UNIVERSIDAD DE SALAMANCA

DEPARTAMENTO DE MEDICINA

HEMATOLOGÍA

TESIS DOCTORAL

Caracterización molecular de la Macroglobulinemia de Waldenström: Implicaciones en el diagnóstico, pronóstico y transformación histológica

Cristina Jiménez Sánchez

Salamanca, 2017

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Tutor: Marcos González Díaz

La presente tesis doctoral corresponde a un compendio de 4 trabajos previamente publicados o aceptados para publicación, que se detallan a continuación:

1. **MYD88 L265P is a marker highly characteristic of, but not restricted to, Waldenström's macroglobulinemia**
   Cristina Jiménez, Elena Sebastián, Mª del Carmen Chillón, Pilar Giraldo, José Mariano Hernández, Fernando Escalante, Tomás González-López, Carmen Aguilera, Alfonso García de Coca, Ilda Murillo, Miguel Alcoceba, Ana Balanzategui, Mª Eugenia Sarasquete, Rocío Corral, Luis A. Marín, Bruno Paiva, Enrique M. Ocio, Norma C. Gutiérrez, Marcos González, Jesús F. San Miguel and Ramón García-Sanz.
   Leukemia. 2013 Aug;27(8):1722-8. doi: 10.1038/leu.2013.62.

2. **Detection of MYD88 L265P Mutation by Real-Time Allele-Specific Oligonucleotide Polymerase Chain Reaction**
   Cristina Jiménez, María del Carmen Chillón, Ana Balanzategui, Noemí Puig, Elena Sebastián, Miguel Alcoceba, María E. Sarasquete, Isabel P. Conde, Rocío Corral, Luis A. Marín, Bruno Paiva, Montserrat Ruano, Alicia Antón, Rebeca Maldonado, Jesús F. San Miguel, Marcos González and Ramón García-Sanz.
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3. **Unraveling the heterogeneity of IgM monoclonal gammopathies: a gene mutational and gene expression profiling study**
   Cristina Jiménez, Mª Isabel Prieto-Conde, María García-Álvarez, Miguel Alcoceba, Fernando Escalante, Mª Carmen Chillón, Alfonso García de Coca, Ana Balanzategui, Alberto Cantalapiedra, Carlos Aguilar, Rocío Corral, Tomás González-López, Luis A. Marín, Abelardo Bárez, Noemí Puig, Arancha García-Mateo, Norma C. Gutiérrez, Mª Eugenia Sarasquete, Marcos González, Ramón García-Sanz.
   Br J Cancer, en revisión

4. **From Waldenström's macroglobulinemia to aggressive diffuse large B-cell lymphoma: a whole-exome analysis of abnormalities leading to transformation**
   Cristina Jiménez*, Sara Alonso-Álvarez*, Miguel Alcoceba, Gonzalo R. Ordóñez, María García-Álvarez, Mª Isabel Prieto-Conde, Mª Carmen Chillón, Ana Balanzategui, Rocío Corral, Luis A. Marín, Norma C. Gutiérrez, Noemí Puig, Mª Eugenia Sarasquete, Marcos González, and Ramón García-Sanz.
   Blood Cancer J, en revisión

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CERTIFICAN:

Que el presente trabajado titulado "**Caracterización molecular de la Macroglobulinemia de Waldenström: Implicaciones en el diagnóstico, pronóstico y transformación histológica**" y realizado por D.ª Cristina Jiménez Sánchez bajo su dirección en el Departamento de Medicina, reúne, a su juicio, las condiciones de originalidad y calidad científica requeridas para su presentación y defensa para optar al grado de Doctor en Medicina por la Universidad de Salamanca.

Y para que así conste a los efectos oportunos, firmamos el presente certificado

En Salamanca, a 30 de mayo de 2017

Dr. Ramón García Sanz   Dra. Mª Eugenia Alonso Sarasquete   Prof. D. Marcos González Díaz

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Abreviaturas

2-CdA	Cladribina
ABC	Célula B activada (Activated-B cell)
Akt	Protein kinase B
ARID1A	AT-rich interactive domain-containing protein 1A
ARID1B	AT-rich interactive domain-containing protein 1B
ASO-RQ-PCR	Reacción en cadena de la polimerasa cuantitativa en tiempo real específica de alelo (Allele-specific oligonucleotide real-time quantitative polymerase chain reaction)
ATM	Ataxia telangiectasia mutated serine/threonine kinase
ATXN1	Ataxin 1
B2M	β_2-microglobulina
β-arr	β-arrestinas
BCL2	B-cell lymphoma 2
BCR	Receptor de la célula B (B-cell receptor)
BLIMP1/PRDM1	B-lymphocyte-induced maturation protein 1
BLNK	B-cell linker
BTK	Tirosina quinasa de Bruton (Bruton's tyrosine kinase)
CARD11	Caspase recruitment domain-containing protein 11
CART	Chimeric antigen receptor T-cell
CCND3	Ciclina D3
CD	Cluster of differentiation
CD79A	B-cell antigen receptor complex-associated protein alpha chain
CD79B	B-cell antigen receptor complex-associated protein beta chain
CDR	Región determinante de la complementariedad (Complementarity-determining region)
CGH	Hibridación genómica comparada (Comparative genomic hybridization)
CELSR2	Epidermal growth factor-like protein 2
CXCR4	C-X-C motif chemokine receptor 4
DNTT	Deoxyribonucleic acid nucleotidylexotransferase
EMA	Agencia Europea de Medicamentos (European Medicines Agency)
EMR	Enfermedad mínima residual
ERK	Extracellular signal-regulated kinase
FAM135B	Family with sequence similarity 135 member B

FDA Administración de medicamentos y alimentos (Food and drug administration)

FISH Hibridación *in situ* fluorescente (Fluorescence *in situ* hybridization)

FMOD Fibromodulin

FRYL Furry homolog-like transcription coactivator

GEP Perfil de expresión génica (Gene expression profiling)

GMSI Gammapatía monoclonal de significado incierto

GPSM2 G-protein signaling modulator 2

GRK G protein-coupled receptor kinases

HAT Histona acetiltransferasa

HCK Hematopoietic cell kinase

HCLS1 Hematopoietic cell-specific lyn substrate 1

HDAC Histona deacetilasa

HIST1H1 Histone cluster 1 H1 family member

HIST1H13B Histone cluster 1 H3 family member B

HMS Hipermutación somática

HMT Histona metiltransferasa

HNF1B Hepatocyte nuclear factor 1-beta

Ig Inmunoglobulina

IGFN1 Immunoglobulin-like and fibronectin type III domain containing 1

IGH Gen de la cadena pesada de las inmunoglobulinas (Immunoglobulin heavy locus)

IGHV Región variable del gen de la cadena pesada de las inmunoglobulinas (Immunoglobulin heavy variable)

IL Interleucina

IL-6 Interleucina-6

IPSS Sistema pronóstico internacional (International prognostic scoring system)

IRAK1 Interleukin 1 receptor associated kinase 1

IRAK4 Interleukin 1 receptor associated kinase 4

IRF3 Interferon regulatory factor 3

ITAM Dominio de activación (Immunoreceptor tyrosin-based activation motif)

JAK Janus kinase

LAM Leucemia aguda mieloblástica

LAPTM5 Lysosomal protein transmembrane 5

LBDGC	Linfoma B difuso de célula grande
LCM	Linfoma de células del manto
LDH	Lactato deshidrogenasa
LEF1	Lymphoid enhancer binding factor 1
LF	Linfoma folicular
LLC	Leucemia linfática crónica
LLP	Linfoma linfoplasmocítico
LNH	Linfoma no-Hodgkin
LOH	Pérdida de heterocigosidad (Loss of heterozygosity)
LYN	Lck/Yes-related novel protein tyrosine kinase
LZM	Linfoma de la zona marginal
MAPK	Mitogen-activated protein kinase
MARCKS	Myristoylated alanine rich protein kinase C substrate
MEK1/MAP2K1	Mitogen-activated protein kinase kinase 1
MLL2	Lysine methyltransferase 2D
MM	Mieloma múltiple
MO	Médula ósea
mTOR	Mammalian target of rapamycin
MW	Macroglobulinemia de Waldenström
MWA	Macroglobulinemia de Waldenström asintomática
MWS	Macroglobulinemia de Waldenström sintomática
MYBBP1A	V-Myb avian myeloblastosis viral oncogene homolog binding protein 1A
MYD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Secuenciación de nueva generación (Next generation sequencing)
NSV	Variantes no sinónimas codificantes (Nonsynonymous variant)
P38/MAPK14	Mitogen-activated protein kinase 14
PCR	Reacción en cadena de la polimerasa (Polymerase chain reaction)
PD-1/PD-L1	Programmed cell death 1/Programmed death-ligand 1
PER3	Period circadian clock 3
PI3K	Phosphatidylinositol 3-kinase
PI3Kδ	Phosphatidylinositol 3-kinase delta isoform
PIM1	Proviral integration site 1 oncogene
PKCβ	Protein kinase C beta

PLCγ2	Phospholipase C gamma 2
PTPRD	Protein tyrosine phosphatase receptor type D
RAG1	Recombination activating 1
RAG2	Recombination activating 2
SDF-1	Stromal cell-derived factor 1
Ser/S	Serina
SLP	Síndrome linfoproliferativo
SMD	Síndrome mielodisplásico
SNP	Polimorfismo de nucleótido único (Single nucleotide polymorphism)
Src	V-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
STAT	Signal transducers and activators of transcription
SWI-SNF	Switch/Sucrose non fermentable
Syk	Spleen tyrosine kinase
TAP2	Antigen peptide transporter 2
Thr/T	Treonina
TLR	Receptor tipo Toll (Toll-like receptor)
TNF	Factor de necrosis tumoral (Tumor necrosis factor)
TNFAIP3/A20	Tumor necrosis factor alpha induced protein 3
TNFRSF14	Tumor necrosis factor receptor superfamily member 14
TNIP1	Tumor necrosis factor alpha induced protein 3 interacting protein 1
TP53	Tumor protein P53
TPH	Trasplante de progenitores hematopoyéticos
TRAF	Tumor necrosis factor receptor associated factor
TRAF2	Tumor necrosis factor receptor associated factor 2
TRAF3	Tumor necrosis factor receptor associated factor 3
UCHL5	Ubiquitin carboxyl-terminal hydrolase L5
USP14	Ubiquitin specific peptidase 14
VCAM1	Vascular cell adhesion molecule 1
V(D)J	Variable (diversity) functional joining
VSG	Velocidad de sedimentación globular
WHIM	Verrugas, hipogammaglobulinemia, infecciones y mielocatexis (Warts, hypogammaglobulinemia, infections, and myelokathexis)
WNK1	Protein kinase with no lysine 1
ZFHX4	Zinc finger homeobox 4

Introducción

1. Macroglobulinemia de Waldenström

1.1. Descubrimiento y definición

La macroglobulinemia de Waldenström (MW) es un síndrome linfoproliferativo (SLP) B poco común caracterizado por infiltración de la médula ósea (MO) por células linfoplasmocíticas y producción de una inmunoglobulina (Ig) monoclonal tipo IgM.[1] Forma parte del grupo de enfermedades denominadas gammapatías monoclonales, en el que también se incluyen el mieloma múltiple (MM) y la gammapatía monoclonal de significado incierto (GMSI). Su nombre deriva de Jan Gosta Waldenström, célebre médico sueco que describió por primera vez dos pacientes con hemorragia de la mucosa oral, epistaxis y linfadenopatía generalizada. Las pruebas de laboratorio de estos pacientes revelaron anemia normocrómica, trombocitopenia, elevación de la velocidad de sedimentación globular (VSG) y disminución del fibrinógeno y la albúmina. Waldenström observó dos diferencias cruciales entre esta afección y el MM: en primer lugar, sus pacientes no presentaban ni dolor ni lesiones óseas y, en segundo lugar, la MO estaba infiltrada por células linfocíticas más que plasmocíticas.[2] Las observaciones preliminares de Waldenström se incluyen en los criterios actuales de diagnóstico para la MW, definidos en el panel consenso que se estableció en la 2ª Reunión Internacional de MW (Atenas, 2002). De acuerdo con ellos, la MW se define por la presencia de una IgM monoclonal (pico M) de cualquier grado, junto con afectación de la MO por infiltrado linfoplasmacítico con patrón predominantemente intertrabecular. Debe considerarse como una entidad clinicopatológica distinta y no como un síndrome clínico secundario a la secreción de IgM.[3]

Para un diagnóstico preciso, es necesario descartar otras neoplasias linfoides de células B pequeñas en las que también puede haber cierta diferenciación plasmocítica, en especial, la leucemia linfática crónica (LLC), los linfomas de la zona marginal (LZM) y el linfoma linfoplasmocítico (LLP) 'puro'. Este último se asocia a paraproteína monoclonal en 2/3 de los casos, pero para no ser etiquetado como MW el componente monoclonal tiene que ser de tipo IgG o IgA y/o carecer de infiltración de la MO.[4] También es fundamental diferenciar la MW de la GMSI tipo IgM (GMSI-IgM), y dentro de los casos con criterios de MW, se debe distinguir entre los que tienen y los que no tienen sintomatología, ya que estos últimos no requieren tratamiento (MW asintomática, MWA).[3] Se considera que los pacientes tienen GMSI si presentan gammapatía monoclonal IgM pero no hay evidencia morfológica de infiltración medular ni extramedular por linfoma. Los pacientes con MW

pueden considerarse asintomáticos si no presentan características clínicas atribuibles a la infiltración tumoral, como por ejemplo, síntomas constitucionales, citopenias u organomegalia, y/o síntomas causados por la proteína monoclonal, como síndrome de hiperviscosidad, crioglobulinemia, amiloidosis o fenómenos autoinmunes tales como neuropatía periférica y enfermedad por aglutininas frías.[5,6] Por último, existen algunos pacientes con características clínicas atribuibles a la proteína monoclonal IgM, pero sin evidencia manifiesta de linfoma, y que se consideran como un grupo clínico distinto denominado 'trastornos asociados a IgM'.[3] Todos estos criterios se resumen en la Tabla 1.

1.2. Epidemiología y predisposición (MW familiar)

La MW es una enfermedad rara, con una tasa de incidencia global ajustada por edad de 3,8 casos por millón de habitantes al año en Estados Unidos,[7] y 3,1 casos por millón en España.[8] Representa el 3% de todas las gammapatías monoclonales (17% de las gammapatías IgM), el 2% de los linfomas y el 6% de los SLP-B leucémicos.[7] Afecta con mayor frecuencia a los hombres (2 veces más que a las mujeres), de raza blanca (2-3 veces más que a los de raza negra) y edad avanzada, siendo la mediana de edad en el momento del diagnóstico entre 63 y 68 años.[9,10]

Tabla 1. Criterios establecidos en la 2ª Reunión Internacional de MW para el diagnóstico diferencial de la MW y entidades relacionadas. Adaptada de Owen et al., *Semin Oncol* 2003.

		Proteína monoclonal IgM[a]	Infiltración de la médula ósea[b]	Síntomas por IgM	Síntomas por infiltración tumoral
MW	Sintomática	+	+	+	+
	Asintomática	+	+	-	-
Trastornos asociados a IgM[c]		+	-	+	-
GMSI-IgM		+	-	-	-
Linfoma linfoplasmocítico[d]		+/-	+/-	+/-	+/-

[a]Aunque no se ha definido una concentración de IgM para distinguir GMSI de MW, es importante tener en cuenta que en la GMSI nunca o rara vez excede los 3 g/dL.
[b]En ocasiones, en ausencia de evidencia morfológica de infiltración de la MO, se pueden detectar pequeñas poblaciones de células linfoides B clonales en pacientes asintomáticos con técnicas más sensibles como el inmunofenotipo o la biología molecular. Alternativamente, los pacientes pueden tener infiltrados equívocos de MO sin estudios fenotípicos confirmatorios. Se considera que estos pacientes deben ser clasificados como GMSI o enfermedad relacionada con IgM hasta que haya más datos disponibles.
[c]Estos pacientes suelen presentarse con crioglobulinemia sintomática, amiloidosis o fenómenos autoinmunes como neuropatía periférica y enfermedad por aglutininas frías.
[d]Pacientes que aun teniendo una lesión extramedular con diagnóstico histológico de LLP, no cumplen los criterios de MW (carecen de componente IgM y/o infiltración medular). Al igual que la MW y otros linfomas indolentes, pueden carecer de síntomas derivados de infiltración o del componente M (si lo tuvieran).

Los factores genéticos pueden ser importantes para la patogénesis de la MW. Varios estudios han demostrado un agrupamiento familiar de MW y otros SLP-B o neoplasias malignas, lo que sugiere una predisposición común. Los casos familiares suelen ser diagnosticados una década antes que los que se presentan esporádicamente y los parientes de primer grado de los pacientes con MW/LLP tienen un riesgo 20 veces mayor de desarrollar este trastorno, como indica un gran estudio poblacional sueco.[11–13] Se ha propuesto que la sobreexpresión de la proteína Bcl-2 (*B-cell lymphoma 2*), que bloquea la muerte celular, así como la presencia de algunas variantes germinales (*Lysosomal Protein Transmembrane 5*, *LAPTM5*, C403T y *Hematopoietic Cell-Specific Lyn Substrate 1*, *HCLS1*, G496A) o genes de susceptibilidad en los cromosomas 1q, 3q, 4q y 6q podrían contribuir potencialmente a la predisposición genética en la MW familiar.[14–16] No obstante, es necesario ampliar los estudios para confirmar estos hallazgos. Además, la predisposición familiar en MW se ha asociado con una peor respuesta al tratamiento y supervivencia libre de progresión, con la notable excepción de los regímenes que contienen bortezomib.[17,18] Estos datos apoyan la teoría de que la susceptibilidad genética predispone a los pacientes a una forma más grave de la enfermedad.

1.3. Origen

El análisis de la naturaleza y distribución de las mutaciones somáticas en las regiones variables de las cadenas pesada y ligera de las Igs en los pacientes con MW mostró un alto índice de mutaciones con cambio de aminoácido en las regiones CDR (región determinante de la complementariedad, por sus siglas del inglés *Complementarity-determining region*), al compararlas con los genes más cercanos de la línea germinal, y sin que existiera variación intraclonal.[19–21] Por ello, se cree que la célula clonogénica de la MW deriva de un linfocito B post-centro germinal que ha sufrido el proceso de hipermutación somática (HMS), pero no el cambio de isotipo (Figura 1), y que muestra preferencia por ciertos segmentos VH en el reordenamiento de los genes V(D)J (*Variable*, *Diversity* y *functional Joining*) de la región variable de la cadena pesada de las Igs, como VH3-23.[21–25]

Además, los cambios genéticos entre diagnóstico y progresión (evolución clonal) y la aparición de subclones cada vez menos secretores, más proliferativos y resistentes al tratamiento sugieren que la MW podría originarse en un proceso multifásico de transformación neoplásica de la

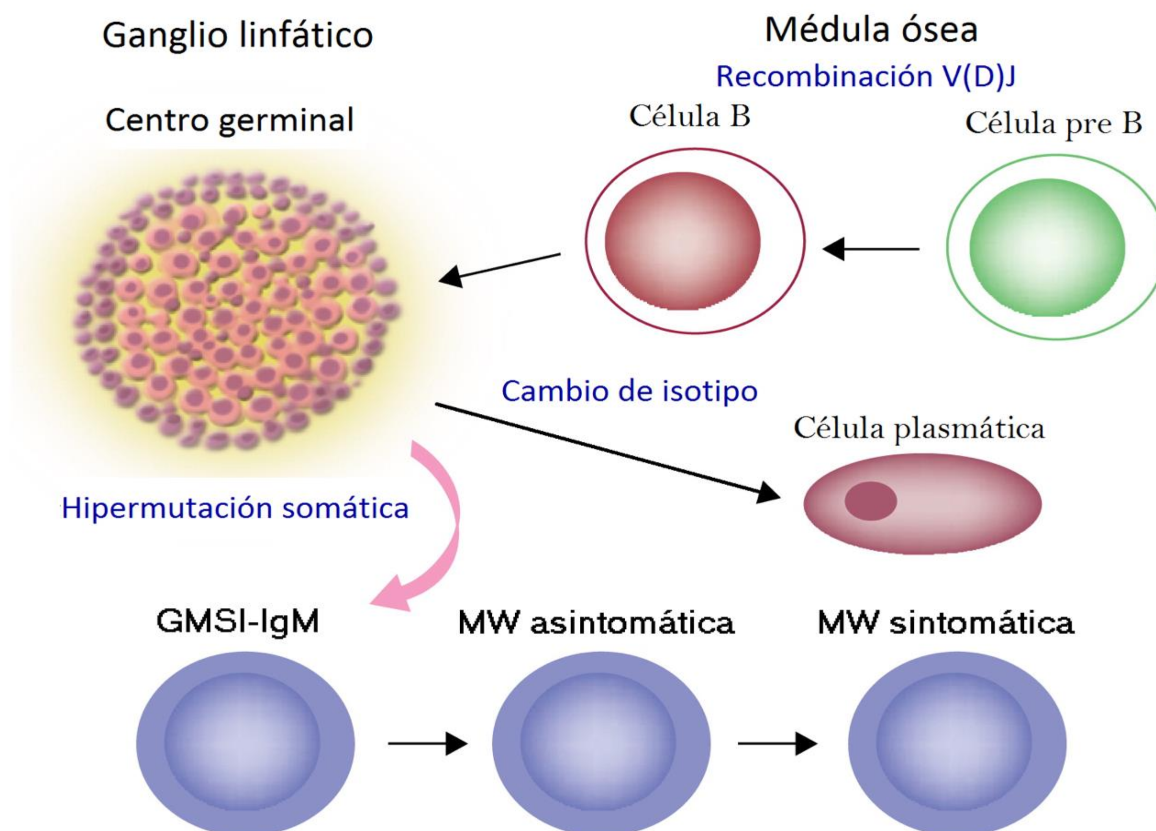


Figura 1. Maduración normal de las células B (modificada de Ghobrial et al., *Lancet Oncol* 2003). Se cree que la célula clonogénica de la MW es un linfocito B que ha sufrido hipermutación somática pero que es incapaz de llevar a cabo el proceso de cambio de clase.

GMSI-IgM por acumulación de fenómenos oncogénicos secuenciales, como comentaremos más adelante.[26]

1.4. Clínica

Hasta una cuarta parte de los pacientes con MW son asintomáticos en el momento del diagnóstico, y sólo el 40-70% de ellos desarrollarán síntomas en un periodo de 3 a 10 años.[8]

1.4.1. Síntomas iniciales

Las manifestaciones clínicas iniciales son típicamente vagas e incluyen síntomas inespecíficos de fatiga, fiebre, malestar y pérdida de peso. El primer síntoma más frecuente es astenia progresiva, generalmente debida a anemia, seguido de manifestaciones hemorrágicas, neurológicas y adenopáticas. Un 10-20% de los casos presentan manifestaciones iniciales inusuales: respiratorias, oculares, insuficiencia renal, síntomas cutáneos, etc. El diagnóstico

también puede ser casual, al observar un proteinograma con componente monoclonal o una VSG elevada en un estudio realizado por cualquier motivo. Por último, un 10% de las MW provienen de una GMSI-IgM previamente diagnosticada.

1.4.2. Manifestaciones clínicas

Las manifestaciones clínicas de la MW pueden clasificarse en las relacionadas con el crecimiento e infiltración directa del tumor y en las relacionadas con la cantidad y propiedades específicas (agregación, polimerización, crioprecipitación y actividad autoinmune) de la paraproteína IgM circulante y su depósito en los tejidos (Tabla 2).

A. Manifestaciones clínicas dependientes del crecimiento o infiltración tumoral

Los síntomas asociados con la infiltración de órganos o del tejido hematopoyético incluyen citopenias (comúnmente anemia normocítica y normocrómica), linfadenopatía o hepatoesplenomegalia en un 20-25% de casos, y, menos frecuentemente, nódulos pulmonares, infiltrados o derrame pleural, hemorragia de la mucosa gastrointestinal, diarrea y síndrome de Bing-Neel.[27–31] El síndrome de Bing-Neel se define por infiltración del sistema nervioso central, presentando gran variedad de manifestaciones neurológicas progresivas, incluyendo cefalea, confusión, convulsiones e incluso coma.[31–34]

B. Manifestaciones clínicas debidas a la paraproteína monoclonal

Hasta un tercio de los pacientes puede manifestar síntomas de hiperviscosidad, atribuibles a los grandes pentámeros de IgM que se confinan principalmente en el compartimento intravascular.[8,35] Entre ellos se incluyen sangrado mucocutáneo, trastornos visuales, cefalea, mareos, ataxia, sordera y, en raras ocasiones, deterioro cognitivo y alteración del estado mental. Además, el examen oftalmoscópico puede revelar un fondo de ojo anormal con venas retinianas distendidas y tortuosas o hemorragias retinianas.[36]

La crioglobulinemia debida a la IgM se observa en el 10-20% de los pacientes, aunque menos del 5% tienen manifestaciones clínicas. Los síntomas se deben a la precipitación en los capilares cutáneos por bajas temperaturas: fenómeno de Raynaud, necrosis acra (oreja, nariz, dedos) y púrpura vascular en extremidades inferiores.[37,38]

Tabla 2. Características de las morbilidades relacionadas con la inmunoglobulina M o la infiltración directa de las células de la MW. Adaptada de Kapoor et al., *Blood Rev* 2015.

Fisiopatología		**Característica clínica**
Por infiltración tumoral	Médula ósea (100%)	Anemia Trombocitopenia Neutropenia
	Tejidos hematopoyéticos extramedulares (25%)	Linfadenopatía Hepatomegalia Esplenomegalia
	Pulmonar (4%)	Nódulos o infiltrados difusos Derrame pleural
	Sistema nervioso central (raro)	Síndrome de Bing-Neel
Por infiltración tumoral y paraproteína monoclonal	Renal (4%)	Infiltración de células neoplásicas Masa perineal Proteinuria de Bence Jones
	Gastrointestinal (4%)	Malabsorción Diarrea Sangrado de la mucosa
	Cutánea (3%)	Lesiones cutáneas Síndrome de Schnitzler
Por paraproteína monoclonal	Hiperviscosidad (35%)	Sangrado Fondo de ojo anormal Síntomas visuales Cefaleas Estado mental alterado (raro) Derrame cerebral (raro)
	Neuropatía por IgM (hasta el 40%)	Típicamente periférica, simétrica y sensorial, con vibración pronunciada y pérdida de pinchazos, ataxia y temblor leves
	Crioglobulinemia (asintomática hasta en el 15% de casos; sintomática en ≤5%)	Fenómeno de Raynaud Neuropatía periférica Púrpura Ulceración o necrosis cutánea Artralgia Hematuria por glomerulonefritis
	Anemia hemolítica por aglutinina fría (10%)	Anemia hemolítica (hemoglobina >7 g/dl normalmente)

La neuropatía periférica es la complicación neurológica más frecuente, y afecta hasta al 40% de los pacientes. Además de por depósito de la IgM, los mecanismos patogénicos de daño nervioso pueden deberse a infiltración de células tumorales en los nervios periféricos, existencia de

anticuerpos contra diversas glicoproteínas o glicolípidos de los nervios periféricos y al depósito de sustancia amiloide.[39–41]

Además, la IgM puede dar lugar a lesiones cutáneas y urticaria (síndrome de Schnitzler) por depósito intraepidérmico,[42,43] o producir diarrea y malabsorción cuando se deposita en el intestino.[44]

Por último, la paraproteína IgM puede funcionar como un autoanticuerpo y unirse a proteínas de: i) eritrocitos, causando anemia hemolítica autoinmune y fenómeno de Raynaud; ii) plaquetas y factor von Willebrand, simulando la púrpura trombocitopénica inmune o la enfermedad de von Willebrand adquirida, iii) membrana basal glomerular, produciendo glomerulonefritis inmune.[8,45–47]

1.5. Pronóstico

La MW tiene un curso crónico y progresivo, con una supervivencia media superior a 5 años y con al menos un 30% de pacientes vivos a los 10 años del diagnóstico. No obstante, su evolución es variable. Hay casos que permanecen estables durante años y otros con comportamiento clínico agresivo y supervivencia inferior a 1 año.[8,48,49] Para determinar el pronóstico (en los casos sintomáticos) se utiliza el Sistema Pronóstico Internacional (IPSS, por sus siglas del inglés *International Prognostic Scoring System*). Este sistema de estadificación se estableció basándose en pacientes tratados con agentes alquilantes y análogos de nucleósidos y consiste en asignar 1 punto a cada parámetro, excepto a la edad, que se le atribuyen 2 puntos porque tiene un profundo impacto en el pronóstico (por definición, los pacientes mayores de 65 años no pueden pertenecer a una categoría de bajo riesgo). La puntuación total de cada paciente al momento del inicio de la terapia los sitúa en la categoría de bajo (puntuación ≤1), intermedio (puntuación =2) o alto riesgo (puntuación ≥3), con tasas de supervivencia a cinco años de 87%, 68% y 36%, respectivamente (Tabla 3).[50]

Aunque la lactato deshidrogenasa (LDH) está ausente del IPSS, sus niveles influyen en el pronóstico de los pacientes de alto riesgo, dividiéndolos en dos subgrupos con resultados significativamente diferentes, de forma que el 90% de los pacientes de alto riesgo con LDH elevada (>250 Unidades Internacionales/litro) mueren por MW frente al 60% de los que la tienen normal/baja.[51–53]

La integración de la calidad de la respuesta en el IPSS agrega valor al pronóstico de los pacientes. De hecho, el IPSS pierde su valor pronóstico en pacientes de alto riesgo que logran una

Tabla 3. Sistema Pronóstico Internacional para la MW. Adaptada de Morel et al., *Blood* 2009.

Factores asociados con el pronóstico	Valor
Edad (años)	>65
Hemoglobina (g/dl)	≤11,5
Plaquetas ($x10^9$/l)	≤100
β_2-microglobulina (mg/l)	>3
IgM monoclonal (g/dl)	>7

Estratificación del riesgo y supervivencia

Grupos de riesgo	Puntuación[a]	Mediana de supervivencia (meses)
Bajo	0-1 (exceptuando la edad)	142,5
Intermedio	2 ó edad >65	98,6
Alto	>2	43,5

[a]Se asigna un punto por cada factor positivo y se suman todos los puntos para establecer el grupo de riesgo.

respuesta parcial sostenida o mejor durante al menos 2 meses después de la terapia de primera línea.[50]

Este sistema se debe utilizar sólo para pacientes que requieren tratamiento y no para determinar si un paciente requiere intervención. Esto sigue siendo una decisión clínica.[5]

1.6. Tratamiento

1.6.1. Indicaciones

No todos los pacientes con diagnóstico de MW necesitan terapia inmediata. En general, se recomienda que el tratamiento se reserve para pacientes con síntomas o signos atribuibles a la MW. El inicio de la terapia no debe basarse únicamente en los niveles de IgM, ya que pueden no correlacionarse con la carga de enfermedad, la sintomatología o el pronóstico. Las indicaciones para comenzar el tratamiento, propuestas en la 2ª Reunión Internacional de MW y confirmadas en la 8ª Reunión Internacional, incluyen complicaciones relacionadas con la IgM y/o síntomas directamente relacionados con la presencia de células tumorales como citopenias (anemia, trompocitopenia o neutropenia), síntomas constitucionales (fiebre, escalofríos, sudores nocturnos, fatiga y pérdida de peso) y enfermedad extramedular. Además, algunos síntomas (hiperviscosidad

sintomática, anemia hemolítica moderada a severa o crioglobulinemia sintomática) requieren terapia urgente.[54–57]

Para los pacientes que no cumplen estos criterios pero que presentan indicios de posible progresión de la enfermedad (disminución en el nivel de hemoglobina con anemia asintomática, ligeros aumentos de IgM, linfadenopatía progresiva o sintomática o esplenomegalia sin molestias para el paciente), se recomienda una observación intensiva.[5] Igualmente, los pacientes con GMSI-IgM o MWA, aunque no presentan síntomas relacionados con la enfermedad, poseen un riesgo diez veces mayor de transformación a enfermedad activa, por lo que deben ser monitorizados más de cerca (cada 3-4 meses).[58]

1.6.2. Opciones de tratamiento disponibles

Dada la escasez de ensayos clínicos aleatorizados, aún no se ha definido un algoritmo terapéutico preciso para el tratamiento de la MW. Entre los agentes activos se incluyen alquilantes (clorambucilo, ciclofosfamida y bendamustina), análogos de purinas (cladribina y fludarabina), anticuerpos monoclonales (rituximab y ofatumumab), inhibidores del proteasoma (bortezomib, carfilzomib e ixazomib), fármacos inmunomoduladores (talidomida, lenalidomida y pomalidomida) e inhibidores de las vías de señalización (everolimus e ibrutinib).

A. Plasmaféresis

En los pacientes que presentan síntomas de hiperviscosidad, el tratamiento de elección es la plasmaféresis precoz, que es muy eficaz dado que la IgM es mayoritariamente intravascular. Al mismo tiempo, se debe iniciar tratamiento citostático para reducir la producción de IgM.[55]

B. Agentes alquilantes

Los alquilantes orales (clorambucilo, melfalán, ciclofosfamida) se utilizan ampliamente en el tratamiento de la MW, tanto solos como en terapia combinada con esteroides (prednisona). Constituyen fármacos de primera elección por su eficacia, comodidad de uso y escaso coste.[59]

C. Análogos de purinas

Los análogos de purinas (fludarabina y cladribina, 2-CdA) forman parte del tratamiento de primera línea en la MW.[60] Las respuestas con estos fármacos son muy rápidas, lo que los hace especialmente útiles en pacientes muy sintomáticos, con síndrome de hiperviscosidad evidente o gran infiltración tumoral. Su mayor desventaja radica en su mielotoxicidad y la inmunosupresión que producen. Al igual que los alquilantes, se asocian con elevado riesgo de síndrome mielodisplásico

(SMD) y leucemia aguda mieloblástica (LAM) secundarios y mayor incidencia de transformación a síndrome de Richter.[57]

La bendamustina tiene similitudes estructurales tanto con los agentes alquilantes como con los análogos de la purina. Se utiliza en 1ª línea en combinación con rituximab.[61]

D. Anticuerpos monoclonales

Rituximab es un anticuerpo monoclonal dirigido contra el antígeno CD20 (*Cluster of Differentiation 20*), presente en la superficie de las células B. CD20 es un miembro de la vía de señalización del factor de necrosis tumoral (TNF, por sus siglas del inglés *Tumor Necrosis Factor*), y su interacción con rituximab conduce a la lisis de la célula B.[62] Constituye un componente fundamental de los regímenes utilizados en la práctica clínica para la MW y se utiliza tanto en monoterapia (con una tasa de respuesta de aproximadamente el 30%),[63] como en combinación con quimioterapia, obteniéndose más y mejores respuestas.[64,65] Rituximab se asocia con un aumento transitorio en los niveles de IgM sérica (fenómeno de *'flare'* o llamarada) que puede dar lugar a complicaciones clínicas.[66]

La actividad observada con rituximab en neoplasias hematológicas ha impulsado la investigación de otros anticuerpos monoclonales. Ofatumumab es un anti-CD20 totalmente humanizado dirigido a un epítopo diferente al del rituximab, con una tasa de respuesta global del 59%,[67] y se ha administrado con éxito a pacientes que demostraron intolerancia al rituximab.

Debido a que CD52 se expresa mucho en las células linfoplasmocíticas,[68] alemtuzumab, un anti-CD52 utilizado en el tratamiento de la LLC y la leucemia de linfocitos grandes granulares, también se ha investigado en pacientes con MW, con una tasa de respuesta global del 75%.[69] Sin embargo, se asoció con una alta incidencia de citopenias de grado ≥3 y trastornos inmunológicos tardíos, por lo que su utilidad clínica en MW debe considerarse en el contexto de estas toxicidades.

Por último, el obinutuzumab, otro anti-CD20 desarrollado más recientemente, ha demostrado su utilidad en MW en algunos estudios tanto en 1ª como en 2ª línea.[70–73]

E. Inhibidores del proteasoma

La inhibición del proteasoma puede afectar a la proliferación celular y la supervivencia en las células B malignas. El bortezomib es un inhibidor reversible de la actividad quimotripsina del proteasoma 26S, citotóxico para las células cancerosas.[74] Se recomienda en pacientes con altos

niveles de IgM, hiperviscosidad sintomática, crioglobulinemia, amiloidosis e insuficiencia renal, o en pacientes jóvenes en los que se desea evitar la terapia con alquilantes o análogos de purinas.[75]

Debido a que el bortezomib produce con frecuencia una neuropatía periférica grave, se ha investigado el uso de carfilzomib, un inhibidor irreversible del proteasoma 20S estructuralmente distinto del bortezomib, sustancialmente menos neurotóxico, en combinación con rituximab y dexametasona, como alternativa al bortezomib y rituximab.[76]

Los inhibidores proteasómicos orales (ixazomib, oprozomib) están actualmente bajo investigación en pacientes con MW y podrían convertirse en opciones prometedoras.[77–79]

F. Agentes inmunomoduladores

Los fármacos inmunomoduladores (talidomida, lenalidomida y pomalidomida) se utilizan ampliamente en MM. Sin embargo, en MW su uso debe considerarse solo en el contexto de un ensayo clínico dado sus efectos adversos potenciales (neurotoxicidad y mielosupresión).[80–82]

G. Inhibidores de la vía PI3K/Akt/mTOR

La vía de señalización PI3K/Akt/mTOR (*Phosphatidylinositol 3-Kinase/Protein Kinase B/mammalian Target Of Rapamycin*) está constitutivamente activada en las células de la MW y regula el metabolismo celular, la proliferación, la supervivencia y la angiogénesis.[83] Por ello, su inhibición ha resultado ser eficaz en el tratamiento de la enfermedad.[84] Everolimus (inhibidor oral de Akt/mTOR), perifosina (inhibidor oral de Akt), enzastaurina (inhibidor oral de PI3K/Akt) e idelalisib (inhibidor oral de PI3Kδ, *Phosphatidylinositol 3-kinase delta isoform*) constituyen potenciales opciones de tratamiento que han demostrado actividad antitumoral en los estudios preclínicos y clínicos de MW.[85–89]

H. Inhibidores de la vía del receptor de la célula B

La tirosina quinasa de Bruton (BTK, por sus siglas del inglés *Bruton's Tyrosine Kinase*) es un componente de la vía de señalización del receptor de la célula B (BCR, por sus siglas en inglés, *B-Cell Receptor*). El BCR está implicado en la tumorigénesis de la MW, pero también constituye un regulador importante de otras vías de supervivencia de las células B que median la apoptosis, la adhesión, la migración y el anidamiento.[90] Ibrutinib es un inhibidor oral de la BTK irreversible y muy potente, que se une covalentemente a un residuo de cisteína-481 en el sitio activo de BTK, dando como resultado una fuerte inhibición de la actividad quinasa. Ha sido aprobado, entre otros, para pacientes con MW, tanto en segunda como primera línea, y en general es muy bien tolerado.[91,92]

Acalabrutinib (ACP-196) es un inhibidor irreversible de BTK de segunda generación que ha demostrado ser más potente y selectivo que ibrutinib en LLC. En MW se está probando en pacientes en recidiva/refractarios en ensayos fase I y II.[93] BGB-3111 es otro inhibidor de BTK de 2ª generación con una gran especificidad por BTK, sin afectar a otras quinasas, lo que permite usar dosis más altas con menor toxicidad. Los resultados iniciales obtenidos son muy prometedores.[94]

I. Inhibidores de histona deacetilasas

Los inhibidores de las histona deacetilasas (HDAC) regulan epigenéticamente la expresión génica acetilando diversos sustratos y modificando la estructura de la cromatina. Vorinostat, un inhibidor de las HDAC, induce la apoptosis de las células de la MW al activar caspasas específicas.[95] Panobinostat también ha demostrado actividad citotóxica contra las células de MW en los estudios preclínicos.[96] Sin embargo, los pobres resultados obtenidos tras su ensayo en pacientes lo han excluido de las actuales pautas de tratamiento para la MW.[97]

J. Trasplante de progenitores hematopoyéticos

El trasplante de progenitores hematopoyéticos (TPH) es una opción para la terapia de rescate en MW, particularmente entre los pacientes más jóvenes que han tenido varias recaídas o tienen enfermedad refractaria primaria. Se han evaluado varias series tanto en experiencias autólogas como alogénicas, con resultados heterogéneos.[98] Hoy por hoy, lo único que se puede decir con claridad es que es una opción posible que debe ser reservada para casos individualizados y ensayos clínicos.

K. Nuevos agentes en desarrollo

Existen diversos fármacos en desarrollo para MW (Figura 2). IMO-8400 un inhibidor de la vía de señalización de los receptores tipo *Toll* (TLR, por sus siglas del inglés *Toll-like Receptors*), clave para la patogenia de la MW; CB-839, un inhibidor de la glutaminasa (se sabe que la glutamina es necesaria para el crecimiento celular y la supervivencia) y venetoclax, un antagonista altamente selectivo de Bcl-2 (proteína antiapoptótica que podría contribuir a la supervivencia de las células de la MW), se están ensayando actualmente en pacientes.[93,99] Otras dianas potenciales para agentes anti-MW que ya se están probando, incluyen MyD88 (*Myeloid Differentiation Primary Response 88*),[100] Syk (*Spleen Tyrosine Kinase*),[101] las enzimas de deubiquitinación asociadas con el proteasoma (*Ubiquitin Specific Peptidase 14*, USP14, y *Ubiquitin Carboxyl-terminal Hydrolase L5*, UCHL5),[102] la interleucina (IL) 6 (IL-6)[103] y la Aurora quinasa A.[104] Finalmente, se está desarrollando también la inmunoterapia con linfocitos T modificados genéticamente para que reproduzcan

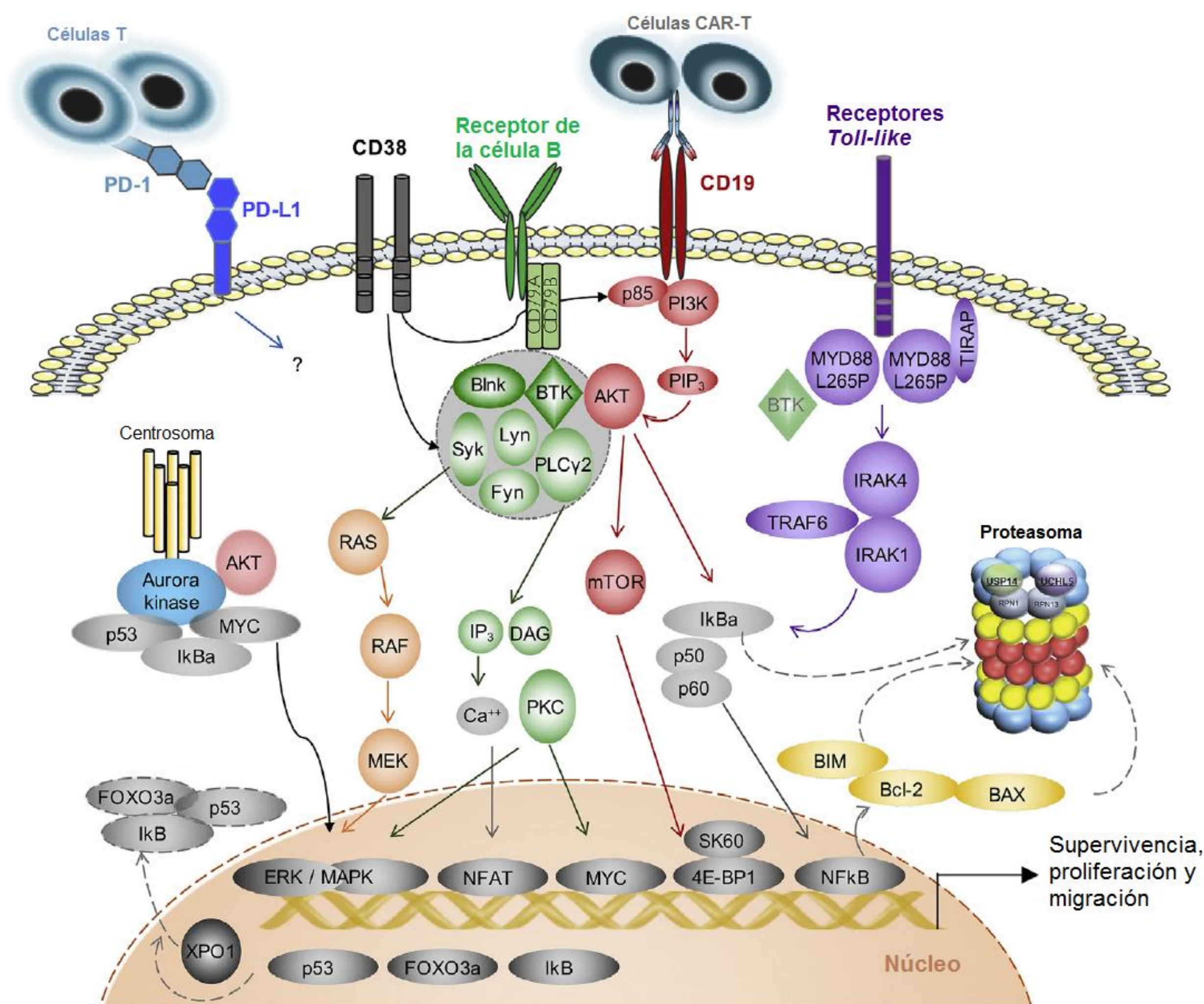


Figura 2. Potenciales dianas farmacológicas que intervienen en la supervivencia de las células de la MW (modificada de Paulus et al., *Best Pract Res Clin Haematol* 2016). Existen nuevas dianas cuya función o presencia ha demostrado ser importante para la supervivencia de las células de la MW que se están investigando actualmente en ensayos clínicos o preclínicos. Entre ellas se incluyen diversas proteínas quinasas y moléculas adaptadoras, tales como MyD88, Syk y Aurora quinasa A; las enzimas de deubiquitinación del proteasoma (USP14 y UCLH15), responsables de una óptima homeostasis celular/proteica en las células de MW, o varios receptores de la superficie celular como CD38 o los TLRs 7, 8 y 9, que transmiten señales de proliferación y previenen la muerte celular por células T citotóxicas, mediante la interacción PD-1/PD-L1 (*Programmed cell death 1/Programmed death-ligand 1*). Por último, CD19 constituye un excelente marcador sobre el que dirigir la inmunoterapia con células CART en MW.

receptores de antígenos quiméricos (o inmunoterapia CART, por sus siglas del inglés *Chimeric Antigen Receptor T-Cell Therapy*), dirigidos en este caso a CD19.[105]

1.6.3. Estrategia terapéutica

El manejo de la MW está guiado por un principio básico: es una enfermedad indolente y tratable, pero incurable con los enfoques terapéuticos actuales. Como tal, los objetivos

fundamentales de las estrategias de tratamiento no son sólo aliviar los síntomas y disminuir el riesgo de daño orgánico, sino también mantener el control a largo plazo de la enfermedad, mejorar la calidad de vida, limitar la toxicidad relacionada con la terapia y mejorar la supervivencia global.

Como acabamos de ver, las opciones de tratamiento para los pacientes con MW han evolucionado rápidamente durante la última década. Sin embargo, y pese a la diversidad de fármacos disponibles, ninguno, exceptuando ibrutinib (inhibidor de BTK), ha sido aprobado por la Administración de Medicamentos y Alimentos (FDA, por sus siglas del inglés *Food and Drug*

Tabla 4. Recomendaciones de tratamiento según las condiciones clínicas para los pacientes con MW, de acuerdo a las indicaciones propuestas por la 8ª Reunión Internacional de MW. Adaptada de Kastritis et al., *Best Pract Res Clin Haematol* 2016.

Condición clínica	Estrategia recomendada
Citopenias	Dexametasona/rituximab/ciclofosfamida Bendamustina/rituximab Bortezomib/dexametasona/rituximab Ibrutinib
IgM elevada[a], hiperviscosidad sintomática[a], crioglobulinemia[a] o aglutinemia fría, candidato a autotrasplante	Bendamustina/rituximab Bortezomib/dexametasona/rituximab
IgM elevada[a], hiperviscosidad sintomática[a], crioglobulinemia[a] o aglutinemia fría, no candidato a autotrasplante	Bendamustina/rituximab Bortezomib/dexametasona/rituximab Ibrutinib
Comorbilidades y citopenias	Rituximab Ibrutinib
Edad avanzada, progresión lenta, candidato a terapia oral	Ibrutinib Fludarabina oral
Neuropatía periférica por IgM	Rituximab Dexametasona/rituximab/ciclofosfamida Ibrutinib Rituximab/fludarabina
Recaída, ≥2-3 años después de la terapia inicial con rituximab	Repetir el régimen primario o uno similar que contenga rituximab
Recaída, <12 meses después de la terapia inicial con rituximab o refractario a rituximab en combinación	Ibrutinib Bendamustina/rituximab (si no ha recibido bendamustina) Rituximab con un inhibidor del proteasoma (si no lo ha recibido antes) Fludarabina/ciclofosfamida/rituximab o fludarabina/rituximab

[a]Debe considerarse la plasmaféresis, ya sea preventivamente o para el manejo inmediato de las complicaciones.

Administration) o la Agencia Europea de Medicamentos (EMA, por sus siglas del inglés *European Medicines Agency*) específicamente para MW.

El tratamiento de los pacientes debe ser individualizado según las características de la enfermedad (niveles de IgM, presencia de citopenias, complicaciones inmunológicas de la IgM, volumen de enfermedad), del paciente (edad fisiológica, estado funcional, comorbilidades y preferencia por la terapia oral frente a la parenteral) y los objetivos de la terapia, ya que no todos requieren una respuesta inmediata con un tratamiento intensivo. En los pacientes jóvenes, se pueden considerar combinaciones intensificadas para aumentar la probabilidad de respuesta completa, a expensas de mayor toxicidad a corto plazo; en cambio, en pacientes ancianos, que son la mayoría, el objetivo no es una respuesta completa y estricta, sino más bien controlar la enfermedad durante un largo período con toxicidad mínima. Las recomendaciones de tratamiento actualizadas para pacientes sintomáticos no tratados y previamente tratados se resumen en la Tabla 4.[55,106]

2. Caracterización de la MW

2.1. Morfológica

La MW presenta infiltración de la MO por una población de células linfoplasmocíticas. El patrón de infiltración puede ser difuso, intersticial o nodular, aunque el más frecuente es el intertrabecular. En el aspirado medular se puede observar una población linfoide polimorfa, en cantidad variable, con linfocitos, linfoplasmocitos (que pueden contener cuerpos de Russell y Dutcher) y células plasmáticas. También es muy característica la presencia de mastocitos o células cebadas, que, probablemente, tienen algún papel en la patogenia de la enfermedad.[3,107]

2.2. Inmunofenotípica

El perfil inmunofenotípico característico de las células linfoplasmocíticas de la MW debe incluir la expresión de los marcadores pan-B CD19, CD20, CD22 y CD79, así como la expresión de IgM de superficie y restricción de cadena ligera intracitoplasmática y de superficie (κ o λ). La expresión de CD25, CD27, FMC7, BCL-2 y CD52 puede observarse también en la mayoría de los casos. En cambio, la reactividad para CD10, CD23, CD103 y CD138 es más rara. CD5 también es infrecuente (5-20% de los casos), y aunque no excluye un diagnóstico de MW, en los casos positivos se debe descartar la LLC y el linfoma de células del manto (LCM). En resumen, el perfil de expresión inmunofenotípica más común para el clon de Waldenström podría definirse como: CD5-/CD10-CD11c-/CD19+/CD20+/CD22+débil/CD23-/CD25+/CD27het/CD38het/CD79b+/CD81+/CD103-/CD200het/CD305-, siendo bastante comparable a las células B de memoria normales, excepto para la expresión de CD200.[108–110]

2.3. Alteraciones cromosómicas

Los estudios de citogenética convencional, hibridación *in situ* fluorescente (FISH, por sus siglas del inglés *Fluorescence In Situ Hybridization*), hibridación genómica comparada (CGH, por sus siglas del inglés *Comparative Genomic Hybridization*) y más recientemente las matrices de polimorfismos de nucleótido único (SNP, por sus siglas del inglés *Single Nucleotide Polymorphism*) han identificado para la MW un cariotipo de baja complejidad, con una mediana de 2-3 anomalías cromosómicas por paciente.[111–117] Estos valores son comparables a otros SLPs de bajo grado, como la LLC y LZM, pero significativamente más bajos que en los linfomas más agresivos y el MM.[118,119] La pérdida de heterocigosidad (LOH, por sus siglas del inglés *Loss Of Heterozigosity*) también es muy rara en el genoma de la MW, y sólo se ha descrito en dos casos, afectando al cromosoma 13

y a las regiones teloméricas en 6q y 17q.[120] Lo mismo ocurre con las deleciones bialélicas, que implicaron a los genes *MIRN15a-16* (microARN inductor de la apoptosis mediada por Bcl-2) y *TRAF3* (*Tumor Necrosis Factor Receptor Associated Factor 3*), regulador negativo de la vía de señalización de NF-kB (*Nuclear Factor Kappa-light-chain-enhancer of activated B cells*) recurrentemente alterado en otras neoplasias malignas de células B como MM, linfoma B difuso de célula grande (LBDCG) y LZM.[111,121,122]

La deleción de 6q (-6q o del6q) es la anomalía cromosómica más frecuente en MW, presente en un 40-50% de los pacientes.[123,124]. Además, se encuentra únicamente en MW y no en GMSI, por lo que probablemente sea un evento secundario. En esta línea, ha demostrado estar asociada con algunos parámetros clínicos desfavorables como anemia, hipoalbuminemia, β_2-microglobulina (B2M) elevada y baja producción de IgM, además de conferir un peor pronóstico.[113,115,124,125] Las regiones suprimidas con mayor frecuencia son 6q21 y 6q23,[112,115,120] donde se han identificado los genes candidatos *BLIMP1* o *PRDM1* (*B-Lymphocyte-Induced Maturation Protein 1*) en 6q21, y *TNFAIP3* o *A20* (*TNF Alpha Induced Protein 3*) en 6q23.[114,126] BLIMP1 es un factor de transcripción con función principalmente represora que desempeña un papel fundamental en la diferenciación de célula B a célula plasmática. Entre los genes silenciados por este factor están los marcadores de superficie de células B (CD19, CD20 y CD45) y varias moléculas de la cascada de señalización del BCR tales como Syk, BLNK (*B-Cell Linker*), CD79A (*B-Cell Antigen Receptor Complex-Associated Protein Alpha Chain*), PKCβ (*Protein Kinase C Beta*) y LYN (*Lck/Yes-Related Novel Protein Tyrosine Kinase*).[127,128] TNFAIP3 es otro regulador negativo de NF-kB, por lo que se cree que su pérdida conduce a la activación constitutiva de dicha vía.[114,129]

Sin embargo, aunque la del6q es común en la MW, no es exclusiva de esta enfermedad, ya que se ha detectado la pérdida de cuatro regiones mínimas en 6q en varias neoplasias derivadas de células B, incluyendo MM y LZM.[118] En cambio, la trisomía del 4, identificada en aproximadamente un 10-20% de pacientes, sí que parece ser específica de la MW, siendo en ocasiones la única lesión genética observada.[113,116] La trisomía del 18, común en el LZM, está presente en un 15% de los pacientes con MW y se asocia con la trisomía del 4. La deleción del 17p, típicamente asociada con la pérdida del gen *TP53* (*Tumor Protein P53*) y con mal pronóstico en LLC, MM y LZM, ocurre en alrededor del 8% de los pacientes. En MW también se correlaciona con una menor supervivencia libre enfermedad aunque sin afectar tanto a la supervivencia global, lo que sugiere que no tiene un papel claro como factor pronóstico negativo en MW.[113]

Otras anomalías recurrentes encontradas en MW (5-20%) incluyen ganancias de los cromosomas 3, 6p, 12 y 18 y deleciones de 7q, 11q23 (*ATM*, *Ataxia Telangiectasia Mutated Serine/Threonine Kinase*, y otros genes), 13q14 (*MIRN15a-16*) y Xq26, muchas de ellas compartidas también con LZM, LLC y MM.[112,113,118,123,130] Sin embargo, al contrario que estas, las traslocaciones que implican el locus de la cadena pesada de las Ig (*IGH*) son raras en MW, encontrándose en menos del 3% de los casos.[113,116,123,131]

2.4. Expresión génica

Los estudios de expresión génica han contribuido a definir algunas de las principales vías moleculares subyacentes a la fisiopatología de la MW, como las de TLR/MyD88, CXCR4 (*C-X-C Motif Chemokine Receptor 4*), NF-κB y BCR.

Contrariamente a lo que se había predicho, los primeros estudios genéticos mostraron que la MW se asemejaba más a la LLC que al MM,[126,132] identificándose cuatro genes capaces de discriminar ambas entidades con gran exactitud: *LEF1* (*Lymphoid Enhancer Binding Factor 1*), *ATXN1* (*Ataxin 1*), *FMOD* (*Fibromodulin*), sobreexpresados en LLC, y *MARCKS* (*Myristoylated Alanine Rich Protein Kinase C Substrate*), sobreexpresado en MW. El análisis del perfil de expresión génica (GEP, por sus siglas del inglés *Gene Expression Profiling*) también reveló una mayor expresión de IL-6 en MW que en MM y LLC y, aunque su papel en la patogénesis de la MW sigue sin estar claro aún,[133] se sabe que la IL-6 activa las vías de señalización MAPK/ERK (*Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase*), siendo responsable de su activación crónica en ausencia de mutaciones activantes,[134] y JAK/STAT (*Janus Kinase/Signal Transducers and Activators of Transcription*), proponiéndose como mecanismo subyacente a la hipersecreción de IgM por las células de la MW.[135,136]

Los estudios subsiguientes se centraron únicamente en la expresión de los genes implicados en la fase tardía de diferenciación de las células B, identificando un patrón heterogéneo en la pérdida de genes reguladores clave en las células linfoplasmocíticas.[137,138] También se han encontrado alteraciones del GEP en regiones con anomalías cromosómicas (especialmente en 6q)[120] y asociadas con el perfil mutacional.[139,140] Así, los pacientes con el gen *CXCR4* mutado mostraron una firma transcriptómica distinta que afectaba a procesos como el ciclo celular, la replicación del ADN, las interacciones célula-célula, el crecimiento celular y la proliferación.[139] Además, un estudio muy reciente de transcriptoma de nueva generación (*RNA Sequencing*) ha contribuido a esclarecer

las diferencias entre los tres tipos de pacientes de MW que sobresalen a día de hoy: *MYD88*^{wt}-*CXCR4*^{wt} (*MYD88* y *CXCR4* no mutados), *MYD88*^{L265}-*CXCR4*^{wt} (*MYD88* mutados y *CXCR4* no mutados) y *MYD88*^{L265}-*CXCR4*^{WHIM} (*MYD88* y *CXCR4* mutados). La agrupación por genes relacionados con la diferenciación de células B es capaz de separar los pacientes en los tres grupos y sugiere que el doble negativo puede representar una etapa más temprana en la diferenciación debido a que presenta una señalización alterada. Por su parte, las mutaciones de *CXCR4* fueron capaces de silenciar los supresores tumorales que se encuentran aumentados en respuesta a la señalización de MyD88 mutada. Y los pacientes sin mutaciones de *MYD88* mostraron una expresión heterogénea, lo que indica diversidad patogénica en esta población.[140]

Por otro lado, la comparación de las células de MW con células B de donantes sanos reveló una mayor expresión de los genes implicados en los mecanismos de reparación del ADN como *DNTT* (*Deoxyriboucleic Acid Nucleotidylexotransferase*), *RAG1* (*Recombination Activating 1*) y *RAG2* (*Recombination Activating 2*), así como miembros de la vía de señalización de CXCR4 (*SDF-1*, *Stromal Cell-Derived Factor 1*, *VCAM1*, *Vascular Cell Adhesion Molecule 1* y el mismo *CXCR4*), resaltando el papel de la señalización de CXCR4 en MW independientemente del estado de mutación del gen.[140]

Por último, existen varios estudios de GEP que analizaron las diferencias entre GMSI-IgM, MWA y MWS, buscando establecer los posibles mecanismos de progresión. Trojani y colaboradores observaron una sobreexpresión de los genes implicados en la regulación de la transcripción, la respuesta inmune, la activación celular y las vías JAK/STAT, PI3K/Akt/mTOR y MAPK en MW en comparación con GMSI.[141] En cambio, Paiva y colaboradores no encontraron un perfil significativamente desregulado entre las células B de los 3 grupos de enfermedad, sino sólo contra las células B normales.[142] Finalmente, Herbaux y colaboradores demostraron que las formas sintomáticas y asintomáticas presentaban diferentes firmas de expresión, afectando principalmente a las vías de diferenciación y activación de células B, interacción con el microambiente y PI3K/Akt.[143]

Sin embargo, todos estos estudios presentan algunas limitaciones, ya sea en cuanto al uso de metodologías poco reproducibles como los chips de ADN (del inglés *DNA microarrays*),[144,145] de células seleccionadas CD19+ sin prestar atención a la heterogeneidad de esta población (es decir, clonal vs. policlonal), o por el bajo número de pacientes evaluados. En consecuencia, se necesitan estudios adicionales en series más grandes, con poblaciones puras y métodos reproducibles.

2.5. Mutaciones somáticas

La citogenética, la FISH y el GEP son capaces de proporcionar información significativa con respecto a la genética de la MW pero hasta ahora no han revelado ninguna firma molecular específica capaz de distinguirla de otras neoplasias B malignas.[111] Esto sugiere que las mutaciones genéticas, los SNPs y/o los mecanismos epigenéticos podrían ser los responsables del fenotipo único de esta enfermedad. Las técnicas de secuenciación de nueva generación (NGS, por sus siglas del inglés *Next Generation Sequencing*) o secuenciación masiva han permitido identificar la presencia de mutaciones somáticas recurrentes, confirmando que prácticamente todos los pacientes tienen alteraciones, lo que ha supuesto un gran avance en el conocimiento de la biología de la MW.[146]

2.5.1. Gen *MYD88*

La mutación L265P en el gen *MYD88 (Myeloid differentiation primary response gene 88)*, situado en el cromosoma 3p22.2, que da como resultado un cambio de leucina a prolina en el aminoácido 265, se identificó por primera vez en 26 de 30 pacientes con MW mediante la secuenciación del genoma completo de las células tumorales y su comparación con las respectivas células normales (n=10) o con bases de datos (n=20).[146] Posteriormente, fue confirmada por múltiples grupos en diversas series, demostrando su presencia hasta en el 91% de casos.[147–149] Mutaciones de *MYD88* distintas a la L265P, como S219C, M232T y S243N, también han sido identificadas, aunque más raramente, en pacientes de MW y otras neoplasias de células B.[150]

A. Función y vía de señalización

MyD88 es una proteína adaptadora clave en la activación canónica de la vía NF-κB a través de los TLR y las familias de receptores de IL-1, que intervienen en los procesos inflamatorios e infecciosos. Tras la activación del receptor, MyD88 se homodimeriza (se une a otras moléculas MyD88), lo que le permite actuar como un puente para el reclutamiento de otras proteínas (IRAK1 e IRAK4, *Interleukin 1 Receptor Associated Kinases 1 and 4*) formando un complejo, el 'Myddosoma', que desencadena la señalización corriente abajo y da lugar a la activación de NF-κ B, responsable del crecimiento y la supervivencia de las células de la MW.[151–153] Las mutaciones en *MYD88* se describieron por primera vez en el LBDCG del subtipo de célula B activada (ABC, por sus siglas del inglés *Activated B-Cell*), donde se demostró que daban lugar a la activación

constitutiva de la vía.[154] En MW, NF-κB también puede activarse constitutivamente a través de BTK (la diana de ibrutinib).[90] Además, se ha visto que la inhibición de la actividad BTK no afecta a IRAK1/4 y viceversa, lo que sugiere que la señalización de MyD88 a través de ambas vías es independiente.[155] El reclutamiento y la activación de IRAK1/IRAK4, así como de BTK, puede impedirse mediante la modificación genética de *MYD88* o el uso de péptidos que bloquean la homodimerización, induciendo la muerte de las células mutadas.[90,100]

MyD88 mutada también puede activar HCK (*Hematopoietic Cell Kinase*), una proteína de la familia Src (familia homóloga a la de la proteína del virus del sarcoma de Rous), bien directamente, o bien a través de la IL-6. HCK contribuye al crecimiento y supervivencia de las células de la MW mutadas a través de múltiples vías de señalización entre las que se incluyen BTK, PI3K/Akt y MAPK/ERK (Figura 3).[156]

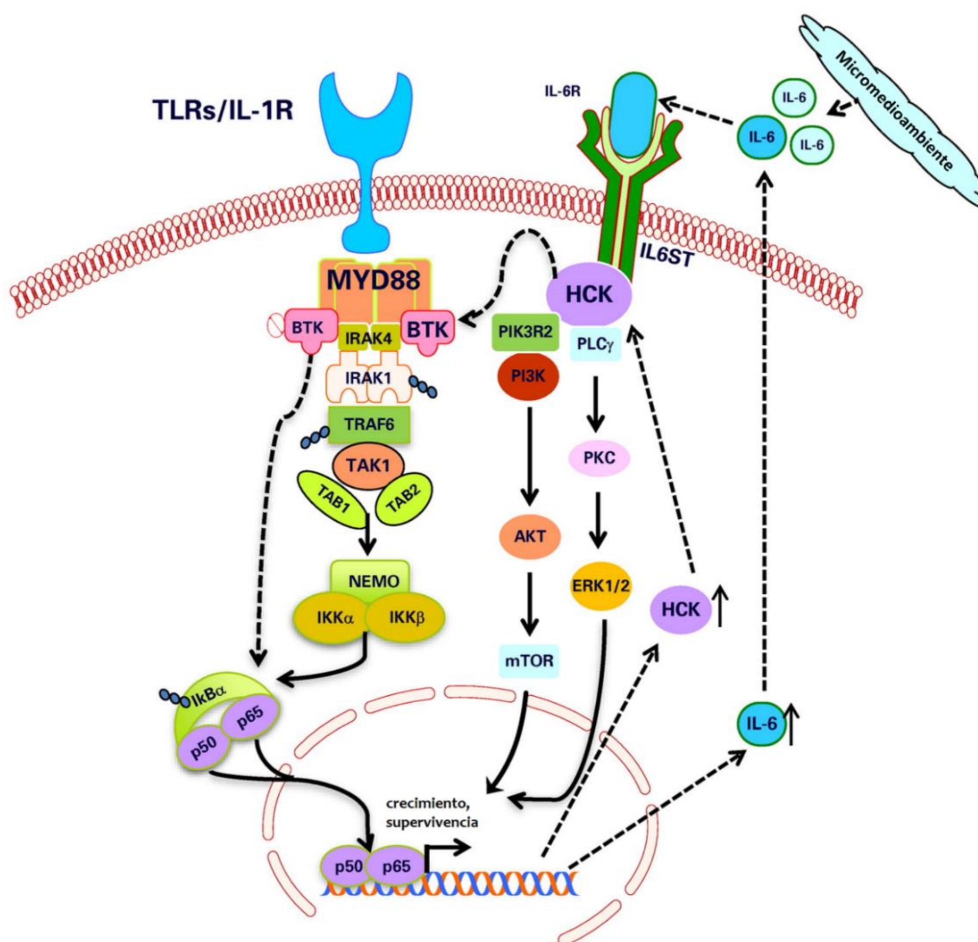


Figura 3. Representación esquemática de la vía TLR/MyD88 (modificada de Hunter et al., *J Clin Oncol* 2017). La proteína MyD88 mutada activa NF-κB a través de vías divergentes que incluyen a los mediadores IRAK1/ IRAK4 o BTK. Además, induce la transcripción y activación de HCK, que puede también promover el crecimiento y la supervivencia de las células de la MW a través de las vías de señalización de BTK, Akt y ERK1/2.

B. *MYD88* como herramienta de diagnóstico

Las mutaciones de *MYD88* están ausentes o se encuentran en baja frecuencia en otras neoplasias de células B que comparten características morfológicas y clínicopatológicas con la MW, como el MM IgM (0%), el LZM (6-10%) y la LLC (3-8%), lo que permite la discriminación molecular en el diagnóstico.[147,148,157–159] Además, cuando se combina con el estado mutacional de la región variable del gen de la cadena pesada de las inmunoglobulinas (*IGHV,* por sus siglas del inglés *Immunoglobulin Heavy Chain Variable region*), la mutación *MYD88* L265P parece delinear una firma genética única que distingue a la MW de las otras neoplasias de células B similares.[160]

La presencia de *MYD88* L265P tanto en el líquido cefalorraquídeo, como en el líquido pleurítico o sangre periférica constituye una ayuda para el diagnóstico en pacientes con enfermedad sintomática fuera de la médula ósea y los ganglios linfáticos.[32,161]

C. Influencia de *MYD88* en la presentación clínica

Desde el punto de vista clínico, la presencia de la mutación de *MYD88* parece distinguir dos poblaciones distintas de pacientes. Aunque ambos grupos muestran una morfología similar de las células tumorales al microscopio, los que carecen de la mutación son más mayores, pero presentan una afectación significativamente menor de la médula ósea y niveles séricos de IgM más bajos. Sin embargo, a pesar de la menor carga de enfermedad, su supervivencia global puede ser más corta con un riesgo de muerte 10 veces mayor que en los pacientes mutados.[147,162,163]

D. *MYD88* como herramienta para el seguimiento y la evaluación de la respuesta

La alta prevalencia de la mutación de *MYD88* en la MW y su correlación con la carga tumoral hacen de ella un marcador adecuado para evaluar la respuesta molecular. De este modo, su detección mediante técnicas de alta sensibilidad podría utilizarse potencialmente para el seguimiento de la enfermedad e incluso para ayudar a definir mejor los criterios de respuesta.[148,164] Un análisis comparativo de la población CD19+ en muestras pareadas de sangre periférica y MO mostró valores comparables de detección de la mutación (85% frente a 89%, respectivamente). Este resultado es muy alentador ya que proporciona un método menos invasivo para el cribado de la mutación, eliminando la necesidad de una biopsia de MO para realizar las pruebas moleculares.[165] Otro estudio demostró una correlación significativa entre el grado de afectación de la MO y los niveles de la mutación medidos por reacción en cadena de la polimerasa alelo específica (AS-PCR,

por sus siglas del inglés *Allele-Specific Polymerase Chain Reaction*). Además, se observó una disminución de los niveles en los casos de respuesta al tratamiento, llegando a ser indetectables en un caso de respuesta completa.[147] Aunque estos resultados tienen que ser validados en series prospectivas más amplias con distintos regímenes de tratamiento, parece que la cuantificación de la carga mutacional podría ayudar a predecir la consecución de remisiones moleculares, así como correlacionarse con la supervivencia global y libre de progresión.[147,162,163] El análisis de la enfermedad mínima residual (EMR) ya ha demostrado tener un importante papel como marcador pronóstico en los trastornos linfoproliferativos, especialmente en el linfoma folicular (LF), el LCM y el MM,[166–168] habiendo incluso estudios alentadores en MW.[169]

La detección de *MYD88* L265P, tanto con fines de diagnóstico como para el seguimiento de la enfermedad durante y después de la terapia, requiere de técnicas altamente sensibles y capaces de cuantificar la carga tumoral. Muchos de los SLP a los que hacíamos referencia previamente se caracterizan por una baja infiltración tumoral, que puede llevar a subestimar la frecuencia real de la mutación si se utilizan metodologías convencionales con sensibilidad relativamente baja, como la secuenciación Sanger, que precisa de frecuencias alélicas superiores al 25%.[170] En cambio, la PCR cuantitativa en tiempo real con cebadores específicos de alelo (ASO-RQ-PCR, por sus siglas del inglés *Allele-Specific Oligonucleotide Real-time Quantitative PCR*) combinada con sondas TaqMan podría constituir una herramienta adecuada para este propósito ya que es capaz de detectar la mutación incluso estando presente en muy pocas células y permite la cuantificación en tiempo real.[171]

E. Influencia de *MYD88* en la evolución de la enfermedad

Las mutaciones de *MYD88* abarcan todo el clon y son detectables en un ~50% de GMSI-IgM, pero no en las de tipo IgG o IgA, lo que sugiere un papel oncogénico temprano en la patogénesis de la enfermedad, pudiendo ser el evento inicial que confiere ventaja competitiva al clon y lo predispone a otras alteraciones genéticas.[147,163,172–175] Además, según varios estudios, los pacientes con GMSI de tipo IgM que presentan la mutación de *MYD88* parecen tener mayor riesgo de progresión a MW (odds ratio: 4,7, intervalo de confianza del 95%: 0,8-48,7, p=0,04). Esto sugiere un papel potencial para *MYD88* L265P en la estratificación de pacientes con GMSI-IgM.[147,163,172] Sin embargo, la implicación pronóstica del estado mutacional de *MYD88* aún necesita ser aclarada, ya

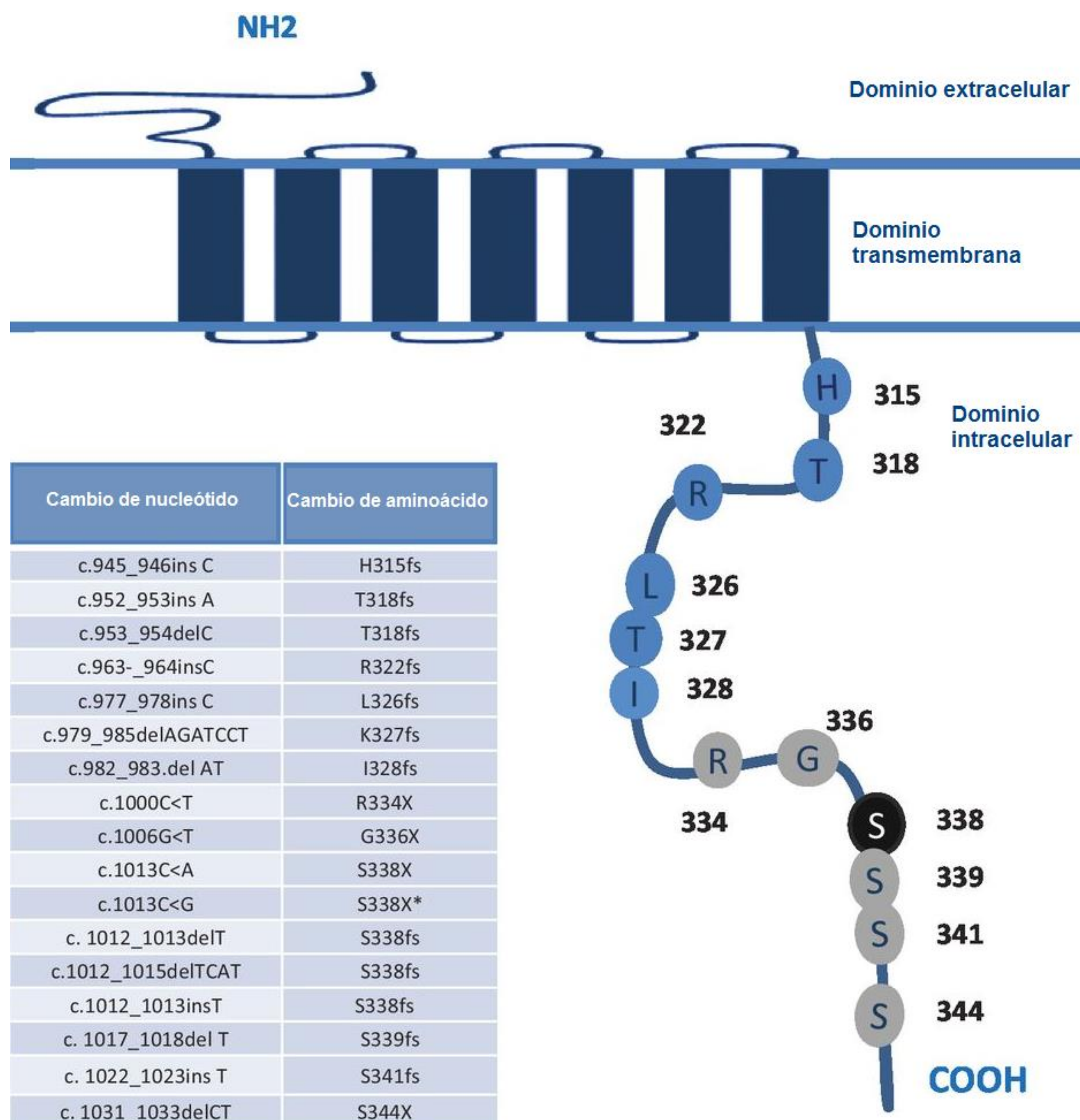
que otras series han demostrado que los casos positivos parecen tener un pronóstico favorable, no sólo en MW,[162] sino también en LLC.[176]

En conjunto, estos datos sugieren que *MYD88* L265P podría ser usada como biomarcador molecular para el diagnóstico, estratificación del riesgo y seguimiento de la respuesta al tratamiento en pacientes con MW. Y dado su papel en la activación constitutiva de la vía de NF-κB, el uso de una terapia dirigida bien frente a MyD88, o bien la combinación de inhibidores de BTK e IRAK, constituyen enfoques farmacológicos prometedores en el tratamiento de la MW.[90,177] La detección de la mutación ayudaría a identificar aquellos pacientes que son más adecuados para recibir estos tratamientos dirigidos.

2.5.2. Gen *CXCR4*

Las segundas mutaciones más frecuentes en la MW afectan al dominio carboxi-terminal (C-terminal) del gen *CXCR4* (receptor de quimiocinas C-X-C tipo 4) y están presentes hasta en un 40% de los pacientes, casi siempre en asociación con las de *MYD88*.[139,178] Estas alteraciones son prácticamente exclusivas de la MW, ya que hasta ahora no se han descrito en otras neoplasias, con la excepción de unos cuantos casos de LZM (7%) y LBDCG (1-7%).[175,179,180] Por contra, son muy similares a las mutaciones hereditarias (germinales) encontradas en los pacientes con síndrome de WHIM (verrugas, hipogammaglobulinemia, infecciones y mielocatexis, por sus siglas del inglés *Warts, Hypogammaglobulinemia, Infections and Myelokathexis*), una forma rara de inmunodeficiencia.[181,182] La base del mecanismo desencadenante de esta enfermedad está en el papel de CXCR4 en el control de la migración de las células hematopoyéticas y los linfocitos B. Por ello, cuando está alterado, la activación por su ligando SDF-1 genera una extensa señalización quimiotáctica que resulta en el secuestro de neutrófilos en la médula ósea (mielocatexis) y la alteración del desarrollo de los linfocitos.[182]

En MW se han descrito más de 30 mutaciones diferentes en el dominio C-terminal del gen (Figura 4), tanto sin sentido (mutaciones que codifican un codón de stop), como mutaciones con desplazamiento o cambio en el marco de lectura (inserciones o deleciones).[139,178,179,183] Esto da como resultado la pérdida de los aminoácidos finales, entre los que se incluyen los residuos de serina (S) y treonina (T), que tienen función reguladora, ya que cuando se fosforilan tras la unión de CXCR4 con su ligando, se produce la internalización del receptor y la interrupción de la señalización (Figura 5).[184,185] Así, la caracterización funcional *in vivo* ha demostrado que las alteraciones de



Cambio de nucleótido	Cambio de aminoácido
c.945_946ins C	H315fs
c.952_953ins A	T318fs
c.953_954delC	T318fs
c.963-_964insC	R322fs
c.977_978ins C	L326fs
c.979_985delAGATCCT	K327fs
c.982_983.del AT	I328fs
c.1000C<T	R334X
c.1006G<T	G336X
c.1013C<A	S338X
c.1013C<G	S338X*
c. 1012_1013delT	S338fs
c.1012_1015delTCAT	S338fs
c.1012_1013insT	S338fs
c. 1017_1018del T	S339fs
c. 1022_1023ins T	S341fs
c. 1031_1033delCT	S344X

Figura 4. Mutaciones somáticas de CXCR4 (modificada de Poulain et al., *Clin Cancer Res* 2016). Las mutaciones somáticas en el dominio C-terminal de CXCR4 fueron identificadas por primera vez mediante NGS en pacientes con MW. Actualmente, se conocen más de 30 mutaciones diferentes, tanto sin sentido o *nonsense* (X) (coloreadas en gris) como de cambio en el marco de lectura o *frameshift* (fs) (señaladas en azul). La más frecuente es la mutación *nonsense* C1013G (indicada con *) que afecta al aminoácido S338 (en negro). S, serina; T, treonina

CXCR4 tienen un papel activador en las células de MW, las cuales exhiben una proliferación y diseminación tumoral significativa a los órganos extramedulares, lo que conduce a la progresión de la enfermedad y disminución de la supervivencia.[180]

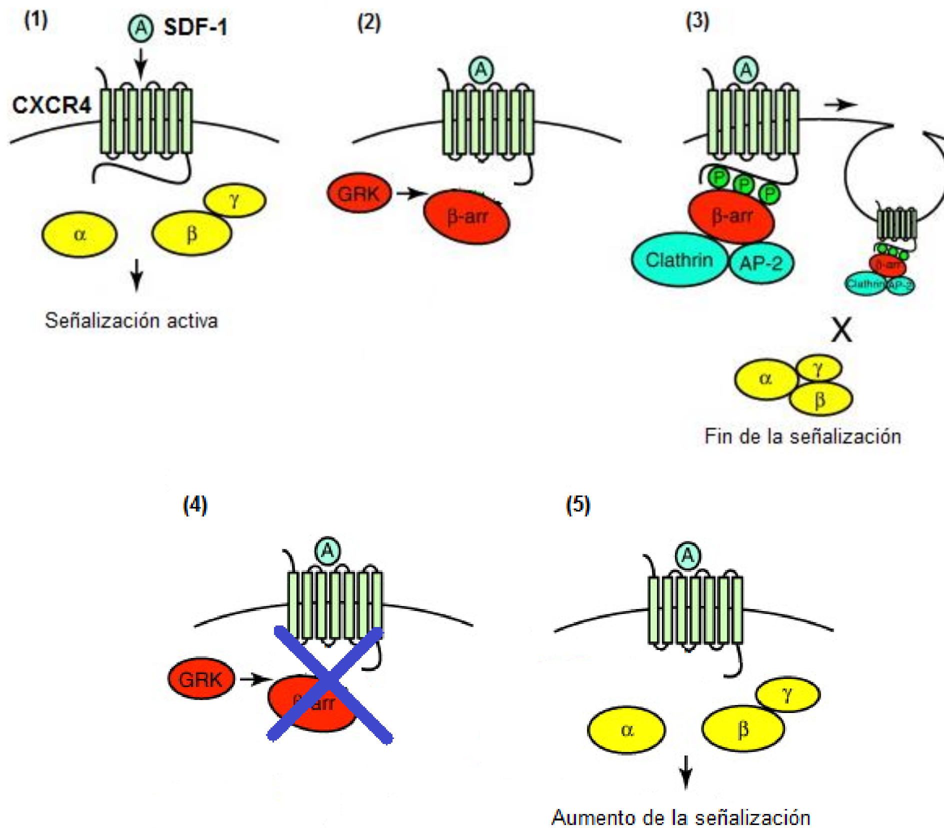


Figura 5. Representación de la regulación de CXCR4 y las consecuencias de las mutaciones en el extremo C-terminal (modificada de Whalen et al., *Trends Mol Med* 2011). (1) La interacción de CXCR4 con su ligando SDF-1 provoca la activación de rutas de señalización dependientes de proteínas G (α, β y γ). **(2-3)** La unión de β-arrestinas (β-arr), inducida por la fosforilación (mediada por GRKs, *G protein-coupled receptor kinases*) de los residuos de serina (Ser) y treonina (Thr) del dominio C-terminal, da lugar a la desensibilización e internalización del receptor, suponiendo el fin de la señalización. **(4)** Las mutaciones de CXCR4 truncan el dominio C-terminal regulador eliminándose los residuos de Ser y Thr. **(5)** Esto disminuye endocitosis del receptor con el consiguiente incremento de la respuesta a SDF-1.

A diferencia de lo que ocurre con *MYD88*, las mutaciones de *CXCR4* son comúnmente subclonales, observándose incluso múltiples mutaciones en el mismo paciente, bien en clones separados o en la misma célula (eventos heterocigotos compuestos),[139,179] lo que sugiere que se originarían después de *MYD88* L265P, aunque también prontamente en la oncogénesis, ya que se han detectado en un ~20% de pacientes con GMSI-IgM.[179,180]

La presencia de mutaciones en *CXCR4* también puede afectar a la presentación clínica de la enfermedad. Los pacientes mutados (que son también *MYD88*^{L265P}) se presentan más comúnmente con una enfermedad más agresiva y sintomática (con mayor afectación de la MO y niveles séricos de IgM y/o riesgo de hiperviscosidad sintomática) que con frecuencia requiere

tratamiento inmediato tras el diagnóstico. Sin embargo, estas diferencias no parecen afectar negativamente a la supervivencia global.[139,162,183]

La utilidad clínica potencial más relevante de estas mutaciones se basa en el hecho de que parecen conferir cierta resistencia a ibrutinib, así como a otros agentes terapéuticos como los inhibidores de mTOR (everolimus) y de PI3K (idelalisib), aunque no a los inhibidores del proteasoma (carfilzomib).[76,91,92,180,186–188] La eficacia de ibrutinib también está condicionada por el estado de *MYD88* debido a que la activación de la proliferación celular se debe al complejo $MyD88^{L265P}$-BTK, lo que hace que las células sean más susceptibles al tratamiento dirigido a inhibir BTK.[90] De este modo, los pacientes con *MYD88* mutado lograron mayores tasas de respuesta (92%) en comparación con los pacientes con ambas mutaciones (en *MYD88 y CXCR4*, 62%), y los dobles negativos $MYD88^{wt}$-$CXCR4^{wt}$ (0%).[150] Por tanto, y teniendo en cuenta el elevado coste de los nuevos enfoques de tratamiento, la evaluación sistemática de las mutaciones en *MYD88* y *CXCR4* debe ser incluida en el algoritmo de diagnóstico de base con el fin de identificar a los pacientes que podrían beneficiarse más de estos tratamientos.

2.5.3. Otros genes

A. ARID1A

Se han encontrado mutaciones somáticas en *ARID1A* (*AT-Rich Interactive Domain-Containing Protein 1A*) en cerca del 20% de los pacientes con MW.[178] ARID1A y su homólogo ARID1B (*AT-Rich Interactive Domain-Containing Protein 1B*) se encuentran en el cromosoma 6q (frecuentemente delecionado) y son componentes de la familia de proteínas SWI-SNF (*SWItch/Sucrose Non Fermentable*), implicadas en varias funciones celulares, incluyendo la remodelación de la cromatina, la regulación de la transcripción y la reparación del ADN.[189] Las mutaciones en *ARID1A* aún no se han estudiado bien en el contexto de las neoplasias hematológicas, pero en el cáncer de ovario, donde se han evaluado más a fondo, se piensa que ARID1A mutada puede modular *TP53*, actuando como un supresor tumoral epigenético.[190,191] En MW, los pacientes con mutaciones en *ARID1A* (y $MYD88^{L265P}$) presentan una enfermedad más agresiva, caracterizada por una mayor infiltración de la MO y menor hematocrito y recuento plaquetario.[140]

B. Genes de la vía del BCR

Existe cada vez más evidencia sobre el posible papel de la vía del BCR en el crecimiento y supervivencia de las células de la MW, ya sea sola o en cooperación con la de MyD88.[192–198] Un estudio reciente ha demostrado que la señalización del BCR está constitutivamente activa en la MW, incluso en ausencia de alteraciones genómicas en los constituyentes de la vía,[199] hecho que justifica el éxito del tratamiento con inhibidores de BTK.[90–92]

CD79A y *CD79B* (*B-Cell Antigen Receptor Complex-Associated Protein Alpha and Beta Chains*) codifican las dos cadenas de una proteína transmembrana que forma un complejo con el BCR y que es responsable de generar una señal interna después de que el BCR reconozca al antígeno.[200] Las mutaciones en el dominio de activación (ITAM, por sus siglas del inglés *Immunoreceptor Tyrosin-based Activation Motif*) de estos genes han sido descritas en el LBDCG del subtipo ABC, donde promueven el crecimiento y la supervivencia a través de la activación de la cascada de señalización del BCR, que incluye Syk, PLCγ2 (*Phospholipase C Gamma 2*) y BTK.[154] Además, se ha visto que las alteraciones en *MYD88* y *CD79B* y la sobreexpresión de CD79B en estos linfomas se asocia con resistencia a ibrutinib.[201,202] En MW, las mutaciones en *CD79A* y *CD79B* afectan al 8-12% de los pacientes, principalmente también mutados en *MYD88*, aunque también se ha observado una mutación de *CD79B* en un paciente con *MYD88* salvaje.[120,139,178] Además, parece que estas alteraciones y las de *CXCR4* son mutuamente excluyentes, lo que indicaría que pueden existir dos poblaciones diferentes con *MYD88* mutado.[139] También se han descrito mutaciones en *BTK*, *PLCγ2* y *CARD11* (*CAspase Recruitment Domain-containing protein 11*) asociadas a progresión en pacientes tratados con ibrutinib.[203]

C. Otras mutaciones

Por último, otros genes afectados con mutaciones somáticas recurrentes son *MYBBP1A* (*V-Myb Avian Myeloblastosis Viral Oncogene Homolog Binding Protein 1A*) (7%), *TP53* (7%), *MLL2* (*Lysine Methyltransferase 2D*) (7%) o *TRAF3* (5%).[112,120,178] Esto significa que, a pesar de su monoclonalidad, las células tumorales albergan una variabilidad importante que podría ser responsable de la heterogeneidad clínica de la MW. Sin embargo, hasta ahora no se ha encontrado una correlación clara entre el perfil mutacional y el comportamiento clínico, ya que ningún estudio previo diferenciaba entre las formas indolentes y sintomáticas de la enfermedad.

3. Transformación a linfoma B difuso de célula grande

3.1. Evolución de Gammapatía monoclonal de significado incierto-MW asintomática-MW sintomática

Los pacientes con GMSI-IgM o MWA no presentan ningún dato clínico de enfermedad. Sin embargo, están asociados con un mayor riesgo de progresión a enfermedad sintomática con necesidad de tratamiento. En el caso de los pacientes con GMSI existe una tasa de progresión de aproximadamente 1,5% al año. Los estudios de seguimiento a largo plazo han identificado la concentración inicial de proteínas monoclonales y los niveles de albúmina sérica como factores de riesgo significativos en la progresión.[204] Para los pacientes con MWA, el riesgo es sustancialmente mayor (10 veces), con una frecuencia anual del 12% aproximadamente durante los primeros 5 años y del 2% durante los 5 siguientes. Ello significa que el 75% de los pacientes requiere tratamiento a los 15 años de seguimiento. Entre los factores de riesgo se incluyen un porcentaje elevado de células linfoplasmocíticas en la MO, un nivel elevado de proteína monoclonal sérica, la presencia de anemia o el aumento de la B2M.[8,58,204,205]

La evolución de GMSI a MWA primero y a MWS después es probablemente un proceso de varias etapas en el que tienen lugar múltiples eventos genéticos capaces de convertir una condición pre-benigna en una enfermedad neoplásica. Como hemos visto anteriormente, la evidencia disponible sugiere que las alteraciones más frecuentes (*MYD88*, *CXCR4*) constituyen eventos tempranos en la patogénesis molecular, sin tener una influencia muy clara en la progresión de GMSI a enfermedad sintomática.[147,163,172–174] En cambio, se desconoce si el resto de mutaciones están ya presentes en GMSI o podrían formar parte de los mecanismos responsables de la evolución a MWS.

Igualmente, los resultados de los estudios del perfil de expresión génica no siempre encontraron diferencias entre las firmas moleculares de los estados precursores y la enfermedad sintomática.[141–143] Además, se ha demostrado que las células B clonales en los tres grupos de pacientes (GMSI, MWA y MWS) son fenotípicamente muy similares, detectándose el clon tumoral ya en las primeras etapas de la enfermedad.[142] La única diferencia notable ha sido la frecuencia de pacientes con alteraciones cromosómicas numéricas, que aumentó significativamente de GMSI (36%) a MWA (73%) y MWS (82%).[142] Más allá de eso, se desconocen los posibles mecanismos y la contribución de estos genes (mutados o desregulados) en la patogenia y progresión de la enfermedad.

3.2. Transformación de MW a LBDCG

El proceso de evolución de GMSI a MW suele ser permanente y no se detiene en la progresión a enfermedad sintomática. La transformación histológica a linfoma agresivo, comúnmente LBDCG, es un evento ampliamente establecido en la historia natural de los pacientes con linfomas indolentes, principalmente LF y LLC.[206–208] Aunque se trata de un proceso lento, la disponibilidad de terapias más eficientes ha permitido que los pacientes vivan más tiempo, por lo que la transformación final a LBDCG se ha convertido en un problema en aumento que, en el caso de la MW/LLP afecta hasta a el 10% de los pacientes.[26,209–213] A esto se añade el problema de que el LBDCG después de MW u otro SLP-B indolente es normalmente muy resistente a la terapia, siendo la principal causa de muerte en estos pacientes, y está asociado con un peor pronóstico y menor supervivencia desde el momento de la transformación (~2 años) que en el caso de los pacientes con LBDCG *de novo*.[213–216]

La transformación puede ocurrir en cualquier momento durante el curso de la enfermedad: en el diagnóstico, antes del tratamiento, durante el tratamiento e incluso 20 años después del diagnóstico inicial,[213] sin que se haya observado ningún rasgo clinicopatológico o factor de riesgo indicativo. Tampoco se han llevado a cabo estudios genómicos en pacientes con MW transformada para identificar algún marcador biológico que pudiera explicar cierta susceptibilidad. A diferencia del LF y la LLC, donde el proceso biológico de transformación a linfoma agresivo se ha estudiado a fondo, los datos de otros SLP-B indolentes son muy limitados.[206,217–225] En el caso de la MW todavía no se han descrito las causas de transformación, y aunque algunos estudios han puesto de relieve un posible papel etiológico en el tratamiento previo con análogos de purinas, cada vez parece más claro que la transformación histológica es un fenómeno complejo y heterogéneo.[212,226] Los mecanismos potencialmente implicados en la transformación se comentan a continuación.

3.2.1. Analogía biológica entre la MW y el LBDCG

La transformación de MW a LBDCG resulta de particular interés, especialmente por tratarse de entidades genéticamente muy distintas, en apariencia. La MW parece una enfermedad más homogénea con una mutación muy recurrente (*MYD88* L265P) y alteraciones secundarias poco frecuentes.[146,178] Por el contrario, el LBDCG alberga una amplia gama de mutaciones somáticas y lesiones genéticas muy diversas que afectan a numerosas vías intracelulares, convirtiéndolo en una entidad intrínsecamente mucho más compleja.[227–229]

Sin embargo, ambas comparten ciertos aspectos moleculares, como la alteración de algunas vías de señalización intracelular que regulan el desarrollo normal y la función de los linfocitos B, como son las vías de TLR, BCR y NF-κB (Figura 6).

A. Vía de los TLR

El descubrimiento de las mutaciones de *MYD88* en un 39% de los LBDCG de subtipo ABC sirvió para identificar la implicación de la desregulación de la vía de los TLR en linfomagénesis. Posteriormente, se demostró que la proteína MyD88 mutada promovía la señalización de NF-κB y JAK/STAT3, confiriendo una ventaja selectiva en la supervivencia celular. Además, la inhibición de la señalización de MyD88 disminuía la actividad de NF-κB y la supervivencia de las líneas celulares que expresaban la mutación, por lo que se sugirió su papel como mutación *driver* o conductora.[154] En la misma línea, se ha demostrado que MyD88 mutada favorece el crecimiento y la supervivencia de las células de la MW a través de NF-κB,[90] considerándose también como un evento oncogénico

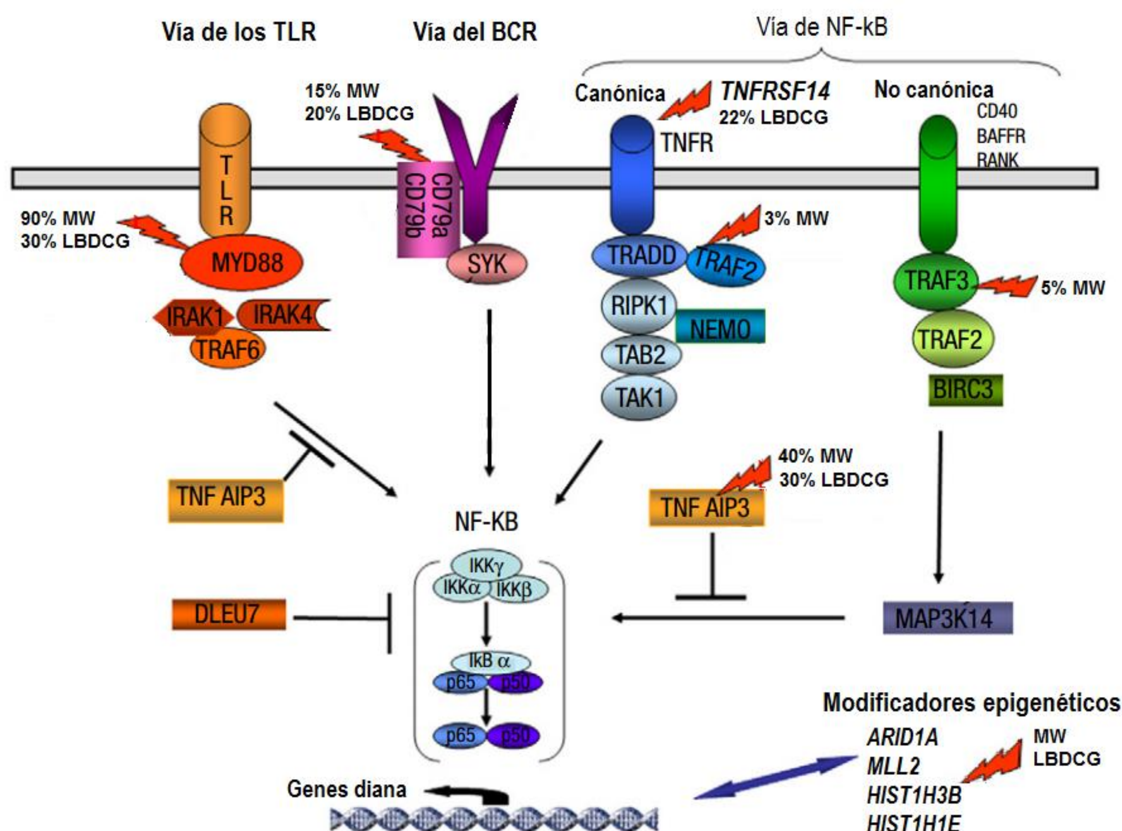


Figura 6. Vías fisiopatológicas comunes a MW y LBDCG (modificada de Poulain et al., *Clin Lymphoma Myeloma Leuk* 2013). La secuenciación del genoma completo ha permitido identificar las vías y mecanismos afectados por alteraciones moleculares recurrentes (representadas con un rayo) en MW y LBDCG. La alteración de la señalización de las vías de TLR, BCR y NF-κB así como de los mecanismos de regulación epigenética es común en la patogénesis de ambas neoplasias y podría estar implicada en el proceso de transformación de MW a LBDCG.

temprano para el desarrollo de esta enfermedad. Por ello, la mutación *MYD88* L265P constituye un componente crítico en la patogénesis de ambas entidades.[230]

Además, en el LBDCG se han identificado mutaciones de pérdida de función en TNFRSF14 (*Tumor Necrosis Factor Receptor Superfamily Member 14*).[227,228] Esta proteína, al igual que otros receptores de la superfamilia del TNF transmite su señal a través de las proteínas TRAF (*Tumor Necrosis Factor Receptor Associated Factors*), que también se han encontrado mutadas en MW.[112]

B. Vía del BCR

El BCR y su vía de señalización intracelular asociada son fundamentales tanto para el desarrollo normal del linfocito B, como para coordinar su respuesta ante la estimulación antigénica. Las mutaciones somáticas que implican genes de la ruta del BCR son de las más frecuentes en pacientes con LBDCG.[227–229] Los estudios de NGS han identificado alteraciones en *CD79A*, *CD79B* y *CARD11* en aproximadamente el 10-15% de los casos, mientras que en otros tipos de linfoma son poco frecuentes.[229,231] Las mutaciones de *CD79A* y *CD79B* parecen ser exclusivas del subtipo ABC de LBDCG y afectan mayoritariamente a la región ITAM.[227,229] Cuando están presentes, aumentan la expresión en superficie del BCR debido a que se disminuye su internalización.[231] En CARD11, las alteraciones tienden a agruparse en el dominio helicoidal de la proteína, responsable de la oligomerización, provocando una ganancia de función.[232] El patrón de activación que resulta de las mutaciones en estos genes conduce a la señalización crónica de la vía del BCR, y se asocia con la activación constitutiva de NF-κB, cuya importancia está bien establecida en este tipo de linfomas. Los estudios funcionales han demostrado que la inactivación de las subunidades del BCR u otros componentes de esta vía resulta letal para el LBDGC, lo que indica su dependencia de las señales constitutivas del BCR.[231,232]

Como hemos dicho anteriormente, en MW también se han descrito mutaciones en *CD79B* y *CD79A*, así como la activación crónica de la señalización del BCR,[120,199] aunque aún no se ha establecido su papel en la patogenia de la enfermedad.

C. Vía de NF-κB

Muchas de las vías de señalización importantes para la homeostasis normal de las células B que se encuentran alteradas en las células tumorales contribuyen a la activación constitutiva de NF-κB, considerándose por ello uno de los principales mecanismos efectores en los tumores de células B.[233] Así, se ha descrito la afectación de esta vía como un mecanismo de transformación en el LF.[221] La activación de la familia de factores de transcripción de NF-κB da como resultado su translocación

al núcleo y la subsiguiente regulación de la transcripción de una amplia gama de genes diana con implicaciones en la oncogénesis. En el LBDCG, las mutaciones de *CARD11*, *CD79A*, *CD79B* y *MYD88* suponen la activación de NF-κB en el ~30-50% de los casos.[154,231,232] Esto sugiere la existencia alteraciones adicionales como las de *TNFAIP3*, un regulador negativo de la señalización,[234] que puede presentar inactivaciones bialélicas, mutaciones y/o deleciones, en aproximadamente un 30% de los casos, causando la activación de la vía.[235] En MW también se han descrito alteraciones que afectan a proteínas clave de esta ruta como TRAF3 (5% de los pacientes), TRAF2 (3%) y TNFAIP3 (40-60%).[112,115,178]

D. Modificaciones epigenéticas

La acetilación y metilación de histonas es una modificación covalente de las colas de las histonas que es capaz de modular la estructura de la cromatina y reclutar factores de transcripción. El diverso grupo de proteínas responsables de estas modificaciones son las histona acetiltransferasas (HAT), las histona deacetilasas (HDAC) y las histona metiltransferasas (HMT). Alteraciones en este grupo de genes así como en otros reguladores epigenéticos han sido identificadas tanto en MW (por ejemplo en *ARID1A y MLL2*) como en LBDCG (*HIST1H3B*, *Histone Cluster 1 H3 Family Member B*, y *MLL2*),[146,178,228] aunque sus implicaciones en linfomagénesis siguen siendo en gran parte desconocidas.

Dada la existencia de eventos patogénicos comunes a ambas entidades, se ha pensado que la transformación histológica podría ocurrir dentro del clon original de células B como consecuencia de la adquisición de alteraciones genéticas adicionales. Sin embargo, los factores de riesgo para desarrollar LBDCG y los mecanismos moleculares de la transformación aún se desconocen, por lo que resultaría muy interesante estudiar este proceso con el objetivo de facilitar el desarrollo de estrategias terapéuticas para mejorar el pronóstico de estos pacientes.

Hipótesis de trabajo

La macroglobulinemia de Waldenström (MW) es un linfoma B indolente, poco común e incurable con la terapia actual. Durante su evolución pueden identificarse tres estados clínicamente diferentes: gammapatía monoclonal de significado incierto IgM (GSMI-IgM), MW asintomática (MWA) y MW sintomática (MWS). Los avances de los últimos años han permitido aumentar nuestro conocimiento sobre la biología de la enfermedad. La secuenciación del genoma completo y los estudios posteriores han identificado la mutación *MYD88* L265P como un evento molecular presente en la mayoría de los casos (~91%), siendo muy específico de la MW en comparación con el resto de trastornos linfoproliferativos. También se han descrito otras alteraciones recurrentes, como las mutaciones de *CXCR4* (29%) y *ARID1A* (17%). Asimismo, los estudios de expresión génica han contribuido a la caracterización del perfil transcriptómico de las células tumorales de la MW.

Toda esta información está ayudando a establecer una firma genómica que sirve para comprender mejor las bases etiopatogénicas de la enfermedad. Así, la transición de GMSI-IgM a MWS se basa en la adquisición de múltiples alteraciones genéticas necesarias para la progresión de un estado pre-maligno a una enfermedad neoplásica. Con el tiempo, este proceso de evolución puede ir más allá de la MWS y dar lugar a la transformación a formas más agresivas, como el linfoma B difuso de célula grande (LBDCG). Estos pacientes suelen ser muy resistentes a la terapia y presentan un pronóstico adverso.

Las causas biológicas y los mecanismos patogénicos responsables de la heterogeneidad clínica de la MW, así como su implicación en la evolución de la enfermedad a formas más activas aún se desconocen.

En este contexto, las hipótesis de esta tesis son:

a. La mutación *MYD88* L265P se postula como un marcador molecular específico de la MW que puede utilizarse en un contexto clínico para confirmar su diagnóstico, así como para evaluar la respuesta a la terapia y monitorizar los niveles de enfermedad mínima residual.

b. La mutación *MYD88* L265P puede detectarse mediante una herramienta sensible, barata y sencilla, como la PCR en tiempo real con cebadores específicos de alelo, que se podría utilizar en el diagnóstico de rutina de los trastornos linfoproliferativos B.

c. La variabilidad en la presentación de la enfermedad se debe a las características biológicas del clon tumoral: presencia de mutaciones en diferentes genes o variaciones en el perfil de expresión génica. Un estudio genómico y transcriptómico de la MW, distinguiendo entre las tres etapas de la enfermedad (GMSI-IgM, MWA y MWS), permitiría identificar los mecanismos moleculares que diferencian las formas indolentes de las sintomáticas o son capaces de predecir la progresión.

d. La transformación de MW a LBDCG es un proceso regido por anomalías genéticas. Presumimos que algunas de estas alteraciones están ya presentes al diagnóstico en una proporción detectable con las metodologías actuales de secuenciación masiva. Su identificación permitirá definir marcadores biológicos que predigan el riesgo de transformación o confieran mayor susceptibilidad para el desarrollo del LBDCG. El estudio completo de la transición de MW a linfoma favorecerá la investigación de nuevas estrategias para el diagnóstico y el desarrollo de terapias dirigidas.

Objetivos

Mediante el presente trabajo doctoral, pretendemos mejorar nuestro conocimiento acerca de la macroglobulinemia de Waldenström, y con esta premisa, nos planteamos los siguientes objetivos:

1) PRIMER TRABAJO: ESTUDIO DE LA MUTACIÓN *MYD88* L265P

- Evaluar la presencia de la mutación *MYD88* L265P en una serie de pacientes con MW y síndromes linfoproliferativos B estrechamente relacionados, utilizando una técnica de PCR altamente sensible y específica.
    - Establecer la frecuencia de la alteración en otros trastornos distintos de la MW con el fin de determinar si puede utilizarse para el diagnóstico diferencial.
    - Evaluar muestras secuenciales de pacientes tras la terapia con el fin de encontrar una relación entre la reducción o desaparición de la mutación y la respuesta y supervivencia, estableciendo su potencial capacidad predictiva de recaída y su utilidad como diana de enfermedad mínima residual.
    - Dilucidar por qué existen casos de MW con un *MYD88* de tipo salvaje y cómo esto puede afectar a la presentación de la enfermedad, la respuesta al tratamiento y la supervivencia.

2) SEGUNDO TRABAJO: DESARROLLO DE UNA TÉCNICA SENCILLA, SENSIBLE Y DE BAJO COSTE PARA LA DETECCIÓN DE LA MUTACIÓN *MYD88* L265P

- Diseñar y validar una PCR alelo específica en tiempo real (ASO-RQ-PCR) para detectar la mutación *MYD88* L265P.
    - Evaluar la reproducibilidad y sensibilidad de la técnica mediante un estudio de diluciones a partir de una muestra positiva previamente conocida.
    - Obtener la curva estándar para cuantificar la carga tumoral de las muestras.
    - Evaluar la aplicabilidad de la metodología analizando pacientes positivos ya conocidos.
    - Comparar la carga tumoral estimada por la ASO-RQ-OCR con la establecida por citometría de flujo (CMF).

3) TERCER TRABAJO: CARACTERIZACIÓN DE LAS GAMMAPATÍAS MONOCLONALES IGM

- Comprender los mecanismos responsables de la heterogeneidad en el comportamiento de la enfermedad entre diferentes pacientes con una condición similar (gammapatía monoclonal IgM).

- Describir el perfil mutacional en todos los pacientes en el momento del diagnóstico para encontrar diferencias entre las tres entidades (GMSI-IgM, MW asintomática y MW sintomática) que pudieran explicar la variabilidad en la presentación de la enfermedad.
- Esclarecer si las anomalías están presentes desde el inicio de la patogénesis (GMSI-IgM) y pueden condicionar la evolución.
- Encontrar diferencias en el perfil de expresión génica que pudieran estar asociadas con los diferentes diagnósticos.
- Recoger toda la información del clon tumoral y relacionarla con las características del paciente con el fin de identificar los mecanismos que condicionen el desarrollo de los síntomas y la respuesta a la terapia.
- Definir perfiles de alto riesgo que pudieran predecir la progresión de pacientes indolentes a sintomáticos o un peor pronóstico en pacientes sintomáticos, estableciendo un modelo de pronóstico clínico/molecular.
- Determinar las posibles aplicaciones diagnósticas, pronósticas o terapéuticas basadas en estos hallazgos.

4) CUARTO TRABAJO: EVALUAR LA TRANSFORMACIÓN DE MW A LBDCG

- Caracterizar genéticamente el proceso de transformación de MW a LBDCG mediante un estudio de secuenciación de todo el exoma.
  - Describir las anomalías más frecuentes presentes al diagnóstico en los pacientes que evolucionan a formas agresivas.
  - Encontrar las similitudes y diferencias en el perfil mutacional al diagnóstico y en la transformación, estableciendo el posible papel en el proceso de las mutaciones comúnmente descritas en MW (*MYD88*, *CXCR4*, *ARID1A, etc*.).
  - Determinar qué alteraciones presentes solo en las muestras de LBDCG podrían haber sido responsables de la transformación agresiva y evaluar si se encontraban ya al diagnóstico en una pequeña proporción de la población tumoral.
  - Definir posibles marcadores de progresión que identifiquen a los pacientes con mayor riesgo de transformación con el objetivo de poder monitorizar el curso de la enfermedad y diseñar estrategias de prevención.
  - Estudiar la dinámica de la evolución clonal en la MW que se transforma a LBDCG y establecer el patrón evolutivo de este proceso.

Material, Métodos & Resultados

En esta sección se describen los pacientes, materiales y métodos empleados, así como los resultados obtenidos en relación con cada uno de los objetivos planteados.

Para ello, se incluyen los cuatro artículos científicos originales redactados y publicados como consecuencia del trabajo realizado.

Cada uno de los artículos referidos está precedido de un breve resumen en castellano que pretende facilitar una revisión rápida de la información contenida en los mismos.

1) PRIMER TRABAJO: ESTUDIO DE LA MUTACIÓN *MYD88* L265P

MYD88 L265P is a marker highly characteristic of, but not restricted to, Waldenström's macroglobulinemia.

Cristina Jiménez, Elena Sebastián, Mª del Carmen Chillón, Pilar Giraldo, José Mariano Hernández, Fernando Escalante, Tomás González-López, Carmen Aguilera, Alfonso García de Coca, Ilda Murillo, Miguel Alcoceba, Ana Balanzategui, Mª Eugenia Sarasquete, Rocío Corral, Luis A. Marín, Bruno Paiva, Enrique M. Ocio, Norma C. Gutiérrez, Marcos González, Jesús F. San Miguel and Ramón García-Sanz.

Introducción

La macroglobulinemia de Waldenström (MW) es una neoplasia hematológica poco frecuente que representa una etapa intermedia entre los síndromes linfoproliferativos (SLPs) y las discrasias de células plasmáticas. Estudios recientes han descrito la presencia de la mutación somática L265P en el gen *MYD88* en el 91% de los pacientes. Esta misma alteración también se ha encontrado, aunque en menor frecuencia, en otros SLPs, como el linfoma B difuso de célula grande (LBDCG) del tipo de célula B activada (ABC) (14-29%), la gammapatía monoclonal de significado incierto IgM (GMSI-IgM, 10%) o el linfoma de la zona marginal (LZM, 7%). Sin embargo, la mayoría de estos estudios se han llevado a cabo con técnicas de baja sensibilidad, como la secuenciación convencional, lo que podría haber llevado a subestimar la frecuencia de mutación.

Pacientes y métodos

Se evaluaron muestras de 38 donantes sanos y de 117 pacientes con MW y 273 con SLPs de célula B relacionados, distribuidos de la siguiente manera: 14 LZM esplénicos, 35 tricoleucemias, 48 LBDCG de tipo ABC, 28 de tipo centro germinal y 13 no subclasificados, 39 leucemias linfáticas crónicas (LLC) (16 con un componente monoclonal asociado), 31 GMSI-IgM, 25 GMSI IgA/IgG, 24 mielomas múltiples (MM) (incluyendo 3 con isotipo IgM), 6 amiloidosis, 9 linfomas linfoplasmocíticos (LLP) y 1 trastorno relacionado con IgM (neuropatía periférica).

Se analizó la presencia de la mutación *MYD88* L265P en el ADN con una variante comercial de la PCR específica de alelo en tiempo real basada en el sistema de mutación refractario a la amplificación por PCR: 'qBiomarker Somatic Mutation Assay for MYD88_85940' (SABiosciences, Qiagene Co., Hilden, Alemania). Para evaluar la sensibilidad de la técnica, se realizó un estudio de diluciones con una muestra de un paciente positivo con un 60% de células IgM-lambda monoclonales por citometría de flujo, diluida en ADN de un control sano carente de la mutación.

Resultados

Análisis de la mutación

El estudio de diluciones demostró una sensibilidad de $2,5x10^{-3}$ (correspondiente a ~1 célula mutada entre 1000 células normales) que se consideró suficiente para evaluar las muestras (>1% de infiltración tumoral). Además, nos permitió obtener una curva estándar para cuantificar la carga tumoral mediante el método del ΔCT.

En cuanto al estado mutacional, ninguna de las 38 muestras de donantes sanos presentó la alteración *MYD88* L265P, mientras que sí se encontró en la mayoría de los pacientes con MW (101/117, 86%, sin diferencias entre los casos sintomáticos y asintomáticos) y GMSI-IgM (27/31, 87%). Además, hubo 3/14 (21%) LZM esplénicos y 9/48 (19%) LBDCG de tipo ABC mutados. El resto de pacientes fueron negativos (LBDCG de tipo centro germinal, LBDCG no subclasificados, tricoleucemias, LLC, GMSI IgA o IgG, MM, amiloidosis, LLP y neuropatía periférica relacionada con IgM).

Estudio de enfermedad mínima residual

En los pacientes mutados, la estimación del número de células clonales fue comparable a los resultados de citometría de flujo, mostrando un coeficiente de correlación de Pearson, R, de 0,548 ($p=2.3x10^{-17}$). Además, debido a su elevada sensibilidad, se decidió probar como herramienta para monitorizar la terapia y la enfermedad residual en MW. Para ello, se evaluaron seis pacientes seleccionados que respondieron muy bien al tratamiento (>90% de reducción del componente monoclonal) y se compararon los resultados obtenidos con la citometría de flujo. La PCR se mantuvo positiva en cinco de ellos, aunque indicando un número muy bajo de células tumorales, similar al de citometría de flujo.

Diferencias entre casos mutados y no mutados en gammapatías IgM (GMSI-IgM, MW asintomática y MW sintomática)

a. Diferencias biológicas

El estudio inmunofenotípico de las células tumorales reveló diferencias menores entre ambos grupos, principalmente en cuanto a la expresión de FMC7 (positivo en el 64% de los casos no mutados frente al 25% de los mutados, p=0,015), CD23 (positivo en el 25% de no mutados frente al 39% de mutados, p=0,098) y CD27 (positivo o fuertemente positivo en el 75% de los no mutados con un patrón homogéneo, frente al 55% de los pacientes mutados y con un patrón heterogéneo p=0,08).

Con respecto a la presencia de mutaciones somáticas en la región variable del gen de la cadena pesada de las inmunoglobulinas (*IGHV*), se encontró que había un 97% de pacientes en el grupo de los *MYD88* mutados con hipermutación somática (HMS) (es decir, con una desviación >2% de la línea germinal) en comparación con el 57% de los pacientes que tenían un *MYD88* de tipo salvaje (p=0,012). También destacó que el repertorio del *IGHV* fue más sesgado en los casos *MYD88* positivos que en los no mutados, ya que el gen IGHV3-23 estaba seleccionado en el 27% de los casos con *MYD88* L265P frente a un 9% de los pacientes sin la mutación (p=0,014).

No se observaron diferencias en el patrón de infiltración de médula ósea, el número de células B o la presencia de anomalías citogenéticas detectadas por FISH.

b. Diferencias clínicas

En cuanto a la posible relación con las características clínicas, de nuevo encontramos pocas diferencias entre los pacientes con y sin mutación. Los casos positivos tuvieron un componente monoclonal ligeramente superior (2,62±2,02 frente a 1,77±2,33 g/dl, p=0,009), niveles séricos de lactato deshidrogenasa más bajos (265±93 vs. 371±189, p=0,002) y menor incidencia de linfocitosis (5 vs. 24%, p=0,022). Este cuadro es coherente con la ausencia de diferencias estadísticamente significativas en términos de tiempo libre de tratamiento, tiempo hasta la progresión y supervivencia global. Tampoco se encontraron diferencias en las tasas de respuesta o en la supervivencia libre de progresión y la supervivencia global después de la terapia entre los pacientes que requirieron tratamiento. Finalmente, no hubo diferencias con respecto al riesgo de progresión de formas asintomáticas a sintomáticas en función del estado de *MYD88*.

Conclusiones

En este estudio hemos demostrado que la mutación *MYD88* L265P está presente en el 87% de los pacientes con MW, lo que la convierte en un marcador molecular muy característico de la enfermedad, especialmente si consideramos que se encontró con mucha menor frecuencia en otros SLPs relacionados. La sensibilidad analítica del método empleado fue crítica ya que estos trastornos se suelen caracterizar por una baja infiltración tumoral.

Globalmente, nuestros resultados no apoyan la idea de que la mutación de *MYD88* separe a un subgrupo de pacientes con un perfil singular, aunque sí observamos que los casos negativos parecen tener una firma de MW menos típica (menor pico monoclonal, linfocitosis más frecuente, niveles séricos más altos de lactato deshidrogenasa, inmunofenotipo atípico y menos HMS).

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ORIGINAL ARTICLE

MYD88 L265P is a marker highly characteristic of, but not restricted to, Waldenström's macroglobulinemia

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We evaluated the MYD88 L265P mutation in Waldenström's macroglobulinemia (WM) and B-cell lymphoproliferative disorders by specific polymerase chain reaction (PCR) (sensitivity $\sim 10^{-3}$). No mutation was seen in normal donors, while it was present in 101/117 (86%) WM patients, 27/31 (87%) IgM monoclonal gammapathies of uncertain significance (MGUS), 3/14 (21%) splenic marginal zone lymphomas and 9/48 (19%) non-germinal center (GC) diffuse large B-cell lymphomas (DLBCLs). The mutation was absent in all 28 GC-DLBCLs, 13 DLBCLs not subclassified, 35 hairy cell leukemias, 39 chronic lymphocytic leukemias (16 with M-component), 25 IgA or IgG-MGUS, 24 multiple myeloma (3 with an IgM isotype), 6 amyloidosis, 9 lymphoplasmacytic lymphomas and 1 IgM-related neuropathy. Among WM and IgM-MGUS, MYD88 L265P mutation was associated with some differences in clinical and biological characteristics, although usually minor; wild-type MYD88 cases had smaller M-component (1.77 vs 2.72 g/dl, $P = 0.022$), more lymphocytosis (24 vs 5%, $P = 0.006$), higher lactate dehydrogenase level (371 vs 265 UI/L, $P = 0.002$), atypical immunophenotype (CD23 − CD27 + + FMC7 + +), less Immunoglobulin Heavy Chain Variable gene (IGHV) somatic hypermutation (57 vs 97%, $P = 0.012$) and less *IGHV*3–23 gene selection (9 vs 27%, $P = 0.014$). These small differences did not lead to different time to first therapy, response to treatment or progression-free or overall survival.

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Keywords: MYD88 mutations; Waldenström's macroglobulinemia; IgM monoclonal gammapathies; diagnosis; prognosis; immunophenotyping

INTRODUCTION

Waldenström's macroglobulinemia (WM) is a rare hematological malignancy with an incidence of 3.6–5.5 cases per million person–years in the EU and US.[1–4] Despite its low frequency, WM is particularly interesting due to its singular pathogenic features, which represent the most genuine intermediate stage between lymphoproliferative disorders (LPDs) and plasma cell dyscrasias, in which clinical and biological features mimic the two ends of the spectrum of such disorders. Moreover, the study of WM may be relevant to better understand the genetic mechanisms involved in those LPDs derived from abnormalities in the terminal B-cell differentiation process.

Although most WM patients have been reported to have genetic aberrancies, few recurrent chromosomal changes have been described in this disease, probably due to the difficulty of obtaining tumor metaphases for karyotype studies.[5] Whole-genome sequencing has confirmed that virtually all WM patients have molecular DNA alterations.[6] In particular, the L265P mutation at the *MYD88* gene (38182641 in chromosome 3p22.2), which results in a leucine to proline change at the L265P amino acid, was reported in 26 of the 30 WM patients initially evaluated with whole-genome sequencing.[6] An extended evaluation of this mutation in a larger series of patients showed it to be present in 91% (49/54) of WM patients, and it was suggested that in IgM monoclonal gammapathies it looked to be almost exclusive of lymphoplasmacytic bone marrow (BM) infiltrative forms of the disorder. This was based on the fact that the mutation was very infrequent in IgM monoclonal gammopathy of uncertain significance (MGUS, 10% of cases) and in marginal zone lymphomas (MZLs, 7%), and it was completely absent from multiple myeloma patients (0/10) and healthy donors.[6] Apart from this, MYD88 L265P mutation is also present in a fraction of diffuse large B cell lymphomas (DLBCLs) of the activated B-cell type (14–29%)[7–9] and leg type (69%),[10] primary central nervous system lymphoma (36–38%)[11,12] and mucosa-associated lymphoid tissue lymphoma (9%).[7] In addition, most of these studies have been carried out with conventional sequencing techniques, such as Sanger sequencing, which has a relatively low sensitivity and requires at least 20–30% of cells to carry the mutation to be able to detect it among the vast majority of normal alleles. As many of the aforementioned tumors can have low infiltration in the tissues available for analysis, this technical limitation could lead to underestimate the mutation frequency. Such an effect is especially important in some entities, such as IgM-MGUS, where the percentage of true monoclonal cells in the BM aspiration could be as low as $<1\%$.[13]

Polymerase chain reaction with allele-specific oligonucleotides (ASO-PCR) is a technique that can discriminate low levels of the mutant sequence against a background high in wild-type DNA.[14] In ASO-PCR, the primer pair is designed so that one of the 3′ ends

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perfectly matches the variant nucleotide in the target sequence—the mutated sequence, in this case. It exploits the lack of 3′ exonuclease activity of *Taq* polymerase, therefore, the enzyme extends the primers bound to their target sequences very inefficiently when the 3′ base is mismatched. Accordingly, the extension of such primers in PCR is a rare event when the mutation is not present, and amplification does not occur or is considerably retarded. The simultaneous use of a TaqMan probe in the same reaction enables it to be quantified in real time. Thus, the use of this method should enable the mutation to be identified with high specificity and sensitivity, and the number of mutated and wild-type alleles in the sample to be estimated.

The aim of the present work was to evaluate the presence of the MYD88 L265P mutation in a series of WM and closely related B-cell LPDs, with a highly sensitive and specific PCR technique.

PATIENTS AND METHODS

We evaluated DNA extracted from whole-peripheral blood of 38 normal donors, tumor specimens (whole samples, mainly BM or lymph nodes) from 117 WM patients and 273 related B-cell LPDs, distributed as follows: 14 splenic MZLs, 35 hairy cell leukemias, 48 non-germinal center (GC) DLBCLs, 28 GC-DLBCLs, 13 DLBCLs not subclassified, 39 chronic lymphocytic leukemias (CLLs) (16 of which had a small monoclonal component observed by conventional electrophoresis), 31 IgM-MGUS, 25 IgA/IgG-MGUS, 24 multiple myeloma (including 3 with an IgM isotype), 6 amyloidosis, 9 lymphoplasmacytic lymphomas and 1 IgM-related disorder (peripheral neuropathy).

Cases were diagnosed using standard criteria of the WHO classification (2008 update), with a review that included the recently published recommendations and concepts for application.[15] For WM and related disorders, these criteria consider what the IWMG agreed in the second International Workshop held in 2002 in Athens, which require the absence of BM infiltration by morphological examination of the bone biopsy in the absence of clinical, morphological or immunophenotypic features of other LPDs.[16] DNA was extracted by conventional methods: manually with the DNAzol reagent (MRC, Cincinnati, OH, USA), or automatically with the MagNA Pure system (Roche Diagnostics, Mannheim, Germany). All samples were evaluated by flow cytometry to ensure a minimum frequency of 1 tumor cell per 100 normal/reactive cells.[13,17]

Immunophenotypic evaluation was done using conventional methods, panels of monoclonal antibodies previously described by our group,[13,18] and following the general recommendations of the EuroFlow group for the immunophenotypic evaluation of hematological malignancies.[19–21] Deletions of 6q were assessed in IgM-MGUS and WM by either simple interphase FISH performed on cell nuclei from whole-BM samples ($n = 53$) or CD19-selected cells ($n = 80$) using our previously published techniques.[22] A minimum of 100 cells were analyzed in all patient samples using Vysis scoring criteria (Vysis, Downers Grove, IL, USA). The cutoff point for the identification of alteration was set at $\geqslant$10% cells with abnormal signal.

DNA was analyzed for the presence of the MYD88 L265P with a commercially available variant of real-time ASO-PCR that combines an allele-specific amplification with Amplification Refractory Mutation System (ARMS) technology and hydrolysis probe detection: 'qBiomarker Somatic Mutation Assay for MYD88_85940' (SABiosciences, Qiagene Co., Hilden, Germany). Dilution experiments were done using a BM sample from a positive patient with 60% of monoclonal IgM-lambda cells, as evaluated by flow cytometry. The presence of monoclonal VDJH rearrangements was evaluated by PCR amplification with the BIOMED-2 strategy[23] and well-established sequentiation methods.[24]

RESULTS

4.1 Dilution studies

The ASO-PCR was first evaluated using a WM patient sample, with 60% tumor cells counted by flow cytometry, in which the heterozygous MYD88 L265P mutation had been previously found by conventional Sanger sequencing. Then, we investigated the sensitivity of the method by analyzing the DNA from the patient diluted in DNA from a healthy control lacking the mutation. We found the usual sensitivity to be 2.5×10^{-3} (Supplementary Figure 1A), which corresponds to $\sim$1 mutated cell among 1000 normal cells. As all samples included in the study had $>$1% of tumor cells, this sensitivity was considered sufficient to evaluate these samples correctly.

4.2 Tumor cell quantification by PCR and the relationship with flow cytometry

For each case, the ASO-PCR provided a CT value that can be considered to be the cycle in which the fluorescence began to be distinguished from the background in the PCR machine. This CT was always $>$45 in controls and negative cases, and between 23 and 40 in positive cases. There was an inverse correlation between the CT value and the percentage of tumor cells. For more accurate tumor cell estimation independent of the DNA quality, the CT was normalized using the CT increment (ΔCT) with respect to the reference CT (ΔCT = CT value − reference CT value), obtained from a non-variable region of the same gene evaluated in the same PCR. Among MYD88 L265P positive patients, there were 81 cases in which the flow -cytometry estimation of tumor cells was available, with a sensitivity of 10^{-3}–10^{-4}; in these cases, the corresponding estimates of the number of clonal cells measured by flow cytometry and by the ΔCT had a Pearson correlation coefficient, *R*, of 0.548 ($P = 2.3 \times 10^{-17}$, Supplementary Figure 1B).

4.3 Mutational status

None of the 38 samples from normal donors featured the MYD88 L265P mutation, whereas it was present in the majority of WM patients (101/117 cases, 86%) and IgM-MGUS (27/31, 87%). In addition, the mutation was present in 3/14 (21%) splenic MZLs and 9/48 (19%) non- GC-DLBCLs. Finally, the mutation was not present in any of the 28 GC-DLBCLs, 13 DLBCLs not subclassified, 35 hairy cell leukemias, 39 CLL (even though 16 of them had a small monoclonal component), 25 IgA or IgG-MGUS, 24 MM (including 3 with an IgM isotype), 6 amyloidosis, 9 lymphoplasmacytic lymphomas and 1 IgM-related peripheral neuropathy. Interestingly, none of the non-IgM-MGUS or lymphoplasmacytic lymphomas harbored the mutation, nor did the CLL or multiple myeloma cases, including those with an IgM monoclonal component. By contrast, some splenic MZLs (all with an IgM monoclonal component and BM infiltration, but without a clear lymphoplasmacytic pattern) and DLBCLs (mainly of the non-GC type) displayed the mutation. Considering all of the evaluated cases, the mutation seems to be highly specific to WM and IgM-MGUS patients (Table 1).

Of the patients fulfilling the WM criteria (IgM monoclonal component plus lymphoplasmacytic BM infiltration demonstrated by cytomorphological examination of a trephine bone biopsy), no major differences in MYD88 status were seen between symptomatic and asymptomatic cases: 49/55 (89%) asymptomatic cases and 53/62 (86%) symptomatic cases had the mutation ($P > 0.1$).

Among the 31 IgM-MGUS patients, 15 had monoclonal lymphoid cells by flow ($\sim$1% or less), whereas 16 patients had small numbers of lymphoid (CD19+) B cells, but with no detectable light chain restriction. The distribution of the MYD88 L265P mutation was similar: 27 mutated (13 with clonal detectable cells and 14 with no detectable monoclonal cells) vs 4 wild type (2 with clonal detectable cells and 2 with no detectable monoclonal cells). The presence of clonal VDJH or DJH rearrangements was evaluated in 14 MGUS cases: 13 with clonal amplification (10 VDJH and 3 DJH) and one with no detectable clonal rearrangement. Among these 14 cases, only one had a wild-type MYD88 gene that showed a complete VDJH rearrangement with a clonal pattern.

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Table 1. Relative frequency of the MYD88 L265P mutation among different B-cell LPDs

Entity	n	*MYD88 L265P*
Waldenström's macroglobulinemia	117	101 (86%)
IgM MGUS	31	27 (87%)
Non-GC diffuse large cell lymphoma	48	9 (19%)
Marginal zone lymphomas	14	3 (21%)
B-CLL (16 with monoclonal component)	39	0 (0%)
Hairy cell leukemia	35	0 (0%)
Multiple myeloma (three IgM)	24	0 (0%)
IgA/IgG-MGUS	25	0 (0%)
Lymphoplasmacytic lymphoma	9	0 (0%)
Amyloidosis	6	0 (0%)
IgM-related disorder (neuropathy + IgM component)	1	0 (0%)
Healthy volunteers	38	0 (0%)

Abbreviations: GC, germinal center; LPDs, lymphoproliferative disorders; MGUS, monoclonal gammapathies of uncertain significance.

4.4 Differences between mutated and unmutated cases in IgM gammapathies

Biological differences. We selected the patients with IgM-MGUS, asymptomatic WM or symptomatic WM, and compared MYD88-mutated- and unmutated patients in order to determine any differences between them. There were some differences in the immunophenotypic characteristics of the tumor cells between these two groups, although they were usually minor (Table 2). The only consistent difference corresponded to the FMC7 expression, as unmutated cells were more frequently positive than the mutated cases, whereas the CD23 + antigen sometimes present in WM was more typical of the mutated cases (Table 2). Interestingly, unmutated cases were usually positive or strongly positive for CD27 with a homogeneous pattern, whereas patients harboring the MYD88 L265P mutation usually exhibited a positive but heterogeneous pattern.

No differences were observed in the pattern of BM infiltration or in the number of BM B cells. In addition, this aspect was not affected by the type of disorder, and the detection rate was similar between MGUS, aWM and sWM despite that their infiltration rate was different (mean of 1.9, 9.7 and 22.7%, respectively). The only relevant difference concerning tumor load was that mutated cases usually had a higher percentage of BM plasma cells (BMPCs) than did unmutated cases (Tables 2 and 4).

With respect to the cytogenetic abnormalities detected by FISH, no important differences were observed in the presence of IgH translocations and del(6q) (Table 2). Thus, 44/117 WM cases harbored the 6q deletion, 37 of which (84%) had the mutation. However, considering the presence of somatic mutations of the *IGHV* gene involved in the VDJH rearrangement, we found that the MYD88-mutated group usually exhibited a much higher degree of somatic mutation compared with the wild-type cases (median, 9 vs 5%, $P < 0.01$). Accordingly, the final percentage of cases with SHM (> 2% deviation from the germline) was lower in the group with a wild-type *MYD88* gene (57%) than in the group harboring the MYD88 L265P mutation (97%, $P = 0.012$). Finally, it was also interesting to discover that the IGHV repertoire was more biased in mutated than wild-type MYD88 cases, whereby the *IGHV*3–23 gene, which is frequently present in WM, was found in 27% of mutated cases, whereas it was selected in only 9% of unmutated cases ($P = 0.014$, Table 2).

Clinical differences. Turning now to the potential clinical relationship with mutation status, we again found few differences between cases with and without the mutation, and those that were observed were not clearly consistent from a clinical point of view (Tables 3 and 4). Globally, cases with the mutation had a slightly higher monoclonal component (monoclonal peak and IgM

Table 2. Clinical and biological characteristics of the IgM-MGUS and WM according to the presence of the MYD88 L265P mutation

Characteristic	*Unmutated* n = *20*	*Mutated* n = *128*	P-(*value*)
CD103 +	0%	3%	NS
CD11b +	0%	5%	NS
BCL2 +	100%	100%	NS
CD10 +	0%	3%	NS
CD11c +	18%	14%	NS
CD138 +	0%	5%	NS
CD19 +	100%	100%	NS
CD20 +	100%	100%	NS
CD22 +	100%	100%	NS
CD22 + [w]	76%	91%	NS
CD23 +	25%	39%	0.098
CD24 +	15%	5%	NS
CD25 +	100%	100%	NS
CD27 +	75%[a]	55%[b]	0.08***
CD38 +	17%	31%	NS
CD45 +	90%	99%	NS
CD45RA +	100%	98%	NS
CD45RO +	0%	13%	NS
CD5 +	7%	9%	NS
CD56 +	0%	6%	NS
FMC7 + [s]	64%	25%	0.015
HLA-DR +	86%	100%	NS
SIgM +	88%	98%	NS
Del(6q)	17%	30%	NS
Mastocytes	33%	45%	NS
SHM (> 2% germline deviation)	57%	97%	0.012
IGHV3–23	9%	27%	0.014
BMPC < 0.5%	30%	73%	0.011

Abbreviations: BMPC, bone marrow plasma cells; WM, Waldenström macroglobulinemia; w, weak positivity; s, strong positivity. ***$P < 0.05$ for heterogeneity. [a]Homogeneous. [b]Hetereogeneous.

Table 3. Clinical and biological characteristics of the IgM-MGUS and WM with respect to the presence of the MYD88 L265P mutation

	Unmutated *n* = *20*	*Mutated* *n* = *128*	P (*MW*)
Age (years)	67 ± 13	69 ± 11	
ECOG	0.89 ± 1.26	0.65 ± 0.81	
Hemoglobin	11.6 ± 2.9	12.0 ± 2.4	
Lymphocytosis ($> 5 \times 10^9$/l)	24%	5%	0.022
Platelet $< 100 \cdot 10^9$/l	6%	6%	
Albumin (g/dl)	3.77 ± 0.60	3.67 ± 0.47	
Gammaglobulin > 30 g/l	23%	54%	0.032
Gamma (g/dl)	2.42 ± 2.39	3.06 ± 1.98	0.007
Total IgM	2736 ± 3118	3098 ± 2553	
Total IgG	751 ± 352	925 ± 507	
Total IgA	173 ± 126	148 ± 144	
M-component (g/dl)	1.77 ± 2.33	2.62 ± 2.02	0.009
ESR*	76 ± 39	79 ± 29	
Proteinuria	1.59 ± 7.29	0.47 ± 2.96	
Creatinine (mg/dl)	1.19 ± 0.60	1.06 ± 0.36	
LDH	371 ± 189	265 ± 93	0.002
B2M (mg/l)	3.66 ± 3.5	3.20 ± 2.45	
CPR higher than normal	50%	52%	

Abbreviations: ESR, erythrocyte sedimentation rate; LDH, lactate dehydrogenase; WM, Waldenström's macroglobulinemia.

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serum level), as well as erythrocyte sedimentation rate (ESR). Such differences were especially evident in cases with low clonality load (i.e., MGUS) (Tables 3 and 4). In addition, mutated cases had lymphocytosis infrequently (5 vs 24%, $P = 0.022$) and lower lactate dehydrogenase serum levels, but always within normal ranges (265 ± 93 vs 371 ± 189, $P = 0.002$; normal range between 220 and 460 U/ml). Taken together, the data suggest that there are no substantive differences in IgM monoclonal disorders with respect to their MYD88 status, although wild-type cases had a less typical WM than mutated cases. This picture is consistent with the absence of any statistically significant differences in terms of time to therapy, progression-free survival and overall survival (Figure 1). Actually, of the patients who finally required some treatment, the percentages achieving a complete or partial response (54 vs 45%), minor response/stable disease (15 vs 33%) or progressive disease (31 vs 22%) showed no statistically significant differences between cases with a wild-type or mutated *MYD88* gene, respectively. Moreover, progression-free survival after therapy and overall survival were similar for mutated and unmutated patients (Figure 1).

Regarding the risk of progression from asymptomatic to symptomatic forms of the disease, no differences were observed depending on the MYD88 status. In the specific case of the 31 IgM-MGUS patients, with a median follow-up of 11 years, only 2 patients have progressed into symptomatic WM at 32 and 39 months, both of them responding to therapy and being alive at 5.4 and 6.3 years since diagnosis. The two cases had the MYD88 L265P mutation and the CT became reduced at progression, which is equivalent to an increase in the number of tumor cells.

Minimal residual disease evaluation. Given the high sensitivity of the ASO-PCR for MYD88 mutation evaluation, it could be a very interesting tool for monitoring therapy and residual disease evaluation in WM. To assess this aspect, we evaluated six selected patients who responded very well (>90% M-component reduction) to the therapy and compared the results yielded by flow cytometry (table 5). PCR persisted positive in five of them, although with an amplification curve with a very high CT, identifying a very low number of tumor cells and with comparable results based on flow cytometry.

DISCUSSION

In this study, we have demonstrated that the MYD88 L265P mutation is present in 87% of WM patients, making this molecular abnormality a highly characteristic marker of the disease, especially if we consider that it was much less frequent in other related LPDs. Previous studies have used the Sanger method, which has a low sensitivity limit for the detection of the MYD88 L265P mutation, whereas the PCR approach employed here is more sensitive and, probably, more easily applied routinely in laboratories.

We first assessed the analytical sensitivity of our ASO-PCR in serial dilutions of DNA from a heterozygous MYD88 L265P sample with DNA from a MYD88 wild-type sample. The detection limit was 0.25% of diploid tumor cells. This is a critical aspect of the study, as immunoproliferative B-cell disorders, such as WM, amyloidosis, myeloma and MGUS, are frequently characterized by low tumor burden infiltration, especially in the BM,[25–28] meaning that sensitive techniques are required to evaluate them.

We then analyzed samples from 117 WM patients with a diagnosis confirmed by standard criteria and found that 101 were positive for the mutation (87% of the entire series), similar to other series.[6,29,30] Interestingly, no relationship was found between the positivity and the percentage of clonal cells present in the sample of source DNA, confirming that the tumor burden did not influence the detection capacity of this technique. In fact, patients with IgM-MGUS, who had ~1% median BM infiltration, were positive at a similar rate (27/31 cases, 89%), in contrast with the initially observed frequency of the mutation (only 10%).[6] This discordance can be easily explained by the low sensitivity of the Sanger sequencing method used in their initial report. This effect has been very well shown in the field of DLBCL, where the use of the Sanger sequencing (25% of sensitivity) yielded a lower efficiency than a PCR technique followed by high-resolution melting analysis (5% sensitivity).[31] In fact, using a more sensitive approach, the group from Boston and others have observed a mutation frequency close to 60% in IgM-MGUS.[30,32,33] In addition, the number of tumor cells could easily be estimated using a real-time ASO-PCR technique, as the CT value was correlated with the number of tumor cells detected by flow cytometry. This opens the

Table 4. Clinical and biological characteristics of IgM-MGUS, asymptomatic (aWM) and symptomatic WM (sWM) according to the presence of the MYD88 L265P mutation

	MGUS, n = 31		*aWM*, n = 55		*sWM*, n = 62	
	Unmutated	*Mutated*	*Unmutated*	*Mutated*	*Unmutated*	*Mutated*
Age (years)	70 ± 14	67 ± 11	64 ± 9	71 ± 9	72 ± 11	68 ± 12
Hemoglobin (g/dl)	12.8 ± 2.5	13.8 ± 1.6	14.2 ± 1.2	13.0 ± 1.3	10.5 ± 3.1	10.4 ± 2.3
Leukocytes (x 10^9/l)	7.02 ± 2.39	7.51 ± 2.30	7.73 ± 2.05	7.52 ± 3.50	12.28 ± 10.63	8.67 ± 8.98
Lymphocytes (x 10^9/l)	2.01 ± 0.60	2.14 ± 0.87	3.24 ± 1.20	2.57 ± 2.53	7.93 ± 9.40	2.87 ± 3.86
Albumin (g/dl)	3.825 ± 0.675	3.968 ± 0.409	3.965 ± 0.819	3.701 ± 0.376	3.933 ± 0.423	3.519 ± 0.505
B2 microglobulin (mcg/l)	1.30 ± 0.88	2.57 ± 2.08	2.49 ± 0.65	2.46 ± 1.06	5.39 ± 4.53	4.09 ± 3.04
Calcium (mg/dl)	9.85 ± 0.54	9.56 ± 0.54	9.66 ± 0.61	9.47 ± 0.49	9.72 ± 0.38	9.46 ± 0.63
Creatinine (mg/dl)	1.17 ± 0.33	1.00 ± 0.40	1.06 ± 0.18	0.96 ± 0.19	1.45 ± 1.17	1.16 ± 0.40
Total proteins (g/dl)	6.85 ± 0.33	7.36 ± 0.77	7.56 ± 0.95	7.82 ± 1.07	9.54 ± 1.62	9.48 ± 1.88
ESR (mm/h)	34 ± 33	35 ± 33	53 ± 17	72 ± 33	93 ± 36	107 ± 27
M-component (g/dl)	0.50 ± 0.28	1.14 ± 0.93	1.30 ± 0.54	1.88 ± 0.98	3.45 ± 2.59	3.97 ± 2.11
Gammaglobulin (g/dl)	1.42 ± 0.36	1.71 ± 0.80	1.83 ± 0.98	2.21 ± 1.00	3.41 ± 3.32	4.28 ± 2.18
IgM (mg/dl)	597 ± 269	1232 ± 878	1509 ± 727	2192 ± 1377	4862 ± 3312	4622 ± 2832
IgG (mg/dl)	793 ± 63	897 ± 262	868 ± 381	917 ± 475	707 ± 232	944 ± 617
IgA (mg/dl)	224 ± 52	148 ± 76	145 ± 75	147 ± 143	193 ± 118	149 ± 170
BMBC (% by FCM)	1.83 ± 1.00	1.91 ± 2.17	7.91 ± 6.03	10.22 ± 13.03	18.77 ± 20.14	23.70 ± 18.51
BMLC (% by morphology)	12.1 ± 1.3	14.1 ± 8.3	16.6 ± 7.3	24.8 ± 19.9	35.7 ± 27.0	35.8 ± 25.3
BMPC (% by FCM)	0.08 ± 0.08	0.31 ± 0.32	1.97 ± 2.06	0.59 ± 0.76	0.99 ± 0.91	0.55 ± 1.04
BMPC (% by morphology)	2.2 ± 1.8	2.4 ± 2.0	1.6 ± 2.1	2.4 ± 2.2	4.5 ± 3.9	3.2 ± 2.6

Abbreviations: BMBC, bone marrow B-cells; BMLC, bone marrow lymphoplasmocytoid cells; BMPC, bone marrow plasma cells; FCM, flow cytometry; MGUS, monoclonal gammapathies of uncertain significance.

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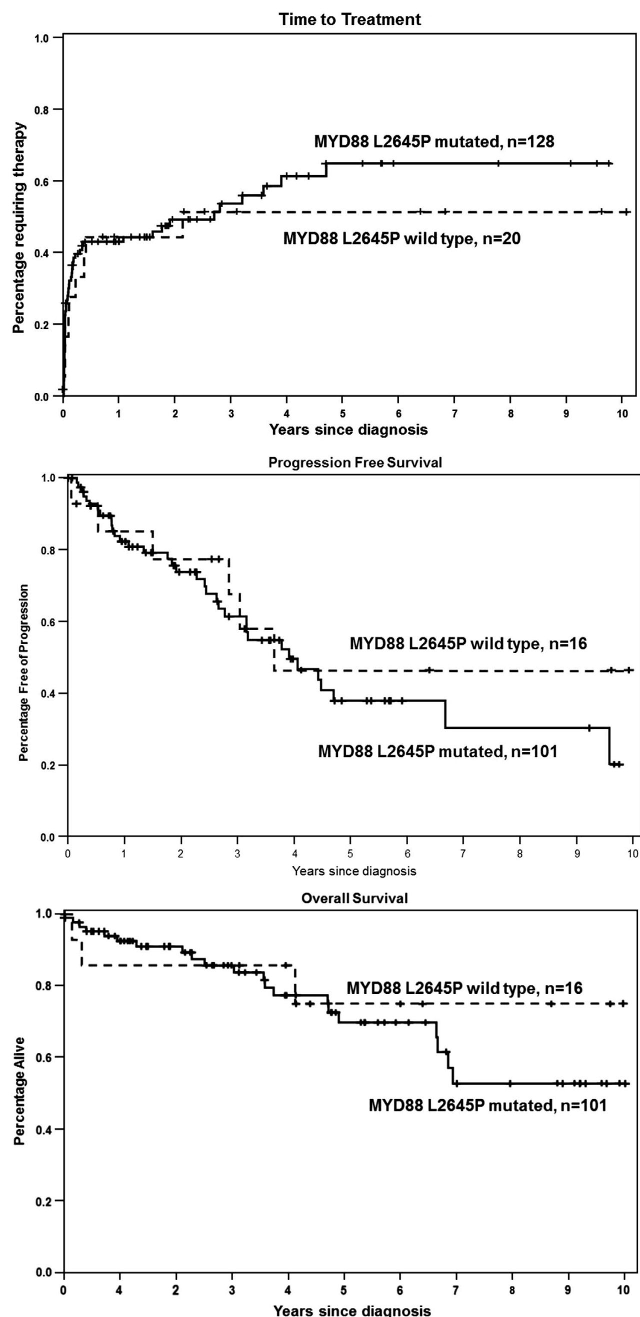

Figure 1. Projected survival of IgM-related disorders with respect to their MYD88 status. Top: time from diagnosis to the start of therapy (including IgM-MGUS, aWM and sWM); middle: survival from diagnosis to first disease progression (only aWM and sWM); bottom: overall survival from diagnosis to death.

possibility to use the PCR quantitative strategy as a monitoring technique for response to therapy and minimal residual disease evaluation that can compete with or complement flow cytometry.[18,32]

According to our findings and those of others,[6] the MYD88 L265P mutation seems to have a key role in the pathogenesis of IgM monoclonal gammapathies. It can activate the NF-κB pathway, and may alter the microenvironment by inducing interleukin-6 and -10 secretion in the tumor cells through the activation of the interleukin-1 receptor-associated kinase 4.[6] However, the presence of the MYD88 L265P mutation in

Table 5. Results of monitoring tests by in six responsive patients evaluated pre- and post therapy (pre-therapy firstst line; post therapy, second line)

Patient no.	*Optical count (%)*	*Flow cytometry*		*MYD88 (ΔCT)*
		Clonal B cells (%)	*Clonal plasma cells (%)*	
4044	16	3.5	1.29	+ (6.91)
	0	0	0.42	+ (10.23)
5309	22	5.84	0.27	+ (5.34)
	0	0	0	+ (11.3)
5606	64	49	0.09	+ (4.3)
	0	0	0	− (>24)
5928	62	27	0	NA
	0	1.02	0	+ (7,96)
6122	96	50	4	+ (2,29)
	0	0	0.91	+ (8.3)
7277	77	35	0.42	+ (4.21)
	0	0	0.37	+ (6.77)

Abbreviation: NA, not available.

indolent forms of these disorders calls into question its role in the development of aggressive forms and indicates that further research is needed to better understand the molecular mechanisms involved in the transformation from indolent to aggressive forms.[5,6] Interestingly, this mutation appears in B-cell LPDs of post-GCcells (activated B-cell type type DLBCL and primary central nervous system lymphoma), non-switched B-cell LPDs (IgM-MGUS and WM), and a small fraction of mucosa-associated lymphoid tissue lymphomas and other marginal zone lymphomas.[7–12] However, it is not present either in B-cell LPDs of pre-GCcells (acute ymphoblastic leukemia,[34] unmutated CLL[35]) or in LPDs driven by cells that have been able to undergo the class switching process of the immunoglobulins (hairy cell leukemias, IgA and IgG-MGUS and multiple myeloma).[32,34] Together, these findings strongly suggest a role for the *MYD88* gene in the terminal differentiation process. In fact, the MYD88 participates as a transducer in the processes taking place downstream of the activation of TACI (transmembrane activator calcium-modulating cyclophilin ligand interactor) and toll-like receptor during the class switch recombination induced via T-cell-independent stimulation.[36] These data imply that the MYD88 L265P mutation could be responsible, at least in part, for the theoretical incapacity for class switching exhibited by WM cells.

Several groups have suggested a potential correlation between MYD88 status, and the clinical and biological characteristics of WM patients. This could be based on some clinical and biological data provided in the two reports.[30,32] However, our results do not support the idea that MYD88 mutation separates a subgroup of patients with a singular profile, and perhaps the only weak association we have observed is that WM cases lacking the MYD88 L265P mutation seem to have a less typical WM signature (small M-component peak, more frequent lymphocytosis, higher lactate dehydrogenase serum level, atypical immunophenotype, less SHM).

Some biologic markers were weakly found to be slightly associated with MYD88 L265P in WM and IgM-MGUS patients. Of note, whereas typical Waldenström's-related phenotypic aberrancies (e.g., $CD22^{+low}$ or $CD25^{+}$)[37] were noted in both groups, patients with unmutated MYD88 showed more frequently a $CD23^{-}$, $FMC7^{+}$ and $CD27^{+}$ phenotype, which may suggest that WM clonal B cells in these specific patients are usually more mature (post-germinal center-restricted cells) as compared with WM patients with MYD88 L265P.[38] More consistent seem to be the differences concerning the IGHV selection and SHM pattern. First, cases with a wild-type MYD88 had an under-representation of the

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typical WM *IGHV*3–23 gene,[24] which reinforces the existence of biological differences between WM MYD88 mutated and unmutated cases. Second, MYD88-negative cases gene had a lower rate of SHM, resulting in a substantial proportion of cases with IGHV in germline configuration (57%), much higher than it has been usually reported in WM.[24,39] This suggests that the transformation event giving rise to these unmutated cases would have targeted cells that have not suffered the SHM process that takes place when crossing the germinal center. This finding would support the view that cells lacking the MYD88 L265P mutation could explain, at least in part, the small fraction of WM cases in whom the clone seem to emerge in a physiopathogenic scenario different to the conventional GCreaction proposed for most LPDs.[24,40,41] However, whether these biological differences have or not an impact on the clinical behavior of the final disorder remains unknown, although the data here reported suggest no relevant consequences.

Another important contribution of this work is that it raises the possibility of using this ASO-PCR as a tool for evaluating minimal residual disease in WM, with interesting results in selected cases, as it has also been shown by others.[32] This is especially important at this time, when the response criteria in WM are under review,[42] especially given the possibility of achieving unprecedentedly high-quality responses.[38,43] We have already shown that residual disease evaluation in BM by flow cytometry can improve response assessment after therapy.[18] Molecular approaches based in VDJH clonal rearrangements have never been tried in WM due to the complexity of currently available methods;[44] however, the ASO-PCR based on the MYD88 L265P mutation seems very promising for this purpose as an inexpensive, sensitive and easily performed procedure.

In summary, we present a study of the MYD88 L265P mutation in B-cell LPDs with a highly sensitive method that confirms its association with WM, and refines our knowledge about its frequency in other related disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS CONTRIBUTIONS

Cristina Jiménez and Ramón García-Sanz were the initial designers of the study. Cristina Jiménez, Elena Sebastián, María del Carmen Chillón and Ana Balanzategui carried out all molecular studies and prepared the database for the final analysis. Ramón García-Sanz desgined the database, and Miguel Alcoceba helped to handle it and to develop the initial analysis. Ramón García-Sanz helped by Luis A Marín developed the statistical analysis. Pilar Giraldo, José Mariano Hernández, Fernando Escalante, Tomás J González-López, Carmen Aguilera, Alfonso García de Coca, Ilda Murillo and Marcos González were clinicians responsible for the patients and those who took care of the protocols' accomplishment, sampling and collection of clinical data. Rocío Corral and María Eugenia Sarasquete helped in the molecular analysis and data collection. Bruno Paiva, Enrique M Ocio and Norma C Gutiérrez were responsible for the immunophenotyping and ctyogenetic analysis of the patients included in this series. Ramón García-Sanz and Cristina Jiménez prepared the initial version of the paper. Jesús F San Miguel and Marcos González were the main responsible of the global and molecular groups, respectively, and they were the persons responsible of the final revision of the draft, as well as the ones who gave the final approval of the version to be published. Ramón García-Sanz reviewed the conception and design of most of the work, made the database and supervised the statistical analysis. He rewrote the paper and made the final upload of the paper.

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2) SEGUNDO TRABAJO: DESARROLLO DE UNA TÉCNICA SENCILLA, SENSIBLE Y DE BAJO COSTE PARA LA DETECCIÓN DE LA MUTACIÓN *MYD88* L265P

Detection of MYD88 L265P mutation by real-time allele-specific oligonucleotide polymerase chain reaction.

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Introducción

La mutación *MYD88* L265P es la alteración que define a la macroglobulinemia de Waldenström (MW) ya que está presente en el ~91% de los pacientes. La identificación de esta anomalía puede resultar muy útil como herramienta para el diagnóstico diferencial frente otras neoplasias malignas de células B que comparten características morfológicas y clínico-patológicas con la MW. Esta determinación se ha llevado a cabo con diferentes estrategias, tales como secuenciación Sanger, análisis de curvas de fusión de alta resolución, o PCR seguida de digestión con enzimas de restricción. Sin embargo, estas técnicas presentan algunas desventajas, principalmente en lo que respecta a la sensibilidad y especificidad, capacidad para cuantificar, coste, laboriosidad o aplicabilidad.

Pacientes y métodos

Se diseñó una PCR en tiempo real específica de alelo (ASO-RQ-PCR) para la mutación L265P basada en el uso de dos cebadores reversos que difieren en el último nucleótido (de la posición 3') de modo que son específicos del alelo salvaje o del mutado. Sus secuencias eran: 5'-CCTTGTACTTGATGGGGATCA-3' (salvaje) y 5'-CCTTGTACTTGATGGGGATGG-3' (mutado). Además, se diseñó un cebador directo común (5'-ACTTAGATGGGGGATGGCTG-3') y una sonda TaqMan específica (5'-FAM-TTGAAGACTGGGCTTGTCCCACC-TAMRA-3').

Para cada experimento se realizaron dos PCRs diferentes: una para la detección de la mutación (con el cebador reverso mutado) y la otra como control de la calidad del ADN (usando el cebador reverso salvaje). Las reacciones se llevaron a cabo en un sistema StepOnePlus Real-Time PCR (Applied Biosystems, Foster City, CA) y consistieron en 10 min a 95ºC y 50 ciclos con las siguientes condiciones: 95ºC durante 15 s y 60ºC durante 60 s. Los datos se analizaron con el software StepOne v2.1 (Applied Biosystems).

Se analizaron muestras de médula ósea de 30 pacientes (10 GMSI-IgM y 20 MW) seleccionados por ser positivos para la mutación, y de sangre periférica de 10 donantes sanos. Los porcentajes medios de células clonales por citometría de flujo fueron de 1,73% (0,5 a 5) en GMSI y de 8,0% (1,7 a 59,1) en MW. Todos los casos evaluados se habían analizado previamente utilizando el ensayo comercial 'qBiomarker Somatic Mutation Assay for MYD88_85940' (SABiosciences, Qiagene Co., Hilden, Alemania).

Resultados

Estudio de sensibilidad

En primer lugar, se realizó un estudio de diluciones para establecer el límite de detección de la técnica. Se analizó el ADN de un paciente con la mutación heterocigota (evaluada por secuenciación Sanger) diluido en ADN normal. La ASO-RQ-PCR pudo detectar la mutación *MYD88* L265P hasta un nivel de dilución del 0,25% con >10 ciclos de diferencia respecto a los controles negativos.

Este experimento también nos permitió obtener una curva estándar para la cuantificación a través del valor del ciclo umbral (CT) (ciclo en el que la fluorescencia es 10 veces la basal), que está inversamente relacionado con la carga tumoral (coeficiente de correlación de 0,966).

Evaluación de la mutación MYD88 L265P

Los resultados del análisis de la mutación en las 40 muestras fueron concordantes con el ensayo comercial. El valor del CT fue siempre >50 en los donantes sanos y entre 29,6 y 38,1 en MW y 33,4 y 38,4 en GMSI (lo que significa que hubo >11 ciclos de diferencia entre las muestras sin y con la mutación).

La comparación entre el porcentaje de células clonales por citometría de flujo y por PCR confirmó la buena correlación entre ambas técnicas (con un coeficiente de correlación de Pearson, R=0,917, p=$1,0 \times 10^{-12}$).

Conclusiones

Hemos desarrollado un método barato, robusto y sensible para la detección de la mutación *MYD88* L265P basado en ASO-RQ-PCR que podría utilizarse como herramienta para la evaluación molecular de los síndromes linfoproliferativos B.

Detection of *MYD88* L265P Mutation by Real-Time Allele-Specific Oligonucleotide Polymerase Chain Reaction

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Abstract: *MYD88* L265P mutation has been reported in ~90% of Waldenström's Macroglobulinemia (WM) patients and immunoglobulin M (IgM) monoclonal gammopathies of uncertain significance (MGUS), as well as in some cases of lymphoma and chronic lymphocytic leukemia. The present study aimed to develop a real-time allele-specific oligonucleotide PCR (ASO-RQ-PCR) to detect the *MYD88* L265P mutation. We first evaluated the reproducibility and sensitivity of the technique with a diluting experiment of a previously known positive sample. Then, we evaluated the applicability of the methodology by analyzing 30 selected patients (10 asymptomatic WM, 10 symptomatic WM, and 10 IgM MGUS) as well as 10 healthy donors. The quantitative ASO-PCR assay could detect the *MYD88* L265P mutation at a dilution of 0.25%, showing an inverse correlation between the tumor cell percentage and the cycle threshold (CT) value, thus allowing for tumor burden quantitation. In addition, mutated cases were distinguished from the unmutated by >10 cycles of difference between CTs. To sum up, ASO-RQ-PCR is an inexpensive, robust, and optimized method for the detection of *MYD88* L265P mutation, which could be considered as a useful molecular tool during the diagnostic work-up of B-cell lymphoproliferative disorders.

Key Words: Waldenström's Macroglobulinemia, *MYD88*, ASO-PCR, diagnosis

(*Appl Immunohistochem Mol Morphol* 2014;00:000–000)

Waldenström's Macroglobulinemia (WM) is a rare lymphoproliferative disorder (LPD) characterized primarily by monoclonal immunoglobulin M (IgM) hypersecretion associated with bone marrow infiltration by lymphoplasmacytic lymphoma.[1] Although the oncogenic basis of WM has not been defined yet, Next-Generation Sequencing techniques have identified disease-associated mutations that could be responsible for the final phenotype and clinical behavior.[2–6] Among such mutations, the c.794 T > C change, that results in an aminoacid substitution from leucine to proline at position L265P in the *MYD88* gene (38182641 in chromosome 3p22.2),[7] has been reported to be present in approximately 90% of WM patients.[7–11]

Once the *MYD88* L265P mutation was described with high throughput sequencing techniques, it was extensively tested in several hematological malignancies and, apart from WM, it was described to be present at variable frequencies in non-IgM–secreting lymphoplasmacytic lymphoma,[7,9] diffuse large B-cell lymphoma (DLBCL) of the activated B-cell type,[4,12–16] DLBCL of leg-type,[17] primary central nervous system lymphoma,[18,19] mucosa-associated lymphoid tissue lymphoma,[8,12] marginal zone lymphoma,[7–10,20,21] IgM monoclonal gammopathy of uncertain significance (IgM-MGUS),[7,9–11,22] and chronic lymphocytic leukemia.[5,9,11] In addition, it has also been studied, although not found, in multiple myeloma,[7,9,11,23] IgG and IgA MGUS,[9,11] hairy cell leukemia,[9] acute leukemia,[23] ocular mucosa-associated lymphoid tissue lymphoma,[24] and amyloidosis.[9]

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C.J. and R.G.-S.: the initial designers of the study. C.J., E.S., M.C.C., and A.B.: carried out all molecular studies. M.R., A.A., and R.M.: provided technical assistance. R.G.-S., M.A., and L.A.M.: carried out the analysis. I.P.C., M.E.S., N.P., and R.C.: helped in the molecular analysis and data collection. B.P.: responsible for the immunophenotyping and cytogenetic analysis of the patients included in this series. C.J.: prepared the initial version of the paper. J.F.S.M. and M.G.: main responsible of the global and molecular groups, respectively, and they were the people responsible of the final revision of the draft as well as the ones who gave the final approval of the version to be published. R.G.-S.: reviewed the conception and design of most of the work and rewrote the paper and made the final upload of the paper.
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Altogether, these data illustrate that the identification of the presence/absence of the *MYD88* L265P mutation can be very useful during the diagnostic work-up of B-cell LPDs. In addition, several reports have suggested that the use of a sensitive technique could be of great help during the follow-up monitoring of the patients,[10,11] even for a better definition of response criteria of WM.[25] Accordingly, the assessment of the *MYD88* L2645P mutation has been carried out with different strategies, such as PCR amplification and Sanger sequencing,[16–22] high-resolution melting analysis,[15] digestion with restriction enzymes,[8] and allele-specific PCR (AS-PCR).[9–11] However, all of them have some disadvantages mainly concerning the sensitivity and specificity, ability to quantitate, simplicity, cost-effectiveness, laboriousness, or applicability. Therefore, we aimed to develop a cheap, rapid, and reliable tool for the detection of *MYD88* L265P mutation, considering that it could be potentially useful for the diagnosis and management of WM and related lymphoid neoplasms.[10,11]

MATERIALS AND METHODS

Samples

We analyzed bone marrow samples from 30 patients with asymptomatic WM (n = 10), symptomatic WM (n = 10), or IgM-MGUS (n = 10) and peripheral blood samples from 10 healthy donors. Diagnoses had been made according to the latest World Health Organization classification of tumors of hematopoietic and lymphoid tissues.[26] Median percentages of clonal cells evaluated by flow cytometry were 1.73% (0.5 to 5) in MGUS and 8.0% (1.7 to 59.1) in WM. All cases evaluated here had been analyzed in our previous study,[9] using the commercial assay "qBiomarker Somatic Mutation Assay for MYD88_85940" (SABiosciences, Qiagene Co., Hilden, Germany). The patient samples selected for the present study were known to be positive for the *MYD88* L265P mutation, whereas the control samples, obtained from healthy donors, were known to be negative. DNA extraction was automatically carried out with the MagNA Pure System (Roche Diagnostics, Manheim, Germany).

Immunophenotypic Analysis and Clonal Cell Quantification

Immunophenotypic evaluation was carried out using conventional methods, previously described by our group,[27,28] and following the general recommendations of the EuroFlow group for the evaluation of Hematological Malignancies.[29–31] These cases were immunophenotyped using 4-color combinations including up to 20 different

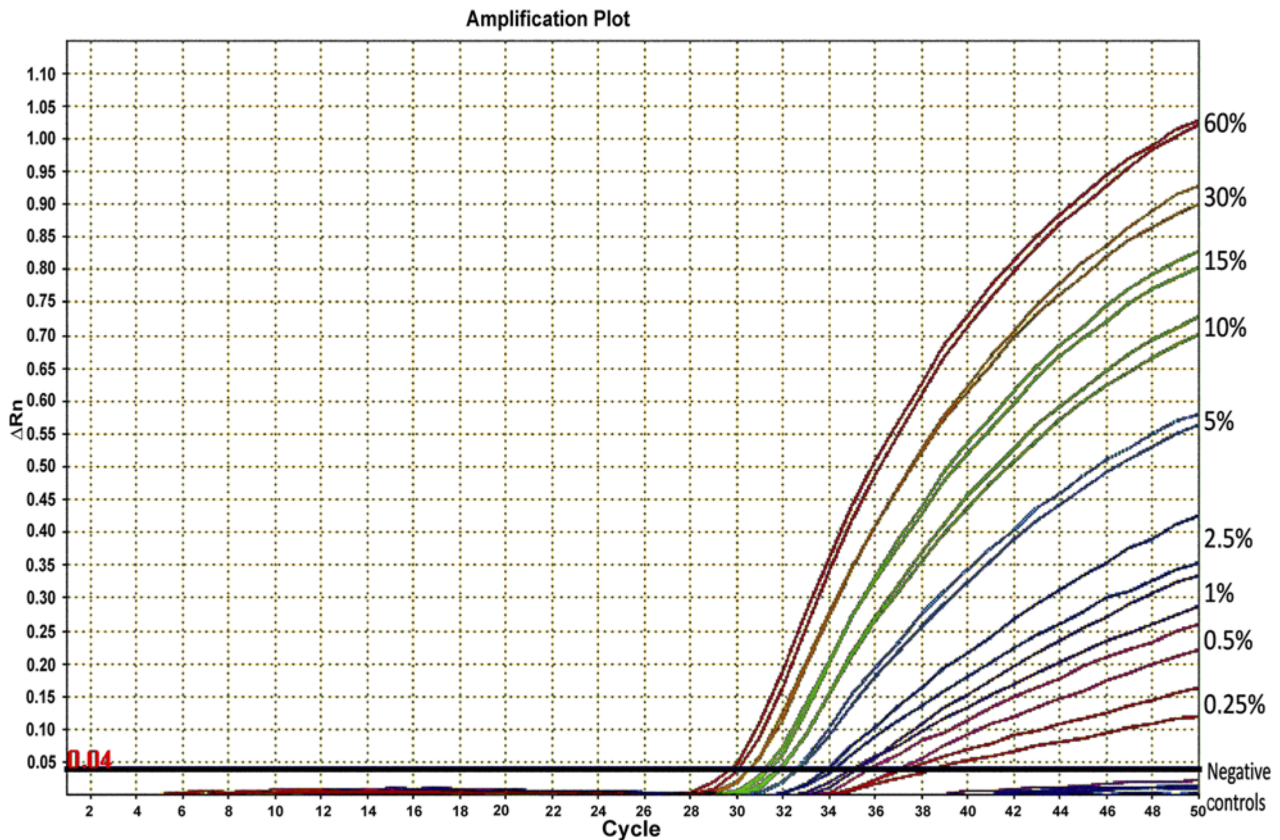

FIGURE 1. Sensitivity plot for quantitative allele-specific polymerase chain reaction. Every serial dilution (60%, 30%, 15%, 10%, 5%, 2.5%, 1%, 0.5%, and 0.25%) was made in duplicate. As it can be observed in the amplification plot, the *MYD88* L265P allele can be detectable to a dilution of 0.25%. Negative controls (also in duplicate) have cycle threshold values >50.

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antibodies in addition to surface IgM (sIgM) and cytoplasmatic Ig λ and κ (cyIgλ and cyIgκ). The percentage of clonal cells was determined in with the tube including the sIgM/CD25/CD19/CD38 MoAb. Data acquisition was performed in a FACSCalibur flow cytometer (Becton Dickinson Biosciences—BDB, San Jose, CA) using the FACSDiva software (version 6.1; BDB), and a 2-step acquisition procedure for total and $CD19^+$-only events. Data analysis was performed using the Infinicyt software (Cytognos, Salamanca, Spain).

PCR Design

The real-time allele-specific oligonucleotide PCR (ASO-RQ-PCR) here developed was based on the use of 2 reverse primers differing in the last nucleotide (at 3′ position) so that they are specific either of the wild-type or the mutated allele. To prevent the amplification of the non-matching primer (increasing specificity), an additional nucleotide mismatch (*C* > *G*) was introduced next to the mutated base. ASO primers were designed using the Oligo 6.0 software (Molecular Biology Insights, Cascade, CO) and were as follows: 5′-CCTTGTACTTGATGGGGATCA-3′ (wild-type) and 5′-CCTTGTACTTGATGGGGATGG-3′ (mutated). The common forward primer was 5′-ACTTAGATGGGGGATGGCTG-3′, for a final 142 bp PCR product. In addition, a specific TaqMan probe was designed for the PCR assay with the Primer Express Software v3.0.1 (Applied Biosystems, Foster City, CA), that yielded the following 6FAM dye—TAMRA labeled probe: 5′-TTGAAGACTGGGCTTGTCCCACC-3′.

Real-Time ASO-PCR Development

Each experiment required 2 different PCR reactions: one for the detection of the mutation (with the mutated reverse primer) and the other one as a control of the DNA quality (using the wild-type reverse primer). Each reaction was carried out in a final volume of 20 µL, containing 300 nM of each primer (forward and reverse wild-type or mutated), 200 nM of the probe, 1× of the TaqMan Universal PCR Master Mix (Applied Biosystems), and 20 ng of genomic DNA.

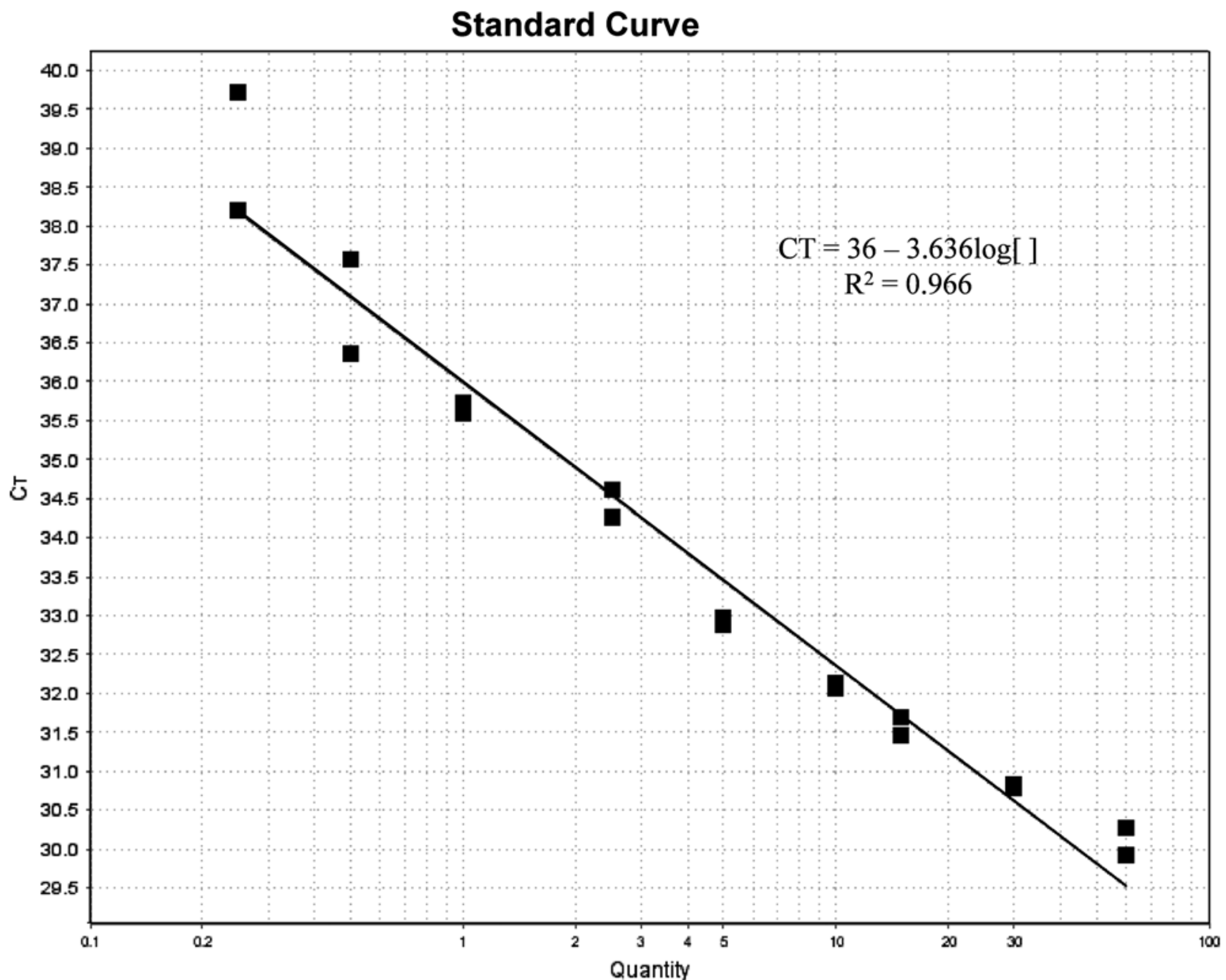

FIGURE 2. Standard curve for AS-RQ-PCR: correlation coefficient, 0.966; slope value = −3.636. CT indicates cycle threshold.

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Experiments were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems) and consisted of an initial denaturation step of 10 minutes at 95°C, followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Data were analyzed with the StepOne Software v2.1 (Applied Biosystems). The cycle in which fluorescent emission reaches 10-fold the basal emission is known as the cycle threshold (CT), a value that is proportional to the copy number of the target gene.

RESULTS

Sensitivity Assay

Firstly, we carried out a dilution study to establish the detection limit of our technique. We analyzed the DNA of a patient with the heterozygous mutation (previously found by Sanger sequencing) diluted in wild-type DNA to different concentrations: 60%, 30%, 15%, 10%, 5%, 2.5%, 1%, 0.5%, and 0.25%. All dilutions were tested in duplicate, including 2 controls lacking the mutation. This experiment allowed us to generate the standard curve (SC) for future quantifications.

As Figure 1 shows, ASO-RQ-PCR could detect the *MYD88* L265P mutation at a dilution of 0.25% with >10 cycles of difference from the wild-type DNA background. There was an inverse correlation between the tumor cell percentage and the CT value, which allowed good tumor burden estimation. The correlation coefficient of the SC was 0.966 with a slope value of -3.636 (Fig. 2).

Evaluation of *MYD88* L265P Mutation

A mutation analysis was carried out in the 40 samples selected for this study. All results were in accordance with those already obtained with the commercial assay. The CT value was always >50 in healthy donors, and between 29.6 and 38.1 in MW and 33.4 and 38.4 in MGUS (what means that there were >11 cycle differences between samples with and without the mutation) (Fig. 3).

A dot plot with the percentage of clonal cells evaluated by multiparametric flow cytometry versus the percentage provided by the SC was done to confirm that both estimations correlated well (Fig. 4). Accordingly, the corresponding estimates of both methodologies provided a Pearson correlation coefficient, R, of 0.917 ($P = 1.0\times10^{-12}$, Fig. 4).

DISCUSSION

In this study, we have developed and validated a simple inexpensive tool for the detection of the *MYD88*

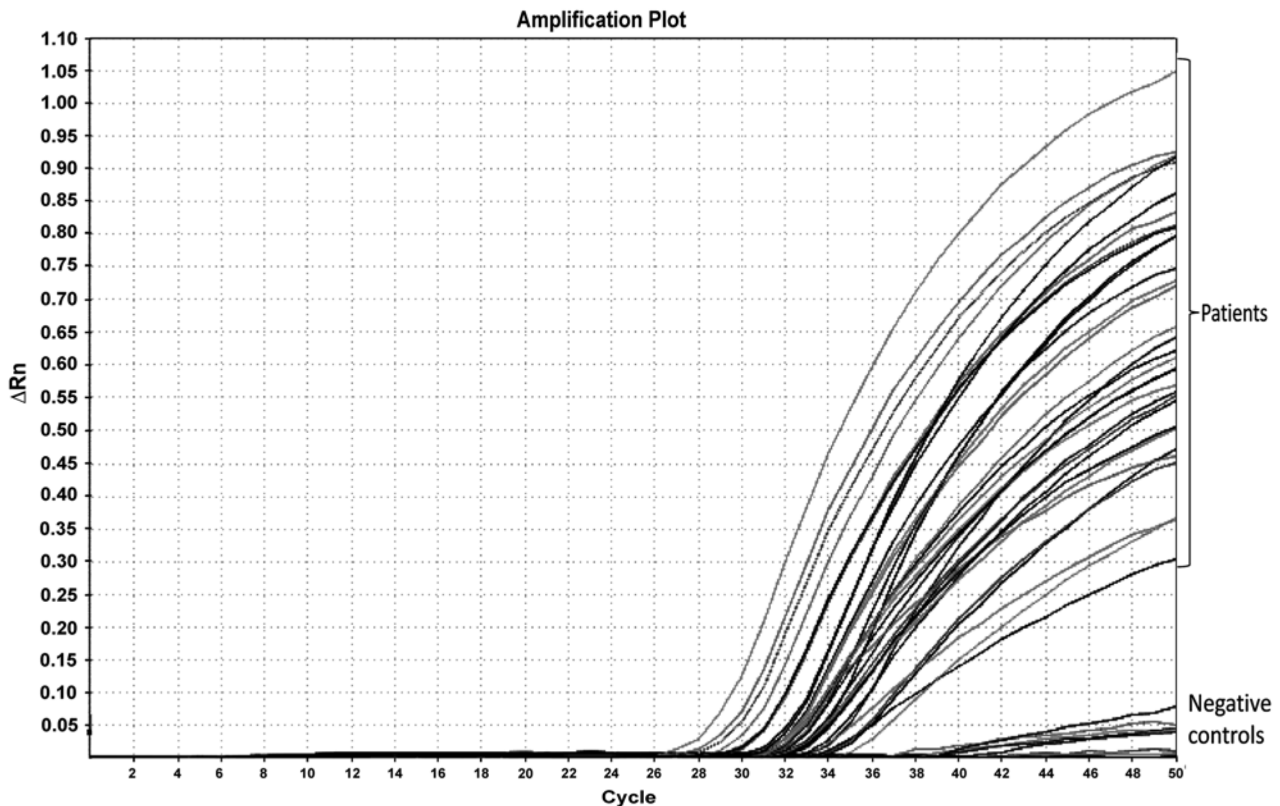

FIGURE 3. Determination of the *MYD88* L265P mutation in 10 asymptomatic MW, 10 symptomatic MW, 10 IgM-MGUS, and 10 healthy donors, by AS-RQ-PCR. Positive cases (in the upper part of the figure) can be distinguished from negatives (at the bottom), whose cycle threshold value is >50.

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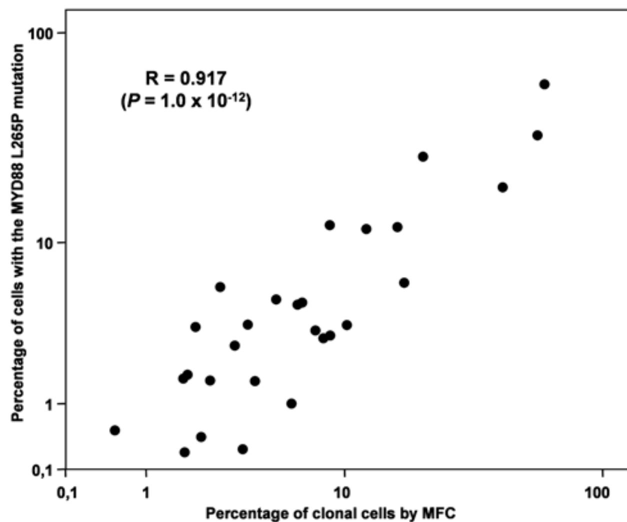

FIGURE 4. Correlation between the percentage of clonal cells evaluated by multiparametric flow cytometry (MFC) versus the percentage provided by the standard curve (SC): Pearson correlation coefficient, $R=0.917$ ($P=1.0\times10^{-12}$).

L265P mutation which may have a potential use in diagnostic discrimination or as a prognostic marker.

For this purpose, we have used a standard methodology for primers and probes design that has demonstrated to produce robust and reproducible PCR experiments in extensively used minimal residual disease studies.[32] However, we have also introduced some modifications in the mutant-specific amplification primer design that are thought to increase the specificity of the PCR reaction, such as the addition of mismatches close to the 3′ end.[33,34] This allowed us to produce a PCR reaction in which the negative result was always 10 CT (or more) higher than the highest positive result. This has to be combined with the high sensitivity that is offered by the use of TaqMan probes in quantitative PCR systems. In our case, PCR experiment rendered a sensitivity close to 10^{-3}, which means that 1 mutated cell can be detected within 1000 wild-type cells.

There are other methods to analyze the *MYD88* L265P mutation, although ASO-RQ-PCR has some advantages.[8–11,15–22] Sanger sequencing has demonstrated the presence of the mutation in several series,[12,17–19,22] but it has a relatively high cost and low sensitivity, requiring at least 10% to 40% of mutated cells (mean 25%).[15] This sensitivity can be improved. Wang et al[15] demonstrated that PCR and high-resolution melting analysis can achieve a sensitivity of 5%, similar to the achieved by Gachard et al,[8] with the use of PCR followed by enzymatic digestion restriction enzymes. However, this sensitivity is still insufficient for certain entities characterized by low tumor burden, even < 1%, such as IgM-MGUS,[28] which is characterized by high prevalence of the mutation (> 50%).[9–11,22] Finally, some reports have used AS-PCR followed by agarose gel electrophoresis, which increases sensitivity to 10^{-2}.[10,11] Although post-PCR handling has some disadvantages in terms of laboriousness and risk of cross-contamination, this detection limit approaches the optimal requirements for diagnostic and follow-up purposes. However, there is another disadvantage, as this methodology could lead to false-negative results, especially in the context of MGUS evaluation or monitoring of residual disease after treatment in WM symptomatic patients. The group from Boston has proposed a variation of this PCR by using SYBR Green and a Real-Time PCR equipment, improving their detection limit up to approximately 10^{-3},[11] which is adequate for diagnostic purposes and probably for most monitoring studies. However, this sensitivity gain is hampered by a relatively loss of specificity, as the highest CT value of the positive cases is only 3 cycles below the CT value of the wild-type cases. This raises the concern of possible false-positive results, especially when we require high sensitive values (ie, posttreatment evaluations). Our strategy, with the use of an additional base mismatch at the very 3′ end of the primer, allows to obtain very high CT values for wild-type samples (beyond 50—absolute lack of amplification, or at least cycle > 10 cycles respect the mutated samples). This makes our system very robust in terms of specificity, without any loss of sensitivity.

The main limitation of the assay here suggested would be the sensitivity, as 10^{-3} is below to the usual sensitivity reported for ASO-PCR assays.[32] However, we have to remind that the discrimination capacity relies on a single-base change, so any trial to improve the sensitivity could result in a reduction of the specificity. In addition, another commonly recognized disadvantage of our approach is the use of TaqMan probes, which could result in a cost increase respect to other experiments. For instance, SYBR Green assays would be less expensive, keeping the suitability for quantitative mutation detection. Nevertheless, the TaqMan assay adds advantages in terms of specificity[35] with a negligible cost increase for optimized PCR assay in a routine laboratory (< 1 €/experiment).

In conclusion, we hereby present an inexpensive, robust and optimized ASO-RQ-PCR assay for the detection of *MYD88* L265P mutation, which could be considered as a useful molecular tool for the evaluation of B-cell LPD.

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3) TERCER TRABAJO: CARACTERIZACIÓN MOLECULAR DE LAS GAMMAPATÍAS MONOCLONALES IGM

Unraveling the heterogeneity of IgM monoclonal gammopathies: a gene mutational and gene expression profiling study

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Introducción

Las gammapatías monoclonales IgM son trastornos inmunoproliferativos que muestran una variabilidad importante, pudiendo presentarse como tres formas diferentes: gammapatía monoclonal de significado incierto IgM (GMSI-IgM), macroglobulinemia de Waldenström asintomática (MWA) y MW sintomática (MWS). Estudios recientes han identificado mutaciones recurrentes en los genes *MYD88*, *CXCR4*, *ARID1A*, *CD79B* y otros, que pueden tener un impacto en el perfil transcripcional, la presentación de la enfermedad, la respuesta a la terapia y la supervivencia global. Sin embargo, poco se sabe sobre las causas de la heterogeneidad clínica o los mecanismos implicados en la progresión de formas asintomáticas a sintomáticas.

Pacientes y métodos

Se estudiaron sesenta y nueve pacientes con MW/GMSI-IgM, distribuidos como se indica: 15 GMSI-IgM, 26 MWA y 28 MWS. El ADN y el ARN se extrajeron de células separadas CD19+ de médula ósea.

La mutación *MYD88* L265P se evaluó mediante PCR específica de alelo en tiempo real (ASO-RQ-PCR) y las de *CXCR4* mediante secuenciación Sanger.

El estudio de expresión génica se llevó a cabo por PCR cuantitativa en tiempo real utilizando chips de ADN de baja densidad con sondas TaqMan (TLDA) (ThermoFisher Scientific, Waltham, MA) en noventa y cinco genes, que fueron seleccionados por su relevancia en la enfermedad. Los resultados se analizaron con el software ExpressionSuite v1.0.3 (ThermoFisher Scientific), que permite la normalización de los datos y la cuantificación relativa para la comparación de muestras a través del método $2^{-\Delta\Delta CT}$. Los valores de expresión génica normalizados se exportaron al SPSS v15.0 (SPSS Inc., Chicago, IL) para el análisis estadístico. Se utilizó el test U de Mann-Whitney para identificar diferencias estadísticamente significativas entre los grupos.

El estudio de secuenciación masiva (NGS) se realizó con un panel personalizado de amplicones compuesto por 12 genes de interés en MW (*ARID1A*, *CD79A*, *CD79B*, *TP53*, *MYBBP1A*, *TRAF2*, *TRAF3*, *RAG2*, *HIST1H1B*, *HIST1H1C*, *HIST1H1D* y *HIST1H1E*). La secuenciación se llevó a cabo en el equipo MiSeq (Illumina, San Diego, CA) y los datos generados fueron pre-procesados con el MiSeq Reporter (software integrado en el MiSeq, Illumina). La detección de variantes se llevó a cabo utilizando el software Illumina VariantStudio 2.2 y el Integrative Genomics Viewer (Broad Institute, Cambridge, MA).

Resultados

Mutación MYD88 L265P

La mutación *MYD88* L265P fue muy frecuente, aunque varió en función del diagnóstico: 67% de GMSI-IgM, 96% de MWA y 100% de MWS (p=0,001). Además, se asoció con niveles más altos de hemoglobina, ya que todos los pacientes no mutados presentaron anemia ([hemoglobina] <11,5 g/dl) en comparación con sólo el 55% de los mutados (p=0,032).

Mutaciones CXCR4 WHIM

Se encontraron 25/69 (36%) pacientes con alteraciones en *CXCR4*, sin diferencias según el diagnóstico: 33% de GMSI-IgM, 42% de MWA y 32% de MWS. Excepto en un caso de GMSI, las mutaciones de *CXCR4* estuvieron siempre asociadas con la de *MYD88* (96%). Curiosamente, los niveles de expresión de *CXCR4* o de su ligando *CXCL12* no se vieron afectados por la presencia de las mutaciones. La comparación entre casos mutados y no mutados no reveló ninguna diferencia en las características clínicas, biológicas o en la supervivencia (global, libre de evento y libre de progresión). Sólo cuando se consideraron únicamente los pacientes con MW (excluyendo GMSI) se observó un componente monoclonal IgM más elevado (4418±3169 mg/dl) y menores niveles de β_2-microglobulina (B2M 2,8±1,9 mg/l) en los casos *CXCR4* mutados frente a los salvajes (2687±2767 mg/dl, p=0,025 y 3,7±1,6 mg/l, p=0,034, respectivamente). Por su parte, la sobreexpresión de *CXCR4* se relacionó con la presencia de adenopatías (en el 33% de los pacientes frente al 5% de los que no lo sobreexpresaron, p=0,029).

Otras mutaciones genéticas

Dado que las mutaciones de *CXCR4* demostraron estar presentes desde el inicio y no tuvieron influencia en la progresión a enfermedad sintomática, decidimos evaluar las mutaciones en otros 12 genes relevantes en MW. Globalmente se encontraron 29 mutaciones en 23/61 pacientes, distribuidos de la siguiente manera: 3/14 (21%) GMSI-IgM, 8/23 (35%) MWA y 12/24 (50%) MWS.

La media de mutaciones por paciente también aumentó progresivamente conforme a la evolución de la enfermedad (0,2, 0,4 y 0,7, respectivamente). Hay que destacar que los pacientes con un gen *MYD88* salvaje (n=6), no presentaron mutaciones adicionales en ninguno de estos genes (p=0,045). Como el número de mutaciones encontradas fue bajo, no se pudieron establecer diferencias en la distribución en función del diagnóstico. Lo más notable fue que las variantes en *HIST1H1C* sólo aparecieron en GMSI (p=0,031).

Las mutaciones en *CD79B* fueron las más frecuentes (8%), pero no se relacionaron con ninguna característica clínica de los pacientes. En cambio, la sobreexpresión de otros genes pertenecientes a la vía de señalización del BCR, como *CD79A*, *SYK*, *BTK* y *BLNK*, se asoció con un mayor nivel de infiltración de la médula ósea por citometría de flujo y más componente monoclonal.

El segundo gen más frecuentemente mutado fue *HIST1H1E*, en el 7% de los pacientes, cifra que aumentó al 13% cuando se consideraron todos los miembros de la familia de histonas (*HIST1H1 B-E*). Análogamente a *CXCR4*, las mutaciones en *HIST1H1E* no afectaron a su expresión y tampoco se observaron diferencias clínicas. Por el contrario, una mayor expresión de *HIST1HE* se relacionó con características clínicas favorables, como niveles más bajos de B2M (2,4±1,7 frente a 3,7±1,8 mg/dl, p=0,007) y de creatinina (0,9±0,2 frente a 1,1±0,4 mg/dl, p=0,044).

Los genes *ARID1A*, *MYBBP1A* y *TRAF3* se encontraron mutados en tres pacientes cada uno (5%). Estas alteraciones se asociaron con una presentación más adversa, con niveles más bajos de albúmina (2,9±0,2 frente a 3,7±0,5 g/dl, p=0,017) y hemoglobina (9,3±2,1 frente a 12,2± 2,1 g/dl, p=0,047) , así como mayor incidencia de adenopatías (66% vs. 17%, p=0,033) en pacientes con *ARID1A* mutado; más alteraciones neurológicas (50% frente a 8%, p=0,045) en pacientes mutados en *MYBBP1A;* y B2M elevada (6,7±0,4 frente a 3,2±1,8 mg/l, p=0,034) en pacientes con *TRAF3* mutado.

Finalmente, los dos pacientes con mutaciones de *TP53* fueron sintomáticos y presentaron anemia en el momento del diagnóstico. Uno de ellos correspondió a una forma muy resistente de la enfermedad que se transformó a LBDCG. Además, el recuento plaquetario fue menor que en los pacientes con *TP53* de tipo salvaje (116±37 frente a 263±121 x10^9/l, p=0,026).

Perfil de expresión génica (GEP)

A continuación, decidimos buscar diferencias en el GEP entre los pacientes indolentes (GMSI y MWA, n=22) y los sintomáticos (MWS, n=18). Los resultados mostraron que había 11

genes con diferente expresión; concretamente, *ADARB1* (responsable del empalme alternativo), *CCND3* (ciclina D3), *GPSM2* (modulador de la señalización de las proteínas G) y *LEF1* (factor de transcripción) estaban sobreexpresados en los casos asintomáticos, mientras que *CD79A* (vía del BCR), *IRF3* (factor de transcripción), *MEK1*, *P38*, *WNK1* (vía de las MAP quinasas), *MYD88* y *TAP2* (transportador de antígenos) lo estaban en los sintomáticos. *IRF3*, *MYD88*, *MEK1* y *P38* son parte de la vía de los receptores tipo *Toll* (TLR), que es fundamental para el crecimiento y supervivencia de las células de la MW, y junto con *CD79A* y *TAP2*, tienen un papel en la regulación de la respuesta inmune. Las diferencias de expresión se reflejaron en la presentación clínica de la enfermedad, ya que el perfil de expresión de los pacientes sintomáticos se asoció con una mayor frecuencia de adenopatías, hepatomegalia, esplenomegalia y síntomas B, así como con anemia, trombocitopenia, hipoalbuminemia y mayor B2M y componente monoclonal ($p \leq 0,05$), como ocurre en los casos con mal pronóstico. En consecuencia, se observó que tres de estos genes (*IRF3*, *MYD88*, *WNK1*) estaban sobreexpresados en el grupo de pacientes de alto riesgo de acuerdo con el sistema pronóstico internacional (IPSS). Sin embargo, no se encontraron diferencias estadísticamente significativas en la supervivencia global y libre de progresión asociadas a la expresión de cualquiera de estos genes.

Conclusiones

En resumen, nuestros datos revelan una mayor incidencia de mutaciones conforme a las sucesivas etapas de evolución (de GMSI-IgM a MW asintomática y a MW sintomática), lo que significa que, a diferencia de las mutaciones en *MYD88* y *CXCR4*, presentes desde el inicio de la patogénesis, la mayoría de estas alteraciones se adquirirían conforme la enfermedad progresa. El estudio de expresión también destacó diferencias significativas entre los pacientes indolentes y sintomáticos, en los genes relacionados con los mecanismos patogénicos de la MW (vías de TLR/MyD88, CXCR4 y BCR), que aportan algunas pistas sobre la heterogeneidad de esta enfermedad y parecen tener impacto en la presentación clínica.

TITLE: UNRAVELING THE HETEROGENEITY OF IgM MONOCLONAL GAMMOPATHIES: A GENE MUTATIONAL AND GENE EXPRESSION PROFILING STUDY

RUNNING TITLE: Genetics and Clinics in Monoclonal Gammopathies

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CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest

ABSTRACT

Background: Immunoglobulin M (IgM) monoclonal gammopathies show considerable variability, involving three stages of presentation: IgM monoclonal gammopathy of undetermined significance (IgM-MGUS), asymptomatic Waldenström's macroglobulinemia (AWM) and symptomatic WM (SWM). Causes responsible for this clinical heterogeneity and mechanisms involved in the progression from indolent to symptomatic stages are unknown**.**

Methods: We analyzed mutations in fourteen genes and performed a gene expression study in a well-characterized cohort of 69 patients in an attempt to establish the relationship with their clinical and biological features.

Results: Our results showed that the frequency of genetic alterations progressively increased from IgM-MGUS to SWM. This means that, in contrast to *MYD88* and *CXCR4* mutations, present from the beginning, most of them would be acquired during the course of the disease. *CD79B*, part of the B-cell receptor (BCR), was the most frequently mutated gene (8%), thus confirming the importance of the BCR signaling pathway in the pathobiology of WM. In addition, the expression study revealed an overexpression of genes belonging to the Toll-like receptor pathway in symptomatic versus indolent forms, which was also reflected in the disease presentation and prognosis.

Conclusion: Our findings showed that IgM Monoclonal Gammopathies present a higher frequency of alterations as disease progresses.

KEY WORDS: IgM Monoclonal Gammopathies, Heterogeneity, Mutations, Gene Expression Profiling, Clinicobiological features

INTRODUCTION

Immunoglobulin M (IgM) monoclonal gammopathies are immunoproliferative disorders that show considerable variability involving three different stages of presentation: IgM monoclonal gammopathy of undetermined significance (IgM-MGUS), asymptomatic Waldenström's macroglobulinemia (AWM) and symptomatic WM (SWM) (Owen *et al*, 2003). Patients with IgM-MGUS have a risk of progression to WM or other lymphoproliferative disorders of 1.5-2% per year (Kyle *et al*, 2003, 2011), while the risk of changing from AWM to SWM is 12% per year (Kyle *et al*, 2012). This process probably involves multiple steps and the acquisition of genetic alterations, although the exact mechanisms are not well established. *MYD88* L265P mutation, the hallmark of WM (Treon *et al*, 2012), is already present in 50-80% of IgM-MGUS (Jiménez *et al*, 2013; Xu *et al*, 2013), suggesting that it may be the initial event that confers a competitive advantage on the clone and predisposes it to the subsequent genetic alterations that are responsible for progression to the next steps in WM.

The second most common alterations in WM (29% of patients) affect the C-terminal domain of the chemokine receptor gene *CXCR4* (Hunter *et al*, 2014). These WHIM syndrome-like mutations have also been found in ~20% IgM-MGUS (Roccaro *et al*, 2014), although they are primarily subclonal, which suggests secondary acquisition, after the *MYD88* L265P, in WM oncogenesis (Xu *et al*, 2016). Moreover, both *MYD88* and *CXCR4* variants may condition the clinical presentation of the disease (Treon *et al*, 2014). However, little is known about other, less frequent alterations described in these disorders. Previous works that identified mutations in genes such as *TRAF3* (5%) (Braggio *et al*, 2009), *ARID1A* (17%) (Hunter *et al*, 2014), or *CD79A*/*B* (15%) (Poulain *et al*, 2013), did not differentiate between the indolent and symptomatic forms of the disease or establish a relationship with clinical and biological characteristics, so further investigations are required.

The improvement in our knowledge of this disease has allowed us to characterize not only the genomic but also the transcriptomic profile of WM cells. Gene expression studies have also helped define some of the key molecular pathways underlying the physiopathology of WM, e.g., the Toll-like receptor (TLR), CXCR4, nuclear factor-kappa B (NF-κB) and B-cell receptor (BCR) signaling pathways. Nevertheless, these studies

have some limitations concerning the use of difficult-to-reproduce methodologies such as microarrays (Kothapalli *et al*, 2002; Draghici *et al*, 2006), and the low number of patients evaluated. In addition, initial studies focused mainly on the comparison of WM with chronic lymphocytic leukemia and multiple myeloma (Chng *et al*, 2006; Gutiérrez *et al*, 2007), or on the expression of genes involved in late-stage B-cell differentiation (Leleu *et al*, 2009). Others combined gene expression and mutation analysis but did not distinguish between AWM and SWM (Poulain *et al*, 2013, 2016; Hunter *et al*, 2016). Finally, Trojani et al. (Trojani *et al*, 2013), Paiva et al. (Paiva *et al*, 2015) and Herbaux et al. (Herbaux *et al*, 2016) compared IgM-MGUS with WM, and AWM with SWM, but with very limited data about mutations and insufficient numbers of patients, so additional validations are required.

Our work is notable for integrating gene expression profiling (GEP) with mutation assessment in a large and well-characterized cohort of WM patients, which allowed us to identify the relationships between the genotype and the clinical and biological features, and to establish a clinical and molecular prognostic model. We also included patients with IgM-MGUS, AWM and SWM, to see if there were differences between the three entities that could explain the heterogeneity in the disease presentation and, at the same time, if the abnormalities were present from the beginning of the pathogenesis and were able to predict its evolution. The combination of several methodologies (multiparametric flow cytometry [MFC], conventional cytogenetics, GEP and sequencing) facilitates the complete characterization of the genetic abnormalities and pathogenic mechanisms typical of each disorder, enabling risk factors to be established and specific inhibitors against these targets to be developed.

MATERIALS AND METHODS

Patients

Sixty-nine patients with WM/IgM-MGUS were evaluated. DNA was extracted from bone marrow (BM) cells after selection of CD19+ cells using immunomagnetic methods. *MYD88* L265P assessment and *CXCR4* sequencing were performed in all patients (15 IgM-MGUS, 26 AWM and 28 SWM). Forty of the 69 patients (3 IgM-MGUS, 19 AWM and 18 SWM) were evaluated by GEP, while a next-generation sequencing (NGS) study of 61 patients (14 IgM-MGUS, 23 AWM and 24 SWM) was carried out.

Cases had been diagnosed using standard WHO classification criteria (2008 update) (Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, 2008), with a review that included the recently published recommendations and concepts for diagnosis (Campo *et al*, 2011). For WM and related disorders, these criteria encompass what the IWMG agreed in the second International Workshop held in 2002 in Athens, which requires the absence of BM infiltration by morphological examination of the bone biopsy in the absence of clinical, morphological or immunophenotypic features of other lymphoproliferative disorders (Owen *et al*, 2003). The clinical characteristics of this cohort were those usually seen in these entities (Table 1).

Sample preparation

CD19-selected cells were isolated from all BM samples using the autoMACS® Pro Separator system (Miltenyi-Biotec, Auburn, CA). The final purity was >90% in all cases. After purification, B-cells were stored in guanidine thiocyanate buffer (Qiagen, Hilden, Germany) at -80°C for subsequent DNA/RNA extraction. Total RNA and DNA were extracted using an AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol.

The reverse transcription reaction was performed with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA) in accordance with the manufacturer's recommendations. The RNA input was 400 ng in a final reaction volume of 20 µl, and the conditions for the reaction were: 10 min at 25°C, 120 min at 37°C and 5 s at 85°C.

MYD88 L265P assessment and CXCR4 sequencing

MYD88 L265P mutation was assessed by real-time allele-specific oligonucleotide PCR (ASO-RQ-PCR), as described elsewhere (Jiménez *et al*, 2014). *CXCR4* WHIM mutations were assessed by Sanger sequencing with the primers used by Hunter et al (Hunter *et al*, 2014).

Gene Expression Profiling (GEP)

Ninety-five genes (plus *GAPDH*) were selected on the basis of the aforementioned studies (Chng *et al*, 2006; Gutiérrez *et al*, 2007; Leleu *et al*, 2009; Poulain *et al*, 2013, 2016; Trojani *et al*, 2013; Paiva *et al*, 2015; Herbaux *et al*, 2016; Hunter *et al*, 2016), or because they belonged to WM-relevant signaling pathways. Gene expression analysis was carried out by real-time quantitative PCR using TaqMan low-density arrays (TLDAs) with a TaqMan Universal PCR Master Mix (ThermoFisher Scientific, Waltham, MA) in an ABI 7900HT Fast Real Time PCR system (Applied Biosystems, Foster City, CA) in accordance with the following thermal cycling conditions for Micro Fluidic Cards: 10 min at 94.5°C and 40 cycles (97°C for 30 s and 59.7°C for 1 min).

Raw data were analyzed with the ABI 7900 Sequence Detection Software version 2.2.2 using automatic baseline correction and a manual quantification cycle (Ct) setting. Resulting Ct data were exported for further analysis with the ExpressionSuite Software v1.0.3 (ThermoFisher Scientific), which allows data normalization, relative quantification for sample comparison through the $2^{-\Delta\Delta Ct}$ method, and graphical visualization of the results. The expression level of each gene was estimated from the mean of the duplicates and normalized with respect to *GAPDH*. Only genes with reproducible amplification curves of both duplicates were considered. Normalized gene expression values expressed as $\log_2$ were exported to an SPSS v15.0 (SPSS Inc., Chicago, IL) file for further statistical analysis using the Mann-Whitney U-test to identify statistically significant differences ($p<0.05$) between groups.

Next-generation sequencing (NGS)

NGS study was performed with a novel custom amplicon-based panel of 12 genes of interest in WM (*ARID1A*, *CD79A*, *CD79B*, *TP53*, *MYBBP1A*, *TRAF2*, *TRAF3*, *RAG2*, *HIST1H1B*, *HIST1H1C*, *HIST1H1D* and *HIST1H1E*). Sequencing was carried out in a MiSeq platform (Illumina, San Diego, CA) using 150-bp paired-end reads and a mean depth of 2000x. The data generated were pre-processed with the MiSeq Reporter (MiSeq integrated software, Illumina), which uses a Burrows-Wheeler Aligner (BWA) (Li & Durbin, 2009) and the Genome Analysis Tool Kit (GATK) (McKenna *et al*, 2010) for variant calling of single-nucleotide polymorphisms (SNPs) and short insertions and deletions (InDels). Secondary data analysis, sequence alignment and variant detection were carried out using Illumina VariantStudio 2.2 (http://www.illumina.com/informatics/research/biological-data-interpretation/variantstudio.html) and the Integrative Genomics Viewer (http://software.broadinstitute.org/software/igv/) (Broad Institute, Cambridge, MA).

Multiparametric flow cytometry (MFC) study

Immunophenotypic evaluation was carried out using conventional methods, as previously described by our group (Ocio *et al*, 2011; Paiva *et al*, 2014), and following the general recommendations of the EuroFlow group for the evaluation of hematological malignancies (Pedreira *et al*, 2008; Rawstron *et al*, 2008; van Dongen *et al*, 2012). These cases were immunophenotyped using 4- to 8-color combinations, including up to 20 antibodies in addition to surface IgM (sIgM) and cytoplasmic Ig λ and κ (cyIgλ and cyIgκ). Data were acquired in a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) using FACSDiva v6.1 (Becton Dickinson Biosciences), and a two-step acquisition procedure for total and CD19+-only events. Data were analyzed using Infinicyt software (Cytognos, Salamanca, Spain).

FISH studies

Deletions of 6q, 11q and 17q, and translocations of 14q32, were assessed in IgM-MGUS and WM by simple interphase FISH performed on cell nuclei from whole-BM samples using our previously published techniques (Ocio *et al*, 2005). For del(6q), a minimum of 100 cells were analyzed in all samples with the probe

‘Vysis CEP 6’, following Vysis scoring criteria (Abbott Molecular, Des Plaines, IL). The cutoff point for the identification of an alteration was set at ≥10% cells with an abnormal signal.

Statistical analyses

The chi-square (X^2) and Mann-Whitney U-tests were used to identify statistically significant differences between groups. Survival and progression were analyzed by the Kaplan–Meier method, using the log-rank test for comparisons.

RESULTS

MYD88 L265P mutation

MYD88 L265P mutation in this specific cohort of patients was recurrent, with a frequency that varied with the diagnosis: 67% (10/15) for MGUS, 96% (25/26) for AWM and 100% (28/28) for symptomatic WM (p=0.001). Moreover, it was associated with higher levels of hemoglobin, since all wild-type patients had anemia ([hemoglobin]<11.5 g/dl) compared with only 55% of the mutated patients (p=0.032). No other clinical differences between the groups were found.

CXCR4 WHIM mutations

The presence of somatic mutations in the C-terminal domain of CXCR4 was evaluated by conventional Sanger sequencing. We found 25/69 (36%) patients to have alterations (12 frameshift and 15 nonsense, two of which were subclonal), showing no differences according to the diagnosis: 5/15 (33%) IgM-MGUS, 11/26 (42%) AWM, and 9/28 (32%) SWM. Most patients with *CXCR4* mutations (24/25, 96%) were also *MYD88* L265P-positive and, in the same way, 5/6 (83%) of the *MYD88* wild-type were also *CXCR4*-unmutated. The comparison of the mutated and unmutated groups did not reveal any difference in the clinical and biological characteristics or in survival (overall, event-free survival and progression-free survival), based on the presence of *CXCR4* mutations. When considering only WM cases (excluding MGUS) did we observe a larger IgM monoclonal component (4418±3169 mg/dl) and lower β_2-microglobulin level (B2M 2.8±1.9 mg/l) in *CXCR4*-mutated compared with wild-type patients (2687±2767 mg/dl, p=0.025, and 3.7±1.6 mg/l, p=0.034, respectively). It was notable that no differences were found in the expression levels of either *CXCR4* or its ligand *CXCL12*, although *ADAM28* (lymphocyte-expressed metalloproteinase) and *ADARB1* (the mRNA editase responsible for alternative splicing) were downregulated in mutated patients (p=0.017 and p=0.022, respectively).

Other gene mutations

Since *CXCR4* mutations were demonstrated to be present from the beginning of the disease and had no influence on progression to a symptomatic stage, we decided to search for mutations in 12 other genes of relevance in WM (*ARID1A*, *CD79A*, *CD79B*, *TP53*, *MYBBP1A*, *TRAF2*, *TRAF3*, *RAG2*, *HIST1H1B*, *HIST1H1C*, *HIST1H1D* and *HIST1H1E*) by next-generation sequencing in CD19+ selected cells. Overall, we found 29 non-synonymous alterations, corresponding to 23/61 (38%) patients, which were distributed as follows: 3/14 (21%) IgM-MGUS, 8/23 (35%) AWM and 12/24 (50%) SWM (p=0.076). The mean number of mutations per patient also increased as the disease evolved (0.2, 0.4 and 0.7, respectively). Interestingly, the mean variant allele frequency (VAF) of the alterations did not follow this trend (33% for MGUS, 27% for asymptomatic WM and 37% for symptomatic WM). Genes affected by mutations were: *CD79B* (n=5, 8%), *HIST1H1E* (n=4, 7%), *ARID1A*, *MYBBP1A*, *TRAF3* (n=3, 5%), *TP53*, *HIST1H1B*, *HIST1H1C* (n=2, 3%), and *HIST1H1D*, *RAG2* and *TRAF2* (n=1, 2%) (Figure 1). Patients with an *MYD88* wild-type gene (n=6), showed no additional mutations in any of the studied genes (p=0.045). This was not the case with *CXCR4* wild-type patients, since 34% of them (n=14) displayed other alterations compared with 66% (n=27) who did not. The low number of mutations prevented any difference being identified in the distribution with respect to the diagnosis. The most remarkable finding was that variants in *HIST1H1C* only appeared in MGUS (p=0.031).

Mutations in *CD79B* were the most frequent (8% patients) and were located in the ITAM domain. Patients with a mutated *CD79B* presented no important clinical features, except hypoalbuminemia (3.3±0.3 g/dl vs. 3.7±0.5 g/dl in *CD79B* wild-type). A lower level of expression of *CASP9* (apoptosis-activating factor) was also observed in these patients (p=0.02).

The second most frequently mutated gene was *HIST1H1E*, which was altered in 7% patients, the value increasing to 13% when we considered all the histone family members (*HIST1H1 B-E*). Mutations in *HIST1H1E* were associated with the overexpression of *ADAM28* (p=0.019), *ATXN1* (p=0.042), *BLNK* (p=0.032), *BTK* (p=0.016), *CDK4* (p=0.032), *CDK6* (p=0.01), *GPER* (p=0.011), *IRF3* (p=0.021), *OSBPL3* (p=0.012) and *XBP1* (p=0.012). However, the expression of *HIST1H1E* itself was not affected. No clinical differences were observed.

The genes *ARID1A*, *MYBBP1A* and *TRAF3* were mutated in three patients each (5%). Although it is not possible to draw any firm conclusion because of the low number of cases, we would nevertheless like to highlight the significantly younger age of the *ARID1A*- and *MYBBP1A*-mutated patients (58±7 and 60±6 years, respectively) compared with the wild-type patients (71±10 years for both; p=0.028 and 0.045, respectively). However, this was the only favorable prognostic feature associated with these alterations, as *ARID1A*-mutated patients had lower levels of albumin (2.9±0.2 vs. 3.7±0.5 g/dl; p=0.017) and hemoglobin (9.3±2.1 vs. 12.2±2.1 g/dl; p=0.047), as well as higher incidence of adenopathy (66% vs. 17%, p=0.033); *MYBBP1A*-mutated patients presented more neurological alterations (50% vs. 8%, p=0.045); and *TRAF3*-mutated patients displayed higher levels of B2M (6.7±0.4 vs. 3.2±1.8 mg/l, p=0.034). There were no deregulated genes.

Finally, there were only two *TP53* mutated patients, both symptomatic and presenting with anemia at diagnosis. One case corresponded to a very highly resistant form of the disease (showing a minor response to ibrutinib after five lines of prior therapy) with *ARID1A* and *CD79B* mutations as well, and who finally transformed into diffuse large B-cell lymphoma (DLBCL). The second *TP53*-mutated patient achieved partial response after DRC (dexamethasone, rituximab and cyclophosphamide) and is now in his first year of follow-up. The only indication of poor prognosis was that the mean platelet count seemed to be lower than in *TP53* wild-type patients (116±37 vs. 263±121 x10^9/l, respectively; p=0.026).

Gene expression profiling

As the distribution of mutations did not significantly differ between MGUS, AWM and SWM, we decided to search for differences, focusing more exclusively on the GEP. We compared all the indolent patients (MGUS plus AWM, n=22) with the SWM patients (n=18), using the Mann-Whitney U-test to establish statistically significant differences between the groups. The comparison between indolent and symptomatic patients highlighted 11 differentially expressed genes; in particular, *ADARB1* (alternative splicing), *CCND3* (cyclin D3), *GPSM2* (G-protein signaling modulator) and *LEF1* (lymphoid enhancer-binding factor expressed in pre-B and T cells) were upregulated in asymptomatic cases, whereas *CD79A* (BCR related), *IRF3* (interferon regulatory transcription factor), *MEK1*, *P38* (MAP kinases), *MYD88*, *TAP2* (antigen peptide transporter) and *WNK1* (Ser/Thr kinase part of the ERK5/MAPK pathway) were overexpressed in symptomatic WM (Table 2). *IRF3*, *MYD88*, *MEK1* and *P38* are part of the TLR pathway, which is essential for WM cell growth and survival, and together with *CD79A* and *TAP2*, have a role in the immune response regulation. These differences in GEP were reflected in the disease presentation: underexpression of *ADARB1* and *CCND3* (as occurs in symptomatic patients) was associated with adenopathy and hepatomegaly, respectively. By contrast, splenomegaly and B symptoms were more frequent in cases who overexpressed *CD79A*, *IRF3*, *MEK1*, *TAP2* and *WNK1* (p≤0.05) (Supplemental Table 1). We then assessed the clinical covariates of the International Prognostic Scoring System (IPSS) for WM: age, hemoglobin, platelets, B2M, and the monoclonal component, also including albumin. Results showed that patients with an expression profile associated with a symptomatic disease (lower expression of *ADARB1*, *CCND3*, *GPSM2* and *LEF1*, and higher expression of *CD79A*, *IRF3*, *MEK1*, *P38*, *MYD88*, *TAP2* and *WNK1*) tended to present lower levels of hemoglobin, platelets and albumin, and higher levels of B2M and monoclonal component (Supplemental Table 2), as occurs in cases with poor prognosis. In line with these results, when we compared the three risk groups with respect to the IPSS (low risk, n=9 vs. intermediate risk, n=14 vs. high risk, n=11), overexpression of *IRF3*, *MYD88*, *WNK1*, and *PIK3CB* (a subunit of the phosphatidylinositol 3-kinases located downstream of CXCR4) was observed in high-risk

patients ($p<0.05$). However, no statistically significant differences in overall and progression-free survival were found to be associated with the expression of any of these genes.

Regarding the other relevant pathway in the pathogenesis of WM (which involves CXCR4 receptor), our results revealed differences in the presence of adenopathy, which occurred in 33% patients overexpressing *CXCR4* compared with 5% of patients underexpressing it ($p=0.029$).

CD79B mutations did not affect the clinical presentation or the GEP, but we decided to investigate other closely related genes involved in the BCR pathway for which expression data were available, such as *CD79A*, *SYK*, *BTK* and *BLNK*. Upregulation of these genes was associated with a higher level of BM infiltration by MFC and the monoclonal component (Supplemental Table 3).

Overexpression of *HIST1H1E* was related to favorable clinical features, such as lower B2M and creatinine (2.4±1.7 vs. 3.7±1.8 mg/dl $p=0.007$, and 0.9±0.2 vs. 1.1±0.4 mg/dl $p=0.044$, respectively), whereas mutations in this gene did not seem to have any effect on the clinical characteristics. No differences were observed with respect to *ARID1A* expression.

DISCUSSION

Recent work to characterize the molecular basis of IgM monoclonal gammopathies has provided new insights into the mechanisms that may contribute to the pathogenesis of the disease. The present work aimed to contribute to our knowledge by focusing on the three stages of the disease (IgM-MGUS, asymptomatic WM and symptomatic WM) in order to elucidate the causes of the clinical heterogeneity and genetic events associated with progression. The most frequently somatic mutations observed in these gammopathies (i.e., *MYD88* L265P and *CXCR4* WHIM) (Treon *et al*, 2012; Hunter *et al*, 2014) are already present in the first stages of these entities (Jiménez *et al*, 2013; Xu *et al*, 2013, 2016; Roccaro *et al*, 2014), suggesting that they are responsible for the initiating process, while other genomic alterations should be responsible for disease progression. Gene expression profiling analyses, on the other hand, have revealed distinct molecular signatures in the comparison between WM and IgM-MGUS or indolent and symptomatic WM (Trojani *et al*, 2013; Herbaux *et al*, 2016). However, further studies in larger cohorts of patients are still needed to establish which genes and pathways distinguish MGUS, smoldering and symptomatic WM in order to identify patients at high risk of progression.

Our results confirmed the presence of *CXCR4* mutations in around one third of the patients (36%), with similar frequencies among the three stages (33% MGUS, 42% AWM and 32% SWM). Except in one MGUS, *CXCR4* mutations were always associated with *MYD88* L265P, as reported earlier (Hunter *et al*, 2014; Poulain *et al*, 2016). Other groups have shown that *CXCR4* alterations are determinants of disease presentation (Treon *et al*, 2014; Poulain *et al*, 2016), but we were not able to confirm this relationship. This could be explained in part because we included MGUS in the analysis. In fact, when we excluded them, mutated *CXCR4* proved to be associated with a higher IgM M-component and a lower B2M. In turn, *CXCR4* overexpression was related to the presence of adenopathy, probably due to the greater cell adhesion associated with CXCR4 activation (Ngo *et al*, 2008), which reflects a greater BM involvement (Hunter *et al*, 2016). However, it must be taken into account that, as previously stated (Hunter *et al*, 2016), *CXCR4* expression is not affected by the presence of the mutations. In our case, only *ADAM28* (cell differentiation) and *ADARB1* (alternative splicing) were downregulated, since other potentially deregulated genes were not included in the current study (Hunter *et al*, 2016; Poulain *et al*, 2016).

The frequency of genomic alterations (i.e., loss of heterozygosity or copy-number abnormalities) increases from IgM MGUS to AWM and SWM (Poulain *et al*, 2013; Paiva *et al*, 2015). This phenomenon was also observed in the present NGS study. Only three patients with MGUS demonstrated additional mutations (21%) in at least one of the studied genes, while those numbers increased by up to eight in AWM (35%), and by 12 in SWM (50%), with a mean of 0.2, 0.4 and 0.7 mutations/patient, respectively. This confirms the association between the clinical behavior and a higher frequency of genetic alterations. Interestingly, a wild-type *MYD88* implied the absence of mutations in the other twelve genes, reinforcing the view that these cases could be a distinct entity (García-Sanz, 2016).

CD79B mutations were present in 8% of the patients, as reported by other groups (Poulain *et al*, 2013, 2016; Hunter *et al*, 2014), but were not mutually exclusive to *CXCR4* alterations. By contrast, *CD79A* was always wild-type. In DLBCL, a mutated *CD79B* (found in 10-15% of patients) (Pasqualucci *et al*, 2011) is considered a driver event that induces a sustained cell survival effect (Davis *et al*, 2010). In WM, cells have been shown to exhibit constitutive activation of BCR-related signaling elements and to express higher levels of sIgM, even in the absence of BCR alterations (Argyropoulos *et al*, 2016), a finding supported by the successful treatment with BTK inhibitors (Dimopoulos *et al*, 2017). In our study, *CD79B* mutations were only related to lower levels of albumin and downregulation of the apoptosis-activating factor *CASP9*, whereas the overexpression of *CD79A*, *SYK*, *BTK* and *BLNK* (genes belonging to the BCR signaling pathway) was associated with a high degree of BM infiltration by MFC and elevated M-component.

Mutations in the linker histone genes *HIST1H1 B-E* were present in 13% of patients, similar to what is observed in DLBCL (Lohr *et al*, 2012; Morin *et al*, 2013). Although these alterations had no apparent effect on the clinical characteristics, a higher level of expression of *HIST1HE* was related to more favorable clinical features, such as lower levels of B2M and creatinine.

Another regulator of chromatin structure, *ARID1A*, was mutated in 5% of patients, a lower value than the 17% previously described (Hunter *et al*, 2014). Although we did not observe the reported increase in BM infiltration, our patients who harbored these mutations had lower levels of hemoglobin and albumin and more adenopathy than those with the wild-type *ARID1A* gene. Mutations in *MYBBP1A* (5%), *TRAF3* (5%) (regulators of the NF-κB pathway), and *TP53* (3%) displayed a similar incidence to those previously described (7%, 3% and 7%, respectively) (Hunter *et al*, 2014), and were associated with advanced disease and poor outcome. Due to the small number of patients, these results need to be confirmed in a larger series of patients.

Focusing on GEP, previous studies had identified deregulation of genes involved in B-cell activation, the immune response, transcription regulation, and part of the JAK/STAT, PI3K/Akt/mTOR and MAPK signaling pathways (Trojani *et al*, 2013; Herbaux *et al*, 2016). In this line, our results showed an upregulation of *ADARB1* (alternative splicing), *CCND3*, *GPSM2* (cell cycle) and *LEF1* (transcription regulation), but not of *BACH2* (Herbaux *et al*, 2016) in AWM. By contrast, *CD79A* (B-cell activation), *IRF3*, *MYD88*, *MEK1*, *P38* (TLR pathway), *WNK1* (MAPK pathway), and *TAP2* (immune response) were overexpressed in SWM, suggesting a more intense activity of the signaling pathways responsible for WM cell growth and survival (Yang *et al*, 2013). These differences were reflected in the clinical characteristics, whereby the SWM expression profile was associated with higher frequencies of adenopathy, hepatomegaly, splenomegaly, and B symptoms, as well as of anemia, thrombocytopenia and hypoalbuminemia, and higher levels of B2M and the monoclonal component. Consequently, we observed that three of these genes were overexpressed in the high-risk group (*IRF3*, *MYD88*, *WNK1*). Other studies also found genes relevant to WM biology and downstream of IL6 signaling to be associated with IgM, hemoglobin and BM disease involvement (Hunter *et al*, 2016).

In summary, we have carried out an extensive study of mutations and gene expression in a well characterized series of 69 patients with IgM monoclonal gammopathies, distinguishing between the three disease presentations. Our data reveal a higher incidence of mutations during the different stages of evolution (from IgM MGUS to asymptomatic WM and symptomatic WM), meaning that, in contrast to *MYD88* L265P and *CXCR4* WHIM alterations, which are present from the beginning of the pathogenesis, most of these mutations would be acquired during the multistep process of WM evolution. The expression study also highlighted significant differences between indolent and symptomatic patients with respect to genes involved in WM pathogenic mechanisms. This sheds some light on the heterogeneity of this disease, and affected the clinical characteristics. The already known MYD88, CXCR4 and BCR pathways are confirmed as playing an important role in the biology and pathogenesis of the disease, thereby reinforcing the value of using therapeutic strategies that block these routes in the treatment of these patients.

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AUTHOR CONTRIBUTIONS

C.J. and R.G.S. designed the initial study and selected the patients. C.J., M.I.P.C. and M.G.A. processed the samples and carried out all the molecular studies. C.J., A.B., M.C.C. and M.E.S. collected and analyzed the data and interpreted the results. R.G.S. and M.A. designed and helped manage the database. R.G.S., R.C. and L.A.M. supervised the statistical analysis.

R.G.S., F.E., A.G.C., A.C., C.A., T.G.L., A.B. and A.G.M. were the clinicians responsible for the patients, ensuring the protocols were correctly followed, sampling, and collecting clinical data.

N.P. and N.C.G. were responsible for the immunophenotyping and cytogenetic analysis, respectively, of the patients included in this series.

C.J. prepared the initial version of the paper. R.G.S. reviewed the conception and design of most of the work and corrected the manuscript. M.G., the head of the group, supervised the final revision of the draft and gave final approval for the version to be published.

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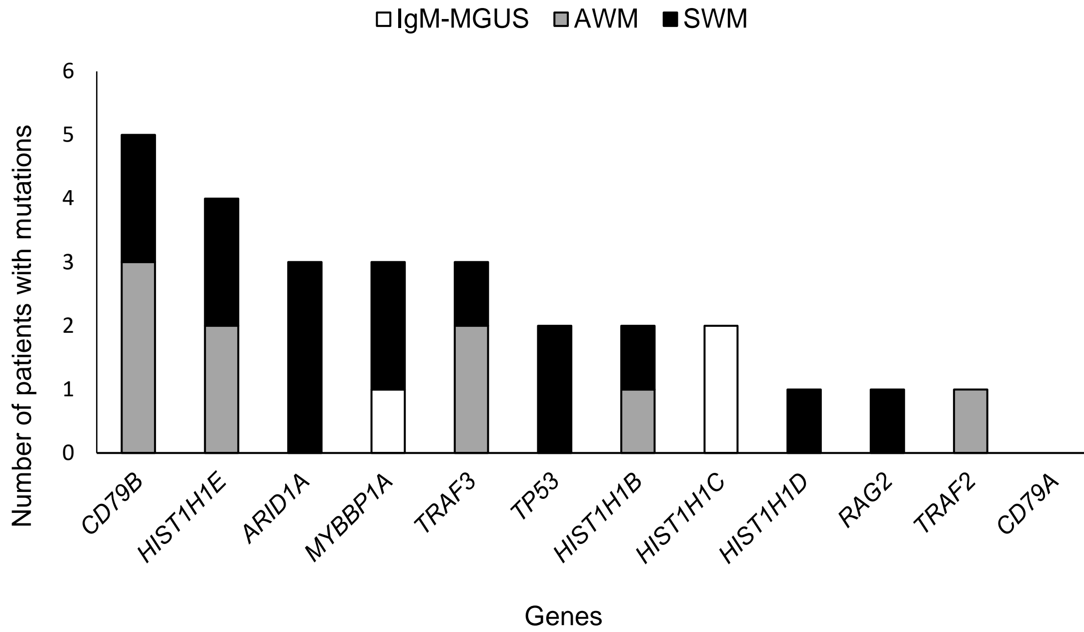
FIGURES

Figure 1.- Distributions of mutations among the studied genes. Bars represent the number of patients mutated for each gene. Colors of the bars indicate the different disease presentations: IgM monoclonal gammopathy of uncertain significance (IgM-MGUS), asymptomatic Waldenström's macroglobulinemia (AWM) and symptomatic Waldenström's macroglobulinemia (SWM).



TABLES

Table 1.- Clinical and biological characteristics of patients. Means and standard deviations of the main clinical and biological features of the patients for the three stages.

	IgM-MGUS[a] (n=15)	**Asymptomatic WM**[b] (n=26)	**Symptomatic WM**[b] (n=28)
Age (years)	71±12	70±10	70±12
Performance status (ECOG)	0.2±0.6	0.4±0.7	1.5±0.9(*)
Hemoglobin (g/dL)	13.5±1.4	13.2±1.9	9.7±1.7(*)
Leukocytes ($x10^9$/L)	7.9±3.2	8.2±4.0	7.1±3.0
Platelets ($x10^9$/L)	290±152	264±84	219±132
Albumin (g/dL)	3.8±0.5	3.8±0.6	3.5±0.5(*)
Gammaglobulin (g/dL)	1.5±0.9	2.8±4.7	4.0±3.3(*)
IgM[c] (mg/dL)	1367±1581	1875±1447	4475±3489(*)
IgG[c] (mg/dL)	1059±408	905±321	1010±761
IgA[c] (mg/dL)	219±180	126±83	187±247
M Component[d] (g/dL)	0.8±0.8	1.7±1.1	3.8±2.5(*)
Proteinuria (g/24h)	0.4±1.0	0.02±0.06	0.7±1.8(*)
Creatinine (mg/dL)	1.1±0.7	1.0±0.2	1.1±0.3
LDH[e] (UI/L)	264±95	259±84	288±131
Calcium (mg/dL)	9.6±0.4	9.4±0.6	9.3±0.6
C-Reactive Protein (mg/dL)	3.7±5.3	5.8±6.4	4.0±4.1
β_2-Microglobulin (mg/L)	2.9±2.1	2.9±2.0	3.7±1.4(*)
Del(6q)	0%	12%	30%(*)
***MYD88* L265P**	67%	96%	100%(*)

(*) $p<0.05$; [a]MGUS: monoclonal gammopathy of uncertain significance; [b]WM: Waldenström's macroglobulinemia [c]Ig: immunoglobulin; [d]M component: monoclonal component; [e]LDH: lactate dehydrogenase.

Table 2.- Comparative expression of most relevant genes between symptomatic and asymptomatic IgM monoclonal gammopathies. This table shows the significantly upregulated genes in asymptomatic patients (on top) and in symptomatic patients (on the bottom) with their relative expression ratio in symptomatic versus asymptomatic cases.

Gene	***n-fold change symptomatic/indolent WM***	***p***
ADARB1	0.4	0.011
CCND3	0.5	0.036
GPSM2	0.5	0.017
LEF1	0.3	0.041
CD79A	1.7	0.019
IRF3	2.1	0.016
MAP2K1 (MEK1)	1.3	0.041
MAPK14 (P38)	1.4	0.011
MYD88	1.5	0.002
TAP2	1.6	0.006
WNK1 (P65)	1.6	0.032

Supplemental Table 1. Relationships between GEP of symptomatic versus asymptomatic patients and clinical findings. Genes significantly dysregulated between indolent and symptomatic patients are shown. Patients were classified into the high or low group, depending on whether their level of expression was, respectively, greater or less than the median level of expression for that gene. Figures in bold represent the expression pattern of symptomatic patients. We then looked for differences in the clinical presentation of the two expression-level groups in each gene by comparing the percentage of patients presenting each symptom. Only clinical features with significant differences between the two groups in at least one gene are displayed. Overall, the expression profile of symptomatic patients was associated with worse disease presentation.

	mRNA expression	Adenopathy	Hepatomegaly	Splenomegaly	B symptoms
ADARB1	high	14%*	6%	6%	20%
	low	**86%***	**11%**	**11%**	**44%**
CCND3	high	15%	0%*	5%	25%
	low	**24%**	**18%***	**12%**	**37%**
GPSM2	high	6%*	6%	6%	18%
	low	**33%***	**11%**	**11%**	**42%**
LEF1	high	22%	6%	11%	32%
	low	**17%**	**11%**	**6%**	**28%**
CD79A	**high**	**30%**	**12%**	**18%***	**47%***
	low	10%	5%	0%*	15%*
IRF3	**high**	**18%**	**12%**	**18%***	**42%**
	low	20%	5%	0%*	20%
MAP2K1 (*MEK1*)	**high**	**28%**	**0%**	**11%**	**47%***
	low	11%	16%	5%	15%*
MAPK14 (*P38*)	**high**	**22%**	**6%**	**11%**	**42%**
	low	16%	11%	5%	20%
MYD88	**high**	**18%**	**12%**	**12%**	**42%**
	low	20%	5%	5%	20%
TAP2	**high**	**24%**	**18%***	**18%***	**47%***
	low	15%	0%*	0%*	15%*
WNK1 (*P65*)	**high**	**18%**	**12%**	**18%***	**47%***
	low	20%	5%	0%*	15%*

*p≤0.05

Supplemental Table 2. Relationships between GEP of symptomatic versus asymptomatic patients, and clinicobiological features determining the International Prognostic Scoring System (IPSS) of WM. The table shows the genes that are significantly dysregulated between indolent and symptomatic patients. Patients with a high or low level of expression were determined by comparison with the median expression value for that gene. Figures in bold correspond to the expression pattern of symptomatic patients. We then compared the mean (and standard deviation) of the clinicobiological covariates of the IPSS and the albumin in the two groups of patients with high or low levels of expression for each gene. Globally, the expression profile of symptomatic patients was associated with worse prognostic features (older, lower hemoglobin, platelets and albumin, and higher β2-microglobulin and serum monoclonal protein).

Gene	mRNA expression	Age (years)	Hemoglobin (g/dl)	Platelet count (x10^9/l)	β_2-microglobulin (mg/l)	Serum monoclonal protein (g/dl)	Albumin (g/dl)
ADARB1	high	70±11	11.9±2.4	290±124	2.5±1.2	1.4±1.0	3.8±0.5
	low	**72±11**	**11.3±2.2**	**232±107**	**3.5±1.8**	**2.9±2.6**	**3.6±0.7**
CCND3	high	71±11	12.0±2.0	223±91	3.1±1.7	2.0±1.7	3.8±0.4
	low	**71±10**	**11.0±2.6**	**294±138**	**3.1±1.8**	**2.8±2.7**	**3.6±0.8**
GPSM2	high	72±11	12.3±2.5*	250±114	2.4±1.1*	1.6±1.5	3.9±0.6
	low	**71±11**	**10.8±2.0***	**267±134**	**3.9±2.0***	**3.0±2.5**	**3.5±0.6**
LEF1	high	68±10	12.3±1.8	272±114	2.4±1.1*	2.1±1.6	3.8±0.5
	low	**73±11**	**11.1±2.2**	**256±120**	**4.0±2.1***	**2.6±2.6**	**3.5±0.7**
CD79A	**high**	**71±9**	**10.5±2.2***	**287±139**	**3.2±1.8**	**3.4±2.7***	**3.4±0.5***
	low	70±12	12.5±2.0*	230±96	3.0±1.7	1.5±1.2*	4.0±0.6*
IRF3	**high**	**71±11**	**10.7±2.4***	**257±148**	**3.2±1.7**	**3.6±2.7***	**3.5±0.5***
	low	71±11	12.3±2.0*	258±91	3.0±1.8	1.5±1.1*	3.9±0.7*
MAP2K1 (*MEK1*)	**high**	**73±10**	**10.8±2.2***	**244±129**	**3.3±1.8**	**3.0±2.5**	**3.7±0.5**
	low	69±11	12.3±2.3*	270±114	2.9±1.7	1.6±1.3	3.7±0.7
MAPK14 (*P38*)	**high**	**71±10**	**11.4±2.5**	**223±117**	**3.2±1.7**	**2.6±2.1**	**3.7±0.5**
	low	70±11	11.7±2.2	290±117	3.0±1.7	2.1±2.4	3.7±0.7
MYD88	**high**	**73±10**	**10.5±2.2***	**245±146**	**3.3±1.4**	**3.1±2.9**	**3.6±0.5**
	low	69±11	12.5±2.0*	270±93	2.9±2.0	1.7±1.2	3.8±0.7
TAP2	**high**	**70±11**	**10.8±2.3**	**255±133**	**3.2±1.7**	**3.5±2.7***	**3.6±0.6**
	low	72±10	12.2±2.2	260±111	3.0±1.8	1.4±0.8*	3.8±0.6
WNK1 (*P65*)	**high**	**72±9**	**10±2.4***	**249±143**	**3.2±1.6**	**3.5±2.8***	**3.5±0.5***
	low	70±12	12.4±2.0*	266±98	3.0±1.8	1.5±1.1*	3.9±0.7*

*p<0.05

Supplemental Table 3. Clinical findings in relation to expression of B-cell receptor (BCR) pathway-related genes. Description of data: This table illustrates the genes that are part of the BCR signaling pathway and that were included in our study. Patients belonging to the high or low expression group were established by comparing their expression level with the median expression value for that gene. We then compared the clinical characteristics of the two groups of patients and found significant differences in the serum monoclonal protein and bone marrow infiltration. Patients with overexpression of those BCR-related genes (being suggestive of activation of the pathway) presented higher levels of monoclonal component and infiltration by multiparametric flow cytometry.

mRNA expression		Serum monoclonal protein (g/dl)	Bone marrow infiltration by MFC (%)
CD79A	high	3.4±2.7*	19±17
	low	1.5±1.2*	14±20
SYK	high	3.2±2.8	18±16
	low	1.8±1.4	15±21
BTK	high	3.2±2.6	23±21*
	low	1.7±1.5	10±13*
BLNK	high	3.6±2.6*	24±22
	low	1.4±1.1*	10±13

*p<0.05

MFC: multiparametric flow cytometry

4) CUARTO TRABAJO: EVALUAR LA TRANSFORMACIÓN DE MW A LBDCG

Waldenström's macroglobulinemia to aggressive diffuse large B-cell lymphoma: a whole exome analysis of gene abnormalities leading to transformation.

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Introducción

La transformación de macroglobulinemia de Waldenström (MW) a histologías más agresivas, como el linfoma B difuso de célula grande (LBDCG) ha sido descrita en hasta un 10% de los pacientes. Además, estos linfomas suelen tener peor pronóstico y menor supervivencia desde el momento de la transformación (mediana de ~2 años). Sin embargo, hasta la fecha se ha investigado poco sobre este proceso, por lo que las causas y mecanismos implicados en la transformación a linfoma agresivo todavía se desconocen. La identificación de los cambios genéticos que impulsan esta transición permitiría desarrollar nuevas estrategias terapéuticas para mejorar el pronóstico de estos pacientes.

Pacientes y métodos

En el estudio se incluyeron cuatro pacientes (#1-4) diagnosticados de MW transformada. Se realizó la secuenciación del exoma completo de las muestras tumorales pareadas al diagnóstico y la transformación, así como del ADN germinal para los estudios comparativos. Se añadió una muestra adicional del paciente #2 (correspondiente a un evento de progresión de MW sin transformación). En el paciente #3, sólo se pudo incluir el ADN tumoral de la transformación y el germinal.

Las librerías se generaron con el kit SureSelectXT2 Human All Exon V5 de 51 MB (Agilent Technologies, Santa Clara, CA). La secuenciación se llevó a cabo en el equipo HiSeq 2000 (Illumina, San Diego, CA). DreamGenics® (Oviedo, España) realizó el procesamiento y análisis bioinformático inicial utilizando algoritmos no comerciales. Se filtraron los polimorfismos de un solo nucleótido (SNP) para incluir sólo las alteraciones no sinónimas y codificantes en los resultados. Las frecuencias alélicas de las variantes (VAFs) se corrigieron de acuerdo al contenido tumoral por citometría de flujo, para estimar el porcentaje de células tumorales afectadas por cada mutación.

Resultados

Variantes no sinónimas codificantes (NSV)

Globalmente, se encontraron 421 variantes no sinónimas codificantes (NSV) en el diagnóstico y la transformación entre los cuatro pacientes, distribuidas en 355 genes. De estos, 39 eran genes mutados exclusivamente en MW (al diagnóstico n=29, o en la progresión n=10), mientras que 267 eran exclusivos de las muestras de LBDGC.

El número de genes mutados fue mucho más elevado en el momento de la transformación (mediana 85, rango 49-165) que en el momento del diagnóstico (mediana 21, rango 20-35). En consecuencia, hubo una ganancia media de 70 variantes (intervalo de 29-144) durante la transición de MW a LBDCG, que no se asoció de forma significativa con el tiempo de transformación (R^2=0,51).

NSV presentes en el diagnóstico y la transformación

Aunque hubo más alteraciones en la transformación, al comparar la VAF corregida por la infiltración tumoral en ambos momentos, se observó que el porcentaje de células tumorales afectadas por cada alteración fue generalmente más alto al diagnóstico. Además, el número de mutaciones conservadas en ambos momentos difirió significativamente entre los pacientes, desde únicamente dos (*MYD88* y *TNIP1* en el paciente #1) a 29 (en el paciente #2). Las más recurrentes fueron *MYD88* L265P, presente en todos los pacientes (como es habitual en MW), y *CD79B* Y196C/H, que se encontró en 3/4 pacientes en la transformación (75%) y en 2/3 al diagnóstico (67%), cifras mucho más elevadas que en series de MW y LBDCG convencionales. *CELSR2*, *FAM135B*, *IGFN1* y *ZFHX4* también fueron genes recurrentemente mutados (presentes en al menos dos pacientes) en ambos momentos. Todas estas alteraciones se encontraron en una alta proporción de células tumorales y se conservaron durante todo el proceso, lo que sugiere un posible papel como eventos conductores (*drivers*) tempranos.

NSV exclusivas de un único momento

A continuación, se consideraron las variantes detectadas solo en uno de los momentos, siendo exclusivas del diagnóstico, la progresión o la transformación. El papel de las NSV exclusivas del diagnóstico es considerado más secundario (mutaciones pasajeras) porque desaparecen en el clon del linfoma, mientras que las variaciones exclusivamente presentes en la etapa de transformación (o que aparecieron más tarde en el clon de Waldenström) se consideran conductoras intermedias o tardías, y se cree que podrían conferir alguna ventaja al clon tumoral. En nuestro caso se encontraron cinco genes recurrentemente mutados en la etapa de transformación que no lo

estaban en el diagnóstico: *FRYL* (pareja de fusión de MLL en la leucemia linfoide), *HNF1B* (factor de transcripción cuya expresión está alterada en algunos tumores), *PER3* (proteína con un papel importante en la activación de puntos de control de la mitosis, la proliferación celular y la apoptosis), *PIM1* (protooncogén con actividad serina/treonina quinasa implicado en la patogénesis del linfoma) y *PTPRD* (supresor tumoral que contribuye al desarrollo de diversos tumores).

Patrón de evolución de MW a LBDCG

La existencia de NSV comunes y exclusivas de los distintos momentos es sugerente de un modelo de evolución ramificado. De acuerdo con él, el clon de linfoma no se derivaría de la mera acumulación de mutaciones en el clon de la MW, sino que representaría la evolución de ciertos subclones que adquieren nuevas mutaciones responsables de la transformación, sin conservar algunas de las detectadas en el momento del diagnóstico. El hecho de que ambos tumores (MW y LBDCG) tengan el mismo reordenamiento V(D)J e idéntica región CDR3 apunta a una célula progenitora común en la que se originan las mutaciones conductoras. Este patrón evolutivo no lineal está bien representado en el paciente #2, que tuvo una progresión sintomática como MW antes de la transformación. En este paciente se observaron alteraciones comunes en todas las muestras (diagnóstico, progresión y transformación) pero también algunas que se detectaron en el evento intermedio (progresión) pero que no estaban en el momento del diagnóstico y no se mantuvieron en la transformación. No obstante, estos datos deben considerarse con precaución debido al bajo nivel de infiltración de las muestras de diagnóstico, que podría conducir a la infradetección de variantes que estuvieran presentes en una baja proporción de la población tumoral.

Conclusiones

El proceso de transformación de MW a LBDCG no se rige por un evento genético único sino por una suma de alteraciones que pueden contribuir a la aparición de la enfermedad agresiva. La existencia de genes frecuentemente mutados (*CD79B*) al diagnóstico en el subgrupo de pacientes que sufren transformación histológica en comparación con su baja incidencia en las MW que no se transforman, sugiere el potencial papel de estos genes como biomarcadores para predecir el riesgo de transformación. Sin embargo, todavía son necesarias más investigaciones para entender mejor la biología de este proceso y poder diseñar terapias preventivas.

TITLE: From Waldenström's macroglobulinemia to aggressive diffuse large B-cell lymphoma: a whole-exome analysis of abnormalities leading to transformation

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CONFLICT OF INTEREST

The authors declare no conflict of interest

RUNNING TITLE: Waldenström's macroglobulinemia transformation

1. ABSTRACT

Transformation of Waldenström's macroglobulinemia (WM) to diffuse large B-cell lymphoma (DLBCL) occurs in up to 10% of patients and is associated with an adverse outcome. Here we performed the first whole-exome sequencing study of WM patients who evolved to DLBCL and report the genetic alterations that may drive this process. Our results demonstrate that transformation depends on the frequency and specificity of acquired variants, rather than on the duration of its evolution. We did not find a common pattern of mutations at diagnosis or transformation; however, there were certain abnormalities, such as *MYD88* and *CD79B*, that were present in a high proportion of clonal tumor cells and that were conserved during this transition, suggesting that they have a key role as early drivers. In addition, recurrent mutations gained in some genes at transformation (e.g., *PIM1, FRYL, HNF1B*) represent cooperating events in the selection of the clones responsible for disease progression. Detailed comparison reveals the gene abnormalities at diagnosis and transformation to be consistent with a branching model of evolution. Finally, the frequent mutation observed in the *CD79B* gene in this specific subset of patients implies that it is a potential biomarker predicting transformation in WM.

2. INTRODUCTION

Waldenström's macroglobulinemia (WM) is a neoplastic disease characterized by bone marrow infiltration with lymphoplasmacytic lymphoma, and the presence of an IgM monoclonal component.[1] Most patients show an indolent clinical course, and survival outcome has improved in recent years.[2] However, this long-term evolution has led to an inherent increased risk of developing other malignancies including acute leukemia[3] or non-Hodgkin lymphoma.[4] Transformation into more aggressive histologies, in this case to diffuse large B-cell lymphoma (DLBCL), has been reported in up to 10% of WM patients.[5,6] Moreover, the prognosis of these patients appears to be worse than that for patients with *de novo* DLBCL, and survival from the time of transformation is usually poor (median survival of ~2 years).[6–8]

The biological process of transformation in follicular lymphoma has been thoroughly studied.[9–15] However, data from other indolent B-cell lymphoproliferative disorders are limited. No causes of WM transformation have yet been described, so understanding this process would be of great interest and would facilitate the development of new therapeutic strategies for improving the outcome of these patients. Recent advances in determining the WM mutational profile have revealed the presence of recurrent mutations, such as those of *MYD88*, *CXCR4* and *ARID1A*.[16,17] However, it is not known whether they are part of the mechanisms underlying the transformation to aggressive lymphoma. This event is of particular interest, especially when it involves such genetically distinct entities in appearance. Accordingly, WM seems to be a very homogeneous disease with a monotonous recurrent driver mutation (*MYD88* L265P),[18,19] and infrequent secondary alterations.[20] Conversely, DLBCL harbors a wide range of diverse somatic mutations and genetic lesions that affect numerous intracellular pathways, thus making it intrinsically much more complex.[21–23] However, the two entities share some alterations, such as *MYD88* L265P (observed in ~90% of WM and in 29% of ABC-type DLBCL),[18,19,21,24] *CD79A*/*CD79B* (15% WM and 10-15% DLBCL),[17,20,21,23,25,26] or copy number variations affecting 6q (40-60% WM and 20-40% DLBCL).[27–30]

Transformation to DLBCL can occur at any time during the course of WM: at diagnosis, before treatment, during therapy, and even 20 years after the initial diagnosis.[6] In addition, no indicative clinicopathological feature or risk factor for developing DLBCL has so far been identified. No genomic studies of transformed WM patients have been carried out, so nothing is known about the biological signals that might explain particular susceptibilities. For this reason, the identification of genetic changes driving transformation is essential for a comprehensive understanding of the transition from WM to DLBCL that could help in the development of new diagnostic strategies and targeted therapies.

Against this background, we decided to perform the first whole-exome sequencing (WES) study focused on WM patients who experienced histological transformation. Integrating our new findings into our existing knowledge about the transformation of indolent lymphoproliferative syndromes to aggressive diseases should enable a transformational biological model to be derived, help define the molecular risk criteria for monitoring

the course of the disease, and develop new preventive strategies. Our results revealed a higher incidence of mutations in the *CD79B* gene than in non-transformed WM patients, which could be interpreted as being a potential mechanism contributing to transformation. Finally, the comparison between diagnosis and transformation allowed us to establish an evolutionary pattern associated with the transforming event.

3. SUBJECTS AND METHODS

3.1. Subjects

Four patients diagnosed with transformed WM were included in the study. In three of them (patients #1, #2 and #4), matched tumor samples from diagnosis and transformation to DLBCL, as well as germline DNA were available for comparative study. One extra sample from patient #2 (corresponding to an event of WM progression without transformation) was also included. For patient #3, only DNA from germinal and transformed tumor cells was available. Cases were diagnosed using standard WHO classification criteria,[31] including the new concepts that appear in the most recent review.[32] The study and all procedures were performed in accordance with the Helsinki Declaration and were reviewed and approved by the Institutional Review Board of the center.

The study group included one woman and three men, with a median age of 76 years (range, 62-82 years). The median time from diagnosis of WM to histological transformation was 52 months (range, 42-153 months). Three of the four patients had received treatment prior to histological transformation, and only one patient (#4), corresponding to an initial asymptomatic form, was treatment-naïve. The complete patient characteristics are shown in Table I.

3.2. DNA extraction and quality assessment

The sample origins were: 1) for WM tumor cells: bone marrow at diagnosis in all patients; 2) for DLBCL cells: spleen (patient #1), inguinal lymph node (patient #2) and bone marrow (patients #3 & #4) at transformation; and 3) for germline cells: peripheral blood samples in which no infiltration (<0.1%) was demonstrated by flow cytometry.

DNA was extracted by conventional methods: manually with the DNAzol reagent (MRC, Cincinnati, OH), or automatically with the Maxwell® system (Promega Corporation, Madison, WI). Quantification and quality control of DNA were evaluated before enrichment and library preparation. DNA concentrations were measured using the Qubit® fluorometer (Thermo Fisher Scientific, Inc., Waltham, MA). DNA sample quality was assessed by gel electrophoresis. At least 1 µg of DNA was required for library preparations.

3.3. Flow cytometry (FCM) studies

Immunophenotypic evaluation was done by conventional methods, using panels of monoclonal antibodies previously described by our group,[33] and following the general recommendations of the EuroFlow group for the immunophenotypic evaluation of hematological malignancies.[34]

3.4. FISH studies

Simple interphase FISH was performed on cell nuclei from whole bone marrow samples using our previously published techniques.[35] Del(6q), *MYC* and *BCL6* translocations, t(14;18) (q21; q32), and del(17p) were analyzed with the probes Vysis CEP 6, LSI MYC Dual Color Break Apart Rearrangement (8q24), LSI BCL6 Dual Color Break Apart Rearrangement (3q27), Vysis IGH/BCL2 Dual Color Dual Fusion Translocation Probe t(14;18)(q32;q21) and LSI TP53 Probe (Abbott Molecular, Des Plaines, IL), respectively. At least 100 cells were analyzed in all patient samples, applying Vysis scoring criteria. The cutoff point for the identification of alteration was set at ≥10% cells with abnormal signal.

3.5. V(D)J clonal rearrangements

V(D)J clonal rearrangements of WM and DLBCL matched samples were amplified and sequenced as described by the BIOMED-2/Euroclonality strategy.[36]

3.6. Whole-exome sequencing (WES)

All diagnostic and transformation samples, as well as those for normal DNA matching, were sent for library construction and WES at Macrogen Inc. (Seoul, South Korea). Enrichment and generation of libraries were performed with SureSelectXT2 Human All Exon V5 of 51 MB (Agilent Technologies, Santa Clara, CA) that uses cRNA probes of 120 nt. Paired-end sequencing was carried out in the Illumina HiSeq 2000 platform (Illumina, Inc., San Diego, CA). The number of reads was set up according to each sample tumor infiltration defined by FCM. Germinal samples were sequenced with a mean depth of 100x, and tumor DNA with a depth of 150x or 200x, depending on whether the infiltration was greater or less than 40%, respectively.

3.7. Sequencing data processing and bioinformatic analysis

DreamGenics® (Oviedo, Spain) supervised the pre-processing and performed the initial bioinformatics analysis using algorithms and non-commercial pipelines to call variants, analyze and compare them. Briefly, data generated by the sequencer were converted to FastQ with the Illumina Consensus Assessment of Sequence and Variation (CASAVA) version 1.8 software (http://www.illumina.com/software/genome_analyzer_software.ilmn#third_party_tools), and aligned to the human reference genome (Genome Reference Consortium human build 37, human genome 19) with BWA software.[37] Variants were called using Atlas-SNP and Atlas-indel,[38] discarding those with suboptimal quality indexes according to the pipeline criteria.

3.8. Interpretation of variants

Only non-synonymous protein-coding alterations were considered. Criteria for filtering out single nucleotide polymorphisms were a frequency lower than 2% in the germinal sample and a population allele frequency <1% (according to the Human Gene Mutation Database).[39] Some mutations were excluded on the basis of their distance with respect to the Agilent V5 probes. All samples were analyzed in pairs, with their corresponding germinal sample taken as reference.

Variant allele frequencies (VAFs) were corrected according to their tumor content defined by flow cytometry, in order to better estimate the percentage of tumor cells affected by each mutation. Variants with a corrected VAF of <10% were not included in the analysis.

3.9. Enrichment analysis

Gene-set enrichment analysis was performed with the WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) (http://www.webgestalt.org/analysis.php) to highlight categories and pathways present in the Gene Ontology and Pathway Commons databases. The statistical method employed was the hypergeometric test, with p-values adjusted by the Benjamini & Hochberg (1995) method.[40]

4. RESULTS

4.1. Global non-synonymous variations

Overall, we found 421 non-synonymous variations (NSVs) at diagnosis and transformation in the four patients, distributed among 355 genes. Of these, only 39 were mutated exclusively in WM (at diagnosis, n=29; on progression, n=10), and 49 were present at the time of both events (diagnosis and transformation). All other genes (n=267) were mutated only in the DLBCL samples.

4.2. Variant allelic frequency and percentage of tumor cells

To understand the possible mechanisms leading to the transformation event, we compared the results in tumor cells of the two events studied. First, we observed a much higher frequency of mutated genes at transformation (median 85, range 49-165) than at diagnosis (median 21, range 20-35) (Fig 1). Accordingly, there was a median gain of 70 variants (range 29-144) per case during the transition from WM to DLBCL. Interestingly, the number of gains was not closely correlated with the interval between diagnosis and transformation (R^2=0.51). However, we noticed that patients #1 and #3, who presented the fastest

transformation (~3 years), contained more variants (165 and 98, respectively) than did patient #2, who transformed in 5 years and had 72 mutations, and patient #4, who exhibited the smoothest transformation, lasting 13 years, and acquired only 49 alterations (Fig 2).

4.3. NSV present at diagnosis and transformation

We found that the variant allele frequency (VAF) was increased at transformation for almost all these common mutations, but this was attributed to the higher tumor infiltration present in the DLBCL compared with the corresponding WM sample (#1, 40% vs. 12%; #2, 51.3% vs. 22.4%; #4, 54.6% vs. 11%). Thus, the corrected VAF using flow cytometry showed the opposite pattern and VAFs were greater at diagnosis than at transformation (Fig 3). This means that, although there were more alterations at transformation, the percentage of tumor cells affected by each alteration was usually lower. This was confirmed using the globally corrected mean VAF for all NSVs present at diagnosis and at transformation (Table II).

Not all patients had the same number of potential early drivers. Patients #2 and #4 had NSVs in 29 and 14 genes in common at diagnosis and transformation, respectively. However, patient #1 only shared two variants at the time of both events: *MYD88* and *TNIP1* (the latter having a higher VAF at diagnosis). This gene codes for a TNFAIP3-interacting protein that plays a role in regulating nuclear factor kappa-B (NF-κB) activation, which is a frequent mechanism of alteration of cell cycle control in all lymphoid neoplasias.

4.4. Recurrent NSV among different patients

MYD88 L265P was present in all patients at both times (3/3 at diagnosis and 4/4 at transformation). The second most frequent was *CD79B* Y196C/H, which was found in 3/4 patients at transformation (75%), and in 2/3 at diagnosis (67%). VAFs of *CD79B* mutations were half of the *MYD88* in two patients and the same as *MYD88* in one patient, and kept that relation at baseline and transformation. *CELSR2*, *FAM135B*, *IGFN1*, and *ZFHX4* also had recurrent variations (in at least two patients).

We then considered variants that could be detected at the time of only one of the events, i.e., exclusively at diagnosis, progression, or transformation (Supplemental Table I). Variations exclusively detected at diagnosis were not present in the lymphoma clone, implying that it does not provide any evolutionary advantage (passenger mutations). In contrast, recurrent variations exclusively present at the transformation stage (or that appeared late in the Waldenström's clone) may be considered as temporally intermediate or late drivers that confer some advantage to the tumor clone. Five genes had recurrent NSVs acquired at the transformation stage: *FRYL* (MLL fusion partner in lymphoid leukemia), *HNF1B* (transcription factor whose expression is altered in some cancers), *PER3* (checkpoint protein that plays an important role in checkpoint activation, cell proliferation and apoptosis), *PIM1* (proto-oncogene with serine/threonine kinase activity involved in the pathogenesis of lymphoma), and *PTPRD* (tumor suppressor that contributes to the development of multiple cancers).

At this point, we carried out gene-set enrichment analysis of the genes modified at transformation (n=314) to search for cellular processes and the pathways affected. Genes were classified into the following functional categories: histone modification (*KMT2D*, *HDAC9*), chromatin modification (*PAX5*, *UBR5*), cell-cell adhesion (*CELSR2*, *TNIP1*), cell development (*WT1*, *CXCR4*), cell differentiation (*KIT*, *EZR*), protein modification (*KMD5C*, *KMD1B*), chromatin organization (*ARID1A*, *SOX1*), protein autophosphorylation (*PIM1*, *PRKD1*), chromosome organization (*HIST1H2BC*, *TP53*), transcription regulation (*RARB*, *PRDM1*), and protein kinase activity (*BCR*, *TGFA*). Focusing on pathways, those most significantly affected were: IGF1 pathway (*CD79B*, *PPM1D*), IL3-mediated signaling events (*TGFBRAP1*, *IFNA14*), insulin pathway (*COPA*, *CAD*), VEGF and VEGFR signaling network (*ACTN1*, *PELP1*), and nectin adhesion pathway (*ROBO1*, *NOS3*).

4.5. Patterns of evolution from WM to DLBCL

As mentioned above, all patients shared some NSVs at both stages, but some others were exclusively detected at diagnosis or transformation. Accordingly, certain subclones present at diagnosis would have evolved by acquiring new mutations responsible for the transformation event (or the progression), while others would have been reduced (disappearing or becoming undetectable). Therefore, and despite both tumors (WM

and DLBCL) having the same V(D)J rearrangement and an identical CDR3 region, suggesting a common progenitor cell, their evolution is consistent with a branching model. These findings should be considered with caution because of the low level of infiltration of diagnostic samples, which may lead to the underdetection of variants that are present at a low level in the tumor population.

This nonlinear evolutionary pattern was well illustrated by patient #2, who had a symptomatic progression of WM before transformation, and was studied at the time of all three events (Fig 4). During the evolution of his condition, we found mutations that were conserved from diagnosis to transformation (n=29). However, many other novel alterations not present at diagnosis appeared at progression (n=17) and transformation (n=38). Almost all of these were different, with five exceptions: *PPM1D*, *SBF2*, *TRAPPC9*, *TRPM7* and *WT1*. In the same way, some mutations present at diagnosis or acquired at progression (i.e., two *TP53* mutations) disappeared or became undetectable (at the level of sensitivity used) by the time of disease transformation. This would mean that the transformed final clone did not come from the intermediate subclone responsible for progression, but from a previous minor subclone that only grew after progression.

5. DISCUSSION

Waldenström's macroglobulinemia (WM) patients eventually experience histological transformation to diffuse large B-cell lymphoma (DLBCL) (2.4% transformation rate at 10 years), being at risk of poor outcome and short survival.[6,7] Understanding the biology and mechanisms underlying this process is important for identifying susceptible patients and for developing therapeutic strategies aimed at cancer control. In this study, we have carried out for the first time a WES study of four cases with paired WM and transformed DLBCL samples in order to evaluate the genetic basis of this transition and to find genomic alterations and pathways that could be therapeutically targeted.

Maybe due to the small sample size, we could not find a unique genetic event responsible for WM transformation to DLBCL. In fact, our findings revealed extensive genetic heterogeneity with a large number of aberrations affecting many genes and pathways, reflecting the complexity associated with the transformation process. Even asymptomatic and chemotherapy-naïve patients, as was the case for patient #4, may develop an aggressive disease after more than 12 years with an untreated indolent WM. Many more alterations were associated with aggressive lymphoma development, although the number was inversely related to the time to transformation. Thus, patients showing the fastest transition (#1 and #3) presented the greatest number of mutations. This could be explained by the onset of new mutations conferring a proliferative advantage on the harboring cell, leading to stronger competition during the evolution of the cancer.[41–43]

However, not all the events would be of equal importance in the pathogenesis of the disease. Mutations present at both events are likely to be spread in nearly all clones and to remain stable over time. In our WM cases, these variants had a higher variant allele frequency, so targeting these presumed early genetic events could lead to the elimination of all oncogenic clones. An example of these mutations would be the *MYD88* L265P, the only one present in all patients and at all times, a mutation with a well established role in both WM and DLBCL.[16,24,44] It is of particular note that, in patient #1, it was the only alteration, together with *TNIP1*, recognized at both times. *TNIP1* is an essential gene for NF-κB activation, so its mutation could be significant in the transformation process, in which NF-κB signaling is known to be involved.[12] Furthermore, this patient also showed other differences with respect to the other cases, such as a much higher frequency of mutations at transformation (n=165), diffuse spleen infiltration by aggressive lymphoma cells, and an unmutated *CD79B*.

Now considering *CD79B*, this B-cell receptor (BCR) associated gene was frequently mutated, appearing in 2/3 cases (67%) at diagnosis and in 3/4 cases (75%) at transformation. VAFs of this mutation in relation to *MYD88* indicated that it was present in half of the tumor clone in two patients and in the whole clone in the other patient, staying the same at both moments. This variant affects the first tyrosine ITAM kinase domain of the receptor and has been described in 12% of ABC-type DLBCLs,[45] in connection with the acquisition of the lymphoma phenotype. However, a limited oncogenic potential has been associated with *CD79B* mutations, since other alterations must occur in order to facilitate the transition to the aggressive lymphoma.[46–48] In conventional WM, *CD79B* has been found to be mutated in ~10%,[17,20,25,26] so the high frequency reported here

(3/4 of cases) is intriguing. Accordingly, we suggest that *CD79B* mutations identify a subgroup of WM with aggressive clinical evolution and a high risk of transformation.

With the exception of *MYD88* and *CD79B*, we observed a notable diversity in the mutational spectrum across samples, with few recurrent genes. Only *CELSR2* (growth factor), *FAM135B*, *IGFN1*, and *ZFHX4* (transcription factor) were present in more than one patient. This recurrence should be taken into account, since another commonly mutated gene in WM (30% patients), *CXCR4*,[17,20] appeared in just one patient. Regardless of their incidence, relevant mutations seem to be present in the initial WM cell that will transform, suggesting that they may be involved in tumor initiation. Conversely, genes exclusively involved in transformation may represent cooperating events that interact with these pathogenic mutations. The most frequently altered genes were *FRYL*, *HNF1B*, *PER3*, *PIM1*, and *PTPRD* (50% of patients). *PIM1* could be targeted with the PIM kinase inhibitors that have already shown activity in myeloma and acute myeloid leukemia.[49,50] However, some of the non-recurrent alterations could have been randomly acquired and may not be causally related to the pathogenesis of the disease.

Aberrations acquired at transformation probably cooperate with early initiating events in the selection of tumor clones responsible for disease progression. Nonetheless, considering the diversity of the alterations, many of them will not confer any advantage on the malignant cell. An illustrative case could be the presence of mutations in *TP53*. Deletions and mutations of this gene have been found to be related to poor prognosis in chronic lymphocytic leukemia,[51] multiple myeloma,[52] and probably in WM.[53] However, in our cases this gene appeared mutated at transformation in patient #1 and at progression in patient #2. Interestingly, this abnormality disappeared at transformation in the latter case, suggesting the loss of any advantage. Therefore, it is not easy to determine what prompts aggressive behavior: the genes, the number of alterations, the clone in which they arise, the pathway affected, the cell function deregulated or, most likely, a combination of all of these factors. These matters should be addressed in further studies with the ultimate aim of developing therapeutic strategies that may disrupt these mechanisms, thereby completely preventing transformation.

Finally, we observed that the transformation process seemed to be consistent with part of a branching model of evolution in which only clones containing driver mutations evolve to more aggressive populations by acquiring new aberrations. Identical scenarios have been reported in multiple myeloma,[54] follicular lymphoma,[12] chronic lymphocytic leukemia,[55] acute myeloid leukemia,[42] acute lymphoblastic leukemia,[56] and even solid tumors.[57] Nevertheless, this needs to be confirmed in further analyses of single cells. Likewise, it would also be interesting to establish whether there is a progenitor tumor cell that is common to both diseases and that is responsible for their pathogenesis. In this study, the analysis of the V(D)J rearrangement and CDR3 region of the immunoglobulin heavy chain gene in the WM and their matched lymphoma samples, confirmed that they belonged to the same clone. The concept of the tumor-initiating cell is already established for leukemia[58] and follicular lymphoma,[59,60] and it would be supported in our case by the presence from the outset of certain mutations that are shared by the entire tumor population, since they remain clonally stable throughout the entire course of the disease. DLBCL would then arise from these precursors, showing a higher (as in patients #2 and #4) or lower (patient #1) degree of genetic similarity to the WM clone.

In conclusion, although this is merely the first step and we were not able to identify a unique genetic event responsible for WM transformation to DLBCL, it appears that certain alterations may contribute to the onset of aggressive disease. Those genes frequently mutated at diagnosis (*CD79B*) should be considered as potential biomarkers for predicting the risk of transformation in prospective studies. Additional research is needed to better understand the biology of this process and to facilitate the design of preventive therapies targeted during the early evolutionary pathways that are responsible for transformation. This knowledge will enable us to improve the outcome of these patients.

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7. CONFLICT OF INTEREST

The authors declare no conflict of interest

8. AUTHOR CONTRIBUTIONS

C.J., S.A.A. and R.G.S. designed the initial study. C.J., S.A.A. and M.A. selected the patients and prepared the samples. G.R.O preprocessed the data for analysis. C.J., S.A.A., M.I.P.C and M.G.A. analyzed the data and interpreted the results. M.C.C., M.E.S., R.C. and A.B. helped collect the data. L.A.M. helped with the statistical analysis.

R.G.S was the clinician responsible for patients, ensuring the protocols were correctly followed, sampling, and collecting clinical data.

N.P. and N.C.G. were responsible for the immunophenotyping and cytogenetic analysis, respectively, of the patients included in this series.

C.J. and S.A.A. prepared the initial version of the paper. R.G.S. reviewed the conception and design of most of the work and corrected the manuscript. M.G. was the head of the group, supervised the final revision of the draft and gave final approval of the version to be published.

Supplementary information is available at Blood Cancer Journal's website.

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FIGURES

Figure 1.- Frequency of mutations at diagnosis (WM) and transformation (DLBCL). Comparison of the median number of mutations of the four patients at diagnosis (n=21) and upon transformation (n=85).

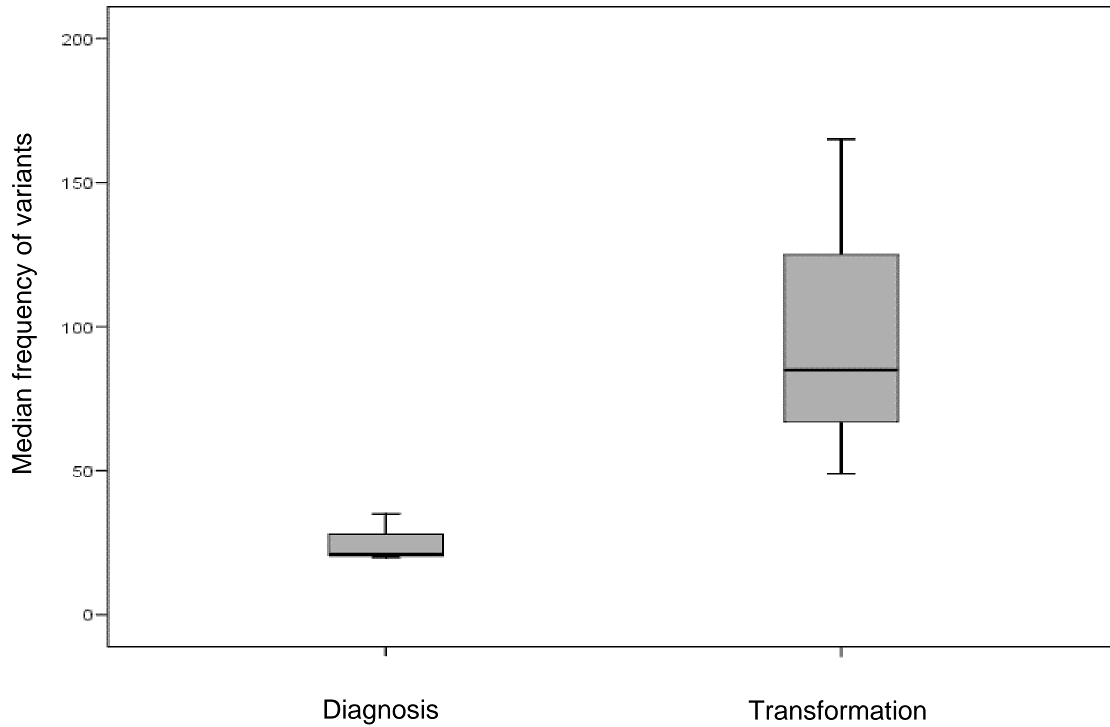


Figure 2.- Representation of the total number of alterations at each moment (diagnosis, progression and transformation) versus the time to transformation. Patients #1 and #3 presented the fastest transformation (~3 years) and the highest frequency of variants (165 and 98, respectively). Patient #2 transformed in 5 years and had 72 mutations. Patient #4 took 13 years to transform and acquired only 49 alterations.

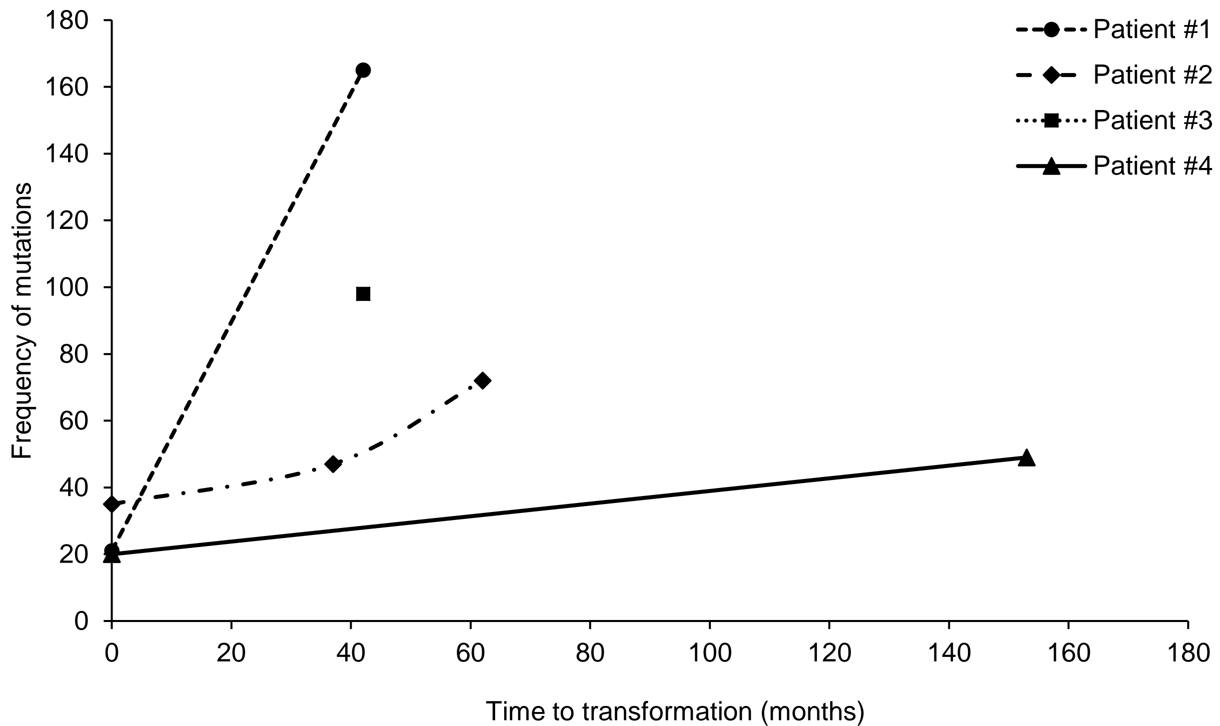


Figure 3.- Variant allele frequency (VAF) of common mutations at diagnosis, progression and transformation for each patient. The percentage of tumor cells affected by a mutation decreased from diagnosis to transformation in most of the cases.

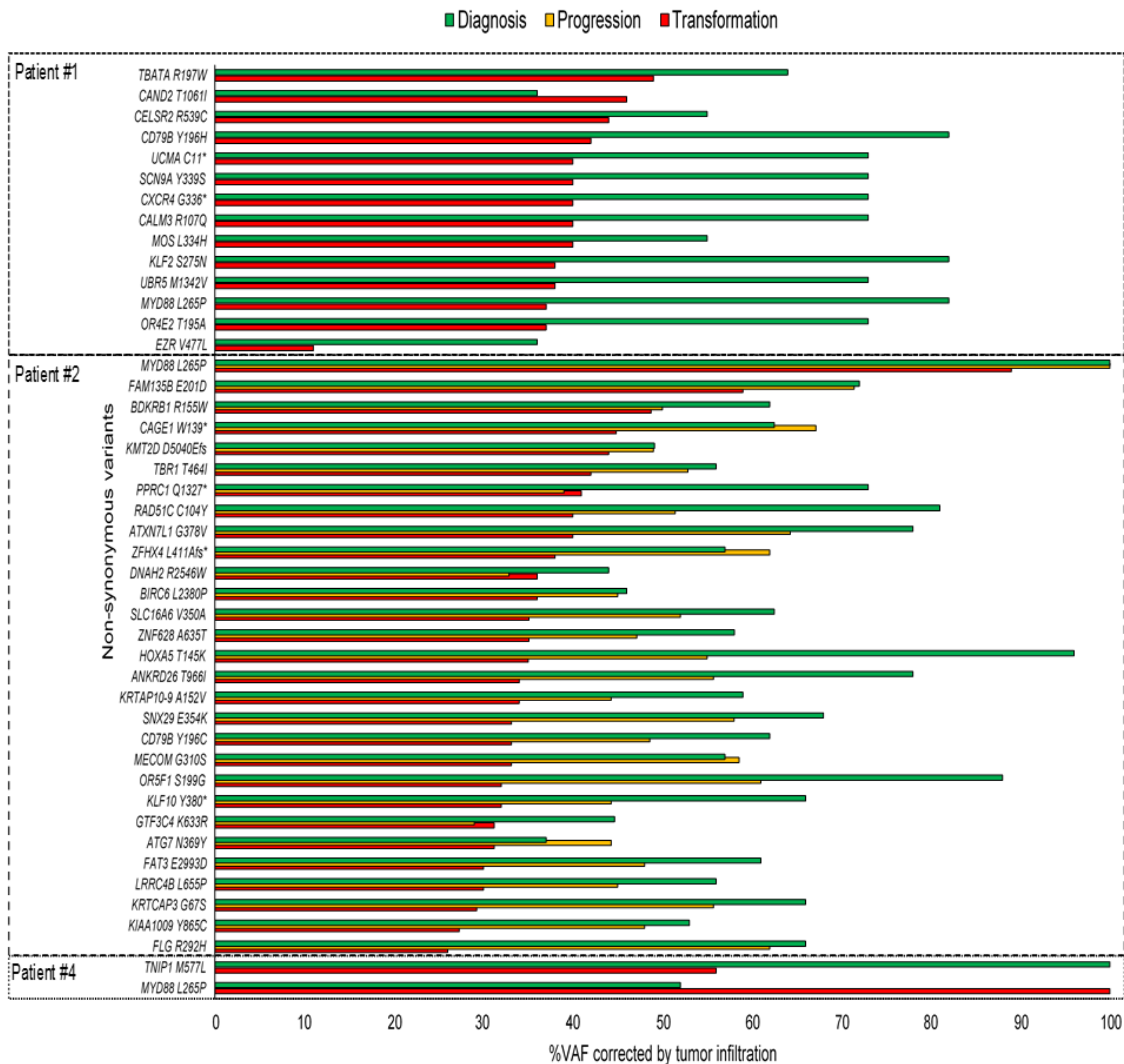
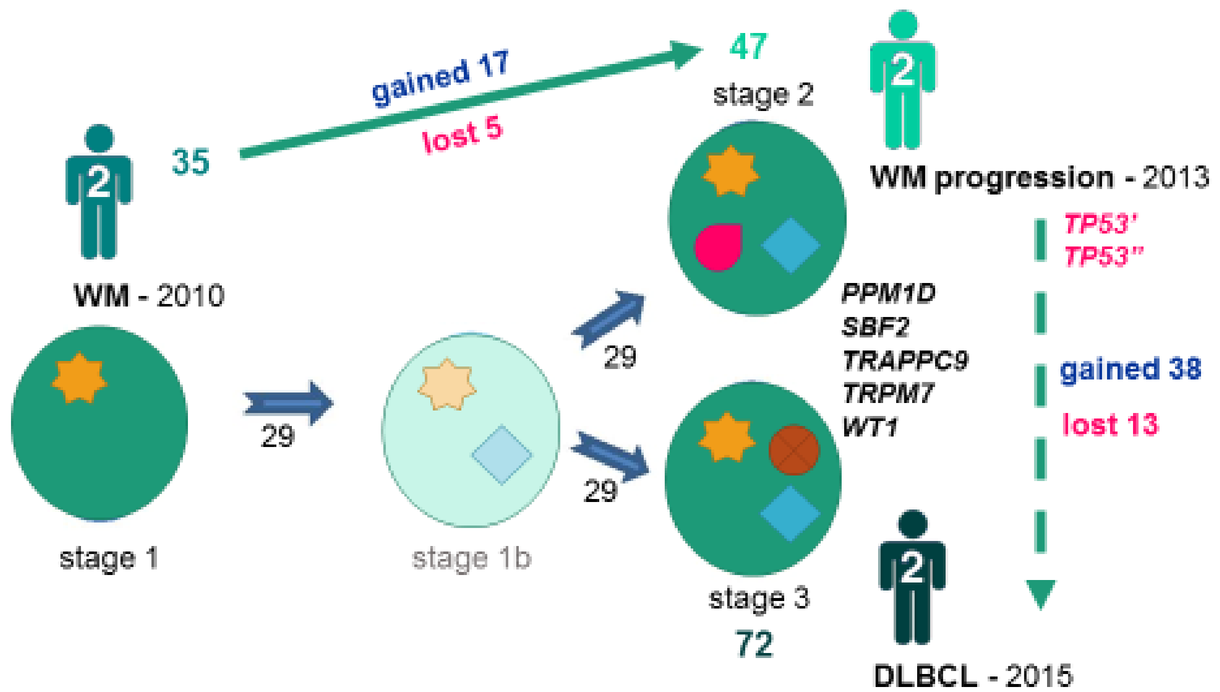


Figure 4.- Evolution of WM in patient #2. This patient was diagnosed with WM in 2010 and transformed to DLBCL in 2015, with a symptomatic progression in 2013 before the transformation. We observed 35 mutations at diagnosis, 47 at relapse and 72 at transformation, including 29 alterations that were conserved at the times of the three events. The *PPM1D*, *SBF2*, *TRAPPC9*, *TRPM7* and *WT1* genes were mutated either at progression or transformation. By contrast, the two mutations found in *TP53* were seen at relapse but were lost by the time of transformation. This implies that the transformed final clone did not evolve from the same subclone as was responsible for progression, but from a previous one that would not yet have acquired the *TP53* mutations (among others).



TABLES

Table 1.- Clinical characteristics of patients

Patient #	**1**	**2**	**3**	**4**
		Waldeström macroglobulinemia		
Age at diagnosis	62 (2009)	82 (2010)	81 (2003)	72 (2002)
Clinical symptoms				
Anemia	Yes	Yes	No	Yes
Polyadenopathies	Yes	No	No	No
Hyperviscosity	No	No	Yes	No
Others	No	No	No	No
Bone marrow infiltration (FCM)	12%	22%	16%	11%
Frontline therapy (year)	R-VD (2009)	R-CD (2010)	Plasmapheresis	W&W
Therapies at relapse (year)	FC (2011)	FC (2013) RB (2013/2014) R-VD (2015)	None	None
		Transformation to DLBCL		
Time to transformation (years)	3	5	3	13
Clinical symptoms				
Anemia	Yes	No	Yes	Yes
Polyadenopathies	No	Yes	No	No
LDH elevation	Yes	Yes	Yes	Yes
B symptoms	Yes	No	Yes	Yes
Others	Splenomegaly			
Tumor infiltration (FCM)	40% (spleen)	50% (adenopathy)	16% (BM)*	43% (BM)
DLBCL therapy	None	GemOx (2015) Ibrutinib (2016)	None	RCOP (2015) (reduced)
Status	Dead	Palliative care	Dead	Dead

R-VD: Rituximab, Bortezomid, Dexamethasone; FC: Fludarabine, Cyclophosphamide; R-CD: Rituximab, Cyclophosphamide, Dexamethasone; RB: Rituximab, Bendamustine; GemOx: Gemcitabine, Oxaliplatin; BM: Bone marrow; W&W: Watch and wait (observation); RCOP: Rituximab, Cyclophosphamide, Vincristine, Prednisone.

* BM: 8% WM and 8% DLBCL

Table 2.- Mean variant allele frequency (VAF) of common and exclusive mutations at diagnosis and transformation

	Patient #1		Patient #2		Patient #4	
	Diagnosis	Transformation	Diagnosis	Transformation	Diagnosis	Transformation
Mean VAF of common mutations	76%	78%	64%	37%	66%	38%
Mean VAF of exclusive mutations	68%	51%	30%	22%	45%	16%

Suplemental table 1.- List of mutations present in each patient at one-time moment.

Gen	Mutation	% VAF	%VAF corrected by tumor infiltration	Patient #	Time point
ABCB4	p.T1255K	22	56	1	Transformation
ACTN1	p.V873M	12	31	1	Transformation
ADAMTS16	p.A514E	16	39	1	Transformation
ADAMTS2	p.N949K	22	55	1	Transformation
ADAMTS9	p.L1420M	12	24	2	Transformation
AGPAT4	p.P295L	11	21	2	Transformation
AIM1	p.G638D	21	52	1	Transformation
AJAP1	p.P146L	10	81	1	Diagnosis
AMER1	p.V633F	16	39	1	Transformation
ANKRD52	p.T665A	7	14	2	Transformation
AP3B2	p.V231M	9	12	2	Progression
ARID1A	p.M872V	17	43	1	Transformation
ASTN2	p.G539V	28	71	1	Transformation
ASXL3	p.P1003S	9	13	2	Progression
AVPR1B	p.S220N	8	68	1	Diagnosis
B3GNT6	p.L316W	9	80	4	Diagnosis
BCCIP	p.L178R	26	64	1	Transformation
BGN	p.R183W	8	68	1	Diagnosis
BHLHE22	p.G217S	7	67	4	Diagnosis
BTG1	p.S43N	13	26	2	Transformation
C10orf82	p.R48G	26	64	1	Transformation
C17orf107	p.A144T	26	64	1	Transformation
C19orf59	p.M111I	13	26	2	Transformation
C21orf49	p.G68*	3	28	4	Diagnosis
C6orf141	p.R218*	14	35	1	Transformation

Gen	Mutation	% VAF	%VAF corrected by tumor infiltration	Patient #	Time point
CAD	p.Y60C	23	57	1	Transformation
CAPN13	p.D541N	18	44	1	Transformation
CASC5	p.T2217I	7	12	4	Transformation
CCDC88A	p.K714E	20	50	1	Transformation
CCNA1	p.T220N	19	49	1	Transformation
CDH7	p.A591V	14	34	1	Transformation
CDH9	p.G250V	23	56	1	Transformation
CEP170B	p.A795T	25	61	1	Transformation
CHST2	p.L68F	17	44	1	Transformation
CHST2	p.L68V	16	41	1	Transformation
CLPB	p.A222T	24	59	1	Transformation
CMYA5	p.E3551*	18	45	1	Transformation
CNTNAP2	p.S53P	19	47	1	Transformation
COPA	p.D351H	6	10	4	Transformation
CRISPLD1	p.S102I	18	44	1	Transformation
CRY2	p.I521T	4	36	4	Diagnosis
CRYGN	p.R96C	18	44	1	Transformation
CTNNA3	p.L814*	19	48	1	Transformation
CXorf65	p.F2L	22	55	1	Transformation
DCHS2	p.G924R	13	100	1	Diagnosis
DDX23	p.R368K	20	51	1	Transformation
DDX53	p.P288S	13	32	1	Transformation
DENND6B	p.F94L	22	54	1	Transformation
DLGAP2	p.R237W	23	58	1	Transformation
DNAH7	p.L3066H	9	18	2	Transformation
DRP2	p.R410H	16	29	4	Transformation
DSC3	p.K653Q	5	10	2	Transformation
DSCAML1	p.V189I	44	63	2	Progression
DSG2	p.T108N	8	11	2	Progression
EHF	p.G14S	8	14	4	Transformation
EIF4G3	p.Q1558K	11	90	1	Diagnosis
ENAH	p.E217D	4	35	1	Diagnosis
ENTPD2	p.G340E	19	48	1	Transformation
ERAP2	p.H145L	18	44	1	Transformation
ERBB3	p.T32A	21	53	1	Transformation
ERCC4	p.T29I	10	18	4	Transformation
ETAA1	p.E25D	29	71	1	Transformation
FAM135B	p.N447K	17	43	1	Transformation

Gen	Mutation	% VAF	%VAF corrected by tumor infiltration	Patient #	Time point
FAM149A	p.R364W	20	100	1	Diagnosis
FAM154A	p.G77W	30	76	1	Transformation
FAM209A	p.S6L	35	88	1	Transformation
FAM83F	p.F204Y	8	15	2	Transformation
FAM84A	p.R220C	22	55	1	Transformation
FARP2	p.V1003I	23	57	1	Transformation
FAT4	p.S4952F	6	11	4	Transformation
FBN3	p.I1504T	21	52	1	Transformation
FEZF1	p.P76T	21	51	1	Transformation
FLJ00104	p.H240Afs*21	17	41	1	Transformation
FLT4	p.T70S	26	37	2	Progression
FNBP4	p.A900T	26	66	1	Transformation
FREM1	p.H1258Q	12	23	2	Transformation
FRYL	p.A355V	16	41	1	Transformation
FRYL	p.N2282K	7	14	2	Transformation
FSD1	p.R332C	17	34	2	Transformation
GABRA3	p.Q235L	31	77	1	Transformation
GLIS3	p.T322M	6	12	4	Transformation
GLOD4	p.F199S	7	14	2	Transformation
GLRA1	p.R57K	13	33	1	Transformation
GOT2	p.A213S	8	16	2	Transformation
GPATCH8	p.S469G	10	84	1	Diagnosis
GPC5	p.S245F	17	42	1	Transformation
GPR116	p.L1198P	13	32	1	Transformation
GPR6	p.L210P	24	60	1	Transformation
GRIPAP1	p.F10S	32	79	1	Transformation
GRM3	p.R68H	16	39	1	Transformation
GRM6	p.R731Q	7	10	2	Progression
HDAC9	p.R663Q	19	48	1	Transformation
HIST1H2BC	p.E72D	20	49	1	Transformation
HIVEP1	p.S2574T	19	48	1	Transformation
HNF1B	p.S115N	22	54	1	Transformation
HNF1B	p.Q147H	16	32	2	Transformation
HOXA3	p.E69D	17	25	2	Progression
IGFN1	p.T1549R	8	34	2	Diagnosis
IGLL5	p.S47R	24	61	1	Transformation
IGSF1	p.M335I	19	48	1	Transformation
IMPA1	p.I27M	23	59	1	Transformation

Gen	Mutation	% VAF	%VAF corrected by tumor infiltration	Patient #	Time point
IRF2BP2	p.Y43D	16	41	1	Transformation
ITIH2	p.C261Y	21	52	1	Transformation
JARID2	p.D744N	12	22	4	Transformation
JMJD6	p.A283T	6	11	4	Transformation
KALRN	p.L1654F	42	100	1	Transformation
KCNJ1	p.F65I	6	12	2	Transformation
KDM1B	p.A720T	19	46	1	Transformation
KDM3B	p.I1683M	19	48	1	Transformation
KDM4C	p.W773*	18	46	1	Transformation
KDM5C	p.S169A	41	100	1	Transformation
KHSRP	p.C436R	19	48	1	Transformation
KIAA0100	p.Q687R	9	74	1	Diagnosis
KIAA0319L	p.L131*	7	12	4	Transformation
KIT	p.S465F	21	52	1	Transformation
KLF17	p.E30K	10	19	2	Transformation
KMT2D	p.L3154*	13	25	2	Transformation
KRT4	p.V133F	20	49	1	Transformation
KRTAP4-2	p.G16R	8	16	2	Transformation
KTN1	p.N970S	14	28	2	Transformation
L1TD1	p.K812E	20	49	1	Transformation
LILRB4	p.S146*	8	14	4	Transformation
LIN54	p.P311R	6	12	2	Transformation
LPCAT1	p.S500C	7	14	4	Transformation
LPPR3	p.L41V	6	10	4	Transformation
LRP4	p.P183S	15	37	1	Transformation
MAGEE1	p.R658*	15	38	1	Transformation
MALRD1	p.W180C	20	49	1	Transformation
MCF2L2	p.R120*	10	83	1	Diagnosis
MCHR2	p.F208Sfs*5	11	19	4	Transformation
MCM8	p.T135P	7	13	2	Transformation
MDN1	p.R3986W	5	10	4	Transformation
MED13	p.P835R	5	38	1	Diagnosis
MELK	p.Y267D	19	47	1	Transformation
MEP1A	p.P676L	20	50	1	Transformation
MLLT3	p.T319Vfs*8	10	26	1	Transformation
MMAA	p.G35R	6	11	4	Transformation
MMP3	p.R53K	31	77	1	Transformation
MPEG1	p.S273F	17	43	1	Transformation

Gen	Mutation	% VAF	%VAF corrected by tumor infiltration	Patient #	Time point
MT-ND5	p.L266Pfs*3	34	62	4	Transformation
MUSK	p.D725N	21	52	1	Transformation
MYO18A	p.S35R	12	23	2	Transformation
MYO5A	p.R178Q	16	41	1	Transformation
NCKAP5	p.E1307K	7	13	4	Transformation
NCOA2	p.P724S	7	33	2	Diagnosis
NDST3	p.I267T	19	47	1	Transformation
NEK4	p.R777K	18	45	1	Transformation
NGRN	p.A63G	14	36	1	Transformation
NOS3	p.R670W	19	48	1	Transformation
NRBP1	p.R104C	20	50	1	Transformation
NRP1	p.R334H	10	84	1	Diagnosis
NSMF	p.Q310K	9	76	1	Diagnosis
NTSR1	p.R372H	21	53	1	Transformation
ODF3B	p.S44P	39	97	1	Transformation
ODF3B	p.P33A	19	49	1	Transformation
OR2T4	p.P127H	15	39	1	Transformation
OR4C6	p.C95*	14	27	2	Transformation
OR4K5	p.I219S	7	12	4	Transformation
OR4K5	p.F211C	6	11	4	Transformation
OR4N4	p.N137T	16	40	1	Transformation
OR5AS1	p.Q193H	7	12	4	Transformation
OR5M9	p.L129F	15	29	2	Transformation
OSBPL10	p.G53V	16	41	1	Transformation
PAPOLA	p.Q17L	7	12	4	Transformation
PATZ1	p.K299Q	18	46	1	Transformation
PAX5	p.P190R	6	12	2	Transformation
PCDH20	p.I544M	21	53	1	Transformation
PCDHA3	p.V464M	17	42	1	Transformation
PCDHGB3	p.T406R	5	10	4	Transformation
PCLO	p.E2925D	3	29	4	Diagnosis
PCLO	p.R1613Q	9	16	4	Transformation
PCNT	p.R169H	10	44	2	Diagnosis
PER3	p.H509Y	15	37	1	Transformation
PHB	p.L241V	16	40	1	Transformation
PIM1	p.K24N	34	85	1	Transformation
PIM1	p.M1_M87del	31	77	1	Transformation
PIM1	p.L25V	20	49	1	Transformation

Gen	Mutation	% VAF	%VAF corrected by tumor infiltration	Patient #	Time point
PIM1	p.L182V	18	45	1	Transformation
PIM1	p.L129F	17	43	1	Transformation
PIM1	p.F116V	16	41	1	Transformation
PIM1	p.Q37P	16	41	1	Transformation
PIM1	p.P125S	16	40	1	Transformation
PIM1	p.L184F	14	35	1	Transformation
PIM1	p.Q37*	13	31	1	Transformation
PIM1	p.G50D	12	30	1	Transformation
PIM1	p.P81S	11	28	1	Transformation
PIM1	p.G55D	9	23	1	Transformation
PIM1	p.S146N	8	20	1	Transformation
PIM1	p.E135*	6	16	1	Transformation
PKHD1	p.N1532*	12	30	1	Transformation
PLEKHA7	p.I157M	31	77	1	Transformation
POLR2F	p.M57K	25	63	1	Transformation
POTEC	p.K36R	3	28	1	Diagnosis
PPL	p.T564M	16	31	2	Transformation
PRDM1	p.A174P	26	65	1	Transformation
PRDM6	p.P317L	22	56	1	Transformation
PRKD1	p.R695W	19	48	1	Transformation
PSG9	p.*427W	16	39	1	Transformation
PTPRD	p.L673H	25	63	1	Transformation
PTPRD	p.R1323H	9	17	4	Transformation
RARB	p.F82L	18	34	2	Transformation
RGS1	p.T94I	22	54	1	Transformation
RGS22	p.R1108W	23	57	1	Transformation
RNPC3	p.Y49D	36	89	1	Transformation
ROBO1	p.P1529S	15	37	1	Transformation
RYR3	p.Q1493H	6	12	2	Transformation
SCN7A	p.F724L	8	16	2	Transformation
SDK1	p.Q874*	28	69	1	Transformation
SEL1L2	p.T639M	20	50	1	Transformation
SEMA3G	p.E186D	6	46	1	Diagnosis
SEMA6D	p.V758L	20	50	1	Transformation
SETBP1	p.Y571Hfs*32	14	34	1	Transformation
SGSM1	p.L861R	21	52	1	Transformation
SHANK2	p.V80M	23	59	1	Transformation
SHROOM3	p.P982T	13	33	1	Transformation

Gen	Mutation	% VAF	%VAF corrected by tumor infiltration	Patient #	Time point
SI	p.T543I	6	47	1	Diagnosis
SIAH2	p.A126V	16	40	1	Transformation
SIK1	p.R432P	11	21	2	Transformation
SLC13A1	p.A383V	13	26	2	Transformation
SLC27A1	p.R462C	18	44	1	Transformation
SLC35F5	p.R44*	5	10	2	Transformation
SLC6A8	p.A345S	11	27	1	Transformation
SLC9A3	p.L299R	19	47	1	Transformation
SLITRK3	p.F943I	6	11	4	Transformation
SLITRK4	p.S358I	21	41	2	Transformation
SOX1	p.Q17*	22	56	1	Transformation
SPAG17	p.H529Y	16	41	1	Transformation
SPATA17	p.A219T	16	39	1	Transformation
SPEF2	p.E116D	15	37	1	Transformation
SPEG	p.N1538D	7	14	2	Transformation
SPHKAP	p.C84F	9	13	2	Progression
SPTBN5	p.A452V	25	61	1	Transformation
SRP54	p.D138G	14	34	1	Transformation
ST6GALNAC6	p.R202Q	9	17	4	Transformation
STARD9	p.C3057Y	7	12	4	Transformation
STEAP1	p.E217*	6	11	4	Transformation
SUN1	p.A556V	23	59	1	Transformation
SYCP2L	p.L161Wfs*8	11	21	2	Transformation
TAS2R10	p.A148V	17	44	1	Transformation
TBCD	p.P485S	18	45	1	Transformation
TBL1XR1	p.G247E	22	32	2	Progression
TBX22	p.G109R	32	63	2	Transformation
TCEAL2	p.Q15P	6	15	1	Transformation
TCHH	p.R953H	8	15	4	Transformation
TDRD6	p.E988*	14	26	4	Transformation
TEX13A	p.R77C	11	95	1	Diagnosis
TEX29	p.G122E	8	11	2	Progression
TGFA	p.A85V	19	48	1	Transformation
TGFBRAP1	p.G74S	20	51	1	Transformation
TJP3	p.G878E	22	54	1	Transformation
TP53	p.T211I	26	65	1	Transformation
TP53	p.R196*	19	27	2	Progression
TP53	p.R248W	11	15	2	Progression

Gen	Mutation	% VAF	%VAF corrected by tumor infiltration	Patient #	Time point
TRANK1	p.K135N	7	12	4	Transformation
TRIM62	p.E52G	24	60	1	Transformation
TRPM3	p.R571H	16	32	2	Transformation
TTC40	p.D2439N	23	57	1	Transformation
TTLL2	p.S329P	9	16	4	Transformation
TYRO3	p.R490C	21	52	1	Transformation
UBE2A	p.F41S	37	93	1	Transformation
UBR7	p.D116G	9	17	2	Transformation
UCMA	p.C11*	22	40	4	Transformation
UGT3A1	p.M114T	19	47	1	Transformation
UNC13B	p.G355C	35	87	1	Transformation
UNC13C	p.S818G	19	48	1	Transformation
UNC93B1	p.N44T	38	95	1	Transformation
VSIG10L	p.A859P	5	42	1	Diagnosis
VWF	p.S1506L	4	20	2	Diagnosis
WDR93	p.L490M	3	28	4	Diagnosis
ZC3H12B	p.V764M	34	84	1	Transformation
ZFHX4	p.K3332R	6	15	1	Transformation
ZMYM2	p.A1258V	7	57	1	Diagnosis
ZNF181	p.H451P	5	22	2	Diagnosis
ZNF324	p.A226S	8	15	4	Transformation
ZNF385D	p.T385S	17	42	1	Transformation
ZNF469	p.R473W	24	59	1	Transformation
ZNF565	p.V296A	23	56	1	Transformation
ZNF84	p.I508S	27	67	1	Transformation

Conclusiones

PRIMER TRABAJO — En relación con el estudio de la mutación *MYD88* L265P:

1. La mutación *MYD88* L265P es característica de la macroglobulinemia de Waldenström, encontrándose en más del 85% de los pacientes, mientras que es poco frecuente o está ausente en otros síndromes linfoproliferativos afines. Esto la convierte en un marcador de gran ayuda en el diagnóstico diferencial.
2. La mutación *MYD88* L265P también está presente en la mayoría de las gammapatías monoclonales de significado incierto tipo IgM, aunque se necesitan metodologías con elevada sensibilidad para su detección.
3. No hay grandes diferencias clínico-biológicas entre los pacientes que presentan o no la mutación de *MYD88*, pero sí se puede decir que los casos que carecen de ella tienen una firma de MW menos típica: menor cuantía de componente monoclonal, más linfocitosis, niveles séricos más elevados de lactato deshidrogenasa, inmunofenotipo atípico y menos hipermutación somática.

SEGUNDO TRABAJO — En relación con la técnica desarrollada para la detección de la mutación *MYD88* L265P:

1. La ASO-RQ-PCR estandarizada en el presente trabajo es una metodología sencilla, específica y sensible para la detección de la mutación *MYD88* L265P.
2. Al añadir una discordancia adicional de una base nucleotídica en el extremo 3' del cebador reverso se favorece que las muestras negativas presenten una amplificación muy tardía respecto a las mutadas, siendo posible detectar hasta una célula mutada entre 1000 no mutadas. Todo ello la convierte en una técnica muy robusta en términos de especificidad y sensibilidad.
3. La metodología estandarizada se postula como técnica de elección para monitorizar la enfermedad, ya que permite cuantificar la carga tumoral con una alta concordancia con la citometría de flujo.

TERCER TRABAJO — En relación con la caracterización molecular de las gammapatías IgM:

1. Las mutaciones del gen *CXCR4* están presentes en un 40% de las gammapatías IgM, pero no parecen tener impacto clínico-pronóstico.

2. La frecuencia de mutaciones somáticas aumenta progresivamente de GMSI-IgM a MW asintomática y a MW sintomática, aunque sin diferencias significativas en su distribución.
3. La vía del receptor de células B (BCR) se postula como un mecanismo importante para la patogénesis de la MW ya que, después de *MYD88* y *CXCR4*, las mutaciones en *CD79B* fueron las más comunes en nuestra serie.
4. La desregulación de la expresión génica tiene mayor impacto en la presentación clínica que la presencia de mutaciones. Así, los pacientes sintomáticos presentaron sobreexpresión de genes pertenecientes a la vía de los receptores Toll-like/MyD88, y este perfil se asoció con características de peor pronóstico: menores niveles de hemoglobina, plaquetas y albúmina y niveles más elevados de β_2-microglobulina y componente monoclonal.

CUARTO TRABAJO — En relación con la transformación a linfoma B difuso de célula grande:

1. La transformación de MW a LBDCG implica la adquisición de numerosas alteraciones moleculares, cuyo número total se correlaciona inversamente (aunque no de forma significativa) con el tiempo de transformación. Sin embargo, nuestros resultados sugieren que lo que provoca la transición en última instancia no es la cantidad de alteraciones ni el tiempo de evolución, sino la adquisición de alteraciones moleculares específicas.
2. Las mutaciones presentes en ambos estadios y que afectan a una elevada proporción de células tumorales, como las de *MYD88* o *CD79B*, se consideran eventos conductores iniciales en el proceso maligno. En cambio, las alteraciones recurrentes adquiridas en la transformación (*PIM1*, *FRYL* o *HNF1B*) representarían eventos cooperantes que participan en la selección de los clones responsables de la progresión de la enfermedad.
3. El gen *CD79B* se encuentra frecuentemente mutado en el subgrupo de MW transformadas. Dada su baja incidencia en la MW convencional, es posible que pueda usarse como biomarcador para predecir el riesgo de transformación.
4. La transformación a LBDCG sigue un patrón de evolución tumoral ramificada ya que hay variantes alélicas presentes en el momento del diagnóstico o durante la progresión de la MW que no se detectan en el momento de la transformación.

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Summary

I. Introduction

Waldenström's macroglobulinemia (WM) is a rare indolent B-cell lymphoproliferative disorder (LPD) characterized by bone marrow infiltration with lymphoplasmacytic lymphoma and presence of an IgM monoclonal component.[1] The incidence in Spain is estimated at around 3.1 cases/1,000,000 inhabitants/year, affecting most commonly the elderly, with a median age at diagnosis of 71 years.[2]

At the clinical level, the disease presents a wide heterogeneity with a behavior ranging from indolent forms to highly symptomatic. Three main stages can be differentiated: 1) IgM monoclonal gammopathy of undetermined significance (IgM-MGUS), lacking on bone marrow infiltration; 2) asymptomatic WM (AWM), an stage fulfilling diagnostic criteria but lacking on symptomatology; and 3) symptomatic WM (SWM), a status in which complications of malignancy appear and make therapy necessary.[1,3,4] The three stages are successive in time, although not all patients go through all steps and remain indolent for years showing no progression.[5] Within symptomatic patients, therapy is usually effective, but the evolution is highly variable, with patients being disease free for long periods and others quickly progressing and needing additional therapy.[6] Causes underlying this clinical heterogeneity, as well as physiopathological mechanisms that lead progression of tumor cells towards aggressive disease remain to be clarified.

Therapy is restricted to symptomatic patients and must consider specific disease characteristics (burden of disease, IgM and β_2-microglobulin levels, presence of cytopenias) and patient characteristics (age, comorbidities, toxicity). Current therapeutic approaches include alkylators, nucleoside analogs, proteasome inhibitors, immunomodulatory agents or B-cell receptor pathway inhibitors, as well as monoclonal antibodies such as anti-CD20 (e.g., rituximab), which are used in monotherapy or as backbone for combinations. Rituximab-based therapy is used in virtually all patients and can be combined with an alkylating agent (i.e., dexamethasone, rituximab, cyclophosphamide−DRC; or bendamustine plus rituximab−BendaR) or a proteasome inhibitor (e.g., bortezomib, rituximab, dexamethasone−BRD; carfilzomib, rituximab, dexamethasone−CaRD). Purine nucleoside analogues (fludarabine and cladribine) were widely used in Europe,[7–9] although they have currently been relegated for 2nd or later lines of therapy. In January 2015, ibrutinib (a first-in-class inhibitor of Bruton's tyrosine kinase−BTK) was approved by the Food and Drug Administration (FDA) as a new option for selected newly diagnosed or relapsing patients.[10,11] It was also immediately

approved by the European Medicines Agency (EMA), so it is now available for most patients in Spain. Investigational therapies include agents against BCL2,[12–14] Nampt[15] and the mammalian target of rapamycin (mTOR),[16,17] as well as new proteasome inhibitors (oprozomib and ixazomib)[18,19] and other anti-CD20 monoclonal antibodies (ofatumumab).[20] Additional potentially druggable targets that play a role in WM cell survival and are already being tested include MyD88,[21] Syk,[22] proteasome-associated deubiquitinating enzymes (USP14 and UCHL5),[23] Interleukin 6 (IL-6),[24] and the activator of mutant p53 (PRIMA-1Met).[25] Finally, CD19-directed chimeric antigen receptor-modified (CAR) T-cell immunocellular therapy is under development.[26]

Genetic characterization

In recent years, progress has been made to characterize the genetic profile of WM tumor cells. *MYD88* L265P mutation was identified as a unifying event in most WM cases (~91%)[27] and is also present in 50-80% IgM-MGUS,[28–30] where it has proven to be an independent risk factor for progression.[31] This suggests that it may be the tumor-initiating event that confers a competitive advantage to the clone and predisposes it to further genetic alterations responsible for progression to WM. However, the prognostic implication of *MYD88* mutational status still needs to be clarified, because some clinical data have demonstrated a better prognosis for cases that harbor the mutation, not only in WM,[32] but also in chronic lymphocytic leukemia (CLL).[33]

The strength of this mutation lies in its potential use as a tool for differential diagnosis between WM and other lymphoproliferative neoplasms with overlapping clinical features, such as IgM-related disorders, the pure lymphoplasmacytic lymphoma (without WM), marginal zone lymphoma or CLL with plasmacytic differentiation.[34,35] However, for that purpose it is necessary to first evaluate the presence of the *MYD88* L265P mutation in those closely related B-cell LPDs. Initial studies were carried out with conventional techniques, such as Sanger sequencing, which has a relatively low sensitivity, requiring at least 20-30% of mutated cells.[30,36,37] As many of the aforementioned tumors can have a low tissue infiltration, this technical limitation could lead to underestimate the mutation frequency.

The high prevalence of *MYD88* mutation in WM makes it a suitable marker for evaluating molecular response. Several reports have suggested that the use of a sensitive technique could be of great help during the follow-up monitoring of patients,[28,29] even for a better definition of response criteria.[38] Actually, minimal residual disease (MRD) analysis has been demonstrated to be a strong

predictor of outcome in LPD, especially in follicular and mantle cell lymphoma.[39,40] This highlights the need of specific and highly sensitive assays to identify the mutation and quantify the tumor burden. Real-time quantitative polymerase chain reaction with allele-specific oligonucleotides (ASO-RQ-PCR) is a technique that can discriminate low levels of the mutant sequence within a high wild-type DNA background.[41] The use of a TaqMan probe in the same reaction enables to quantify mutated alleles in real time. Thus, this method could be easily used to identify the mutation for diagnostic purposes and to monitor disease evolution during and after the therapy.

The second most common alteration in WM (29% of patients) affects the C-terminal domain of the chemokine receptor CXCR4. There are several types but all are similar to the germline mutations that are present in patients suffering from the WHIM syndrome (Warts, Hypogammaglobulinaemia, Infections and Myelokathexis), a rare form of immunodeficiency.[42] This protein plays a crucial role in modulating cell trafficking of hematopoietic stem cells and clonal B cells.[43] Currently, more than 30 different mutations (either nonsense or frameshift) have been described in WM, the most frequent being the C1013G (S338X) point mutation.[44] In IgM-MGUS are rare, since they affect to less than 20% of patients. Moreover, it is very likely that *CXCR4* WHIM mutations are primarily subclonal and probably acquired after the *MYD88* mutation along WM oncogenesis.[45,46] Interestingly, *CXCR4* mutations are closely linked to *MYD88* L265P, and almost none WM patient with a *CXCR4* mutated gene has been reported in the absence of the *MYD88* mutation. *In vivo* functional characterization has demonstrated that *CXCR4* alterations have an activating role in WM cells, which exhibited significant tumor proliferation and dissemination to extramedullary organs, leading to disease progression and decreased survival.[46] The potential clinical impact of these mutations is based on the fact that they seem to confer resistance to ibrutinib, as well as to other WM therapeutic agents, such as mTOR and PI3K inhibitors, but not to proteasome inhibitors.[47] Therefore, the systematic evaluation of *CXCR4* would allow identifying patients that might benefit from certain treatments.

Nonetheless, the efficacy of ibrutinib is primarily determined by *MYD88* status because the complex MyD88 L265P–BTK (the target of ibrutinib) promotes cell proliferation and makes cells more susceptible to this therapy.[48] Thus, patients positive for *MYD88* mutation alone achieve higher partial response rates (92%) compared to patients with *MYD88* plus *CXCR4* mutations (62%), while patients with a wild-type *MYD88* gene do not achieve an objective response alone (0%).[11,49]

On the other hand, patients with mutations in both genes present more commonly with a more aggressive and symptomatic disease (with higher bone marrow involvement and serum IgM levels), immediately requiring therapy at diagnosis. However, the overall survival is only impacted by *MYD88*, as wild-type patients have a worse survival than patients with *MYD88* L265P, while patients with and without *CXCR4* mutations have a similar survival in the presence of the *MYD88* L265P mutation.[32]

Other recurrent mutations found in WM concern genes such as *ARID1A* (17% of patients), *CD79A/B* (15%), *TP53* (7%), *MYBBP1A* (7%), or *TRAF3* (5%).[50,51,43] This means that, in spite of their monoclonality, tumor cells harbor an important variability that could be responsible for the clinical heterogeneity of WM. However, up to now, no clear correlation has been found between the mutational profile and the clinical behavior of IgM gammopathies, since previous works did not differentiate between the indolent and symptomatic forms of the disease. Furthermore, these mutations could have a role in the multistep oncogenic process that drives the transition from IgM-MGUS to first AWM, and later to SWM. Therefore, it would be interesting to evaluate the presence of these alterations in IgM-MGUS to see whether they appear at the beginning of the pathogenesis or if they are part of the potential mechanisms leading to progression.

Gene expression profiling

The improvement in our knowledge of this disease has allowed to characterize not only the genomic but also the transcriptomic profile of WM cells. Gene expression studies have also helped in the definition of some key molecular pathways underlying the physiopathology of WM, e.g., Toll-like receptor (TLR)/MyD88, CXCR4, nuclear factor-kappa B (NF-κB) and B-cell receptor (BCR). Initial studies mainly focused on the comparison between WM with CLL and multiple myeloma (MM). Results showed that the transcription profile of WM was more similar to CLL and normal B cells than to MM and normal plasma cells.[52,53] Other studies only addressed the expression of genes involved in the late stage of B-cell differentiation, identifying a heterogeneous pattern of loss in key regulatory genes in lymphoplasmacytic cells.[54,55] Alteration of gene expression profiling (GEP) was also demonstrated in areas with DNA copy number changes in WM (especially in chromosome 6q),[51] and associated with the mutational profile.[44,56] Thus, *CXCR4* mutated patients displayed a differential transcriptomic signature, affecting several functional networks such as cell cycle, DNA replication, cell-to-cell interaction and signaling, cellular growth, and proliferation.[44] In addition, a very recent

report using next generation sequencing (NGS) and transcriptome analysis has elucidated the different mechanisms underlying the three type of WM cases: *MYD88*^{wt}-*CXCR4*^{wt}, *MYD88*^{L265P}-*CXCR4*^{wt}, and *MYD88*^{L265P}-*CXCR4*^{WHIM}.[56] Clustering by genes related to B-cell differentiation effectively sorted samples in the three groups, suggesting that the double negatives may represent an earlier stage of B-cell differentiation. In addition, *CXCR4* mutations appeared to diminish B-cell differentiation and hamper the tumor suppressors up-regulated in response to mutant MyD88 signaling, and *MYD88*^{wt} patients showed an heterogeneous expression, indicating a high pathogenic diversity in this population.[56]

Finally, several GEP studies identified differences between IgM-MGUS, AWM and SWM, as well as potential mechanisms of progression. Trojani et al. found an overexpression of genes involved in regulation of transcription, immune response, cell activation and JAK/STAT, PI3K/Akt/mTOR and MAPK signaling pathways in WM compared with IgM MGUS.[57] On the contrary, Paiva et al. did not observe a significantly deregulated profile between clonal B-cells from the 3 disease groups, and they only found numerical differences in terms of clonal versus polyclonal B-cells.[58] Lastly, Herbaux et al. demonstrated that AWM and SWM displayed different expression signatures, mainly concerning the pathways of B-cell differentiation and activation, microenvironment interactions and PI3K/AKT.[59]

All these studies display some limitations either concerning the use of difficult-to-reproduce methodologies such as microarrays[60,61] and CD19-selected cells without paying attention to the heterogeneity of this population (i.e. clonal plus polyclonal), or because of the low number of patients included. Accordingly, more studies evaluating larger series, with pure populations and reproducible methods are strongly needed.

Transformation to diffuse large B-cell lymphoma

The multistep evolution process of WM is usually permanent and does not stop at the progression to symptomatic disease. The availability of more effective and less toxic therapies allows patients to live for long periods, which, as in other indolent B-cell LPDs, increases the probability to transform into aggressive forms, usually diffuse large B-cell lymphoma (DLBCL), in up to 10% of the patients.[62–66] This initially unusual phenomenon is now an increasing clinical worry, because these lymphomas are very resistant forms and the leading cause of death and shortened survival (<2 years) in these patients.[67–69] Transformation to DLBCL can occur at any time during the course of the disease:

at diagnosis, before treatment, during therapy, and even 20 years after the initial diagnosis.[68] In addition, there is no indicative clinicopathological feature or risk factor for developing DLBCL recognized up to now. Actually, there are no genomic studies of WM transforming patients that had searched for biological signals explaining this susceptibility. For this reason, the identification of genetic changes driving transformation would be essential for a comprehensive understanding of the transition from WM to DLBCL that could help in the development of new diagnostic strategies and targeted therapies.

Through the present doctoral work, we have attempted to improve our knowledge about the biology and pathogenesis of WM. Thus, we have characterized the genomic landscape of WM by carrying out mutational and expression studies, differentiating between the three forms of the disease, in order to find the potential causes responsible for this clinical heterogeneity and describe the role of certain alterations and pathways in the evolution and prognosis of WM. In addition, we have studied the process of transformation into DLBCL, by analyzing the whole exome of four WM transformed patients, to elucidate mechanisms underlying the transition from an indolent to an aggressive form of the disease. We have identified several of these possible mechanisms as well as a potential biomarker for predicting the risk of transformation in these patients.

II. Working hypothesis

Waldenström's macroglobulinemia (WM) is a rare, indolent B-cell lymphoma, incurable with current therapy, which shows important variability at the clinical level involving three different stages of presentation: IgM monoclonal gammopathy of undetermined significance (IgM-MGUS), asymptomatic WM (AWM), and symptomatic WM (SWM). Important advances in the understanding of the biology of WM have been made in the last few years. Whole-genome sequencing and further studies have identified the *MYD88* L265P mutation as a molecular event present in most WM cases (~91%), being highly specific respect to other lymphoproliferative disorders. Other recurrent alterations, such as *CXCR4* WHIM-like mutations (29%) and *ARID1A* mutations (17%), among others, have also been described. In the same way, gene expression studies have provided new insights into the characterization of the transcriptomic profile of WM tumor cells.

This increasing body of data is helping to delineate a genomic signature that will serve for a better understanding of the etiopathogenic bases of the disease. Accordingly, transition from IgM-MGUS to SWM is hypothesized to be based on the acquisition of multiple genetic hits required for progression from a pre-benign condition to a neoplastic disease. Eventually, this multistep evolution process can go beyond symptomatic WM and lead to transformation into more aggressive forms, as diffuse large B-cell lymphoma (DLBCL). These DLBCL patients are usually very resistant to therapy and have a very poor prognosis.

Biological causes and pathogenic mechanisms responsible for the clinical heterogeneity of WM, as well as their potential role in the evolution of the disease to more active stages still remain to be clarified.

In this context, the hypotheses of this thesis are:

a. A reliable diagnosis of WM, as well as a discrimination from other lymphoproliferative disorders, may be done in the clinical setting based on the determination of *MYD88* L265P. Moreover, assessment of this mutation can be used as a tool for monitoring response to therapy and minimal residual disease evaluation.

b. *MYD88* L265P can be detected by an inexpensive, easy-to-perform and sensitive tool, such as real-time allele-specific PCR, which could be routinely used for the diagnostic work-up of B-cell lymphoproliferative disorders.

c. The heterogeneity in the disease presentation is due to variable biological characteristics present in the tumor clone: presence of mutations in different genes or variations in gene expression profiling. We propose to analyze the genomic and transcriptomic landscape of WM, distinguishing between the three stages of the disease (IgM-MGUS, AWM and SWM), to identify molecular mechanisms that may differentiate indolent from symptomatic forms, or predict progression.

d. Transformation from WM to DLBCL is an ordered process governed by genetic abnormalities. We presume that some of these alterations are already present at diagnosis in a proportion that could be detected by the current NGS methodologies. Identifying these genetic changes will allow to define biological markers predicting the risk of transformation or explaining a certain susceptibility for developing DLBCL. A comprehensive review of the transition from WM to DLBCL will help to investigate new diagnostic strategies and targeted therapies.

III. Aims

Through the present doctoral study, we attempt to improve our knowledge of Waldenström's macroglobulinemia. With this premise, we consider the following objectives:

1) To evaluate the presence of the *MYD88* L265P mutation in a series of WM and closely related B-cell LPDs, with a highly sensitive and specific PCR technique.

- To establish the prevalence of the alteration in WM and other closely related disorders, so it would be of help in the differential diagnosis.
- To evaluate sequential samples of patients after therapy in order to find a relationship between the reduction or disappearance of such abnormality and the response and survival, establishing its potential capacity to predict relapse and to monitor residual disease.
- To elucidate why WM cases with a wild-type *MYD88* exist and how this may affect disease presentation, response to treatment and survival.

2) To develop and validate a real-time allele-specific oligonucleotide PCR (ASO-RQ-PCR) to detect the *MYD88* L265P mutation.

- To assess the reproducibility, accuracy and sensitivity of the technique with a diluting experiment of a previously known positive sample.
- To establish a standard curve for the accurate quantification of tumor burden.
- To evaluate the applicability of the methodology by analyzing known positive patients.
- To compare the tumor load estimated by the RQ-PCR vs. multiparametric flow cytometry.

3) To understand the mechanisms of WM behavior heterogeneity across different patients with a presumed similar condition (IgM monoclonal gammopathy).

- To describe the mutational profile in all patients at diagnosis in order to find differences between the three entities that could explain the variability in the disease presentation.
- To clarify whether or not the abnormalities are present from the beginning of the pathogenesis (i.e. in IgM-MGUS) and may determine its evolution.
- To find out differences in GEP that could be associated with the different diagnoses.

- To collect all the information of the tumor clone and relate it to the patients characteristics in order to identify the mechanisms explaining the development of symptoms and the response to therapy.
- To define high-risk signatures that could predict progression from indolent to symptomatic patients or a worse outcome in symptomatic patients, establishing a clinical/molecular prognostic model.
- To determine possible diagnostic, prognostic or therapeutic applications based on these findings.

4) To characterize the genomic landscape of the transformation process from WM to DLBCL by performing a whole-exome sequencing study.

- To describe the most frequent abnormalities present at diagnosis in patients who evolve to aggressive forms.
- To find similarities and differences in the mutational profile at diagnosis and transformation, establishing the potential role in transformation of mutations conventionally described in WM (*MYD88*, *CXCR4*, *ARID1A*…).
- To elucidate which alterations present in DLBCL samples could have been responsible for the aggressive transformation and to assess whether these mutations may be already present at diagnosis in a low proportion of the tumor population.
- To define potential biomarkers of progression that identify patients at a higher risk of transformation, with the aim of monitoring the course of the disease and designing preventive strategies.
- To study the dynamics of clonal evolution in WM transforming to DLBCL and establish the evolutionary pattern for this process.

IV. Results

The results presented in this doctoral thesis have been published in peer-reviewed scientific journals. These papers and some supplemental material are included in this thesis.

PAPER 1: *MYD88* L265P is a marker highly characteristic of, but not restricted to, Waldenström's macroglobulinemia. Leukemia. 2013;27(8):1722-8. PMID: 23446312.

Background

Waldenström's macroglobulinemia (WM) is a rare hematological malignancy that represents an intermediate stage between lymphoproliferative disorders (LPDs) and plasma cell dyscrasias. Recent studies have reported the presence of the L265P somatic mutation at the *MYD88* gene in 91% of patients. The same alteration has also been described, although with lower frequencies in other LPDs, such as diffuse large B cell lymphomas (DLBCLs) of the activated B-cell type (14–29%), IgM monoclonal gammopathy of uncertain significance (MGUS, 10%) or marginal zone lymphomas (MZL, 7%). However, most of these studies have been carried out with conventional sequencing techniques with relatively low sensitivity, which could have led to underestimate the mutation frequency.

Patients and methods

We evaluated samples from 38 normal donors and tumor specimens (mainly bone marrow or lymph nodes) from 117 WM patients and 273 related B-cell LPDs, distributed as follows: 14 splenic MZLs, 35 hairy cell leukemias, 48 non-germinal center (GC) DLBCLs, 28 GC-DLBCLs, 13 DLBCLs not subclassified, 39 chronic lymphocytic leukemias (CLLs) (16 with a small monoclonal component), 31 IgM-MGUS, 25 IgA/IgG-MGUS, 24 multiple myeloma (MM) (including 3 with an IgM isotype), 6 amyloidosis, 9 lymphoplasmacytic lymphomas and 1 IgM-related disorder (peripheral neuropathy).

DNA was analyzed for the presence of the *MYD88* L265P mutation with a commercially available variant of the real-time ASO-PCR that combines an allele-specific amplification with the amplification refractory mutation system technology and hydrolysis probe detection: 'qBiomarker Somatic Mutation Assay for MYD88_85940' (SABiosciences, Qiagene Co., Hilden, Germany). To

evaluate the sensitivity of the technique, we performed a dilution experiment with a sample from a positive patient with 60% of monoclonal IgM-lambda cells evaluated by flow cytometry, diluted in DNA from a healthy control lacking the mutation.

Results

Mutation assessment

The dilution experiment demonstrated a sensitivity of 2.5×10^{-3} (corresponding to ~1 mutated cell among 1000 normal cells) which was considered sufficient to evaluate the samples correctly (>1% tumor infiltration). Moreover, it allowed us to establish a standard curve to quantify the tumor cells with the ΔCT method.

Regarding the mutational status, none of the 38 samples from normal donors had the *MYD88* L265P alteration, whereas it was present in most of WM patients (101/117, 86%, with no differences between symptomatic and asymptomatic cases) and IgM-MGUS (27/31, 87%). In addition, there were 3/14 (21%) splenic MZLs and 9/48 (19%) non-GC-DLBCLs mutated patients. Finally, we did not find the mutation in any of the remaining diseases (GC-DLBCLs, DLBCLs not subclassified, hairy cell leukemias, CLL, IgA or IgG-MGUS, MM, amyloidosis, lymphoplasmacytic lymphomas and IgM-related peripheral neuropathy).

Minimal residual disease evaluation

Among *MYD88* L265P positive patients, the estimation of the number of clonal cells was very comparable to the flow cytometry results, showing a Pearson correlation coefficient, R, of 0.548 ($p=2.3 \times 10^{-17}$). In addition, the high sensitivity of the methodology encouraged us to test it as a tool for monitoring therapy and residual disease in WM. To assess this aspect, we evaluated six selected patients who responded very well (>90% M-component reduction) to the therapy and compared the results yielded with flow cytometry. PCR persisted positive in five of them although with a very low number of tumor cells, similar to results based on flow cytometry.

Differences between mutated and unmutated cases in IgM gammopathies (IgM-MGUS, asymptomatic WM and symptomatic WM)

a) Biological differences

The immunophenotypic study of the tumor cells revealed minor differences between both groups, mainly concerning the expression of FMC7 (positive in 64% of unmutated versus 25% of mutated cases, p=0.015), CD23 (positive in 25% unmutated vs. 39% mutated, p=0.098) and CD27 (positive or strongly positive in 75% of wild-type with a homogeneous pattern vs. 55% of mutated patients with an heterogeneous pattern, p=0.08).

As far as the presence of somatic mutations in the *IGHV* (immunoglobulin heavy chain variable region) gene was concerned, we found that there were 97% of patients among the *MYD88*-mutated group with somatic hypermutation (SHM) (>2% deviation from the germline) compared to 57% in the wild-type *MYD88* group (p=0.012). It was also interesting to discover that the *IGHV* repertoire was more biased in mutated than wild-type *MYD88* cases, since the *IGHV3–23* gene was selected in 27% of mutated cases, vs. 9% of the unmutated (p=0.014).

No differences were observed in the pattern of bone marrow infiltration, the number of B-cells or in the presence of cytogenetic abnormalities detected by FISH.

b) Clinical differences

Turning now to the potential clinical relationship, we again found few differences between patients with and without the mutation. Globally, positive cases had a slightly higher monoclonal component (2.62±2.02 vs. 1.77±2.33 g/dl, p=0.009), lower lactate dehydrogenase serum levels (265±93 vs. 371±189, p=0.002) and less frequency of lymphocytosis (5 vs. 24%, p=0.022). This picture is consistent with the absence of any statistically significant differences in terms of time to therapy, progression-free and overall survival. No differences were found either in response rates or in progression-free survival and overall survival after therapy among patients who required treatment. Finally, regarding the risk of progression from asymptomatic to symptomatic forms of the disease, there were no differences depending on the *MYD88* status.

Conclusions

In this study, we have demonstrated that the *MYD88* L265P mutation is present in 87% of WM patients, making this molecular abnormality a highly characteristic marker of the disease,

especially if we consider that it was much less frequent in other related LPDs. The analytical sensitivity of the method employed was critical since these disorders are frequently characterized by low tumor burden infiltration.

In summary, our results do not support the idea that *MYD88* mutation separates a subgroup of WM patients with a singular profile in the outcome, although we observed that negative cases seem to have a less typical WM signature (small M-component peak, more frequent lymphocytosis, higher lactate dehydrogenase serum level, atypical immunophenotype, and less SHM), which could be the basis for considering *MYD88*^{wt} WM as a different entity.

PAPER 2: Detection of *MYD88* L265P mutation by real-time allele-specific oligonucleotide polymerase chain reaction. Appl Immunohistochem Mol Morphol. 2014; 22:768-73. PMID: 24992174.

Background

MYD88 L265P mutation is the disease-associated alteration of Waldenström's macroglobulinemia (WM) as it is present in ~91% of the patients. The identification of this abnormality can be very useful for the molecular discrimination from other B-cell malignancies that share similar morphological and clinicopathological features with WM. This has been carried out with different strategies, such as Sanger sequencing, high-resolution melting analysis, or PCR followed by digestion with restriction enzymes. However, these techniques have some disadvantages mainly concerning the sensitivity and specificity, laboriousness, accuracy for quantification, cost-effectiveness, or applicability.

Patients and methods

A real-time allele-specific oligonucleotide PCR (ASO-RQ-PCR) was developed for *MYD88* L265P mutation based on the use of 2 reverse primers that differed in the last nucleotide (at 3' position) so that they were specific either of the wild-type or the mutated allele. Sequences were as follows: 5'-CCTTGTACTTGATGGGGATCA-3' (wild-type) and 5'-CCTTGTACTTGATGGGGATGG-3' (mutated). In addition, a common forward primer (5'-ACTTAGATGGGGGATGGCTG-3') and a specific TaqMan probe (5'-FAM-TTGAAGACTGGGCTTGTCCCACC-TAMRA-3') were designed.

Each experiment required two different PCR reactions: one for the detection of the mutation (with the mutated reverse primer) and the other one as a control of the DNA quality (using the wild-type reverse primer). Reactions were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) and consisted of 10 min at 95ºC and 50 cycles with the following conditions: 95ºC for 15 s and 60ºC for 60 s. Data were analyzed with the StepOne Software v2.1 (Applied Biosystems).

We analyzed bone marrow samples from 30 patients (10 MGUS and 20 WM) known to be positive for the L265P, and peripheral blood samples from 10 healthy donors. Median percentages of clonal cells evaluated by flow cytometry were 1.73% (0.5 to 5) in MGUS and 8.0% (1.7 to 59.1) in

WM. All cases evaluated had been previously analyzed using the commercial assay "qBiomarker Somatic Mutation Assay for MYD88_85940" (SABiosciences, Qiagene Co., Hilden, Germany).

Results

Sensitivity of the assay

First, we carried out a serial dilution study to establish the detection limit of our technique. We analyzed the DNA of a patient with the heterozygous mutation (previously found by Sanger sequencing) diluted in wild-type DNA. ASO-RQ-PCR could detect the *MYD88* L265P mutation at a dilution level of 0.25% with >10 cycles of difference from the wild-type DNA background.

This experiment also allowed us to generate the standard curve (SC) for the relative quantification through the CT (cycle threshold) value (cycle in which fluorescence reaches 10-fold the basal emission), which was inversely correlated to the tumor burden (correlation coefficient of 0.966).

Evaluation of MYD88 L265P mutation

Results of the mutation analysis of the 40 samples were concordant with the commercial assay. The CT value was always >50 in healthy donors, and between 29.6 and 38.1 in WM and 33.4 and 38.4 in MGUS (which means that there were >11 cycles differences between samples with and without the mutation).

The comparison between the percentage of clonal cells evaluated by multiparametric flow cytometry and the percentage provided by the RQ-PCR confirmed that both techniques correlated well (Pearson correlation coefficient, $R=0.917$, $p=1.0 \times 10^{-12}$).

Conclusions

We have developed an inexpensive, robust and accurate ASO-RQ-PCR assay for the detection of *MYD88* L265P mutation, which could be considered as a useful molecular tool for the evaluation of B-cell LPD.

PAPER 3: Unraveling the heterogeneity of IgM monoclonal gammopathies: a gene mutational and gene expression profiling study. Br J Cancer, under review.

Background

Immunoglobulin M (IgM) monoclonal gammopathies are immunoproliferative disorders that show important variability, involving three different stages of presentation: IgM monoclonal gammopathy of undetermined significance (IgM-MGUS), asymptomatic Waldenström's macroglobulinemia (AWM), and symptomatic WM (SWM). Recent studies have identified recurrent mutations in *MYD88*, *CXCR4*, *ARID1A*, *CD79B* and other genes, which may have an impact on the transcriptional profile, disease presentation, therapeutic outcome and overall survival. However, little is known about the causes of the clinical heterogeneity or the mechanisms involved in the progression from asymptomatic to symptomatic forms.

Patients and methods

Sixty-nine patients with WM/IgM-MGUS were included in the study. Distribution according to diagnosis was as follows: 15 IgM-MGUS, 26 AWM and 28 SWM. DNA and RNA were extracted from bone marrow CD19+ cells selected with immunomagnetic methods.

MYD88 L265P mutation was assessed by real-time allele-specific oligonucleotide PCR (ASO-RQ-PCR) and *CXCR4* WHIM mutations by both ASO-PCR and Sanger sequencing.

Gene expression analysis was carried out by real-time quantitative PCR using TaqMan low-density arrays (TLDAs) (ThermoFisher Scientific, Waltham, MA) in ninety-five genes that were selected based on their relevance in the disease. Results were analyzed with the ExpressionSuite Software v1.0.3 (ThermoFisher Scientific) that allows data normalization and relative quantification for sample comparison through the $2^{-\Delta\Delta CT}$ method. Normalized gene expression values were exported to the Statistical Package for Social Sciences (SPSS) version 15.0 (SPSS Inc, Chicago, IL) for further statistical analysis. The Mann-Whitney U-test was used to identify statistically significant differences between groups.

A next generation sequencing (NGS) study was performed with a custom amplicon-based panel including 12 genes of interest in WM: *ARID1A*, *CD79A*, *CD79B*, *TP53*, *MYBBP1A*, *TRAF2*, *TRAF3*,

RAG2, *HIST1H1B*, *HIST1H1C*, *HIST1H1D*, and *HIST1H1E*. Sequencing was carried out in a MiSeq platform (Illumina, San Diego, CA). Data generated were pre-processed with the MiSeq Reporter (MiSeq integrated software, Illumina) and variant detection was carried out using the Illumina Variant Studio 2.2 software and Integrative Genomics Viewer software (Broad Institute, Cambridge, MA).

Results

MYD88 L265P mutation

MYD88 L265P mutation was recurrent, with a frequency that varied with the diagnosis: 67% for MGUS, 96% for AWM and 100% for SWM (p=0.001). Moreover, it was associated with higher levels of hemoglobin, since all wild-type patients had anemia ([hemoglobin] <11.5 g/dl) compared with only 55% of the *MYD88* mutated patients (p=0.032).

CXCR4 WHIM mutations

There were 25/69 (36%) patients with *CXCR4* alterations, showing no differences according to the type of diagnosis: 33% IgM-MGUS, 42% AWM, and 32% SWM. In all except one MGUS, *CXCR4* mutations were always associated with *MYD88* L265P (96%). Interestingly, expression levels of either *CXCR4* or its ligand *CXCL12* were not affected by the presence of mutations. The comparison of the mutated and unmutated groups did not reveal any differences in the clinical and biological characteristics or in the survival (overall, event-free survival and progression-free survival). Only when we considered just WM cases (MGUS excluded), we observed a larger IgM monoclonal component (4418±3169 vs. 2687±2767 mg/l, p=0.025) and lower β_2-microglobulin level (B2M 2.8±1.9 vs 3.7±1.6 mg/l, p=0.034) in *CXCR4*-mutated compared with wild-type patients, respectively. In turn, *CXCR4* overexpression was related to the presence of adenopathy, since 33% patients with high mRNA values of *CXCR4* had lymphadenopathy compared with only 5% among the group with low *CXCR4* mRNA values (p=0.029).

Other gene mutations

Since *CXCR4* mutations were demonstrated to be present from the beginning of the disease (IgM MGUS already presented this mutation) and had no influence on progression to the symptomatic stage, we decided to search for mutations in other 12 genes of relevance in WM. Overall, we found 29 non-synonymous alterations in 23/61 patients, distributed as follows: 3/14 (21%) IgM-MGUS, 8/23 (35%) AWM and 12/24 (50%) SWM. Interestingly, the mean number of

mutations per patient also increased progressively as the disease evolved (0.2, 0.4 and 0.7 for IgM-MGUS, AWM and SWM, respectively). In addition, patients with a wild-type *MYD88* gene (n=6), showed no additional mutations in any of the genes that we studied here (p=0.045). The low number of mutations made hard to find any difference in the distribution with respect to the diagnosis. The most remarkable finding was that variants in *HIST1H1C* only appeared in MGUS (p=0.031).

Mutations in *CD79B* were the most frequent (8% patients) but were not related to any important clinical feature. On the contrary, the overexpression of other genes belonging to the BCR signaling pathway, such as *CD79A*, *SYK*, *BTK* and *BLNK*, was associated with a higher level of bone marrow infiltration by flow cytometry and of the monoclonal component.

The second most frequently mutated gene was *HIST1H1E*, altered in 7% patients, the value increasing to 13% when we considered all the histone family members (*HIST1H1 B-E*). Analogously to *CXCR4*, mutations in *HIST1H1E* did not affect the expression of *HIST1H1E* itself. No clinical differences were further observed. However, a higher expression of *HIST1HE* was related to favorable clinical features, such as lower B2M and creatinine (2.4±1.7 vs. 3.7±1.8 mg/dl, p=0.007, and 0.9±0.2 vs. 1.1±0.4 mg/dl, p=0.044, respectively).

ARID1A, *MYBBP1A* and *TRAF3* genes were mutated in three patients each (5%). These alterations were associated with worse presentation features such as lower levels of albumin (2.9±0.2 vs. 3.7±0.5 g/dl, p=0.017) and hemoglobin (9.3±2.1 vs. 12.2±2.1 g/dl, p=0.047), as well as higher incidence of adenopathy (66% vs. 17%, p=0.033) for *ARID1A*-mutated patients; more neurological alterations (50% vs. 8%, p=0.045) in *MYBBP1A*-mutated patients; and higher levels of B2M (6.7±0.4 vs. 3.2±1.8 mg/l, p=0.034) in *TRAF3*-mutated patients.

Finally, the two *TP53*-mutated patients, were symptomatic and presented with anemia at diagnosis. Moreover, one of two corresponded to a very highly resistant form of the disease who transformed to diffuse large B-cell lymphoma. Platelet count was lower than in *TP53* wild-type patients (116±37 vs. 263±121 x10_9/l, p=0.026). Nevertheless, these results need to be confirmed in larger series of patients.

Gene Expression Profiling (GEP)

Then, we decided to search for differences on the GEP comparing indolent (MGUS plus AWM, n=22) with symptomatic patients (SWM, n=18). The comparison highlighted 11 differently expressed genes; in particular, *ADARB1* (alternative splicing), *CCND3* (cyclin D3), *GPSM2* (G-protein signaling

modulator) and *LEF1* (transcription regulator) were upregulated in asymptomatic cases, whereas *CD79A* (BCR related), *IRF3* (transcription regulator), *MEK1*, *P38, WNK1* (MAPK pathway), *MYD88*, and *TAP2* (antigen peptide transporter) were overexpressed in symptomatic WM. *IRF3*, *MYD88*, *MEK1* and *P38* are part of the Toll-like receptor (TLR) pathway, essential for WM cell growth and survival, and together with *CD79A* and *TAP2*, have a role in the immune response regulation. These differences were reflected in the disease presentation: the expression profile of symptomatic patients was associated with a higher frequency of adenopathy, hepatomegaly, splenomegaly, and B symptoms, as well as anemia, thrombocytopenia, hypoalbuminemia and higher B2M and monoclonal component ($p \leq 0.05$), as occurs in cases with poor prognosis. Consequently, we observed that three of these genes (*IRF3*, *MYD88*, *WNK1*) were overexpressed in the high-risk group according to the International Prognostic Scoring System. However, no statistically significant differences in overall and progression-free survival were found to be associated with the expression of any of these genes.

Conclusions

In summary, our data reveal a higher incidence of mutations during the different stages of evolution (from IgM MGUS to asymptomatic WM and symptomatic WM), meaning that, in contrast to *MYD88* L265P and *CXCR4* WHIM alterations, present from the beginning of the pathogenesis, most of these mutations would be acquired during the progression to the symptomatic stages. The expression study also highlighted significant differences between indolent and symptomatic patients, regarding genes involved in WM pathogenic mechanisms (TLR/MYD88, CXCR4 and BCR pathways), which provided some clues about the heterogeneity of this disease and had an impact on the clinics.

PAPER 4: Waldenström's macroglobulinemia to aggressive diffuse large B-cell lymphoma: a whole exome analysis of gene abnormalities leading to transformation. Blood Cancer J, under review.

Background

Transformation of Waldenström's macroglobulinemia (WM) into more aggressive histologies, such as diffuse large B-cell lymphoma (DLBCL), has been reported in up to 10% of WM patients. Moreover, these patients usually have worse prognosis and short survival from the time of transformation (median survival below 2 years). However, this process has been poorly studied and the causes and mechanisms involved in the transformation to aggressive lymphoma have not been described yet. The identification of genetic changes driving this transition would allow developing new therapeutic strategies that could improve the outcome of these patients.

Patients and methods

Four patients (#1-4) diagnosed with transformed WM were included in the study. We performed whole-exome sequencing of matched tumor samples from diagnosis and transformation, as well as germline DNA for comparative studies. One extra sample of patient #2 (corresponding to an event of WM progression without transformation) was added. For patient #3, only DNA from germinal and transformed tumor cells was available.

Enrichment and generation of libraries were performed with SureSelectXT2 Human All Exon V5 of 51 MB kit (Agilent Technologies, Santa Clara, CA). Paired-end sequencing was carried out in the Illumina HiSeq 2000 platform (Illumina, Inc., San Diego, CA). DreamGenics® (Oviedo, Spain) supervised the pre-processing and performed the initial bioinformatics analysis using algorithms and non-commercial pipelines. Single nucleotide polymorphisms were filtered out and in the final results only non-synonymous protein-coding alterations were included. Variant allele frequencies (VAFs) were corrected according to the tumor content defined by flow cytometry to better estimate the percentage of tumor cells affected by each mutation.

Results

Global non-synonymous variations

Overall, we found 421 non-synonymous protein-coding variations (NSVs) at diagnosis and transformation in the four patients, distributed among 355 genes. Of these, 39 were mutated exclusively in WM (at diagnosis n=29, or progression n=10), whereas 267 appeared only in the DLBCL samples.

First, we observed a four times higher frequency of mutated genes at transformation (median 85, range 49-165) than at diagnosis (median 21, range 20-35). Accordingly, there was a median gain of 70 variants (range 29-144) per case during the transition from WM to DLBCL. Interestingly, the number of variants was not closely correlated with the interval time between diagnosis and transformation (R^2=0.51).

NSV present at diagnosis and transformation

Although there were more alterations at transformation, the percentage of tumor cells affected by each alteration was usually lower, as it was observed when we compared the corrected VAFs of the variants present in the two events. In addition, the number of these common mutations (present at diagnosis and transformation) significantly differed between patients, ranging from two (*MYD88* and *TNIP1* in patient #1) to 29 (in patient #2). The most recurrent were *MYD88* L265P, present in all patients, and *CD79B* Y196C/H, which was found in 3/4 patients at transformation (75%), and in 2/3 at diagnosis (67%). *CELSR2*, *FAM135B*, *IGFN1* and *ZFHX4* also had recurrent variations (in at least two patients). All these alterations were present in a high proportion of clonal tumor cells and were conserved in both events, which suggests having a potential role as early drivers.

NSV exclusive of one event

We then considered variants that could be detected at the time of only one of the two events, being exclusive of either diagnosis, (progression) or transformation. The role of NSVs that were exclusively present at diagnosis was thought to be secondary (passenger mutations) because they disappear in the lymphoma clone, whereas those recurrent variations exclusively present at the transformation stage (or that appeared late in the Waldenström's clone) can be considered as intermediate or late drivers that confer some advantage to the tumor clone. Five genes had recurrent NSVs acquired at the transformation stage: *FRYL* (MLL fusion partner in lymphoid leukemia), *HNF1B*

(transcription factor whose expression is altered in some cancers), *PER3* (checkpoint protein that plays an important role in checkpoint activation, cell proliferation and apoptosis), *PIM1* (proto-oncogene with serine/threonine kinase activity involved in the pathogenesis of lymphoma), and *PTPRD* (tumor suppressor that contributes to the development of multiple cancers). Therefore, *FRYL*, *HNF1B*, *PER3*, *PIM1* and *PTPRD* could be considered as key genes with a crucial role in the Richter transformation of WM.

Pattern of evolution from WM to DLBCL

The existence of both common and exclusive NSVs at diagnosis and at transformation is suggestive of a branching model of evolution. Accordingly, the lymphoma clone is not derived from the mere accumulation of mutations in the WM clone, but represents the evolution of certain subclones that acquire new mutations responsible for the transformation event and that do not conserve some of those detected at diagnosis. The fact that both tumors (WM and DLBCL) had the same V(D)J rearrangement and an identical CDR3 region, points out to a common progenitor cell in which driver mutations arise. This nonlinear evolutionary pattern is well illustrated by patient #2, who had an initial response and subsequent symptomatic progression of WM before transformation: there were common alterations in all samples (diagnosis, progression and transformation) but some of the variants detected in the intermediate event had not been observed at diagnosis, and were not either maintained at transformation. Nonetheless, these findings should be considered with caution because of the low level of infiltration of some diagnostic samples, which may lead to the underdetection of variants that are present at a low level in the tumor population.

Conclusions

Although we could not identify a unique genetic event responsible for WM transformation to DLBCL, it appears that certain alterations may contribute to the onset of the aggressive disease. The existence of frequently mutated genes (*CD79B*) at diagnosis in the subgroup of patients undergoing histological transformation, compared with the low incidence in non-transformed WM, suggests their potential role as biomarkers for predicting the risk of transformation. However, additional research is still needed to better understand the biology of this process and allow the design of preventive targeted therapies.

V. General discussion

Despite recent advances in understanding the biology of Waldenström's macroglobulinemia, certain aspects, mainly concerning its high variability, are still unknown. In this thesis, we have characterized the WM tumor cell trying to improve our knowledge of the ontogeny, pathogenesis and evolution of the disorder.

MYD88 L265P is a marker highly characteristic of, but not restricted to, Waldenström's macroglobulinemia

Waldenström's macroglobulinemia (WM) is characterized by lymphoplasmacytic infiltration of the bone marrow together with the presence of serum monoclonal immunoglobulin M (IgM).[1] As a lymphoplasmacytic lymphoma, it shares important morphologic and immunophenotypic features with other B-cell lymphoproliferative disorders (LPDs).[34,35] Therefore, the recently identified *MYD88* L265P mutation underlying WM[27] could be very useful for the differential diagnosis. In this work, we have demonstrated that this alteration is present in 86% of WM patients, making this molecular abnormality a highly characteristic marker of the disease. Previous studies performed to detect this mutation had used Sanger sequencing,[27,30,70–72] which has a low sensitivity limit (25%),[73] whereas the ASO-PCR that we employed is more sensitive and easier to apply in routine laboratories. This is a critical aspect of the study, as certain B-cell disorders, such as WM, amyloidosis, myeloma and MGUS, are frequently characterized by low tumor burden infiltration, especially in the bone marrow,[74–76] meaning that sensitive techniques are required to evaluate them. In fact, our approach was able to identify up to 87% IgM-MGUS with the *MYD88* L265P mutation, compared to the lower frequency (6-56%) reported before with other techniques.[27–30]

The high prevalence of *MYD88* mutation in WM makes it a suitable marker for evaluating molecular response. This ASO-PCR could be used as a tool for assessing minimal residual disease and monitoring response to therapy, competing with or complementing flow cytometry.[28,77,78] The number of tumor cells could be easily estimated with the cycle threshold (CT) value and correlated well with the data reported by flow cytometry.

Regarding other related B-cell LPDs, *MYD88* mutation was much less frequent, as in splenic marginal zone lymphomas (21%) and non-germinal center diffuse large B-cell lymphomas (DLBCLs) (19%), or absent, like in IgM secreting myeloma, chronic lymphocytic leukemias (even with IgM

monoclonal component), non-IgM-MGUS, and lymphoplasmacytic lymphomas. Therefore, the mutation seems to be highly specific to WM and IgM-MGUS, enabling molecular discrimination.[28]

Finally, several groups have suggested a potential correlation between *MYD88* status, and the clinical and biological characteristics as unmutated patients presented with significantly lower bone marrow disease involvement and serum IgM levels.[28,29,32] However, our results did not completely support the idea that the mutation separates a subgroup of patients with a singular profile, although the association observed between negative cases and a non-typical WM signature (small M-component peak, more frequent lymphocytosis, higher lactate dehydrogenase serum level, atypical immunophenotype, and less somatic hypermutation) could be the initial basis for a discrimination between *MYD88*^{wt} and *MYD88*^{L265P} WM patients. Likewise, in contrast to the shortened overall survival of wild-type patients reported by others,[32] the absence of the mutation in our series did not have an impact on the clinical behavior (i.e.: time to first therapy, response to treatment, progression-free and overall survival), although differences in the clinical management could be the reason for this discrepancy.

Detection of *MYD88* L265P mutation by real-time allele-specific oligonucleotide polymerase chain reaction

Assessment of *MYD88* L265P mutation is a potential tool for diagnostic discrimination and prognosis prediction. In the previous study, we had employed a commercial assay based on ASO-RQ-PCR to detect this alteration. However, this method was not economically feasible to be used in daily routine, so we have developed and validated a simple and economic tool for the same purpose.

RQ-PCR based approaches had already demonstrated to produce robust and reproducible experiments in minimal residual disease studies in acute lymphoblastic leukemia.[79] We have introduced a mismatch close to the 3′ end in the mutant-specific primer to increase the specificity of the reaction.[80] This allowed us to obtain a PCR with more than 10 amplification cycles of difference between positive and negative samples. Moreover, the use of TaqMan probes conferred a higher sensitivity, close to 10^{-3}, which means that 1 mutated cell can be detected within 1000 wild-type cells.

There are other methods to analyze the *MYD88* L265P mutation, but ASO-RQ-PCR has more advantages. Sanger sequencing has demonstrated the presence of the mutation in several series,[81,30] but it has a relatively high cost and requires at least 10 to 40% of mutated cells.[73] High-resolution melting analysis or PCR followed by enzymatic digestion with restriction enzymes can achieve a

sensitivity of 5%,[82,73] however, it is still insufficient for certain entities characterized by low percentage of tumor cells, even <1%, such as IgM-MGUS.[74] Finally, some reports have used AS-PCR followed by agarose gel electrophoresis, which increases sensitivity to 10^{-2}.[29] However, post-PCR handling has some disadvantages in terms of laboriousness and risk of cross-contamination. Another group has proposed a variation of this PCR using SYBR Green, which demonstrated a detection limit up to approximately 10^{-3}, being adequate for diagnostic purposes and monitoring studies. Nevertheless, this sensitivity gain was hampered by a relatively loss of specificity, with a difference of only 3 cycles between mutated and wild-type cases, raising the possibility of false-positive results.[28]

Unraveling the heterogeneity of IgM monoclonal gammopathies: a gene mutational and gene expression profiling study

Progress recently achieved in the characterization of the molecular basis of WM has provided new insights about the genes and pathways contributing to the pathogenesis of the disease. However, we still do not know much about the causes responsible for its clinical heterogeneity or the mechanisms involved in the progression from indolent to symptomatic stages. We have combined mutation analysis and gene expression profiling in an attempt to find a molecular signature able to distinguish symptomatic from indolent forms or to predict disease progression.

Starting by *CXCR4* mutations, our results confirmed their presence in many patients (36%), most with *MYD88* L265P, but showing no differences according to diagnosis (33% MGUS, 42% AWM and 32% SWM), or correlations with the disease presentation and prognosis.[44,32,43,45] In turn, *CXCR4* overexpression was related to the presence of adenopathy, probably due to the greater cell adhesion associated with CXCR4 activation[83] and that in a previous study had been reflected in a greater bone marrow involvement.[56] However, *CXCR4* expression was not affected by the presence of the mutations in any case.

Similar to *CXCR4*, no differences were found in the distribution of the mutations among the different diagnoses of the other twelve genes studied by NGS. Nevertheless, the global frequency did increase from IgM-MGUS (21% of mutated patients) to AWM (35%) and SWM (50%), as it has been described in the literature for genomic alterations (like copy-number abnormalities or loss of heterozygosity), and which confirmed the association between an aggressive clinical behavior of the disease and a higher frequency of alterations.[51,58] In addition, patients with a wild-type *MYD88* showed no additional mutations, reinforcing the view that these cases could be a distinct entity.[84]

There is increasing evidence about the role of the B-cell receptor (BCR) pathway in the pathobiology of WM, either alone or in cooperation with the MYD88 signaling axis.[85–91] Our results showed that *CD79B* mutations were the most recurrent, affecting to 8% of the patients, as reported by other groups.[51,44,43] Moreover, overexpression of *CD79A*, *SYK*, *BTK* and *BLNK* (genes belonging to the BCR signaling pathway) was associated with more bone marrow infiltration by flow cytometry and elevated monoclonal component. WM cells had been shown to exhibit constitutive activation of BCR-related signaling, even in the absence of BCR alterations,[92] a finding supported by the successful treatment with BTK inhibitors.[48,10,11] Nonetheless, it should be also reminded here that dual *MYD88* and *CD79B* mutations and CD79B overexpression have been associated with ibrutinib resistance in DLBCL.[93,94]

Mutations in the linker histone genes *HIST1H1 B-E* were present in 13% of patients, the same frequency observed in DLBCL.[95,96] However, alterations in the other genes were less frequent: *ARID1A* (5% of patients), *MYBBP1A* (5%), *TRAF3* (5%) and *TP53* (3%), with similar incidences than previously described,[43] except for *ARID1A*, whose reported frequency was as high as 17%. Although the number of affected patients was small, these abnormalities seemed to be associated with worse disease presentation (lower levels of hemoglobin, platelets, albumin, higher of β_2-microglobulin and more incidence of adenopathy and neurologic alterations) and outcome, as was also suggested by other authors.[56,97] Nevertheless, these results need to be confirmed in larger series of patients.

Regarding the gene expression study, we found an upregulation of genes belonging to the toll-like receptor (TLR) (*IRF3*, *MYD88*, *MEK1* and *P38*) and BCR pathways (*CD79A*) in symptomatic compared with indolent patients, thus suggesting a more intense activity of the routes triggering WM cell growth and survival.[48] Furthermore, this molecular profile was associated with higher frequencies of adenopathy, hepatomegaly, splenomegaly and B symptoms, as well as with anemia, thrombocytopenia, hypoalbuminemia and higher levels of β_2-microglobulin and monoclonal component. Other studies had also found genes relevant to WM biology to be associated with IgM, hemoglobin and bone marrow disease involvement.[56]

From Waldenström's macroglobulinemia to aggressive diffuse large B-cell lymphoma: a whole-exome analysis of abnormalities leading to transformation

WM patients eventually experience histological transformation to DLBCL (2.4% transformation rate at 10 years), being at risk of poor outcome and short survival.[68,98] We have

performed a whole-exome sequencing study of four cases with paired WM and transformed DLBCL samples trying to understand the biology and mechanisms underlying this transition and to identify susceptible patients as well as targetable alterations and pathways.

Our findings have revealed that this is a quite heterogeneous event with a large number of genetic aberrations that affect many pathways, similar to what has been reported for the transformation of other lymphomas.[99–102] The complexity of the process was reflected on the number of alterations found in the lymphoma with respect to WM, much higher in the former. Patients showing the fastest transition (#1 and #3) presented the greatest number of mutations, probably as a result of the proliferative advantage conferred by the onset of new mutations, that led to stronger competition.[103–105]

However, not all alterations would be of equal importance in the pathogenesis. Mutations present at both events were likely to be spread in nearly all clones (as reflected their high variant allele frequency) remaining stable over time. An example of these mutations would be the *MYD88* L265P which, with a well-established role in both WM and DLBCL,[48,27,81] was the only one present in all patients and at all times. Interestingly, in patient #1, *MYD88* mutation was the only alteration, together with *TNIP1*, recognized at both moments. *TNIP1* is an essential gene for NF-κB activation, pointing out to this pathway to be involved in the transformation process.[106,107]

CD79B, whose product is part of the B-cell receptor, was the second most frequently mutated gene, appearing in 2/3 cases (67%) at diagnosis and in 3/4 cases (75%) at transformation. Its variants have been described in 12% of ABC-type DLBCLs[108] in connection with the acquisition of the lymphoma phenotype together with other alterations.[109,110] In conventional WM, *CD79B* has been found to be mutated in ~10%,[51,44,43,111] so the high frequency reported here (2 out of 3 cases) is very remarkable. Accordingly, we suggest that *CD79B* mutations could identify a subgroup of WM with aggressive clinical evolution and higher risk of transformation.

Except for *MYD88* and *CD79B*, we observed a notable diversity in the mutational spectrum across samples, with few recurrent genes. Only *CELSR2*, *FAM135B*, *IGFN1*, and *ZFHX4* were present in more than one case. Even another commonly mutated gene in WM (30% patients), *CXCR4*,[44,43] appeared just in one patient. Regardless of their incidence, these mutations are thought to be present in the WM cell that suffers the transformation, suggesting that they may be involved in tumor initiation.

Genes exclusively found in transformation may represent cooperating events that interact with these pathogenic mutations. The most frequently mutated genes at transformation were *FRYL*, *HNF1B*, *PER3*, *PTPRD*, and *PIM1* (targetable with the PIM kinase inhibitors[112,113]). However, considering the amount of alterations acquired at transformation, many of them may be not determinant for the pathogenesis of the disease. This could be the case of the two different *TP53* mutations observed during progression in patient #2 but that disappeared at transformation, suggesting that they were not able to confer a proliferative advantage to the malignant cells. Therefore, it is not easy to determine what promotes the final aggressive behavior of the transformation: the genes, the number of alterations, the clone in which they arise, the pathway affected, the cell function deregulated or, most likely, a combination of all of these factors. These hypotheses should be addressed in larger studies with the ultimate aim of developing therapeutic strategies that may disrupt these mechanisms.

Finally, we observed that the transformation process seems to be consistent with a branching model of evolution in which only clones containing driver mutations evolve to more aggressive populations by acquiring new aberrations. Identical scenarios have been reported in multiple myeloma,[105,114] follicular lymphoma,[102] chronic lymphocytic leukemia,[115] acute myeloid leukemia,[116,117] acute lymphoblastic leukemia,[118,119] and even solid tumors.[120] Moreover, analysis of the V(D)J rearrangement and CDR3 region of the immunoglobulin heavy chain gene in the WMs and their matched lymphoma samples, confirmed that they belonged to the same clone. This, together with the presence of certain mutations shared by the entire tumor population that remain clonally stable throughout the entire course of the disease, points out to the existence of a common progenitor tumor cell for both diseases and responsible for their pathogenesis. Thus, WM evolution would not profoundly differ from what has already been shown for leukemia[121] and follicular lymphoma.[122,123]

VI. Conclusions

FIRST ARTICLE

1) The *MYD88* L265P mutation is present in more than 85% of patients with Waldenström's macroglobulinemia, while it is rare in other related lymphoproliferative disorders. Thus, it is a useful marker for differential diagnosis between them.
2) The *MYD88* L265P mutation is present in most IgM-MGUS as well, although high sensitive methodologies are needed to detect it in this entity.
3) There are no major clinico-biological differences between patients depending on the *MYD88* status. The only association observed is that wild-type cases display a less typical WM signature: small M-component, higher lymphocytosis and lactate dehydrogenase, atypical immunophenotype, and less somatic hypermutation.

SECOND ARTICLE

1) The ASO-RQ-PCR standardized in the present work is a simple, specific and sensitive tool for the detection of the *MYD88* L265P mutation.
2) The use of an additional base mismatch at the 3′ end of the primer conditions a very late amplification of the negative samples compared to the mutated ones, allowing the detection of up to one mutated cell among 1000 unmutated. All this makes our system very robust in terms of specificity and sensitivity.
3) The standardized methodology can be considered of choice to monitor tumor burden, since it allows to quantify the tumor load with a high concordance with flow cytometry.

THIRD ARTICLE

1) Somatic activating mutations in *CXCR4* are present in nearly 40% of IgM monoclonal gammopathies, but they do not seem to have any clinico-prognostic impact.
2) The frequency of molecular alterations progressively increases from IgM-MGUS to asymptomatic and symptomatic WM, although without significant differences in the distribution among the three entities.

3) The B-cell receptor pathway could be considered a remarkable mechanism involved in WM pathogenesis given that, after *MYD88* and *CXCR4*, mutations in *CD79B* were the most common in our series.
4) Gene dysregulation rather than gene mutations had more impact on the clinical presentation. Thus, symptomatic patients presented with an overexpression of certain genes belonging to the Toll-like receptor/MyD88 pathway, profile that was also associated with poor prognostic clinical characteristics (low hemoglobin levels, platelets and albumin, as well as higher levels of β_2-microglobulin and monoclonal component).

FOURTH ARTICLE

1) WM transformation to DLBCL implies the acquisition of many novel alterations whose total number is inversely correlated (although not significantly) with the time to transformation. However, our results suggest that what ultimately prompts the transformation process is not the amount of alterations or the elapsed time, but the acquisition of specific alterations.
2) Mutations present at both stages in a high proportion of tumor cells, such as *MYD88* or *CD79B*, are thought to be early drivers of the malignant process. On the other hand, recurrent mutations gained at transformation (e.g. *PIM1*, *FRYL*, or *HNF1B*) would represent cooperating events participating in the selection of the clones responsible for disease progression.
3) *CD79B* is more frequently mutated in patients who suffer disease transformation compared to conventional WM, thus suggesting its potential role as biomarker for predicting the risk of transformation.
4) Transformation to high-grade lymphoma results from a branching pattern of tumor evolution, because there are some molecular variants present at diagnosis or acquired at WM progression that are no longer detected at transformation.

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