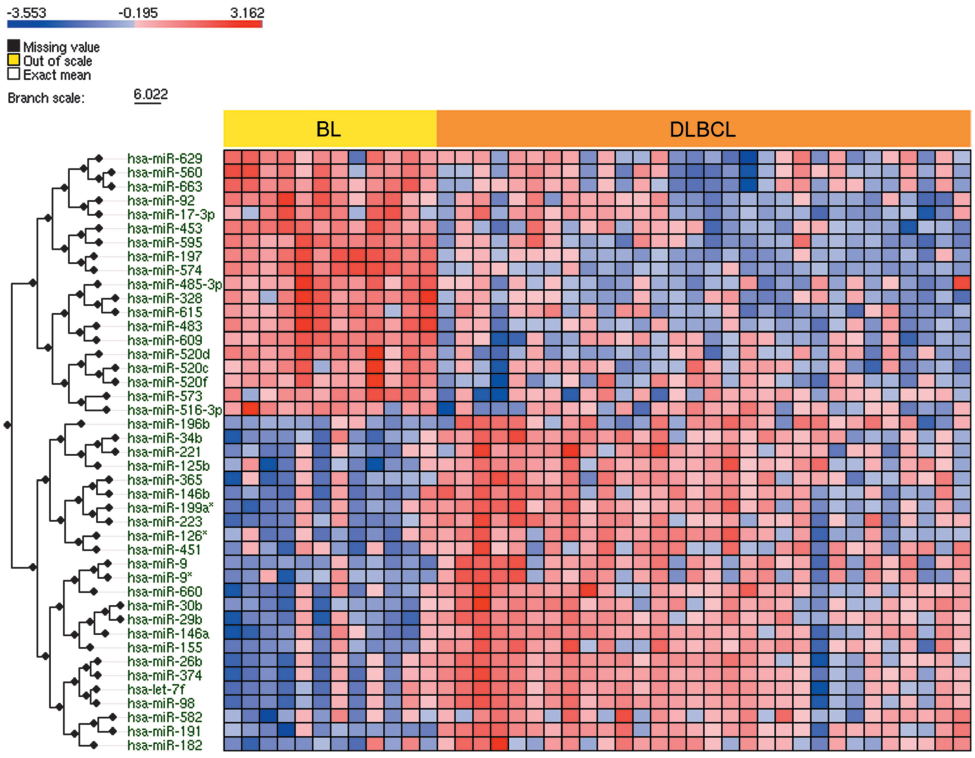

Figure 3. MiRNAs differentially expressed between BL and DLBCL. In all, 43 miRNAs were significantly (FDR <0.01 and fold change (FC) >1.5) differentially expressed in a SAM analysis comparing microarray data of 12 BL vs 29 DLBCL.

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

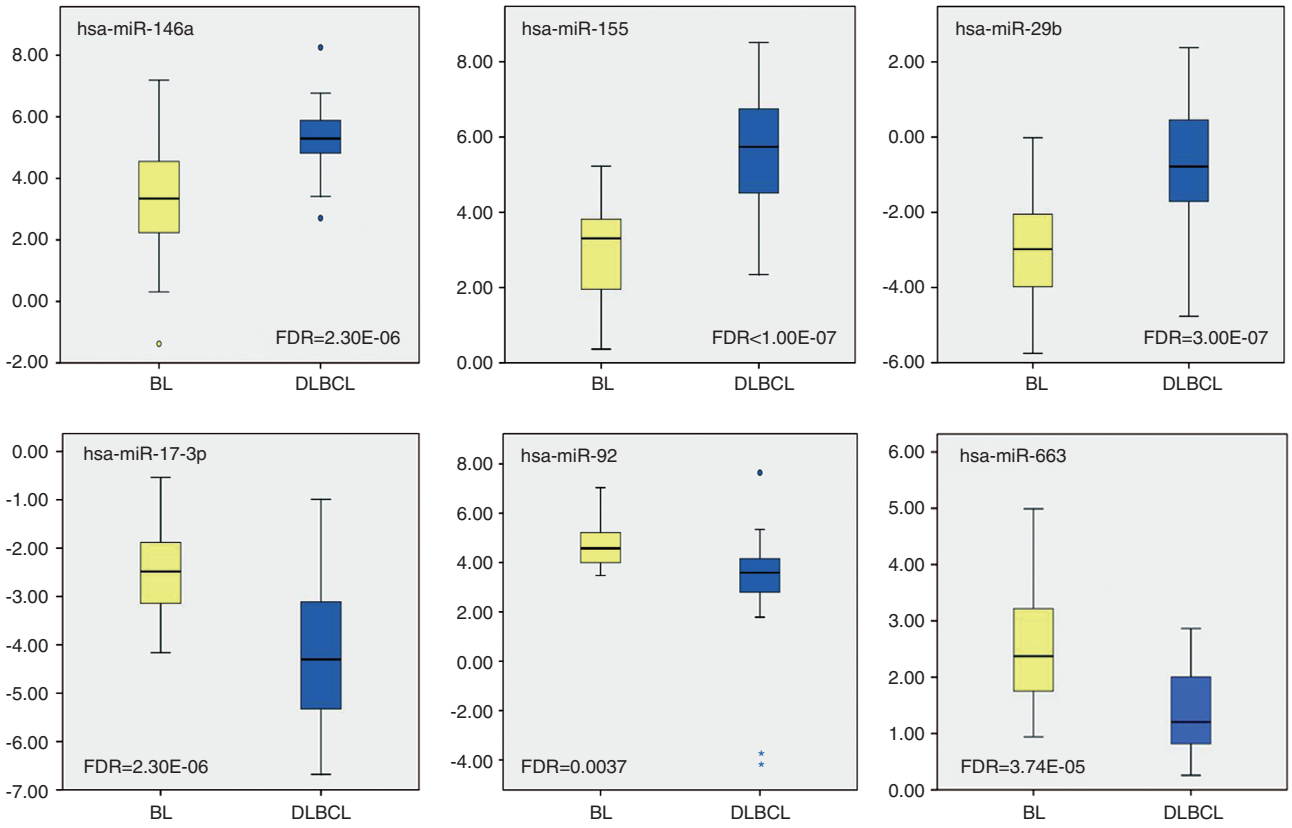

Figure 4. Box plots of qRT-PCR results. A selection of significant miRNAs (BL vs DLBCL) confirmed by qRT-PCR is illustrated. Mir-146a, miR-155 and mir-29b were upregulated in the DLBCL group, whereas miR-17-3p, miR-92 and miR-663 were upregulated in the BL group. $-\Delta Ct$ values are represented in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

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Epstein-Barr virus microRNAs repress BCL6 expression in diffuse large B-cell lymphoma

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Diffuse large B-cell lymphoma (DLBCL) is the most common form of lymphoma, accounting for 30–40% of all newly diagnosed lymphomas. DLBCL is considered a heterogeneous disease, with some specific clinicopathological variants of DLBCLs being associated with the presence of the EBV.[1] EBV is a lymphotropic virus that has been implicated in the development of several lymphoid malignancies, mainly Burkitt lymphoma (BL) and Hodgkin's lymphoma and with low prevalence in DLBCL.[1] BCL6 is a key transcriptional repressor during normal B-cell differentiation that has been shown to repress NF-kB in some DLBCLs.[2] In some B-cell lymphomas, BCL6 expression was inversely correlated with LMP1 expression, and some evidences suggest that LMP1 can cause downregulation of BCL6(ref. 3), but other possible mechanisms have not been studied. We have found a strong inverse correlation between BCL6 protein expression and EBV infection ($P<0.001$, Figures 1a and b) in a series of 149 DLBCL samples, where only one out of 34 EBV-positive cases (2.94%) expressed BCL6, although 87 out of 115 EBV-negative cases expressed BCL6 (75.65%). However, this correlation was independent of LMP1 because 54% of EBER-positive samples were LMP1-negative (Spearman, *P*-value: 0.18). Little is known about the mechanisms that cause the absence of BCL6 in EBV-positive DLBCL; however, the possibility that EBV-encoded miRNAs could contribute to BCL6 repression has never been explored.

We have studied the miRNA expression profile in 36 DLBCL samples using miRNA microarrays. Prevalence of EBV in this series (33 EBV-negative and 3 EBV-positive) was close to normal prevalence in DLBCL.[1] Twenty-two out of thirty-two analysed viral miRNAs were significantly upregulated in EBV-positive samples (>two-fold change; *t*-test corrected *P*-value<0.05, Figure 1c, Supplementary table 2 and Supplementary table 3). None of the miRNAs belonged to the BHRF1 cluster, a finding that confirms previous results suggesting that the highest levels of these miRNAs are reached during the lytic cycle.[4] To test the hypothesis that some of these miRNAs can potentially downregulate BCL6, we made a bioinformatic prediction using the miRanda algorithm. This prediction indentified 21 binding sites in the 3′ UTR of BCL6 for 18 EBV-encoded miRNAs (Supplementary table 1). More than 70% of these predictions were also found with other algorithms (Supplementary Figure 1). Ten of these miRNAs were also differentially expressed between EBV-positive and EBV-negative DLBCL cases. This high proportion of miRNAs potentially targeting BCL6 suggests a physiological mechanism of the virus to reduce the amount of BCL6. Consequently, we selected four EBV-encoded miRNAs for further validation. These miRNAs were selected according to the following parameters: 1) attainment of a high score from miRanda and 2) differential expression in the EBV-positive DLBCL cases. In this way, we selected ebv-miR-BART3, ebv-miR-BART9 and ebv-miR-BART17-5p. We also selected ebv-miR-BART7 because, although it does not achieve one of the highest scores from miRanda, the program predicts two binding sites for the miRNA in the 3′ UTR of BCL6, increasing the probability of it being a *bona fide* regulator of BCL6 (Supplementary Figure 2). To analyse these miRNAs in a larger series of cases, we selected 40 cases (15 EBV-positive and 25 EBV-negative) from the 149-patients series to check their expression by RT–PCR. The mean of the viral miRNAs expression among EBER-positive patients was 10- to 100-fold higher compared with EBER-negative patients (Figure 1d). We also analysed EBV-encoded miRNA levels by RT–PCR in BL and DLBCL-derived cell lines and observed a correlation between BCL6 protein and miRNA levels (Figures 1e and f). These results indicate that a relatively low level of expression of these miRNAs could enable the high level of expression of BCL6 observed in EBV-positive BL,[5] thus explaining the differences between BL and DLBCL.

Therefore, to validate the putative BCL6-regulatory role of these miRNAs, we transfected synthetic miRNAs and measured the luciferase activity in a reporter system in which we cloned the 3′UTR of BCL6 (Supplementary Materials & methods). With this approach, we noted a significant reduction in luciferase signal for three of the four viral miRNAs assayed (Figure 2a) when compared with either the reporter transfected with a miRNA mimic negative control sequence or the vector without the 3′ UTR of BCL6. Ebv-miR-BART3, ebv-miR-BART9 and ebv-miR-BART17-5p reduced the luciferase signal by at least 60%. The effect of these miRNAs on the endogenous BCL6 protein was investigated in lymphoid EBV-negative BCL6-expressing cell lines. We used three DLBCL-derived cell lines (DB, SU-DHL-4 and SU-DHL-6) and one BL-derived cell line (Ramos) to test the effect of these miRNAs in different lymphoid models. In DLBCL-derived cell lines transfection of the viral miRNAs ebv-miR-BART3, ebv-miR-BART9 and ebv-miR-BART17-5p led to a reduction in the levels of BCL6 protein ranging from 25 to 72%, 48 h after transfection (Figure 2b). In contrast, ebv-miR-BART7 was able to reduce BCL6 protein expression only in SU-DHL-4 and SU-DHL-6 whereas it had little or no effect in DB. In Ramos cell line, transfection of the miRNAs led to a significant reduction of BCL6 protein levels in the case of ebv-miR-BART7, ebv-miR-BART9 and ebv-miR-BART17-5p, whereas the cells showed no response to ebv-miR-BART3. This may reflect a cell context-dependent phenomenon that could be related to target-site accessibility. Moreover, we used EBV-positive DLBCL-derived cell lines to allow inhibition of endogenous EBV-encoded miRNAs by miRNA inhibitors. Inhibition of ebv-miR-BART9 and ebv-miR-BART17-5p, led to a moderate increase in the expression of BCL6 in the Farage cell line but not in the DoHH-2 cell line, which expressed the highest levels of EBV-encoded miRNAs (Figure 2c).

EBV is more frequently found in tumors with a plasmablastic phenotype in which other factors (for example, expression of BLIMP1) could be repressing BCL6 at the transcriptional level.[6] Our data support a role of EBV microRNAs that could help to diminish BCL6 expression facilitating BCL6 transcriptional repression by BCL6 targets such as BLIMP1.[7] However, the reason by which EBV downregulates BCL6 in DLBCL remains unknown. It is known that several EBV-driven lymphomas rely on the activation of the NF-kB pathway[8] as well as ABC-type DLBCL.[9] Viral proteins such as LMP1 and LMP2A can activate the NF-κB pathway directly or indirectly to promote the survival of the host cell.[8,10–12] Because BCL6 represses NF-κB under

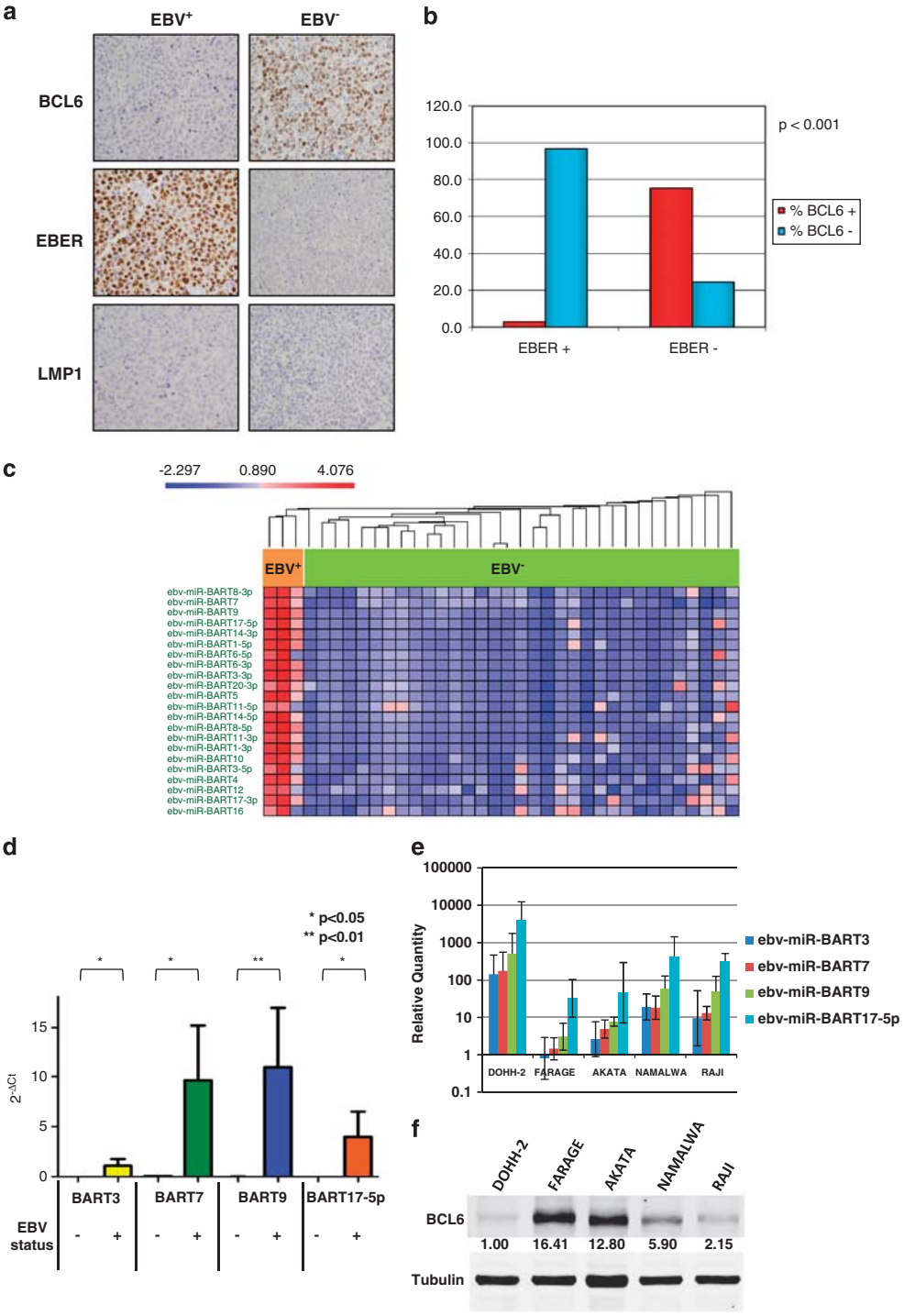

Figure 1 (**a**) Immunohistochemical staining of two cases of DLBCL showing an opposite pattern of BCL6 expression and EBV presence. (**b**) Graphic representation and statistical analysis by χ^2-test of the immunohistochemical results in a series of 149 DLBCL patients. (**c**) Gene clustering reveals the pattern of expression of EBV-encoded miRNAs in EBV-positive DLBCL patients. (**d**) RT–PCR was carried out on 40 DLBCL samples using probes for five miRNAs. Black bars indicate standard deviation. (**e**) RT–PCR analysis of five EBV-encoded miRNAs in DLBCL and BL cell lines. Expression data is represented in a logarithmic scale and has been normalized using the Ramos EBV-negative cell line as the background signal. (**f**) Levels of BCL6 protein expression in the same cell lines show an inverse correlation with the levels of EBV-encoded miRNAs.

normal and pathogenic conditions,[2] BCL6 downregulation may be necessary to promote survival of the EBV-positive neoplastic cells in some DLBCLs. EBV does not repress BCL6 in Burkitt lymphoma; however, in this tumor type, activation of the NF-κB pathway has been shown to induce apoptosis,[13] and so the role of the virus might be different in this disease.

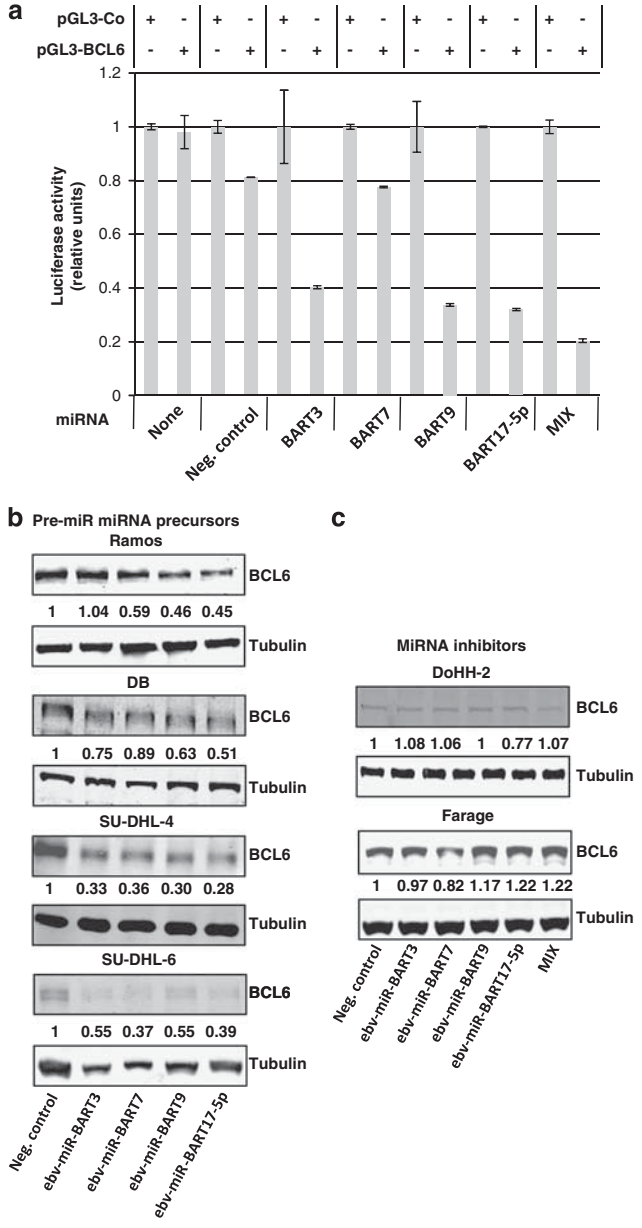

Figure 2 (**a**) Some predicted miRNAs can downregulate BCL6. Co-transfection of several miRNAs with a luciferase reporter with (pGL3-BCL6) or without (pGL3-Co) the 3′UTR of BCL6 led to specific inhibition for miRNAs ebv-miR-BART3, ebv-miR-BART9 and ebv-miR-BART17-5p when compared with the effect of a miRNA mimic negative control sequence. (**b**) Transfection of the EBV-negative DLBCL and BL cell lines with several miRNAs led to decreased BCL6 expression. BCL6 signal was normalized using tubulin. (**c**) Transfection of miRNA inhibitors in EBV-positive cell lines led to a moderate upregulation of BCL6 in the Farage cell line when ebv-miR-BART9 and ebv-miR-BART17-5p were inhibited. An equimolar mixture of the four anti-miRNAs (200 nM final concentration) was also able to increase BCL6 levels. DoHH-2 cell line showed no response to miRNA inhibition. BCL6 signal was normalized using tubulin.

Conflict of interest

The authors declare no conflict of interest.

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D Martín-Pérez[1], P Vargiu[1], S Montes-Moreno[1], EA León[2], SM Rodríguez-Pinilla[1], LD Lisio[1], N Martínez[1], R Rodríguez[3], M Mollejo[3], J Castellvi[4], DG Pisano[2], M Sánchez-Beato[1,5] and MA Piris[1,5]
[1]*Lymphoma Group, Molecular Pathology Programme; Spanish National Cancer Research Centre (CNIO), Madrid, Spain;*
[2]*Bioinformatics Unit (UBio), Structural Biology and Biocomputing Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain;*
[3]*Department of Pathology. Hospital Virgen de la Salud, Toledo, Spain and*
[4]*Department of Pathology, Vall d'Hebron University Hospital, Barcelona, Spain*
E-mail: mapiris@cnio.es
[5]*Senior authors.*

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Obesity and the risk of chronic myelogenous leukemia: is this another example of the neoplastic effects of increased body fat?

Leukemia (2012) **26,** 183–184; doi:10.1038/leu.2011.190; published online 22 July 2011

The risk of cancers of varied tissues is increased in persons who are overweight or obese.[1–4] The increased relative risk (RR) of cancer in overweight and obese subjects has also included the hematological malignancies, although the major types of leukemia and lymphoma have not been stratified in several of these large epidemiological studies.[1,2,5–7] Myeloma also has been associated with overweight and obesity as has its antecedent, essential monoclonal gammopathy.[8,9] The evidence for an increased risk of leukemia in the aggregate[1,2,5–7] and of the four major types of leukemia is noteworthy.[10] Given the broad range of tissues for which a risk of neoplasia is increased in overweight and obese persons, there appears to be a global effect of the metabolic, endocrinologic and inflammatory changes resulting from obesity on either the induction of neoplastic clones, or the selection and enhanced growth and survival of preexisting, dormant transformed clones.

BCR-ABL-positive chronic myelogenous leukemia is a very uncommon secondary leukemia. Unlike secondary acute myelogenous leukemia, which can occur as a result of exposure to high-dose ionizing radiation, certain types of chemotherapy (especially, alkylating agents and topoisomerase inhibitors), prolonged inhalation of tobacco smoke or higher-dose prolonged exposure to benzene, only ionizing radiation is an established cause of secondary chronic myelogenous leukemia.[11] In addition, there is little evidence that either a familial predisposition gene (non-syndromic) or an inherited predisposing disorder (syndromic predisposition gene) is associated with chronic myelogenous leukemia, as it is with acute myelogenous leukemia.[11] Although a proportion of the healthy population carries low copy numbers of the *BCR-ABL* oncogene in its blood cells, it is not known whether these clones are susceptible to clonal evolution to a clinically overt neoplasm, either spontaneously or under provocation. The apparent effect of increased fat mass on an increased RR of chronic myelogenous leukemia should be of research interest to students of carcinogenesis and leukemogenesis. The relationship of overweight to cancer is a major challenge to the practice of preventive medicine and the soft (cajoling) and hard (pharmacotherapy) science of behavior modification.

In a search of the literature, I have identified eight studies that have examined the relationship of overweight or obesity to the risk of chronic myelogenous leukemia, either as a primary research effort or as a meta-analysis of prior studies. In these studies, the body mass index (BMI) was used as a measure of fat tissue mass. The World Health Organization and the United States Public Health Service considers a BMI of $<18\,kg/m^2$ as underweight, 18–24.9 kg/m^2 as a normal body fat mass, 25 to 29.9 kg/m^2 as overweight and $>29.9\,kg/m^2$ as obesity. Obesity may be stratified into Class I (BMI = 30–34.9 kg/m^2), Class II (BMI = 35–39.9 kg/m^2), Class III (BMI $\geqslant 40\,kg/m^2$).

In 2005, investigators in Melbourne, Victoria, Australia found a five-fold increase in the hazard ratio (HR) for myeloid leukemias (acute and chronic myelogenous leukemia) in overweight, HR = 5.3 (95% confidence interval (CI), 1.9–15.2), and obese, HR = 5.0 (95% CI, 1.6–15.5), persons as compared with those with a normal BMI ($<25.0\,kg/m^2$). The HR was higher for chronic than acute myelogenous leukemia, but the number of cases of chronic myelogenous leukemia was not sufficient to apply statistical tests of homogeneity between the two leukemias.[12]

In a study of 2 000 611 Norwegian men and women, reported in 2006, the trend of RR of chronic myelogenous leukemia using a normal range of BMI of 18.5–24.9 kg/m^2 as the referent value (1.0), and studying degrees of BMI from underweight, to normal, to overweight, to obese, to massively obese, found a significant trend for increased risk of chronic myelogenous leukemia from RR = 0.78 in underweight (BMI $<18.5\,kg/m^2$) to RR = 1.65 in obese men (BMI $\geqslant 30.0\,kg/m^2$).[13] In women, the same significant trend was observed ranging from RR = 0.85 in underweight women to a RR = 1.89 in women with Class III obesity (BMI $\geqslant 40\,kg/m^2$). The results were ambiguous in that the trends by BMI were highly significant and the RR of chronic myelogenous leukemia increased generally with increased BMI; however, the increased RR at each level of increased BMI was not statistically significant in several cases.

Three reports between 2004 and 2007 found an increased RR of chronic myelogenous leukemia in overweight or obese men of European or African descent,[14] and in men and women,[13,15] but the RR (1.15 to 1.65) was either not significant or just missed statistical significance. However, a meta-analysis, published in 2008, of the three studies,[13–15] which included five populations, found a significantly increased meta-relative risk (MRR) of chronic myelogenous leukemia (MRR = 1.26 (95% CI, 1.09–1.46)) in the obese study subjects as compared with those with a normal BMI.[10]

In a population-based case–control study conducted in eight Canadian provinces, published in 2005, an association was found with increased BMI and an increased RR of chronic myelogenous leukemia with a significant dose-response relationship; the increment in risk for chronic myelogenous leukemia from normal to overweight (odds ratio (OR) = 1.4; 95% CI, 1.0–2.0) and from normal to obese (OR = 2.3; 95% CI, 1.5–3.4) subjects was highly significant.[16]

Overweight and obesity and the risk of hematological malignancies were studied in a cohort of Swedish and Finnish twins (70 067 persons), who were followed prospectively. An increased RR of chronic myelogenous leukemia was observed in those who were overweight or obese (RR = 2.5 95% CI, 1.0–6.2).[17]

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Combinatorial effects of microRNAs to suppress the Myc oncogenic pathway

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Combinatorial effects of microRNAs to suppress the Myc oncogenic pathway

María J. Bueno,[1,2] Marta Gómez de Cedrón,[1] Gonzalo Gómez-López,[3] Ignacio Pérez de Castro,[1] Lorena Di Lisio,[4] Santiago Montes-Moreno[4] Nerea Martínez,[4] Manuel Guerrero,[1,2] Ruth Sánchez-Martínez,[1] Javier Santos,[2,5] David G. Pisano,[3] Miguel Angel Piris,[4] José Fernández-Piqueras,[2,5] and Marcos Malumbres[1]

[1]Cell Division and Cancer Group, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain; [2]Centro de Biología Molecular Severo Ochoa CSIC-Universidad Autónoma de Madrid and Dept Biología, Universidad Autónoma de Madrid, Madrid, Spain; [3]Bioinformatics Unit, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain; [4]Lymphoma Group, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain; and [5]Ciber de Enfermedades Raras, Centro de Investigaciones Biomédicas en Red de Enfermedades Raras, ISCIII, Madrid, Spain

Many mammalian transcripts contain target sites for multiple miRNAs, although it is not clear to what extent miRNAs may coordinately regulate single genes. We have mapped the interactions between down-regulated miRNAs and overexpressed target protein-coding genes in murine and human lymphomas. *Myc,* one of the hallmark oncogenes in these lymphomas, stands out as the up-regulated gene with the highest number of genetic interactions with down-regulated miRNAs in mouse lymphomas. The regulation of *Myc* by several of these miRNAs is confirmed by cellular and reporter assays. The same approach identifies *MYC* and multiple Myc targets as a preferential target of down-regulated miRNAs in human Burkitt lymphoma, a pathology characterized by translocated *MYC* oncogenes. These results indicate that several miRNAs must be coordinately down-regulated to enhance critical oncogenes, such as Myc. Some of these Myc-targeting miRNAs are repressed by Myc, suggesting that these tumors are a consequence of the unbalanced activity of Myc versus miRNAs. (*Blood.* 2011;117(23):6255-6266)

Introduction

Tumor development is accompanied by a variety of genetic and epigenetic alterations in protein-coding genes and small, non-coding RNA genes. miRNAs are a diverse family of small RNAs that regulate the stability and translational efficiency of partially complementary target mRNAs.[1,2] By regulating specific oncogenes or tumor-suppressor molecules, these small RNAs may have profound effects in tumor development.[3] A few target genes have been validated for some miRNAs indicating that each individual miRNA can target a few, or possibly, multiple genes and participate in diverse physiologic or pathologic functions. Thus, the miR-15a–miR-16–1 cluster controls prostate cancer by targeting proliferation, survival, and invasion regulators.[4] Let-7 miRNAs act as tumor suppressors by modulating major oncogenes, such as Ras or Myc, among many other targets.[5] On the other hand, each human gene can be modulated putatively by several miRNAs. However, the relevance of this multiplicity in tumorigenesis is not clear.

In this work, we have investigated the relationship between the expression of miRNAs and their putative targets in murine and human lymphomas. The pioneer molecular studies on γ-irradiation-induced lymphomas led to the identification of N-*ras* and K-*ras* as critical oncogenes in these tumors.[6,7] Additional cytogenetic studies demonstrated recurrent chromosomal alterations, such as specific translocations and trisomy of mouse chromosome 15 where the Myc oncogene is located.[8] In addition to Ras and Myc oncogenes, other tumor-promoting or tumor-suppresing genes, such as Notch1, p53, pRb, cyclin D, $p16^{INK4a}$ and $p15^{INK4b}$, $p19^{ARF}$, Pten, or Ikaros, have been suggested to play a significant role in the development of these malignancies.[9-15] Many of these oncogenic events are common to high proliferative lymphomas in humans. Burkitt lymphoma (BL) is a unique hematologic malignancy remarkable for its biologic characteristics, including its highly aggressive nature and its requirement for intensive treatment regimens.[16] Human BLs possess chromosomal rearrangements of the *MYC* oncogene (a genetic hallmark of these neoplasms), which contributes to lymphomagenesis through alterations in cell cycle regulation, cellular differentiation, apoptosis, cellular adhesion, and metabolism.[17]

In this work, we initially identified a panel of 41 miRNAs consistently down-regulated in γ-irradiation-induced lymphomas. Although some of these miRNAs, such as mir-203, mir-134, or mir-154, map to a region that suffers frequent DNA losses, most repressed miRNAs map to regions without DNA alterations. A few of these miRNAs, such as miR-203, are silenced by aberrant hypermethylation of the promoter region,[18] whereas other miRNAs may be repressed by Myc.[19] Interestingly, our data suggest that miRNAs may have a combinatorial effect to suppress the activity of relevant oncogenes. Indeed, Myc is predicted to be the major target of the miRNAs silenced in these malignancies. The regulation of Myc by several miRNAs is validated by reporter and cellular assays. A similar analysis also identifies *MYC* as a major target for the panel of miRNAs down-regulated in human BLs. The down-regulation of multiple miRNAs in these malignancies is predicted to favor not only the overexpression of Myc but also the up-regulation of multiple Myc targets involved in proliferation or differentiation, thus suggesting that the balance between Myc and miRNAs is critical for lymphomagenesis and may be further explored for therapy of these neoplasms.

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Methods

Mouse and human tumors

C57BL/6J and RF/J F1 hybrid mice and pure C57BL/6J animals were maintained in our animal facilities following the appropriate ethical recommendations from our institutions. For tumor induction, 4-week-old mice of both sexes were exposed to 4 weekly doses of 1.75 Gy/dose of ionizing γ-radiation.[6] Treated mice were observed daily until moribund and then killed and autopsied. Retroviral mutagenesis was performed as published previously.[20] In short, newborn mice were injected intraperitoneally with 10^5 infectious units of murine leukemia virus, and animals were monitored in time for the development of tumors. Moribund mice were killed and tumors isolated. Tumor and normal (age-matched) tissues were processed for histologic analysis (paraffin embedding and hematoxilin and eosin staining) following standard protocols. DNA, RNA, and proteins were isolated from these samples as described previously.[18]

All human BLs were obtained from the Spanish Tumor Bank Network of the Spanish National Cancer Research Center (Centro Nacional de Investigaciones Oncológicas [CNIO]). Institutional review board approval was obtained for these studies, and all participants provided written informed consent in accordance with the Declaration of Helsinki.

Transcriptional profiles, comparative genome hybridization, and statistical analysis

Comparative genome hybridization, cDNA, and miRNA array experiments were performed essentially as described previously on mouse γ-irradiation-induced lymphomas[18] or BLs.[21] Differentially expressed cDNAs were obtained using Pomelo tool (www.pomelo2.bioinfo.cnio.es), which implements the limma *t* test using the limma package[22] from Bioconductor project. The estimated significance level (*P* value) was corrected to account for multiple hypotheses testing using Benjamini and Hochberg False Discovery Rate (FDR) adjustment. Genes with FDR less than or equal to 0.01 were selected as differentially expressed between controls and tumors. Gene set enrichment analyses (GSEA) were applied using annotations from Biocarta, KEGG, and GeneMAPP pathway databases. Those miRNAs showing 3′-untranslated region (UTR) binding sites in human *MYC* were tested as a whole gene set using GSEA. We used FDR < 0.25 as significance threshold for the identification of biologically relevant gene sets.[23] Data were also analyzed through the use of Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). Those pathways showing FDR < 0.25, which is a well-established cut-off for the identification of biologically relevant gene sets,[23] were considered enriched in tumors. Myc targets were obtained from the Myc Cancer Gene Database (www.myccancergene.org/site/mycTargetDB.asp),[24] and only those targets characterized in mammals were used for statistical analysis. Significantly deregulated miRNAs were computed using the Significant Analysis of Microarray analysis from the TM4 pathway[25] and the limma package. Precomputed miRNA targets were obtained from miRBase Targets Database, Version 5 (www.microrna.sanger.ac.uk). For miRNA studies in these samples, median between-array normalization was applied to make microarrays comparable. Statistical significance was analyzed using the Fisher exact or χ^2 tests and Prism v 5.01 (GraphPad) software, and networks of interactions were represented using Cytoscape (www.cytoscape.org). Additional details on the statistical analysis of expression data and miRNA-target networks are provided as supplemental Data (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). All microarray data are available on the Gene Expression Omnibus under accession numbers GSE10861 and GSE23026.

Validation of coding-gene and miRNA expression by quantitative reverse-transcribed polymerase chain reaction and protein analysis

To validate miRNA or cDNA expression data, real-time quantitative reverse-transcribed polymerase chain reaction was performed in triplicate using the TaqMan MicroRNA assays kit (Applied Biosystems) according to the manufacturer's instructions in an Applied Biosystems 7900HT Fast Real-Time polymerase chain reaction apparatus. To normalize for differences in the amount of total input DNA, amplification at a reference protein-coding gene (Actin) was performed once per plate in triplicate for each individual DNA. Amplification of RNU19 was used for normalization of miRNA expression. The data analysis was done using the SDS (Sequence Detection Systems), Version 2.2.2 program (Applied Biosystems). Additional primer sets (TaqMan probes; Applied Biosystems) were used to amplify deregulated mRNAs or miRNAs in triplicate on the ABI 7900HT instrument (Applied Biosystems). Differences in gene expression were estimated using Student *t* tests.

Protein lysates were obtained as reported previously.[18] Proteins were transferred to nitrocellulose membranes (Bio-Rad) and probed with antibodies against the following proteins: Myc (Sigma-Aldrich), Mcm2 and Mcm4 (a gift of Juan Méndez, CNIO), Rcc1 (Nventa Biopharmaceuticals), $p27^{Kip1}$ (BD Biosciences), Mad2 (Marine Biologica Laboratory), and Bcl2, cyclin B1, and Cdk4 (Santa Cruz Biotechnology). In addition, antiactin or antitubulin antibodies (Sigma-Aldrich) were used as a loading control. After washing, blots were incubated with the appropriate secondary antibodies coupled to AlexaFluor 680 and 800 (Invitrogen). Subsequently, the membrane was scanned in Odyssey Infrared Imaging System (Li-Cor Biosciences).

Cell culture, transfections, and reporter assays

Jurkat and MOLT-4 (T-cell lymphoblastic lymphoma) and Raji (BL) cells were obtained from the ATCC. These cells were transfected using the Amaxa nucleofection apparatus following the manufacturer's recommendations. Luciferase assays were performed as described previously.[18] The Renilla luciferase, green fluorescent protein, or small RNAs labeled with red fluorescent protein (sigloRed; Dharmacon RNA Technologies) were used to normalize the expression values among different transfections. The 3′-UTRs of mouse and human Myc were cloned in pGL vectors downstream of the luciferase gene. Several mutants to alter the miRNA target sites were obtained using the mutagenesis kit from Stratagene. Most mutants were designed to alter 3 positions in the seed sequence of the target site (mutant sequences and oligonucleotides are available on request). miRNA genes were expressed using the pMirVec vector.[26]

Results

mRNA and miRNA expression profiles in murine lymphomas

To understand the molecular alterations underlying lymphoma development in γ-irradiated mice, we first analyzed mRNA expression profiles in 15 of these tumors (supplemental Data). Mouse γ-irradiation-induced lymphomas overexpress critical oncogenes, such as Myc, as well as growth factor and chemokine signaling molecules, cell cycle regulatory proteins, DNA replication markers, mitotic proteins, translation initiation factors, and ribosomal genes (Figure 1A, supplemental Figure 1; supplemental Table 1). Down-regulated molecules include histocompatibility proteins and lymphocyte differentiation proteins, as well as cell cycle inhibitory proteins and apoptotic markers. Some of these genes are targets of master transcription factors deregulated in these tumors. Thus, whereas Myc targets represent 6.8% (923 of 13 763) of the genes in the array, 98 (17.6%) of the 556 significantly up-regulated transcripts are bona-fide Myc targets, as defined by Zeller et al,[24] suggesting a significant enrichment ($P < .0001$) of up-regulated Myc targets in these γ-irradiated lymphomas. The deregulation of Myc (both at the mRNA and protein levels; Figure 1 and supplemental Figure 1) and Myc targets is a predominant hallmark in the transcriptional alterations in proliferative, apoptotic, and lymphocyte differentiation pathways (Figure 1A; supplemental Data).

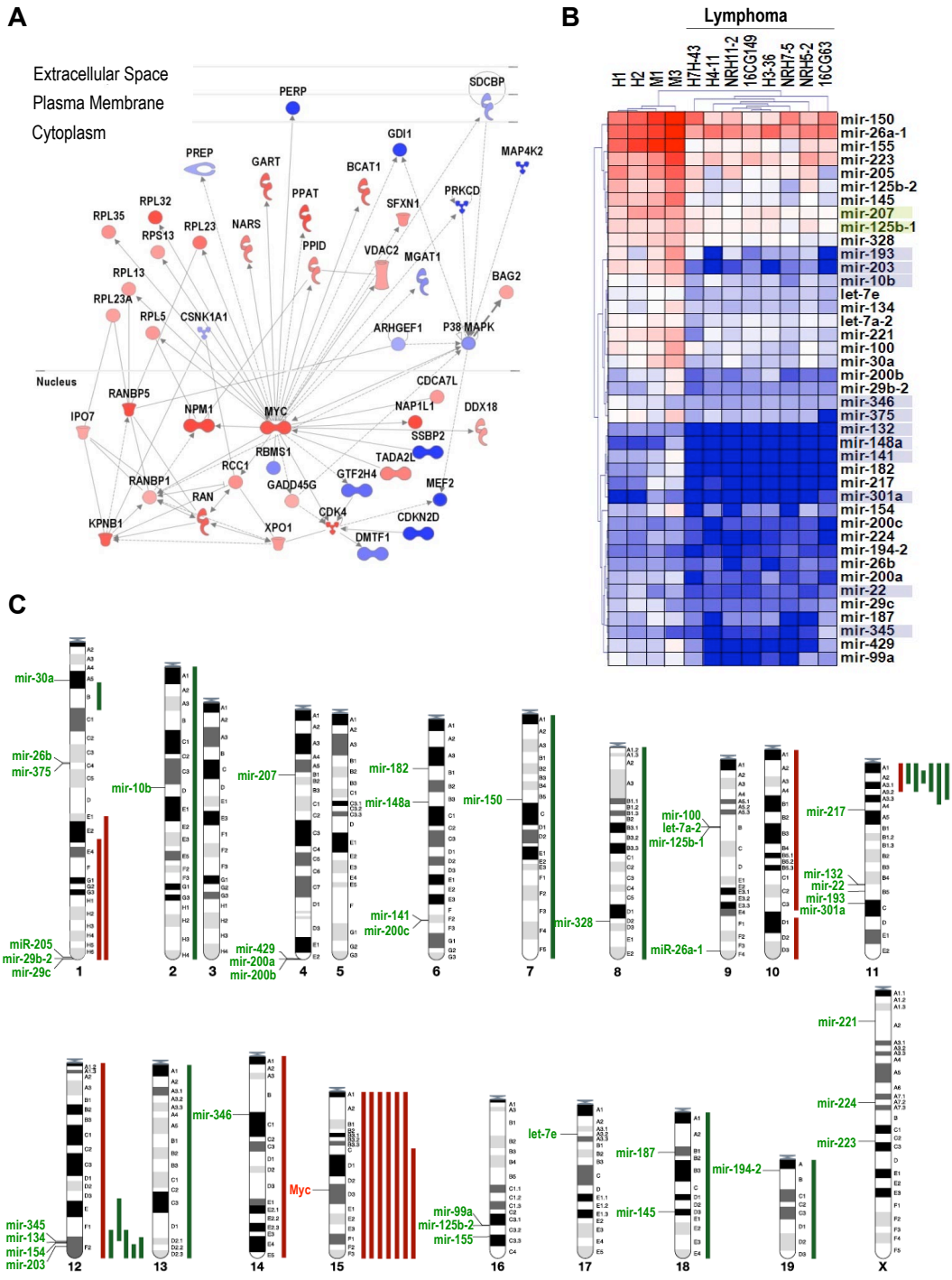

Figure 1. General overview of the transcriptional and genetic alterations in γ-irradiation-induced lymphomas. (A) Myc is a central node in the molecular interactions between up-regulated (red) or down-regulated (blue) proteins, including cell cycle regulators (Cdk or Ran pathways), protein synthesis molecules (Rpl and Rps proteins), and other signaling cascades. (See also supplemental Data for further details.) (B) Transcriptional profiling of miRNAs in normal thymuses (H1, H2, M1, and M3) or T-cell lymphomas. Unsupervised clustering of these data clearly discriminates normal thymuses vs tumor samples. Only significantly deregulated miRNA genes are shown. Blue shadows in miRNA names indicate the presence of a CpG island upstream of the corresponding human or mouse miRNA genes. Green shadows indicate that the CpG island is only present in the mouse sequence. (C) Summary from the comparative genome hybridization analysis of γ-irradiation-induced T-cell lymphomas showing the chromosomal position of down-regulated miRNA genes. Major DNA losses (green bars) and gains (red bars) are indicated to the right of the corresponding chromosomes. The location of *Myc* in chromosome 15 is also indicated.

We then analyzed the miRNA transcriptional profile in these γ-irradiation-induced lymphomas. Forty-one miRNA genes displayed a significant reduction in their expression levels in these tumors (FDR < 0.01; Figure 1B). No miRNA was found overexpressed in these tumors under similar statistical significance. The expression of several of these down-regulated miRNAs was validated using real-time quantitative reverse-transcribed polymerase chain reaction (supplemental Figure 1). Some of these miRNAs have been reported to have tumor suppressor activity. Thus, the let-7 family down-regulates Ras,[27] a major oncogene

in this malignancy, and miR-203 suppresses lymphomas and leukemias by inhibiting Abl1.[18] miR-223 negatively regulates proliferation of hematopoietic progenitors,[28] and it is repressed in hepatocellular carcinomas.[29] A translocation in specific leukemias is likely to inactivate miR-125b-1, suggesting its tumor suppressor function.[30]

Genetic and transcriptional alteration of miRNAs

We first analyzed DNA copy number (by comparative genome hybridization in 12 tumor samples) to test whether miRNA down-regulation could be the result of specific chromosomal aberrations. Trisomy of chromosome 15 or amplification of the Myc locus in this chromosome occurs in 7 samples (58% of γ-irradiated tumors; Figure 1C). Consistent DNA losses were observed in the centromeric region of chromosome 11 (6 samples; 50% of γ-irradiated tumors) and the telomeric end of chromosome 12 (5 tumors; 42%). Some of these chromosome alterations are in agreement with previous results.[31] Although some candidate genes have been suggested, the major target genes in these regions are mostly unknown. A combined analysis of comparative genome hybridization and cDNA expression patterns suggest that *Znfn1a1* (also known as *Ikaros*) may be a candidate gene in the chromosome 11 deletion because it is the only gene down-regulated in this region (data not shown). The target gene in the deleted telomeric region in chromosome 12 is less obvious as 3 different genes in this region, *Bcl11B*, *Siva*, and *Crip2*, are down-regulated at the mRNA level. However, none of these genes is silenced by promoter hypermethylation (data not shown), and the molecular mechanism behind the decreased expression of these genes is unknown at present. This region also contains a high density of miRNA genes, and we have recently reported that one of them, *mir-203*, is methylated in several mouse and human leukemias and behaves as a tumor suppressor miRNA in these malignancies.[18] Apart from the chromosome 12 miRNAs, all the other miRNAs down-regulated in these lymphomas are not preferentially located to DNA regions with frequent loss of heterozygosity (Figure 1C).

Although the transcriptional control of miRNAs is not well understood yet, a few transcription factors, such as E2F1,3, may modulate miRNA expression.[32,33] In addition, Myc has been shown to induce the oncogenic mir-17–92 cluster[34] and results in the repression of a significant number of miRNAs.[19] Indeed, some known Myc target miRNAs, such as miR-125b or miR-150, are down-regulated in K562 leukemic cells expressing a Myc-ER fusion gene inducible by tamoxifen (supplemental Figure 2), confirming that some of the miRNAs down-regulated in mouse and human lymphomas are Myc targets. Because Myc is highly overexpressed in these lymphomas, the observed down-regulation of several Myc targets, including mir-150, mir-22, mir-26a-1, and mir-26b, and the miRNA clusters mir-100–125b-1, mir-99a-125b-2, and mir-29b-2–29c, is likely to be a consequence of increased Myc signaling in these tumors. All together, these observations suggest that a combination of genetic or epigenetic alterations may mediate the loss of certain miRNAs, such as miR-203,[18] and many other miRNAs may be repressed by specific oncogenic transcription factors, such as Myc.

Correlation between miRNA down-regulation and target gene overexpression

Because each miRNA can target multiple genes in the genome, we reasoned that the down-regulation of these 41 miRNA genes in T-cell lymphomas might have important consequences in tumor transcriptional profiles. With a few exceptions (miR-200b, miR-203, miR-429, and miR-205), the set of targets of each individual miRNA was not significantly enriched in overexpressed genes. However, the set of 41 miRNA genes silenced, when considered as a whole, displayed a more dramatic effect on the transcriptome of T-cell lymphomas because the targets of these miRNAs were significantly over-represented in up-regulated genes (supplemental Table 2). Whereas 40.6% of the genes in the array (4997 of 12 322) are putative targets of the 41 miRNAs down-regulated, these targets represent 46.3% (258 of 557) in the subset of overexpressed genes ($P = .0001$).

We next analyzed which genes were most likely to benefit from the combined down-regulation of several miRNA genes. All the putative genetic interactions (GIs; number of tumor-down-regulated miRNA genes that can potentially target specific sequences in the 3′-UTRs of the protein-coding gene indicated) between the 557 up-regulated genes and the 41 down-regulated miRNA genes were obtained from the miRBase Targets, Version 5 database. Several up-regulated genes were predicted to be targeted by more than one of these miRNAs. Interestingly, *Myc* was the gene with the highest number of target sites for different down-regulated miRNA genes (Figure 2). Despite its relatively small size, the 3′-UTR of the murine *Myc* transcript (453 nt; approximately half of the average 3′-UTR size in the genome) is potentially targeted by 13 different miRNA genes silenced in these T-cell lymphomas (32% of the down-regulated miRNA genes). In addition to *Myc*, additional overexpressed genes controlled by multiple miRNA genes included *Ncor2* (11 GI), *Fkbp3* (10 GI), *Cad, Grwd1*, and *Rrp12* (9 GI), as well as other genes with a reduced number (≤ 8 GI) of mRNA-miRNA gene interactions.

Individual and combinatorial effects of miRNAs on Myc expression

To directly evaluate the regulation of Myc by miRNAs, we tested the ability of several of the down-regulated miRNAs to repress Myc. The regulation of Myc by miR-145 has been reported in detail previously[35] and is not further analyzed here. Twelve different additional miRNAs putatively targeting Myc were used to analyze their effect on *Myc* sequences (Figure 3). In addition, we also used the mir-148b gene, which is not silenced in these tumors but expresses a miR-148b mature sequence highly similar to that of miR-148a but with one mismatch. As additional controls, we used 2 miRNA genes, mir-200c and mir-101, which are not predicted to target the murine *Myc* sequence and are down-regulated or unaltered, respectively, in the T-cell tumors analyzed.

We first studied the effect of these miRNAs using the murine *Myc* 3′-UTR cloned downstream of a luciferase reporter. As shown in Figure 3B, 5 miRNA genes, including mir-132, mir-125b-1, let-7e, let-7a, and mir-154, were highly efficient down-regulating luciferase expression in the luc-*Myc*-3′-UTR construct. Because let-7 miRNAs have been previously reported to target Myc,[36,37] we consider the miRNAs in this group (group 1) as highly efficient down-regulators of Myc. In addition, mir-301a, mir-148a, and mir-134 (group 2) displayed an intermediate but consistent effect, whereas mir-150 (Figure 3B), mir-26b, mir-207, and mir-223 (not shown) did not have any significant effect. Similarly, the control vectors expressing mir-148b, mir-200c, or mir-101 did not have any effect on the luciferase signal. To additionally test the effect of these miRNAs on Myc expression, the Myc protein levels were quantified by immunoblotting after transient expression of the individual miRNAs. As shown in

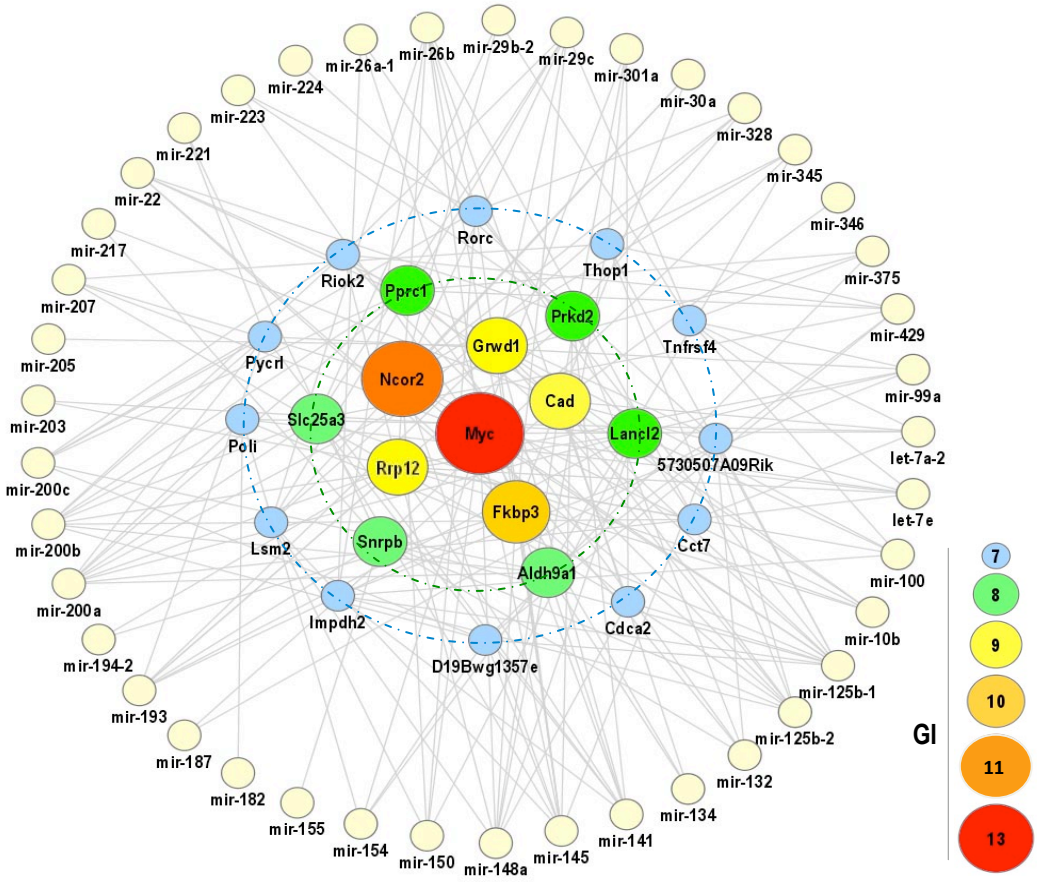

Figure 2. GIs between overexpressed genes and down-regulated miRNA genes. GIs are defined as the number of down-regulated miRNA genes that can potentially target specific sequences in all possible 3′-UTRs of the overexpressed gene indicated. (See supplemental Data for further details.) *Myc* is the overexpressed gene with the highest number of GIs in γ-irradiation-induced T-cell lymphomas. Thirteen of the 41 miRNA genes down-regulated in these tumors can potentially target the *Myc* 3′-UTR. Other genes potentially targeted by multiple miRNAs, such as *Ncor2* (11 GI) and *Kkbp3* (10 GI), are indicated in the figure. Only genes up-regulated in irradiation-induced lymphomas with more than or equal to 7 GI are represented.

Figure 3C, several of these miRNA genes, including mir-132, mir-125b-1, and mir-154, were able to significantly down-regulate Myc expression even stronger than the validated let-7 miRNAs. The reduction in Myc protein levels correlated with a significant decrease in the proliferation rate of Jurkat cells, suggesting a tumor suppressor activity for many of these miRNAs in leukemia cells (supplemental Figure 3). Some miRNAs displayed a dramatic antiproliferative effect despite its minimal effect of Myc protein levels, suggesting additional critical targets for its antiproliferative function. In general, the down-regulation of Myc by miRNAs (mostly group 1) correlated with the down-regulation of Myc target genes, such as Mcm4 or Cyclin B1 (Figure 3C), in agreement with a functional inactivation of Myc transcriptional targets in response to these miRNAs.

To further validate the direct control of Myc by group 1 miRNAs, we performed individual mutagenesis of miRNA target sites in the Myc 3′-UTR. The predicted miR-132, let-7, and miR-154 target sites were mutated by substituting 3 different nucleotides in each corresponding seed sequences. In addition, a double miR-125b mutant was obtained by mutating the 2 predicted target sites for this miRNA. As indicated in Figure 3D, expression of individual group 1 miRNAs reduced luciferase activity in wild-type *Myc* 3′-UTR but not on mutation of the corresponding target sites, suggesting direct down-regulation by miRNAs through these sequences.

We also went to analyze the combined effect of several of these miRNAs in controlling luciferase expression or Myc protein levels. Different pools of miRNAs belonging to group 1 (efficient targeting), group 2 (intermediate effect), and group 3 (miRNAs with no effect on Myc) were prepared. Cotransfection with the group 1 pool resulted in a slightly stronger reduction of luciferase activity (Figure 3E) and Myc protein levels (Figure 3F), although this effect may be limited by technical constraints of these assays (transfection efficiency or saturation of overexpressed miRNAs). Group 2 pools also displayed a stronger effect than individual group 2 miRNAs both in the luciferase assays and in the levels of Myc proteins. No effect was observed in the group 3 pools in agreement with the lack of effect of these individual miRNAs on Myc expression.

Combined down-regulation of MYC-targeting miRNAs in human BL

To validate the strong correlation between deregulation of Myc and miRNAs levels in humans, we studied cDNA and miRNA expression profiles in BL, a human pathology mediated by specific genetic translocations that lead to overexpression of *MYC*. In most BL cases, a reciprocal translocation has moved the proto-oncogene *MYC* from its normal position on chromosome 8 to a location

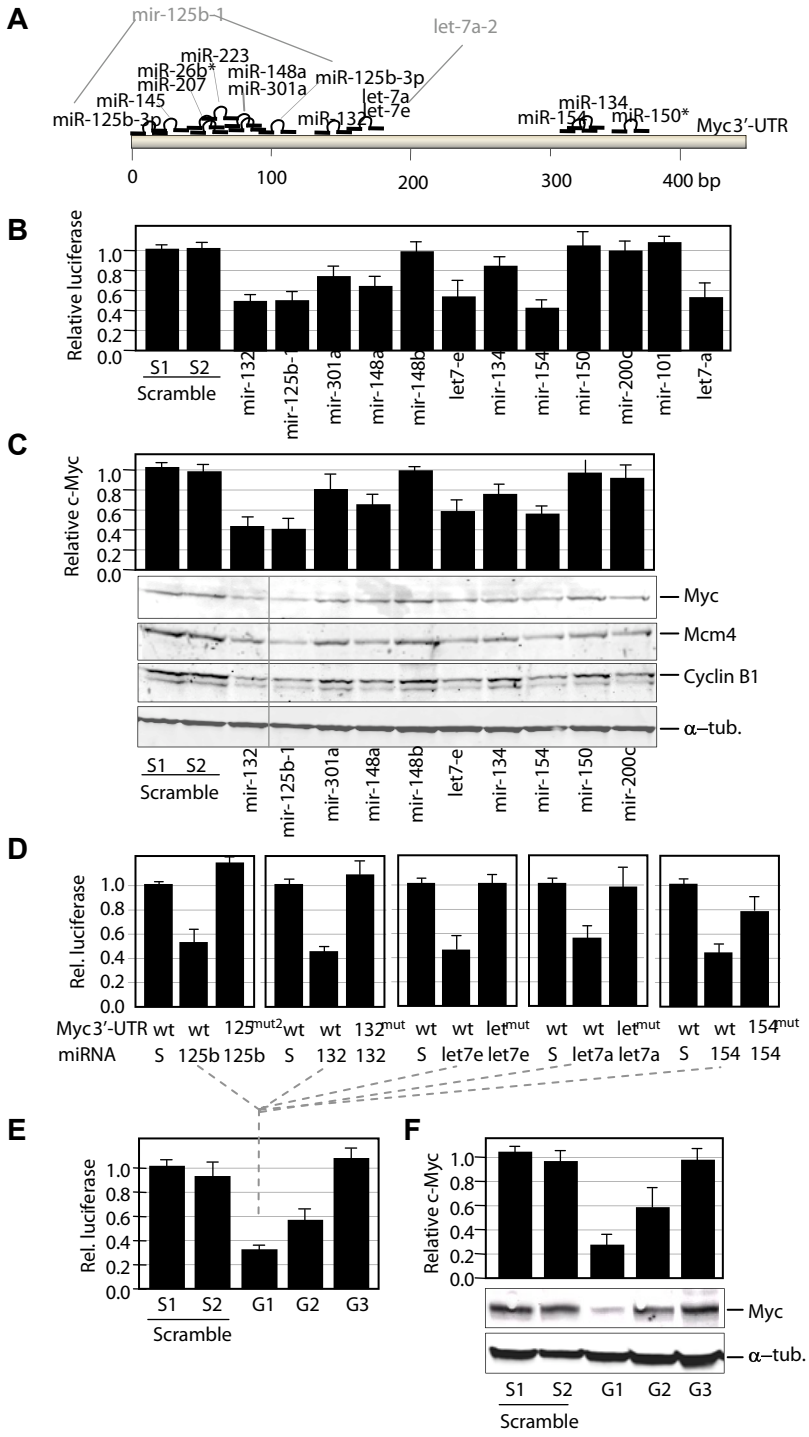

Figure 3. Control of Myc expression by miRNAs. (A) Potential target sites for mature miRNAs in the mouse *Myc* 3′-UTR. Only mature miRNAs produced by miRNA genes down-regulated in γ-irradiation-induced lymphomas are shown in this scheme. Gene nomenclature is also indicated when different from the mature form (eg, mir-125b-1 is the gene that generates miR-125–3p). (B) Luciferase activity of a reporter construct carrying the *Myc* 3′-UTR downstream of the luciferase gene. The construct was cotransfected with a vector expressing each of the indicated miRNA precursors. All data are normalized versus the luciferase levels generated by scramble sequences. (C) Effect of miRNA genes on Myc protein levels. Transfection with miRNA genes was performed as described earlier in the Figure 3 legend, but cells were processed for immunoblot analysis for Myc or 2 different Myc targets, Mcm4 and Cyclin B1. A vertical line has been inserted to indicate repositioned gel lanes. The relative levels of Myc proteins were normalized using α-tubulin (α-tub.) as a loading control. (D) Luciferase activity of wild-type (wt) or mutant (mut) *Myc* 3′-UTRs in the presence of scrambled sequences (S) or the corresponding miRNAs. wt, indicates wild-type *Myc* 3′-UTR sequence; mut, single mutants for the indicated miRNA; and mut2, double mutant for the miR-125b-3p target sites. (E) Luciferase assays of wild-type *Myc* 3′-UTRs in the presence of pools of group 1 (G1; mir-132, mir-125b-1, let-7e, let-7-a, and mir-154), group 2 (G2; mir-301a, mir-148a, and mir-134), or group 3 (G3; mir-26b, mir-150, mir-207, and mir-223) miRNAs. In these pools, the sum of all miRNA vectors also equals 10 μg as in the scramble vectors or the previous assays. (F) Effect of G1–3 pools on Myc protein levels. Transfection with miRNA genes was performed as described earlier in the Figure 3 legend, but cells were processed for immunoblot analysis for Myc. The relative levels of Myc proteins were normalized using α-tubulin (α-tub.) as a loading control.

downstream to the enhancers of the antibody heavy chain genes on chromosome 14. These translocated *MYC* oncogenes maintain the endogenous *MYC* 3′-UTR and are therefore sensitive to miRNAs. Because the expression of miRNAs has not been reported in detail in these tumors, we analyzed the expression profiles of protein-coding genes and miRNAs in the same 12 BL samples compared with normal lymph nodes (Figure 4; supplemental Figure 4) or normal $CD10^+CD19^+$ B cells (supplemental Figure 5). These BL samples displayed the t(8;14)(q24;q32) translocation (data not shown) that is likely to enhance the transcription of *MYC*. Gene expression profiling analysis confirmed a significant up-regulation of *MYC* (FDR < 0.01) in these BL samples (data not shown). In addition, 43 miRNAs were deregulated (FDR < 0.01) in these BLs compared with normal lymph nodes, including 26 up-regulated (including the known oncogenic cluster mir-17–92; supplemental Figure 4) and 17 down-regulated miRNAs (Figure 4A). We then applied GSEA using *MYC*-targeting miRNAs list (as predicted by miRBase) as a gene set. GSEA revealed a significant enrichment (FDR < 0.085) of the *MYC*-targeting miRNAs in control samples compared with BLs (Figure 4B), thus suggesting a BL-specific down-regulation of miRNAs that can potentially target the *MYC* mRNA.

We then mapped GI between up-regulated protein-coding genes (1219 genes) and down-regulated miRNAs (17 miRNAs) in the

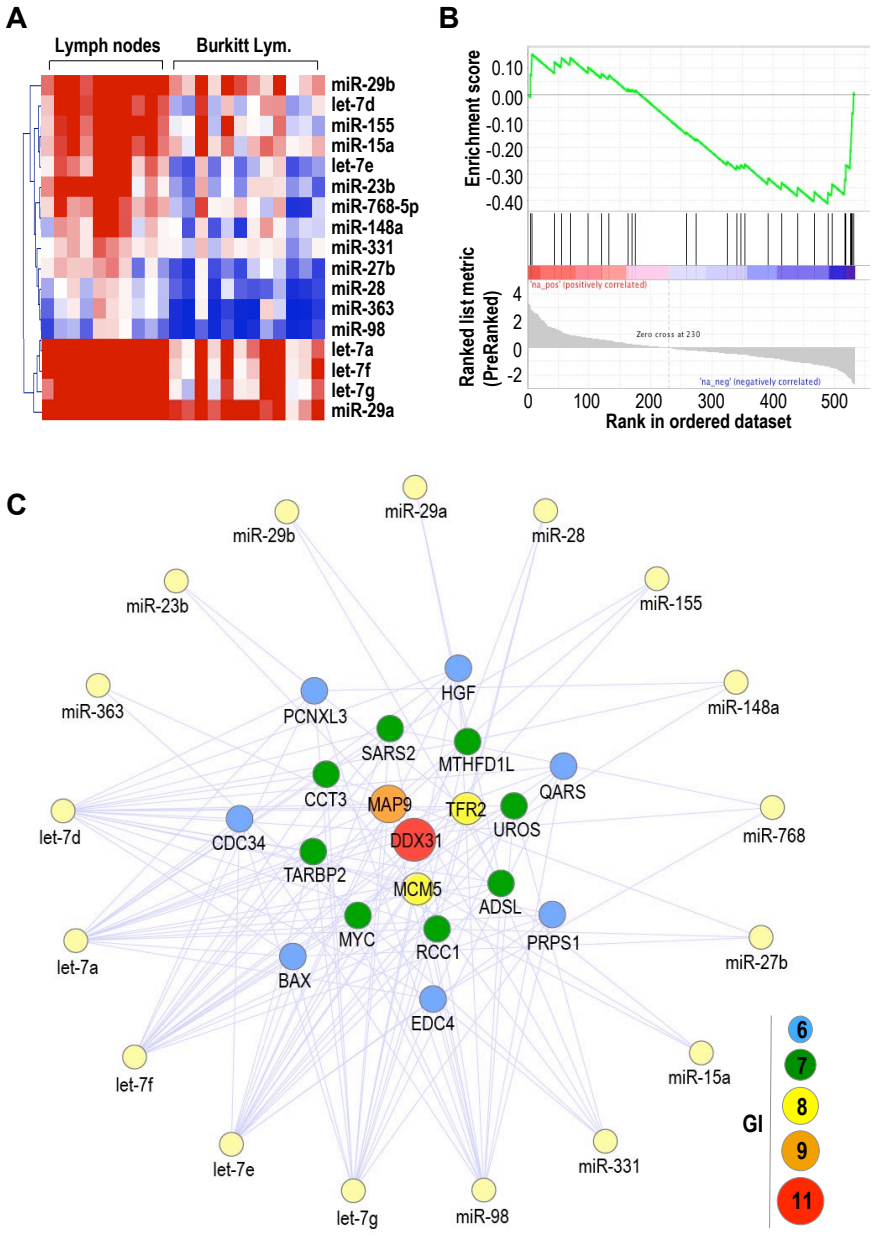

Figure 4. Genetic interactions between protein-coding genes and miRNAs in BL. (A) Heat map of significantly down-regulated miRNAs in BL compared with normal lymph nodes. (B) GSEA for down-regulated miRNAs targeting the *MYC* 3′-UTR. (C) Genetic interactions (GI) between up-regulated protein-coding genes and down-regulated miRNAs in BL. Only protein-coding genes with GI ≥ 6 are shown.

same 12 BL samples. As shown in Figure 4C, *MYC* was one of the most common targets (7 GI) of down-regulated miRNAs in these tumors, along other overexpressed genes, such as *DDX31* (11 GI), *MAP9* (9 GI), and *TFR2* (8 GI), or the replication protein *MCM5* (8 GI). Other genes with multiple target sites for silenced miRNAs included *CDC34*, an ubiquitin-conjugating enzyme known to be modulated by let-7.[38] We also compared the expression of miRNAs of BLs versus normal B cells expressing CD19 and CD10, 2 markers of the normal germinal centers that are also expressed in BL.[39,40] A total of 33 miRNAs were deregulated in BLs using a comparison similar to $CD19^{+}CD10^{+}$ cells, including 11 up-regulated and 22 down-regulated miRNAs (supplemental Figure 5). Common targets of these down-regulated miRNAs also included DDX31 and MAP9 (8 GI), MCM5 (6 GI), or MYC (5 GI), among other overexpressed genes (supplemental Figure 5). These findings indicate that preferential down-regulation of *MYC*-targeting miRNAs probably supports the high expression levels of *MYC* transcripts as a consequence of genetic translocation in human BL.

Because the human *MYC* 3′-UTR only displays a moderate conservation with the mouse sequence, we then validated the control of human *MYC* by miRNAs down-regulated in BLs. Figure 5A shows the localization of target sites for BL-specific down-regulated miRNAs in the human *MYC* 3′-UTR. As shown in Figure 5B, all these miRNAs, with the only exception of miR-155, were able to down-regulate the *MYC* 3′-UTR in reporter assays. In all these 6 cases, a specific mutation in the miRNA target site rescued this repression, suggesting the specificity in this interaction (Figure 5C). Moreover, all these 6 miRNAs were able to down-regulate *MYC* transcripts when overexpressed in the human BL cell line Raji. This down-regulation is even stronger when a pool of these 6 miRNAs

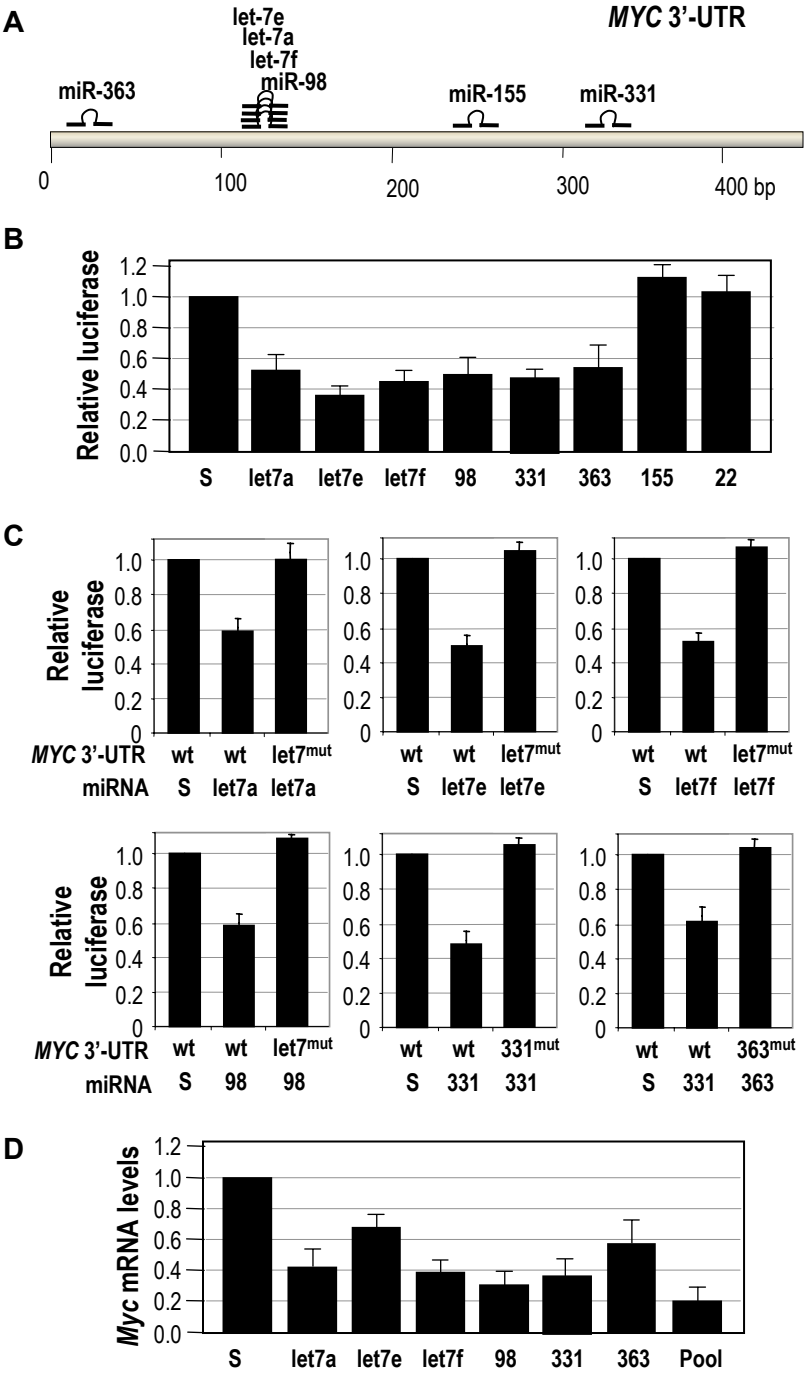

Figure 5. Control of human MYC by multiple miRNAs. (A) Schematic representation of the human *MYC* 3′-UTR and the localization of target sites for the miRNAs down-regulated in BLs. (B) Luciferase reporter assays to test the effect of the indicated miRNAs in the *MYC* 3′-UTR. Vectors expressing scrambled sequences (S) or mir-22 (not predicted to target MYC) were used as controls. (C) Mutagenesis of miRNA target sites in the human *MYC* 3′-UTR. Luciferase activity in the presence of the wild-type (wt) or a mutated (mut) *MYC* 3′-UTR in which 3 positions of the seed sequence have been mutated for each specific miRNA target site indicated. These constructs were assayed in the presence of vectors expressing the indicated miRNAs or scrambled sequences. (D) Effect of the indicated miRNAs in the protein levels of MYC in Raji BL cells. The pool contains an equimolar mixture of all indicated miRNAs in which the sum of all these miRNAs equals 10 μg as in the scramble vectors or the previous assays.

(using a total amount of miRNA DNA similar to the individual transfections) was used (Figure 5D).

The Myc pathway is a significant target of miRNAs down-regulated in lymphomas

The combined effect of down-regulated miRNAs on Myc activity can contribute to the induction of Myc targets and the corresponding downstream pathways in murine and human lymphomas. In both murine and human tumors, there is a significant enrichment of Myc targets among up-regulated genes. As reported in the first section of the "Results," Myc targets were overrepresented among up-regulated genes in γ-irradiation-induced tumors. Similarly, 135 Myc targets were included among the 1219 up-regulated genes (11.1%) in BLs versus 949 Myc targets in the 16 203 genes (5.8%) represented in the array ($P < .0001$). In addition, the set of up-regulated Myc targets was significantly enriched in target sites for down-regulated miRNAs. In murine tumors, 75 of 98 up-regulated Myc target genes (76.5%) can be potentially down-regulated by the silenced miRNA genes (Figure 6A), whereas the corresponding frequency for up-regulated non-Myc targets is 43.9% (207 of 481; $P < .0001$). Similarly, 63.7% (86 of 135) of up-regulated Myc targets were predicted targets of silenced

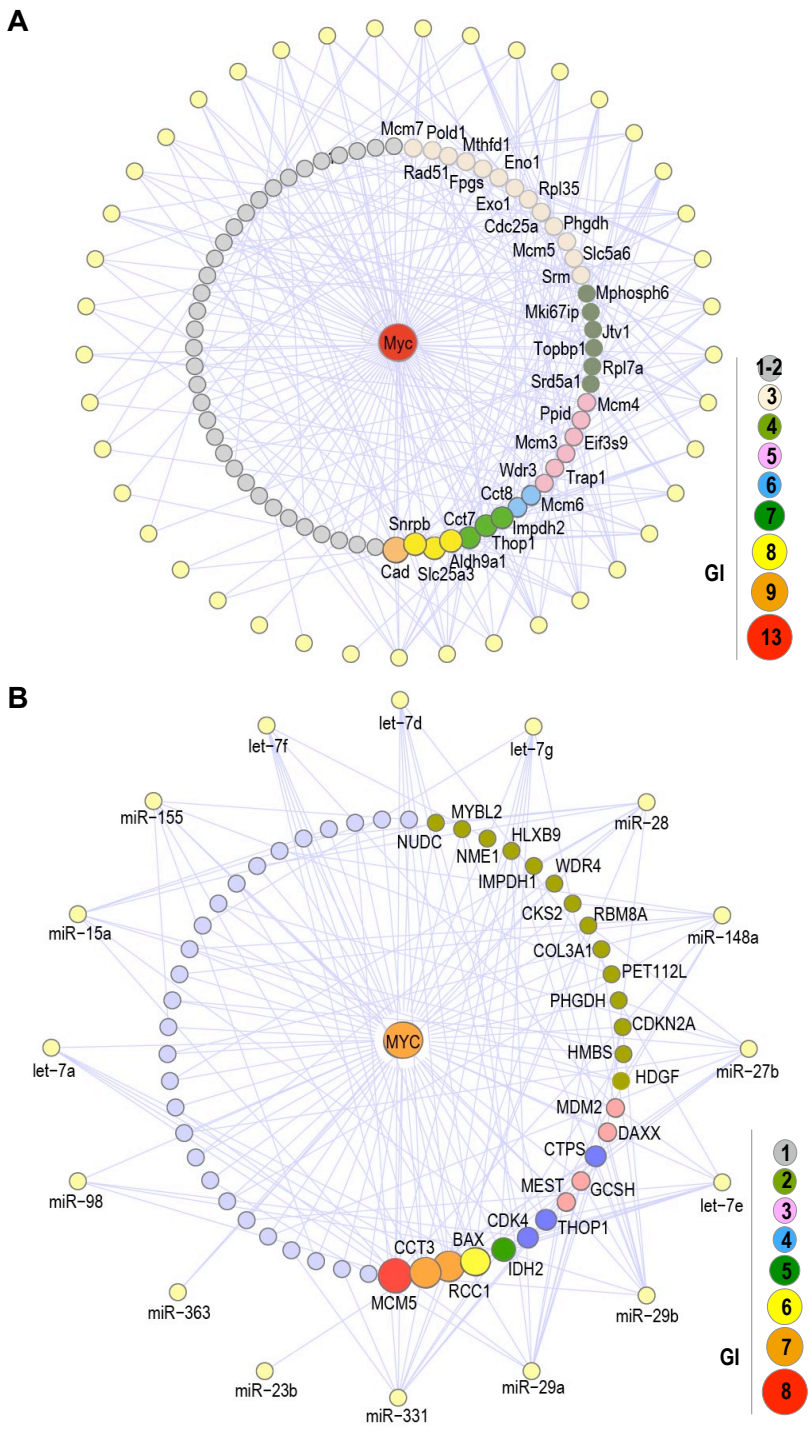

Figure 6. Significant effect of mouse or human down-regulated miRNAs on Myc targets. (A) GIs between Myc target genes and down-regulated miRNA genes in mouse irradiation-induced lymphomas. Peripheral yellow nodes represent down-regulated miRNA genes (as in Figure 2), whereas Myc targets are distributed as a circle around Myc (the interaction between Myc and Myc targets is not shown for clarity). A total of 72 Myc targets are potentially targeted by down-regulated miRNA genes, and only genes with more than or equal to 3 GI are shown. A complete list of these interactions is provided in the Supplemental data. (B) Similar analysis of the GI between MYC target genes and down-regulated miRNAs in BLs. Peripheral yellow nodes represent down-regulated miRNA genes (as in Figure 7), whereas MYC targets are distributed as a circle around MYC.

miRNAs in BLs versus 39.9% (433 of 1084) of non-Myc targets ($P < .0001$; Figure 6B). As an example, mouse *Cad*, *Snrp*, *Slc25a3*, *Aldh9a1,* as well as human *MCM5, CCT3, BAX,* or *RCC1* were among the Myc target genes with a highest number of predicted target sites for down-regulated miRNAs (supplemental Table 3). Thus, the combined effect of Myc overexpression and amplification (Figure 1), down-regulation of miRNAs that control Myc levels in mouse (Figures 2-3) and humans (Figures 4-5; supplemental Figure 5), and down-regulation of miRNAs that control Myc target levels (Figure 6) is likely to have a significant effect in the Myc oncogenic pathways in murine γ-irradiation-induced or human BLs.

Discussion

In mammals, miRNAs are predicted to control the activity of a significant fraction of all protein-coding genes.[41,42] The sets of 41 miRNAs down-regulated in γ-irradiation-induced lymphomas (Figure 1) and 17 miRNAs down-regulated in BLs (Figure 4) are predicted to target approximately 46% (256 of 556) or 43% (519 of 1219), respectively, of the up-regulated genes in these lymphomas, suggesting that miRNA deregulation may have a significant impact in tumor transcription profiles. The analysis of the major targets

of these miRNAs indicates that some genes may be repeatedly targeted by several different miRNAs. Interestingly, *Myc*, a major oncogene in these tumors,[8,17] stands up as the gene in which lack of expression of miRNAs may have the highest influence in γ-irradiation-induced tumors and one of the major targets in BLs. Thus, the silencing of approximately one-third of the miRNA genes down-regulated in these murine or human tumors may help to accumulate Myc proteins. Whereas the sets of miRNA targets may vary using different algorithms, the enrichment of target sequences for down-regulated miRNAs is commonly found in different algorithms, and several of these target sequences have been validated through reporter and mutagenesis assays. Several of these miRNAs, including miR-132, miR-125b-1, let-7 family, and miR-154 are able to decrease Myc levels and the proliferative potential of tumor cells.

Because tumor samples were compared with normal age-matched thymuses or lymph nodes, these results may also indicate that these miRNAs help to maintain low levels of Myc proteins in adult quiescent differentiated cells compared with other proliferative stages. In any case, given the major role of the Myc transcription factor in driving leukemogenesis,[43] it is not surprising that tumors select cells with inactivation of several of the miRNAs that may decrease Myc levels. In addition, proliferating cells frequently express mRNAs with shortened 3′-UTRs and fewer miRNA sites, suggesting the relevance of avoiding miRNA function in cell proliferation.[44] We have failed to detect point mutations in the *MYC* 3′-UTR of human lymphoma patients (N = 38; data not shown). However, given the number of miRNA target sites in that sequence, it is possible that a single point mutation does not confer enough resistance to the combined effect of all miRNAs targeting *MYC* in these cells. Thus, tumor cells preferentially down-regulate inhibitory miRNAs instead of selecting individual or multiple mutations in the *MYC* 3′-UTR.

Myc up-regulates many molecular routes crucial for malignant transformation, including cell survival, proliferation, and translation pathways.[24] Myc is also known to specifically induce the oncogenic mir-17–92 cluster,[34] and several miRNAs expressed in this cluster are up-regulated in BL (supplemental Figure 4). However, the predominant consequence of Myc activation in miRNA biology is thought to be a widespread miRNA repression as recently reported.[19,45] In γ-irradiation-induced or BLs, several silenced miRNAs, including miR-15a, miR-22, miR-23b, miR-125b, miR-150*, miR-26a, or miR-26b, miR-29a, and miR-29b, and several let-7 family members may be repressed as a consequence of Myc signaling.[19,45] Interestingly, some of the silenced miRNAs also have the potential to target the *Myc* transcript, suggesting regulatory loops between Myc and miRNAs (supplemental Figure 6). It has been also previously reported that let-7a down-regulates *Myc* and reverts Myc-induced growth in BL cells.[36] Thus, the inactivation of several of these miRNAs enhances Myc overexpression, which in some cases is originated by trisomy of chromosome 15 (mouse lymphomas) or specific translocations (human neoplasias). Tumor-suppressor miRNAs that are not the target of chromosomal deletions or epigenetic modifications may be directly repressed by Myc in a feedback loop that enhances Myc protein levels and favors malignant transformation (Figure 7). Because a threshold level of c-Myc expression is required to maintain the neoplastic state in Myc-driven tumors,[46] these regulatory loops suggest a bidirectional mechanism to regulate the balance between the Myc pathway and miRNA regulation in normal and tumor cells.

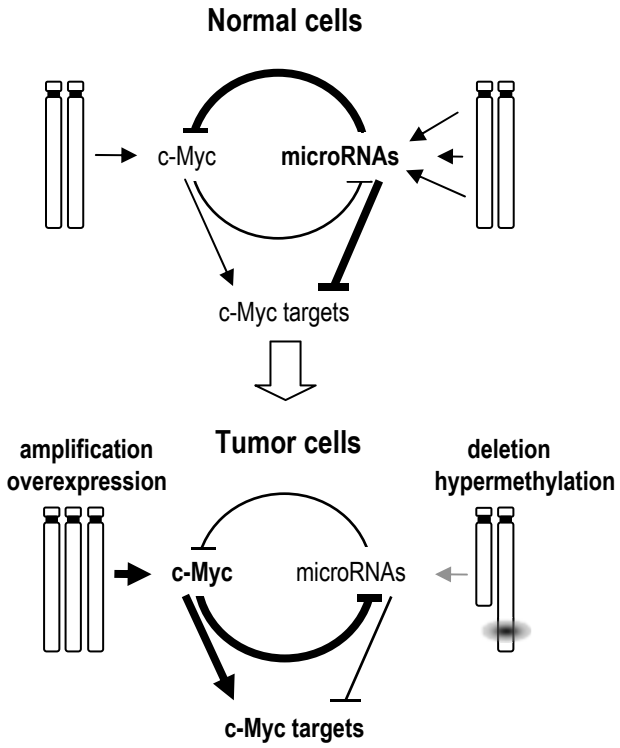

Figure 7. A model of the GIs between Myc, its targets, and miRNAs in normal and tumor cells. In normal cells, multiple miRNAs are expressed that can repress Myc or Myc targets. miRNA genes are not repressed by Myc because of the low levels of Myc signaling in normal cells. Tumor cells acquire diverse genetic or epigenetic alterations that result in the overexpresssion of Myc and Myc targets to facilitate tumor development through diverse cellular processes. On one hand, Myc can be amplified and overexpressed. On the other hand, miRNAs are silenced by genetic (loss of heterozygosity), epigenetic (hypermethylation), or regulatory (repression by Myc) mechanisms. Because of the low levels of these miRNAs, signaling by Myc and Myc targets is enhanced resulting in dramatic deregulation of the cell cycle, protein translation, and metabolism among other cellular processes. This model is mostly based on the results obtained by Chang et al[19] on the repression of miRNAs by Myc and the control of Myc by miRNAs reported here.

Despite the relevant oncogenic function of Myc in several malignancies and the therapeutic value observed in genetic models,[47] its complex function as a transcription factor has made it difficult to design therapeutic approaches to inhibit its activity in human tumors.[48,49] The control of Myc by miRNAs may therefore add some new possibilities for the therapeutic inhibition of Myc in tumors either by reexpressing endogenous miRNAs (eg, through epigenetic drugs that restore the expression of hypermethylated miRNAs) or by introducing exogenous small RNAs that target the Myc transcript.

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Authorship

Contribution: M.J.B. performed most research assays; M.G.d.C., R.S.-M., I.P.d.C., and M.G. contributed with specific assays with mouse samples; G.G.-L., D.G.P., and M.M. performed the statistical analysis; L.D.L., S.M., N.M., and M.A.P. contributed with specific analysis of human samples; J.S. contributed vital new reagents; J.F.-P. and M.M. designed and supervised this research; and M.M. wrote the paper.

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Correspondence: Marcos Malumbres, Centro Nacional de Investigaciones Oncológicas, Melchor Fernández Almagro 3, E-28029 Madrid, Spain; e-mail: malumbres@cnio.es.

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Tumorigenesis and Neoplastic Progression

Deregulated Expression of the Polycomb-Group Protein SUZ12 Target Genes Characterizes Mantle Cell Lymphoma

Daniel Martín-Pérez,* Esther Sánchez,† Lorena Maestre,‡ Javier Suela,§ Pierfrancesco Vargiu,* Lorena Di Lisio,* Nerea Martínez,* Javier Alves,¶ Miguel A. Piris,* and Margarita Sánchez-Beato*

From the Lymphoma Group, Molecular Pathology Programme, the Monoclonal Antibodies Unit,‡ Biotechnology Programme, and the Cytogenetics Unit,§ Human Genetics Programme, Spanish National Cancer Research Centre (CNIO), Madrid; the Department of Pathology,† Hospital Nuestra Señora del Prado, Talavera de la Reina, Toledo; and the Pathology Department,¶ Hospital La Paz, Madrid, Spain*

Polycomb proteins are known to be of great importance in human cancer pathogenesis. SUZ12 is a component of the Polycomb PRC2 complex that, along with EZH2, is involved in embryonic stem cell differentiation. EZH2 plays an essential role in many cancer types, but an equivalent involvement of SUZ12 has not been as thoroughly demonstrated. Here we show that SUZ12 is anomalously expressed in human primary tumors, especially in mantle cell lymphoma (MCL), pulmonary carcinomas and melanoma, and is associated with gene locus amplification in some cases. Using MCL as a model, functional and genomic studies demonstrate that SUZ12 loss compromises cell viability, increases apoptosis, and targets genes involved in central oncogenic pathways associated with MCL pathogenesis. Our results support the hypothesis that the abnormal expression of SUZ12 accounts for some of the unexplained features of MCL, such as abnormal DNA repair and increased resistance to apoptosis. ***(Am J Pathol 2010, 177:930–942; DOI: 10.2353/ajpath.2010.090769)***

The Polycomb group of proteins (PcG) are transcriptional repressors essential for regulation of embryogenesis, tissue development, stem cell self-renewal, and preservation of cell identity (reviewed in[1]). PcG proteins modify histone tails to repress gene expression. Two major PcG complexes have been described in humans: the polycomb repressive complex 1 (PRC1), which contains BMI1, MEL18, RING1, RNF2, HPC1, and others, and the polycomb repressive complex 2 (PRC2), which typically contains EZH2, SUZ12 and various isoforms of EED.[2] PRC2 has histone methyltransferase (HMTase) activity that allows the complex to trimethylate chromatin specifically at lysine 27 of histone H3. PRC1 recognizes this mark and recruits the machinery necessary to remodel chromatin structure.[3–6]

There is mounting evidence of the pathogenic role of PcG in human cancer.[7–10] This is the case for murine Bmi1, which collaborates with c-Myc in transforming lymphoid cells.[11,12] Human BMI1 has been found to be deregulated in mantle cell lymphoma (MCL) and in Hodgkin's and diffuse large B-cell lymphomas.[10,13–16] EZH2 is involved in progression in prostate cancer and in neoplastic transformation of breast epithelial cells.[17,18] This member of the PRC2 complex has HMTase activity and is therefore essential for gene transcription regulation. SUZ12, another important member of this complex, in conjunction with EED and RBAP48, is up-regulated in colon and breast tumors,[19] but its specific function in human cancer is unknown. SUZ12 is a zinc finger protein that has been found at the breakpoints of a recurrent chromosomal translocation in endometrial stromal sarcoma.[20] SUZ12 is essential in mouse development and is required for the proliferation of cultured cells.[21] Within the PRC2 complex, SUZ12 is required for the HMTase activity of the complex.[21,22]

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Address reprint requests to Dr. Margarita Sanchez-Beato, Ph.D., Lymphoma Group, Molecular Pathology Programme, Centro Nacional de Investigaciones Oncológicas (CNIO), C/ Melchor Fernández Almagro 3, E-28029 Madrid, Spain. E-mail: msbeato@cnio.es.

MCL is a lymphoid malignancy with an aggressive clinical behavior, whose study has critically improved our understanding of the pathogenic role of multiple oncogenes and survival pathways.[23–25] It accounts for around 5% to 8% of non-Hodgkin's lymphomas, and is associated with a chromosomal translocation t(11;14)(q13;q32) that puts the *CCND1* gene under the control of the immunoglobulin heavy chain locus regulatory elements.[23] However, this characteristic molecular event does not explain fully the clinical and biological features of the tumor and is not sufficient for tumoral transformation, as has been demonstrated in experimental models.[26] Several studies suggest that other molecular events play a pathogenic role in MCL pathogenesis, such as *ATM* loss or nuclear factor κB pathway activation.[24,27] Nevertheless, there are still various MCL oncogenic features that are not explained by the alterations so far identified.

In this study we have investigated the expression pattern of SUZ12 and EZH2 in a large cohort of human normal tissues and tumors in search of patterns associated with transformation events. We demonstrate that SUZ12 is anomalously expressed in several human primary tumors, and that it is especially relevant in specific tumors such as MCL, melanoma and pulmonary carcinomas, where it is associated with gene amplification in some cases. The use of an integrated approach combining genome-wide location assays, functional studies, and gene expression profiling, leads us to conclude that SUZ12 may be involved in MCL pathogenesis.

Materials and Methods

Production of SUZ12 Monoclonal Antibody

A cDNA encoding the full-length human SUZ12 protein was obtained from the laboratory of Dr Yi Zhang (pGEX-KG-SUZ12). The human SUZ12 gene was amplified by polymerase chain reaction (PCR) and introduced into the pDEST-TH1 expression vector (Invitrogen, Carlsbad, CA) by means of Gateway technology. The MBP-SUZ12 fusion protein was then expressed in *Escherichia coli* strain BL21 (DE3) with 0.4 mmol/L IPTG overnight at 30°C. The bacteria were lysed with BugBuster reagent (Novagen, Madison, WI). The soluble fraction was purified with amylase resin (New England Biolabs, Ipswich, MA), and the joined protein was eluted with 10 mmol/L maltose. The protein-containing fractions were concentrated by Vivaspin ultrafiltration (Sartorius Stedim Biotech, Aubagne, France) and used as an immunogen.

Three BALB/c mice were injected intraperitoneally (three times at 14-day intervals) with 100 μg 6 × MBP-SUZ12 fusion protein and Freund's adjuvant. A 150-μg booster of the recombinant SUZ12 protein was injected intraperitoneally, and fused three days later, as described previously.[28,29] Hybridoma supernatants were screened by enzyme-linked immunosorbent assay. The mouse mAb raised against SUZ12 (220A/A3) was cloned by the limiting dilution technique. Animal experiments were performed under the experimental protocol approved by the Institutional Committee for Care and Use of Animals, CEUCA no. 001/02.

To confirm that 220A/A3 mAb recognized the human SUZ12 protein, immunohistochemistry on frozen cytospin preparations of V5-tagged human SUZ12 expressed in HEK-293T cells was performed. Labeling with the anti-V5 mAb confirmed the efficiency of transfection. A cytospin preparation of V5-tagged human SOX4 protein was used as a negative control (Supplemental Figure 1 at *http://ajp.amjpathol.org*).

Tissue Microarrays and Immunostaining Techniques

Immunohistochemical expression of SUZ12 and EZH2 were assessed using tissue microarray (TMA) technology for 150 normal and 569 tumoral samples. To this end, we used a tissue array device (Beecher Instruments, Sun Prairie, WI), as previously described.[13,30] An additional TMA including 76 MCL cases was also used.[31] Immunohistochemical staining was performed on these TMA sections using the following antibodies: McAb SUZ12 (220A/A3) and EZH2 polyclonal antibody (Zymed, San Francisco, CA).[32,33] Proliferation indices in MCL cases were evaluated by means of Ki-67 expression using MIB1 monoclonal antibody by DAKO (DAKO, Glostrup, Denmark). M.S.-B. and E.S. evaluated the staining of TMA sections for SUZ12, EZH2, and Ki-67 proteins using uniform criteria. Discrepancies in the scoring of cases were resolved after joint examination on a multiheaded microscope. To ensure the reproducibility of this method, we used straightforward, clear-cut criteria, and cases were scored as positive (1) or negative (0) for SUZ12 and EZH2 antibodies. The threshold was 5% of positive cells for both antibodies. For Ki-67, the values were scored as negative (0) for fewer than 5% of proliferating cells, positive (1) for 5% to 25%, and strongly positive (2) if more than 25% of cells were positive for Ki-67.[13] Whole-tissue sections from reactive lymph node, thymus, spleen, and tonsillectomy specimens were used for the examination of benign lymphocyte subpopulations.

FISH

Fluorescence *in situ* hybridization (FISH) was used to detect *SUZ12* copy number changes, as previously reported.[34] To study *SUZ12* amplification we used the bacterial artificial chromosome clones RP11-290N17 and RP11-640N20 from the BACPAC resources center (Children's Hospital Oakland Research Institute, Oakland, CA), which spans the entire 17q11.2 genomic region, and a commercial centromeric probe for chromosome 17 (Vysis Inc., Downers Grove, IL), which was used as a control for the ploidy level of chromosome 17.

FISH evaluation was performed by J.S. with no previous knowledge of other genetic, clinical, or immunohistochemical results. Fluorescence signals were scored in each sample by counting the number of single-copy gene and centromeric signals in an average of 130 (60–210) well-defined nuclei. *SUZ12* amplification was recog-

nized if the *SUZ12*/chromosome 17 ratio was greater than 2 in at least 20% of tumor cells. A sample was considered to feature a *SUZ12* gene gain if the *SUZ12*/chromosome 17 ratio was greater than 1.5 but less than 2.[35,36]

Cell Lines

Human cell lines derived from MCL patients Jeko-1 and Z138 were kindly provided by Dr. Martínez-Climent and cultured in RPMI 1640 medium supplemented with 1% L-glutamine, 10% fetal bovine serum (Invitrogen), 0.5% penicillin/streptomycin, and 0.1% Fungizone. Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

The HEK293T cell line was obtained from the American Type Culture Collection and was cultured in Dulbecco's modified Eagle's medium supplemented with 1% L-glutamine, 10% fetal bovine serum (Invitrogen), 0.5% penicillin/streptomycin and 0.1% Fungizone. Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Virus Production and Cell Line Infection

Viruses were produced by transient transfection in the HEK293T cell line. Plasmids were produced in the TOP10 *E. coli* strain (Invitrogen) and grown in low-salt Luria Bertani medium. Plasmids were isolated using Qiagen EndoFree Plasmid Maxi Kit (Qiagen, Venlo, The Netherlands).

For co-transfection, plasmids pCMVdeltaR8.91 (derived from pCMVR8.9[37]), pMD.G and the lentiviral vector pA179.Helix[38] containing the shRNAi sequences were used. Plasmids were co-transfected using FuGene 6 (Roche, Basel, Switzerland) following the manufacturer's recommendations. Target cells (5×10^5) were transduced by spinoculation using the viral supernatant.

Vectors and shRNA Design

Different shRNAs were designed using the SIDE program (*http://side.bioinfo.cipf.es*, last accessed December 4, 2008). The shRNAs were designed within the ORF sequence of *SUZ12* to avoid off-target effects associated with imperfect matching in the 3′ UTR of the target gene. shRNAs were cloned as previously described.[39] shRNA sequences used for control and SUZ12 knockdown were as follows: Scramble: (5′-GAGGAACCAAACCATAACA-3′); shSUZ12.783: (5′-GGATGTAAGTTGTCCAATA-3′); shSUZ12.2076: (5′-GCTGACAATCAAATGAATCAT-3′).

Cell Competition Assays

GFP expression was analyzed by FACS 72 hours after infection of the cell lines with lentivirus carrying either the empty vector, or a scrambled control or either of the two shRNAs against SUZ12. This was done every 2 to 4 days. The evolution of GFP expression was compared with the third day using the following formula:

$$\%\ \text{initial ratio} = \frac{\text{Ratio GFP}^+/\text{GFP}^-_{\text{day n}}}{\text{Ratio GFP}^+/\text{GFP}^-_{\text{day 3}}} \times 100$$

Cell Growth and Apoptosis

Cell growth was assessed by counting cells in a Neubauer chamber and using trypan blue dye to exclude dead cells. For cell cycle analysis, 10^6 cells were washed with PBS and fixed with chilled 70% ethanol added drop by drop and incubated in the cold for at least 1 hour. The cells were then washed again with PBS and resuspended in 500 μl of PBS. RNase A was added at a final concentration of 200 ng/μl and incubated for 30 minutes. Cells were stained with 10 μl propidium iodide (1 mg/ml) before acquisition in the cytometer.

Cell death was quantitated by annexin V–APC (BD PharMingen, Franklin Lakes, NJ) staining according to the manufacturer's protocol. Briefly, cells were washed in PBS, resuspended in 500 μl of binding buffer (BD PharMingen) containing 0.5 μg/ml annexin V-APC and 25 μg/ml propidium iodide, and then analyzed by flow cytometry. Cell cycle and apoptosis assays were analyzed with a FACSCalibur flow cytometer (BD PharMingen).

Histone Extraction

Histones were isolated by acidic extraction in 0.25 mol/L HCl and precipitation with acetone. Briefly, 10^6 cells were harvested and incubated overnight in 200 μl of 0.25 mol/L HCl at 4°C with shaking. For histone precipitation, 8 volumes of acetone were added to the supernatant, and histones were pelleted by centrifugation and washed with acetone. Histones were air-dried and resuspended in 0.25 mol/L HCl for subsequent immunoblot analysis.

Western Blot Analysis

Total protein extracts were prepared using radioimmunoprecipitation assay lysis buffer supplemented with protease inhibitors. Antibody detection was performed using fluorescent-labeled secondary antibodies (Alexa 680 and Alexa 800, Rockland, Gilbertsville, PA) and an Odyssey infrared system scanner (LI-COR Biosciences, Lincoln, NE).

Antibodies for immunoblot analysis included mouse anti-SUZ12 mAb (clone 220A/A3), rabbit anti-histone H3 trimethylated at lysine 27 (07-499, Upstate Biotechnology, Lake Placid, NY), mouse anti-H3 mAb (clone 6.6.2, Upstate Biotechnology), mouse anti-PARP (P248, Sigma-Aldrich Inc., St. Louis, MO), and mouse anti-α-tubulin (clone DM1A, Sigma-Aldrich). Band intensities were quantified using ImageJ 1.34S software (National Institutes of Health, Bethesda, MD).

ChIP-on-Chip

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was assayed using the ChIP assay kit (Upstate Biotechnology, Billerica, MA) following the manufacturer's recommendations. The antibodies used here were specific for SUZ12 (220A/A3 and Upstate, 07-379), histone H3 trimethylated at lysine 27, and IgG (Upstate Biotechnology).

For ChIP-on-chip experiments, three biological replicates were amplified using the GenomePlex whole genome amplification kit (Sigma-Aldrich) following the protocol provided by the manufacturer. The same quantity of each replicate was mixed in a single tube. Input material and samples were labeled with Cy3 and Cy5, respectively, and hybridized onto the human promoter ChIP-on-chip microarray set (Agilent Technologies Inc., Santa Clara, CA).

DNA Microarray Analysis ChIP-on-Chip

The human promoter ChIP-on-chip microarray set covers −5.5 kb upstream to +2.5 kb downstream of the transcriptional start sites with a total of 487,008 probes. Agilent's ChIP analytics program (v. 1.3.1) was used for the analyses.

A whole-chip error model was used to calculate confidence values from the enrichment ratio and the signal intensity of each probe (probe *P* value) and of each set of three neighboring probes (probe set *P* value). Probe sets with significant probe set *P* values ($P < 0.001$) and significant individual probe *P* values ($P < 0.01$) were judged to be bound. Bound regions were assigned to genes if they were within 1 kb of the transcription start site registered in at least one of five genomic databases.

PCR of Immunoprecipitated Material

Original sequences for primer design were extracted from the March 2006 human reference sequence (NCBI Build 36.1). Immunoprecipitated DNA was subjected to semiquantitative PCR using the following primers: *ATM*: sense strand 5′-GTTGTGCAAAGGGGTCAACT-3′, antisense strand 5′-TTGGCGGAACTGAAAGAAG-3′; *BCOR*: sense strand 5′-GCAAAAGACAGGCGAGCAAG-3′, antisense strand 5′-ACCCCCAGAAAGACCAGGAA-3′; *BIRC2*: sense strand 5′-CCCAGGTGCATTTTTGGAAG-3′, antisense strand 5′-TGCCTGCCAGTCAGTCACAG-3′; *CBX2*: sense strand 5′-TTCTCCCCGCTGTAACCTGA-3′, antisense strand 5′-GCCCGAGATCCAGAACAATG-3′; *E2F5*: sense strand 5′-TGGATTGCAGTGGCAGGA-3′, antisense strand 5′-GGCGTGGTAGTGCACACTTG-3′; *GADD45G*: sense strand 5′-GTGCCAGCGTGTATGGTCAA-3′, antisense strand 5′-CGAGTAAGGGCTGCAAAACG-3′; *H2AFZ*: sense strand 5′-AGGGCCTGGGAGTTTTCTTG-3′, antisense strand 5′-CTGTGTACAGCGCAGCCATC-3′; *HDAC2*: sense strand 5′-CTGGAGAAGGAGGCCGTTTC-3′, antisense strand 5′-GCAGACCTGAGGGGGAGAAC-3′; *JMJD2D*: sense strand 5′-AAATATGTACGGGGCAACCA-3′, antisense strand 5′-TGACATCTCCCCTCCCACTA-3′; *VAV3*: sense strand 5′-GCTCAGCGCACCTAGACGTT-3′, antisense strand 5′-GGCTCAGGTGTTCGACCTTG-3′.

Functional Gene Classification with Ingenuity Pathways Analysis

We identified functions/pathways classification terms enriched for SUZ12-bound genes using Ingenuity Pathways analysis (Redwood City, CA). The probability associated with a biological process is a measure of its statistical significance with respect to the functions/pathways/lists eligible molecules for the dataset and a reference set of molecules that defines the molecules that could possibly have been functions/pathways/lists eligible. The probability is that associated with a right-tailed Fisher's exact test.

Gene Expression Profile

For gene expression profiling, total RNA was extracted from cell lines and MCL frozen tumoral samples using the Qiagen RNeasy kit (Qiagen). 500 ng of RNA were labeled with cyanine 5-conjugated dUTP (Cy5) and hybridized onto the Agilent 44K whole genome microarray chip (Agilent Technologies) against a universal human reference RNA (Stratagene, La Jolla, CA) previously labeled with cyanine 3-conjugated dUTP (Cy3). Slides were scanned in an Agilent G2565AA microarray scanner system and data were extracted with feature extraction software (Agilent Technologies).[39]

Statistical Analysis

To validate ChIP-on-chip results functionally in MCL tumoral samples, Pearson correlations between identified SUZ12 target genes and *SUZ12* expression were calculated using the T-Rex program included in the Gene Expression Pattern Analysis Suite (*http://www.gepas.org/*).[40]

Gene Set Enrichment Analysis

The gene set enrichment analysis (GSEA) tool (*http://www.broad.mit.edu/gsea*, last accessed April 7, 2008)[41,42] was used to explore functional gene sets, allowing the interpretation of complete gene expression data in relation to SUZ12 expression. The gene sets co-regulated with SUZ12 expression were identified using Pearson correlation, with a minimum of 10 and a maximum of 500 genes in a gene set being required to qualify them for further analysis. The selected gene sets corresponded to Biocarta pathways (*http://www.biocarta.com*, last accessed April 7, 2008), excluding those that were not relevant to either lymphoid cell biology or cancer. Gene sets with an false discovery rate of less than 0.25 were considered significant.

Results

SUZ12 Protein Expression Is Restricted to Proliferating Cells in Normal Human Tissues

To screen for potential abnormalities in the expression of SUZ12, we compared expression patterns in tumoral samples with those in normal tissues. To this end, we first generated a monoclonal antibody (mAb) against SUZ12 protein (clone 220A/A3; described in *Materials and Methods*). Demonstration of 220A/A3 mAb specificity against SUZ12 in cytospin preparations can be found in the

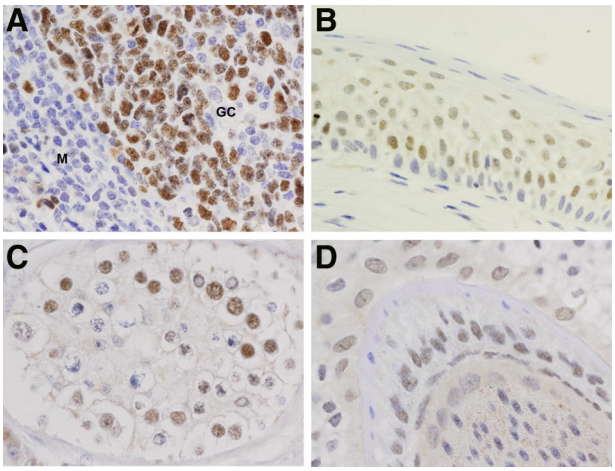

Figure 1. Expression of SUZ12 in non-tumoral human tissues: SUZ12 protein is preferentially detected in proliferating cells in various non-tumoral human tissues such as proliferating cells in tonsil germinal center (M, mantle zone; GC, germinal center) (**A**); proliferating cells in tonsil epithelium (**B**); germinal cells of the testis (**C**); and the hair follicle (**D**). Objective, ×100, immersion).

supplementary information (Supplemental Figure 1 at *http://ajp.amjpathol.org*). These results were confirmed by Western blot using cell lysate of *SUZ12* and *SOX4*-transfected cells (data not shown). The specificity of the mAb generated is also demonstrated in the shRNA experiments described below. The antibody was found to be suitable for immunohistochemistry, immunofluorescence (data not shown), Western blot detection, and chromatin immunoprecipitation.

With this mAb, we characterized the expression of SUZ12 in a large cohort of non-tumoral human tissues using a TMA containing a panel of around 50 different normal tissues.[13] As SUZ12 binds to EZH2 in PRC2 complex, we also characterized EZH2 protein expression in these same tissues. Surprisingly, we found that whereas EZH2 protein is widely detected in a large proportion of tissues, with few exceptions, SUZ12 expression is restricted to proliferating cells in reactive lymphoid tissue, germinal cells in the testis, and the epithelium of various organs (Figure 1,A–D, and Supplemental Table 1 and Supplemental Figure 2 at *http://ajp.amjpathol.org*).

SUZ12 Is Overexpressed in a Subset of Human Tumors

We also studied SUZ12 and EZH2 expression in TMAs containing representative paraffin sections from 569 cases of multiple (up to 69) human tumor types.[13] EZH2 and SUZ12 expression patterns were also different in tumoral samples and we found no significant association between these two proteins. While EZH2 protein was almost ubiquitously expressed in most tumors (489 out of 521, 94%, were positive) although with different intensities, SUZ12 was more restricted to lymphoid, lung, vascular, germinal ,and skin tumors (250 out of 497, 50.3%, had detectable expression). Therefore, those cases positive for SUZ12 are usually EZH2-positive, but not vice versa. We also observed a small fraction of tumors of different types (16/497, 3.2%) in which we could detect SUZ12 but not EZH2. Results are summarized in Supplemental Table 2 and Supplemental Figure 2 at *http://ajp.amjpathol.org*.

Essentially, SUZ12 expression was more frequent in lymphomas (91%) (notably in MCL); germinal cell-derived tumors (70%) (seminomas, teratocarcinomas, and embryonal carcinomas); skin tumors (88%) (melanomas and skin carcinomas); vascular tumors (83%); and pulmonary neuroendocrine small-cell carcinomas (70%) (Supplemental Table 2, A and B, and Supplemental Figure 2 at *http://ajp.amjpathol.org)*. Therefore, SUZ12 was expressed in tumors characterized by a high growth fraction.

SUZ12 Overexpression Is Associated with Gene Locus Amplification

SUZ12 gene is located at the 17q11.2 locus, which is frequently found to be translocated in endometrial stromal tumors.[20] Other cytogenetic alterations have been described at this locus in various types of tumor, such as additions, deletions and translocations,[43–47] meaning that these cytogenetic alterations could be related to tumorigenesis.

To determine whether *SUZ12* is amplified in primary human tumors, we used FISH analysis in TMA paraffin sections. 17q11.2 amplification or gain was analyzed in the same TMAs as were used for IHQ analysis. Five cases showed gene amplification as determined by the standard criterion[35,36] (*SUZ12*/centromeric 17 ratio >2.0) and two cases showed increased gene copy number (ratio > 1.5) distributed in several tumoral types such as mesothelioma (amplification in 1/10), melanoma (amplification in 1/10), skin basal cell carcinoma (gain in 1/10), thyroid follicular carcinoma (amplification in 1/8), leiomyosarcoma (gain in 1/6), MCL (amplification in 1/10), and ovary serous cystoadenocarcinoma (amplification in 1/6). The results are summarized in Supplemental Table 2, A and B, at *http://ajp.amjpathol.org*.

Although not exclusive to MCL, the findings of the anomalous expression of SUZ12 in most MCL cases (9/10) compared with its absence in non-tumoral mantle zone cells, and the detection of *SUZ12* locus amplification associated with strong SUZ12 expression prompted us to extend the study to a larger cohort of cases for further validation.

Additionally, comparing SUZ12 expression in MCL with that in other lymphoma types, it was detected in those lymphomas with a high growth fraction and that are derived from germinal center B cells (diffuse large B-cell and Burkitt lymphomas), or was restricted to proliferating cells in chronic lymphocytic leukemia, follicular lymphoma, and splenic marginal zone lymphoma cases (Figure 2A). However, in MCL, a tumor characterized by lower or intermediate proliferation, we observed a high level of expression of SUZ12 in most (9/10) cases, in contrast with the absence of SUZ12 from the mantle zone cells in reactive lymphoid tissue, which is the normal counterpart of this tumor type (Figure 2B and Supplemental Figure 2 at *http://ajp.amjpathol.org*).

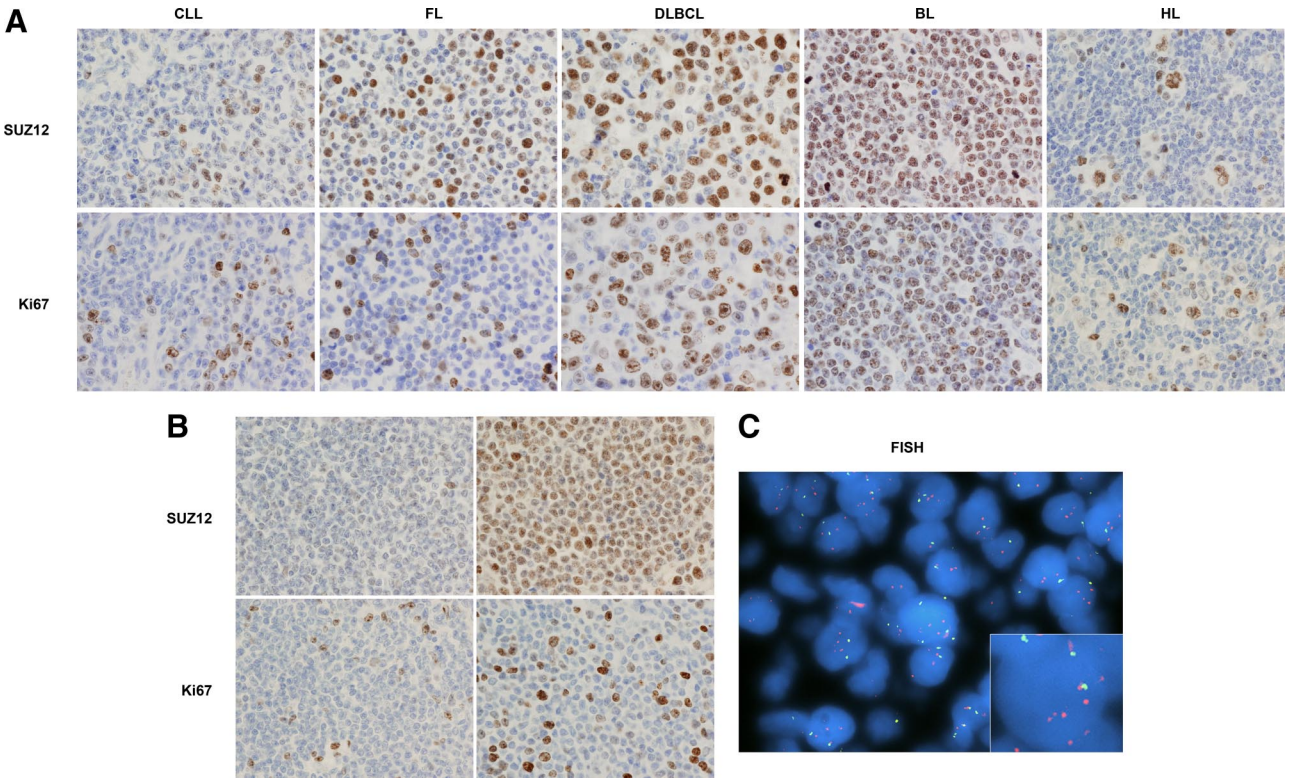

Figure 2. SUZ12 in B-cell lymphomas. **A:** SUZ12 and Ki-67 expression in different types of B-cell lymphoma, showing the correlation between the strength of SUZ12 expression and proliferation index. **B:** However, in samples of MCL, a tumor with a relatively low proliferation index, some cases showed increased SUZ12 expression, in contrast to the absence of its expression from normal mantle zone cells (Figure 1A), which are the benign counterpart of MCL. **C:** *SUZ12* gene amplification detected by FISH in an *SUZ12*-positive MCL case. Several copies of *SUZ12* (in red) are detected, compared with only two centromeric copies for chromosome 17 (green). (Objective, ×100, immersion).

SUZ12 in Mantle Cell Lymphomas

Therefore, we checked SUZ12 expression and gene locus alterations in an additional group of 76 MCL cases (Supplemental Table 2C at *http://ajp.amjpathol.org*). In total, 46 out of 81 (56%) MCL cases (10 in the general multitumor TMA and 71 new evaluable cases in MCL-specific TMA) showed SUZ12 protein expression.

Sixty-seven new MCL samples gave valuable results in the FISH study. In total, three MCL samples (the previous one and two cases in the new TMA) showed gene amplification (Figure 2C) and four cases had a high gene copy number (Supplemental Table 2, B and C at *http://ajp.amjpathol.org*). The three cases with 17q11.2 locus amplification showed SUZ12 expression in more than 80% of tumoral cells and, remarkably, two of these cases were diagnosed as aggressive (blastoid) MCL cases. These data indicate that around 9% of MCL cases had cytogenetic alterations at the *SUZ12* locus. Additionally, polysomy of chromosome 17 was found in 12 MCL samples.

SUZ12 Silencing by shRNA Compromises Cellular Viability

To further assess the significance of SUZ12 overexpression in MCL we performed an RNAi analysis using a lentiviral (HIV)-based RNA interference vector in two MCL-derived cell lines: Jeko-1 and Z138. The vector contains EGFP as a selection marker.

Two sequences (shSUZ12.783 and shSUZ12.2076) correctly induced SUZ12 silencing with a residual expression of less than 20% (Figure 3A). Specificity of the shRNAs was also assessed by measuring trimethylation levels at lysine 27 of histone H3 (H3K27me3), a hallmark of PRC2 activity. Levels of H3K27me3 were significantly lower 72 hours after infection with the lentivirus carrying shRNAs against SUZ12 compared with controls (Figure 3B). To determine whether SUZ12 inhibition had any effect on cell survival or cell growth we designed a strategy based on competitive proliferation similar to that previously described (see Materials and Methods).[48] Thus, we infected Jeko-1 and Z138 cells and a slight but constant decrease in GFP+ cell number was observed, specifically in those cells transduced with lentivirus carrying any of the shRNAs against SUZ12 (Figure 3C).

Additionally, to assess the effect of SUZ12 silencing on cell growth directly, we sorted the GFP+ fraction in each case and measured cell number by trypan blue exclusion counting. Results showed that cells expressing shRNAs against SUZ12 grew less than cells either expressing a scramble control or infected with the empty vector (Figure 3D). Cell cycle analysis by FACS in these GFP+-sorted cells only showed subtle differences: either a slight decrease in G2/M or an increase in SubG1 phase in cells deficient in SUZ12 (data not shown). Levels of ap-

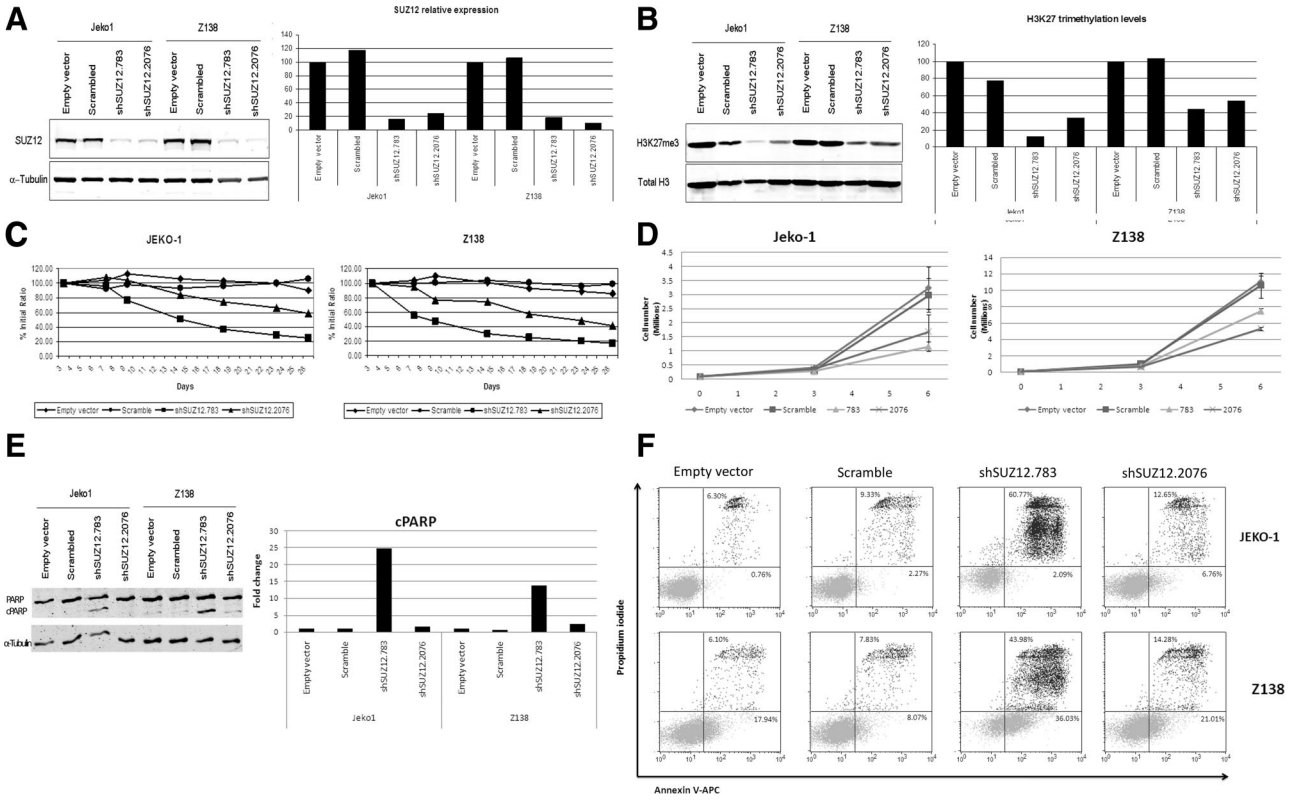

Figure 3. Effects of SUZ12 depletion in MCL cell lines. **A: Left**, anti-SUZ12 immunoblot of Jeko-1 and Z138 cell lines transduced either with the empty vector, a scrambled sequence or with two different hairpins against SUZ12. Band signals were normalized with tubulin as a loading control (**right**). **B:** Depletion of SUZ12 affects the function of the PRC2 complex as assessed by immunoblot using an antibody against histone H3 trimethylation at lysine 27 (**left**). Total histone H3 was used to normalize band signals (**right**). Cropped blots are shown. **C–F:** SUZ12 knockdown compromises cell viability: **C:** Expression of EGFP that marks transduced cells was tracked over time to observe differences in viability between cells transduced either with the empty vector or with a scramble control and cells transduced with two different hairpins against SUZ12. **D:** GFP+-sorted Jeko1 and Z138 proliferation was assessed by counting viable cells using trypan blue exclusion along the time. Mean and SD are shown. **E:** Immunoblot showing specific cleavage of poly (ADP-ribose) polymerase (cPARP) in SUZ12-deficient cells after sorting. **F:** Flow cytometric analysis of apoptosis using double staining of Annexin V and propidium iodide after cell sorting.

optosis were augmented on silencing of SUZ12 as demonstrated by specific annexin V staining and cleavage of PARP (Figure 3, E and F). One of the hairpins was more efficient in killing the cells, and this was associated with the levels of H3K27me3 demethylation. In fact, we were not able to detect PARP cleavage in Jeko-1 with one of the hairpins, probably because of the sensitivity of the antibody, since we could still detect a mild increment in apoptosis with annexin V staining.

Identification and Functional Classification of SUZ12 Genomic Target Genes in MCL

To look for potential targets that could explain the role of SUZ12 in MCL we performed ChIP and subsequent hybridization on Agilent's human promoter microarray (ChIP-on-chip) with the Z138 MCL-derived cell line. We found 17,605 (3.6%) bound probes (peak $P < 0.01$) corresponding to 1806 genes. Those probes with a normalized $\log_2$ ratio >1 were considered to be potential SUZ12 targets (1424 genes including known SUZ12 target genes such as *CDKN2A*, *GADD45G*, *BMP2*, and *WNT*[49–51]).

To determine the accuracy of SUZ12 target gene discovery, single-locus semiquantitative PCR was performed on the immunoprecipitated material with SUZ12 and H3K27me3 antibodies on 18 candidate SUZ12 target genes, including genes known to be relevant in MCL or lymphoma pathogenesis (*ATM*, *BCOR*, *VAV*), in the control of significant processes such as apoptosis and DNA repair (*BIRC2*, *GADD45*), and transcriptional regulation (*JMJD2*). *GADD45G* (a previously described target)[49] was used as the positive control (Figure 4). Eleven of 18 were validated for SUZ12 and H3K27me3 antibodies, including *ATM*, *CBX2*, *VAV3*, *JMJD2*, and *BIRC2*.

To determine which relevant pathways could be altered by SUZ12 overexpression, we examined the functions of SUZ12 target genes using the Ingenuity Pathway Analysis program. Of 1424 genes identified by ChIP-on-chip, 658 have annotated functions in the Ingenuity Pathway Analysis database and were functionally classified. The results showed significant enrichment of genes controlling gene expression, cell cycle and proliferation, DNA replication and repair, and development (Figure 5), consistent with the findings of other studies.[49,50]

The group of SUZ12 target genes controlling gene expression includes transcription factors and regulators, such as E2F5, POU domain proteins, and SUV39H1, and enzymes regulating transcription and translation, such as DNA and RNA polymerases, jumonji domain (JMJD) pro-

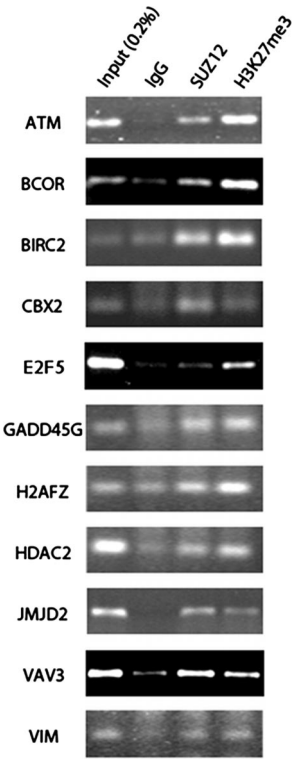

Figure 4. Validation of SUZ12 target genes by semiquantitative ChIP. Single-locus semiquantitative PCR on ChIP samples was performed on several SUZ12 candidate target genes, with SUZ12 and H3K27me3 antibodies. Mouse IgG was used as a negative control.

teins, and several eukaryotic translation initiation factors. Development regulators have also been found among SUZ12 targets in MCL, some of which have been previously published as SUZ12 targets in embryonic cells, such as SOX and FOX family genes, POU domain transcription factors, and BMP2. Another relevant finding is the detection of SUZ12 in the promoter region of several miRs genes, two of which have been identified as SUZ12 targets (hsa-mir-124a and hsa-mir-183[49,52]) (identified genes listed in Supplemental Table 3 at *http://ajp.amjpathol.org*).

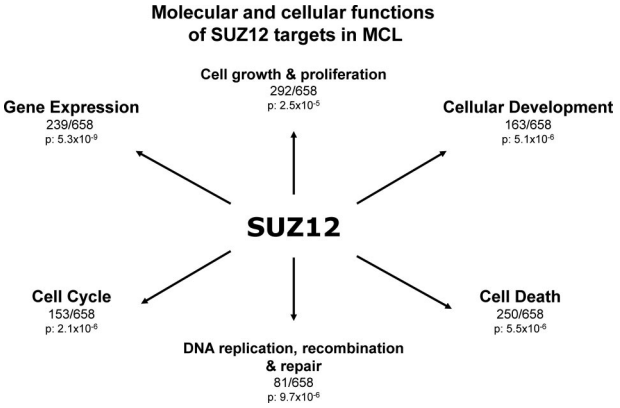

Figure 5. Functional classification of SUZ12-targeted genes. Functions of SUZ12 target genes were analyzed using the Ingenuity Pathway Analysis program. Of 1424 genes identified by ChIP-on-chip, 658 have annotated functions in the Ingenuity Pathway Analysis database. The number of genes identified as belonging to each category is included. The probability is that associated with a right-tailed Fisher's exact test.

However, the most noteworthy finding was that there were significant genes among the top SUZ12 targets that are known to be involved in MCL pathogenesis, some of which were not previously known to be SUZ12 targets. These included those regulating cell cycle (*CDKN2A* and other INK4 family genes, cyclins, CDKs, *CHEK1*, *MAD2L1*, and *BUB3*); DNA damage and repair genes (*ATM*, *GADD45*, several DNA polymerases and topoisomerases, *MLH1*, XRCC family genes, and ERCC family genes); apoptosis regulators (*BCL2* and BCL2 regulator proteins, *BID*, several BIRC family members, and others); and we also found members of nuclear factor κB pathway (*BCL10*, *NFKB2*, and *IKBKG*) to be regulated by SUZ12.

SUZ12 Target Gene Expression in Silenced SUZ12 MCL-Derived Cell Lines and MCL Tumoral Samples

To validate functionally and elucidate the relevance of these ChIP-on-chip findings, we looked at the changes in expression of the targets associated with SUZ12 expression in both Z138 cell line after SUZ12 silencing and in tumoral samples from MCL cases using whole genome expression microarrays.

SUZ12 Targets in SUZ12-Silenced Z138 Cell Line

We first analyzed the expression of SUZ12 targets in SUZ12-silenced cells, comparing the expression profile of SUZ12-depleted cells with those infected with the control vector. After SUZ12 silencing in the Z138 cell line, some SUZ12 targets were actually unrepressed. 140 transcripts showed an up-regulation or down-regulation of at least 0.6 ($\log_2$ scale) and were considered to be significantly deregulated after SUZ12 silencing. These included, among others, CDKN2A, GADD45G, genes involved in development, such as BMP2, several GATA binding proteins or differentiation factors like MLLT3 and CBX2 (Figure 6A).

SUZ12 Targets in MCL Samples

We also examined, in MCL tumoral samples, the relation between SUZ12 expression and that of genes identified by ChIP-on-chip. This analysis revealed that 188 of 642 known genes suitable for the analysis (30%) were significantly correlated with SUZ12 expression (Pearson $R > \pm 0.4$, false discovery rate <0.15). Many of the SUZ12 targets were actually down-regulated in SUZ12-positive MCL samples (126 inversely correlated with SUZ12 expression versus 62 with a direct correlation) (Figure 6, B and C).

Pathways Co-Regulated with SUZ12 in MCL

Finally, we wanted to determine which characteristics of tumors were associated with changes in SUZ12 levels, identifying functional pathways co-regulated with the ex-

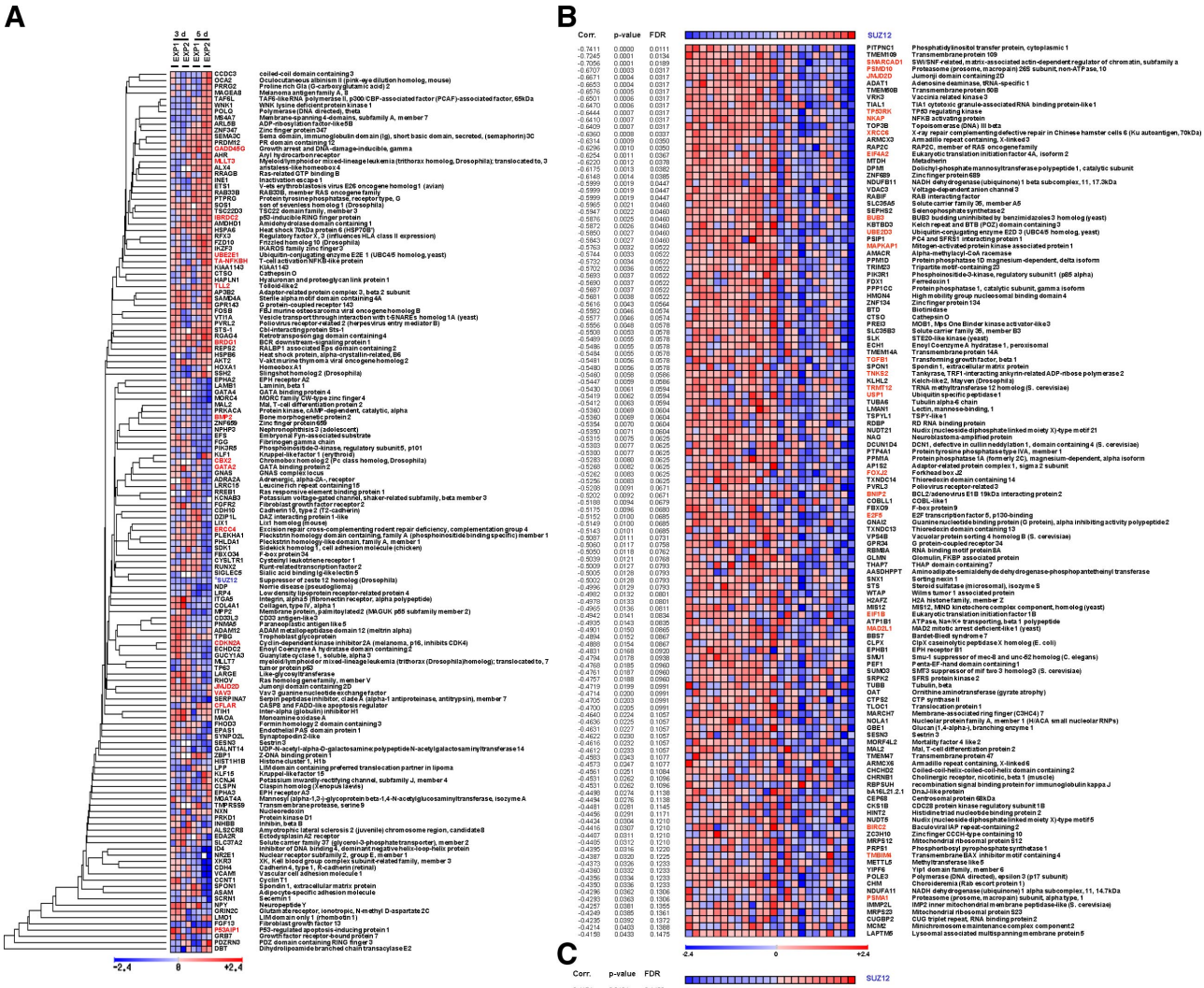

Figure 6. SUZ12 target gene expression was affected in SUZ12-silenced cell lines and MCL cases. **A:** Gene expression profiling was performed in duplicate 3 and 5 days after SUZ12 depletion in Z138, with two different shRNAs (2076 and 783) versus empty vector. The heat map represents expression levels for each sample. A total of 140 significant genes [$\log_2$ (Cy5/Cy3) cut-off = $\pm$0.6 in at least three samples] were ranked by Euclidean squared distance. **B:** Relative expression levels of 126 inversely correlated SUZ12 genes in MCL tumoral samples (false discovery rate $<$ 0.15). **C:** Relative expression levels of 62 directly correlated SUZ12 genes in MCL tumoral samples (false discovery rate $<$ 0.15). Pearson correlation coefficients between identified SUZ12 target genes and SUZ12 expression were calculated using the T-Rex program available from the GEPAS site (v. 3.1).

pression of SUZ12. To this end we performed a gene set enrichment analysis of SUZ12 expression with all of the genes in MCL tumoral samples, not restricted to SUZ12 targets identified by ChIP, using GSEA software (*http://www.broad.mit.edu/gsea/*). This analysis revealed a direct correlation with pathways associated with proliferation, such as cell cycle and caspase-apoptosis pathways and an inverse correlation with the proteasome pathway (Table 1). We also found an inverse correlation with the MAPK pathway, but some specific genes included in this pathway such as *JUN*, *FOS*, *MAPK4, MAPK7*, and *BRAF* showed stronger expression in SUZ12-expressing tumors.

Table 1. Pathways Co-Regulated with SUZ12 Expression in MCL

Name	Size	Enrichment score	Normalized enrichment score	*P*	False discovery rate
Gene sets positively correlated with SUZ12 expression					
Mitochondria pathway	21	0.5952	2.4673	0.0000	0.0053
Cell cycle pathway	21	0.5622	2.3809	0.0000	0.0075
ARAP pathway	19	0.5528	2.1412	0.0020	0.0283
Caspase pathway	21	0.5233	2.1399	0.0040	0.0213
CA2+ CAM pathway	12	0.6362	1.9820	0.0043	0.0513
D4GDI pathway	11	0.6060	1.8114	0.0169	0.1118
Gene sets negatively correlated with SUZ12 expression					
MAPK pathway	83	−0.4378	−3.4409	0.0000	0.0000
Proteasome pathway	19	−0.7375	−2.8678	0.0000	0.0000
GH pathway	26	−0.4999	−2.2277	0.0000	0.0395
IL1R pathway	29	−0.4244	−2.0417	0.0080	0.0779
CHREBP pathway	15	−0.5951	−2.0912	0.0038	0.0832
CK1 pathway	12	−0.6534	−2.0431	0.0056	0.0916
ARF pathway	16	−0.5428	−1.9669	0.0056	0.1074
RARRXR pathway	14	−0.5157	−1.7385	0.0177	0.1367
RAB pathway	10	−0.5956	−1.7550	0.0220	0.1392
ERK pathway	30	−0.3620	−1.7652	0.0203	0.1402
AKAP-centrosome pathway	10	−0.6027	−1.7413	0.0198	0.1413
TPO pathway	22	−0.4373	−1.8955	0.0096	0.1444
PPARA pathway	51	−0.2753	−1.7689	0.0249	0.1465
Cytokine pathway	15	−0.5077	−1.7904	0.0183	0.1473
PDGF pathway	26	−0.3973	−1.7772	0.0149	0.1479
VEGF pathway	26	−0.3992	−1.7929	0.0135	0.1557
TOLL pathway	32	−0.3657	−1.8513	0.0134	0.1642
GATA3 pathway	13	−0.5419	−1.7935	0.0080	0.1678
TOB1 pathway	16	−0.4963	−1.7958	0.0189	0.1797
P38MAPK pathway	37	−0.3407	−1.8065	0.0132	0.1876

GSEA reporting on statistically significant functionally relevant pathways ($P < 0.05$, false discovery rate < 0.25) and on positive and negative Pearson correlation coefficients with a high level of SUZ12 expression. *P*, nominal probability.

Discussion

SUZ12 is a core component of the Polycomb PRC2-HMTase complex that has been shown to be involved in stem cell maintenance and development. Although some studies have demonstrated overexpression of SUZ12 in colon and breast tumors,[19,53] its real relevance in human cancer is yet to be established.

In this study, we first explored EZH2 and SUZ12 protein expression in non-tumoral samples. While EZH2 was widely detected in almost every tissue analyzed, SUZ12 was restricted mainly to those tissue compartments with proliferating cells, such as germinal centers in reactive lymphoid tissue, thymic cortex, epithelial basal cells and germinal cells in the testis. All these tissues are characterized by their regenerative capacity, suggesting a role for SUZ12 in tissue homeostasis and in cell cycle and proliferation.

Analysis of tumoral human samples revealed that EZH2 and SUZ12 are not always expressed simultaneously. Actually, those cases positive for SUZ12 are usually EZH2-positive, but not vice versa. We also observed a small fraction of cases in which we could detect SUZ12 but not EZH2. In these cases we cannot rule out the possibility that SUZ12 might have additional EZH2-independent functions.

There was a high level of expression of SUZ12 in a subset of tumoral samples including germinal cell-derived tumors, melanomas, skin basal cell carcinomas, lung neuroendocrine small-cell carcinoma, pituitary and parathyroid adenomas, and lymphomas, most remarkably in MCL, where the high expression of SUZ12 contrasts with its absence in the non-tumoral mantle zone cells in reactive lymph node. Therefore, our results extend previous observations of the strong expression of SUZ12 in human tumors.[19,53]

SUZ12 locus (17q11.2) has been found amplified, associated with protein overexpression in a small subset of tumors. This finding is especially relevant in MCL, where it seems to be more frequent in blastoid MCL, the aggressive variant of this type of lymphoma, since two of four blastoid-MCL cases showed this amplification. Therefore, our finding of *SUZ12* amplification in MCL or melanoma among others types of tumors, along with the presence of *SUZ12* translocations in endometrial sarcomas of the cervix,[20,54] supports the hypothesis that SUZ12 has an oncogenic function and contributes to tumor formation and maintenance. The findings described here for SUZ12, and the previous results for EZH2,[7,17,18] suggest that alteration of the PRC2 complex is a frequent event in human carcinogenesis.

Given the anomalous expression of SUZ12 in MCL tumoral cells compared with the lack of expression in their normal counterparts and the amplification associated with high levels of expression, we decided to perform functional analysis in MCL cell lines as a model to depict SUZ12 role in tumorigenesis. To this end, we silenced SUZ12 expression by RNAi in MCL-derived cell

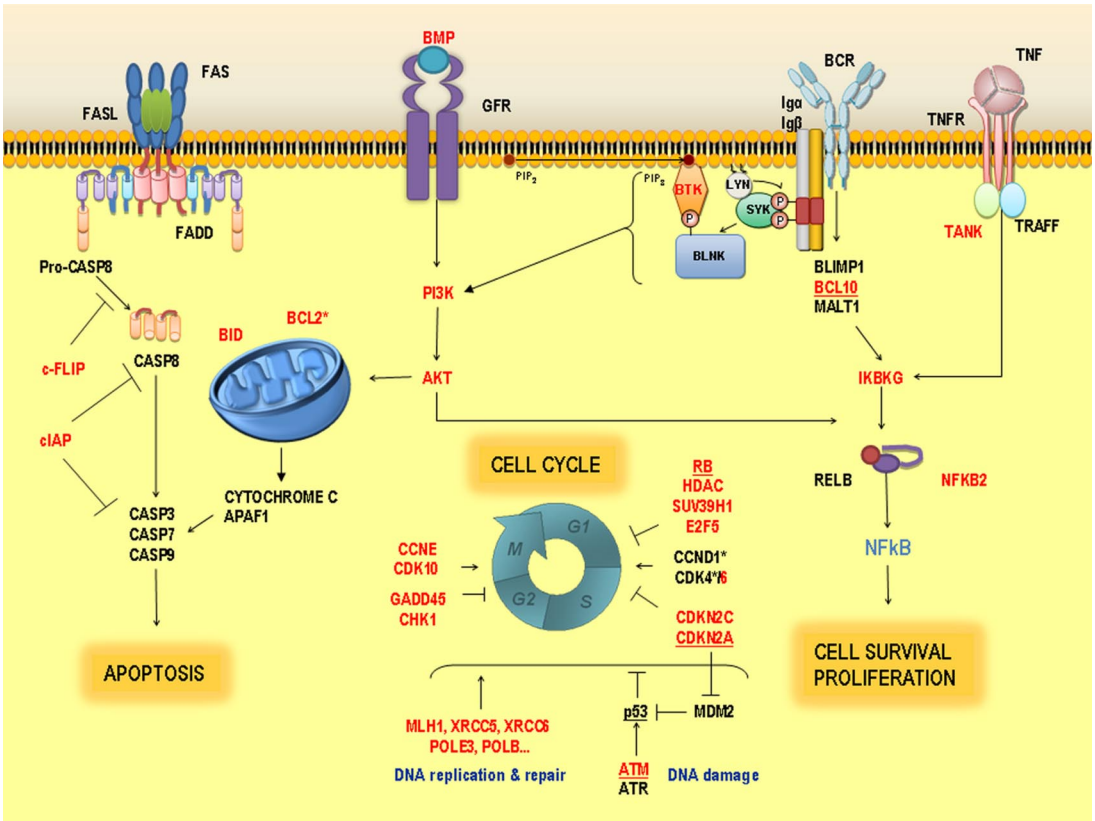

Figure 7. SUZ12 regulates the expression or function of multiple important pathways controlling MCL pathogenesis. Genes identified as SUZ12 targets by ChIP-on-chip in red. Genes marked with an asterisk suffer translocation or overexpression in MCL cases. Genes underlined are frequently deleted in MCL.

lines, and evaluated its effect on levels of H3K27me3, cell proliferation, apoptosis, and cell survival. A clear decrease in H3K27me3 was detected after SUZ12 silencing, demonstrating the interference with PRC2 activity due to the lower levels of SUZ12 in accordance with previous reports.[21] SUZ12 knockdown resulted in an increased apoptosis, as demonstrated by annexin V and PARP cleavage analysis. When we studied the SUZ12 silencing effect over time, we observed that loss of SUZ12 compromised cell viability, as demonstrated by cell counting and competition assays. These results suggest that SUZ12 expression contributes to cell survival in MCL cell lines, avoiding apoptosis and increasing cell proliferation.

SUZ12 is known to exert its function through the direct repression of many target genes. Other studies have shown that SUZ12 targets vary among developmental states, tissues, and cell types.[55] Therefore, we decided to identify SUZ12 targets that could mediate the effect on MCL-derived cell line viability and explain their role in MCL pathogenesis. We identified a group of SUZ12 targets with functions in apoptosis (BIRC family genes, *BCL2*, *BID*, and *cFLIP*); cell proliferation (several INK4 family genes, cyclins, and CDKs) and checkpoint regulators (*CHK1*, *BUB3*, *MADL2*, *GADD45*, and *CDK6*); DNA damage and repair genes (*ATM*, *GMNN*, and *MLHL1*); gene expression; and cell development. Several of these targets are genes of relevance in MCL (Figure 7). For instance, *CDKN2A, ATM*, *BCL10*, and *RBL1*, identified here as being SUZ12 targets, are frequently deleted or lost in MCL.[23] SUZ12 could collaborate to inactivate these genes by epigenetic means. Other molecules, members of pathways of relevance in MCL, are also targets of SUZ12. For example, nuclear factor κB pathway components (*BLIMP1*, *IKBKG*, and *NFKB2*) and proteasomal pathway members have been found to be targets of SUZ12. Some of these targets have been previously found in other cell lines (such as *BMP2*, *CDKN2A*, *SOX3*, and *GADD45G*) but others have not been described before and could be MCL-specific. This is the case for several of the top identified targets such as *ATM*, BIRC family genes, *BTK*, *BUB3*, *MAD2L1*, and *RBL1*, among others, some of which have also been demonstrated with classic ChIP (Figure 4), suggesting that they are bona fide SUZ12 targets, and possibly specific to MCL cells. We also found that SUZ12 targets several microRNAs, some of which, for example, hsa-miR-148a[56] and hsa-miR-223,[57] were already known to be involved in cancer and differentiation.

Many of these target genes were inversely correlated with SUZ12 expression levels in tumoral samples from MCL patients (Figure 6B), such as BIRC2, TMBIM4, XRCC6, JMJD2D, MAD2L1 and BUB3, among others. Additionally, validation in SUZ12-depleted cell lines revealed that several target genes were re-expressed after SUZ12 silencing (Figure 6A). The fact that some of the SUZ12 targets remained unaltered after SUZ12 depletion has several possible explanations. For instance, many SUZ12 targets may require not only the depletion of

SUZ12 but also additional events like DNA demethylation or the presence of an activator to be expressed again.[1,50]

All these findings indicate that SUZ12 could collaborate in deregulating the expression of many important pathways controlling MCL pathogenesis (Figure 7). We propose that the abnormal expression of SUZ12 may account for some of the still unexplained features of MCL, including abnormal DNA repair and increased resistance to apoptosis.

Interestingly, recent publications have described the capacity of several drugs to block the HMTase activity of PRC2 complexes.[58,59] In fact, LBH589 has proved to be effective in acute myelogenous leukemia cells. Patients suffering from other tumors, like MCL and pulmonary neuroendocrine small-cell carcinoma, in which PRC2 alterations are detected, might also benefit from this therapy.

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ORIGINAL ARTICLE

Mantle cell lymphoma: transcriptional regulation by microRNAs

L Di Lisio[1], G Gómez-López[2], M Sánchez-Beato[1], C Gómez-Abad[1], ME Rodríguez[1], R Villuendas[1], BI Ferreira[3], A Carro[2], D Rico[2], M Mollejo[4], MA Martínez[5], J Menárguez[6], A Díaz-Alderete[7], J Gil[7], JC Cigudosa[3], DG Pisano[2], MA Piris[1] and N Martínez[1]

[1]*Lymphoma Group, Molecular Pathology Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain;* [2]*Bioinformatics Unit, Structural Biology and Biocomputing Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain;* [3]*Molecular Cytogenetics Group, Human Cancer Genetics Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain;* [4]*Department of Pathology, Hospital Virgen de la Salud, Toledo, Spain;* [5]*Department of Pathology, Hospital Doce de Octubre, Madrid, Spain;* [6]*Department of Pathology, Hospital Gregorio Marañon, Madrid, Spain and* [7]*Department of Immunology, Hospital Gregorio Marañon, Madrid, Spain*

Mantle cell lymphoma (MCL) pathogenesis is still partially unexplained. We investigate the importance of microRNA (miRNA) expression as an additional feature that influences MCL pathway deregulation and may be useful for predicting patient outcome. Twenty-three MCL samples, eight cell lines and appropriate controls were screened for their miRNAs and gene expression profiles and DNA copy-number changes. MCL patients exhibit a characteristic signature that includes 117 miRNA (false discovery rate <0.05). Combined analysis of miRNAs and the gene expression profile, paired with bioinformatics target prediction (miRBase and TargetScan), revealed a series of genes and pathways potentially targeted by a small number of miRNAs, including essential pathways for lymphoma survival such as CD40, mitogen-activated protein kinase and NF-κB. Functional validation in MCL cell lines demonstrated NF-κB subunit nuclear translocation to be regulated by the expression of miR-26a. The expression of 12 selected miRNAs was studied by quantitative PCR in an additional series of 54 MCL cases. Univariate analysis identified a single miRNA, miR-20b, whose lack of expression distinguished cases with a survival probability of 56% at 60 months. In summary, using a novel bioinformatics approach, this study identified miRNA changes that contribute to MCL pathogenesis and markers of potential utility in MCL diagnosis and clinical prognostication.
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Introduction

Mantle cell lymphoma (MCL), a tumor accounting for 6–7% of non-Hodgkin's lymphomas, is distinguished by its resistance to chemotherapy and poor outcome. It originates from follicular mantle zone cells and is characterized by deregulation of multiple survival signaling pathways.[1–3] It is recognized by the t(11;14)(q13;q32) translocation, which results in cyclin D1 (*CCND1*) overexpression.[4–6] Other alterations have been identified,[2] nevertheless, MCL pathogenesis has yet to be fully explained, as the genetic changes so far identified cannot account for the increased survival signaling that characterizes this tumor.[3]

In this context, a group of post-transcriptional regulators, the microRNAs (miRNAs), has been proposed as candidates that could complete our understanding of tumor pathogenesis. They are small, noncoding RNAs that regulate the expression of multiple mRNAs[7,8] and have a key role in the control of the various biological processes involved in cancer pathogenesis.[9–11] Specific miRNA signatures have been identified for some tumor types,[12–15] and they are thought to function as metastasis regulators.[16,17] Altered miRNA expression is also known to have a role in hematopoietic malignancies such as chronic lymphocytic leukemia (miR-15a, miR-16)[18,19] and diffuse large B-cell lymphoma.[20] Moreover, a recent study has shown that miRNA losses and gains could have a significant role in MCL by regulating CCND1 mRNA expression.[21]

In this study, we have explored whether miRNA losses and gains can help explain MCL pathogenesis. We profiled miRNA and mRNA expression in a series of MCL patients and cell lines. miRNAs making up the MCL signature were then related to the MCL mRNA signature through the computational prediction of miRNA targets. Correlation between miRNA expression and patient outcome was also investigated.

Materials and methods

Patients, cell lines and control tissues

The series included 23 frozen MCL lymph nodes (which contain at least 80% of tumoral cells) and 11 reactive lymph nodes (seven) or tonsils (four) for control purposes. Follow-up was obtained for 22 cases. An independent set of 54 formalin-fixed paraffin-embedded MCL diagnostic samples was analyzed for confirmation of survival studies.[22] All the samples were recovered at the time of diagnosis, and MCL diagnosis was performed according to the World Health Organization criteria.[23] The study was carried out under the supervision of the corresponding local ethics committees.

The study also included eight MCL cell lines (REC-1, Jeko, UPN1, UPN2, Granta, Z138, MINO and Hbl2). Sorted mantle-zone B cells ($CD19^+/IgD^+/CD27^-$)[2,3,24] from three routine tonsillectomy samples were used as controls (procedure described in Supplementary text).

Microarray procedures

For miRNA hybridization, 100 ng of total RNA was hybridized on an Agilent 8×15K human miRNA one-color microarray for detecting 470 human and 64 viral miRNAs, following the manufacturer's instructions (Agilent Technologies Inc, Santa Clara, CA, USA).[25]

Correspondence: Dr MA Piris, Lymphoma Group, Molecular Pathology Programme, Centro Nacional de Investigaciones Oncológicas, C/ Melchor Fernández Almagro no. 3, Madrid E-28029, Spain.
E-mail: mapiris@cnio.es
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Gene expression was carried out with Agilent Technology microarrays.[26,27] RNA extraction, details of microarray and hybridization procedures, and gene expression profiling are described in the Supplementary text.

miRNA expression profiling. Between-array median normalization was carried out to render miRNA expression data sets comparable. Significantly deregulated miRNAs were computed using Significant Analysis of Microarray analysis (an Excel macro, in this case, that provides *q*-values directly).[28,29] The *q*-value corresponds to the false discovery rate (FDR).[30] miRNAs with FDR <0.05 were taken as being differentially expressed in controls and tumors.

Significant miRNAs (FDR <0.05 and more than twofold up- or downregulation) were represented by a heatmap using SOTArray (http://www.gepas.es).

Associations between miRNAs, gene expression signatures and biological pathways. For each differentially expressed miRNA, a contingency table relating the miRNA and its predicted gene targets (whose probe was included in GE platform, Agilent Technologies Inc) was produced using miRBase Targets Release v. 5.0 (http://www.mirbase.org/; Faculty of Life Sciences, University of Manchester) and TargetScan v. 5.1 (http://www.targetscan.org/; Whitehead Institute for Biomedical Research) (including conserved and nonconserved target sites predictions), taking into account whether these targets were included in a consistent gene expression signature (downregulated targets for upregulated miRNAs and vice versa).

Fisher's exact test was used in the miRBase and TargetScan analyses. Those miRNAs whose Fisher's exact test result indicated an FDR <0.05 were selected for further analysis on the basis of their nonrandom association with the gene expression signature of interest.[31]

To identify statistically significant associations between differentially expressed miRNAs (both Significant Analysis of Microarray and Fisher's exact test FDR <0.05) and enriched pathways (FDR <0.26), a ranked list was built that included only the targets predicted for significant miRNAs using the gene expression profiling statistic value obtained from the microarray data. As miRNAs function as repressors, a gain in miRNAs is usually associated with the downregulation of the target RNA or protein, whereas miRNA loss is associated with upregulation of the RNA or protein target.[32] Thus, downregulated miRNAs were tested for their association with upregulated genes, whereas upregulated miRNAs were tested for their association with downregulated genes. The ranked target list was subjected to gene set enrichment analysis (GSEA).[33] Annotations were taken from a curated version of the Biocarta, KEGG and CCG pathway databases[34] with minor modifications (Supplementary Table S1). The analysis was carried out independently using miRBase and TargetScan. Only miRNA pathways with significant associations identified by both miRBase and TargetScan predictions were finally considered.

Interaction networks were depicted using Cytoscape bioinformatics software (http://www.cytoscape.org). Figure 1 provides a flowchart of the entire data analytical approach.

Survival analysis

In the first set of 22 patients, Gene Spring software v. 9.0 (Agilent Technologies Inc) was used to normalize miRNA intra-array data at the 75th percentile, as recommended by the manufacturer.[25] Next, a Random Forests algorithm (available from the SIGNS website http://signs.bioinfo.cnio.es/)[26,35] was used to select a set of 12 miRNAs related to patient survival, which were analyzed in a new set of 54 paraffin-embedded cases for the confirmation step using reverse transcriptase PCR.[36–38]

Univariate Cox regression, available in SPSS v.15.0, (SPSS Inc, Chicago, IL, USA) was used to analyze the confirmation data set. Samples were evaluated for the expression of the 12 miRNAs and other standard clinical features (age, performance status, gender, stage at diagnosis and proliferation activity of the tumor).[39] The comparison of the variables was considered worthwhile in those groups with at least five patients in each category.

Overall survival was plotted by GrapPad Prism software (GraphPad Software, Inc, v. 5, La Jolla, CA, USA) for the Kaplan–Meier method, stratifying the samples into low- and high-risk groups according to the median value of the miR-20b expression. Curves were compared by a log-rank test.

Reverse transcriptase PCR

The expression of 19 selected miRNAs (Supplementary Table S2) was validated in frozen MCL tissues by quantitative PCR (qPCR) (ABI PRISM HT 7900 Real-Time Sequence detection system; Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol (see Supplementary text for details). qPCR of 12 selected miRNAs (Supplementary Table S2) was tested in 54 formalin-fixed paraffin-embedded samples and $-\Delta Ct$ values were used for survival analyses.

miRNA electroporation and immunofluorescence

Electroporation. miR-26a and controls (pre-miR-26a and pre-miR-negative control 1, Applied Biosystems) were electroporated at 60 nM concentration using the Neon Transfection System (Invitrogen, Carlsbad, CA, USA) in the MINO and REC-1 cell lines, using the following settings: 1 pulse, 40 ms, 1000 V. Cells were recovered after 24, 48, 72 and 96 h of electroporation.

Immunofluorescence. Cells were fixed and permeabilized with 100% acetone. p65 (RelA) mouse antibody 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; SC-8008) was used for primary staining. Secondary antibody staining was carried out with anti-mouse 1:200 (Invitrogen, Alexa Fluor 488). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Images were obtained by Leica TCS-SP2 (AOBS) confocal microscopy (Leica Microsystems, Germany) with LCS v. 2.61 software (Leica Microsystems).

Results

miRNA profile in MCL cases and MCL-derived cell lines

An miRNA signature for MCL, including all the miRNAs differentially expressed in the 23 MCL cases compared with the 11 reactive lymphoid tissues (FDR <0.05), was identified (Supplementary Table S3). It includes 117 human miRNAs: 85 downregulated and 32 upregulated; 56 out of 85 were downregulated (>twofold) and 16 out of 32 were upregulated (>twofold) (Figure 2). The most significant miRNAs that were lost in all cases were miR-31, miR-148a and miR-27b (FDR <0.001), whereas the list of upregulated miRNAs was headed by miR-617, miR-370 and miR-654 (FDR <0.001).

Nineteen selected miRNAs were analyzed by qPCR to validate the microarray data further (see Supplementary text).

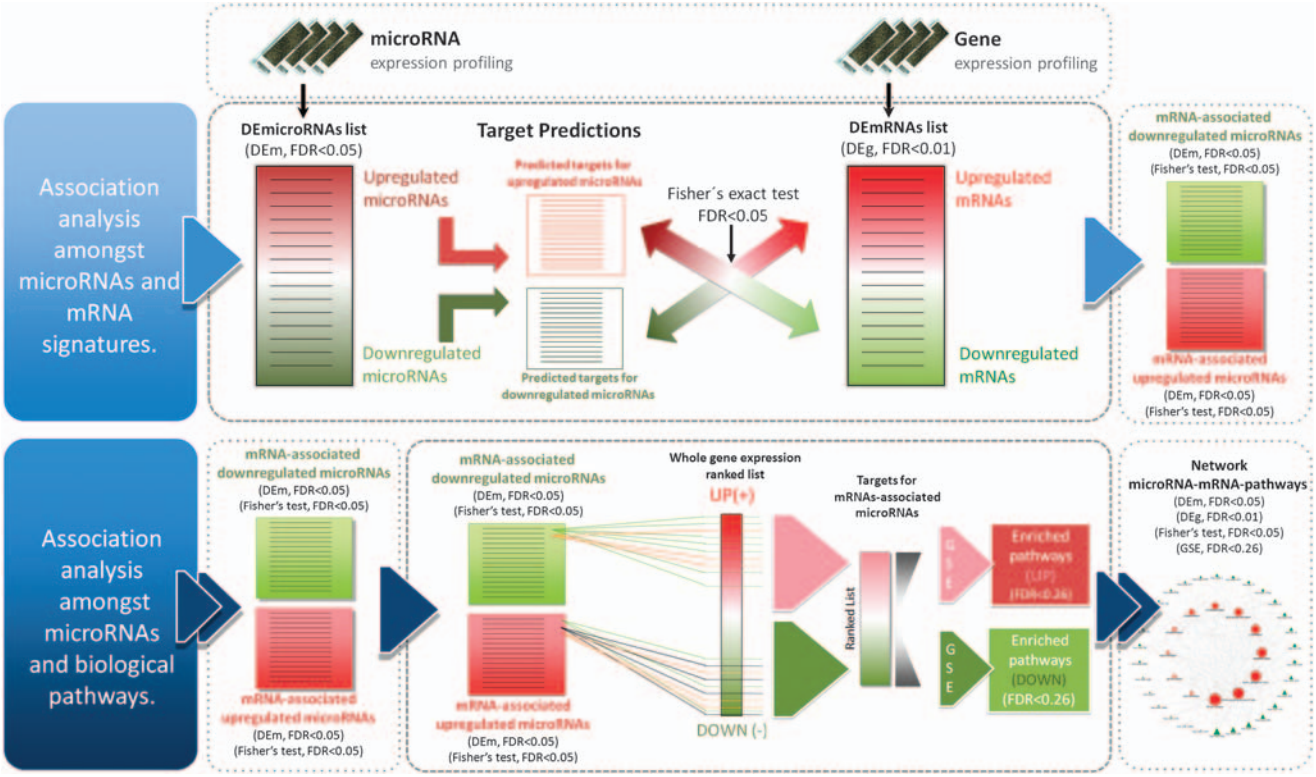

Figure 1 Bioinformatics approach to association analyses between miRNAs, mRNA signatures and molecular pathways. To identify statistically significant associations between differentially expressed miRNAs (DEm, FDR <0.05) and gene expression signatures (DEg, FDR <0.05), we tested whether predicted miRNA–mRNA targeting pairs were randomly associated or not. Thus, for each differentially expressed miRNA, we produced a contingency table relating every miRNA and its predicted gene targets, taking into account whether these targets were included in a consistent gene expression signature (downregulated targets for upregulated miRNAs and vice versa). Those miRNAs found to be significant on the basis of the Fisher's exact test (DEm FDR <0.05, but also Fisher's test, FDR <0.05) were selected on the basis of their nonrandom association with the gene expression signature of interest (DEg, FDR <0.01). Gene target predictions for human miRNAs were obtained using miRBase Targets Release v5.0 and TargetScan v5.1. To find statistically significant associations between differentially expressed miRNAs and enriched pathways, we compiled a ranked list including only the targets predicted for significant miRNAs (DEm FDR <0.05, Fisher's exact test FDR <0.05) using the gene expression profiling statistic value (limma moderated *t*-statistic) obtained from differential expression analysis. Next, a gene set enrichment method (GSE, for example, GSEA) was applied using the ranked target list. Thus, pathways enriched in targets of the selected miRNAs are revealed (GSE, FDR <0.26). Using this approach, the experimental microarray gene expression data are inferred to predict the effects of miRNA expression on the global behavior of the biological pathways.

Eight MCL cell lines were also investigated by comparing their miRNA expression signature with that of CD19+/IgD+/CD27− lymph node-sorted B cells. The miRNA signature (FDR <0.05) identified in MCL cell lines included one upregulated miRNA (>twofold), miR-182, which was also significantly upregulated in MCL cases, and 14 downregulated miRNAs (>twofold) (Supplementary Table S4), six of which were also lost in MCL cases, among them miR-26a and miR-150 as already described (Figure 3).

With less significant FDRs, several components of the let-7 family (f, c, g, e), which are known to regulate multiple stem cell-like properties by silencing multiples targets, including *RAS* and *HMGA2*,[40] were downregulated in MCL cases and cell lines. Interestingly, miR-29a and miR-29c, which regulate *TCL1A*[41] (upregulated in MCL), were lost in the MCL cell lines. MiR-31, which showed one of the greatest losses of all the miRNAs in this series, is predicted to be a regulator of *MAP3K14* (*NIK*) expression, a gene essential for the activation of the alternative NF-κB pathway.[42]

A cluster of miRNAs made up of miR-106b, miR-93 and miR-25, and located in 7q22, were significantly upregulated in our MCL series. This cluster functionally overlaps with the mir-17-92 polycistron,[43,44] known as *oncomir-1*. Its amplification in lymphoma and other tumor types has been linked to accelerated c-Myc-induced tumor development by suppression of the expression of the tumor suppressor *PTEN* and the proapoptotic protein Bim.[45] The gain of function of the miR-106b cluster promotes cell-cycle progression by silencing the cyclin-dependent kinase inhibitor p21/CDKN1A, a direct target of miR-106b. Interestingly, miR-106b overrides a doxorubicin-induced DNA damage checkpoint.[46] Consistent with this finding, many of the MCL cell lines showed stronger expression of the mir-17-92 polycistron.

The series of MCL patients also showed increased expression of miR-372 and miR-373, which are both involved in promoting proliferation and tumorigenesis in primary human cells that harbor active wild-type p53,[47] as found in most MCLs.[2] Finally, miR-210 has been shown to be induced by hypoxia in various tumor types.[48]

Association between miRNAs and mRNA signature genes and pathways

To identify statistically significant associations between differentially expressed miRNAs and gene expression signatures, we investigated whether predicted miRNA (FDR <0.05)–mRNA (FDR <0.01) targeting pairs were consistent with the pair

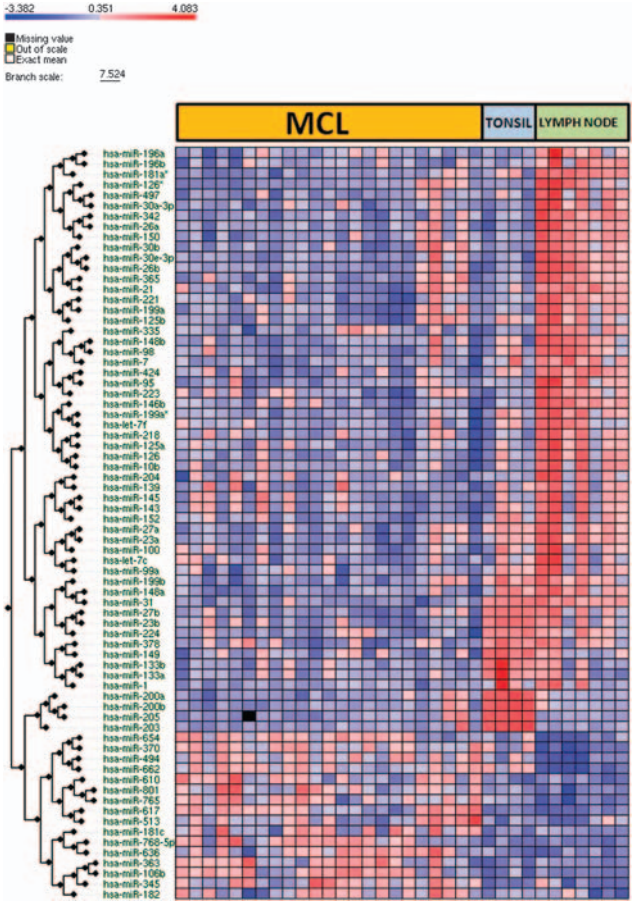

Figure 2 miRNA expression heatmap of 23 MCL samples and 11 control tissues. Significant miRNAs (FDR <0.05 and >twofold change) are illustrated: downregulated in blue, upregulated in red.

component inverse regulation, and whether the consistent pairs were nonrandomly associated. Gene expression results proceeding from the hybridization of 23 MCL samples and 11 controls (lymph nodes and tonsils) are reported in Supplementary text and Supplementary Table S5.

Target genes predicted with miRBase and TargetScan software were listed and Fisher's exact test was applied to significant miRNAs. miRBase and TargetScan jointly identified 21 downregulated and 4 upregulated miRNAs with FDR <0.05 on the basis of a significant results of Fisher's exact tests (Results in Supplementary Tables S6–S8 and Supplementary text).

At this point, connections between the miRNA signatures and the MCL-deregulated pathways were also examined using GSEA. Thus, miRNAs found to be significant by Fisher's exact test were matched with the target genes predicted by miRBase and TargetScan and grouped by significantly enriched GSEA pathways (FDR <0.26). A ranked list containing 3712 nonredundant upregulated genes predicted by miRanda (http://www.mirbase.org; Faculty of Life Sciences, University of Manchester) as targets for the downregulated miRNAs was used. The same approach was adopted for TargetScan predictions using a preranked list of 3861 nonredundant upregulated genes. While using GSEA with upregulated miRNAs, we followed the inverse approach, building a ranked list of predicted downregulated targets (537 miRanda targets and 1951 TargetScan targets).

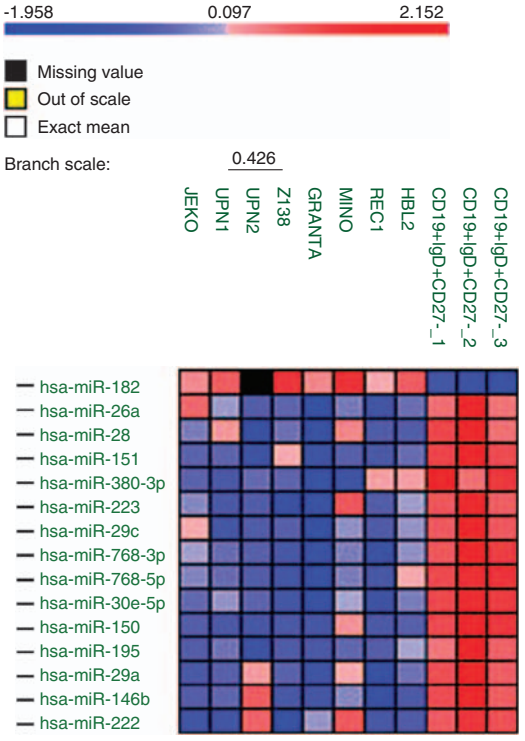

Figure 3 miRNA expression heatmap of MCL cell lines. Significant miRNAs (FDR <0.05 and >twofold change) are illustrated: downregulated in blue, upregulated in red.

Results are included in Table 1, Supplementary Table S9 and illustrated in Figure 4. The most remarkable upregulated pathways associated with losses of miRNAs targeting the genes included in the corresponding pathway were those of the CD40, NF-κB and mitogen-activated protein kinase (MAPK) pathways (and Supplementary Figure S1). We also performed GSEA using the whole gene expression list instead of target-oriented lists. We identified up to 23 significant gene sets related to MCLs, including three out of six gene sets whose expression seems to be closer to miRNA activity.

Interestingly, differentially expressed miRNAs that were significantly associated with gene expression profiles, as revealed by Fisher's exact test, were also capable of targeting more than one gene included in these pathways, which suggests a direct regulatory role in the aforementioned pathways.

No significant relations were found between upregulated miRNAs and downregulated pathways.

miRNA functional validation

MiR-26a was downregulated in MCL cases and cell lines. It was also significant in Fisher's exact test and in pathway analysis, thus it was chosen for functional validation. One of its predicted targets of greatest interest was *MAP3K2*. This was found to be upregulated in our samples and is already known as an NF-κB pathway-activating kinase.[49] NF-κB activation is a common and important finding in MCL cells, but the mechanism of activation is still essentially unknown.

The MINO and REC-1 MCL cell lines proved to be the best model for validation experiments because they have very low levels of miR-26a expression coupled with NF-κB activation, as demonstrated by RelA (p65) nuclear translocation: a finding commonly observed in MCL cases. MiR-26a and negative control

Table 1 GSEA contingency table

Number of genes included in the annotated pathways	*Number of miRNAs targeting the selected gene sets*	Gene set	*FDR of GSEA analysis using miRanda prediction*	*FDR of GSEA analysis using TargetScan prediction*
8	8	Bcells_IgMIgDCd27plus	0.15	0.16
9	7	Blimp1.targets	0.12	0.21
2	2	Blood.PanBcell	0.08	0.19
6	4	CD40.signalling.during.GC.dev	0.22	0.01
5	4	MAPKPATHWAY	0.26	0.14
9	8	NFKBtotalPATHWAY	0.24	0.16

Abbreviations: FDR, false discovery rate; GSEA, gene set enrichment analysis, miRNA, microRNA.
Pathway enrichment results (FDR <0.26) linked to differentially expressed genes (FDR <0.01) and differentially expressed miRNAs (FDR <0.05 and Fisher's exact test FDR <0.05).

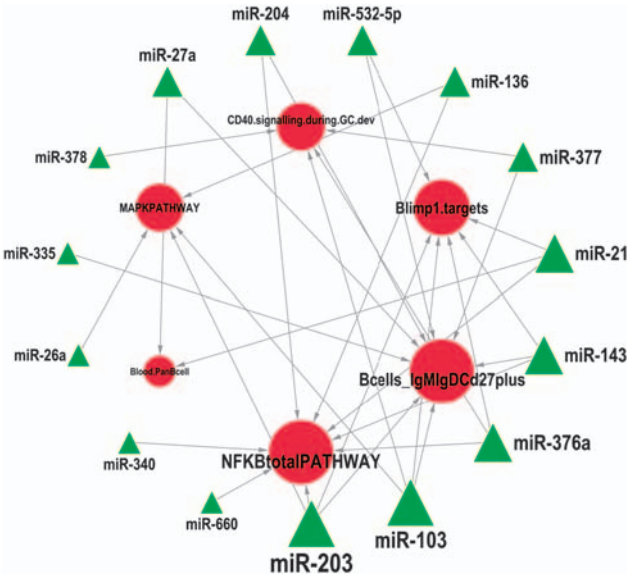

Figure 4 Downregulated miRNAs with connections to upregulated pathways. miRNAs are indicated by triangles, whereas pathways are represented by circles. Their size is proportional to their degree of connectivity. Red and green nodes represent upregulated and downregulated elements, respectively. All the connections represent significant relationships between the downregulated miRNAs and upregulated pathways targeted by the miRNAs.

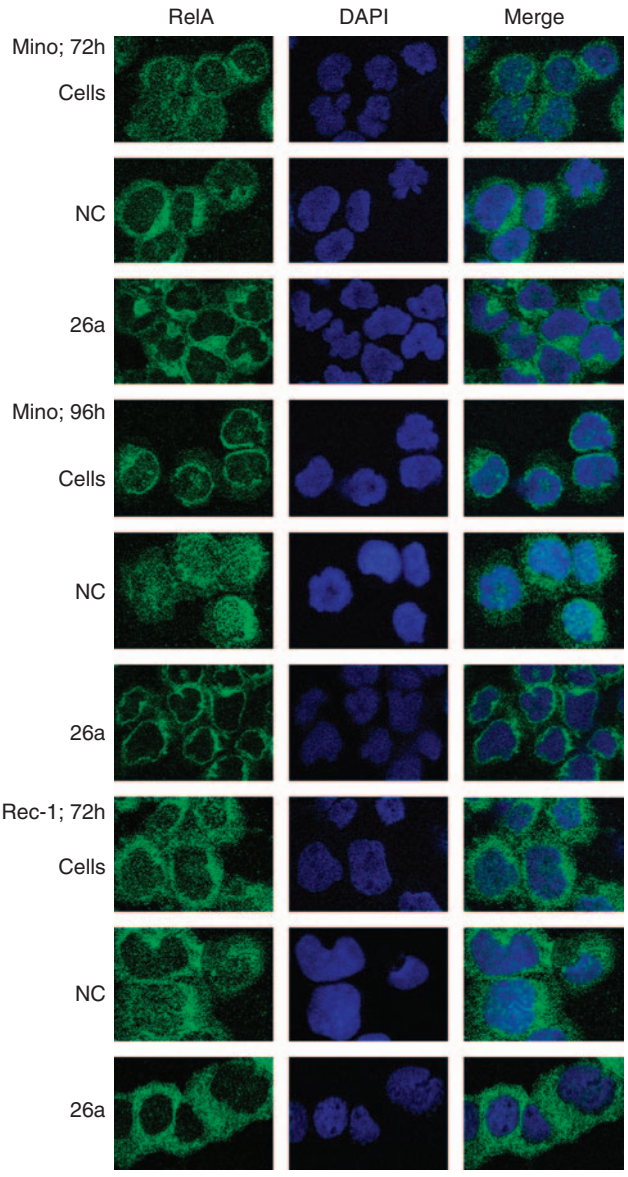

Figure 5 NF-κB pathway activation. RelA (p65) NF-κB subunit nuclear translocation after miR-26a, negative control miR microporation (60 nM) or untreated Mino cells at 72 and 96 h, Rec-1 cells at 72 h. RelA nuclear translocation is shown by Alexa Fluor 488 staining. Nuclei are stained with 4,6-diamidino-2-phenylindole (DAPI). Restoration of miR-26a reduces RelA nuclear translocation at 72 and 96 h after treatment.

miRNA were electroporated at 60 nM concentration and RelA translocation to the nucleus was checked at 24, 48, 72 and 96 h after electroporation. Induced expression of miR-26a abrogated the nuclear translocation of RelA at 72 and 96 h after treatment in the MINO cell line and at 72 h in the REC-1 cell line (Figure 5).

Clinical variability

To identify miRNAs of potential clinical prognostic value, miRNA microarray data of 22 MCL cases were analyzed with the Random Forest predictor.[36–38] The analysis yielded a set of miRNAs that gave a Kaplan–Meier survival curve (log-rank $P<0.001$) in which 12 miRNAs were statistically significant ($P<0.05$) (Supplementary Table S10). These miRNA were selected to confirm their expression in a second group of 54 formalin-fixed paraffin-embedded cases by quantitative reverse transcriptase PCR. MiR-198 was excluded from the analysis because it had a low efficiency of amplification in qPCR. After endogenous normalization, $-\Delta Ct$ values were used for overall survival analysis by Cox regression using SPSS v.

Table 2 Survival analysis by Cox regression

miRNA	*Univariate model*	
	P-*value*	*HR*
miR-130b	0.222	1.150
miR-454	0.239	1.154
miR-99b	0.756	0.964
miR-7	0.630	1.057
miR-181c	0.828	0.979
miR-532	0.880	0.986
miR-362	0.999	1.000
miR-363	0.300	1.149
miR-625	0.300	1.112
miR-20b	0.013	1.388
miR-660	0.527	1.065

Abbreviations: HR, hazard ratio; miRNA, microRNA.
Cox regression models: validation group, 54 samples. Individual results of Cox univariate regression models using the 11 miRNAs are shown, including *P*-value and HRs. Only miR-20b was significant ($P=0.013$).

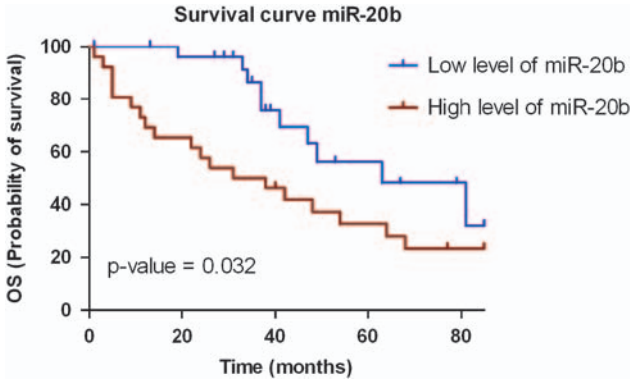

Figure 6 Correlation between miR20b expression and overall survival: confirmation group of 54 cases. Cox regression model was derived from the univariate analysis. The Kaplan–Meier survival curve (log-rank $P=0.032$) was calculated by stratifying the 54 samples into two subgroups according to median expression of the miR-20b. This function estimated a 56% survival rate at 60 months.

15.0. Univariate Cox regression analysis confirmed the significance of miR-20b as a prognostic marker ($P=0.013$) (Table 2), whereas other clinical variables (age <60 years, $P=0.499$; gender, $P=0.592$; performance status, $P=0.916$; clinical stage at diagnosis, $P=0.743$; and proliferation activity of the tumor, $P=0.317$) were found not to be the significant prognostic markers for survival in this group. Results were plotted in a Kaplan–Meier survival curve (log-rank $P=0.032$) (Figure 6), dividing the samples into two risk groups according to their median miR-20b expression. Cases lacking miR-20b expression had a survival probability of 56% at 60 months, whereas only 33% of patients included in the high-risk group (high level of expression of miR-20b) survived for >60 months.

Discussion

Gene expression profiling studies of MCL have revealed increased survival signaling,[2,3,50] but have not identified the mechanisms responsible. miRNA profiling identifies additional genes whose deregulation may enable us to explain MCL pathogenesis more fully, as indicated by the gene pathways targeted by the deregulated miRNAs. CD40, MAPK and NF-κB are among the most significantly deregulated pathways whose increased expression is known to be relevant in MCL pathogenesis.[1] In addition, MCL has a downregulated GC signature (including BCL6, LMO2, SERPIN9 and GCET2 genes) coupled with increased expression of the miRNAs targeting these genes. This suggests that absence of GC differentiation by MCL cells could depend on changes in the expression of multiple miRNAs that regulate the GC signature.

The most essential pathways and genes identified here are potentially targeted simultaneously by multiple miRNAs, suggesting that transcriptional regulation by miRNAs in MCL is the result of the concurrent deregulation of multiple miRNAs with similar targets. This is consistent with what is known about the role of miRNAs as fine-tuning regulators.[51]

Deregulation of the MAPK pathway is one of the cardinal findings in MCL, presumably in relation to CD40 signaling, as shown by this gene expression analysis and other functional studies.[3,52] These findings indicate that constitutive CD40 signaling in B cells selectively activates the noncanonical NF-κB pathway[53] and the MAP kinases, JNK and ERK. The data presented here show that the miRNAs deregulated in MCL characteristically target the CD40 signaling pathway and MAPK genes.

Most of these changes have been detected in MCL cases and MCL cell lines, although there are some intriguing minor variations. MCL cell lines showed gain of *oncomir-1* (17–92 polycistron), confirming previous observations in the Jeko1 cell line.[54] In contrast, MCL cases showed increased expression of the miRNAs miR-106b, miR-93 and miR-25, which are functionally homologous to the 17-92 polycistron, known as *oncomir-1*.[55] MCL cell lines are more representative of the blastoid form than of classic MCL, thus we may hypothesize that aggressive transformation is accompanied by changes in the expression of the miRNAs included in the 17-92 polycistron.

Some of these results coincide with other recently published findings in MCL, such as the increased expression of miR-124a, miR-155, miR-302c, miR-345, miR-373* and miR-210, together with loss of miR-150 and miR-142-3p (the latter with a less significant FDR).[56,57]

Selected results have been functionally validated. Thus, the restoration of miR-26a expression in the Mino and Rec-1 cell lines inhibited RelA nuclear translocation at 72 and/or 96 h, which is consistent with there being an indirect effect of the MAPK pathway and, in particular, of MAP3K2 protein on NF-κB activation. Interestingly, miR-26a, whose expression in normal mantle cells is confirmed by other groups,[58] is already known to have a role in other types of cancer.[59,60]

These data also identify potential new diagnostic and prognostic markers. MCL diagnosis requires some additional tools to enable CCND1-negative forms and blastic variants to be better recognized. In addition, MCL response to therapy is not uniformly unfavorable, and some MCL cases follow a relatively indolent clinical course. Interestingly, weak miR-20b expression can be useful for predicting clinical behavior, enabling a group of MCLs with higher survival probability to be distinguished. MiR-20b expression has been found to have a role in other type of cancers,[61–63] in which its high level of expression was associated with a worse prognosis, as is the case for what we found in MCL. It should be noted that miR-20b is localized in a cluster (X chromosome) that shares some similarities with *oncomir-1*, which is already known to be strongly expressed in MCL cell lines, and is homologous at 21 out of 23 nucleotides with miR-20a, a member of the aforementioned cluster.

The miRNA changes detected here can be explained only in a few cases by chromosomal gains and losses such as the loss of miR-31 (9p21), although the results were not significant (data not shown). Other researchers have already demonstrated that only the levels of miRs included in the miR-17-92 cluster were significantly related to genetic alterations at 13q31.[56] It is therefore likely that most of the changes require other explanations, such as epigenetic regulation or oncogene targeting.

These results are in accordance with those showing that individual and miRNA clusters regulate gene expression with overlapping patterns.[64] It is important to emphasize that the approach followed here, looking at the association of mRNA and miRNA expression, accounts for only a part of the ability of miRNA to modulate protein expression; as miRNA also regulates mRNA translation. However, these findings are consistent with others showing that mRNA destabilization is usually the main component of repression in more highly regulated targets[64] and the same approach can be used to investigate others malignancies. These results also identify miRNAs that could be targeted in future therapeutic experiments and suggest miR-20b as an important component in MCL survival to investigate more in depth.

Conflict of interest

The authors declare no conflict of interest.

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