Leukemia (2013) **27**, 1722–1728
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www.nature.com/leu

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.

Leukemia (2013) **27**, 1722–1728; doi:10.1038/leu.2013.62

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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Received 29 December 2012; revised 9 February 2013; accepted 13 February 2013; accepted article preview online 28 February 2013; advance online publication, 22 March 2013

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 ~ 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).

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 ($>5\times10^9$/l)	24%	5%	0.022
Platelet $<100\cdot10^9$/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.

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

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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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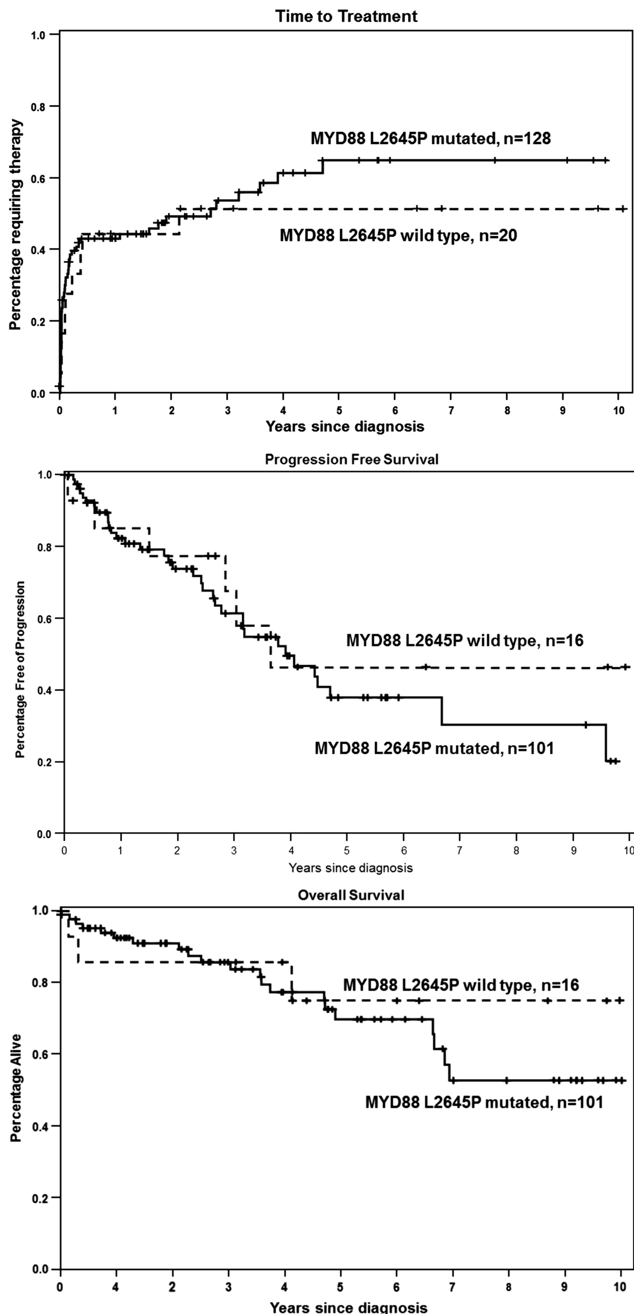

Figure 1. Projected survival of IgM-related disorders with respect to their MYD88 status. Top: time from diagnosis to the start of therapy (including IgM-MGUS, aWM and sWM); middle: survival from diagnosis to first disease progression (only aWM and sWM); bottom: overall survival from diagnosis to death.

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.

ACKNOWLEDGEMENTS

We thank Alicia Antón and Rebeca Maldonado for their technical assistance and Phill Mason for checking the English usage and grammar of the manuscript. This work has been partially supported by the grant number PS09/01450 from the Spanish 'Fondo de Investigaciones Sanitarias' and the grant reference HUS412A12-1 from the 'Consejería de Educación de la Junta de Castilla y León'.

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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