

Instituto de Biología Molecular y Celular del Cáncer



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D SALAMANCA**

CAMPUS OF INTERNATIONAL EXCELLENCE

Doctoral Dissertation

**Analysis of the molecular heterogeneity and
disease evolution in chronic lymphocytic
leukemia by combining high-throughput genomic
and transcriptomic technologies**

With the approval of Salamanca University, this thesis will be defended on 31st May
2017, in the Lecture Hall, *Centro de Investigación del Cáncer*, Salamanca

Supervisors:

Prof. Dr. Jesús María Hernández Rivas
Dra. Ana Eugenia Rodríguez-Vicente
Dra. M^a Rocío Benito Sánchez

María Hernández Sánchez

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Esta tesis doctoral corresponde a un compendio de artículos, tres de ellos han sido publicados en revistas internacionales y el cuarto está en preparación:

Artículo 1:

Título: **MiRNA expression profile of chronic lymphocytic leukemia patients with 13q deletion.**

Autores: María Hernández-Sánchez¹, Ana E. Rodríguez-Vicente¹, José-Ángel Hernández², Eva Lumbreras¹, María-Eugenia Sarasquete¹, Ana-África Martín¹, Rocío Benito¹, Carlos Vicente-Gutiérrez¹, Cristina Robledo¹, Natalia de las Heras³, Juan-Nicolás Rodríguez⁴, Miguel Alcoceba¹, Alfonso García de Coca⁵, Carlos Aguilar⁶, Marcos González¹, Jesús-María Hernández-Rivas¹.

Afiliación de los autores: 1. Hematology Department, Centro de Investigación del Cáncer, IBSAL, IBMCC, Universidad de Salamanca-CSIC, Hospital Universitario de Salamanca, Salamanca, Spain; 2. Hematology Department, Hospital Universitario Infanta Leonor, Universidad Complutense de Madrid, Madrid, Spain; 3. Hematology Department, Hospital Virgen Blanca, León, Spain; 4. Hematology Department, Hospital Juan Ramón Jiménez, Huelva, Spain; 5. Hematology Department, Hospital Clínico Universitario, Valladolid, Spain; 6. Hematology Department, Hospital Santa Bárbara, Soria, Spain.

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Artículo 2:

Título: **A Low Frequency of Losses in 11q Chromosome Is Associated with Better Outcome and Lower Rate of Genomic Mutations in Patients with Chronic Lymphocytic Leukemia.**

Autores: José Ángel Hernández^{*1}, María Hernández-Sánchez^{*2}, Ana Eugenia Rodríguez-Vicente², Vera Grossmann³, Rosa Collado⁴, Cecilia Heras¹, Anna Puiggros⁵, Ana África Martín⁶, Noemí Puig⁶, Rocío Benito², Cristina Robledo², Julio Delgado⁷, Teresa González⁸, José Antonio Queizán⁹, Josefina Galende¹⁰, Ignacio de la Fuente¹¹, Guillermo Martín-Núñez¹², José María Alonso¹³, Pau Abrisqueta¹⁴, Elisa Luño¹⁵, Isabel Marugán¹⁶, Isabel González-Gascón¹, Francesc Bosch¹⁴, Alexander Kohlmann^{3,17}, Marcos González^{2,6}, Blanca Espinet⁵, Jesús María Hernández-Rivas^{2,6,18}, Grupo Cooperativo Español de Citogenética Hematológica (GCECGH) and Grupo Español de Leucemia Linfática Crónica (GELLC).

*These authors contributed equally to this work

Afiliación de los autores: 1. Hematology Department, Hospital Universitario Infanta Leonor, Universidad Complutense de Madrid, Spain; 2. IBSAL, IBMCC, Centro de Investigación del Cáncer, Universidad de Salamanca, CSIC, Hospital Universitario de Salamanca, Spain; 3. MLL

Munich, Germany; 4. Hematology Department, Hospital General, Valencia, León, Spain; 5. Pathology Department, Hospital del Mar, Barcelona, Spain; 6. Hematology Department, Hospital Universitario, Salamanca, Spain; 7. Hematology Department, Hospital Clinic I Provincial, Barcelona, Spain; 8. Fundación Pública Galega de Medicina Xenómica, Santiago de Compostela, Spain; 9. Hematology Department, Hospital General, Segovia, Spain; 10. Hematology Department, Hospital del Bierzo, Ponferrada, Spain; 11. Hematology Department, Hospital Universitario Río Hortega, Valladolid, Spain; 12. Hematology Department, Hospital Virgen del Puerto, Plasencia, Cáceres, Spain; 13. Hematology Department, Hospital Río Carrión, Palencia, Spain; 14. Hematology Department, Hospital Vall d'Hebron, Barcelona, Spain; 15. Hematology Department, Hospital Central de Asturias, Oviedo, Spain; 16. Hematology Department, Hospital Clínico, Valencia, Spain; 17. AstraZeneca, Personalized Healthcare and Biomarkers, Innovative Medicines, Macclesfield, United Kingdom; 18. Department of Medicine, Universidad de Salamanca, Spain.

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Artículo 3:

Título: ***TET2* overexpression in chronic lymphocytic leukemia is unrelated to the presence of *TET2* variations.**

Autores: María Hernández-Sánchez¹, Ana Eugenia Rodríguez¹, Alexander Kohlmann², Rocío Benito¹, Juan Luis García³, Alberto Risueño^{4,5}, Encarna Fermiñán⁶, Javier de las Rivas⁵, Marcos González^{1,7}, Jesús-María Hernández-Rivas^{1,7}.

Afiliación de autores: 1. IBSAL, IBMCC, Centro de Investigación del Cáncer, IBSAL, IBMCC, Universidad de Salamanca-CSIC, Salamanca, Spain; 2. Department of Molecular Genetics, MLL Munich Leukemia Laboratory, Munich, Germany; 3. Instituto de Estudios de Ciencias de la Salud de Castilla y León (IESCSCYL) and Hospital Clínico Universitario de Salamanca (HUSAL), Salamanca, Spain; 4. Celgene Institute for Translational Research Europe (CITRE), Sevilla, Spain; 5. Unidad de Bioinformática y Genómica Funcional, IBMCC, Centro de Investigación del Cáncer, Universidad de Salamanca-CSIC, Salamanca, Spain; 6. Unidad de Genómica, IBMCC, Centro de Investigación del Cáncer, Universidad de Salamanca-CSIC, Salamanca, Spain; 7. Servicio de Hematología y Departamento de Medicina, Hospital Clínico Universitario de Salamanca, Salamanca, Spain.

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Autores: María Hernández-Sánchez¹, Ana E Rodríguez¹, María Abáigar¹, David Tamborero², Rocío Benito¹, Miguel Quijada¹, Ana-África Martín¹, Alfonso García de Coca³, Nuria López-Bigas², Jesús-María Hernández-Rivas¹.

Afiliación de autores: 1. Hematology Department, IBSAL, IBMCC-Cancer Research Center, Hospital Universitario Salamanca, University of Salamanca, Salamanca, Spain; 2. Research Program on Biomedical Informatics, IMIM Hospital del Mar Medical Research Institute and Universitat Pompeu Fabra, Barcelona, Spain; 3. Hematology Department, Hospital Clínico Universitario of Valladolid, Valladolid, Spain.

Estado del artículo: manuscrito **en preparación**.

D. **Jesús María Hernández Rivas**, Doctor en Medicina, Profesor Titular del Departamento de Medicina de la Universidad de Salamanca, Médico Adjunto del Servicio de Hematología del Hospital Clínico Universitario de Salamanca e Investigador del Centro de Investigación del Cáncer de Salamanca,

D.^a **Ana Eugenia Rodríguez Vicente**, Doctora por la Universidad de Salamanca e investigadora del Centro de Investigación del Cáncer e Instituto de Investigación Biomédica de Salamanca,

D.^a **María del Rocío Benito Sánchez**, Doctora por la Universidad de Salamanca e investigadora del Centro de Investigación del Cáncer e Instituto de Investigación Biomédica de Salamanca,

CERTIFICAN:

Que D.^a **María Hernández Sánchez**, licenciada en Biotecnología por la Universidad de Salamanca, ha realizado bajo mi dirección el trabajo de Tesis Doctoral titulado *“Analysis of the molecular heterogeneity and disease evolution in chronic lymphocytic leukemia by combining high-throughput genomic and transcriptomic technologies”*, y que éste reúne, a nuestro juicio, las condiciones de originalidad y calidad científica requeridas para su presentación y defensa ante el tribunal correspondiente para optar al grado de Doctor, con mención “Doctor Internacional”, por la Universidad de Salamanca.

La tesis doctoral ha sido escrita en inglés y, de acuerdo con la normativa de la Universidad de Salamanca para la obtención del título de Doctor, la doctoranda presenta un resumen significativo en castellano de la misma.

Y para que así conste a los efectos oportunos, firmamos el presente certificado en Salamanca, a 28 de febrero de 2017.

Fdo.:

Prof. Dr. Jesús M. Hernández Rivas

Dra. Ana E. Rodríguez Vicente

Dra. M. Rocío Benito Sánchez

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List of abbreviations

ABT	Single-agent Bcl-2 inhibitor	CRISPR	Clustered regularly interspaced short palindromic repeats
aCGH	Array comparative genomic hybridization	cRNA	Complementary RNA
ADAM29	A disintegrin and metalloprotease domain 29	C-terminal	Carboxy terminal
AKT	V-Akt murine thymoma viral oncogene homolog 1	CTLA-4	Cytotoxic T-lymphocyte antigen 4
allo-HSCT	Allogeneic stem cell transplantation	DDX3X	DEAD-box helicase 3 X-linked
AML	Acute myeloid leukemia	del	Deletion
ASXL1	Additional sex combs like transcriptional regulator 1	DICER1	Double-stranded RNA-specific endoribonuclease 1
ATM	Ataxia telangiectasia mutated	DLEU7	Deleted in lymphocytic leukemia 7
BAX	B-cell CLL/lymphoma 2 associated X Protein	DNA	Deoxyribonucleic acid
BAZ2A	Bromodomain adjacent to zinc finger domain 2A	dNTP	Deoxynucleosidetriphosphates
BCL2	B-cell lymphoma 2	E2F	E2 factor
BCL-7A	B-cell CLL/lymphoma 7A	ECOG	Eastern Cooperative Oncology Group
BCOR	B-cell CLL/lymphoma 6 corepressor	EGR2	Early growth response 2
BCR	B-cell receptor	ERIC	European Research Initiative on Chronic lymphocytic leukemia
BIC	B cell integration cluster	ESP	Exome sequencing project
Bim	Bcl-2-like protein 11	ExAC	Exome Aggregation Consortium
BIRC3	Baculoviral inhibitors of apoptosis protein repeat-containing 3	FAT	Focal adhesion targeting
bp	Base pair	FBXW7	F-box and WD repeat domain containing 7
BR	Bendamustine plus rituximab	FC	Fludarabine with cyclophosphamide
BRAF	V-raf murine sarcoma viral oncogene homolog B	FCR	Fludarabine with cyclophosphamide and rituximab
BTK	Bruton's tyrosine kinase	FDA	Food and Drug Administration
BWA	Burrows-wheeler aligner	FDR	False discovery rate
C/EBPβ	CCAAT/enhancer binding protein beta	FISH	Interphase fluorescence in situ hybridization
CARD11	Caspase recruitment domain family member 11	FOXO3a	Forkhead box O3
CART	Chimeric antigen receptor T	fs	Frame-shift
CCND2	G1/S-specific cyclin D2	FUBP1	Far upstream element binding protein 1
CD	Cluster of differentiation	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
CDC73	Cell division dycle 73	GATA3	GATA Binding Protein 3
cDNA	Complementary DNA	GATK	Genome analysis toolkit
CGC	Conventional G-banding cytogenetics	GCECGH	Spanish Group of Cytogenetics
CGI	Cancer Genome Interpreter	GELLC	Spanish Group of Chronic Lymphocytic Leukemia
CHD2	Chromodomain helicase DNA binding protein 2	GEP	Gene expression profile
Clb	Chlorambucil	GNAQ	G protein subunit alpha Q
CLL	Chronic lymphocytic leukemia	GS	Genome sequencer
CLL-IPi	International prognostic index of CLL	Hb	Hemoglobin
CMML	Chronic myelomonocytic leukemia	HDAC	Histone deacetylases
COSMIC	Catalogue of somatic mutations in cancer	HDAC2	Histone deacetylase 2
		HEK293T	Human embryonic kidney cell-line 293T

HIST1H1B	Histone cluster 1 H1 family member B	MYD88	Myeloid differentiation primary response 88
hmC	Hydroxymethylcytosine	NCI	National Cancer Institute
hsa	Homo sapiens	nCLL	CLL with normal FISH
HSCs	Hematopoietic stem cells	NF-kB	NF-kappa-B
ID	Identifier	NFKB2	Nuclear factor kappa B subunit 2
IGH	Immunoglobulin heavy chain	NFKBIE	NF-kappa-B inhibitor epsilon
IGHV	Immunoglobulin heavy-chain variable region	NGS	Next-Generation Sequencing
IKBR	I-kappa-B-related protein	NOTCH1	Neurogenic locus notch homolog 1
IKZF3	Ikaros family zinc finger protein 3	NRAS	Neuroblastoma RAS Viral Oncogene Homolog
INPP5D	Inositol polyphosphate-5-phosphatase D	NRIP1	Nuclear receptor-interacting protein 1
ins	Insertion	NT	Non-truncating mutation
IPA	Ingenuity Pathway Analysis	NXF1	Nuclear RNA Export Factor 1
IRAK1/4	Interleukin-1 receptor-associated kinase-1/4	OS	Overall survival
IRF4	Interferon regulatory factor 4	PABPC3	Poly(A) binding protein cytoplasmic 3
IRON	Interlaboratory Robustness of Next-Generation Sequencing	PCR	Polymerase chain reaction
iwCLL	International working group of chronic lymphocytic leukemia	PEST	Peptide sequence enriched in proline, glutamic acid, serine, and threonine
JAK2	Janus kinase 2	PGM	Personal Genome Machine
KRAS	Kirsten rat sarcoma viral oncogene homolog	PI3K	Phosphoinositide-3-kinase
LDH	Lactate dehydrogenase	PLCG2	Phospholipase C gamma 2
LDT	Lymphocyte doubling time	PLK1	Polo like kinase 1
LPL	Lipoprotein lipase	POT1	Protection of telomeres protein 1
MACS	Magnetically activated cell sorting	PRC1	Protein regulator of cytokinesis 1
MAPK	Mitogen-activated protein kinase	pre-miRNA	Precursor microRNA
MAPK12	Mitogen-activated protein kinase 12	PTEN	Phosphatase and tensin homolog
MAPK7	Mitogen-activated protein kinase 7	PTPN11	Protein tyrosine phosphatase non-Receptor Type 11
Mb	Megabase	qRT-PCR	Real time polymerase chain reaction
MBL	Monoclonal B-cell lymphocytosis	R	Rituximab
MCL1	Myeloid cell leukemia 1	RB1	Retinoblastoma 1
MDM2	Mouse double minute 2 homolog	REL	V-rel avian reticuloendotheliosis viral oncogene homolog
MDR	Minimal Deleted Region	RhoA	Ras homolog family member A
MDS	Myelodysplastic syndromes	RMA	Robust microarray analysis
MED12	Mediator of RNA polymerase II transcription subunit 12	RNA	Ribonucleic acid
MGA	Max Dimerization Protein 5	RPS15	Ribosomal protein S15
MIDs	Multiplex identifiers	ROR1	Receptor tyrosine kinase like orphan receptor 1
miRNA/miR	Micro ribonucleic acid	SAM	Significance analysis of microarray
MLL2	Myeloid/lymphoid or mixed-lineage leukemia protein 2	SAMHD1	Sterile alpha motif domain and HD domain-containing protein 1
MPN	Myeloproliferative neoplasms	SCALE	Scandinavian population-based case-control study
MRD	Minimal residual disease	SET2	SET Domain Containing 2
mRNA	Messenger RNA	SF3B1	Splicing factor 3B subunit 1
MYC	Myelocytomatosis viral oncogene	sgRNA	Short guide RNA

SHIP	SH2-containing inositol 5'-phosphatase	TP53	Tumor protein p53
SIFT	Scale-invariant feature transform	TP53INP1	Tumor protein P53 inducible nuclear protein 1
slg	Surface membrane immunoglobulin	TRAF3	Tumor necrosis factor receptor associated factor 3
SLL	Small lymphocytic lymphoma	TRAF6	TNF receptor-associated factor 6
SNP	Single-nucleotide polymorphism	UTR	Untranslated region
SNV	Single-nucleotide variant	vs.	Versus
SOCS1	Suppressor of cytokine signaling 1	WES	Whole-exome sequencing
SOLiD	Sequencing by Oligonucleotide Ligation and Detection	WGS	Whole-genome sequencing
SP	Sequencing primer	WHO	World Health Organization
SPEN	Spen family transcriptional repressor	Wnt3	Wnt family member 3
SRA	Sequence Read Archive	XPO1	Exportin 1
SSA	Spliceosome inhibitor spliceostatin A	ZAP70	Zeta-chain-associated protein
STAT3	Signal transducer and activator of transcription 3	ZC3H18	Zinc finger CCCH-type containing 18
TCF7	T-cell-specific transcription factor 1	ZMYM3	Zinc finger MYM-type containing 3
TCL1	T-cell leukemia/lymphoma protein 1	β2M	β2-microglobulin
TET2	Tet methylcytosine dioxygenase 2	+12	Trisomy 12
TFT	Time to first treatment	11q-	11q deletion
TK1	Thymidine kinase 1	11q-H	11q deletion in 40% or more cells
TLR	Toll-like Receptors	11q-L	11q deletion in less than 40% cells
TLR2	Toll-like receptor 2	13q-	13q deletion
TM	Time-point	13q-H	13q deletion in 80% or more cells
TNFAIP3	Tumor necrosis factor alpha-induced protein 3	13q-L	13q deletion in less than 80% cells
TOP2A	Topoisomerase (DNA) II Alpha	17p-	17p deletion

Introduction

1. General characteristics of CLL

Chronic lymphocytic leukemia (CLL) is a hematological malignancy characterized by the presence of mature clonal B lymphocytes that accumulate in the blood, bone marrow and other lymphoid tissues. The World Health Organization (WHO) classification of hematological malignancies describes CLL as a leukemic, lymphocytic lymphoma, being only distinguishable from small lymphocytic lymphoma (SLL) by its leukemic appearance.^{1,2}

1.1. Epidemiology

CLL is the most common leukemia in the Western World, accounting for approximately 11% of all hematologic neoplasms and 40% of all adult leukemias. The incidence is about 3 per 100,000 inhabitants in Europe and North American while is rare among Asian or African people.³⁻⁷ CLL is mainly a disease of the elderly with a median age around 70 years. The incidence also varies with sex, being males affected more often than females (1.7-1).⁸⁻¹² The prevalence of CLL is likely to increase further due to the demographic changes in society in the forthcoming decades. Moreover, the proportion of younger patients with early stage CLL and minimal symptoms seems to increase due to more frequent blood testing.^{13,14}

1.2. Etiology

The etiology of CLL remains unknown. The basis for geographical differences of incidence is still elusive and may reflect a combination of genetic and environmental factors.¹⁵ CLL can occur as a sporadic event but there is strong evidence that a genetic component contributes as a family history of CLL or other lymphoid malignancies is stated a risk factor.¹⁶⁻²⁷

1.3. Diagnosis and clinical aspects

The international working group of CLL (iwCLL) has provided precise guidelines about the diagnosis and management of CLL. The diagnosis of CLL is defined by the presence of $\geq 5 \times 10^9/L$ B lymphocytes in peripheral blood for at least three months. The clonality of the circulating B-lymphocytes needs to be confirmed by flow cytometry.^{1,13} The immunophenotype of CLL distinguishes it from other B hematological malignancies by the expression of B cell markers such as CD19, CD23, and weak CD20, along with CD5, a T cell antigen, and low expression levels of surface membrane immunoglobulin (sIg).²⁸

The CLL malignant cells found in the blood smear are characteristically small and mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin. Large atypical cells, cleaved cells, or prolymphocytic cells, which may be up to 55% of the blood lymphocytes could also be observed.²⁹ Grumprecht nuclear shadows, or smudge cells, found as cell debris, are other characteristic morphologic features in CLL.

Despite the homogeneous morphological and immunological phenotype, the clinical presentation of CLL at diagnosis is variable: patients with CLL are generally diagnosed with an asymptomatic disease by blood tests performed during a routine physical exam; however, others patients showed symptoms such as fatigue, fever, lymphadenopathy, hepatomegaly, splenomegaly, bone marrow failure, recurrent infections and/or weight loss.^{30,31}

CLL could be preceded by an asymptomatic condition termed monoclonal B-cell lymphocytosis (MBL), in which CLL-like cells are found in the peripheral blood of otherwise healthy individuals with a count of $<5 \times 10^9/L$ clonal B lymphocytes.^{32,33} Progression to frank CLL occurs in ~1–2% of individuals with high-count MBL ($>0.5 \times 10^9/L$ monoclonal B cells) per year, probably as a result of progressive accumulation of genetic and epigenetic lesions as well as environmental factors.³⁴⁻³⁶ In addition, CLL can rarely transform into clonally related or unrelated diffuse large B cell lymphoma (Richter's transformation), Hodgkin lymphoma or interdigitating dendritic cell sarcoma (2–10% cases).^{37,38}

1.4. Prognostic factors

The natural course of CLL is highly heterogeneous. Whereas some patients suffer from aggressive disease with early need for treatment and significantly shorter overall survival, inferior to 2-3 years, others have indolent disease without any need of treatment at all and identical survival to their CLL free counterparts of the same age.³⁹ Therefore, there has been always a huge interest in defining prognostic markers for patient management. A brief enumeration of them and their implication in prognosis is detailed below (Table 1).

Table 1. Classical and new prognostic factors in patients with CLL

Prognostic factor	Predict poor prognosis
Clinical	
Binet stage	Advanced clinical stage
Rai stage	Advanced clinical stage
Lymphocytes morphology	Atypical and presence of prolymphocytes (>10%)
LDT	Short (<6 months with 30×10^9 lymphocytes/L)
Bone marrow infiltration	Diffuse pattern
Biological	
Serum	
LDH	High levels
$\beta 2M$	High levels
TK1	High levels
Genetic	
<i>IGHV</i> mutational status	Unmutated
<i>IGHV</i> rearrangements	VH3-21
Cytogenetic alterations	17p-/11q- High % of altered cells
Karyotype	Complex
Somatic mutations	<i>TP53</i> , <i>ATM</i> , <i>SF3B1</i> , <i>NOTCH1</i> , <i>BIRC3</i> mutated High number of mutated driver genes
Protein	
ZAP70	High expression ($\geq 30\%$)
CD38	High expression ($\geq 20\%$)
CD49d	High expression ($\geq 30\%$)
Other	
MRD	Positive
miRNA	Overexpression of miRNA-155

LDT: Lymphocyte doubling time; LDH: Lactate dehydrogenase; $\beta 2M$: $\beta 2$ -microglobulin; TK1: Thymidine kinase 1; *IGHV*: Immunoglobulin heavy-chain variable region; MRD: Minimal residual disease; miRNA: microRNA

▪ Clinical markers

Clinical staging systems for CLL were developed by Rai and Binet around 40 years ago and remain widely used in clinical practice.^{40,41} They are based on physical examination and blood counts, being inexpensive and easy to apply. Both staging systems are still the most common and validated prognostic factors in the patients with CLL. However, there is considerable variation in terms of outcome within each clinical stage, and these systems do not fully reflect the high variability of CLL and do not predict survival and response to therapy, particularly in low-risk patients. Therefore, additional clinical factors are more commonly considered in predicting individual patient prognosis and stratifying patient risk: i) lymphocytes morphology in peripheral blood -atypical morphology of CLL cells, a high number of prolymphocytes are linked to a poor prognosis-;^{42,43} ii) blood lymphocyte doubling time

(LDT) -short LDT less than 6 months when lymphocyte counts above $30 \times 10^9/L$;^{1,44,45} iii) bone marrow infiltration degree -diffuse pattern of infiltration is associated with a poor outcome-⁴⁶⁻⁴⁸

- **Biological markers**

Over the last ten years, several biological markers such as serum markers, immunoglobulin heavy-chain variable region (*IGHV*) mutation status, cytogenetic abnormalities or expression of specific proteins on CLL cells have become important prognostic factors.⁴⁹⁻⁵² They are available in routine clinical practice and may even guide treatment decisions. Recently, the improvements in the Next-Generation Sequencing (NGS) technologies have revealed novel genomic markers with important prognostic value.

- **Serum markers**

Most available serum markers providing useful prognostic information in CLL are: lactate dehydrogenase (LDH), $\beta 2$ -microglobulin ($\beta 2M$), thymidine kinase 1 (TK1) and soluble CD23. A high level of them was found in CLL patients with a more aggressive disease.⁵³⁻⁶⁵ Despite these inputs into CLL prognostication, the genetic and protein biomarkers advent has shifted the focus to factors providing more disease-specific information regarding survival and treatment response.

- **Genetic markers**

- ***IGHV* mutation status**

The somatic hypermutation of the variable region of the *IGHV* has become one of the most important molecular parameters to define the outcome in CLL patients. Based on the cutoff value of 98% sequence identity with the corresponding germline *IGHV*, two different subsets of CLL can be identified: *IGHV*-mutated CLL and *IGHV*-unmutated CLL.^{66,67} *IGHV* somatic mutations occur in approximately half of the cases and usually present with non-progressive disease. By contrast, unmutated *IGHV* patients have a more aggressive disease with a shorter time to first treatment (TFT) and overall survival (OS) and correlate with other poor prognostic features.

Furthermore, irrespective of mutation status, some heavy-chain variable regions have been associated with specific clinical outcomes like the rearrangement *IGHV3-21* linked to a poor prognosis.⁶⁸⁻⁷⁰ Moreover, *IGHV4-39* gene usage has been recently reported to predispose to increased risk of Richter's transformation.⁷¹

➤ Cytogenetic alterations

The most common cytogenetic abnormalities which can be used as prognostic factors are deletions of the long arm of chromosome 13 (13q-), long arm of chromosome 11 (11q-) and short arm of chromosome 17 (17p-) as well as trisomy 12 (+12). These cytogenetic abnormalities can be assessed by interphase fluorescence in situ hybridization (FISH) allowing to stratify CLL patients into five prognostic categories with different survival times: patients with 17p- have the worst prognosis, followed by 11q-, trisomy 12 and normal diploid karyotype, whereas patients with 13q- have the best prognosis.⁷² This model has been widely confirmed by other studies.⁷³⁻⁷⁸

Moreover, recent studies have described that the percentage of cells with a cytogenetic alteration defined by FISH has a prognostic value in CLL.⁷⁹ This fact was showed for the first time by our group in patients with 13q deletion as the sole aberration.⁷⁸ More in detail, we demonstrated a significantly shorter OS and TFT in the group of patients with losses in 13q in 80% or more of cells as compared to those cases with less than 80% of 13q- cells. Later, three independent studies, using different cutoffs, corroborated that the percentage of cells displaying 13q deletion influences the outcome of CLL patients.⁸⁰⁻⁸² Analogously to 13q deletion, a high percentage of cells with other cytogenetic alterations such as 11q-, 17p- or +12 has a negative prognostic impact.⁸³⁻⁸⁶

Conventional karyotype studies have recognized that patients with complex karyotype (defined by three or more alterations) have inferior prognosis as compared to those with diploid cytogenetics.⁸⁷⁻⁹² However, conventional cytogenetic analysis of genomic aberrations by chromosome banding has turned out as not easy due to the low proliferation rate of the CLL cells. Recently, several publications have underscored the value of conventional cytogenetics using novel mitogens to stimulate the proliferation of CLL cells.⁹³⁻⁹⁵

In addition, the high-throughput technologies such as array comparative genomic hybridization (aCGH) and high resolution single-nucleotide polymorphism (SNP) arrays have showed other genetic abnormalities which are present in a low proportion of CLL cases but also have prognostic impact.⁹⁶⁻⁹⁸

➤ Somatic mutations

Additional information has come from the presence of mutations in genes, which could be also deleted in CLL such as *TP53* and *ATM*, mapping on 17p13 and 11q22, respectively:

i) Disrupted *TP53* function due to 17p deletion and/or *TP53* gene mutation is an important predictor of chemorefractoriness and is associated with reduced survival.^{77,99-103} In fact, the only biomarker that currently changes the treatment strategy is the presence of *TP53* abnormalities (17p-/*TP53* mutations), being clinically important markers that should be assessed before every new treatment line.^{1,104-106} Luckily, a number of novel biological drugs such as B-cell receptor inhibitors have been developed and incorporated in the treatment of these patients with encouraging results.^{104,107}

ii) *ATM* mutations are associated with a decreased OS, with unfavorable features (unmutated *IGHV* and 11q-), and poor response to chemotherapy.^{108,109} Noteworthy is the fact that patients with biallelic inactivation of *ATM* (11q- and mutation in *ATM*) have a poorer outcome than those with a monoallelic *ATM* alteration.¹⁰⁹

With the advent of NGS, novel gene mutations were discovered in CLL patients. Among them, mutations which have shown an adverse prognostic impact include *NOTCH1*, *SF3B1* and *BIRC3* while the presence of *MYD88* mutations has a favorable impact.^{71,110-118} More recent studies have also identified additional gene mutations that may confer a worse outcome in CLL (*NKFB1E*, *EGR2*, *RPS15* and *MED12*), although they have been less studied.¹¹⁹⁻¹²² In a recent multicenter study conducted within the European Research Initiative on CLL (ERIC), sequencing of *TP53*, *NOTCH1*, *SF3B1*, *BIRC3* and *MYD88* was performed in a large patient series, revealing that, apart from *TP53*, *SF3B1* mutations also remained as independent prognostic markers of shorter TFT in multivariate analysis, even among unmutated *IGHV* patients.¹²³ Moreover, the mutational complexity can be informative as it dissects a specific prognosis for each combination of mutated drivers. In fact, the accumulative number of driver alterations (0 to ≥ 4) discriminated between patients with different clinical behavior.¹²⁴ Specifically, multiple mutations (most often *TP53*+*NOTCH1*+*ATM*) conferred a significantly poorer outcome.¹²⁵

In addition, the enormous capacity of NGS can be also directed to detect mutations existing at very low subclonal levels. In CLL, subclonal populations are linked to poorer clinical outcome, implying that an active evolutionary process is the

underlying basis of aggressive disease.^{126,127} In particular, subclonal mutations in *TP53* are of prognostic importance.¹²⁸⁻¹³¹

- **Protein markers**

Extensive efforts have been made to find protein markers which can substitute *IGHV* mutation status as its analysis is laborious. Among them, CD38, zeta-chain-associated protein (ZAP70) and CD49d stand out. CD38 and ZAP70 were firstly described as surrogate markers for *IGHV*, but this has been controversial.^{66,132} However, further studies demonstrated that both are independently associated with poor outcome regardless of the mutational status.^{66,133-135} Nevertheless, the assessment of a prognostic statement has been difficult due to technical aspects like the choice of an optimal cutoff for the number of CD38 or ZAP70 positive cells determined by flow cytometry. Furthermore, CD38 expression of the leukemia clone may change during the course of the disease.¹³⁶ In addition, CD49d has also been correlated with shorter survival times when expressed at high levels and has recently emerged as the strongest flow cytometry predictor for OS.^{137,138}

- **Other markers**

The assessment of minimal residual disease (MRD) lately has become an important endpoint with prognostic impact as the achievement of lower levels of MRD in response to fludarabine with cyclophosphamide and rituximab (FCR) therapy was shown to be independently predictive for superior progression free and overall survival.^{139,140} MRD assessment is recommended in clinical trials using standardized protocols of either four-color flow cytometry or allele specific oligonucleotide PCR (one CLL cell in 10,000 leukocyte sensitivity),¹⁴¹ although evaluation of MRD is currently not recommended for routine clinical practice.¹ Interestingly, the combination of several markers has permitted a lower detection limit and this could translate into better clinical outcome.¹⁴² In line with this, high-throughput sequencing can reliably detect disease below the levels that can be assessed by flow cytometry. It is likely therefore that sequencing, either by itself or in combination with flow cytometry, may prove to be a valuable resource to improve MRD detection.¹⁴³

Looking into the non-coding genome, the dysregulation of some microRNAs (miRNAs, miRs), such as miR-21, miR-29, miR-34a, miR-181b, miR-223, miR-155, has been suggested as prognostic markers.¹⁴⁴⁻¹⁵⁰ More recently, patients showing recurrent mutations in the 3' UTR of *NOTCH1* behaved similarly to patients with coding mutations in *NOTCH1* in terms of the TFT and OS.^{124,151}

■ Prognostic models

The management of CLL patients is currently undergoing improvements due to a high number of biological and, especially, genetic variables that add prognostic information to the classic clinical staging systems.^{40,41} The current challenge is to build a prognostic model that is clinically relevant, easily applicable, oriented to take therapeutic decisions and feasible in the clinical practice setting. With these goals, some attempts have been done and several comprehensive prognostic models combining clinical, biological, and genetic information have been proposed (Table 2).^{110,152-156}

Table 2. Summary of prognostic models for CLL

System	Markers used	Prognostic groups
Rai (1975) ⁴⁰		
	Hematological blood counts (lymphocytes, Hb, platelets), physical examination (lymphadenopathy, hepatomegaly, splenomegaly)	Low risk stage 0: only lymphocytosis (>5,000 monoclonal B-lymphocytes/μl) in peripheral blood and bone marrow
		Intermediate risk stage I: lymphocytosis and lymphadenopathy (≥1)
		Intermediate risk stage II: lymphocytosis, lymphadenopathy (≥1), and hepatomegaly or splenomegaly (≥1)
		High risk stage III: lymphocytosis together with anemia (Hb<11g/dl)
		High risk stage IV: lymphocytosis together with anemia (Hb<11g/dl) and/or thrombocytopenia (platelets <100,000/μl)
Binet (1981) ⁴¹		
	Hematological blood counts (Hb, platelets), physical examination (lymph node regions)	Stage A (low risk): <3 lymph node regions, Hb ≥10 g/dl and platelets ≥100,000/μl
		Stage B (intermediate risk): ≥3 lymph node regions, Hb ≥10g/dl and platelets ≥100,000/μl
		Stage C (high risk): Hb<10g/dl and/or platelets <100,000/μl
Döhner (2000) ⁷²		
	FISH cytogenetic alterations	13q- (low risk)
		Normal karyotype (low risk)
		+12 (intermediate risk)
		11q- (high risk)
		17p- (high risk)
Wierda (2007) ¹⁵²		
	For OS: age (<50 years =score 1; 50-65 years =score 2; >65 years =score 2), absolute lymphocyte count (20,000-50,000/μl =score 1; >50,000/μl =score 2), β2M (1-2 X upper limit of normal =score 1; >2 x upper limit of normal =score 2), Rai stage (0-II =score 0; III-IV =score 1), sex (male =score 1), lymph node regions (>3 = score 1)	Low risk: score 1-3
		Intermediate risk: score 4-7
		High risk: score ≥8

Haferlach (2010) ¹⁵⁴		
	For OS: age (≥65 years =score 1), lymphocyte count (≥20,000 leucocytes/μl = score 1), <i>IGHV</i> mutation status (unmutated =score 1), TP53 deletion (score 2), IGH translocations (score 2), number of cytogenetic alterations (0 =score 0; 1 or 2=score 1; 3 =score 2)	Favorable risk =score 0-3
		Intermediate risk =score 4-5
		Unfavorable risk> score 5
Rossi (2013) ⁷¹		
	FISH cytogenetic alterations, gene mutations	Very low risk: 13q- only
		Low risk: +12 or a normal FISH
		Intermediate risk: <i>NOTCH1</i> mutations and/or <i>SF3B1</i> mutations and/or 11q-
		High risk <i>TP53</i> and/or <i>BIRC3</i> abnormalities
Pflug (2014) ¹⁵⁶		
	Sex (male =score 1), TK (>10u/l =score 2), β2M (1.7-3.5 mg/dl =score 1; >3.5 mg/dl =score 2), IGHV mutation status (unmutated =score 1), ECOG (>0 =score 1), FISH aberrations (11q- =score 1; 17p- =score 6), age (>60 years =score 1)	Low risk: score =0-2
		Intermediate risk: score =3-5
		High risk: score =6-10
		Very high risk: score =11-14
IPI Project (2016) ¹⁵⁷		
	TP53 status (17p- and/or TP53 mutation =score 4), IGHV mutational status (unmutated =score 2), β2M (>3.5 mg/l =score 2), clinical stage (Binet B-C or Rai I-IV =score 1), age (>65 years =score 1)	Low risk: score =0-1 (watch-and-wait approach)
		Intermediate risk: score =2-3 (do not treat except when the patient is symptomatic)
		High risk: score =4-6 (treat except when the patient is asymptomatic)
		Very high risk: score =7-10 (treat in experimental protocol or with non-cytotoxic drugs if possible (no chemotherapy or chemoimmunotherapy))

Hb: hemoglobin; ECOG: Eastern Cooperative Oncology Group

The inclusion of mutational data together with the cytogenetic model has allowed the proposal of an integrated prognostic model, improving discrimination of low- and high-risk patients and substantially enhancing the capacity to predict survival, compared with the possibilities of the cytogenetic model alone. The model proposed by Rossi *et al.*⁷¹ adds significantly to the ‘Döhner’ model,⁷² due to the coexistence of poor-risk gene mutations in low-risk groups defined solely on the basis of FISH. According to this model, high-risk CLLs are those with *TP53* and/or *BIRC3* disrupted (Table 2). Subsequent studies have confirmed the utility of such a model, although with partially discordant results, which may be related to differences in the design

and analysis of the studies.^{111,123} Further comprehensive prospective multi-institution NGS studies of larger cohorts of patients will allow us the integration of the newly discovered genetic lesions into a comprehensive prognostic model to improve patient prognostication and facilitate introduction of testing into general practice.

So far, none of these models has gained wide international acceptance. Therefore, recently an international consortium of researchers has recently created international prognostic index (CLL-IPI) using information from prospective, randomized trials. The study included a very large cohort of treatment-naïve patients with a median follow-up of 80 months, randomly divided into training (n=2,308), internal (n=2,308) and two external (n=845, Mayo Clinic; n=416, SCALE (Scandinavian population-based case-control study) validation datasets. The CLL-IPI, developed based on the analysis of 27 prognostic factors, could serve as a reliable and easily applicable method of risk stratification for CLL patients. Five independent predictors for OS were identified: age, clinical stage, *TP53* status, *IGHV* mutation status and β 2M level. Adding all these factors together results in a prognostic score ranging from 0-10, with four different risk groups with different treatment recommendations (Table 2).¹⁵⁷

1.5. Therapy strategies

CLL is still an incurable disease, but various treatment approaches are used to control symptoms and prolong survival of patients. CLL treatment has come a long way since the advent of monotherapy with alkylating agents such as chlorambucil with or without prednisone, purine analogues such as fludarabine and pentostatin, followed by the combination of fludarabine with cyclophosphamide (FC) and later on rituximab (FCR), until the newer anti-CD20 monoclonal antibodies (obinutuzumab or ofatumumab), B-cell receptor (BCR) signaling kinase inhibitors (ibrutinib, idelalisib), BCL2 antagonist such as Venetoclax/ABT-199, and chimeric antigen receptor T (CART) cell therapy.⁵¹

Risk stratification is useful to decide a particular treatment approach (Figure 1).¹⁵⁸ Fortunately, most CLL patients (~85-90%) do not need to be treated at diagnosis, being mostly asymptomatic.⁴⁹ Instead, treatment is initiated only in cases with “active disease” as defined by the iwCLL criteria.^{1,13} The criteria include evidence of progressive bone marrow failure, bulky disease, non-responsive autoimmune anemia and/or thrombocytopenia, constitutional symptoms, and progressive lymphocytosis.

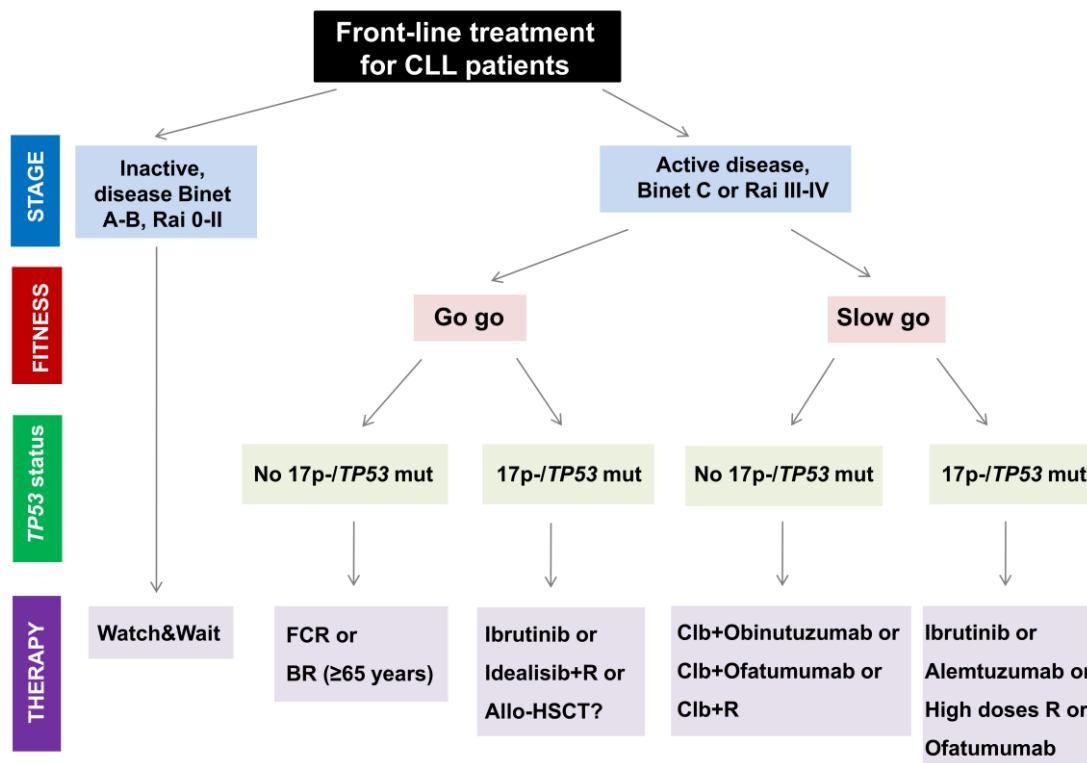


Figure 1: Treatment algorithm for first-line therapy of CLL.¹³ FCR: fludarabine, cyclophosphamide and rituximab, BR: bendamustine plus rituximab; R: rituximab; allo-HSCT: allogeneic hematopoietic stem cell transplantation; Clb: chlorambucil.

For asymptomatic patients, watchful waiting is still the treatment of choice, since these patients show no survival benefit from an early initiation of treatment.¹⁵⁹⁻¹⁶¹ By contrast, for patients who need to be treated, the main parameters currently influencing the choice of treatment are their physical condition and the genetic status of *TP53*.¹³ Thus, CLL patients can be classified as follows:

i) **“Go go” group** (CLL patients with a good physical condition): FCR is now considered the gold standard,¹⁶²⁻¹⁶⁴ achieving an overall response rate of >90%. Recently, long-term results of the FCR regimen showed prolonged and sustained remissions in *IGHV*-mutated CLL patients.^{165,166} This represents the first time in the history of CLL that a non-transplant therapeutic option has resulted in sustained disease-free intervals and raises the possibility of a cure for a subset of patients with generally good-risk disease and excellent response to therapy. However, FCR is too toxic for elderly and the combination of bendamustine with rituximab has proven quite effective, with limited toxicity among elderly patients.¹⁶⁷⁻¹⁷⁰ Similarly, patients with *TP53* aberrations (17p- and/or *TP53* mutation) do not have an optimal benefit from FCR. Therefore, it is recommended that patients with *TP53* deletion/mutation

will be treated with novel BCR inhibitors (ibrutinib, idelalisib) and rituximab.^{171,172} An allogeneic stem cell transplantation (allo-HSCT) should be offered and discussed at the first or second relapse.^{107,173,174}

ii) **“Slow go” group** (those with borderline impaired physical conditions): as FCR is too toxic for elderly and fragile patients who constitute a large fraction of CLL cases, it is recommended the combination of chlorambucil with an anti-CD20 monoclonal antibody (obinutuzumab or ofatumumab better than rituximab) as the first line treatment.¹⁷⁵⁻¹⁷⁸ The presence of *TP53* abnormalities also influences and the optimal treatments are either ibrutinib or anti-CD52 (alemtuzumab). However, in such cases allo-HSCT is not an option.

In the last decade, there has been a dramatic change in the number of agents available with very exciting developments for CLL treatment. Recently, a number of new agents have shown promising clinical activity and some have been approved for the treatment of CLL.¹⁷⁹⁻¹⁸¹ Of note, acalabrutinib, a BCR inhibitor, seem to improve the safety and efficacy of first-generation BTK inhibitors.¹⁸² In addition, venetoclax, a small molecular inhibitor of the BCL2 protein, recently received Food and Drug Administration (FDA) approval for the treatment of CLLs with 17p deletion previously treated with at least one prior therapy.^{183,184} Finally, CLL cellular therapy also took a big step forward with the successful application of CART-cells therapy allowing us to reach remarkable durability of the negative MRD remissions in a subset of CLL patients.¹⁸⁵

2. Biological abnormalities of CLL

2.1. Cytogenetic alterations

While CLL is not associated with a specific genetic aberration, more than 80% of CLL patients carry cytogenetically detectable recurrent chromosomal abnormalities at diagnosis,⁷² being trisomy 12 the most common recurrent ones as well as deletions of 13q, 11q and 17p. FISH is the current method of choice in clinical setting using a specific probe set.

▪ 13q14 deletion

This is the most frequent chromosomal aberration detected in CLL affecting around 43-55% of patients and entailing a group of patients with good prognosis.^{72,75,78} With regard to the biologic basis underlying 13q deletions, miR-15a and miR16-1, located in the minimal deleted region (MDR), have been described to exhibit a tumoral

suppressor function in CLL by targeting the *BCL2* oncogene. Besides these miRNAs, other genes located in 13q, such as *DLEU7*, could cooperate in the tumoral suppressor activity.

Deletions at this chromosome are not homogeneous and neither is the prognosis for this subgroup of patients. There are several factors which should be taken into account:

i) The size of the deletion varies being proposed two types of deletion: 13q- type I or short deletions not comprising *RB1* locus; and 13q- type II or large deletions including the *RB1* locus which has been associated with a more aggressive clinical course.^{186,187}

ii) The number of deleted 13q alleles can be different: monoallelic deletion is present in most cases but the presence of biallelic losses has been described in nearly 30% of 13q- CLL patients. Their clinical impact is controversial: whereas some authors suggest that biallelic 13q- represents a more aggressive abnormality, most of studies revealed that it does not seem to entail a worse clinical outcome.^{73,80-82,188-190}

iii) The size of the abnormal clone detected by FISH has been reported to have a clinical impact, as patients with a higher percentage of 13q- cells (isolated deletion) showed shorter TFT and OS. The optimal cutoff point that defines a poorer outcome for 13q deletion differs between studies.^{78-82,190} Subsequent molecular studies have provided biological evidences underlying the clinical heterogeneity related to the size of the abnormal 13q- clone, such as a distinct gene expression profile leading to decreased apoptosis and increased proliferation in CLLs with a high number of 13q losses.¹⁹¹

▪ 11q deletion

Deletion of the long arm of chromosome 11 is detected in 9–18% of CLL patients and is associated with poor outcome.^{72,75,78} The deletion is exclusively monoallelic and the MDR includes 11q22.3-q23.1 harboring *ATM*. Mutations in *ATM* are observed in approximately 30% of cases with 11q-.¹⁰⁸ Patients with biallelic *ATM* alterations (deletion and mutation) have worse outcome than those with *ATM* mutations in one allele or wild-type *ATM*.¹⁹² 11q deletion could also include *BIRC3*, which is more frequently mutated in fludarabine-resistant CLL patients. Of note, when *BIRC3* and *ATM* appear mutated together, *ATM* mutation remains the most important marker of poor outcome in del(11q) CLL.¹⁹³ As 13q deletion group, 11q- patients can exhibit variable clinical outcomes.⁸⁴

▪ Trisomy 12

Trisomy 12 is detected in around 15% of CLL patients and often appears as the unique cytogenetic alteration (40-60% of cases with +12).^{72,75,78} Its pathogenesis has been difficult to understand as the whole chromosome is affected, instead of a small critical region. Nonetheless, RNA and protein expression analysis have suggested a gene dosage effect associated with the upregulation of genes mapped in chromosome 12, like *CD4* or *MDM2*.^{194,195} The presence of +12 is considered as an intermediate prognostic marker although it corresponds with a clinical heterogeneous entity.⁷² *NOTCH1* mutations have been detected in around 30% of +12 CLL cases and are strongly associated with worse prognosis allowing us to define the prognosis of this group.^{117,118,196} By contrast, trisomy 12 can occur together with trisomies 18 and 19 entailing a subgroup of patients with a favorable complex karyotype.¹⁹⁷

▪ 17p deletion

Deletion of 17p is found in approximately 2–7% of CLL patients at diagnosis,^{72,75,78} although higher occurrences (~40%) have been reported in patients with advance or relapsed disease.¹⁹⁸ The extent of the 17p13 deletion is invariably associated with loss of *TP53*, representing the cytogenetic alteration with the worst impact in prognosis for CLL. Moreover, most of cases with 17p- also carry *TP53* mutations on the second allele (80-90%),⁷⁷ whereas a low frequency of *TP53* mutations were detected in patients without 17p-, suggesting the idea of a selective pressure for cells carrying biallelic inactivation of *TP53*.⁷⁷ Of note, both *TP53* deletion and/or mutation are strong predictors of poor survival and FCR refractoriness in CLL^{77,103,199} and represent the only biomarker which changes the treatment strategy.¹³ As other cytogenetic abnormalities, a high percentage of 17p deleted cells has been associated with a worse outcome.⁸³

▪ Other cytogenetic abnormalities

Other recurrent genomic aberrations have been described in lower proportion of CLL patients. These include deletions of 3p, 6p, 6q, 8p, 10q and 15q, as well as gains of 2p, 3q, trisomy 18 and trisomy 19.²⁰⁰⁻²⁰³ Of note, integrative analysis of copy number variations and somatic mutations revealed that some of these genomic alterations involve genes that could be mutated in CLL.¹²⁴

Translocations are rare in CLL. It is noteworthy to mention those in which *IGH* or *MYC* are involved, as well as the 13q14 region.²⁰⁴⁻²⁰⁹

2.2. Transcriptomic landscape

The comparison of global gene expression profile (GEP) analysis of B CLL cells compared with normal B cells demonstrated that CLL shows a homogeneous gene expression profile differing from other lymphoid cancers.^{210,211} However, despite sharing a common signature, unmutated *IGHV* CLLs had a distinct GEP compared with mutated *IGHV* CLLs in more than 100 genes. Among these, the overexpression of genes encoding ZAP70, lipoprotein lipase (LPL), BCL-7A, dystrophin and gravin were observed in the unmutated cases, while the mutated cases over-express Wnt3, CTLA-4, NRIP1 nuclear receptor gene, ADAM29 and the transcription factor TCF7.²¹² ZAP70 appeared to be one of the most significant.²¹¹ Subsequently, the correlation of ZAP70 expression with the mutational status of the *IGHV* genes was assessed in larger series of CLL patients, where ZAP70 was mostly found expressed in unmutated CLL.²¹³ Further clinical studies revealed that ZAP70 was also an independent prognostic marker.²¹⁴ Another relevant finding derived from expression arrays was the discovery of *ROR1* as one of the most CLL-specific surface antigens.²¹⁵

Microarray expression profiling of CLL has been used to define the patterns of gene expression related to different chromosomal abnormalities^{96,191,216-218} or even mutational status of new drivers such as *NOTCH1*.^{219,220}

Recently, RNA-sequencing (RNA-seq) technology has been widely adopted as an attractive alternative to microarray-based methods to study global gene expression.²²¹ More in detail, RNA-seq analysis have shown that transposable elements are globally downregulated in CLL cells whereas genes involved in metabolic pathways showed higher expression. By contrast, genes related to spliceosome, proteasome, and ribosome were among the most downregulated in CLL.²²²

2.3. MicroRNAs

MiRNAs are non-coding RNAs that have the capacity for simultaneous regulation of genes through direct targeting of UTR.²²³⁻²²⁵ MiRNAs are important for biological processes, including proliferation, apoptosis, development, and cellular differentiation²²⁶ and can function as either oncogenes or tumor suppressors.²²⁴

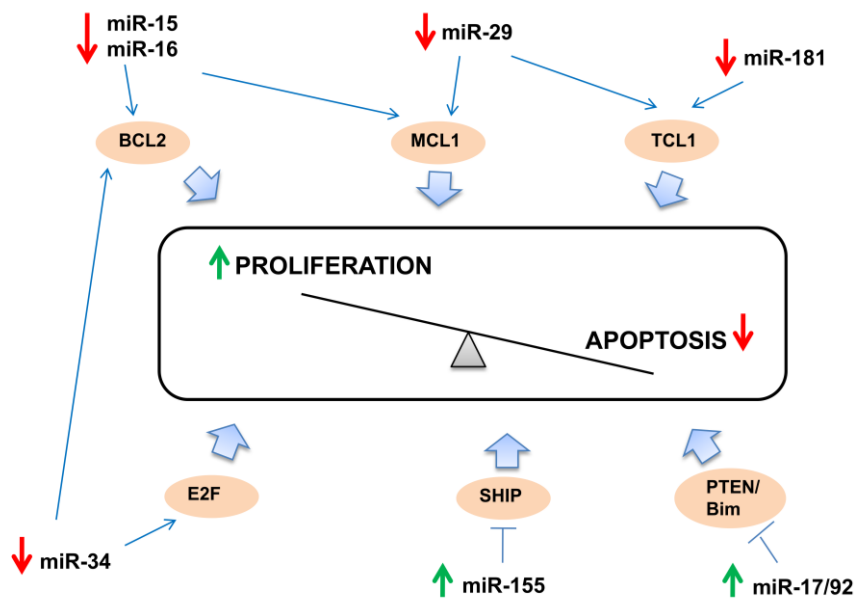


Figure 2: Role of the main miRNAs in CLL pathogenesis. Red arrows mean downregulation whereas green arrows mean upregulation of the miRNAs. Downregulation of miR-15/16, miR-29, miR-181 and miR-34 results in overexpression of gene targets. By contrast, overexpression of miR-155 and miR-17/92 causes inhibition of their targets. Genes in light orange circles represent validated targets for select miRNAs.

The first description of a miRNA associated to cancer was reported in CLL: miR-15a/16-1, located in 13q14.3, are either deleted or downregulated in 68% of CLL patients²²⁷ and target the antiapoptotic *BCL2*, leading to its upregulation and inhibiting apoptosis.²²⁸ Downregulation of miR-29 and miR-181b in aggressive CLL contributes to the overexpression of *TCL1*.¹⁴⁴ Activation of the *TCL1* oncogene is a central initiating event in the pathogenesis of aggressive CLL with functions of a coactivator of PI3K/AKT signaling, and high *TCL1* expression correlates with aggressive phenotype.^{229,230} By contrast, overexpression of miR-155, which represents a biomarker for the risk of progression,¹⁴⁹ targets *SHIP1* by reducing its expression and interfering with the BCR induced pathways.¹⁵⁰ Similarly, miR-17/92 has also an oncogenic function in CLL inhibiting the expression of the tumor suppressor *PTEN* and the proapoptotic protein *Bim*.²³¹ MiR-34 family has been also implicated in CLL as its expression can be induced by *TP53*. A low expression of miR-34a has been associated with chemotherapy-refractory regardless of 17p-/TP53 mutation.^{145,232} Several genes such as *E2F* or *BCL2* have been experimentally validated as miR-34 direct targets.²³³ Therefore, the role of miRNAs in CLL pathogenesis is clear, being mainly involved in the regulation of proliferation/anti-apoptotic signals (Figure 2).^{234,235}

A link between miRNAs and exosomes has been recently reported in CLL. Expression of exosome miR-150 and miR-155 was elevated in CLL cells compared with normal B cells and further increased in response to BCR activation, indicating that the BCR signaling is involved in CLL exosome secretion.²³⁶ Another contribution to CLL could be the development of miRNA-based therapeutic approaches. In vivo studies demonstrated that miR-181b can be effective reducing leukemic cell expansion and increasing mice survival.²³⁷ Moreover, a synergic effect of miR-181b with fludarabine was observed in human primary CLL cells,²³⁸ providing an additional evidence for a potential role of miR-181b as a therapeutic agent in CLL. However, it remains to be seen if cellular or exosome miRNAs can be used in clinical practice to track CLL as well as the possibilities of miRNA-based therapies.

2.4. Pathways significantly affected by mutated genes

Genomic studies performed by NGS have provided a detailed genetic landscape of CLL.^{124,126,127,220,239-242} These studies demonstrated a highly genetic heterogeneity with a small number of significantly recurrent mutated genes and a relatively larger number of rarely mutated genes.¹²⁵ Comparing the largest recent CLL cohorts, common mutations were identified in 29 CLL drivers (Figure 3), although there is a marked variability in the frequency of mutations most probably due to the different clinical characteristics of both cohorts.^{124,127} Landau et al. analyzed matched pretreatment samples from patients who required initial treatment and noted mutations in *SF3B1* (21%), *ATM* (15%), *TP53* (7%), *NOTCH1* (6%) or *BIRC3* (4%).¹²⁷ The other study performed by Puente et al. assessed patients with early stage CLL and identified *NOTCH1* (13%), *ATM* (11%), *BIRC3* (9%), and *SF3B1* (8%) as the most frequent mutated genes.¹²⁴

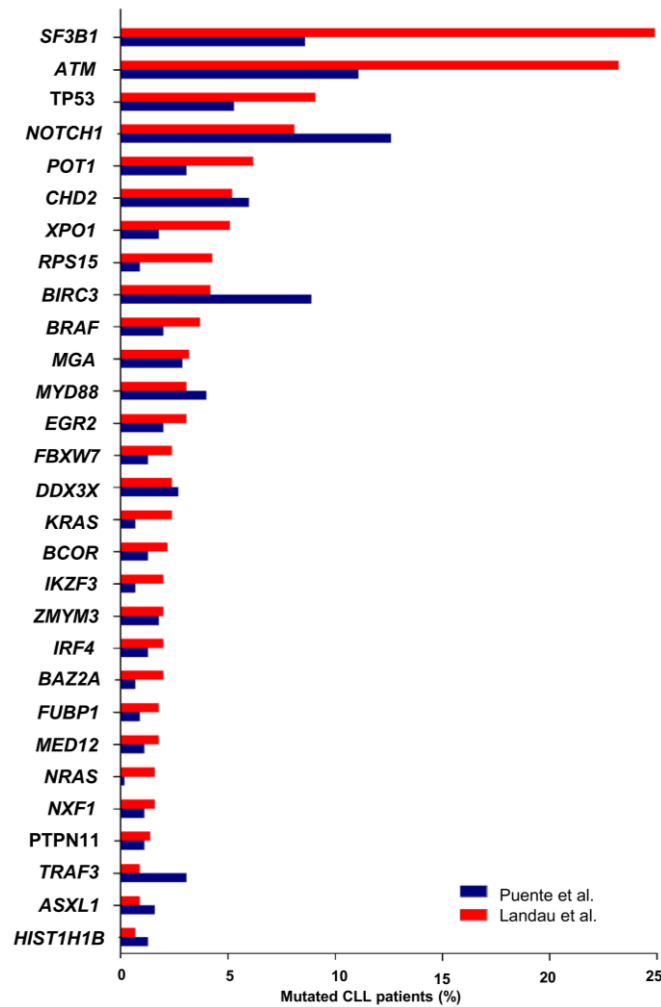


Figure 3: List of CLL mutated driver genes with their corresponding frequency from Puente *et al.*¹²⁴ (blue) and Landau *et al.*¹²⁷ (red) studies. Genes are labeled with different colors according to the biological pathways involved (Notch signaling: orange; RNA metabolism: green; B-cell signaling: red; DNA damage, apoptosis and cell cycle: blue; Genome/chromatin structure: purple).

Globally, these mutations can be grouped in main signaling pathways: NOTCH1 signaling, RNA metabolism, B-cell signaling, genome/chromatin structure, DNA-damage response, apoptosis and cell cycle. Therefore, the assignment of the identified target genes to signaling networks revealed a set of commonly disrupted pathways in CLL (Figure 4).^{124,127}

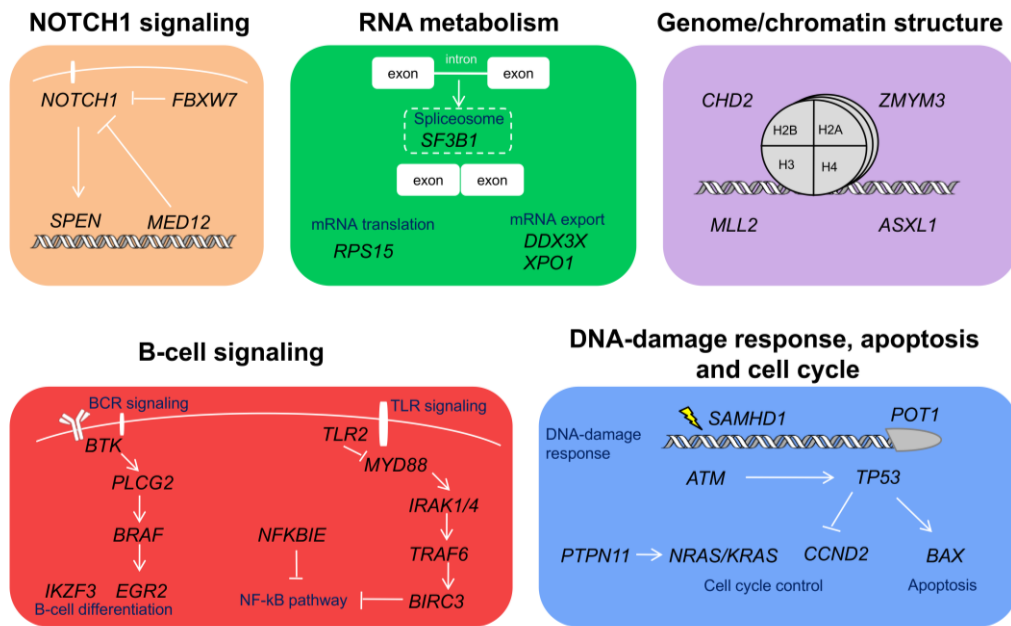


Figure 4: Signaling pathways commonly affected by mutations in patients with CLL (modified from Puente et al.¹²⁴)

▪ Notch signaling

Several genes encoding proteins that control the Notch signaling pathway have emerged as recurrently mutated in CLL. *NOTCH1* mutations occur in around 12% of CLL patients at diagnosis,^{111,115,118,123,220,239} with a higher frequency in fludarabine-refractory CLL¹¹⁵ and in advanced disease such as Richter's syndrome.^{37,117} *NOTCH1* mutated patients frequently belong to *IGHV*-unmutated subgroup and display trisomy 12.²⁴³ Some studies suggested that *NOTCH1* mutated cases exhibit adverse prognostic features with shorter TFT and OS but its role as an independent prognostic marker is still controversial.^{111,115,123,124,239,243} The mutations mostly affect the functional PEST domain (C-terminal region, exon 34) of *NOTCH1*, and are mainly represented by frameshift or nonsense events, with the most frequent mutation being c.7544_7545delCT deletion (approximately 80–95% of all mutations). This pattern of mutations disrupts the PEST domain of the protein, generating a truncated protein and leading to the accumulation of a stable and active NOTCH1 isoform, which causes a constitutive activation of the NOTCH1 pathway.²²⁰ Recently, recurrent non-coding mutations in the 3'UTR region of *NOTCH1* have been reported in 3% of CLL cases, leading to similar functional deregulation.¹²⁴

Mutations in additional genes of the Notch pathway have been described, although observed in lower frequencies. Among them, inactivating mutations of *FBXW7*, that encode an E3 ligase targeting *NOTCH1* for ubiquitination and degradation, lead to *NOTCH1* stabilization by the inability of the *FBXW7* mutants to bind *NOTCH1* PEST domain.²⁴⁴ *FBXW7* may also regulate MYC protein stability²⁴⁵ and is mutated in approximately 2% of CLL patients.^{111,124,127} *MED12* mutations (<2%)¹²⁰ and *SPEN* mutations (~1%)¹²⁴ potentially also lead to an increase in Notch signaling.^{246,247} All these genetic alterations result in an independent enhanced NOTCH1 signaling, whose consequences are not yet completely known. Up to now, NOTCH1 signaling has been shown to interfere with BCR signaling and could affect B-cell differentiation.²⁴⁸

Targeting Notch signaling has emerged as a promising therapeutic strategy for CLL. In this line, ex vivo studies have revealed that γ -secretase inhibitor PF-03084014 inhibits the constitutive Notch activation and induces selective apoptosis in CLL cells carrying *NOTCH1* mutations.²⁴⁹ In addition, the presence of *NOTCH1* mutations has been associated with a relative resistance to anti-CD20 immunotherapy comparing the effectiveness of the FC regimen vs. the FCR regimen.²⁵⁰ *NOTCH1* mutations have been reported recently to be associated with low CD20 levels in CLL and responsible of a dysregulation of histone deacetylases (HDAC)-mediated epigenetic repression of CD20 expression.²⁵¹ Therefore, drugs interfering with the NOTCH1 pathway and/or inhibiting HDACs might have a role to increase CD20 expression in vivo, thus overcoming the relative resistance of *NOTCH1*-mutated CLL to rituximab-containing therapies.

▪ RNA metabolism

SF3B1 is mutated in approximately 10% of CLL patients at diagnosis,^{111,220,240,242} with a higher frequency (17%) in patients with fludarabine-refractory disease. *SF3B1* mutations are more common in unmutated *IGHV* and 11q- CLLs.^{111,123,242,252} Several studies have demonstrated that *SF3B1* mutated patients have shorter OS and TFT compared with *SF3B1* wild-type patients,^{111,123,240,242} being an independent prognostic marker in some cohorts.^{111,123}

Most mutations are missense mutations localized within the HEAT domain repeats, indicating a gain of function, being K700E the most frequent mutation (approximately 50% of all mutations).²⁵³ In various cellular contexts, *SF3B1* mutations are associated with abnormal splice events.²⁵⁴ In CLL, *SF3B1* mutation induces subtle but broad changes in the splicing of transcripts involved across

multiple CLL-associated pathways including RNA splicing, DNA damage, Notch signaling, telomere maintenance, cell proliferation, apoptosis and metabolism.^{255,256}

In addition to *SF3B1*, other genes involved in splicing as well as RNA processing and transport are mutated in CLL at low frequencies. Among them, *DDX3X*, mutated in 2.5% CLL and recently associated with poor outcome,²⁵⁷ is involved in many steps of RNA metabolism.²⁵⁸ In addition, *XPO1* participates to the nucleocytoplasmic export of proteins and RNA²⁵⁹ and its mutations are present in around 3.4% of CLLs.¹¹¹ Mutations in *RPS15* (1-4%) which play also a role during RNA processing and export have been recently described.^{121,127} Assuming that all these lesions affect the same pathway, they account for up to ~30% of patients with CLL, suggesting that the dysregulation of RNA processing may represent a common mechanism in CLL pathogenesis.²⁵² Based on this, new drugs such as SF3B1 inhibitors (Spliceosome inhibitor spliceostatin A, SSA)²⁶⁰ or XPO1 inhibitors²⁶¹ are currently being assessed in order to target this pathway.

▪ B-cell signaling: BCR/TLR and NF-κB pathway

A heterogeneous set of genetic lesions associated with CLL seems to activate positive regulators or disrupt negative regulators of pathways such as BCR, TLR and NF-κB pathways.²⁶²

BCR signaling has long been recognized as a major player in CLL development, based on structural restrictions of the BCR, and BCR-dependent survival and growth of the malignant B cells.²⁶³⁻²⁶⁵ Genes encoding for components of the BCR signaling machinery are not usually targeted by somatic mutations in unselected CLL. However, the introduction of BCR inhibitors such as ibrutinib has disclosed the acquisition of previously unexpected drug resistant mutations in *BTK* or *PLCG2*.^{266,267} These resistant subclones have been detected before ibrutinib therapy when evaluating single cells at high throughput using the droplet-digital amplification technology.²⁶⁸ Interestingly, a hotspot mutation in *IKZF3* (p.L162R, <2%) has been reported. This transcription factor plays an important role in B-cell differentiation, proliferation and maturation.^{124,127} Mutations in genes involved in BCR downstream pathways such as MAPK signaling have been reported: *EGR2* (2-3%) and *BRAF* (2-3%).¹¹⁹

MYD88, a crucial adaptor of intracellular signaling downstream of TLR complex, is mutated in around 4% of CLL cases,^{112,124,126,127,220,240,242} being L265P the most frequent mutation. *MYD88* missense mutations are gain-of-function mutations that result in the constitutive activation of the NF-κB and others signaling pathways.²⁶⁹

Despite NF- κ B upregulation, these mutations confer favorable outcome in CLL patients as they are associated with good characteristics such as mutated *IGHV* or low expression of CD38 and ZAP-70.¹¹² Other genes (*TLR2*, *IRAK1/4* or *TRAF6*) of the TLR pathway leading to potential activation of NF- κ B signaling have been reported to be mutated in CLL.¹²⁴

The NF- κ B pathway is known to be continuously activated in CLL cells through the BCR and TLR receptors and downstream signaling cascades.²⁷⁰ *BIRC3* gene, which is a negative regulatory element of non-canonical NF- κ B signaling, is mutated in 2-9% of CLL.^{114,124} These mutations cause the truncation of the RING domain, leading to a constitutive non-canonical NF- κ B activation. From a clinical standpoint, *BIRC3* mutations identify a genetic subgroup of cases characterized by poor risk disease.¹¹⁴ Similarly, frequent inactivating mutations of another negative regulator of NF- κ B, NFKBIE, have been reported in advanced stage CLL.¹²² Moreover, mutations in other genes of the NF- κ B core complex such as *NFKB2*, *TNFAIP3*, *IKBR* and *REL* have been identified in CLL patients.¹²²

- **DNA-damage response, apoptosis and cell cycle**

TP53 codes for a central regulator of the DNA-damage response pathway or, alternatively, apoptosis if the DNA damage cannot be repaired. *TP53* abnormalities can be mutations (mainly from exon 4 to 11, with a frequency of 5-10%), deletions (17p-) or a combination of both, and are associated with poor responses to DNA-damaging chemotherapeutic regimens, consistent with their dominant- negative function. In fact, their presence directs the choice of the therapy and is associated with dismal prognosis.^{77,99,101-103,232} More recently, subclonal *TP53* mutations were also shown to be associated with poor prognosis, and therefore they should be tested for with highly sensitive methods in routine clinical settings.^{105,128,129,131}

As for *TP53*, the *ATM* gene is inactivated in CLL by both deletion and/or somatic mutations, which result in impaired DNA damage response. *ATM* mutations have been described in approximately 12% of CLL patients at diagnosis¹⁰⁹ and 30% of patients with 11q deletion.^{108,109} Similarly to cases with *TP53* disruption, *ATM*-disrupted CLLs are associated with genomic instability, the acquisition of additional genetic lesions and poor response to chemotherapy.²⁷¹

Other genes involved in DNA damage response are *SAMHD1* (mutated in 3-4% of CLLs)²⁷² and *POT1* (mutated in around 5%), whose mutations have an impact on DNA structure affecting telomere maintenance.²⁴¹

Apart from the role of *TP53* and *ATM* in apoptosis and/or cell cycle, other genes that can be mutated and deregulate these essential pathways are *PTPN11*, *KRAS*, *NRAS* and *CCND2* for cell cycle and *BAX* for apoptosis, all mutated in less than 5% of CLL patients.^{124,127}

▪ **Genome/chromatin structure**

A large number of mutated genes are involved in genome and chromatin structure,¹²⁴ being *CHD2* the most frequent mutated genes (6%), mainly in *IGHV*-mutated CLL patients.²⁷³ *CHD2* mutants show altered nuclear distribution, and its DNA-binding domain exhibits defective association with active chromatin.²⁷³ Other genes mutated at lower frequencies (<5%) are *ZMYM3*, *SETD2*, *MLL2* and *ASXL1*.

Together with *ASXL1*, *TET2*, which is rarely mutated in B-lineage lymphoid malignancies (~2%),²⁷⁴⁻²⁷⁶ is also detected and associated with an increased risk of hematologic disorders including CLL.²⁷⁷⁻²⁷⁹ The mechanism underlying the participation of *TET2* mutations to B-cell transformation will have to be determined. *TET2* encodes for a protein involved in converting 5-methylcytosine to 5-hydroxymethylcytosine, an important step in DNA demethylation. Mutations in *TET2* block the DNA demethylation.²⁸⁰

2.5. Epigenetic basis

The development of genome-wide technologies provided an unbiased picture of the epigenetic make-up of CLL.²⁸¹⁻²⁸⁴ Initial studies highlighted that the DNA methylation status in CLL is remarkably stable over time,²⁸⁵ revealing differential methylation of a few known CLL prognostic genes between the *IGHV*-mutated and *IGHV*-unmutated CLL subgroups.^{281,285} CLL epigenome is characterized by widespread hypomethylation of DNA in genes and enhancer loci, combined with local hypermethylation.²⁸² Of note, it has been shown that high levels of intra-sample methylation heterogeneity correlate with high-risk genetic lesions, CLL clonal evolution and poor prognosis.^{283,284}

2.6. Clonal evolution

Clonal evolution is characterized by the acquisition of new genetic aberrations during the course of the disease, and is a key point for CLL development and relapse. FISH and conventional G-banding cytogenetics (CGC) have been useful complementary methods for studying clonal evolution in CLL samples, describing a wide range of clonal evolution frequencies (10-45%) and being the most frequently

acquired abnormalities 17p- and 11q-.^{76,200,286-290} However, these methods can only detect a relatively small number of genetic alterations, whereas NGS can quantify thousands of somatic mutations per sample, and track subclones.

Whole-genome sequencing (WGS) showed different temporal patterns of repopulation after therapy that deviate from a stable equilibrium among subpopulations, to marked shifts in which one minor subclone replaced the dominant clone entirely over time.²⁹¹ In particular, whole-exome sequencing (WES) data on matched samples collected at the time of first progression and relapse of FCR therapy have revealed marked clonal evolution with large clonal shifts between pre-treatment and relapse samples in the majority of cases (~95%).¹²⁷ By contrast, the absence of an intervening therapy was largely associated with stable subclonal composition over time.²⁹² Nevertheless, the impossibility of applying this methodology in large cohorts of patients still leaves much to be elucidated.

Moreover, through NGS-based studies, a temporal order of clonal and subclonal mutations corresponding to earlier and later events, respectively, has been suggested. Thus, 13q deletion, mutations of *MYD88* and trisomy 12 were identified as consistently clonal while mutations in *ATM*, *TP53* or *RAS* were identified as subclonal genetic alterations.^{126,127} These studies also demonstrated that genetic features of subclonal populations are linked to poorer clinical outcome, implying that an active evolutionary process is the underlying basis of aggressive disease.^{126,127}

3. Applications of high-throughput technologies for CLL study

3.1. Gene expression arrays

Microarrays emerged as powerful tools to increase the potential of standard methods through genome-wide biological studies. GEP using genome-wide high-density microarrays is a powerful approach to measure the gene expression changes by comparing the expression levels of genes from two groups of patients. This can be mainly used for the identification of genes that could be involved in pathological processes or gene expression patterns that define disease subtypes or may represent disease stages.

A gene expression microarray is typically a slide that contains thousands of oligo probes fixed in an orderly manner at specific locations. These probes are organized in probe sets, consisting of a collection of probes designed to interrogate a specific

sequence and a single gene can be targeted by more than one probe set. These probes act as targets for sample RNA binding. By this way, any mRNA sequence in the sample can hybridize to specific probes on the microarray by its complementary sequence. The amount of RNA bound to a probe set is proportional to the initial amount of mRNA present in the sample.^{293,294}

Many different analytic platforms are available. In this thesis, gene expression studies were carried out using GeneChip Human Exon 1.0 ST array (Affymetrix, Santa Clara, CA, USA) (see Results: Chapter 3). This microarray contains 5,362,207 oligonucleotide features with over 1.4 million probe sets to interrogate over one million exons from human genes. Briefly, total mRNA is converted to double-stranded cDNA by reverse transcription, and then to cRNA by in vitro transcription. Then, this cRNA is converted to cDNA by reverse transcription and finally, single-stranded cDNA is labeled with biotin. This labeled cDNA is then randomly fragmented, and hybridized onto the array. The array is then washed, stained with streptavidin-phycoerythrin conjugate, and scanned. Raw gene expression data are digitally captured and pre-processed before extracting any biological information. These pre-processing steps include background correction, summarization and normalization, to remove systematic variation between chips and allow data to be compared from one array to another (group comparisons). After normalization, data are typically reported as expression ratios, where positive values represent over-expressed genes and negative values under-expressed genes.

As for other cancers, expression microarrays have contributed to the molecular characterization of CLL, describing gene signatures for the different molecular subtypes of CLL and identifying individual genes or pathways related to the clinical and biological evolution of the disease (See Introduction: Section 2.2).^{210,211,218,295,296} The most clinically relevant result derived from the application of this technology was the identification of *ZAP70* expression levels as a prognostic marker^{132,211} or *ROR1* as one of CLL-specific surface antigen.²¹⁵

▪ miRNA expression arrays

The rapid advancement of miRNA research experienced a rapid growth in methodology associated with miRNA profiling analysis. Diverse techniques are available for studying miRNA expression profiling.^{297,298} Similarly to gene expression arrays, miRNA expression arrays contain probes that interrogate the mature miRNA and precursor-miRNA (pre-miRNA). The workflow is simpler than gene expression arrays, following three main steps: poly-A tailing of total RNA (including miRNAs), a

reaction of ligation to label polyA-tailed RNA with biotin, and its hybridization onto the array (Figure 5). As a result, miRNAs of the sample can hybridize to specific probes on the microarray by its complementary sequence.

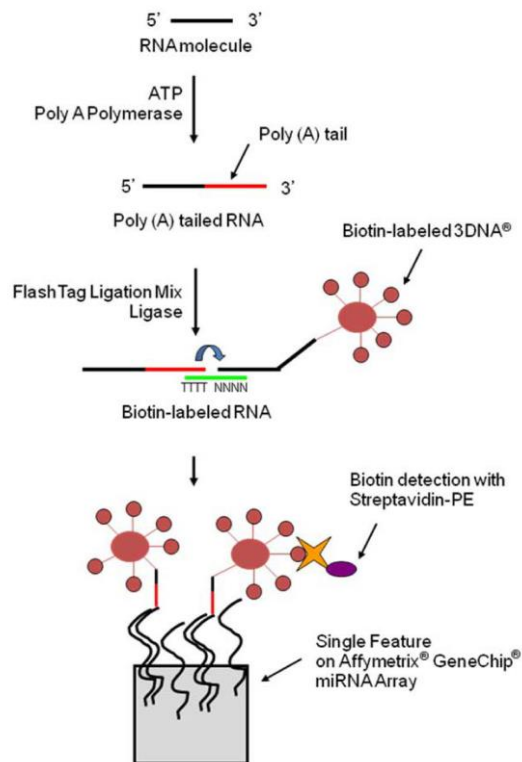


Figure 5: Schematic overview of the steps involved in an Affymetrix miRNA expression array experiment (taken from User guide FlashTag[™] Biotin HSR RNA Labeling kit for Affymetrix[®] GeneChip[®] miRNA Arrays).

The discovery of miRNAs has broadened our understanding of the mechanisms that regulate gene expression with the addition of an entirely novel level of gene expression control. In CLL, miRNA expression profiling can distinguish normal B cells from malignant CLL cells, as well as aggressive and indolent CLLs which exhibit a different miRNA profile.^{299,300} The interaction between miRNAs, target genes, and pathways in CLL is clearly complex (Figure 2, see Introduction: Section 2.3) and miRNAs remain one of the most exciting new avenues for CLL research.

Among the available platforms of miRNA expression microarrays, the Gene Chip Micro 3.0 Array (Affymetrix, Inc, Santa Clara, CA, USA) provides for universal miRNA coverage (100% miRBase v17 coverage (www.mirbase.org)). In this thesis, miRNA expression profiling was performed using this array (see Results: Chapter 1). Specifically, this microarray contains a total probe set of 19,724 for detecting 1,733 mature human miRNAs and 1,658 human pre-miRNAs.

3.2. Next-Generation Sequencing

NGS technologies, also known as massive parallel sequencing, have the ability to perform millions of sequencing reactions in parallel, generating millions of sequencing reads in one single experiment. Therefore, NGS offers much greater throughput and sensitivity than previous techniques such as Sanger sequencing.^{301,302} NGS platforms have been typically represented by Genome Analyzer/HiSeq 2000/MiSeq from Illumina (<http://www.illumina.com>), and GS FLX Titanium/GS Junior from Roche (<http://sequencing.roche.com/>), SOLiD/Ion Torrent PGM from Life Sciences (www.appliedbiosystems.com).³⁰³⁻³⁰⁵

NGS allows a few to thousands of genes or genomic regions to be analyzed in many samples, if properly barcoded, in a relatively efficient and effective manner. NGS technologies enable the analysis of complete cancer genomes, exomes, or targeted sequencing of a panel of genes in one sequencing run.³⁰⁶

- **Whole genome and exome sequencing**

WGS offers genomic coverage of all sequenciable regions in the genome, however usually with depth of coverage (defined as the number of reads per region) of only approximately 30x, being mutations with low allelic frequencies difficult to be detected. To focus on protein-coding sequences, WES is based on a target capture method to enrich the sequences of the coding regions of the genome, rendering superior depth of coverage compared to WGS (80-100x).

Nowadays, many large-scale sequencing efforts have used the Illumina sequencing technique (Illumina, San Diego, CA). In this thesis (see Results: Chapter 4), WES experiments were performed following TruSeq Exome Enrichment protocol (20,794 genes; 201,121 exons) (Figure 6).³⁰⁷

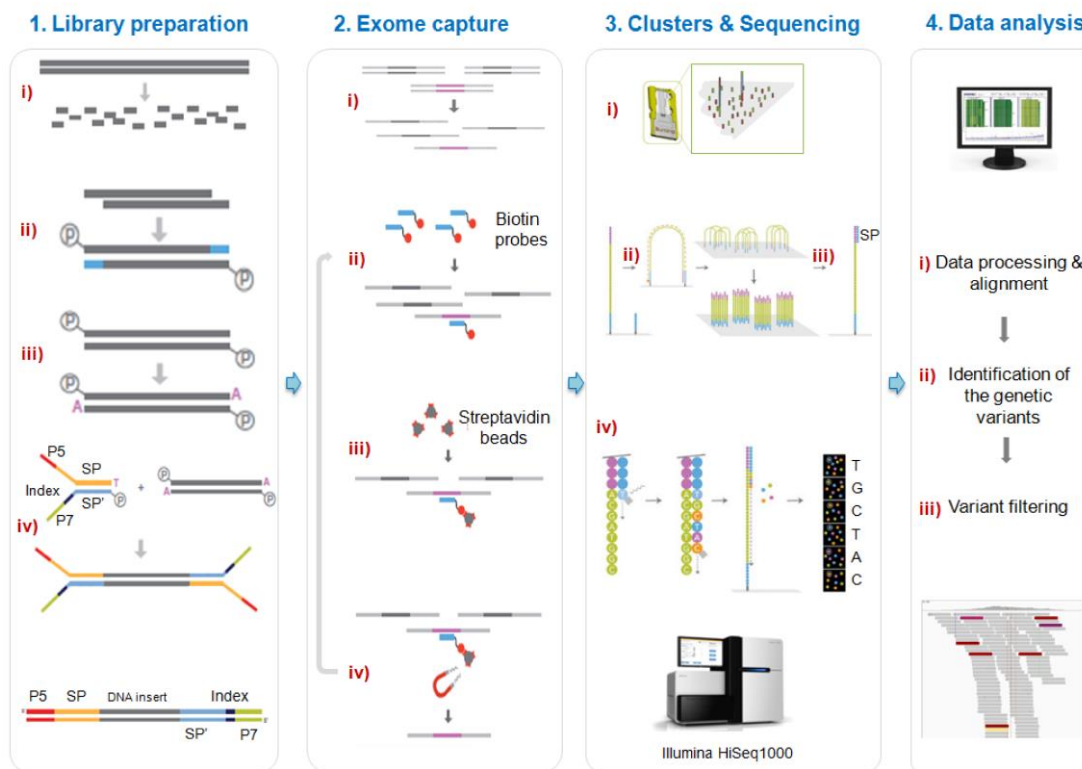


Figure 6: Schematic representation of the WES workflow by Illumina (adapted from <https://www.illumina.com/index-d.html>). The TruSeq Exome workflow includes four main steps (library preparation, exome capture, clusters generation and sequencing, and data analysis): 1) The library preparation begins with DNA mechanically fragmentation, preparation of DNA fragments for ligation to the indexed adapters (P5 and P7: adaptors; SP: sequencing primer). 2) The exome enrichment consists in the capture of coding regions by hybridization to biotinylated probes, and then isolated by magnetic pull-down. 3) Cluster generation and sequencing is based on the library loading into a flow cell where each fragment is amplified through bridge amplification and sequenced by synthesis technology. 4) Data analysis: the sequence data generated from the sequencer are analyzed using a pipeline of bioinformatics analysis.

WGS and WES have enabled the thorough exploration of the mutational landscape of CLL. WGS and WES data are now available for an extended patient series, comprising more than 800 published CLL exomes or genomes, revealing a vast genetic inter- and intra- heterogeneity in CLL patients.^{124,126,127,220,239,240,242,291,308,309} These studies further uncovered a number of novel frequent somatic mutations and showed the most commonly mutated genes (*SF3B1*, *ATM*, *TP53*, *NOTCH1*...) in CLL patients. Compared with other cancers, CLL is a disease with a relatively low genomic complexity, with a mutation rate of only 0.8/Mb, similar to other hematological malignancies like acute leukemias, but lower than that of solid cancers, such as melanoma (mutation rate of 15/Mb).³¹⁰ Indeed, the average numbers of somatic mutations affecting protein-coding genes and copy-number alterations in CLL are around 25 and 2 per sample, respectively.^{124,127}

▪ Targeted sequencing

The enormous capacity of NGS can be also directed to selected genomic regions. With targeted sequencing, the regions of interest are isolated and sequenced. Such targeted analysis can include the exome (see Figure 6), specific genes of interest or targets within genes (custom content). Recent developments in custom targeted sequencing approaches are significantly impacting medical research, as it enables increased coverage than WES (>1000x) to be reached, allowing the detection of subclonal mutations. These NGS strategies facilitate for multiplexing of many samples which reduces cost.

Targeted enrichment methods fall broadly into two categories: (1) capture-based target enrichment in which specific regions of interest are capture by hybridization to biotinylated probes, and isolated by magnetic pull-down; (recommended for a panel of many genes or even whole exome); (2) amplicon generation which involves the amplification and purification of regions of interest using highly multiplexed oligo sets (recommended for a panel of few genes).³¹¹ In this thesis (see Results: Chapter 2 and 3), the amplicon-based deep sequencing workflow was followed using Roche/454 FLX platform (Figure 7). The 454 platform is based on pyrosequencing in which the incorporation of nucleotides complementary to the template strand triggers an enzymatic cascade, which in turn produces a chemiluminescent signal.^{312,313}

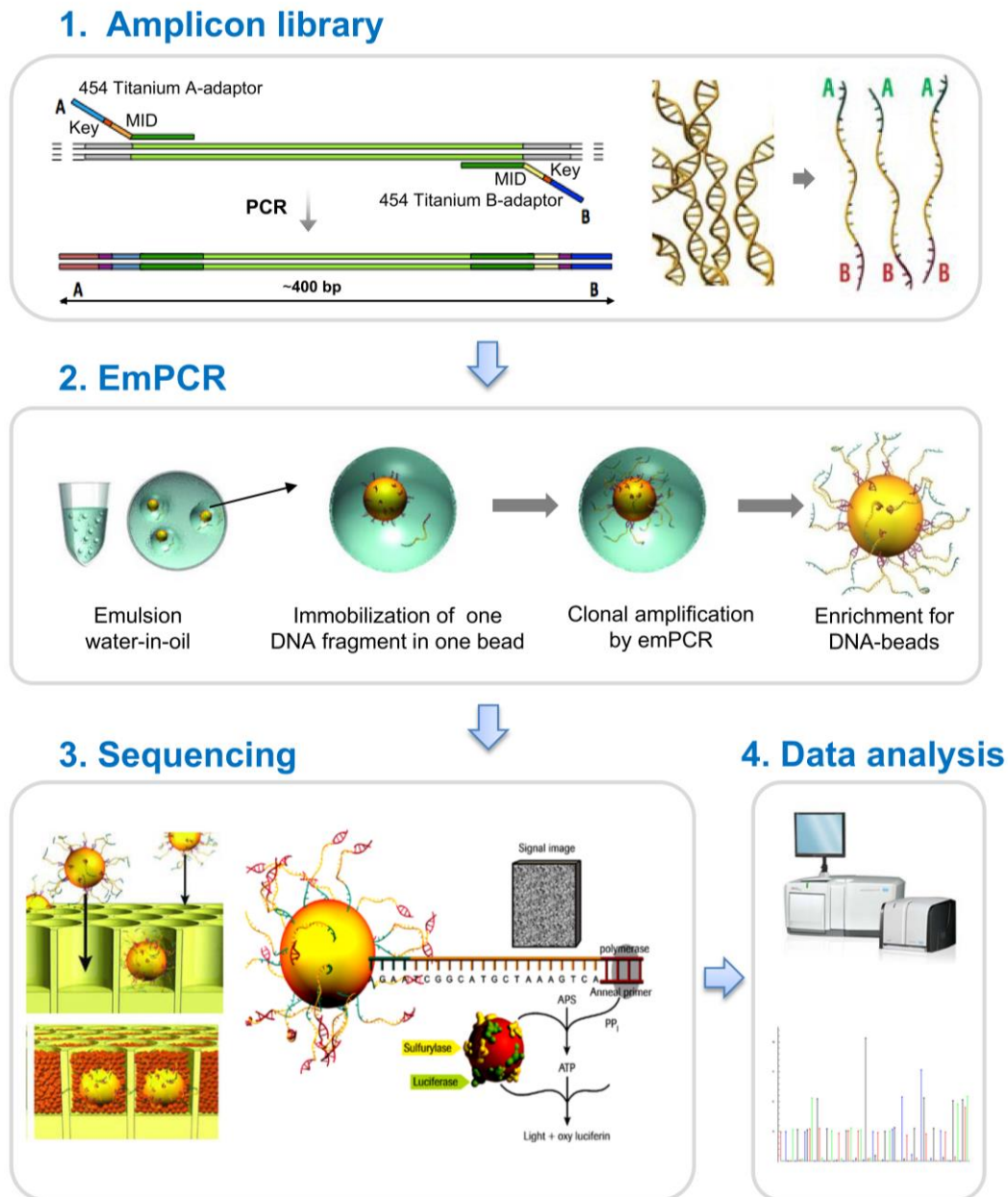


Figure 7: Workflow of amplicon-based NGS by 454 system (modified from Mardis *et al.*³⁰¹). 1) Amplicon library: each amplicon is PCR-amplified by using specific oligonucleotide primer pairs, which include "A" and "B" 454-adaptor sequences, a "key" sequence of 4 nucleotides "TCAG", multiplex identifiers (MIDs) and the template specific primer sequence. 2) Emulsion PCR (emPCR): pooled amplicons are clonally amplified in droplet emulsions. 3) Sequencing: isolated DNA-carrying beads are loaded into individual wells on a plate and surrounded by the enzymes required for the pyrosequencing. 4) Data analysis: all data generated during the sequencing run, on the 454 Sequencing System (GS FLX or GS Junior) are automatically processed for image processing and amplicon pipeline data analysis.

Targeted NGS is particularly suitable to be used in the clinical setting, as it may allow the concurrent evaluation of the most prognostically relevant genes for CLL. So far, different groups have used this approach based on a custom set of genes in CLL, being *TP53*, *NOTCH1* and *SF3B1* assessed commonly in all gene panels.^{129,314-317} The efforts to design a gene panel that could be widely used for CLL

prognostication and, more importantly, treatment-effect prediction, are still on-going. The importance of subclonal lesions has been further elucidated in recent studies by ultra-deep targeted NGS.^{128,129,131} These studies clearly documented the clinical significance of *TP53*-mutated microclones and their clonal evolution in CLL. Moreover, the targeted approach has been used to identify mutations in genes involved in a particular pathway that could be relevant for CLL pathogenesis.^{122,318,319}

In summary, despite the increasing knowledge of the mechanisms involved in the pathogenesis of CLL, driven by advancing technology in recent years, there are still questions to be resolved for a comprehensive understanding of CLL and its vast heterogeneity. Nowadays, the prognostic utility of the hierarchical Döhner classification is widely used and has allowed us to identify different subgroups of CLL patients based on the cytogenetic alteration. The application of high-throughput genomic technologies will enable us to gain knowledge in the biological heterogeneity within these well-defined subgroups of CLL patients. Moreover, the assessment of clinico-biological characteristics using large cohorts of patients will allow us to identify new variables with potential prognostic value in order to improve CLL prognostication accuracy. Finally, the dynamics of genetic changes in CLL patients have not been extensively studied during disease evolution without therapy pressure, and thereby longitudinal studies applying WES could be valuable for a better understanding of the molecular mechanisms of clonal evolution and for predicting clinical progression.

Hypothesis

Chronic lymphocytic leukemia is a heterogeneous disease with a highly variable clinical outcome. The course of patients ranges from very indolent with a nearly normal life expectancy to rapidly progressive leading to early death. Large-scale NGS analyses have revealed clear evidence of the genetic heterogeneity across samples potentially driving the variable clinical course of this disease. In this line, the use of high-throughput molecular analytical techniques could contribute to a better knowledge of molecular basis of the clinical heterogeneity in CLL.

Considerable efforts have been directed towards the identification of prognostic factors that can be used in clinical practice for a better patient management. Recurrent cytogenetic alterations have an established role in outcome prediction allowing us to stratify CLL patients in subgroups according to a specific chromosomal abnormality defined by FISH. However, clinical differences have been observed even within these well-defined subgroups. In particular, several studies have reported that 13q-, traditionally associated with a favorable prognosis, entails a heterogeneous group with different outcome depending on the percentage of cells displaying this aberration. However, the molecular mechanisms underlying the clinical heterogeneity are not fully understood. As 13q deleted region includes miRNAs, the evaluation of miRNA expression profile could provide new biological evidence of the clinical variability observed in this subgroup of CLL patients. Furthermore, taking into account that the quantification of the number of cells with a genetic abnormality has a prognostic value, it would be interesting to assess the impact of the number of cells with other cytogenetic alterations, such as 11q deletion, in order to improve CLL prognostication accuracy. 11q- usually implies the loss of *ATM* which is involved in the repair of damaged DNA and its deficiency causes genomic instability. Thus, the clinical heterogeneity observed within this subgroup of CLL patients could be a reflex of its genetic heterogeneity.

The advent of NGS has been transformative for understanding the molecular underpinnings of blood malignancies with the discovery of novel mutated genes, some of them commonly affected among different hematological malignancies. Moreover, little is known about how gene mutations can influence gene expression. Therefore, an integrative analysis of transcriptomic networks as well as the mutational status could be highly useful for CLL study, specifically in target genes playing a role in other hematological neoplasms.

NGS technologies have also described marked clonal changes at genetic level during the course of the disease. In fact, clonal evolution is considered as a hallmark of progression and relapse in CLL. The acquisition of driver mutations accompanied

by selectively neutral passenger changes may be essential to understand the transformation from diagnosis to later more aggressive stages. However, the key genetic events that likely fuel the clonal evolution during the clinical progression prior any therapy are still largely unknown.

Therefore, I hypothesize that the combination of different transcriptomic and genomic high-throughput molecular techniques such as expression arrays and NGS will allow us to gain insight into miRNA expression deregulation and mutational status within CLL subgroups. The integration of these molecular studies can better explain the biological evidence underlying the clinical heterogeneity and disease evolution in CLL patients.

Aims

General aim

To improve understanding of the molecular basis of clinical heterogeneity in CLL patients displaying different cytogenetic alterations as well as the molecular mechanisms that could be involved in disease evolution of CLL patients, through a combined analysis of the genome and transcriptome of B clonal lymphocytes.

Specific aims

1. To define the miRNA expression profile of B clonal lymphocytes by miRNA microarrays in CLL patients with 13q deletion according to the percentage of cells displaying this aberration, in order to characterize the biological mechanisms responsible for the clinical heterogeneity observed in this subgroup of patients.
2. To assess the clinico-biological characteristics and outcome of CLL patients with 11q deletion, analyzing whether the percentage of losses in 11q can have an impact on the overall survival and time to the first therapy.
3. To characterize the mutational status of *ATM*, *TP53*, *NOTCH1*, *SF3B1*, *MYD88*, *FBXW7*, *XPO1* and *BIRC3* in CLL patients with 11q deletion by Next-Generation Sequencing (NGS), in order to gain insights into the genetic mechanisms underlying the different clinical course in this subgroup of patients.
4. To evaluate the role of *TET2* in CLL analyzing its expression level and the presence of variations by the combination of expression microarrays and Next-Generation Sequencing in B clonal lymphocytes from CLL patients.
5. To analyze the clonal evolution during the clinical progression in CLL, identifying the genetic changes by whole-exome sequencing in a longitudinal study.

Results

This section includes the experimental work performed on this thesis, including Material and Methods, Results and Discussion. This section has been divided into four chapters:

Chapter 1. M Hernández-Sánchez, AE Rodríguez-Vicente, JA Hernández, E Lumbreras, ME Sarasquete, AA Martín, R Benito, C Vicente-Gutiérrez, C Robledo, N de las Heras, JN Rodríguez, M Alcoceba, A García de Coca, C Aguilar, M González, JM Hernández-Rivas. **MiRNA expression profile of chronic lymphocytic leukemia patients with 13q deletion.** Leuk Res. 2016 Jul;46:30-6. doi: 10.1016/j.leukres.2016.04.008. Pubmed PMID: 27111859.

Chapter 2. JA Hernández*, M Hernández-Sánchez*, AE Rodríguez-Vicente, V Grossmann, R Collado, C Heras, A Puiggros, AA Martín, N Puig, R Benito, C Robledo, J Delgado, T González, JA Queizán, J Galende, I de la Fuente, G Martín-Núñez, JM Alonso, P Abrisqueta, E Luño, I Marugán, I González-Gascón, F Bosch, A Kohlmann, M González, B Espinet, JM Hernández-Rivas, Grupo Cooperativo Español de Citogenética Hematológica (GCECGH) and Grupo Español de Leucemia Linfática Crónica (GELLC). **A Low Frequency of Losses in 11q Chromosome Is Associated with Better Outcome and Lower Rate of Genomic Mutations in Patients with Chronic Lymphocytic Leukemia.** PLoS One. 2015 Dec 2;10(11):e0143073. doi: 10.1371/journal.pone.0143073. PMID: 26630574.

*These authors contributed equally to this work.

Chapter 3. M Hernández-Sánchez, AE Rodríguez, A Kohlmann, R Benito, JL García, A Risueño, E Fermiñán, J de las Rivas, M González, JM Hernández-Rivas. **TET2 overexpression in chronic lymphocytic leukemia is unrelated to the presence of TET2 variations.** Biomed Res Int. 2014;2014:814294. doi: 10.1155/2014/814294. PMID: 24693539.

Chapter 4. M Hernández-Sánchez, AE Rodríguez, M Abáigar, D Tamborero, R Benito, M Quijada, AA Martín, A García de Coca, N López-Bigas, JM Hernández-Rivas. **Analysis of clonal evolution in chronic lymphocytic leukemia from diagnosis to symptomatic disease using whole-exome sequencing.** Manuscript in preparation.

All of them have been developed to accomplish the general aim of this work and give the title to this doctoral dissertation: **Analysis of the molecular heterogeneity and disease evolution in chronic lymphocytic leukemia by combining high-throughput genomic and transcriptomic technologies.**

A General Discussion, with additional data and which comprises all research, is addressed in a separate section of this thesis.

In addition, supplementary data corresponding to each of the above chapters are collected at the end of the thesis.

MiRNA expression profile of chronic lymphocytic leukemia patients with 13q deletion

María Hernández-Sánchez¹, Ana E. Rodríguez-Vicente¹, José-Ángel Hernández², Eva Lumbreras¹, María-Eugenia Sarasquete¹, Ana-África Martín¹, Rocío Benito¹, Carlos Vicente-Gutiérrez¹, Cristina Robledo¹, Natalia de las Heras³, Juan-Nicolás Rodríguez⁴, Miguel Alcoceba¹, Alfonso García de Coca⁵, Carlos Aguilar⁶, Marcos González¹, Jesús-María Hernández-Rivas¹

¹Hematology Department, Centro de Investigación del Cáncer, IBSAL, IBMCC, Universidad de Salamanca-CSIC, Hospital Universitario de Salamanca, Salamanca, Spain

²Hematology Department, Hospital Universitario Infanta Leonor, Universidad Complutense de Madrid, Madrid, Spain

³Hematology Department, Hospital Virgen Blanca, León, Spain

⁴Hematology Department, Hospital Juan Ramón Jiménez, Huelva, Spain

⁵Hematology Department, Hospital Clínico Universitario, Valladolid, Spain

⁶Hematology Department, Hospital Santa Bárbara, Soria, Spain

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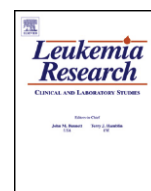
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Research paper

MiRNA expression profile of chronic lymphocytic leukemia patients with 13q deletion



María Hernández-Sánchez^a, Ana E. Rodríguez-Vicente^a, José-Ángel Hernández^b, Eva Lumbreras^a, María-Eugenia Sarasquete^a, Ana-África Martín^a, Rocío Benito^a, Carlos Vicente-Gutiérrez^a, Cristina Robledo^a, Natalia de las Heras^c, Juan-Nicolás Rodríguez^d, Miguel Alcoceba^a, Alfonso García de Coca^e, Carlos Aguilar^f, Marcos González^a, Jesús-María Hernández-Rivas^{a,*}

^a Hematology Department, Centro de Investigación del Cáncer, IBSAL, IBMCC, Universidad de Salamanca-CSIC, Hospital Universitario de Salamanca, Salamanca, Spain

^b Hematology Department, Hospital Universitario Infanta Leonor, Universidad Complutense de Madrid, Madrid, Spain

^c Hematology Department, Hospital Virgen Blanca, León, Spain

^d Hematology Department, Hospital Juan Ramón Jiménez, Huelva, Spain

^e Hematology Department, Hospital Clínico Universitario, Valladolid, Spain

^f Hematology Department, Hospital Santa Bárbara, Soria, Spain

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ABSTRACT

Deletion 13q (13q-) is the most common cytogenetic aberration in chronic lymphocytic leukemia (CLL) and is associated with the most favorable prognosis as the sole cytogenetic abnormality. However, it is heterogeneous whereby CLL patients with higher percentages of 13q- cells (13q-H) have a more aggressive clinical course and a distinct gene expression profile. The microRNA (miRNA) expression profile of CLL gives additional biological and prognostic information, but its expression in 13q- CLL has not been examined in detail. The miRNA expression of clonal B cell lymphocytes (CD19+ cells) of 38 CLL patients and normal B cells of six healthy donors was analyzed. CLL patients with higher percentages of 13q- cells ($\geq 80\%$) showed a different level of miRNA expression from patients with lower percentages ($< 80\%$). Interestingly, miR-143 was downregulated and miR-155 was overexpressed in 13q-H. This deregulation affected important validated target genes involved in apoptosis (*BCL2*, *MDM2*, *TP53INP1*) and proliferation (*KRAS*, *PI3K-AKT* signaling), that could lead to decreased apoptosis and increased proliferation in 13q-H patients. This study provides new evidence about the heterogeneity of the 13q deletion in CLL patients, showing that miRNA regulation could be involved in several significant pathways deregulated in CLL patients with a high number of losses in 13q.

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1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adult populations of Western countries. It is characterized by the progressive accumulation of mature, monoclonal B lymphocytes in the blood, bone marrow and secondary lymphoid tissues [1]. CLL is a hematological disease with a highly variable clinical course that can be predicted by the presence of specific cytogenetic abnormalities. Thus, patients with complex karyotypes

or deletions of the 11q22-q23 or 17p13 regions have a worse prognosis than those with a normal karyotype or with the 13q14 deletion as the sole genetic abnormality [2,3].

13q deletion is the most common cytogenetic abnormality detected by FISH. Recent findings by our group and others suggest that patients with del(13q) are a heterogeneous group, with a different outcome depending on the number of cells displaying this aberration [4–7]. Specifically, CLL patients with higher percentages of 13q- cells (13q-H) have more aggressive clinical courses with a shorter time to first therapy (TFT) and overall survival (OS). Subsequent molecular studies have yielded biological evidence including the identification of a distinct gene expression profile for 13q-H CLL patients underlying the clinical heterogeneity related to the size of the abnormal 13q- clone [8].

* Corresponding author at: Hematology Department, Centro de Investigación del Cáncer, IBSAL, IBMCC, Universidad de Salamanca-CSIC, Hospital Universitario de Salamanca, Paseo de San Vicente s/n, 37007 Salamanca, Spain.

E-mail address: jmhr@usal.es (J.-M. Hernández-Rivas).

MiRNAs are small, non-coding RNAs that mediate the expression of target genes through sequence-specific base pairing with target mRNA [9]. Deregulation of miRNAs has been implicated in human tumorigenesis and many miRNAs are located in genomic regions involved in cancer [10], such as miR-15a and miR-16-1 in the minimal deleted region in 13q14. Moreover, the miRNA expression profile has been used to identify signatures in CLL [11–15], although not all of them have been performed in isolated CD19 cells. Recent studies have demonstrated a decrease in miR-29c and miR-223 levels during disease progression [16], and overexpression of miR-21 or miR-155 and low miR-181b expression have been reported as unfavorable prognostic factors [13,17,18]. However, few studies have attempted to profile miRNA expression in clonal B lymphocytes regarding specific cytogenetic aberrations [19,20]. Although some miRNAs have been suggested to be deregulated in 13q-H patients [8], the miRNA signature of 13q- CLL patients has not been so far analyzed in detail depending on the number of cells displaying this aberration by specific miRNA arrays.

The present study aimed to analyze whether 13q- CLL cases may differ in their miRNA signature with respect to the number of cells harbouring the aberration. Differences in the expression of several miRNAs, such as miR-143 and miR-155, were observed between the two 13q- CLL subgroups. These data provide new insights into the biological mechanisms underlying the different clinical course of 13q- CLL patients.

2. Methods

2.1. Patients

Samples were collected from 38 untreated CLL patients and 6 healthy donors. CLL diagnosis was performed according to the World Health Organization (WHO) classification [21] and the Working Group of National Cancer Institute (NCI) criteria [22]. A complete immunophenotypic analysis by flow cytometry [23] and FISH studies were carried out for all CLL cases. Only samples with low-risk cytogenetics (deletion in 13q as the sole cytogenetic abnormality and normal FISH) were included in the study. Based on our previous results [5,8], two groups of patients with 13q- were chosen for study: those in whom 80% or more cells showed 13q- (13q-H) and those in whom fewer than 80% of cells showed 13q losses (13q-L). The distribution of patients was: 13q-H (n = 14; 37%), 13q-L (n = 13; 34%) and normal FISH (nCLL, n = 11; 29%). The main biological features of the CLL patients are summarized in Supplementary Table 1. Monoallelic 13q14 deletions were found in 22 13q- CLL samples, whereas biallelic deletions were detected in 5 patients. An independent group of 18 CLL patients has been used to validate the results of miRNA expression. Among them, 10 were 13q-H whereas 8 were 13q-L. The study was approved by the local ethical committee (Comité Ético de Investigación Clínica, Hospital Universitario de Salamanca). Written informed consent was obtained from all participants before they entered the study.

2.2. B cell isolation

B lymphocytes (CD19-positive cells) from CLL patients and healthy donors were positively selected using magnetically activated cell sorting (MACS) CD19 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. B cell purity was greater than 98%, as determined by flow cytometry.

2.3. miRNA expression profile using high-density microarrays

The expression of miRNAs in the isolated samples was analyzed using the Gene Chip® miRNA 3.0 Array (Affymetrix, Inc., Santa

Clara, CA, USA). RNA isolation, labeling and microarray hybridization were carried out following the manufacturer's protocols for the Affymetrix GeneChip platform. Detailed methods are described in the Supplementary methods.

2.4. Microarray data analysis

The Robust Microarray Analysis (RMA) algorithm was used for background correction, intra- and inter-microarray normalization, and expression signal calculation [24]. After filtering the probe sets corresponding to mature human miRNA and human pre-miRNA, subsequent analyses were performed using MeV v4.3.2 software [25]. Initially, Significance Analysis of Microarray (SAM) [26] was used to identify the miRNAs differentially expressed in low-risk CLL patients compared with healthy donors. A false discovery rate (FDR) cut-off of <0.01 was used. The Welch approximate *t*-test was then applied to identify those miRNAs that were differentially expressed between 13q-H and 13q-L patients ($P < 0.05$).

2.5. Functional analysis

Functional analysis was done and potential miRNA targets identified using Ingenuity Pathway Analysis (IPA Ingenuity Systems; <http://www.ingenuity.com>). In order to select the targets with an experimental observation of the miRNA-gene interaction, only the category “experimentally validated targets” were considered, using miRecords (<http://mirecords.biolead.org/>), Tarbase (<http://microrna.gr/tarbase>) or TargetScan (<http://www.targetscan.org/>). To gain insights into the potential mechanisms affected by the downregulation of miR-143 and the overexpression of miR-155 in B lymphocytes of 13q-H compared with 13q-L patients, deregulated genes were mapped to regulatory networks using Ingenuity Pathway Analysis (IPA Ingenuity Systems, <http://www.ingenuity.com>).

Selected mature miRNA expression was validated using TaqMan MicroRNA Assays (Applied Biosystems) specific for miR-15a, miR-16-1 and miR-143 in 20CLL patients (10 13q-H and 10 13q-L), following the manufacturer's recommendations (Supplementary methods). For miR-155, the expression in 10 nCLL and 6 healthy donors was also assessed.

Moreover, expression data for selected target genes were validated in a subset of CLL patients (n = 26) by semi-quantitative SYBRgreen PCR in triplicate with SYBR® Green PCR Master Mix (Applied Biosystems) using the IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad), following the manufacturer's recommendations (Supplementary methods). Primer sequences were shown in Supplementary Table S2.

2.6. Statistical analysis

Statistical analysis was performed using SPSS (v20). Differences between the results of the qRT-PCR experiments with CLL patients were analyzed with the Mann–Whitney *U* test. Values of $P < 0.05$ for the tests were considered statistically significant.

3. Results

3.1. MiRNA expression profiles differ between 13q- CLL subgroups

The comparative analysis of miRNA expression in the two subgroups of 13q- CLL patients with respect to the percentage of 13q- cells revealed the differential expression of 76 miRNAs (*t* test, $P < 0.05$). Patients with a high number of 13q losses showed 35 and 41 miRNAs overexpressed and downregulated, respectively, relative to 13q-L CLL patients (Supplementary Table 3).

MiRNA target analysis identified that 18 (8 overexpressed and 10 underexpressed) of the 76 deregulated miRNAs had experimen-

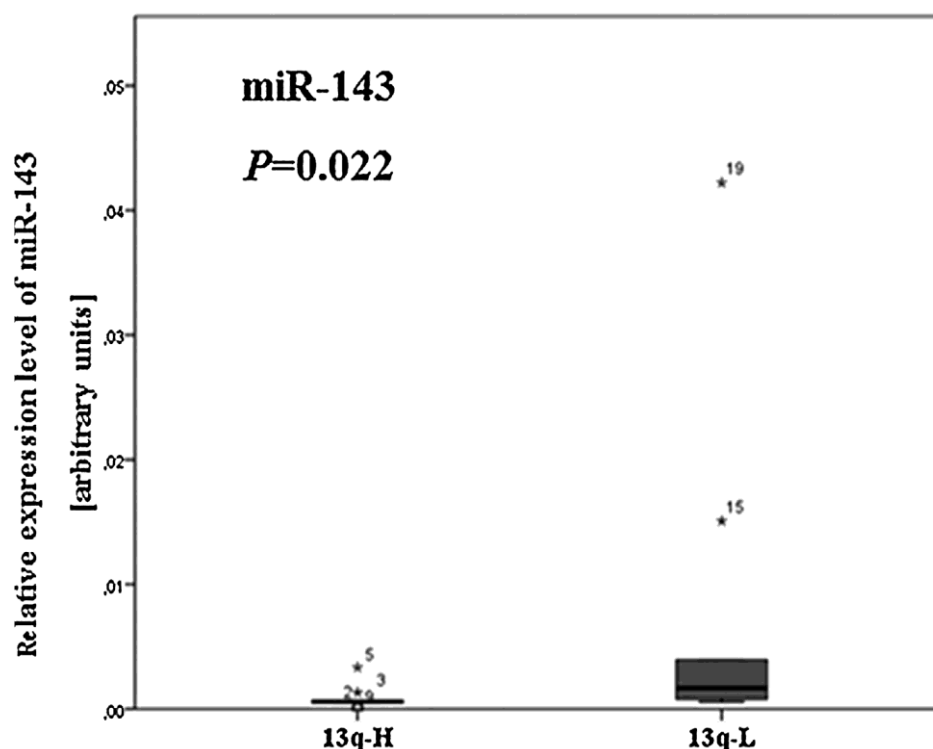


Fig. 1. Quantitative RT-PCR validation for miR-143 in 13q- CLL with respect to percentage losses of 13q. MiR-143 was found to be significantly downregulated in B lymphocytes from 13q-H vs. 13q-L CLL patients. Relative expression of miR-143 (expressed in arbitrary units) was evaluated by individual TaqMan miRNA assays performed in duplicate and normalized with respect to RNU43 ($2^{-\Delta\Delta C_t}$) in 20CLL patients (10 13q-H and 10 13q-L). Box plots indicate the median value (horizontal line) and the 25th–75th percentile range (box). Whiskers show the maximum and minimum values. Outliers are shown as open circles. *P*-values were determined by the Mann–Whitney *U* test.

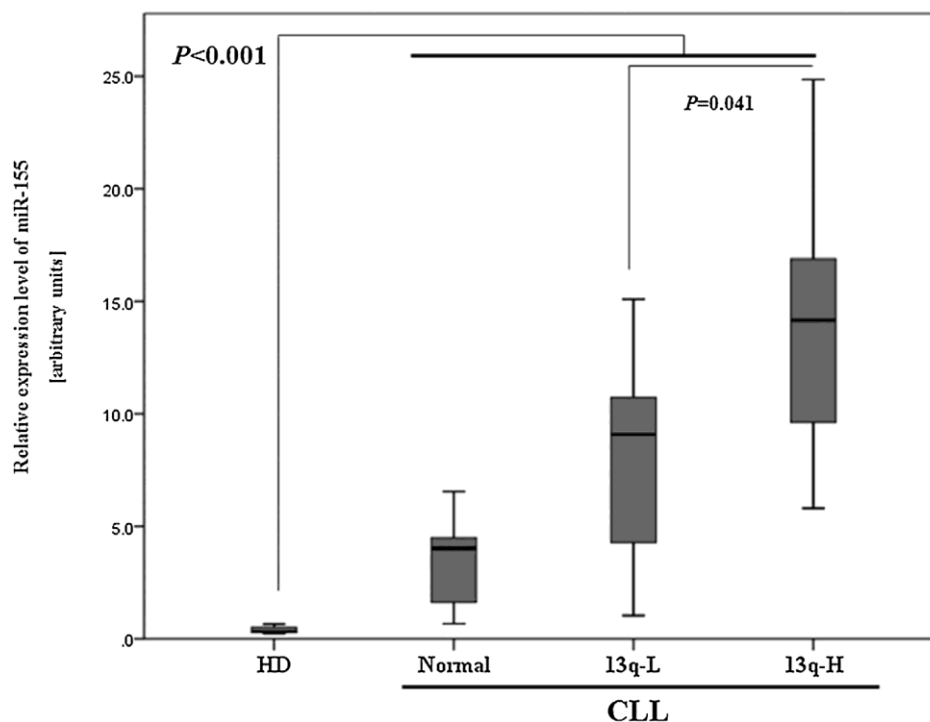


Fig. 2. Quantitative RT-PCR validation for miR-155 in CLL patients (13q-H vs 13q-L vs normal FISH) and healthy donors. MiR-155 was found to be significantly upregulated in B lymphocytes of 13q-H CLL patients relative to B cells of 13q-L CLL patients and in 13q- CLL patients relative to normal FISH CLL patients. MiR-155 was also found to be significantly upregulated in B lymphocytes of CLL patients relative to B cells of healthy donors (HD) by quantitative RT-PCR. Relative expression of miR-155 (expressed in arbitrary units) was evaluated by individual TaqMan miRNA assays performed in duplicate and normalized to RNU43 ($2^{-\Delta\Delta C_t}$). Box plots indicate the median value (horizontal line) and the 25th–75th percentile range (box). Whiskers show the maximum and minimum values. Outliers are shown as open circles. *P*-values were determined by the Mann–Whitney *U* test.

Table 1

MiRNAs with experimentally validated targets that are significantly deregulated in B lymphocytes from 13q-H compared to 13q-L patients.

miRNA	Expression	P-value	Number of targets ^a
miR-181a	Down	0.006	17
miR-143	Down	0.007	10
miR-130a	Down	0.010	8
miR-223	Down	0.016	9
miR-145	Down	0.021	43
miR-675	Down	0.022	1
miR-100	Down	0.026	6
miR-503	Down	0.026	1
miR-199a-5p	Down	0.043	7
miR-3687	Down	0.047	1
miR-515-3p	Up	0.002	3
miR-499a-5p	Up	0.025	1
miR-362-3p	Up	0.026	3
miR-155	Up	0.031	125
miR-133b	Up	0.037	27
miR-302d	Up	0.046	85
miR-521	Up	0.046	1
miR-23b	Up	0.048	18

^a Number of targets represents how many experimentally validated targets these 18 miRNAs have, basing on experimental observations that have demonstrated the miRNA-gene interaction and identified using Ingenuity Pathway Analysis (IPA Ingenuity Systems; <http://www.ingenuity.com>). Upregulation or downregulation refers to 13q-H relative to 13q-L CLL patients.

tally validated mRNA targets (Table 1). In total, 344 genes were potentially regulated by miRNAs (Supplementary Table 4). Two of these miRNAs were analyzed in 13q- CLL patients by qRT-PCR, confirming the downregulation of miR-143 ($P=0.022$) (Fig. 1) and the overexpression of miR-155 ($P=0.041$) in B lymphocytes from 13q-H (Fig. 2). In addition, these results were validated in an independent group of 13q- CLL patients (Supplementary Fig. 1).

3.2. Deregulated genes by the underexpression of miR-143 and overexpression of miR-155 in 13q-H patients

MiR-143 was one of the most significantly downregulated miRNAs ($P<0.01$) in the 13q-H CLL group (Table 1). This miRNA is located on chromosome 5 and is cotranscribed as a cluster with miR-145, which is also downregulated in 13q-H (Table 1). *In silico* analysis showed that miR-143 had 10 predicted targets (Table 1 and Supplementary Table 4), distinguishing two antiapoptotic genes (*BCL2*, *MDM2*) and four kinases (*KRAS*, *MAPK7*, *MAPK12* and *PLK1*), most of which are involved in cell proliferation. Other targets of miR-143, such as *PRC1* and *TOP2A*, play a role in the cell cycle.

By contrast, miR-155 was overexpressed in B lymphocytes from 13q-H compared with 13q-L CLL patients (Table 1). A total of 125 of the 344 genes (36.4%) reported in Table 1 and described in Supplementary Table 4 are predicted target mRNAs of miR-155, being the miRNA with the largest number of targets. The *in silico* analysis revealed that there were three tumor suppressor genes (*TP53INP1*, *RhoA*, *SOCS1*) among these targets. In fact, two proapoptotic molecules, such as *FOXO3a* and *TP53INP1*, are targeted by this miRNA. MiR-155 is also involved in cell cycle through *SOCS1* and in phosphatidylinositol 3-kinase (PI3 K)-AKT signaling through *INPP5D*.

The semi-quantitative PCR results of some miRNAs targets indicated the overexpression of *BCL2* and *PLK1* and the downregulation of *INPP5D* and *SOCS1* in 13q-H CLLs compared with 13q-L (Supplementary Fig. 2).

Thus, the analysis showed that the miRNA expression profile of 13q- CLLs is different depending on the percentage of cells displaying this aberration. Among the deregulated miRNAs, miR-143 and miR-155 provided great insights into the biological mechanisms underlying the clinical differences in this CLL subgroup (Supplementary Table 5).

3.3. MiR-15a and miR-16-1 are both downregulated in 13q-H CLL with biallelic deletion

Significant decreases in the expression of hsa-miR-15a (t test, $P=0.012$) and hsa-miR-16 (t test, $P=0.00007$), which are both colocalized in the 13q14.3 region, were observed in the subgroup of CLL patients with biallelic 13q14 deletion (CLL patients 11, 12, 13, 14 and 27) with respect to the patients with monoallelic losses. The greatest decrease in the expression of hsa-miR-15a was detected in three patients (CLL 12, 13 and 14), all of them with more than 80% of cells with biallelic 13q14 deletion. By contrast, no significant differences in expression were observed in either of the miRNAs with respect to the percentage of cells with 13q- (Supplementary Fig. 3).

Due to the design of the Gene Chip® miRNA 3.0 Array used in this study, miR-15a expression could be specifically analyzed by a probeset (hsa-miR-15a_st), whereas that measuring miR-16-1 expression is not specific for this miRNA, since it could also detect the expression of hsa-miR-16-2, located on chromosome 3. To confirm the microarray results and assess specifically the expression of miR-16-1, both miRNAs were also analyzed by qRT-PCR. The results were closely correlated with the microarray data (Fig. 3). As expected, miR-15a and miR-16-1 expression was significantly lower in cases with biallelic deletion than in those with monoallelic losses ($P=0.007$).

The levels of miR-15a and miR-16-1 expression detected by the miRNA microarray differed among individual patients of the cohort (Supplementary Fig. 3), even though both miRNAs were colocalized in the 13q14.3 region. This result suggests that the two miRNAs exhibit distinct regulation of expression that is likely to be indicative of their functional independence.

3.4. Differential expression of miRNAs in low-risk CLL patients

Global miRNA expression profiles were determined in CD19+ cells from low-risk CLL patients and healthy donors. A total of 381 mature miRNAs significantly distinguished the two groups (SAM, FDR<0.01). These comprised 96 overexpressed and 285 underexpressed miRNAs in the low-risk CLL patients. Supplementary Table 6 reports the top 100 differentially expressed miRNAs in clonal CLL lymphocytes, along with the corresponding fold-change (FC) values in their expression relative to that in normal B lymphocytes.

In low-risk CLL patients, the three most highly upregulated miRNAs (FC>30) were miR-1295, miR-21 and miR-34a, whereas the three most significantly downregulated miRNAs (FC<0.01) were miR-432, miR-181a and miR-181c. One of the deregulated miRNAs (miR-155) was further assayed by qRT-PCR for validation purposes in 30 CLL patients and 6 healthy donors. These results confirmed that clonal B cells from CLL patients have a significantly higher expression of miR-155 than B cells from healthy donors ($P<0.001$) (Fig. 2). Moreover, within the CLL patients, 13q-H had a higher level of expression of miR-155 than 13q-L, and this subgroup showed stronger expression of this miRNA than did CLL patients with normal FISH (Fig. 2).

4. Discussion

Deletion 13q is the most common chromosomal aberration in CLL and patients with 13q- as the sole abnormality are typically associated with favorable outcome [2]. However, several studies have indicated that CLL patients with higher percentages of 13q- cells have a poorer outcome in terms of TFT and OS [4–7]. Subsequent molecular studies have indicated that the gene expression profile of 13q- CLL differs between 13q-H and 13q-L patients [8]. As a next step towards elucidating the biological differences within the 13q- subgroup, this study shows the deregulated miRNAs in CLL

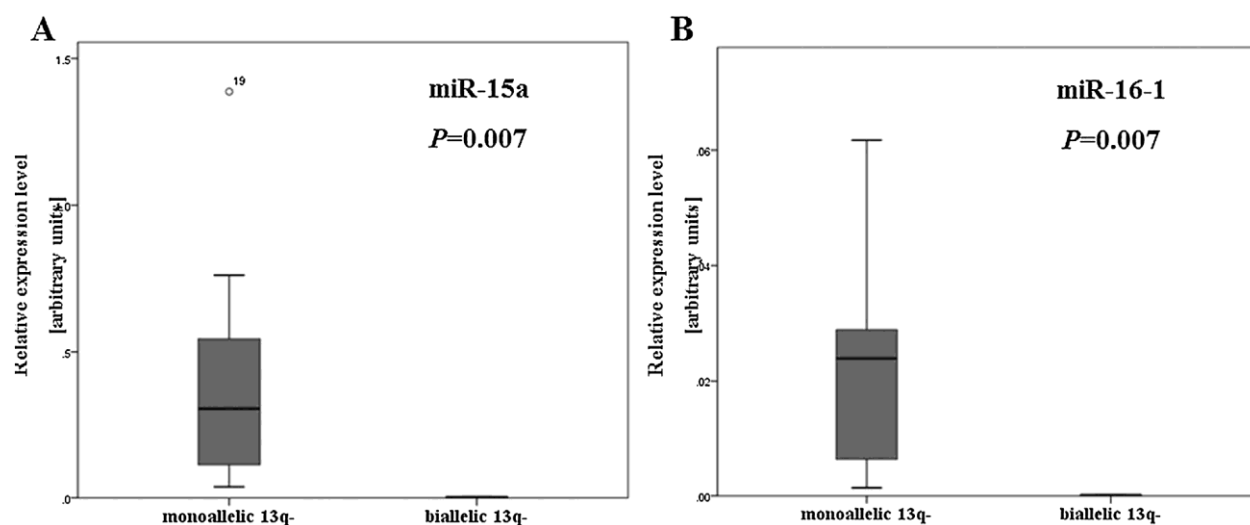


Fig. 3. Quantitative RT-PCR validation for miR-15a (A) and miR-16-1 (B) in 13q-patients showing monoallelic and biallelic 13q deletion.

MiR-15a (A) and miR-16-1 (B) were significantly downregulated in B lymphocytes from biallelic 13q- CLL patients relative to B cells from monoallelic 13q- CLL patients, as shown by quantitative RT-PCR. This technique allowed us specifically to measure miR-16-1 from 13q- patients. Relative expression of miR-15a and miR-16-1 (expressed in arbitrary units) was evaluated by individual TaqMan miRNA assays performed in duplicate and normalized with respect to RNU43 ($2^{-\Delta\Delta C_t}$). Box plots indicate the median value (horizontal line) and the 25th–75th percentile range (box). Whiskers show the maximum and minimum values. Outliers are shown as open circles. *P*-values were determined by the Mann–Whitney *U* test.

patients with a high number of losses in 13q by miRNA microarrays. Among the novel deregulated miRNAs, miR-143 was the second most significantly downregulated miRNA with validated targets after miR-181 (Table 1). By contrast, miR-155 was upregulated in 13q-H and has also recently been described as a marker of poor prognosis [17]. In addition, several important miRNAs, such as miR-223, miR-29c and miR-181, were downregulated in 13q-H, as in the high-risk cytogenetic subgroups of CLL [14,16]. Therefore, miRNA regulation could be related to the worse outcome in 13q-H patients and may be involved in several significant pathways deregulated in 13q-H CLL patients [8].

MiR-143 is one of the main deregulated miRNAs in cancer (<http://atlasgeneticsoncology.org/Deep/MicroRNAandCancerID20101.html>). It is transcribed concomitantly with miR-145 (which is also downregulated in 13q-H, Table 1) as a cluster in the 5q32 chromosomal region. Downregulation of miR-143 has previously been described in B cell malignancies [27,28]. More specifically, relative downregulation of miR-143 has been reported in unmutated *versus* mutated *IGHV* CLL patients [29]. However, this is the first study in which miR-143 has been observed to be downregulated in 13q-H patients, a subgroup of CLL patients with worse outcome than 13q-L. MiR-143 was found to be a tumor-suppressive miRNA [30]. This anti-oncomir works through targeting genes (Supplementary Table 4), that could result in increased cell proliferation by higher activation of *KRAS* and *MAPK* genes, as previously described in colorectal cells [30], and more defective apoptosis by the upregulation of antiapoptotic *BCL2* and *MDM2* genes, both of which play an important role in the tumorigenicity and disease progression of CLL (Supplementary Table 5) [31,32]. Thus, 13q-H patients may have an imbalance between proliferative and apoptotic signals, which could explain the higher level of lymphocytosis and the poor outcome previously described in these patients [5].

MiRNA expression profiling has been performed in CLL patients by several methods: qRT-PCR, cloning and microarrays of defined miRNAs [11–15]. However, few studies have been carried out with purified B lymphocytes from CLL patients using miRNA microarrays and considering cytogenetic alterations. In this work, we also report results of extensive genome wide profiling of miRNA expression in

CD19+ cells from low-risk CLL patients. Several miRNAs of known importance in the biology of CLL were observed deregulated in 13q-H patients. Of these, this study confirmed a relative overexpression of miR-155 in B cells from 13q-H *versus* 13q-L patients, as previously reported [8]. This miRNA is also confirmed to be upregulated in CLL (Fig. 2) [13,14,17]. MiRNA-155 is processed from the B cell integration cluster (*BIC*) non-coding gene located on chromosome 21 and its expression is induced by B-cell receptor (BCR) activation [33]. Therefore, overexpression of miR-155 could be related to enhanced BCR-activation, previously described as one of the most significant affected pathways in 13q-H CLL patients, due to the overexpression of genes mainly involved in BCR signaling [8]. Moreover, this miRNA is a well-known oncomir involved in various cancers [34]. More than 100 genes have so far been confirmed as being directly targeted by this miRNA. Notably, inhibition of two proapoptotic molecules such as *FOXO3a* and *TP53INP1* by miR-155 [35,36] could lead to a decrease in apoptosis, one of the most important pathways affected in 13q-H CLL patients [8]. In addition, miR-155 also targets *SOCS1*, whose inhibition stimulates the activity of the JAK2/STAT3 pathway, leading to a greater cell survival [37]. Interestingly, it has been recently reported that *STAT3* can induce miRNA-155 expression in CLL cells [38]. Moreover, another important deregulated pathway in CLL, PI3K-AKT signaling, could be more activated in these patients through inhibition of its negative regulator *INPP5D*, as has been reported in CLL [39] and diffuse large B cell lymphoma (Supplementary Table 5) [40]. Its prognostic value has recently been reported in monoclonal B-cell lymphocytosis and CLL [17]. In this study, we found that the level of miR-155 expression is higher for worse outcomes in CLL patients (Fig. 2).

Furthermore, miR-155 and miR-143 are connected to each other by *C/EBPβ* [41], which is a validated target of miR-155 [42] that may play a role as a transcriptional activator for miR-143. Therefore, overexpression of miR-155 could repress miR-143, which is infra-expressed in 13q-H patients, by targeting *C/EBPβ* and subsequently deregulating miR-143 targets.

The location of miR-15a and miR-16-1 in the 13q deletion suggests that the role of these miRNAs in the pathogenesis of CLL is not negligible [43,44]. Earlier studies reported decreased expression of miR-15a and miR-16-1 in CLL patients [10–12,43,45]. However,

more recent studies have not confirmed a link between underexpression of these miRNAs and the presence of 13q- [46,47]. This study shows significant infraexpression of miR-15a and miR-16-1 in B lymphocytes with biallelic 13q- cells compared with those with monoallelic deletion, as previously reported [29,48,49]. This could be related to a worse outcome of the biallelic 13q- patients [50], although differences in prognosis between monoallelic and biallelic deleted cases are difficult to confirm [6]. Although no significant decrease in expression was observed in either of these miRNAs with respect to the percentage of cells with monoallelic 13q14 deletion, the three samples with the most significant underexpression of both miRNAs were 13q-H patients with biallelic deletion.

In summary, this study provides new evidence about the heterogeneity of the 13q deletion in CLL patients, showing that miRNA regulation could be involved in several significant pathways deregulated in 13q-H CLL patients. Notably, infraexpression of miR-143 and overexpression of miR-155 could trigger the deregulation of genes involved in apoptosis (*BCL2*, *MDM2*, *TP53INP1*) and proliferation (*KRAS*, *PI3K-AKT* signaling), leading to lower apoptosis and higher proliferation rates in B cells of 13q-H patients. This study improves our understanding of why the subgroup of CLL patients with a high number of losses of 13q has a poor outcome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.leukres.2016.04.008>.

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A low frequency of losses in 11q chromosome is associated with better outcome and lower rate of genomic mutations in patients with chronic lymphocytic leukemia



José Ángel Hernández^{*1}, María Hernández-Sánchez^{*2}, Ana Eugenia Rodríguez-Vicente², Vera Grossmann³, Rosa Collado⁴, Cecilia Heras¹, Anna Puiggros⁵, Ana África Martín⁶, Noemí Puig⁶, Rocío Benito², Cristina Robledo², Julio Delgado⁷, Teresa González⁸, José Antonio Queizán⁹, Josefina Galende¹⁰, Ignacio de la Fuente¹¹, Guillermo Martín-Núñez¹², José María Alonso¹³, Pau Abrisqueta¹⁴, Elisa Luño¹⁵, Isabel Marugán¹⁶, Isabel González-Gascón¹, Francesc Bosch¹⁴, Alexander Kohlmann^{3,17}, Marcos González^{2,6}, Blanca Espinet⁵, Jesús María Hernández-Rivas^{2,6,18}, Grupo Cooperativo EspaHematológica (GCECGH) and Grupo Español de Leucemia Linfática Crónica (GELLC)

¹Hematology Department, Hospital Universitario Infanta Leonor, Universidad Complutense de Madrid, Spain; ²IBSAL, IBMCC, Centro de Investigación del Cáncer, Universidad de Salamanca, CSIC, Hospital Universitario de Salamanca, Spain; ³MLL Munich, Germany; ⁴Hematology Department, Hospital General, Valencia, León, Spain; ⁵Pathology Department, Hospital del Mar, Barcelona, Spain; ⁶Hematology Department, Hospital Universitario, Salamanca, Spain; ⁷Hematology Department, Hospital Clinic I Provincial, Barcelona, Spain; ⁸Fundación Pública Galega de Medicina Xenómica, Santiago de Compostela, Spain; ⁹Hematology Department, Hospital General, Segovia, Spain; ¹⁰Hematology Department, Hospital del Bierzo, Ponferrada, Spain; ¹¹Hematology Department, Hospital Universitario Río Hortega, Valladolid, Spain; ¹²Hematology Department, Hospital Virgen del Puerto, Plasencia, Cáceres, Spain; ¹³Hematology Department, Hospital Río Carrión, Palencia, Spain; ¹⁴Hematology Department, Hospital Vall d'Hebron, Barcelona, Spain; ¹⁵Hematology Department, Hospital Central de Asturias, Oviedo, Spain; ¹⁶Hematology Department, Hospital Clínico, Valencia, Spain; ¹⁷AstraZeneca, Personalized Healthcare and Biomarkers, Innovative Medicines, Macclesfield, United Kingdom; ¹⁸Department of Medicine, Universidad de Salamanca, Spain

*These authors contributed equally to this work

RESEARCH ARTICLE

A Low Frequency of Losses in 11q Chromosome Is Associated with Better Outcome and Lower Rate of Genomic Mutations in Patients with Chronic Lymphocytic Leukemia



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José Ángel Hernández¹*, María Hernández-Sánchez², Ana Eugenia Rodríguez-Vicente², Vera Grossmann³, Rosa Collado⁴, Cecilia Heras¹, Anna Puiggros⁵, Ana África Martín⁶, Noemí Puig⁶, Rocío Benito², Cristina Robledo², Julio Delgado⁷, Teresa González⁸, José Antonio Queizán⁹, Josefina Galende¹⁰, Ignacio de la Fuente¹¹, Guillermo Martín-Núñez¹², José María Alonso¹³, Pau Abrisqueta¹⁴, Elisa Luño¹⁵, Isabel Marugán¹⁶, Isabel González-Gascón¹, Francesc Bosch¹⁴, Alexander Kohlmann^{3,17}, Marcos González^{2,6}, Blanca Espinet⁵, Jesús María Hernández-Rivas^{2,6,18*}, Grupo Cooperativo Español de Citogenética Hematológica (GCECGH) and Grupo Español de Leucemia Linfática Crónica (GELLC)[¶]

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1 Hematology Department, Hospital Universitario Infanta Leonor, Universidad Complutense de Madrid, Madrid, Spain, **2** IBSAL, IBMCC, Centro de Investigación del Cáncer, Universidad de Salamanca, CSIC, Hospital Universitario de Salamanca, Spain, **3** MLL Munich, Germany, **4** Hematology Department, Hospital General, Valencia, Spain, **5** Pathology Department, Hospital del Mar, Barcelona, Spain, **6** Hematology Department, Hospital Universitario, Salamanca, Spain, **7** Hematology Department, Hospital Clinic i Provincial, Barcelona, Spain, **8** Fundación Pública Galega de Medicina Xenómica, Santiago de Compostela, Spain, **9** Hematology Department, Hospital General, Segovia, Spain, **10** Hematology Department, Hospital del Bierzo, Ponferrada, León, Spain, **11** Hematology Department, Hospital Universitario Río Hortega, Valladolid, Spain, **12** Hematology Department, Hospital Virgen del Puerto, Plasencia, Cáceres, Spain, **13** Hematology Department, Hospital Río Carrión, Palencia, Spain, **14** Hematology Department, Hospital Vall d'Hebron, Barcelona, Spain, **15** Hematology Department, Hospital Central de Asturias, Oviedo, Spain, **16** Hematology Department, Hospital Clínico, Valencia, Spain, **17** AstraZeneca, Personalized Healthcare and Biomarkers, Innovative Medicines, Macclesfield, United Kingdom, **18** Department of Medicine, Universidad de Salamanca, Spain

* These authors contributed equally to this work.

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* jmhr@usal.es

Abstract

To analyze the impact of the 11q deleted (11q-) cells in CLL patients on the time to first therapy (TFT) and overall survival (OS), 2,493 patients with CLL were studied. 242 patients (9.7%) had 11q-. Fluorescence *in situ* hybridization (FISH) studies showed a threshold of 40% of deleted cells to be optimal for showing that clinical differences in terms of TFT and OS within 11q- CLLs. In patients with $\geq 40\%$ of losses in 11q (11q-H) (74%), the median TFT was 19 months compared with 44 months in CLL patients with $< 40\%$ del(11q) (11q-L) ($P < 0.0001$). In the multivariate analysis, only the presence of 11q-L, mutated *IGHV* status, early Binet stage and absence of extended lymphadenopathy were associated with longer

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Competing Interests: The authors have declared that no competing interests exist. Oligonucleotide primer plates for amplicon deep-sequencing were provided by Roche Diagnostics, Penzberg, Germany, as part of the IRON-II study. VG and AK were employed by MLL Munich Leukemia Laboratory and AK is employed by AstraZeneca. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

TFT. Patients with 11q-H had an OS of 90 months, while in the 11q-L group the OS was not reached ($P = 0.008$). The absence of splenomegaly ($P = 0.02$), low LDH ($P = 0.018$) or $\beta 2M$ ($P = 0.006$), and the presence of 11q-L ($P = 0.003$) were associated with a longer OS. In addition, to detect the presence of mutations in the *ATM*, *TP53*, *NOTCH1*, *SF3B1*, *MYD88*, *FBXW7*, *XPO1* and *BIRC3* genes, a select cohort of CLL patients with losses in 11q was sequenced by next-generation sequencing of amplicons. Eighty % of CLLs with 11q- showed mutations and fewer patients with low frequencies of 11q- had mutations among genes examined (50% vs 94.1%, $P = 0.023$). In summary, CLL patients with <40% of 11q- had a long TFT and OS that could be associated with the presence of fewer mutated genes.

Introduction

In chronic lymphocytic leukemia (CLL) the presence of cytogenetic aberrations assessed by fluorescence *in situ* hybridization (FISH) influences the prognosis, in terms of time to first therapy (TFT), response to treatment, and overall survival (OS) [1]. Deletions on 11q are observed in 9–18% of CLL patients [1,2]. These patients are younger, have abdominal bulky lymph node involvement [3,4], and often show ZAP-70 expression, unmutated status of *IGHV* and shorter survival, although with a highly variable clinical outcome. The use of chemoimmunotherapy, including rituximab and cyclophosphamide to fludarabine schedules, can improve the response in patients with 11q deletion [5].

Deletions of 11q almost invariably include the ataxia telangiectasia mutated (*ATM*) gene [6]. This important tumor suppressor gene plays a crucial role in DNA repair and recombination, and regulates cell cycle progression [7]. Although mutations of this gene have been linked to poor prognosis and are associated with 11q deletions in CLL patients, due to its extreme size (62 coding exons) with lack of well characterized (hot-spot) mutations, *ATM* sequencing studies in CLL have been challenging, leaving several issues unresolved [8–10].

The application of next-generation sequencing (NGS) allows the detection of new candidate genes with frequent mutations in CLL patients as detected by whole-exome and whole-genome sequencing [11–14]. Besides *TP53* mutations [15,16], *NOTCH1* and *SF3B1*, found in around 10% of newly diagnosed CLL patients, are the most frequently mutated genes [17,18]. Patients with mutations in some of these genes have been associated with shorter TFT and OS [19,20]. Other recurrent mutations in *MYD88*, *FBXW7*, *XPO1* and *BIRC3* genes have been reported at frequencies below 10% [19,21]. Moreover, *BIRC3*, a negative regulator of NF κ B signaling pathway, is located near to *ATM* gene, at 11q22 [9].

In the last few years, it has been reported that patients with CLL and 13q deletion may differ in their outcomes depending on the percentage of cells displaying this aberration [2,22–24]. To assess the potential prognostic value of the number of cells with deletion on 11q and to gain insight into the molecular basis of this abnormality in CLL, we have performed a multicenter study of a large series of patients diagnosed with 11q- CLL to determine whether the frequency of losses in 11q has an influence on OS and TFT. Furthermore, NGS studies were carried out, in a subset of patients, to analyze the mutational status of *ATM*, *TP53*, *NOTCH1*, *SF3B1*, *MYD88*, *FBXW7*, *XPO1* and *BIRC3* in this group of patients.

Methods

Patients

A total of 2,493 patients registered in the DataBase of CLL of the Spanish Group of Cytogenetics (GCECGH) and the Spanish Group of CLL (GELLC) were included. The diagnosis of CLL

was made according to the International Workshop on CLL (IWCLL) criteria [25]. In all cases, an immunophenotypic analysis was performed by flow cytometry. FISH studies, including specific probes for at least the 11q22.3–23.1, 12p11.1–q11, 13q14, and 17p13 regions were carried out.

A total of 242 patients (9.7%) had an 11q deletion. The final analysis was limited to 197 cases, including 11q deletion performed at diagnosis of CLL, after excluding cases with monoclonal B-cell lymphocytosis, clonal evolution or inappropriate follow-up (Table A in [S1 File](#)). Basic clinical and biological data were recorded by reviewing the GCECGH and GELLC Data-Base. The study was approved by the local ethical committees “Comité Ético de Investigación Clínica, Hospital Universitario de Salamanca”. Written informed consent was obtained from each patient before they entered the study.

Fluorescence *in situ* hybridization (FISH)

Interphase FISH was performed in peripheral blood samples using commercially available probes for the 13q14, 12p11.1–q11 (alpha satellite), 11q22/ATM and 17p13/P53 regions (Vysis/Abbott Co, Abbott Park, IL, USA). Dual-color FISH using differently labeled control probes and test probes were performed. The methods used for FISH analysis have been described elsewhere [26]. Signal screening was carried out in at least 200 cells with well-delineated fluorescent spots. In cases with 11q deletion a score of $\geq 10\%$ was considered positive, according to the cut-off of our laboratories.

Next-generation sequencing analysis

A total of 25 11q- CLL patients were included in sequencing studies. Samples were obtained at diagnosis in all cases. NGS was performed using 454 Titanium Amplicon chemistry (Roche Applied Science, Penzberg, Germany) [27] to investigate the ATM, TP53, NOTCH1, SF3B1, MYD88, FBXW7, XPO1 and BIRC3 mutations in 11q- CLL patients. Information about primer sequences is shown in Table E in [S1 File](#) and the PCR conditions are described in Table F in [S1 File](#). The oligonucleotide design was performed as part of the IRON-II network.

All data were generated using the GS FLX and Junior Sequencer Instrument software version 2.7 (Roche Applied Science). To detect variants, filters were set to display sequence variants occurring in more than 2% of bidirectional reads per amplicon in at least one patient. Table G in [S1 File](#) shows the median number of reads generated for each gene, allowing variants to be identified down to a detection limit of 2% [28].

Detailed methods are described in the [S1 File](#). The sequencing data are uploaded to the Sequence Read Archive (SRA) (<http://trace.ncbi.nlm.nih.gov/Traces/sra/>) under accession number PRJNA297249. All the information is accessible with the following link <http://www.ncbi.nlm.nih.gov/bioproject/297249>.

Statistical analysis

Statistical analysis were performed using SPSS 20.0 for Windows (SPSS, Chicago, IL, USA). TFT and OS were analyzed on the date of the initial FISH study, coinciding in all of cases with CLL diagnosis. The number of losses in 11q-deleted nuclei was divided into deciles to better define the most significant cut-off point for TFT and OS. The chi-square test was used to assess associations between categorized variables, while continuous variables were analyzed with the Mann-Whitney U test. Statistically significant variables related to TFT and OS were estimated by the Kaplan-Meier method, using the log-rank test to compare the curves of each group. Univariate and multivariate analyses of the TFT and OS employed the Cox regression method. Results were considered statistically significant for values of $P \leq 0.05$.

Results

Clinical and biological characteristics of CLL patients carrying 11q deletion

One hundred ninety-seven patients with 11q deletion were selected for the analysis. There was a predominance of males (76.6%), and the median age was 65 years (range: 28–97 years). Most patients (61%) were in Binet stage A and only 14.9% had B symptoms. In 46.4% of patients the lymphocyte blood count was $\geq 20 \times 10^9/L$, while 31.8% and 28.9% patients, respectively, had high serum β_2 -microglobulin and high serum LDH levels. A total of 68.5% of patients had lymph node involvement, and splenomegaly was detected in 23.4% of cases. Regarding biological characteristics, *IGHV* unmutated cases were present in 66.1% of cases, while CD38 $\geq 30\%$ and ZAP-70 $\geq 20\%$ were detected in 55% and 55.7% of patients, respectively (Table A in [S1 File](#)).

Fifty-one patients (25.9%) had $<40\%$ of 11q-deleted cells, while 146 cases (74.1%) had $\geq 40\%$ of such cells. Different cut-off points were analysed, and 40% 11q deleted nuclei was selected to better separate patients with different disease outcome. In 82 out of 197 patients (41.6%) 11q- was the sole cytogenetic aberration, while 115 patients (58.4%) had 11q deletion plus other cytogenetic abnormalities (108 cases had a 13q deletion, 14 had trisomy 12, and 6 patients showed a 17p deletion).

No significant differences in clinical or biological features were found between patients with low ($<40\%$) and high ($\geq 40\%$) frequencies of 11q- cells, except for the number of lymphocytes, Binet stage, *IGHV* mutational status, need for therapy, and death during follow-up (Table 1).

CLL patients with a low number of 11q- cells have a prolonged time to first therapy (TFT)

All 197 patients were evaluable for analysis of TFT, response to therapy and OS. By the time of analysis, 151/197 (76.6%) had received treatment, with a median TFT of 25 months (95% CI, 31–44 months) (Fig A in [S1 File](#)). In terms of TFT, no differences in the group of 11q deletion as unique FISH cytogenetic aberration compared with the group of 11q deletion plus other FISH alterations were observed. A significantly longer TFT was detected in the cohort of patients with $<40\%$ of 11q deleted cells (median, 44 months; 95% CI, 33–55 months) vs those patients $\geq 40\%$ losses in 11q (median, 19 months; 95% CI, 12–24 months) ($P < 0.0001$) (Table 1A). Of note, 52% of patients in the former group required treatment while 70.5% of patients with $\geq 40\%$ of 11q-deleted nuclei were treated. Variables associated with a longer TFT were early clinical stage ($P = 0.024$), absence of extended lymphadenopathy (<2 node areas involved) ($P < 0.0001$), absence of splenomegaly ($P = 0.045$), low serum LDH ($P = 0.045$), low serum β_2M ($P = 0.019$), low CD38 expression ($P = 0.023$), low ZAP70 expression ($P = 0.025$), mutated *IGHV* status ($P < 0.0001$) and del(11q) in $<40\%$ of cells ($P < 0.0001$) (Table B in [S1 File](#)). In the multivariate analysis, only the presence of del(11q) in $<40\%$ of cells (Hazard Ratio, HR, 4.475; 95% CI, 1.813–7.171; $P = 0.001$), mutated *IGHV* status (HR, 3.659; $P = 0.005$), early Binet stage (HR 2.492; $P = 0.023$) and absence of extended lymphadenopathy (HR 1.854; $P = 0.016$) identified independent risk factors associated with longer TFT (Table 2).

In addition, in patients with del(11q) as the sole cytogenetic aberration, a longer TFT was observed in patients with 11q-L (median 45 months vs 15 months, $P < 0.0001$).

CLL patients with a low number of 11q- cells have longer overall survival (OS)

By the time of analysis, 60/197 patients (30.5%) had died. The median OS of the global series was 106 months (95% CI, 97–128 months) (Fig A in [S1 File](#)). Significantly longer survival was

Table 1. Characteristics of 197 patients with 11q deletion with respect to the number of losses detected by FISH: <40% (n = 51) or ≥40% (n = 146).

Characteristic	Category	del(11q) <40%, N = 51 (26%)	del(11q) ≥40%, N = 146 (74%)	P
Age, years		62 (28–84)	65 (33–91)	0.13
White blood cells, range /μL		17,900 (7,800–98,100)	28,000 (6,600–365,000)	0.008
Lymphocytes, range /μL		12,600 (5,100–84,8500)	21,100 (5,200–364,000)	0.007
Lymphocytes > 20 x 10 ⁹ /L	Yes	14	75	0.005
	No	35	68	
Hemoglobin, range g/dL		14 (6–17)	14 (5–17)	0.91
Platelet count, range /μL		195,000 (63,000–352,000)	182,000 (2,000–412,000)	0.44
<i>IGHV</i> (n = 56)*	Mutated	9	10	0.024
	Unmutated	6	31	
ZAP-70 (n = 79)*	+	12	32	0.44
	-	11	24	
CD38 (n = 130)*	+	16	40	0.139
	-	19	56	
del(11q) as sole cytogenetic aberration	Yes	20	62	0.74
	No	31	84	
del(11q) + del(13q)	Yes	29	77	0.63
	No	22	69	
Sex	Male	40	111	0.84
	Female	11	35	
LDH (n = 187)*	Normal	34	99	0.49
	High	13	41	
β microglobulin (n = 170) *	Normal	33	80	0.19
	High	11	43	
Binet stage (n = 195)*	A	36	83	0.17
	B	10	44	
	C	4	14	
Lymphadenopathy (n = 193)*	No	20	41	0.12
	≤ 2 nodal areas	16	43	
	> 2 nodal areas	13	60	
Hepatomegaly (n = 193)*	Yes	3	15	0.57
	No	46	129	
Splenomegaly (n = 193)*	Yes	10	36	0.43
	No	39	108	
B symptoms (n = 195)*				

(Continued)

Table 1. (Continued)

Characteristic	Category	del(11q) <40%, N = 51 (26%)	del(11q) ≥40%, N = 146 (74%)	P
	Yes	6	23	0.34
	No	44	122	
Second Cancer (n = 172)*	Yes	4	22	0.23
	No	42	104	
Died during follow-up	Yes	10	50	0.04
	No	41	96	
Therapy during follow-up	Yes	27	104	0.025
	No	24	42	

*Number of cases.

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observed in patients with a low frequency of losses in 11q-. Thus, in patients with loss of 11q in ≥40% of cells, the OS was 90 months (95% CI, 57–123 months), while in the group with <40% of losses in 11q, the median OS had not been reached (95% CI, 114–157 months) ($P = 0.006$) (Fig 1B). In the univariate analysis, early clinical Binet stage ($P = 0.001$), asymptomatic disease ($P = 0.034$), absence of hepatomegaly ($P = 0.025$) or splenomegaly ($P < 0.0001$), lymphocyte count $< 20 \times 10^9/L$ ($P = 0.032$), low serum of either LDH level ($P < 0.0001$) or β_2M ($P < 0.0001$), the presence of an association of 11q deletion and 13q deletion ($P = 0.045$), and a low number (<40%) of cells with 11q- ($P = 0.006$) were associated with longer OS (Table C in S1 File). In the multivariate analysis, the variables independently related to longer OS were the absence of splenomegaly (HR, 1.786; $P = 0.023$), low serum LDH (HR, 2.076; $P = 0.018$), low serum β_2M (HR, 2.448; $P = 0.006$) and the presence of del(11q) in <40% of cells (HR, 3.145; 95% CI, 1.474–6.691; $P = 0.003$) (Table 3).

Regarding the patients with del(11q) as the unique cytogenetic aberration, a longer OS was observed in patients with 11q-L (median not reached vs 70 months, $P = 0.007$)

The analyses of other cut-offs for the number of 11q- cells (<40% vs 40–59% vs ≥60%) showed similar results for TTF and OS (Fig B in S1 File).

Table 2. Multivariate Cox regression analysis of time to first therapy in 11q- CLL patients with respect to the number of losses detected by FISH: <40% (n = 51) or ≥40% (n = 146).*

Variable	Hazard Ratio	95% CI	P (log-rank test)
del(11q) <40%	4.475	1.813–7.171	0.001
Mutated <i>IGHV</i>	3.659	1.478–9.057	0.005
Early Binet stage	2.492	1.137–5.463	0.023
Non-extended lymphadenopathy (≤ 2 nodal areas)	1.854	1.121–3.065	0.016

*The following covariates were included in the final model: age, sex, Binet stage, splenomegaly, extended lymphadenopathies, LDH, β_2 microglobulin, CD38, ZAP70, *IGHV* mutation status and percentage 11q deleted nuclei.

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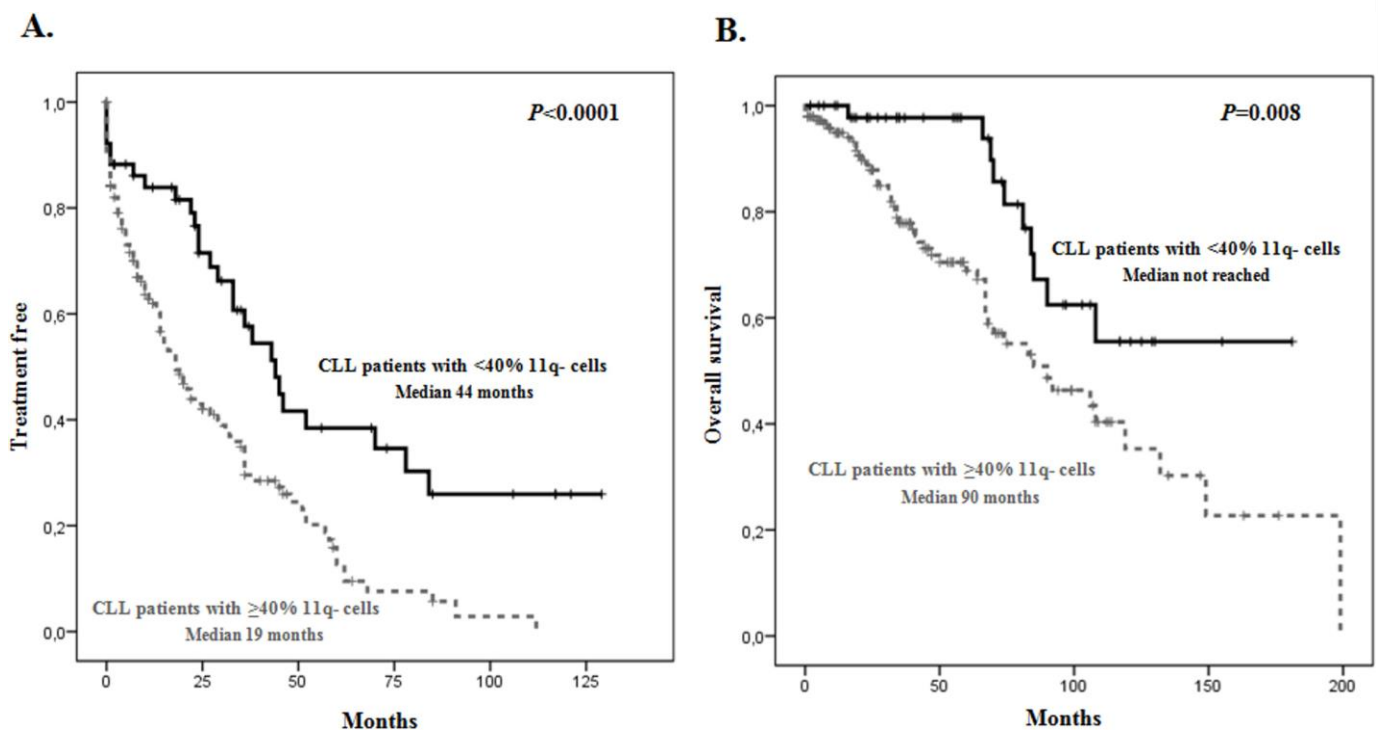


Fig 1. (A) Time to first therapy (TFT) and (B) overall survival (OS) of 197 patients with 11q deletion CLL and <40% or ≥40% FISH losses.

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Biallelic inactivation of the *ATM* gene is observed in one-third of 11q- CLL patients

ATM mutations were found in eight (32%) of 25 patients with 11q-. In total, 14 different mutations were detected by *ATM* molecular mutation screening: 11 point mutations (7 missense and 4 nonsense; 78.6%) and three frameshift mutations (2 deletions and 1 insertion; 21.4%). These mutations are shown in Fig 2 and listed in Table 4. All patients with mutated *ATM* had at least one truncating or damaging mutation. Interestingly, four of the eight patients with *ATM* mutations carried more than one type of mutation. Thus, two patients with *ATM* mutations carried two different mutations while two other patients with *ATM* mutations carried three mutations. It is of note that the patients with several *ATM* mutations had different mutational loads, suggesting the presence of independent clones or clonal evolution with the

Table 3. Multivariate Cox regression analysis of overall survival in 11q- CLL patients with respect to the number of losses detected by FISH: <40% (n = 51) or ≥40% (n = 146).*

Variable	Hazard Ratio	95% CI	P (log-rank test)
Del 11q <40%	3.145	1.474–6.691	0.003
Low serum β_2 microglobulin	2.448	1.260–4.753	0.006
Low serum LDH	2.076	1.061–4.064	0.018
Absence of splenomegaly	1.786	1.044–4.481	0.023

*The following covariates were included in the final model: age, sex, Binet stage, splenomegaly, extended lymphadenopathies, LDH, β_2 microglobulin, CD38, ZAP70, *IGHV* mutation status and percentage 11q deleted nuclei.

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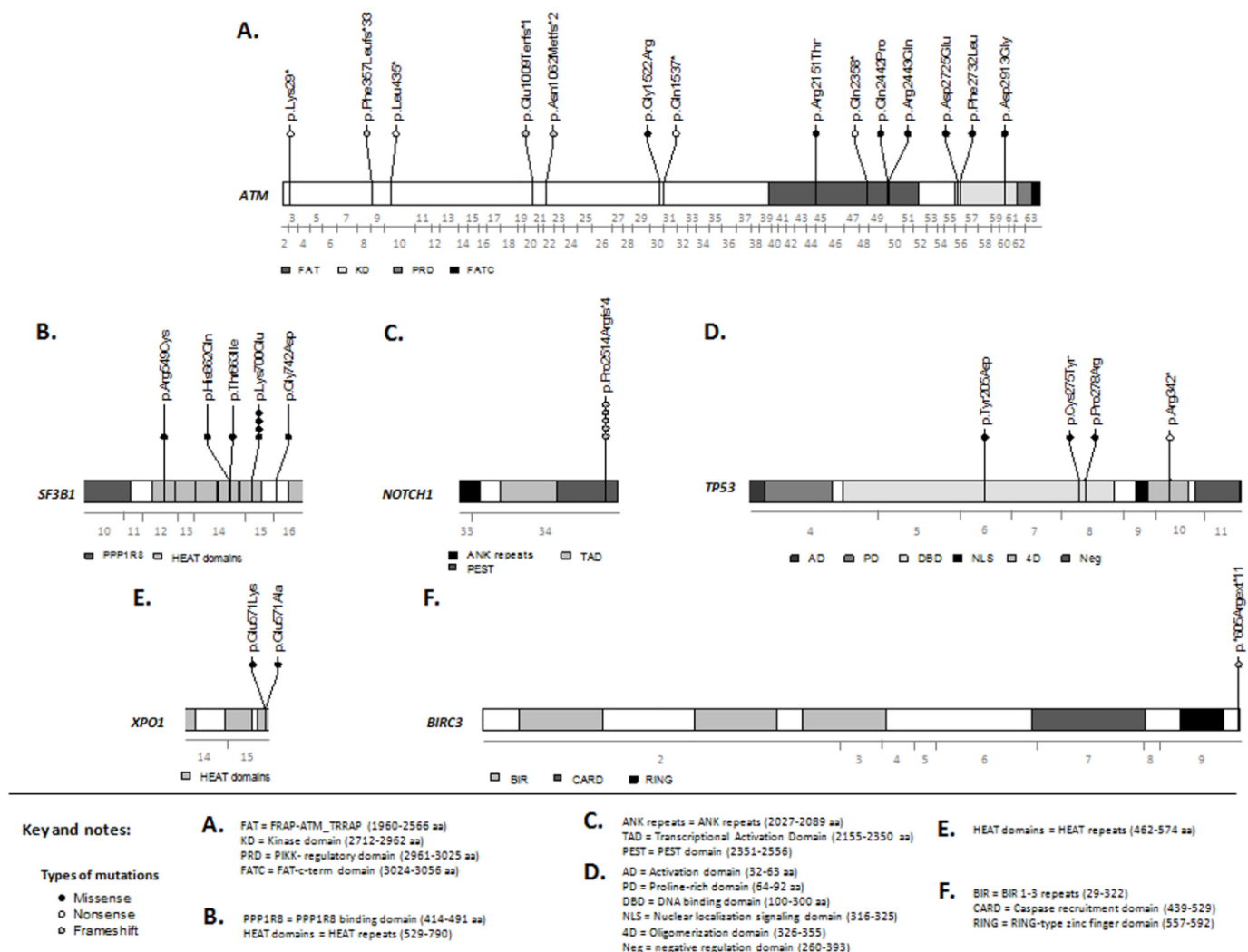


Fig 2. Localization and frequencies of mutations in *ATM*, *SF3B1*, *NOTCH1*, *TP53*, *XPO1* and *BIRC3* genes. Mutations are indicated at the amino-acid level; each detected alteration is represented by a dot. Mutation type is represented by a dark (missense), white (nonsense) or grey (frameshift) dot. The sequenced exons for each gene are represented with a grey line below each schematic protein organization.

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acquisition of a second mutation. However, it could not be confirmed whether the mutations from one patient belonged to different clones, since they were located on distinct sequencing reads in different amplicons.

All the mutations were located in exons 3–60, between amino acids 29 and 2913, and involved the FAT and PI3K domains of the ATM protein. Five mutations resulted in a truncated form of the ATM protein (termed ‘truncating’) while the remaining mutations resulted in the expression of the full-length, but mutated form of the ATM protein (termed ‘non-truncating’). These missense mutations were analyzed with SIFT algorithms, which predicted six of them to be damaging. The median mutational burden was 14.3% (range, 2.0–92.5%). Eight of 14 (57.1%) variants had a mutational load of $\leq 15\%$ and thus would not have been detected by capillary Sanger sequencing.

Table 4. *ATM* mutations in 25 CLL patients with del(11q).

Patient ID	FISH			<i>ATM</i> mutations							
	% 11q-	11q- Group	Other abnormalities	Mutational load (%)	Sequence change	Exon	Protein change	Domain protein	Type of mutation	Consequence (SIFT)	Reported previously
14	27	11q-L	30% 13q-	13	c.85A>T	3	p.Lys29X	-	nonsense	T	-
14	27	11q-L	30% 13q-	24	c.6452G>C	44	p.Arg2151Thr	FAT	missense	NT (Tolerated)	-
2	33	11q-L	-	56.5	c.7325A>C	50	p.Gln2442Pro	FAT	missense	NT (Damaging)	1, 2, 3, 4
3	38	11q-L	89% 13q-	5.5	c.3024insT	20	p.Gln1009fs	-	frameshift	T	-
3	38	11q-L	89% 13q-	14.5	c.7072C>T	48	p.Gln2358X	FAT	nonsense	T	-
18	70	11q-H	37.5% 13q-	7	c.1304T>A	10	p.Leu435X	-	nonsense	T	-
18	70	11q-H	37.5% 13q-	2	c.4564G>C	30	p.Gly1522Arg	-	missense	NT (Damaging)	-
18	70	11q-H	37.5% 13q-	26	c.7328G>A	50	p.Arg2443Gln	FAT	missense	NT (Damaging)	5, 6
6	73	11q-H	-	43	c.3185delA	22	p.Asn1062fs	-	frameshift	T	-
6	73	11q-H	-	7	c.8196C>A	56	p.Phe2732Leu	PI3K	missense	NT (Damaging)	-
6	73	11q-H	-	3.5	c.8738A>G	60	p.Asp2913Gly	PI3K	missense	NT (Damaging)	-
23	82.5	11q-H	-	14	c.1067delT	9	p. Phe357LeufsX33	-	frameshift	T	-
16	83	11q-H	56% 13q-	92.5	c.8175T>A	56	p.Asp2725Glu	PI3K	missense	NT (Damaging)	-
15	88.5	11q-H	85% 13q-	79.5	c.4609C>T	30	p.Gln1537X	-	nonsense	T	-

11q-L: 11q- with <40% 11q-

11q-H: ≥40% 11q-

T: Truncating mutation

NT: Non-truncating mutation

1 Fujimoto A et al Nature Genetics 2012

2 Ding et al Nature 2008

3 Davies H et al Cancer Research 2005

4 Vorechovsky Nature Genetics 1997

5 Yip S et al. J Pathol 2012

6 Greiner TC et al Proc Natl Acad Sci USA 2006.

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The TFT and OS were assessed in all patients and related to the mutational status of the *ATM* gene. There was no difference in TFT or OS between 11q- patients with and without *ATM* mutations ($P < 0.970$ and $P < 0.623$, respectively). Interestingly, bivariate analyses indicated that the presence of *ATM* mutations was a useful characteristic for identifying CLL patients with a different TFT in the subgroup of CLL patients with low frequencies of 11q-. Thus, CLL with *ATM* mutations had a shorter TFT than patients without *ATM* mutations (36 vs 46 months; $P = 0.046$). By contrast, 11q patients harboring ≥40% of 11q- had shorter TFT independently of *ATM* mutational status. Moreover, there was no significant association between the presence of an *ATM* mutation and other clinical or biological prognostic factors (Table D in S1 File).

SF3B1 is a frequently mutated gene in CLL patients with 11q-

Mutations in *TP53*, *NOTCH1*, *SF3B1*, *MYD88*, *FBXW7*, *XPO1* and *BIRC3* were analyzed in the entire cohort of 25 CLL patients. In total, 20 mutations were detected: eight patients had *SF3B1* mutations, five cases had mutations in *NOTCH1*, four in *TP53*, two in *XPO1*, while one CLL had a mutation in *BIRC3*. Most of them had previously been described as mutations in the COSMIC database. The median mutational burden was 27% (range, 3–81%). In 7/20 (35%) variants the mutation load was ≤15%. The frequency of 11q- CLL with associated mutations

was 8/25 (32%) for *SF3B1*, 5/25 (20%) for *NOTCH1*, 4/25 (16%) for *TP53*, 2/25 (8%) for *XPO1* and 1/25 (4%) for *BIRC3*. These mutations are shown in Fig 2 and listed in Table 5. Confirming previously published sequencing data, the most frequent *SF3B1* mutation was p.Lys700Glu (4/8, 50%) while the presence of p.Pro2514Argfs*4 was the most frequent *NOTCH1* mutation (5/5, 100%). In addition, 50% of the 11q- CLL patients with *TP53* mutations also showed 17p-.

In terms of prognostic relevance, significant differences were observed only in TFT between 11q- patients with and without *NOTCH1* mutations (5 vs 36 months; $P = 0.031$) and in OS between patients with and without *TP53* mutations (1 vs 197 months; $P < 0.003$) (Fig C in S1 File).

Genetic mutations are associated with a higher percentage of 11q- cells

As a next step towards understanding the clinical differences within the 11q- subgroup, the association between the presence of genetic mutations and the percentage of 11q- cells in CLL patients was examined. The incidence of *ATM* mutations was similar in the two groups. Thus, 29.4% of patients with a high frequency of 11q- exhibited *ATM* mutations while 37.5% of patients with a low frequency of 11q losses had *ATM* mutations ($P = 0.513$). However, considering the mutations of all the genes analyzed, fewer patients with low frequencies of 11q- had mutations among genes examined compared with the subgroup of a high number of losses

Table 5. Mutations in other genes in 25 CLL patients with del(11q).

Patient ID	FISH			Mutations					
	% 11q-	11q-group	Other abnormalities	Gene	Mutational load (%)	Sequence change	Exon	Protein change	COSMIC database
10	26	11q-L	-	<i>SF3B1</i>	17	c.2225G>A	16	p.Gly742Asp	COSM145923
14	27	11q-L	30% 13q-	<i>NOTCH1</i>	6	c.7541_7542delCT	34	p.Pro2514ArgfsX4	COSM12774
22	48	11q-H	-	<i>SF3B1</i>	4	c.2098A>G	15	p.Lys700Glu	COSM84677
22	48	11q-H	-	<i>TP53</i>	28	c.824G>A	8	p.Cys275Tyr	COSM10893
21	48.5	11q-H	81.5% 13q-	<i>SF3B1</i>	25	c.1645C>T	12	p.Arg549Cys	COSM1014502
21	48.5	11q-H	81.5% 13q-	<i>TP53</i>	16	c.1024C>T	10	p.Arg342X	COSM11073
25	62	11q-H	-	<i>NOTCH1</i>	68.5	c.7541_7542delCT	34	p.Pro2514ArgfsX4	COSM12774
18	70	11q-H	37.5% 13q-	<i>SF3B1</i>	34	c.2098A>G	15	p.Lys700Glu	COSM84677
6	73	11q-H	-	<i>SF3B1</i>	39	c.2098A>G	15	p.Lys700Glu	COSM84677
6	73	11q-H	-	<i>NOTCH1</i>	4	c.7541_7542delCT	34	p.Pro2514ArgfsX4	COSM12774
20	78	11q-H	63% 13q-	<i>SF3B1</i>	46.5	c.1988C>T	14	p.Thr663Ile	COSM145921
16	83	11q-H	56% 13q-	<i>SF3B1</i>	4	c.2098A>G	15	p.Lys700Glu	COSM84677
13	83	11q-H	86% 13q-	<i>TP53</i>	7.5	c.613T>G	6	p.Tyr205Asp	COSM43844
9	83.5	11q-H	61% 13q-	<i>NOTCH1</i>	3	c.7541_7542delCT	34	p.Pro2514ArgfsX4	COSM12774
5	84.5	11q-H	87% 17p-	<i>NOTCH1</i>	45	c.7541_7542delCT	34	p.Pro2514ArgfsX4	COSM12774
19	84.5	11q-H	85% 13q-	<i>XPO1</i>	47.5	c.1711G>A	15	p.Glu571Lys	COSM96797
4	89	11q-H	87% 17p-	<i>TP53</i>	81	c.833C>G	8	p.Pro278Arg	COSM10887
4	89	11q-H	87% 17p-	<i>BIRC3</i>	12	c.1813T>C	9	p.X605ArgextX11	-
1	90	11q-H	87% 13q-	<i>SF3B1</i>	50.5	c.1986C>G	14	p.His662Gln	COSM110692
17	97	11q-H	97% 13q-	<i>XPO1</i>	51	c.1712A>C	15	p.Glu571Ala	COSM1291526

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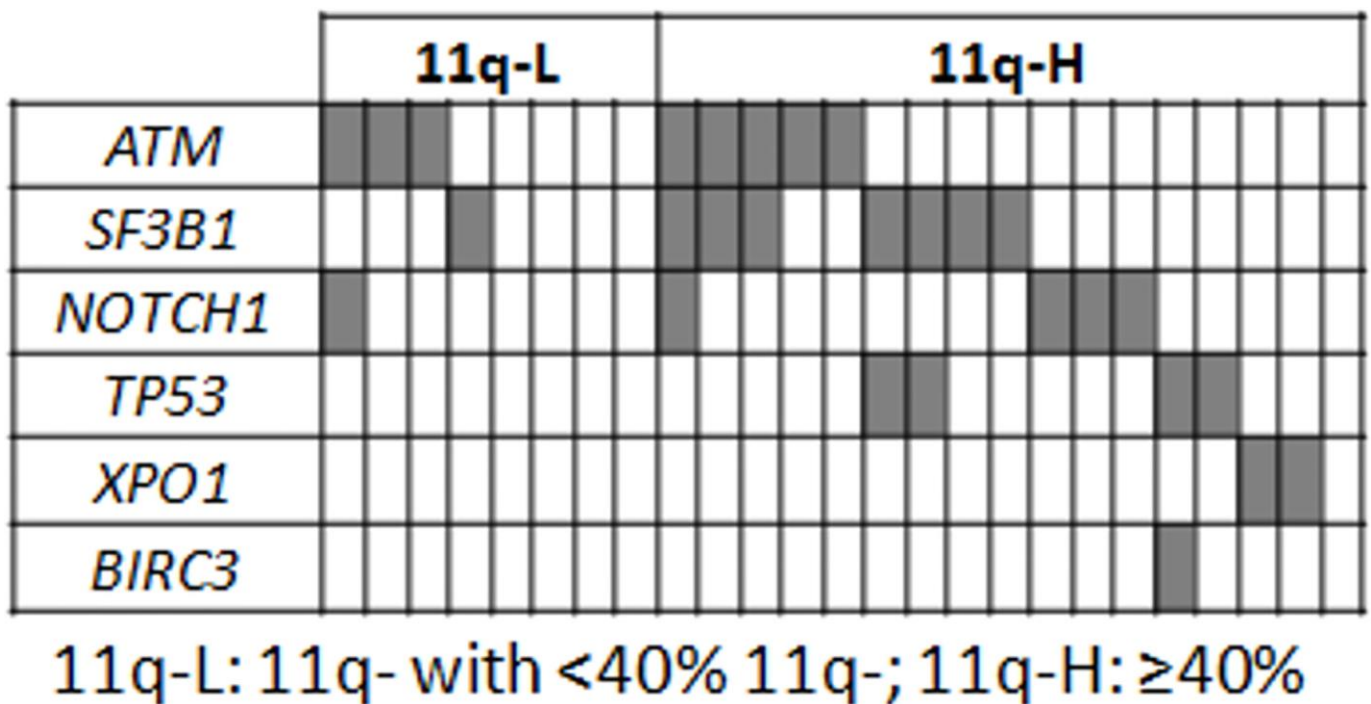


Fig 3. Distribution of mutations of *ATM*, *SF3B1*, *NOTCH1*, *TP53*, *XPO1* and *BIRC3* among 11q- CLL patients with respect to the percentage of 11q- cells. In the heat maps, rows correspond to identical genes, and columns represent individual patients color-coded on the basis of gene status (white: wild type; grey: mutated gene).

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11q- (4/8, 50% vs 16/17, 94.1%; $P = 0.023$) (Fig 3). Interestingly, among the CLL patients without a gene mutation, the median proportion of the CLL tumor population with an 11q deletion was significantly lower than that of CLL patients with mutated genes (20.5%, range, 12–71.5% vs 80.3%, range, 26–97%; $P = 0.007$). *TP53* mutations were present only in patients with a high frequency of 11q- cells.

Discussion

In this study, we analyzed the outcome of 11q-deleted CLL patients at diagnosis with respect to TFT and OS, and the presence of mutations in the most relevant genes to date in CLL. The importance of the percentage of cells displaying a genetic aberration determined by FISH, as an independent prognostic factor in CLL, has recently been recognized in 17p-, 13q- and +12 [2,22–24,29,30], whereby a high number of cells with 13q deletion has been associated with a worse outcome [2,22–24]. In fact, patients displaying a high degree of infiltration of 13q- had an intermediate prognosis, with a shorter time to first therapy and survival than those with normal cytogenetics or trisomy 12 [2,22–24]. In addition, patients with fewer losses in 17p or patients with a low number of trisomy 12 have a better outcome [29,31]. Several reports have shown the 11q deletion to be a factor predicting poor prognosis in CLL [1,25,32]. In the present study, we have confirmed these previous observations, whereby CLL patients with 11q- had a median time of 2 years to receipt of first therapy and an overall survival of 8 years. Although the present study is a retrospective and multicenter analysis of a series of CLLs, the characteristics of the patients agree real-world basis. Thus, a median age of 65 years, predominance of male sex (76%) and frequent lymphadenopathy (69%) were observed, as previously described

[1]. In addition, 66% of patients had unmutated *IGHV* status with more than 50% of cases expressing CD38 and/or ZAP-70. Of note, we have observed that the number of cells carrying 11q- influences the disease outcome in terms of time to first therapy and overall survival. These results are consistent with those of two recently published series that reported a longer TFT in patients with 11q deletion and a low number of losses [33,34]. However, one of these studies found no improvement in the overall survival in this group of patients [34]. Therefore, the present study confirms that patients with 11q deletion do not comprise a homogeneous subgroup. We identified a threshold of 40% of deleted cells to be optimal for showing that a low number of losses in 11q is associated with a better outcome in terms of TFT and OS. However, the use of other cut-off points (i.e., <40% vs 40–59% vs ≥60%) yielded similar results.

Interestingly, this study showed that CLL with losses in 11q with early clinical stage, mutated *IGHV* status and/or a low number of losses in the 11q chromosome had a TFT of more than two years compared with cases with >40% of cells with 11q deletion (median, 44 vs 19 months), similar to previously reported results [34]. Therefore, we identified the presence of advanced clinical stages, unmutated *IGHV*, and a high number of losses in 11q as the main predictors indicating therapy in the group of CLL displaying 11q-.

Moreover, CLL patients with a lower percentage of 11q deletion had a better OS than those with ≥40% of 11q-deleted nuclei (median, not reached vs 90 months), with an estimated 3 years longer survival in patients with less than 40% of 11q deletions. In addition, clinical (absence of splenomegaly) or biological variables (low 11q-, low LDH and β₂M) were included in the final multivariate model of OS. Therefore, the assessment of the number of 11q- cells should be included to better define survival in patients with CLL displaying this genetic abnormality and could be incorporated into the design of clinical trials to define their influence on the response to new therapies and on survival.

To better define the molecular characteristics of the CLL patients displaying losses in 11q, a mutational study performed by next-generation sequencing, including the most frequently mutated genes in CLL, was carried out. The results showed that patients with a low number of 11q losses displayed fewer mutations. Interestingly, *ATM* mutations were present in both cohorts of 11q- patients at a similar frequency to those previously reported [9,35–37]. We found no significant impact of *ATM* mutations on prognosis for all the 11q- patients, as described by other authors [9,37]. However, focusing on the group of patients with a low number of 11q losses, *ATM* mutations were useful for identifying CLL patients with a shorter TFT. Thus, our results suggest that the integration of molecular markers, such as *ATM* mutations, and the FISH analysis, in patients showing loss of 11q could provide a better prognostic stratification than has been recently demonstrated in other CLL patients [20].

The presence of gene mutations has been widely demonstrated in CLL [11–14]. Mutations of *TP53*, *NOTCH1*, *SF3B1* and *BIRC3* are known to be associated with a worse prognosis, while mutations in *MYD88* are related to a better outcome [18,19,21,38,39]. However, some of the results concerning the incidence and independent prognostic value of these mutations are controversial [12,14]. We observed a higher percentage of CLL patients with *XPO1* mutations in our cohort of patients than in those described by others [12,13,19,40]. However, it should be taken in account the limitation of the size of our sequencing samples cohort. Consistent with previous studies, mutations of *NOTCH1* and *TP53* occurred as mutually exclusive events [13,15]. Of note, *TP53* mutations were only detected in patients with a high frequency of 11q losses. Furthermore, *NOTCH1* and *SF3B1* mutations were more frequent in this group of 11q- CLLs (Fig 3). Therefore, our study provides new insights into the molecular basis of the worse outcome of CLL patients who have losses in 11q. It should be noted that the high frequency of gene mutations did not involve the *ATM* gene, suggesting that the clonal evolution

(heterogeneity) affecting any CLL-related gene could be the basis of the dismal prognosis of patients with a high frequency of 11q- [12,40,41].

In summary, our results suggest that in patients with CLL, the frequency of 11q-deleted cells influences the clinical outcome, and a low number of 11q- is associated with a longer time to progression and overall survival. In addition, this study shows that fewer CLL patients with low frequencies of 11q- had mutations among genes examined. Our findings, derived from a large retrospective cohort of CLL patients from several Spanish institutions, need to be validated in prospective clinical trials.

Supporting Information

S1 File. Supplementary methods data. Characteristics of the series of 197 CLL patients with 11q deletion (**Table A**). Univariate analysis of time to first therapy in 11q- CLL patients with respect to the number of losses detected by FISH: <40% (n = 51) or ≥40% (n = 146) (**Table B**). Univariate analysis of overall survival in 11q- CLL patients with respect to the number of losses detected by FISH: <40% (n = 51) or ≥40% (n = 146) (**Table C**). Main clinical and biological characteristics of 25 CLL patients with 11q- with respect to *ATM* mutational status (**Table D**). PCR primers used for next-generation sequencing studies (**Table E**). A: PCR amplification protocol for *ATM*. B: PCR amplification protocol for the remaining genes (**Table F**). Median frequency of reads generated by next-generation sequencing (NGS) (**Table G**). A. Time to first therapy (TFT) and B. Overall survival (OS) of the global series of 197 CLL patients with 11q deletion (**Fig A**). A. Time to first therapy (TFT) and B. Overall survival (OS) of patients with CLL and 11q deletion and a percentage of FISH losses <40%, 41–59% or ≥60% (**Fig B**). Kaplan-Meier plots of time to first therapy (TFT) (A) and overall survival (OS) (B) from diagnosis for 11q- CLL patients sequenced for *NOTCH1* and *TP53*, respectively (**Fig C**) (DOC)

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Author Contributions

Conceived and designed the experiments: JAH MHS AERV JMHR. Performed the experiments: MHS AERV CR RB. Analyzed the data: JAH MHS AERV CR RB. Contributed reagents/materials/analysis tools: VG AK RC CH AP AAM NP JD TG JAQ JG IF GMN JMA PA EL IM IG MG. Wrote the paper: JAH MHS AERV CR RB VG FB BE MG JMHR.

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***TET2* overexpression in chronic lymphocytic leukemia is unrelated to the presence of *TET2* variations**

3

María Hernández-Sánchez¹, Ana Eugenia Rodríguez¹,
Alexander Kohlmann², Rocío Benito¹, Juan Luis García³, Alberto
Risueño^{4,5}, Encarna Fermiñán⁶, Javier de las Rivas⁵, Marcos
González^{1,7}, Jesús-María Hernández-Rivas^{1,7}

¹*IBSAL, IBMCC, Centro de Investigación del Cáncer, IBSAL, IBMCC, Universidad de Salamanca-CSIC, Salamanca, Spain*

²*Department of Molecular Genetics, MLL Munich Leukemia Laboratory, Munich, Germany*

³*Instituto de Estudios de Ciencias de la Salud de Castilla y León (IESCSCYL) and Hospital Clínico Universitario de Salamanca (HUSAL), Salamanca, Spain*

⁴*Celgene Institute for Translational Research Europe (CITRE), Sevilla, Spain*

⁵*Unidad de Bioinformática y Genómica Funcional, IBMCC, Centro de Investigación del Cáncer, Universidad de Salamanca-CSIC, Salamanca, Spain*

⁶*Unidad de Genómica, IBMCC, Centro de Investigación del Cáncer, Universidad de Salamanca-CSIC, Salamanca, Spain*

⁷*Servicio de Hematología y Departamento de Medicina, Hospital Clínico Universitario de Salamanca, Salamanca, Spain*

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Research Article

TET2 Overexpression in Chronic Lymphocytic Leukemia Is Unrelated to the Presence of TET2 Variations

María Hernández-Sánchez,¹ Ana Eugenia Rodríguez,¹ Alexander Kohlmann,² Rocío Benito,¹ Juan Luis García,³ Alberto Risueño,^{4,5} Encarna Ferminán,⁶ Javier De Las Rivas,⁵ Marcos González,^{1,7} and Jesús-María Hernández-Rivas^{1,7}

¹ IBSAL, IBMCC, Centro de Investigación del Cáncer, Universidad de Salamanca-CSIC, 37007 Salamanca, Spain

² Department of Molecular Genetics, MLL Munich Leukemia Laboratory, 81377 Munich, Germany

³ Instituto de Estudios de Ciencias de la Salud de Castilla y León (IECSCYL) and Hospital Clínico Universitario de Salamanca (HUSAL), 37007 Castilla y León, Spain

⁴ Celgene Institute for Translational Research Europe (CITRE), 41092 Sevilla, Spain

⁵ Unidad de Bioinformática y Genómica Funcional, IBMCC, Centro de Investigación del Cáncer, Universidad de Salamanca-CSIC, 37007 Salamanca, Spain

⁶ Unidad de Genómica, IBMCC, Centro de Investigación del Cáncer, Universidad de Salamanca-CSIC, 37007 Salamanca, Spain

⁷ Servicio de Hematología y Departamento de Medicina, Hospital Clínico Universitario de Salamanca, Paseo San Vicente 58, 37007 Salamanca, Spain

Correspondence should be addressed to Jesús-María Hernández-Rivas; jmhr@usal.es

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TET2 is involved in a variety of hematopoietic malignancies, mainly in myeloid malignancies. Most mutations of *TET2* have been identified in myeloid disorders, but some have also recently been described in mature lymphoid neoplasms. In contrast to the large amount of data about mutations of *TET2*, some data are available for gene expression. Moreover, the role of *TET2* in chronic lymphocytic leukemia (CLL) is unknown. This study analyzes both *TET2* expression and mutations in 48 CLL patients. *TET2* expression was analyzed by exon arrays and quantitative real-time polymerase chain reaction (qRT-PCR). Next-generation sequencing (NGS) technology was applied to investigate the presence of *TET2* variations. Overexpression of *TET2* was observed in B-cell lymphocytes from CLL patients compared with healthy donors ($P = 0.004$). In addition, in CLL patients, an overexpression of *TET2* was also observed in the clonal B cells compared with the nontumoral cells ($P = 0.002$). However, no novel mutations were observed. Therefore, overexpression of *TET2* in CLL seems to be unrelated to the presence of genomic *TET2* variations.

1. Introduction

The Ten-Eleven-Translocation 2 (*TET2*) gene encoding a 2-oxoglutarate/Fe²⁺ oxygenase catalyses mainly the conversion of methylcytosine to hydroxymethylcytosine. *TET2* is implicated in a variety of hematopoietic malignancies, particularly myeloid malignancies. Mutations of *TET2* have recently been identified in 12–26% in MDS/MPN disorders [1–5], 8–19% of adult acute myeloid leukemias (AML) [6, 7]. Its highest incidence has been found in chronic myelomonocytic

leukemia (CMML) patients (50%) [8]. However, the presence of somatic mutations of the *TET2* gene in human mature lymphoid neoplasms has been recently described, whereby *TET2* mutations were present in 2–12% of B-cell and 12% of T-cell neoplasms [9–11].

In contrast to the considerable information about mutations of *TET2*, data about expression of this gene are scarce. *TET2* showed a broad expression pattern in different tissues in healthy donors, highlighting a 10- to 100-fold higher expression in hematological cells, the highest values being

seen in granulocytes. In addition, *TET2* expression was lower in the granulocytes from MDS cases compared with healthy donors, irrespective of the *TET2* mutation status [3].

TET2 expression and mutations have been less studied in chronic lymphocytic leukemia (CLL) and only rare cases of this disease showed *TET2* mutations [12]. In the present study overexpression in CLL patients compared with healthy donors was observed. In addition, Next-generation sequencing studies confirmed that the presence of *TET2* mutations is rare in CLL.

2. Material and Methods

2.1. Patients. In total, 48 samples from CLL patients at diagnosis and 6 healthy donors were analyzed. CLL diagnosis was performed according to the World Health Organization (WHO) classification [13] and Working Group of National Cancer Institute (NCI) criteria [14]. In all cases, a complete immunophenotypic analysis by flow cytometry [15] and FISH studies were carried out. Main biological features of the 48 CLL patients included in the study are shown in Table 1. The study was approved by the local ethical committees “Comité Ético de Investigación Clínica, Hospital Universitario de Salamanca.” Written informed consent was obtained from each patient before they entered the study.

Both CLL B lymphocytes and normal B lymphocytes were purified using magnetically activated cell sorting (MACS) CD19 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD19 selection resulted in >98% purity, as analyzed by flow cytometry.

Genomic DNA and total RNA were obtained from clonal B-cell lymphocytes (CD19+ cell fraction) and the remaining cells (CD19– cell fraction). DNA was isolated by QIAgen (Qiagen, Valencia, CA, USA), following the manufacturer’s recommendations. RNA isolation was carried out using TRIzol reagent.

2.2. Expression Analysis. Genome-wide expression analysis of the isolated samples of 27 CLL patients and 5 healthy donors was performed using Human Exon 1.0 microarrays (Affymetrix, Inc., Santa Clara, CA, USA) following the manufacturer’s protocols for the GeneChip platform by Affymetrix, as previously reported [16]. Hybridized Affymetrix arrays were scanned with an Affymetrix GeneChip 3 000 scanner. Image generation and feature extraction were performed using Affymetrix GCOS Software.

SYBR Green quantitative Real-Time PCR was done in triplicate with SYBR Green mix (Applied Biosystems, Foster City, CA) using the IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) in a subset of CLL patients ($n = 23$) and healthy donors ($n = 5$) with the following gene-specific primers: *GAPDH*, forward 5′-CAG-GGCTGCTTTTAACTCTGG-3′ and reverse 5′-GGGTGG-AATCATATTGGAACA-3′, and *TET2*, forward 5′-GGG-TGGAATCATATTGGAACA-3′ and reverse 5′-TGGACA-CAACCACAAATTCA-3′. The *GAPDH* gene was used as the

TABLE 1: Characteristics of the 48 CLL patients included in the study.

Parameter	Category	%
Age (years), median (range)		65 (37–84)
Gender	Male	54
White blood cells ($\times 10^9/L$), median (range)		24,980 (10,130–96,510)
Lymphocytes ($\times 10^9/L$), median (range)		21,070 (5,900–86,320)
Hemoglobin ($\times 10^9/L$), median (range)		13.6 (11.5–15.8)
Platelet ($\times 10^9/L$), median (range)		176,500 (70,000–333,000)
Binet stage	A	67
	B	27
	C	6
Lactate dehydrogenase	High	7
$\beta 2$ microglobulin	High	25
<i>IGHV</i>	Unmutated	45
FISH abnormality*	13q deletion	56
	11q deletion	15
	17p deletion	2
	Trisomy 12	17
	No FISH abnormalities	29

*Some patients have more than one cytogenetic aberration.

internal control and the quantification of relative expression (reported as arbitrary units (a.u.)) were performed using the comparative Ct method. For analytical purposes, cut-off values were adopted according to median-expression levels for *TET2*.

2.3. Next-Generation Sequencing. NGS was carried out using the Roche GS FLX Titanium sequencing platform to investigate the *TET2* mutations in 26 CLL patients. First, NimbleGen Sequence Capture was applied to sequence the entire *TET2* gene ($n = 4$) with a median coverage more than 20X. Then, the 27 amplicons of the complete *TET2* coding region ($n = 24$) were sequenced using 454 FLX amplicon deep-sequencing chemistry with a median coverage of 689 reads, as previously described [17, 18]. *TET2* was sequenced by both strategies in 2 patients. Moreover, the gene expression profile of 5 out of 26 CLL patients was also analyzed by expression arrays.

Sanger sequencing was performed in CD19-positive and -negative cell fractions. Moreover, in 6 healthy donors a region of exon 11 was sequenced to analyze the association of one polymorphism in CLL. Primers used for Sanger sequencing are shown in Table 2.

TABLE 2: Sequences of primers used for Sanger sequencing.

Primer designation	Sequence (5'-3')
<i>TET2</i> exon 3 (ENSE00002471221)	
Forward	GGAACACACACATGGTGAAC
Reverse	TGGAACAGTCATTGTCCCTG
<i>TET2</i> exon 11 (ENSE00002415078)	
Forward	TCCCATGAACCCTTACCCTG
Reverse	ACGCTTGCACACTCAATG

2.4. Bioinformatic Analysis. For the exon array analysis, the robust microarray analysis (RMA) algorithm was used for background correction, intra- and intermicroarray normalization, and expression signal calculation [19]. Significance analysis of microarray (SAM) [20] was used to calculate significant differential expression. All bioinformatic analyses were performed with the statistical program R, as previously described [21].

The expression data from quantitative SYBR Green PCR were not normally distributed, so nonparametric tests were used. Expression levels of *TET2* in the different groups were analyzed using the Mann-Whitney test with a two-tailed value of $P < 0.05$ taken as indicating statistical significance. All tests were performed using SPSS v19.0.

Sequencing data from the Sequence Capture experiments were analyzed using GS Run Browser and GS Reference Mapper software, version 2.0.01 (Roche Diagnostics, Mannheim, Germany). All putative variants were compared with published single-nucleotide polymorphism (SNP) data (dbSNP build 130).

Amplicon deep-sequencing data were generated using GS FLX Sequencer Instrument, version 2.3, and analyzed with GS Amplicon Variant Analyzer, version 2.3 (Roche Diagnostics). The results were further processed and visualized following a previously described pipeline [17].

3. Results and Discussion

3.1. Overexpression of *TET2* in B Cells of CLL Compared with Healthy Donors. The expression levels of *TET2* were analyzed in CLL patients ($n = 27$) and healthy donors ($n = 5$) using oligonucleotide microarrays. Overexpression of the mRNA levels of *TET2* in CLL patients compared with healthy donors was observed ($P = 0.004$).

These results were confirmed in 23 CLL patients and 5 healthy donors by qRT-PCR. Clonal B cells from CLL patients had a significantly higher expression of *TET2* than B cells from healthy donors ($P = 0.033$) (Figure 1(a)).

In myeloid malignancies, *TET2* expression has been shown to be lower in MDS cases than in healthy controls, irrespective of the *TET2* mutation status [3]. However, there have been no studies addressing the expression levels of *TET2* in CLL. *TET2* plays an important role in the metabolism of 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC) [22]. In addition, inactivation of *TET2* is related to low levels of 5-hmC, as it has been reported in HEK293T cells [23]. In the opposite way, *TET2* overexpression could lead to an increase of 5 hmC in CLL patients.

Moreover, *TET2* overexpression in CLL was not associated with other biological markers with well-known prognostic value in CLL such as *IGHV* mutational status ($P = 0.67$) or cytogenetic alterations ($P = 0.197$).

3.2. Differences in *TET2* Expression in B Clonal Cells and Normal Cells in CLL Patients. The expression differences between the B-cell lymphocytes (CD19+) and the remaining cells (CD19-) were studied. In healthy donors ($n = 5$), CD19- cells had a higher level of *TET2* expression than CD19+ cells (Figure 1(b)), consistent with the previously described highest expression of *TET2* in granulocytes [3]. As expected, *TET2* expression depends on the cell type [3]. By contrast, overexpression was observed in the clonal CD19+ cells of CLL patients compared with CD19- cells ($P = 0.002$) (Figure 1(c)). To our knowledge, such results have not been previously reported.

TET2 overexpression in CLL patients could be explained by the deregulation of expression of other members of the TET family in B cells. Compensatory action in the TET family has also been suggested by a previous study [24]. However, a *TET2* deficient murine model has recently been proposed in which the loss of *TET2* during adult hematopoiesis is not compensated by increased transcription of *TET1* and *TET3* [9].

3.3. *TET2* Overexpression Is Unrelated to the Presence of the Gene Mutations. To determine whether *TET2* overexpression could be related to the presence of mutations or polymorphisms in *TET2*, sequencing studies were carried out. No novel variants in *TET2* were revealed from the deep sequencing study following the Sequence Capture strategy.

To further assess the presence and prevalence of *TET2* mutations in CLL, the *TET2* coding sequence was also analyzed in 24 CLL patients by 454 amplicon deep-sequencing. Sequencing data of all coding exons of *TET2*, represented by 27 amplicons, confirmed the presence of known polymorphisms in *TET2*. However, we did not detect any mutations that might affect the protein.

Therefore, NGS did not enable any novel and relevant mutations in *TET2* in CLL patients to be detected. Our sequencing results are consistent with the data provided by the International Cancer Genome Consortium and those recently published in which the *TET2* mutations were rarely present in CLL [12, 25]. Thus, the overexpression of *TET2* in chronic lymphocytic leukemia was unrelated to the presence of *TET2* mutations.

However, a large number of single nucleotide variations described in the SNP database were detected, confirming that the *TET2* gene is a polymorphic gene. Most of these polymorphisms were localized in exons 3 and 11 (Table 3). Due to the primers used in NGS, three variations were also detected in noncoding regions near the analyzed exons. One of these polymorphisms, rs2454206, situated in exon 11, was found in 54% (14/26) of CLL patients. To determine whether this polymorphism could be associated with CLL, 6 healthy donors were sequenced by Sanger. The same polymorphisms were found in 83% (5/6) of these cases.

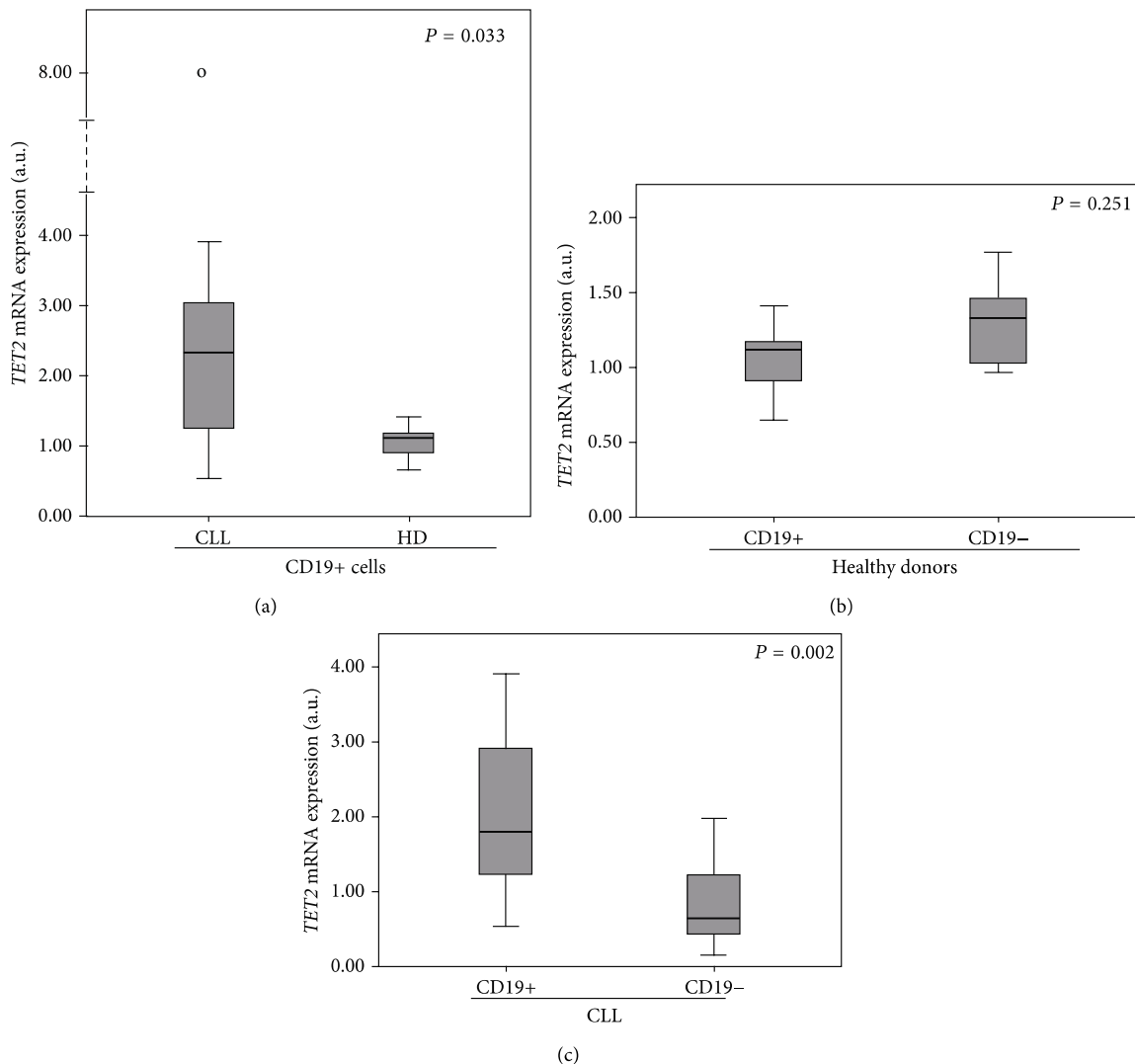


FIGURE 1: (a) Expression of *TET2* in B cells of CLL patients compared with healthy donors (HD). Box plot of the expression levels represented as arbitrary units (a.u.) of *TET2* showing significantly different levels of expression between CLL patients ($n = 23$) and healthy donors (HD) ($n = 5$), assessed by qRT-PCR. They indicate the overexpression of *TET2* in B cells (CD19+ cells) of CLL patients compared with healthy donors ($P < 0.05$). The thick line inside the box plot indicates the median expression levels and the box shows the 25th and 75th percentiles, while the whiskers show the maximum and minimum values. Outliers are represented by open circles. (b) Expression of *TET2* in CD19+ and CD19- cells of healthy donors. It shows a tendency towards overexpression of *TET2* in CD19- cells compared with CD19+ cells in healthy donors ($n = 5$) ($P > 0.05$). (c) Expression of *TET2* in CD19+ and CD19- cells of CLL patients. It illustrates the overexpression of *TET2* in B clonal cells (CD19+ cells) compared with normal cells (CD19- cells) in CLL patients ($n = 15$) ($P < 0.05$).

Recently, the correlation between the presence of polymorphisms in *IDH1* gene and the overexpression of the gene has been described in MDS [26]. To determine whether the presence of *TET2* polymorphisms could influence the overexpression of this gene, the association between gene expression and genetic data was investigated. The expression was analyzed in the clonal CD19+ cells of CLL patients showing polymorphisms ($n = 16$) and in CLL patients without polymorphisms ($n = 2$). The results showed similar *TET2* expression levels in both CLL groups (data not shown).

NGS analysis of the 15 patients analyzed by qRT-PCR showed that all but one had polymorphisms in *TET2* in

both CD19+ and CD19- cells. The clonal CD19+ lymphocytes of CLL patients showing polymorphisms ($n = 14$) were also compared with corresponding CD19- cells. As expected, the tumoral cell fraction of these cases was more strongly expressed ($P = 0.001$) than the nontumoral cell fraction (data not shown). Thus, *TET2* expression could not be related to the presence of polymorphisms and the functional effects of harboring these polymorphisms are unclear.

In conclusion, *TET2* overexpression in CLL patients relative to healthy donors and overexpression of *TET2* in B clonal cells compared with nonclonal B cells in CLL patients have been demonstrated. In addition, *TET2* overexpression

TABLE 3: Known variations of *TET2* detected in 26 CLL cases by next-generation sequencing.

SNP	rs	Region	Protein	n (%)
86C>G	rs12498609	exon 3	Pro29Arg	3 (12)
100C>T	rs111948941	exon 3	Leu34Phe	2 (8)
521C>A	rs146031219	exon 3	Pro174His	1 (4)
652G>A	rs6843141	exon 3	Val218Met	2 (8)
1088C>T	rs17253672	exon 3	Pro363Leu	2 (8)
1105C>T	rs150072691	exon 3	Arg369Trp	1 (4)
2599T>C	rs144386291	exon 3	Tyr867His	1 (4)
5162T>G	rs34402524	exon 11	Leu1721Trp	4 (15)
5167C>T	rs146348065	exon 11	Pro1723Ser	1 (4)
5284A>G	rs2454206	exon 11	Ile1762Val	14 (54)
5333A>G	rs62621450	exon 11	His1778Arg	3 (12)
3803 + 45G>A	rs17319679	Intron 7	—	2 (8)
4045 – 35C>A	rs59519484	Intron 8	—	1 (4)
*74G>A	rs60786079	3'UTR	—	3 (12)

*N = nucleotide N 3' of the translation stop codon.

is unrelated to the presence of the gene mutations and is independent of the presence of polymorphisms.

The relevance of *TET2* overexpression in CLL remains unknown. Given the role of *TET2* in the conversion of methylcytosine to hydroxymethylcytosine, it could be related to epigenetic mechanisms.

4. Conclusions

To the best of our knowledge, this is the first study about the role of *TET2* in CLL: this gene was overexpressed in clonal B cells in CLL. However the overexpression of *TET2* in CLL is not related to *TET2* variations. This report suggested that not only loss of function but also hyperfunction of *TET2* could be related to tumorigenesis.

Conflict of Interests

The authors declare no conflict of interests.

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Analysis of clonal evolution in chronic lymphocytic leukemia from diagnosis to symptomatic disease using whole-exome sequencing

4

María Hernández-Sánchez¹, Ana E Rodríguez¹, María Abáigar¹, David Tamborero², Rocío Benito¹, Miguel Quijada¹, Ana-África Martín¹, Alfonso García de Coca³, Nuria López-Bigas², Jesús-María Hernández-Rivas¹

¹Hematology Department, IBSAL, IBMCC-Cancer Research Center, Hospital Universitario Salamanca, University of Salamanca, Salamanca, Spain

²Research Program on Biomedical Informatics, IMIM Hospital del Mar Medical Research Institute and Universitat Pompeu Fabra, Barcelona, Spain

³Hematology Department, Hospital Clínico Universitario of Valladolid, Valladolid, Spain

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General discussion

CLL is a heterogeneous disease with marked variability in the clinical course. Several molecular studies have suggested that this clinical heterogeneity is a clear reflex of a marked biological diversity.³²⁰ No single genetic mutation or abnormality responsible for CLL development has been identified. Considering the molecular heterogeneity of CLL, high-throughput technologies such as expression arrays and NGS are powerful tools for the global analysis of genetic alterations in CLL, rather than the study of single targets. The combination of these technologies has allowed us to improve our understanding of the molecular mechanisms that could be involved in its pathogenesis and disease evolution, as well as to discover the biological evidence yielding the clinical heterogeneity of CLL. Moreover, the identification of new molecular markers with potential prognostic and therapeutic significance will have an important effect on the clinical management of the disease.

Cytogenetic abnormalities have been widely studied in CLL.³²¹⁻³²⁵ For more than 15 years the Döhner hierarchical classification based on FISH has been the gold standard for the genetic characterization and assessment of prognosis for CLL patients, in which patients with 17p- had by far the worst prognosis, followed by those with 11q-, +12, and normal karyotypes, whereas patients with 13q- as the sole abnormality had the longest estimated survival time.⁷² This prognostic model has been validated by several groups.^{73-75,78,216,326-332} Moreover, recent studies have reported that it is prognostically important not only the cytogenetic alteration *per se*, but also the percentage of cells carrying it.⁷⁸⁻⁸⁶ The biologic diversity which underlies the clinical heterogeneity within the well-defined subgroups of CLL patients according to Döhner's model has been further explored by the first two studies of this thesis.

In the first study, we focused on the subgroup of patients with 13q-, based on previous results obtained by our group. Although 13q- as the sole chromosomal abnormality has been traditionally associated with good prognosis,⁷² we have previously demonstrated that the number of malignant cells carrying this genetic lesion strongly correlates with the disease outcome, establishing two prognostic subgroups according to the percentage of cells with 13q-: the patients with a high proportion (>80%) of 13q- cells, 13q-H, had both a shorter OS and TFT than those of patients with 13q-deleted cells. These results regarding the relationship between the number of 13q- cells and the outcome have been corroborated by other independent groups.⁷⁹⁻⁸² A further molecular characterization of both subgroups of patients by gene expression profiling was carried out, showing that several pathways were deregulated leading to an imbalance between the proliferative and

apoptotic signals that could explain the poor outcome described in 13q-H patients.¹⁹¹ As a next step towards gaining insight into the molecular mechanisms responsible for the different outcome within 13q- CLL patients, here we assessed their miRNA expression profile using a specific high density miRNAs microarray with more than 1700 mature human miRNA.

First, we observed that the miRNA expression profiles differ between 13q- CLL subgroups according to the percentage of cells carrying this chromosomal aberration. MiRNA expression profiling has been previously used to identify signatures in CLL,^{296,299,333-335} although not all of them have been performed in isolated B lymphocytes as it was carried out in our study. In addition, examining the expression levels of these miRNAs in more detail, we identified miR-143 as the second most significantly downregulated miRNA, after miR-181, in 13q-H CLL patients. Interestingly, downregulation of miR-143 has been reported in unmutated *IGHV* CLL patients.³³⁶ Specifically, the infraexpression of miR-143 could increase cell proliferation by different kinases (*KRAS*, *PLK1* and MAPK family)³³⁷ and decrease apoptosis by a higher activation of two known antiapoptotic genes (*BCL2* and *MDM2*).^{338,339} Other important miRNAs (miR- 223, miR-29c and miR-181), whose downregulation has been related to high-risk cytogenetic subgroups of CLL patients,^{146,296} were also downregulated in 13q-H. By contrast, we identified that miR-155, whose overexpression has been related to poor prognosis,¹⁴⁹ was upregulated in 13q-H, showing the largest number of targets. In addition, the upregulation of miR-155, which could be related to enhanced BCR activation,¹⁹¹ could lead to a greater cell survival by *SOCS1*,³⁴⁰ and by *INPP5D*, a negative regulator of phosphatidylinositol 3-kinase (PI3K)-AKT signaling, as well as a decrease in apoptosis by the inhibition of two proapoptotic targets (*FOXO3a* and *TP53/INP1*).^{341,342} In this study, we also observed that higher levels of miR-155 expression correlated with worse outcomes in CLL patients: 13q-H had a higher expression level of miR-155 than 13q-L, and this subgroup showed stronger expression than did CLL patients with normal FISH. Recently, miR-155 has been described as a new prognostic marker.¹⁴⁹

Therefore, miRNA deregulation can affect important validated target genes involved in apoptosis and proliferation that could lead to decreased apoptosis and increased proliferation in 13q-H patients. These results are in line with our previous study in which gene expression profile was evaluated,¹⁹¹ and are also consistent with the poor outcome observed in the CLL patients with a high load of 13q- cells.⁷⁸ Therefore, we have demonstrated that the clinical heterogeneity within 13q- CLL

patients is clearly related to a biological heterogeneity in terms of deregulation of transcriptomic networks. These studies raise the question whether there is also a heterogeneous mutational background within CLL patients with 13q-. In this line, NGS studies have shown that cellular processes such as apoptosis or proliferation are affected by mutated CLL drivers.^{124,127} Furthermore, the co-occurrence of 13q- with other driver mutations such as *TP53*, *BIRC3*, *NOTCH1* or *SF3B1* might confer a more aggressive phenotype.¹²³ Thus, with the emergence of NGS technology, we will be close to know if there are significant differences about gene mutations between both subgroups of 13q deletion according to the percentage of 13q- cells. It will be necessary to analyze large series as the expected genetic instability and the mutational rate of CLL patients with 13q- as sole abnormality are relatively low.^{124,127}

In the second study, we focused on the heterogeneity observed within the group of CLL patients carrying 11q deletion. This chromosomal alteration is frequent in CLL patients and is traditionally associated with poor prognosis.^{72,322} Analogously to other chromosomal alterations,^{78-83,86} we observed clinical differences within CLL patients according to the percentage of 11q- cells. Specifically, a threshold of 40% of 11q deleted cells was established as the optimal one for showing significant differences in terms of TFT and OS in this subgroup. More in detail, patients with <40% of losses in 11q (11q-L) showed a longer TFT and OS compared with patients with ≥40% 11q- cells (11q-H). Therefore, we identified a subgroup of patients, 11q-L, with a better outcome within a group of patients, 11q-, traditionally associated with poor prognosis. It is noteworthy to mention that these results were also in line with other two independent studies,^{84,85} although the optimal threshold to classify patients was different. No significant differences in terms of OS were observed in one of these studies, probably due to limited number of patients in their series.⁸⁴ Recently, a retrospective large cohort confirmed a statically significant association between TFT and the percentage of abnormal nuclei for all cytogenetic alterations assessed by routinely by FISH, except 11q deletion.⁷⁹ Comprehensive prospective multi-institution studies of larger cohorts of CLL patients should be performed in order to better evaluate their impact and to establish the optimal cut-off point to stratify patient risk.

In CLL, 11q deletion is mostly monoallelic and encompasses *ATM* gene.³⁴³ More than one third of 11q- CLL patients carry mutations in the remaining *ATM* allele.¹⁹² Similarly to cases with *TP53* disruption, *ATM* gene is involved in the repair of damaged DNA³⁴⁴ and its deficiency causes genomic instability and allows the accumulation of additional genetic mutations during disease course.²⁷¹ In this

context, high-throughput sequencing technologies helped us to evaluate whether the clinical differences within the 11q- subgroup could be related to genomic instability and genetic heterogeneity. Thus, we analyzed the mutational status of a panel of the most recurrently mutated genes (*ATM*, *BIRC3*, *FBXW7*, *MYD88*, *NOTCH1*, *SF3B1*, *TP53* and *XPO1*) in a subset of CLL patients with 11q- by an amplicon-based NGS approach. Recently, NGS technologies have enabled the thorough exploration of the mutational landscape of CLL discovering a highly comprehensive list of frequently mutated genes,^{124,126,127,220,239,240,242,291} which contains the genes included in our study. Although the incidence of *ATM* mutations was similar in both subgroups (11q-L and 11q-H), considering all the analyzed genes, we observed that fewer patients with a low number of 11q losses had gene mutations compared with the subgroup of patients with a high number of 11q- cells. To our knowledge, this is the first study showing which biological differences can underlie the clinical heterogeneity within 11q- CLL patients based on the number of losses in 11q. Furthermore, *TP53*, *NOTCH1* or *SF3B1* mutations, which have been associated with poor outcome,^{71,111,115,118,345} were more frequent in 11q-H CLL patients.

Based on the first two studies of this thesis deciphering the heterogeneity in CLL, the percentage of cells with a cytogenetic alteration allows us to distinguish subgroups of CLL patients showing different clinical outcome within the well-established subgroups with the same chromosomal alteration according to FISH prognostic model.^{78-86,191} Therefore, as the quantification of the number of cells with a genetic abnormality has a prognostic value, we suggest that the hierarchical model proposed by Döhner could be improved by integrating the percentage of cells with a cytogenetic alteration defined by FISH.

CLL is a highly genetically heterogeneous disease, and then, high-throughput technologies are powerful tools to analyze it, rather than the study of single targets. Here, we have shown the biological diversity yielding the clinical heterogeneity in CLL, even within well-established groups of patients with a specific cytogenetic alteration. This supports the need to integrate genome-wide approaches. Moreover, the value of gene expression profiling is widely accepted in the study of CLL, and the emergence of the NGS techniques has enabled a more thorough characterization of CLL genome by revealing somatic mutations in previously unknown genes in CLL. Thus, in the third study, we combined gene expression arrays and NGS to analyze in depth *TET2*, a gene with a role widely reported in other hematological malignancies.^{274,275} In contrast to myeloid malignancies,³⁴⁶ we observed a significant overexpression of *TET2* in B lymphocytes of CLL patients

compared with healthy donors. Recent sequencing data in larger cohort of patients have revealed a very low frequency of *TET2* mutations in CLL (~2%),^{124,127} as well as in B-lineage derived lymphoid malignancies (2-6%).^{277,347} By contrast, *TET2* mutations were more frequent in T-cell lymphomas (15-33%)³⁴⁸⁻³⁵¹ or myeloid malignancies (13-37%).^{346,352-356} In our study, targeted deep sequencing did not show any *TET2* mutations in CLL patients, but this could be due to the limited number of patients.

Noteworthy is the fact that the enormous capacity of NGS based on the strategy of targeted deep sequencing allowed us the analysis of particularly long genes without known hot-spots regions such as *TET2* or *ATM* sequenced in our studies. In the case of *ATM* gene with a potential clinical impact in CLL, its size of 62 coding exons has hampered its routine analysis up to that point.³⁵⁷ Moreover, the coverage of sequencing reads reached by the targeted approach allowed us to identify subclonal mutations at low frequencies of the cells. The clinical impact of subclonal mutations has been recently reported¹²⁸⁻¹³¹ and the presence of aggressive subclonal mutations has been linked with clonal evolution and poorer outcome.¹²⁶

CLL is a valuable model to study clonal evolution due to a high prevalence, initially slow progression and the easy availability of samples. Clonal evolution still represents a central feature of tumor progression and relapse, in which evolutionary advantageous driver lesions are accompanied by selectively neutral passenger changes.^{358,359} Recent large-scale sequencing studies have uncovered the vast intratumoral genetic heterogeneity that likely fuels clonal evolution and can contribute to the variable clinical course of CLL patients.^{126,127} WGS suggested different temporal patterns of repopulation after therapy²⁹¹ and, more in detail, further longitudinal WES studies revealed marked clonal evolution with large clonal shifts between pre-treatment and FCR relapse samples in the majority of CLL patients.¹²⁷

In the last part of this thesis, we focused on characterizing the genetic events and clonal evolution during the clinical progression prior any therapy in CLL by FISH and WES using paired longitudinal samples taken from CLL patients both at diagnosis and at clinical progression. Moreover, we also assessed a control group analyzing two tumoral paired samples from patients with a stable disease with a median of follow-up almost 11 years. Despite the limitation of the number of patients, our results showed that CLL patients with progression had a higher intra-tumoral heterogeneity than cases without progression. Therefore, we confirmed the presence of subclones within a CLL patient, which could shape tumor evolution

through different mechanisms.^{126,127,291,360} In fact, we observed either acquired mutations or with a significantly increased allele frequency that could be involved in disease evolution in CLL patients with clinical progression. Interestingly, driver mutations in *TP53*, *BIRC3* and *CARD11* were reported as potential candidates to be involved in disease evolution since they were detected exclusively as acquired or with a significant increase of its allele frequency in patients with clinical progression. Previous studies also suggested clonal evolution as a key feature for CLL progression,^{292,361} although this fact is more common in CLL relapse under pressure of therapy.^{127,182}

NGS approaches help us define the evolutionary trajectories through CLL course. Mainly, two common patterns of clonal evolution have been suggested in CLL patients: a) linear evolution in which one dominant clone acquires driver events over time, and b) branched evolution in which several tumoral subclones coexist and evolve over time.^{126,257,288,291,362,363} In our study, we applied exome sequencing; therefore, we were limited in detecting subclonal mutations because of its coverage. Further targeted deep sequencing will allow us to better define the dynamics of mutated clones as well as to determine the presence of these mutations in very small subclones at diagnosis. We detected five acquired driver mutations according to WES data, all of them in patients with an active disease. However, it is worthy to mention that it has been reported that the majority of driver mutations could be already present at initial stages of the disease, often as small subclones.³⁶¹ Thus, it could be of great interest the analysis of these alleged acquired mutations with ultra-high sensitive NGS techniques.

Given the progress in this field, we anticipate that NGS will become an effective approach to systematically monitor disease progression and also prospectively, directing future clinical trials and therapeutic decisions. Further longitudinal studies with larger cohorts will allow us to define which mutations are essential to be monitored from diagnosis in order to predict clinical progression. Moreover, the integrative analysis of coding and non-coding mutations,^{124,361} transcriptomic networks,²²² methylation levels,²⁸³ and chromatin accessibility³⁶⁴ will allow us to gain insight into the key events that can take place from an early CLL leading up to the need for treatment.

Noteworthy is the fact that all the results obtained in this thesis were analyzed in B lymphocytes. However, the cellular origin of this disease is still debated since it has been recently suggested that CLL pathogenesis may start on a previous maturational cell stage of B lymphocytes. In fact, xenotransplantation studies

reported that hematopoietic stem cells (HSCs) from CLL patients were able to reproduce CLL phenotype in murine models³⁶⁵ and it has been addressed that CLL mutations could also appear in HSC.¹¹⁹ Therefore, as chromosomal abnormalities and gene mutations are important events in CLL pathogenesis, it would be also interesting to define which events take place earlier in hematopoiesis or which ones appear in mature B cells in order to establish a hierarchical model of the appearance of genomic alterations in this disease. The knowledge of the cell stage in which CLL is originated will allow us to achieve a better understanding about this disease.

Additional unanswered questions are about how different drivers cooperate and whether there are differences on cellular fitness that account for the clinical heterogeneity observed between CLL patients, even those with the same cytogenetic alteration. Understanding how multiple mutations interact and promote the outgrowth of increasingly fit cells as well as the different evolutionary trajectories will provide the clues of which mechanisms promote the onset of the disease, as well as the progression and the evolution into more aggressive variants. In this line, models applying genomic engineering will serve as valuable tools to study the effects of CLL mutations on cellular fitness. With the development of improved site-specific genome engineering methods such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology,³⁶⁶ we will be able to generate *in vitro* and *in vivo* models harboring the same genetic alterations described in CLL patients. CRISPR/Cas9, which relies on two key components—the bacteria-derived Cas9 nuclease, and a short guide RNA (sgRNA),³⁶⁷ has been discovered as a rapid, efficient and highly powerful tool for manipulating genomic sequences in order to mimic the somatic mutations observed in patients.³⁶⁸⁻³⁷⁰ Hence, with the creation of these disease models, we will be able of measuring the effects of both individual and multiple alterations on B cell biology and clonal competition in an *in vitro* and *in vivo* B cell-based cellular context. In a similar fashion, it would be interesting to edit CD34+ cells to evaluate the impact of events in cells of early B cell lineage, recently suggested as affected in CLL.^{119,365}

In summary, the research carried out during the development of this thesis through a combined analysis of the genome and transcriptome of B lymphocytes has improved our knowledge of the complex biology underlying the heterogeneity of CLL as well as the molecular events involved in its evolution. Clinical analysis suggested that the quantification of the number of cells with a genetic abnormality should be included in the study of the prognostic factors of CLL. The integration of clinical and biological data has shed light into the heterogeneity within well-defined subgroups of

CLL patients, through the identification of deregulated miRNAs and targets and mutated genes which could be further explored as potential therapeutic targets in the future. Finally, genetic changes underlying CLL disease progression were characterized, suggesting the importance of monitoring tumoral clones by the use of NGS in CLL patients.

Concluding remarks

1. CLL patients with 13q deletion as the sole abnormality showed a distinct miRNA expression profile depending on the number of cells carrying 13q-. Thus, clonal B lymphocytes from CLL patients with a high number of 13q losses showed infraexpression of miR-143 and overexpression of miR-155, which can lead to lower apoptosis and higher proliferation rates. These results provide new insights into the transcriptomic networks underlying the different clinical course of 13q- CLL patients and improve our understanding about the clinical poorer outcome of CLL patients with a high load of 13q- cells
2. CLL patients carrying 11q deletion are a heterogeneous group and the number of cells displaying this aberration is related to the outcome. CLL patients with a low frequency of 11q losses (<40%) are characterized by both a longer overall survival and time to first therapy than cases with a high load of 11q- cells.
3. Fewer patients with a low number of 11q losses displayed gene mutations compared with the subgroup of patients with a high number of 11q- cells. Thus, clinical differences within the 11q- subgroup could be due to a genetic heterogeneity in terms of the mutational background.
4. An integrative analysis of gene expression arrays and Next-Generation Sequencing revealed an overexpression of *TET2* gene in clonal B lymphocytes of CLL patients but lack of somatic mutations in the coding regions of this gene, suggesting that *TET2* gene could be a novel candidate involved in CLL. These data bring biological rationale for further investigations on *TET2* deregulation in CLL.
5. Whole-exome sequencing from paired longitudinal CLL samples prior any therapy showed a higher intra-tumoral heterogeneity in CLL patients with an active progressive disease than that of cases with a clinical stable CLL. The disease evolution is accompanied by new appearance or selection of driver mutations such as mutations of *TP53*, *BIRC3* and *CARD11*, highlighting clonal evolution as a key feature for CLL progression.

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Supplementary Appendix

SUPPLEMENTARY MATERIAL



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MiRNA expression profile of chronic lymphocytic leukemia patients with 13q deletion

María Hernández-Sánchez^a, Ana E. Rodríguez-Vicente^a, José-Ángel Hernández^b, Eva Lumbreras^a, María-Eugenia Sarasquete^a, Ana-África Martín^a, Rocío Benito^a, Carlos Vicente-Gutiérrez^a, Cristina Robledo^a, Natalia de las Heras^c, Juan-Nicolás Rodríguez^d, Miguel Alcoceba^a, Alfonso García de Coca^e, Carlos Aguilar^f, Marcos González^a, Jesús-María Hernández-Rivas^{a,*}

Supplementary Methods

miRNA expression profile using high density microarrays

A procedure for manual extraction of total RNA containing miRNA was carried out using a guanidinium-based buffer, which comes with the RNeasy isolation KitR[®] (Qiagen, Valencia, CA, USA). All total RNAs were of high quality according to their RIN score [1] determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA, USA).

The Gene Chip Micro 3.0 Array (Affymetrix, Inc, Santa Clara, CA, USA), which provides for 100% miRBase v17 coverage (www.mirbase.org) by a one-color approach, was used for universal miRNA coverage [2]. The microarray contains a total probe set of 19,724 for detecting 1733 mature human miRNAs and 1658 human pre-miRNAs. RNA labeling and microarray hybridization were carried out following the manufacturer's protocols for the GeneChip platform by Affymetrix. Hybridized Affymetrix arrays were scanned with an Affymetrix Gene-Chip 3000 scanner. The scanned images were processed using the AGCC (Affymetrix GeneChip Command Console) software.

Quantification of miRNA expression levels

10 ng of total RNA was reverse-transcribed using a microRNA reverse transcription kit (Applied Biosystems) and a specific reverse transcription stem-loop primer. The TaqMan MicroRNA Assays for U43 RNA (RNU43, Applied Biosystems) was used to normalize the relative abundance of miRNA using the $2^{-\Delta Ct}$ method. All reactions were performed in duplicate. The expression of each miRNA relative to RNU43 was determined using the $\Delta\Delta$ cycle threshold (Ct) method and differences of expression levels of the selected miRNAs between the distinct groups were then determined by the Mann-Whitney U test calculated in SPSS v19.0. Values of $P < 0.05$ were considered to be statistically significant.

Gene-specific semi-quantitative PCR

Semi-quantitative SYBRgreen PCR was done in triplicate with SYBR[®] Green PCR Master Mix (Applied Biosystems) using the IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Expression data for selected genes were validated in a subset of CLL patients (n=26). Primers were designed based on the probe-sets used by Affymetrix to synthesize the GeneChip Primer sequences (Supplementary Table 2). The *ABL1* gene was used as the internal control and the expression data were analyzed by the comparative Ct method. The data were not normally distributed, so non-parametric tests were used. Expression levels of the selected genes in both groups (13q-H and 13q-L) were analyzed using the Mann-Whitney U test with a two-tailed value of $P < 0.05$ for statistical significance.

Supplementary Data

Supplementary Table 1. Main clinical and biological features of all CLL patients included in the study.

Feature	Category	CLL patients
Median age, years (range)		70 (46-89)
Gender	Male	60%
	A	88%
Binet stage	B	12%
	C	0%
White blood cells /mL (range)		16260 (8300-369000)
Lymphocytes /mL (range)		9300 (5400-355000)
Hemoglobin g/dL (range)		13.9 (10-18)
Platelet count /mL (range)		182500 (73000-408000)
<i>IGHV</i>	Unmutated	30%
LDH	High	0%
$\beta 2$ microglobulin	High	12%
	13q deletion	
FISH abnormality	$\geq 80\%$ of losses	37%
	$< 80\%$ of losses	34%
	Normal	29%

Supplementary Table 2. Sequences of primers used for SYBR Green detection.

Primer designation	Sequence (5'-3')
<i>ABL1</i>	
Forward	GCCCTAGCTTTACGCTCATCAC
Reverse	GCTGTAAGAACCGCATAAAACGAT
<i>BCL2</i>	
Forward	CCCATCAATCTTCAGCACTC
Reverse	CTGGAATCGATCTGGAAAT
<i>PLK1</i>	
Forward	TGCACCGAAACCGAGTTATT
Reverse	TTGGTTGCCAGTCCAAAATC
<i>INPP5D</i>	
Forward	GGTGACCCATCTGCAATACC
Reverse	GGGTGGAGACACGACACTTT
<i>SOCS1</i>	
Forward	TGGTAGCACACAACCAGGTG
Reverse	AGGAGGAAGAGGAGGAAGGTT

Supplementary Table 3. MiRNAs differentially expressed between B lymphocytes from 13q-H and 13q-L CLL patients at $P < 0.05$. Overexpression and underexpression refers to 13q-H relative to 13q-L CLL patients.

miRNAs overexpressed in 13q-H CLL patients	<i>P</i>	miRNAs underexpressed in 13q-H CLL patients	<i>P</i>
miR-513c	6.1E-04	miR-4782-5p	7.3E-05
miR-4686	1.6E-03	miR-3668	2.4E-04
miR-515-3p	1.9E-03	miR-19b-1-star	2.9E-03
miR-3529	8.8E-03	miR-181a	5.7E-03
miR-574-3p	8.9E-03	miR-143	7.1E-03
miR-4804-3p	1.0E-02	miR-181b	9.1E-03
miR-585	1.1E-02	miR-130a	1.0E-02
miR-3922-5p	1.1E-02	miR-323-3p	1.2E-02
miR-138-2-star	1.2E-02	miR-223	1.6E-02
miR-4501	1.3E-02	miR-4425	1.6E-02
miR-1293	1.4E-02	miR-760	1.6E-02
miR-891a	1.5E-02	miR-4714-5p	2.0E-02
miR-423-3p	1.6E-02	miR-523	2.0E-02
miR-629	1.7E-02	miR-145	2.1E-02
miR-4475	2.0E-02	miR-151-3p	2.1E-02
miR-4666-3p	2.0E-02	miR-542-5p	2.2E-02
miR-4445	2.2E-02	miR-675	2.2E-02
miR-124-star	2.5E-02	miR-4441	2.3E-02
miR-499a-5p	2.5E-02	miR-151b	2.3E-02
miR-362-3p	2.6E-02	miR-1233	2.5E-02
miR-466	2.7E-02	miR-3621	2.5E-02
miR-4252	3.0E-02	miR-100	2.6E-02
miR-4669	3.0E-02	miR-876-3p	2.6E-02
miR-4793-5p	3.0E-02	miR-503	2.6E-02
miR-155	3.1E-02	miR-4503	2.9E-02
miR-1296	3.4E-02	miR-1298	2.9E-02
miR-1183	3.4E-02	miR-3647-3p	3.0E-02
miR-4659a-5p	3.7E-02	miR-4293	3.2E-02
miR-133b	3.7E-02	miR-1283	3.2E-02
miR-2278	3.8E-02	miR-492	3.6E-02
miR-4748	3.8E-02	miR-922	3.7E-02
miR-616	3.8E-02	miR-199a-5p	4.3E-02
miR-3689c	3.8E-02	miR-3180	4.6E-02
miR-4781-5p	4.1E-02	miR-3687	4.7E-02
miR-1257	4.2E-02	miR-1909	4.8E-02
miR-4304	4.2E-02		
miR-302d	4.6E-02		
miR-521	4.6E-02		
miR-4709-5p	4.7E-02		
miR-23b	4.8E-02		
miR-1270	4.9E-02		

Supplementary Table 4. miRNAs and their experimentally validated targets that are significantly deregulated in 13q-H CLL patients.

By applying an integrated miRNA-mRNA analysis, mRNA targets with an experimental observation of the miRNA-gene interaction were identified for the list of miRNAs deregulated in 13q-H CLL patients.

Downregulation or upregulation refers to 13q-H relative to 13q-L CLL patients.

miRNA: microRNA

miRNA	Experimentally validated targets
Downregulated	
miR-181a	<i>AICDA, BCL2, CD69, CDKN1B, CDX2, ESR1, GATA6, GRIA2, HOXA11, KRAS, NLK, NOTCH4, PLAG1, TCL1A, TIMP3, TRA, VSNL1</i>
miR-143	<i>BCL2, DNMT3A, FNDC3B, KRAS, MAPK12, MAPK7, MDM2, PLK1, PRC1, TOP2A</i>
miR-130a	<i>ATG2B, CSF1, DICER1, HOXA5, MAFB, MEOX2, TAC1, ZPFM2</i>
miR-223	<i>CNTN4, IRS1, LMO2, MEF2C, NFIA, RHOB, Scn3A, STMN1, VIM</i>
miR-145	<i>ABRACL, ACBD3, AHNAK, C11orf58, CBFB, CCDC25, CCNA2, CDK4, CES1, CLINT1, DDR1, DFFA, DTD1, EIF4E, EIF4BP2, F11R, FBXO28, FLI1, FSCN1, IGF1R, IRS1, KLF5, KRT7, LAMP2, MAPK7, MDFI, MDM2, MMP1, MUC1, MYC, NDUFA4, PADI1, PARP8, PPP3CA, RASA1, ROBO4, RTKN, SPTB, SWAP70, TPM3, UNG, USP46, VASN</i>
miR-675	<i>RB1</i>
miR-100	<i>FGF16, FGFR3, IGF1R, MTOR, PLK1, RPTOR</i>
miR-503	<i>CCND1</i>
miR-199a-5p	<i>ALOX5AP, DYRK1A, ETS1, HIF1A, LAMC2, SET, SIRT1</i>
miR-3687	<i>BDNF</i>
Upregulated	
miR-515-3p	<i>BCL11B, BCOR, BTG1</i>
miR-499a-5p	<i>SOX6</i>
miR-362-3p	<i>BDNF, MAP2K2, MAPK1</i>
miR-155	<i>ABHD16A, AGTR1, AICDA, AMIGO2, ANKFY1, ARFIP1, ARFIP2, ARL10, ARL5B, ATG3, ATP6V1C1, BACH1, BET1, BRPF3, CBFB, CCND1, CD47, CDK5RAP3, CEBPB, CHAF1A, CLDN1, CSF1R, CTLA4, CTNNB1, CUL4B, CUX1, CYP51A1, CYR61, DCAF7, DHX40, DNAJB1, DNAJC19, DPP7, DSG2, ETS1, F2, FADD, FADS1, FAR1, FGF7, FMNL2, FOXO3, GNA13, HSD17B12, HSDL1, IKBKE, IL13RA1, INPP5D, JARID2, LCLAT1, LDOC1, LPL, LY6K, MAF, MARC1, MATR3, MECP2, MEIS1, MET, METTL7A, MOSPD2, MPZL1, MSI2, MYB, MYD88, MYO10, NARS, NT5E, PDE3A, PDLIM5, PHC2, PICALM, PKN2, PLXND1, PODXL, POLE3, POLE4, PPL, PPP5C, PRAF2, PRKCI, PTPRJ, RAB23, RAB34, RAB5C, RAB6A, RAI14, RCN2, RCOR1, RHEB, RHOA, RIPK1, SATB1, SCAMP1, SDCBP, SH3BP4, SLA, SLC30A1, SMAD1, SMAD2, NAP29, SOCS1, SIP1, SYNE2, SYPL1, TAB2, TACSTD2, TBCA, TCF7L2, TM6SF1, TNFRSF10A, TP53INP1, TRAM1, TRIM32, TRIP13, TXNDC12, TXNRD1, UBE2J1, UFL1, VAMP3, WDFY1, WEE1</i>
miR-133b	<i>BCL2L2, CASP9, CDC42, CDK13, CPNE3, CTGF, FSCN1, HCN2, HCN4, IGF1R, KCNE1, KCNH2, KCNQ1, KLF15, KRT7, MCL1, NELFA, NFATC4, PITX3, PKM, PTBP2, PTPRK, RB1CC1, RHOA, RUNX2, SRF, STK3</i>
miR-302d	<i>ADAM9, ANKRD13B, ANKRD533, APP, ARHGEF3, BAZ1A, C12orf23, C9orf78, CCND1, CCND2, CD24, CD44, CD46, CD83, CDK11A/CDK11B, CDK19, CDKN1A, CENPF, CFL2, CMTM4, CNOT6, CYB5R4, DKK1, ERBB4, ESR1, FAM13B, FITM2, FYCO1, GBAS, GBP3, GLTP, GPSM2, HERPUD1, HSPA14, INSIG2, KDM1B, KIAA1919, KIF23, KLF13, KLH12, LATS2, LEFTY1, LMNB1, LUC7L2, MARCH4, MBNL2, MED28, MICA, MKRN1, MYBL1, NIN, NR4A2, NUPL1, PACRGL, PBK, PCGF5, PDIK1L, PHC2, PRC1, PRKACB, RECK, RELA, RNF149, RPIA, SEPT2, SLC25A23, SLC35F6, STK4, STX11, TBC1D2, TEX30, TFAP4, TMEM14A, TMEM9B, TNFAIP1, TP63, TRPS1, TTC8, TUSC2, UBXN1, USP12, VEGFA, VPS26A, ZHX1, ZNF226</i>
miR-521	<i>ERCC8</i>
miR-23b	<i>ATAT1, C21orf33, CXCL12, FBXO32, HES1, IL6R, LMNB1, MDH2, MET, NOTCH1, PLAUI, POU4F2, SEPT3, SMAD3, SMAD4, SMAD5, TRIM63, TRPS1</i>

Supplementary Table 5. The most important deregulated functions that could be deregulated by infraexpression of miR-143 and overexpression of miR-155 in 13q-H patients. The gene targets marked in bold were confirmed by semi-quantitative PCR.

Pathway*	Genes**	miRNA	P-value***
Decreased apoptosis	BCL2 , MDM2, FOXO3a, TP53INP1	miR-143; miR-155	0.007; 0.031
Increased proliferation	KRAS	miR-143	0.007
Altered cell cycle	PLK1 , PRC1, and TOP2A	miR-143	0.007
Increased cell survival	SOCS1	miR-155	0.031
Increased PI3K-AKT signaling	INPP5D	miR-155	0.031

*These pathways are deregulated in 13q-H patients by the overexpression and downregulation of miR-155 and miR-143, respectively, based on the references 30-32, 35-37 and 39.

**Genes represents the experimentally validated targets identified using Ingenuity Pathway Analysis (IPA Ingenuity Systems; <http://www.ingenuity.com>) basing on experimental observations.

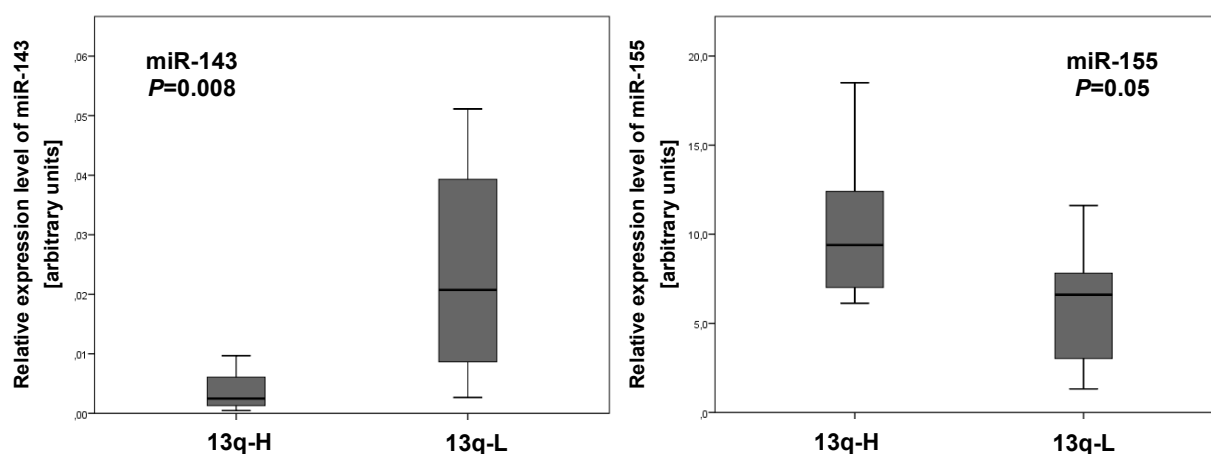
***P-value refers to the deregulation of each miRNA comparing 13q-H vs 13q-L.

Supplementary Table 6. Top differentially expressed miRNAs between B lymphocytes from low-risk CLL patients and healthy donors at FDR<0.01.

Top 50 miRNAs with increased expression in low-risk CLL patients compared with healthy donors		Top 50 miRNAs with decreased expression in low-risk CLL patients compared with healthy donors	
miRNAs overexpressed in CLL	Fold Change	miRNAs underexpressed in CLL	Fold Change
miR-1295	70.33	miR-432	0.03
miR-21	38.51	miR-181a	0.08
miR-34a	32.09	miR-181c	0.09
miR-29b	20.38	miR-3150b-3p	0.11
miR-148a	16.24	miR-1909	0.12
miR-192	14.83	miR-4634	0.12
miR-660	13.91	miR-193b	0.13
miR-146b-5p	12.82	miR-4532	0.14
miR-195	10.40	miR-4739	0.15
miR-140-5p	10.11	miR-650	0.15
miR-19b	9.72	miR-181b	0.15
miR-29c	9.29	miR-3663-3p	0.16
miR-28-5p	8.78	miR-370	0.18
miR-223	7.71	miR-4689	0.19
miR-4524-star	7.55	miR-3195	0.19
miR-532-5p	7.30	miR-4534	0.21
miR-362-5p	7.22	miR-1469	0.22
miR-30e	7.12	miR-3188	0.22
miR-18a	6.80	miR-4492	0.22
miR-30b	6.40	miR-1231	0.23
miR-4454	6.31	miR-4281	0.23
miR-128	6.04	miR-4788	0.23
miR-19a	6.01	miR-4270	0.23
miR-194	5.94	miR-3180-3p	0.24
miR-500a-star	5.93	miR-193b-star	0.24
miR-27a	5.92	miR-663	0.24
miR-27b	5.46	miR-1225-5p	0.25
miR-1271	5.41	miR-3911	0.25
miR-502-3p	5.29	miR-939	0.25
miR-106b	5.14	miR-4463	0.25

miR-3128	4.97	miR-193a-5p	0.26
miR-26b	4.86	miR-762	0.26
miR-335	4.57	miR-4707-5p	0.26
miR-30e-star	4.56	miR-4665-5p	0.26
miR-501-3p	4.31	miR-4763-3p	0.26
miR-29a	4.22	miR-4298	0.26
let-7f	4.22	miR-3135b	0.26
miR-628-3p	4.15	miR-1207-5p	0.27
miR-18b	4.02	miR-4669	0.27
miR-30a	4.01	miR-3197	0.27
miR-17-star	3.94	miR-4745-5p	0.27
miR-30c	3.83	miR-4687-3p	0.27
miR-7-1-star	3.67	miR-4488	0.27
miR-20a	3.49	miR-134	0.27
miR-24	3.22	miR-1224-5p	0.28
miR-155	3.21	miR-4530	0.28
miR-500a	3.13	miR-1915	0.28
let-7g	3.07	miR-4695-5p	0.28
miR-93-star	3.06	miR-3177-3p	0.28
miR-345	2.99	miR-371b-5p	0.28

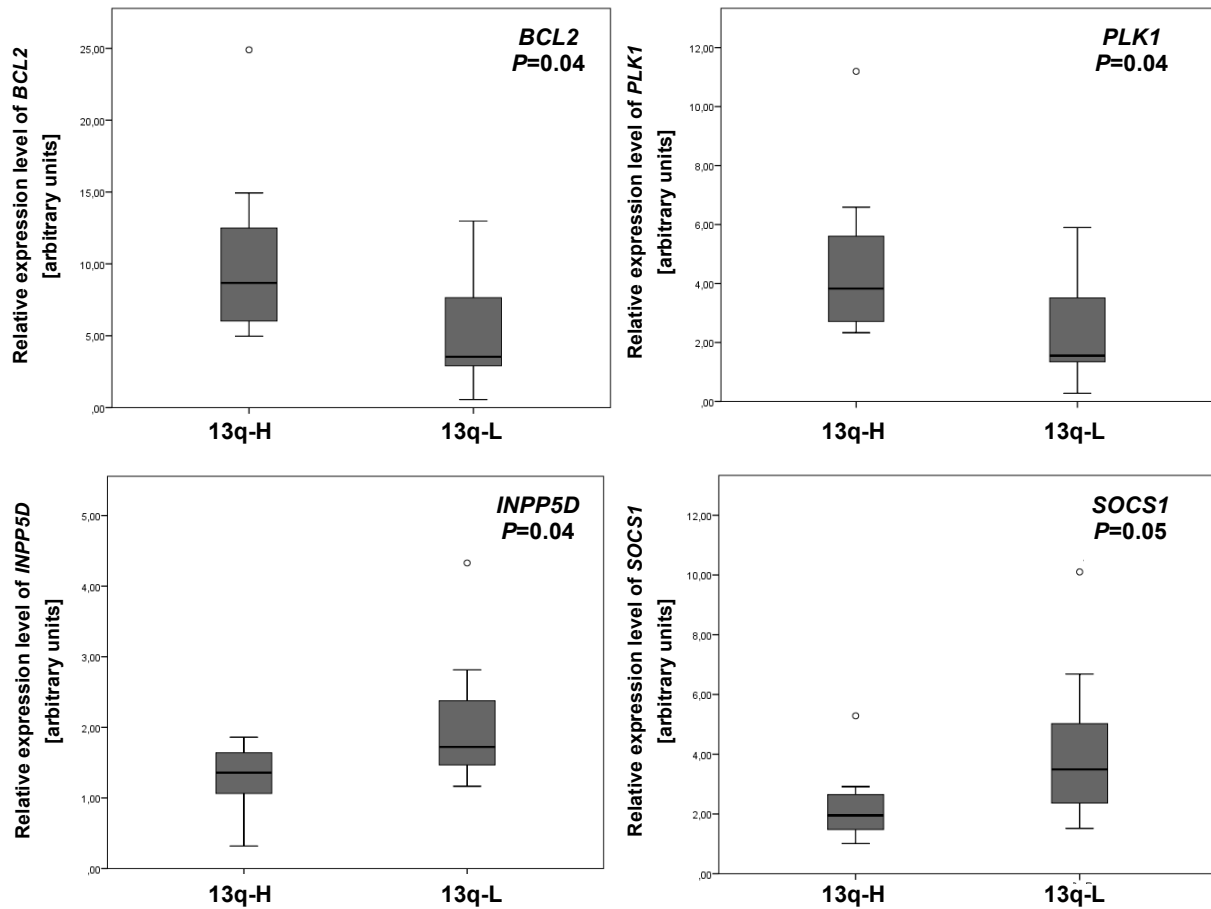
Supplementary Figure 1.



Supplementary Figure 1. Quantitative RT-PCR validation for miR-143 and miR-155 in an independent group of 13q- CLL patients with respect to percentage losses of 13q.

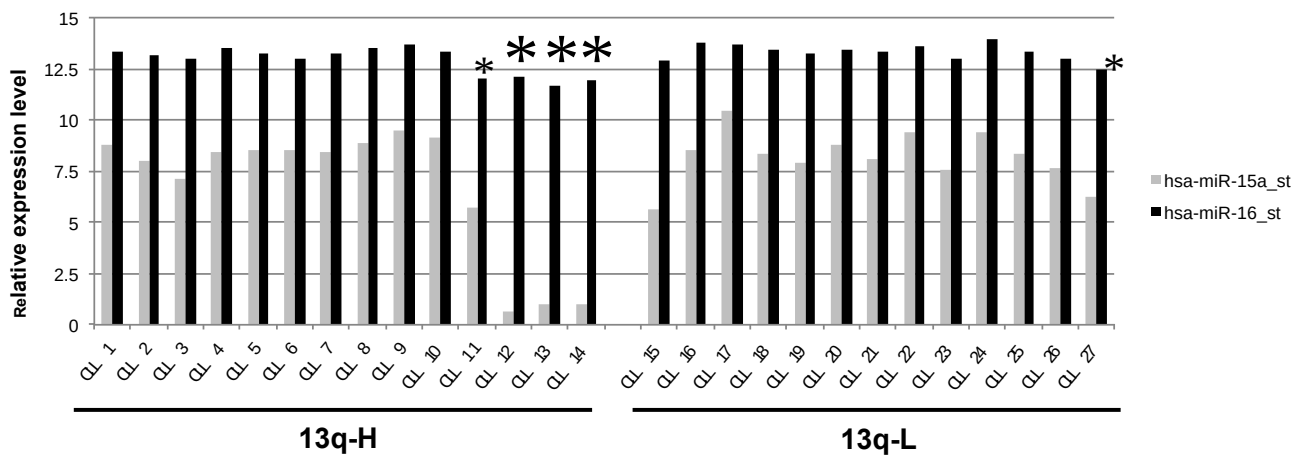
MiR-143 was found to be significantly downregulated in B lymphocytes from 13q-H vs. 13q-L CLL patients, whereas miR-155 was upregulated in B cells from 13q-H vs 13q-L CLL patients. Relative expression of miR-143 (expressed in arbitrary units) was evaluated by individual TaqMan miRNA assays performed in duplicate and normalized with respect to RNU43 ($2^{-\Delta\Delta C_t}$) in 18 CLL patients (10 13q-H and 8 13q-L). Box plots indicate the median value (horizontal line) and the 25th–75th percentile range (box). Whiskers show the maximum and minimum values. P-values were determined by the Mann-Whitney U test.

Supplementary Figure 2.



Supplementary Figure 2. Gene expression levels of target genes significantly deregulated in 13q-H CLL patients.

Box plot of the expression levels of four genes with significantly different expression between 13q-H and 13q-L patients, assessed by semi-quantitative PCR analysis. Box plots show the relative upregulation of *BCL2* and *PLK1* and the downregulation of *INPP5D* and *SOCS1* in patients with a high number of 13q-cells compared with CLL patients with lower percentages of losses in 13q. The thick line inside the box plot indicates the median expression levels and the box shows the 25th and 75th percentiles, while the whiskers show the maximum and minimum values. Outliers (extreme values falling out of the main distribution) are represented by open circles. Statistical significance was determined using the Mann-Whitney U test ($P < 0.05$).

Supplementary Figure 3.**Supplementary Figure 3. Expression level of miR-15a (grey) and miR-16 (black) in 13q- CLL patients, determined by miRNA microarray.**

The subgroup of patients with biallelic 13q losses (CLL 11, 12, 13, 14 and 27), which are marked with an asterisk, shows a significant decrease in the expression of miR-15a and miR-16 compared with the patients with monoallelic losses. This is less clear in miR-16 due to the design of the array: miR-15a expression can be specifically analyzed by a probeset (hsa-miR-15a_st, grey). However, the hsa-miR-16_st probeset (black), which measures miR-16-1 expression, is not specific to this miRNA, but can also detect miR-16-2 expression. Notably, the three patients with more than 80% of cells with biallelic 13q14 deletion, indicated with an asterisk, showed a greater decrease of miRNA-15a expression.

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A Low Frequency of Losses in 11q Chromosome Is Associated with Better Outcome and Lower Rate of Genomic Mutations in Patients with Chronic Lymphocytic Leukemia

José Ángel Hernández^{1☯}, María Hernández-Sánchez^{2☯}, Ana Eugenia Rodríguez-Vicente², Vera Grossmann³, Rosa Collado⁴, Cecilia Heras¹, Anna Puiggros⁵, Ana África Martín⁶, Noemí Puig⁶, Rocío Benito², Cristina Robledo², Julio Delgado⁷, Teresa González⁸, José Antonio Queizán⁹, Josefina Galende¹⁰, Ignacio de la Fuente¹¹, Guillermo Martín-Núñez¹², José María Alonso¹³, Pau Abrisqueta¹⁴, Elisa Luño¹⁵, Isabel Marugán¹⁶, Isabel González-Gascón¹, Francesc Bosch¹⁴, Alexander Kohlmann^{3,17}, Marcos González^{2,6}, Blanca Espinet⁵, Jesús María Hernández-Rivas^{2,6,18*}, Grupo Cooperativo Español de Citogenética Hematológica (GCECGH) and Grupo Español de Leucemia Linfática Crónica (GELLC)¹¹

Supplementary Methods

Next-generation sequencing (NGS) analysis

Genomic DNA was extracted from CLL fixed cells from peripheral blood of 25 11q- CLL patients using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. Due to the large quantity of DNA required for this study, 100-ng inputs of samples were amplified with the REPLI-g Midi Kit (Qiagen, Valencia, CA, USA) [1]. To ensure good quality, amplified DNA was quantified using a standardized PicoGreen fluorescence assay (Life Technologies, Carlsbad, CA, USA). The integrity of the DNA was visually inspected on a 1% agarose gel.

NGS was performed using 454 Titanium Amplicon chemistry (Roche Applied Science, Penzberg, Germany) [2] to investigate the *ATM*, *TP53*, *NOTCH1*, *SF3B1*, *MYD88*, *FBXW7*, *XPO1* and *BIRC3* mutations. The eight candidate genes were screened for mutations as follows: 62 *ATM* coding exons (Transcript-ID ENST00000278616) represented by 65 amplicons; exons 4-10 of *TP53* (8 amplicons, Transcript-ID ENST00000269305); the C-terminal PEST domain (exons 33-34) of *NOTCH1* (7 amplicons, Transcript-ID ENST00000277541); in exons 10-16 of *SF3B1* (7 amplicons, Transcript-ID ENST00000335508); exons 4 and 5 of *MYD88* (2 amplicons, Transcript-ID ENST00000396334); exons 8-12 of *FBXW7* (5 amplicons, Transcript-ID ENST00000281708); exons 14 and 15 of *XPO1* (2 amplicons, Transcript-ID ENST00000401558); and exons 2-9 of *BIRC3* (11 amplicons, Transcript-ID ENST00000263464). Information about primer sequences is shown in S5 Table. The oligonucleotide design was performed as part of the IRON-II network.

A total of 96 amplicon preparations across 25 samples (2400 individual PCR reactions) were processed using the FastStart High Fidelity PCR System kit (Roche Applied Science). The PCR conditions are described in S6 Table. After all 25 samples had been prepared in 96-well plates, each PCR product was individually purified using Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany) and checked by electrophoresis on 1.2% agarose gels.

A single patient-specific *ATM* library was generated for each patient, pooling a specific volume for each of the 65 *ATM* amplicons. Subsequent 454 emulsion PCR and amplicon sequencing were performed with the Genome Sequencer FLX System instrument (Roche Applied Science), following the manufacturer's recommendations [3,4].

Libraries of *TP53*, *NOTCH1*, *SF3B1*, *MYD88*, *FBXW7* and *XPO1* genes were separately generated, pooling 31 amplicons for 3 patients. Furthermore, libraries of *BIRC3* gene were generated pooling 11 amplicons for 8 patients. In these cases, subsequent Agencourt AMPure XP bead purification was needed to remove short fragments. Sequencing runs of these genes were carried out on a Genome Sequencer Junior System instrument (Roche Applied Science).

All sequencing data were generated using the GS FLX and Junior Sequencer Instrument software version 2.7 (Roche Applied Science). Sequence alignment and variant detection were performed using the GS Amplicon Variant Analyzer software version 2.7 (Roche Applied Science). The results were further processed and visualized using the Sequence Pilot software version 3.5.2 (JSI Medical System, Kippenheim, Germany) [5]. To detect variants, filters were set to display sequence variants occurring in more than 2% of bidirectional reads per amplicon in at least one patient. S7 Table shows the median number of reads generated for each gene, allowing variants to be identified down to a detection limit of 2% [4].

All variants were first compared with published single nucleotide polymorphism data (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). Mutations within introns were not scored. Mutations in coding regions were compared with previously described mutations in the COSMIC database (<http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>) [6]. In addition, all missense, frameshift and nonsense mutations were resequenced on unamplified DNA to rule out artifacts arising from whole-genome amplification. For *ATM*, mutations were defined as pathogenic if they were “truncating” due to a sequence alteration that was predicted to terminate the protein prematurely, or “non-truncating” if they were missense and predicted to be damaging by the SIFT algorithm [7].

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Supplementary Data

Table A. Characteristics of the series of 197 CLL patients with 11q deletion.

Characteristic	Number of patients (n = 197)	%
Age, years		
Median	65	
Range	28-97	
Gender		
Male	151	77
Female	46	23
Binet clinical stage		
A	121	61
B	58	30
C	18	9
Lymphadenopathy		
No	62	32
Yes*	135	68
Splenomegaly (n = 192)		
No	147	77
Yes	45	23
Hepatomegaly (n = 192)		
No	174	91
Yes	18	9
Lymphocyte count (>20,000/μL) (n = 192)		
Yes	89	46
No	103	54
Serum LDH (n = 187)		
Normal	133	71
High	54	29
Serum β2 microglobulin (n = 170)		
Normal	116	68
High	54	32
IGHV mutational status (n = 56)		
Unmutated (<2%)	37	66
Mutated ($\geq$ 2%)	19	34
CD38 expression (n = 131)		
Negative (<30%)	59	45
Positive ($\geq$ 30%)	72	55
ZAP-70 expression (n = 79)		
Negative (<20%)	35	44
Positive ($\geq$ 20%)	44	56
FISH alterations in addition		
11q- only	82	42
11q- and other(s)	115	58
13q-	108	55
+12	14	7
17p-	6	3
First therapy		
Yes	151	77
No	46	23
Median TFT** (months)	25	
Survival		
Yes	131	69.5
No	60	30.5
Median OS (months)	106	

* $\geq$ 2 territories (extended lymphadenopathy): 75 patients (38.1 %). **TFT: Time to first therapy. ***OS: Overall survival.

Table B. Univariate analysis of time to first therapy in 11q- CLL patients with respect to the number of losses detected by FISH: <40% (n = 51) or ≥40% (n = 146).

Variable	Median time to first therapy, months	P (log-rank test)
Sex, male/female	27/23	0.270
Binet A vs B vs C	33/10/10	0.024
Lymphadenopathy No vs ≤2 node areas vs >2 node areas	45/33/10	< 0.0001
Splenomegaly , yes/no	8/31	0.045
Lymphocytes >20 x 10 ⁹ /L, yes/no	15/33	0.220
Serum LDH , high/normal	15/30	0.045
β₂ microglobulin , high/normal	12/29	0.019
CD38 expression , high/normal	23/38	0.023
ZAP70 expression , high/normal	24/45	0.025
IGHV mutation status , unmutated/mutated	10/52	<0.0001
del(11q) as sole cytogenetic aberration , yes/no	22/29	0.550
del(11q) and del(13q) , yes/no	31/20	0.250
del(11q) , <40% vs ≥40%	44/18	<0.0001

Table C. Univariate analysis of overall survival in 11q- CLL patients with respect to the number of losses detected by FISH: <40% (n = 51) or ≥40% (n = 146).

Variable	Median overall survival, months	P (log-rank test)
Sex, male/female	108/90	0.680
Binet A vs B vs C	119/74/62	0.001
B symptoms , yes/no	77/108	0.034
Lymphadenopathy No vs ≤2 node areas vs >2 node areas	132/92/85	0.087
Splenomegaly , yes/no	68/119	< 0.0001
Hepatomegaly , yes/no	68/108	0.025
Lymphocytes >20 x 10 ⁹ /L, yes/no	83/119	0.032
Serum LDH , high/normal	67/108	< 0.0001
β₂ microglobulin , high/normal	66/132	< 0.0001
CD38 expression , high/normal	90/109	0.155
ZAP70 expression , high/normal	106/152	0.133
IGHV mutation status , unmutated/mutated	106/122	0.818
del(11q) + del(13q) , yes/no	85/108	0.045
del(11q) as sole cytogenetic aberration , yes/no	88/106	0.425
del(11q) , <40% vs ≥40%	90/NR*	0.006

*NR: not reached

Table D. Main clinical and biological characteristics of 25 CLL patients with 11q- with respect to *ATM* mutational status.

Variable	Category	Subgroup with <i>ATM</i> mutations (n=9)	Subgroup without <i>ATM</i> mutations (n=16)	P
Gender	Male	6/8 (75%)	14/17 (82.4%)	0.525
Age (years), median (range)		69 (48-83)	70 (29-98)	0.789
Binet stage	A	5/8 (62.5%)	10/17 (58.8%)	0.607
	B or C	3/8 (37.5%)	7/17 (41.2%)	
White blood cells (x 10⁹/L), median (range)		19945 (12200-190900)	19600 (5960-91000)	0.56
Lymphocytes (x 10⁹/L), median (range)		14520 (7200-177537)	14280 (5010-80860)	0.485
Hemoglobin (x 10⁹/L), median (range)		14.9 (10.4-16.8)	13.7 (6.8-18.7)	0.366
Platelet (x 10⁹/L), median (range)		207000 (151000-269000)	173000 (108000-257000)	0.062
<i>IGHV</i>	unmutated	8/8 (100%)	11/14 (78.6%)	0.236
LDH	>UNL	2/7 (28.6%)	5/17 (29.4%)	0.682
β2 microglobulin	>UNL	1/5 (20%)	4/14 (28.6%)	0.603
LDT	<1 year	1/6 (16.7%)	7/14 (50%)	0.187
Bone marrow pattern	Diffuse	2/5 (40%)	2/9 (22.2%)	0.455
Hepatomegaly	Yes	2/7 (28.6%)	1/16 (6.3%)	0.209
Splenomegaly	Yes	2/7 (28.6%)	4/16 (25%)	0.618
B symptoms	Yes	0/8 (0%)	2/17 (11.8%)	0.453
Died during follow-up	Yes	4/8 (50%)	4/17 (23.5%)	0.193
Therapy during follow-up	Yes	7/8 (87.5%)	11/17 (64.7%)	0.246

IGHV unmutated: $\geq$ 98% homology with germline; LDH: lactate dehydrogenase; UNL: upper normal level; LDT: lymphocyte doubling time

Table E. PCR primers used for next-generation sequencing studies.

Gene	Strand	Exon	Amplicon	Forward Sequence 5' -> 3'	Reverse Sequence 5' -> 3'	Length (bp)
ATM	+	2	1	TGATGTGTCTCTGAAATTGTGA	ACACACAAAAGTAATATCACAAACAGAA	369
ATM	+	3	2	TGCCGTCAACTAGAACATGATAG	TGCCAAATTCATATGCAAGG	370
ATM	+	4	3	GCTCTTTGTGATGGCATGAA	AACAACTTATGCAACAGTTAAGTCC	342
ATM	+	5	4	GCCATAATTGCGCAATTTCTTC	GACAGAGTGCTTCTTTGGTGA	380
ATM	+	6	5	CCTTTTCTGTATGGGATTATGGA	TTACTGAGTCTAAAACATGGTCTTGC	345
ATM	+	7	6	CCCCCTGTTATACCCAGTTG	TCAACCAGAGAAATCCAGAGG	396
ATM	+	8	7	AGGTTGGACCAGGTGCTTTC	CAGGAAATTTCTAAATGTGACATGA	322
ATM	+	9	8	GTGCTGTTCCACTCCAACCT	GGTTGAGATGAAAGGATTCCAC	352
ATM	+	10	9	ATGGAATGATGGTGATTCTCTAAT	TGCTCAGAACTTATACCACGAAAG	380
ATM	+	10	10	GGTCAAAACCTAGAAAGCTCACAA	AAGTCTTCTCGGCCAAACAA	354
ATM	+	11	11	GCCAGGCACGTCTCTGATA	AAAGCCATCTGGCATCAAT	357
ATM	+	12	12	CAATGGTTGTCCTCCTTAAATTG	TAAGATGCAGCTACTACCCAGCTA	370
ATM	+	13	13	AGGCAAGCATTAGGTACTTGG	TTCTCCTTCCTAACAGTTTACCAAAG	348
ATM	+	14	14	CCAGGATATGCCACCTTTAACT	TTTCATTCAAATTTATCCGAAAC	308
ATM	+	15	15	TTGCTTATACTGTATGACTACGTGGA	ACTCCAGCCTGGGTGACA	361
ATM	+	16	16	TTGTTGCTTGGTCTTTTGTG	CGTGTAGCCAGGATGGTCT	355
ATM	+	17	17	ACAGATGTGAGCCACTGTGC	GGCCTCTTATACTGCCAAATCA	340
ATM	+	18	18	ATATGGCTGTTGTGCCCTTC	TCITCAAAGACACCATGTGATTTC	350
ATM	+	19	19	CTCCCAAAGTGCTGGGATTA	CATCTTGGTCACGACGATACA	337
ATM	+	20	20	GCCAATGGAAGATGTTCTTGA	GCATTCGTATCCACAGATAGCA	357
ATM	+	21	21	TGGCAAGGTGAGTATGTTGG	TGGCCCATTTTGAATAAGGA	370
ATM	+	22	22	CAGGCATCTAACAAAGGAGAGG	TACTGCCATCTGCAGCATTC	374
ATM	+	23	23	TGCTTTGGAAAGTAGGGTTTG	TGATTTGACCCATTGTGACC	375
ATM	+	24	24	TCTGGAGTTCAGTTGGGATTTA	AAGTGCCACTCAGAAAATCTAGC	346
ATM	+	25	25	AGTTGAATGAATGTTGTTCTAGTCT	TGGTATGTGTTGCTGGTG	369
ATM	+	26	26	TTATGGTGGTGGTATGTTCTAAGC	GAAGACTTTTCCAGTCCCTCTTGA	320
ATM	+	26	27	GATGAGGTGAAGTCCATTGCT	AATTCATGGTGGCTATGC	348
ATM	+	27	28	TGCCTTTTGAGCTGCTTGA	CCTCAATTCAAAGGTGGCTATT	335

ATM	+	28	29	GAAGTTCACTGGTCTATGAACAAAAC	CAATTAATGCTGACAAGTAAATACCA	368
ATM	+	29	30	TTGTAGCCGAGTATCTAATTAACAAG	GCGACAGAGTGAGTCTTTG	360
ATM	+	30	31	GAGATGCTGAACAAAAGGACTTC	CCAAATAAACATAAAACACTCAAATCC	354
ATM	+	31	32	TGGCTTACTTTAAAATTATTTCTCTCC	CCAATGTGCCTGGCCTAC	364
ATM	+	32	33	TCACAGGCTTAACCAATACGTG	CAGGAAGAGTTCAGGGTGAGA	370
ATM	+	33	34	TTTCTCTGTTGTACACATATTGCTAATC	ACGCAGCGCATGACTGTA	340
ATM	+	34	35	TCGGCCTTAAGGTTAATTCCTG	TGTGTGAAGTATCATTTCTCCATGA	362
ATM	+	35	36	TGGTGTACTTGATAGGCATTGA	TCTGAGCTTTTCCACACTGC	377
ATM	+	36	37	AGGAAAGGTACAATGATTTCCACT	TGAAGATGATGTGCAGTATCACAG	321
ATM	+	37	38	ATATGTCAACGGGGCATGA	TGGGATTCCATCTTAAATCCAT	345
ATM	+	38	39	GGTACTGCCCCACCAGAACCT	CCTGAATATGGATTACTGCAAGG	360
ATM	+	39	40	TTCTGTTAAGCAGTCACTACCATTG	GGGAACAGGAGGCAAAATAA	351
ATM	+	40	41	TTGTTTGCCACCTTCATTAGTTT	GATGCTTAGTCCAGTAAGTAAATTCAG	319
ATM	+	41	42	GAGTTGGGAGTTACATATTTGGTAATG	CCACATTGCTTCGTGTTTAT	321
ATM	+	42	43	CTGTGGTGGAGGGAAGATGT	TGGCTGTGTAATAATATCCACCAA	323
ATM	+	43	44	CACCACACCCAGCTGATATTT	TGTTGTTTAGAATGAGGAGAGAGG	353
ATM	+	44	45	TTTGTCCTTTGGTGAAGCTATTT	ACATCTGATCACAGCCACCA	324
ATM	+	45	46	AGTAGCAAGCCTATGATGAGAAC	GCTAGGATTACAGGCATGAGC	340
ATM	+	46	47	TCATTTCTCTTGCTTACATGAACCTC	AAAGGAAAGTCAAGAGGTAAGATGAC	324
ATM	+	47	48	GTGGGGAGATGTCATGCAG	CAGTAAACACTAATCCAGCCAAT	364
ATM	+	48	49	GGGCAGTTGGGTACAGTCAT	TTTCAAAATTTGGCTGCTTTG	368
ATM	+	49	50	ATTTAAATTTGGTTGTGTTTCTTGA	ATATATAGTTAAGCCGACCTTTAGAGC	376
ATM	+	50	51	CCCTTTATAATCCTTAGAAGTTTGC	GGGTAGAATATTGGGCTGAGTAAC	380
ATM	+	51	52	TGAATCCAGTTTAAATTTAGGACCAC	GAAATCCTAGGCCCTCCCATC	333
ATM	+	51	53	GAATGGGACCAAGATGATG	TGCAGTGGGTAGAGCGTGTA	273
ATM	+	52	54	TGTTAAGCAAAATGAAAAATATGGA	CCAGCCTTGAACCCGATTTTA	348
ATM	+	53	55	GAGAAGTTTAAATGTTGGGTAGTTCC	AAAGTAACCCAGGGAATGCTGAA	355
ATM	+	54	56	CAATCAGAGCCTGAACCACA	GGCTTGGGCAAGGAAATA	370
ATM	+	55	57	TCACATCGTCATTTGTTTCTCTG	AAGACAAAATCCCAATAAAGCAG	350
ATM	+	56	58	ATTGTTTGAGTGCCTTTG	CTGAGGCTGGTCTCGAACTC	358
ATM	+	57	59	GCTGAATGATCATCAAAATGCTCT	TTTGACAAATTACCTGATGAAATTAAG	336

ATM	+	58	60	TGCTTCCCTGTCCAGACTGT	TGCCAAACAACAAAAGTGCTC	366
ATM	+	59	61	AACTCAACATGGCCGGTTA	TTGGTAGGCAACAACATTCC	320
ATM	+	60	62	CATGTGGTTTCTTGCCCTTG	ACTCAAACTCCTGGGCTCAA	348
ATM	+	61	63	AACGTCTAATGAAAGCCCACTC	TGAAGCAGTGTCTTTCACATC	326
ATM	+	62	64	GGAACATGAAGTGTGCATGA	CACAAAGGTTTCAGTGAGGTGA	377
ATM	+	63	65	GCAGATGACCAAGAATGCAA	TCTAAAGGCTGAATGAAAGGGTA	361
NOTCH1	-	33	1	CCGTAGATGACCTGGGTGAG	CGAGACCCGTGGGTGCTAG	338
NOTCH1	-	34	2	ACTGTGCTTCTCTCTGGTGA	CTTGAGGCTGCCCAGGTA	358
NOTCH1	-	34	3	AGGCTGCTGGACGAGTACAA	CACGGACGGAGACTGCTG	357
NOTCH1	-	34	4	CCATGGCTACCTGTCTCAGACG	TATTGGTTCGGCACCCTATGC	351
NOTCH1	-	34	5	GAATGGTCAATGCGAGTGG	CTGAGCTCACGCCCAAGGT	349
NOTCH1	-	34	6	TGCAGCCACAAAACCTTACAGA	TGTCCACAGGGGAGGAGTAG	333
NOTCH1	-	34	7	CTGCCATCCTCGCTGGT	ATCCACAGAGCGCACACA	335
SF3B1	-	10	1	GTGCAAAATATTGTTCAATTATGCTGT	TGTTAAGGGAAGTTGAAATGTTATGA	345
SF3B1	-	11	2	TCATAACATTTCAACTTCCCTTAACA	ACATGGCCAGGTGCAGAG	331
SF3B1	-	12	3	TGGAAAATCTTCTTTGAGTAATTTG	GTGCAAGGAAAAAGGTCTAGGA	364
SF3B1	-	13	4	TCTTAAACAGTTCGTCCCTTGA	GTAGCCAGACCAGCAGCCTA	360
SF3B1	-	14	5	CCAACTCATGACTGTCTCTTCT	CATAGTAAGACCCCTGTCTCTTAAAGA	369
SF3B1	-	15	6	TGAGAGAACTGGATGATATTGTGT	TTCAGAAAGCAGCCCAACC	336
SF3B1	-	16	7	GCAACTCCTTATGGTATCGAATC	GAACCATGAAACATATCCAGTTTACA	369
TP53	-	4	1	ACCTGGTCCTCTGACTGCTC	CAGGCATTGAAGTCTCATGG	361
TP53	-	5	2	CACCTTGCGCCCTGACTTTCA	CACTCGGATAAGATGCTGAGG	343
TP53	-	6	3	CAGATAGCGATGGTGAGCAG	TTGCACATCTCATGGGGTTA	335
TP53	-	7	4	GCACTGGCCTCATCTTGG	AAGAGGTCCCAAGGCCAGAG	334
TP53	-	8	5	GGACAGGTAGGACCTGATTTC	TCTCCATCCAGTGGTTTCTTC	346
TP53	-	9	6	AAAGGGAGCCCTCACCCAC	TGCTTTTGAGGCATCACTGC	342
TP53	-	10	7	GCTGTATAGGTACTTGAAGTGCAG	CTGCCCTTGACCATGAAGG	349
TP53	-	11	8	AGGGAAAAGGGGCACAG	CCCCACAACAAAACACCAGT	339
FBXW7	-	8	1	TTACTGTTCCCTGTTTATGCGTTCA	TGAGCTCTGATATTGCTATCCA	341
FBXW7	-	9	2	TCTGAAGAGGCCAAACAATAAATAAG	GGCCCCAAATTCACCAATAATAG	360

<i>FBXW7</i>	-	10	3	TTGACTAAATCTACCATGTTTCTCA	TTCATCAGGAGAGCATTTAAGG	359
<i>FBXW7</i>	-	11	4	GGTCAGTAATTGATAGGAAGAGTATCC	TGATGCTAAGGCTCCATATTTTC	349
<i>FBXW7</i>	-	12	5	AACTCATAGCCATTATTTCTAACCCAG	GGAGTATATCGTCTACACAATTGGA	369
<i>MYD88</i>	+	4	1	GCCCTTCTGAAAGCTATTCC	CCTGGCACACATTCACTCAC	347
<i>MYD88</i>	+	5	2	GTTGAAGACTGGGCTTGTC	GAAGTTGGCATCTCCAGGAA	354
<i>XPO1</i>	-	14	1	GCTCATTATTTGCATTTGAAACC	CTTTGCCTCTTTTCTGTTACAT	343
<i>XPO1</i>	-	15	2	TGTGAACAGAAAAGAGGCAAAAG	CCTGAACCTGAACGAAATGC	355
<i>BIRC3</i>	+	2	1	AAGCAAAGCCCATGCACAAA	CGAATCTGCAGCTAGGATACAA	356
<i>BIRC3</i>	+	2	2	GTGGCCTGATGCTGGATAAC	GTCATGGCCCATGTCTGAAA	340
<i>BIRC3</i>	+	2	3	CCACTGTGCAATGAATAACGA	CTTGCAAGCTGCTCAGGATT	348
<i>BIRC3</i>	+	3	4	AGCATGCAGACACATGCAG	CAAGAATTTGCAAAGAGAATTTCA	340
<i>BIRC3</i>	+	4	5	TGATTGAGGCAAAACAACCA	CTTTCTAAATCATGTCAGGTTTGAT	351
<i>BIRC3</i>	+	5	6	CCTTAATGAAAATGGGTTTGT	TTCAATATTAAACAGTACCAAATGACTC	360
<i>BIRC3</i>	+	6	7	CCAAGTTGTTATTTAATGACTGAACG	TCTATAATTCTCTCCAGTTGCTAGGAT	349
<i>BIRC3</i>	+	6	8	GCAATCATGATGAATACTCCTGT	TTGAATTCAGAAATACTAAACATGAAAA	346
<i>BIRC3</i>	+	7	9	GGAAGTTTGTGAGCAGAGTTTG	AAGAGCGTATTTTCAATTGACTTAGA	367
<i>BIRC3</i>	+	8	10	CCTTGAAATGAGTATTTGGCTA	CCACAAGGAATAAACACTATGGA	358
<i>BIRC3</i>	+	9	11	GAAGAAAGAACAATGTAAGTGTGTATG	CAAAAGCCATATCATTTCTTTAGTTTC	359

Table F.**A: PCR amplification protocol for *ATM*.**

Cycles	Time (min)	Temperature (°C)
1	05:00	95.0
10	00:30	95.0
	00:30	63.0 → 58.0
	00:30	72.0
28	00:30	95.0
	00:30	58.0
	00:30	72.0
1	07:00	72.0

B: PCR amplification protocol for the remaining genes.

Cycles	Time (min)	Temperature (°C)
1	10:00	95.0
10	00:30	95.0
	00:30	63.0 → 58.0
	00:30	72.0
25	00:30	95.0
	00:30	58.0
	00:30	72.0
1	07:00	72.0

Table G. Median frequency of reads generated by next-generation sequencing (NGS).

Gene	Median of reads generated by NGS
<i>ATM</i>	890 (151-6608)
<i>TP53</i>	1037(263-3743)
<i>NOTCH1</i>	635 (90-2314)
<i>SF3B1</i>	951 (224-3565)
<i>MYD88</i>	962.5 (297-3008)
<i>FBXW7</i>	1050 (233-3777)
<i>XPO1</i>	945.5 (163-2635)
<i>BIRC3</i>	1116 (84-4130)

Figure A. A. Time to first therapy (TFT) and B. Overall survival (OS) of the global series of 197 CLL patients with 11q deletion.

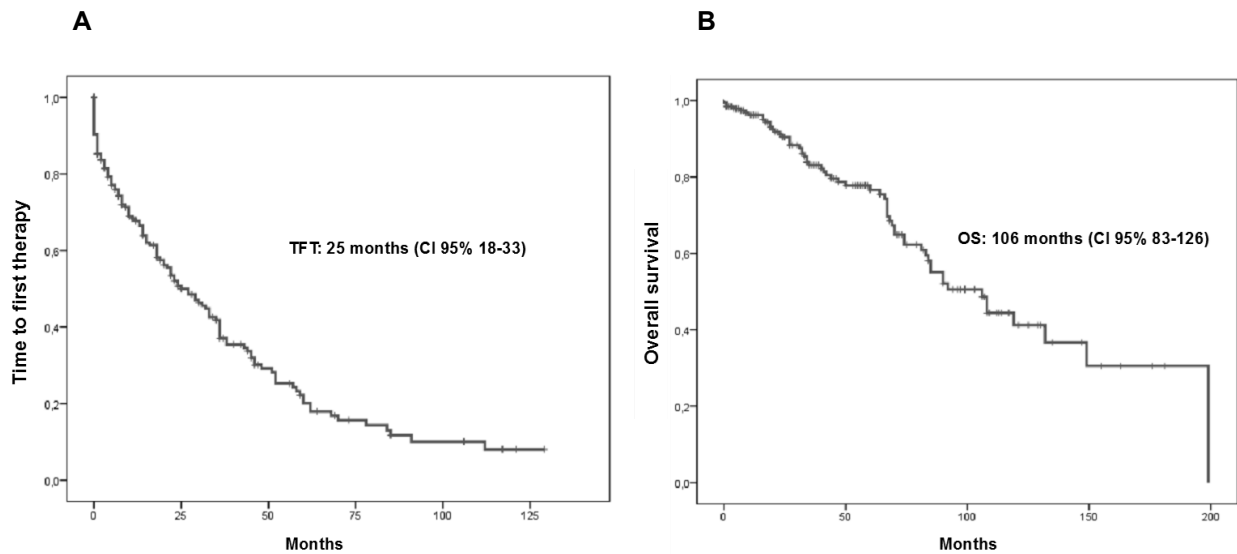


Figure B. A. Time to first therapy (TFT) and B. Overall survival (OS) of patients with CLL and 11q deletion and a percentage of FISH losses <40%, 41-59% or ≥60%.

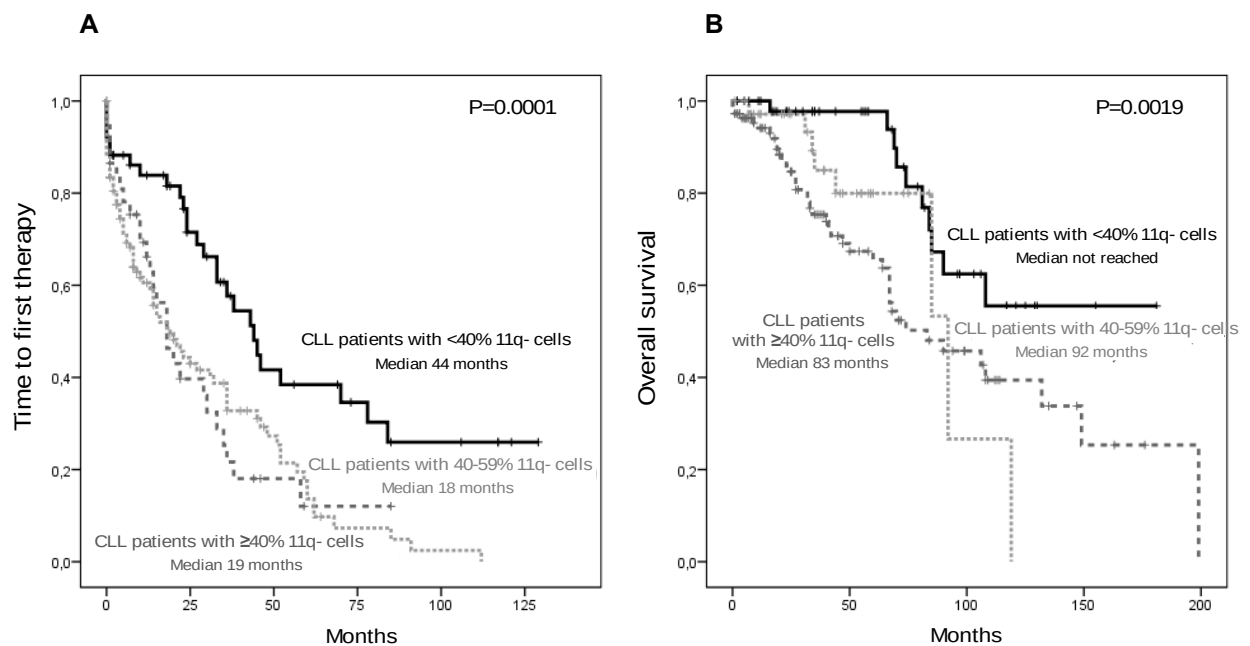
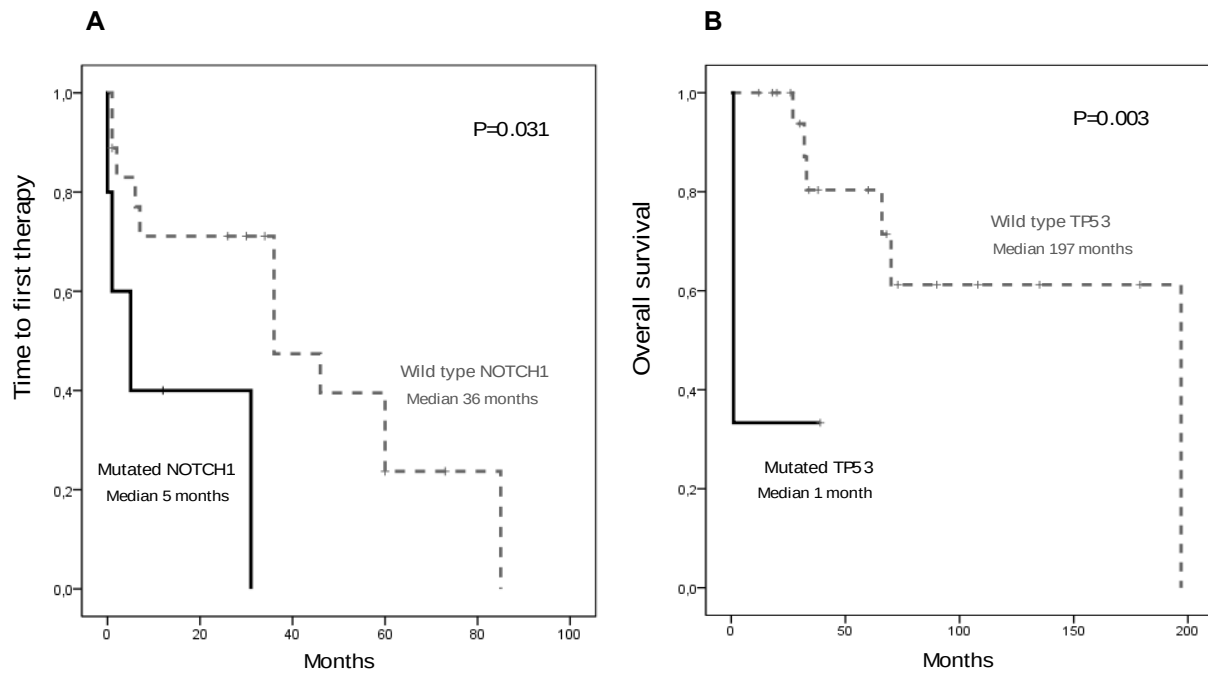


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