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ORIGINAL ARTICLE

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

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Abstract

Immunoglobulin M (IgM) monoclonal gammopathies 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). Despite recent findings about the genomic and transcriptomic characteristics of such disorders, we know little about the causes of this clinical heterogeneity or the mechanisms involved in the progression from indolent to symptomatic forms. To clarify these matters, we have performed a gene expression and mutational study in a well-characterized cohort of 69 patients, distinguishing between the three disease presentations in an attempt to establish the relationship with the clinical and biological features of the patients. Results showed that the frequency of genetic alterations progressively increased from IgM-MGUS to AWM and SWM. This means that, in contrast to *MYD88* p.L265P and *CXCR4* WHIM mutations, present from the beginning of the pathogenesis, most of them would be acquired during the course of the disease. Moreover, the expression study revealed a higher level of expression of genes belonging to the Toll-like receptor (TLR) signaling pathway in symptomatic versus indolent forms, which was also reflected in the disease presentation and prognosis. In conclusion, our findings showed that IgM monoclonal gammopathies present higher mutational burden as the disease progresses, in parallel to the upregulation of relevant pathogenic pathways. This study provides a translational view of the genomic basis of WM pathogenesis.

Keywords IgM monoclonal gammopathies · Heterogeneity · Mutations · Gene expression · Clinicobiological features

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Introduction

Immunoglobulin M (IgM) monoclonal gammopathies are immunoproliferative disorders that show variable behavior involving three different stages of presentation: IgM monoclonal gammopathy of undetermined significance (IgM-MGUS), asymptomatic Waldenström's macroglobulinemia (AWM), and symptomatic WM (SWM) [1]. Patients with IgM-MGUS have a risk of progression to WM or other lymphoproliferative disorders of 1.5–2% per year [2, 3], while the risk of changing from AWM to SWM is 12% per year [4]. This process probably involves multiple steps and the acquisition of genetic alterations, although the exact mechanisms are not well established. *MYD88* p.L265P mutation, the hallmark of WM [5], is already present in 50–80% of IgM-MGUS [6, 7], 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* [8]. These WHIM syndrome-like mutations have also been found in ~ 20% IgM-MGUS [9], although they are primarily subclonal, which suggests secondary acquisition, after the *MYD88* p.L265P, in WM oncogenesis [10]. Moreover, both *MYD88* and *CXCR4* variants may condition the clinical presentation of the disease [11]. However, little is known about other, less frequent alterations described in these disorders. Previous works that identified mutations in genes, such as *TRAF3* (3/57 patients, 5%) [12], *ARID1A* (5/30, 17%), *MYBBP1A* (2/30, 7%) [8], or *CD79A*/*B* (8/54, 15%) [13], 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 [14, 15] (which for example in myeloma would have led to different molecular classifications) [16, 17], 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 [18, 19], or on the expression of genes involved in late-stage B cell differentiation [20]. Others combined gene expression and mutation analysis but did not distinguish between AWM and SWM [13, 21, 22]. Finally, Trojani et al. [23], Paiva et al. [24], and Herbaux et al. [25] compared the three entities (IgM-MGUS, AWM, and SWM), but with very limited data about mutations and insufficient numbers of patients, so additional validations are required.

Our work is notable for integrating a gene expression study 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 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, qPCR gene expression profiling [GEP], and targeted 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.

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* p.L265P assessment and *CXCR4* sequencing were performed in all patients (15 IgM-MGUS, 26 AWM, and 28 SWM). A subgroup of 40 patients (3 IgM-MGUS, 19 AWM, and 18 SWM) was evaluated by qPCR GEP. In addition, 61 of the total 69 patients (14 IgM-MGUS, 23, AWM and 24 SWM) were studied by targeted sequencing.

Cases had been diagnosed using standard WHO classification criteria (2008 update) [26], with a review that included the recently published recommendations and concepts for diagnosis [27]. 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 presence of BM infiltration by morphological examination of the bone biopsy in the absence of clinical, morphological, or immunophenotypic features of other lymphoproliferative disorders [1]. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008, and informed consent was obtained from all individual participants included in the study. The clinical characteristics of this cohort were those usually seen in these entities (Table 1).

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 ($n = 15$)	Asymptomatic WM ($n = 26$)	Symptomatic WM ($n = 28$)
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 ($\times 10^9$/L)	7.9 ± 3.2	8.2 ± 4.0	7.1 ± 3.0
Platelets ($\times 10^9$/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 (mg/dL)	1367 ± 1581	1875 ± 1447	4475 ± 3489*
IgG (mg/dL)	1059 ± 408	905 ± 321	1010 ± 761
IgA (mg/dL)	219 ± 180	126 ± 83	187 ± 247
M component (g/dL)	0.8 ± 0.8	1.7 ± 1.1	3.8 ± 2.5*
Proteinuria (g/24 h)	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 (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%*
Del(RB1)	0%	4%	0%
IGH translocation	21%	13%	4%
MYD88 L265P	67%	96%	100%*

MGUS, monoclonal gammopathy of uncertain significance; *WM*, Waldenström's macroglobulinemia; *Ig*, immunoglobulin; *M component*, monoclonal component; *LDH*, lactate dehydrogenase

*$p < 0.05$

Sample preparation

CD19-selected cells were isolated from all BM samples using the autoMACS® Pro Separator system (Miltenyi-Biotec, Auburn, CA). Prior to isolation, samples had been confirmed to have monoclonal lymphoid cells by flow. The final purity was also assessed by flow (> 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 as follows: 10 min at 25 °C, 120 min at 37 °C, and 5 s at 85 °C.

MYD88 p.L265P assessment and *CXCR4* sequencing

MYD88 p.L265P mutation was assessed by real-time allele-specific oligonucleotide PCR (ASO-RQ-PCR), as described elsewhere [28]. *CXCR4* WHIM mutations were assessed by Sanger sequencing with the primers used by Hunter et al. [8].

Gene expression study

Ninety-five genes (plus *GAPDH*) were selected on the basis of the aforementioned studies [13, 18–25], 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) 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

A targeted sequencing study was performed with a novel custom amplicon-based panel of 12 genes selected because of evidences reported in the literature (*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 2000×. The data generated were processed with the MiSeq Reporter (MiSeq integrated software, Illumina), which uses a Burrows-Wheeler Aligner (BWA) [29] and the Genome Analysis Tool Kit (GATK) [30] for variant calling of single-nucleotide polymorphisms (SNPs) and short insertions and deletions (InDels). Visualization and interpretation of the results 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). Mutations were considered as non-synonymous protein-coding alterations with at least 10% of variant reads relative to the human reference genome. SNPs were discarded based on the information of the public databases, the population frequency, the percentage of reads with the variation, and the effect predicted by PolyPhen.

Multiparametric flow cytometry study

Immunophenotypic evaluation was carried out using conventional methods, as previously described by our group [31, 32], and following the general recommendations of the EuroFlow group for the evaluation of hematological malignancies [33–35]. 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

Simple interphase FISH was performed on cell nuclei from whole BM samples of most IgM-MGUS and WM using our previously published techniques [36]. Deletions of 6q and *RB1* and translocations of 14q32 were analyzed with the probes "KI-10105 6q21/SE 6" (Kreatech Diagnostics, Amsterdam, The Netherlands), "Vysis LSI 13 RB1, 13q14," and "LSI IGH dual-color, break-apart rearrangement 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 an alteration was set at ≥ 10% cells with an abnormal signal.

Statistical analyses

The chi-square (χ^2) and Mann-Whitney *U*-tests were used to identify statistically significant differences between groups. Since this work had an exploratory nature, no statistical corrections were included in the comparisons. Survival and progression were analyzed by the Kaplan-Meier method, using the log-rank test for comparisons.

Results

MYD88 p.L265P mutation

MYD88 p.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 ($p = 0.001$).

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* p.L265P-positive and, in the same way, 5/6 (83%) of the *MYD88* wild-type were also *CXCR4* unmutated. If we consider just patients with *MYD88* L265P mutation ($n = 63$), 24 of them (24/63; 38%) were *CXCR4* mutated. Among *MYD88* WT ($n = 6$), only one patient was *CXCR4* mutated (1/6; 17%). 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, and progression-free survival), based on the presence of *CXCR4* mutations. When considering only WM cases (excluding MGUS), we did 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 as follows: *CD79B* ($n = 5$, 8%); *HIST1H1E* ($n = 4$, 7%); *ARID1A*, *MYBBP1A*, and *TRAF3* ($n = 3$, 5%); *TP53*, *HIST1H1B*, and *HIST1H1C* ($n = 2$, 3%); and *HIST1H1D*, *RAG2* and *TRAF2* ($n = 1$, 2%) (Fig. 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.

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 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 × 10^9/L, respectively; $p = 0.026$).

Gene expression study

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 expression study. 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

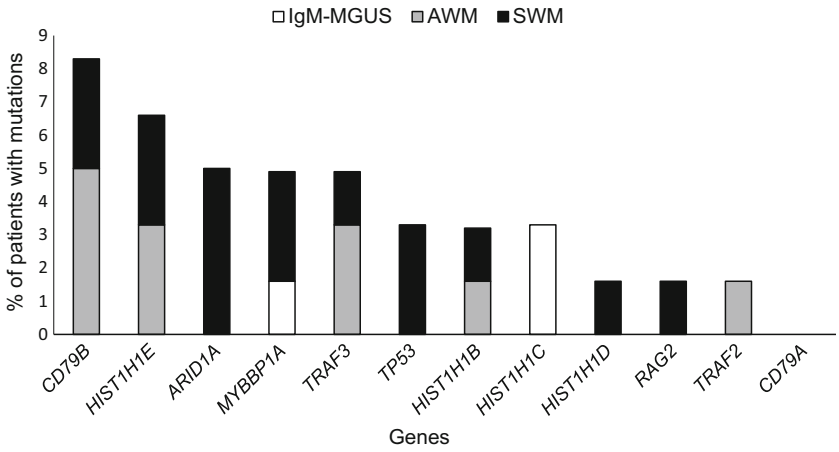

Fig. 1 Distributions of mutations among the studied genes. Bars represent the number of patients mutated for each gene. Colors of the bars indicate the different disease presentations: IgM monoclonal gammopathy of uncertain significance (IgM-MGUS), asymptomatic Waldenström's macroglobulinemia (AWM),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

differences in gene expression 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* ($p \leq 0.05$) (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.

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

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 expression profile, 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* p.L265P and *CXCR4* WHIM) [5, 8] are already present in the first stages of these entities [6, 7, 9, 10], 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 [23, 25]. However, further studies in larger cohorts of patients are still needed to establish which genes and pathways distinguish MGUS, smoldering WM, 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* p.L265P, as reported earlier [8, 22]. Other groups have shown that *CXCR4* alterations are determinants of disease presentation [11, 22], 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 [37]. However, it must be taken into account that, as previously stated [21], *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 [21, 22].

The frequency of genomic alterations (i.e., loss of heterozygosity or copy number abnormalities) increases from IgM-MGUS to AWM and SWM [13, 24]. This phenomenon was also observed in the present sequencing 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 would indicate an 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 12 genes, reinforcing the view that these cases could be a distinct entity [38].

CD79B mutations were present in 8% of the patients, as reported by other groups [8, 13, 22], 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) [39] is considered a driver event that induces a sustained cell survival effect [40]. 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 [41], a finding supported by the successful treatment with BTK inhibitors [42]. 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 [43, 44]. 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 [8]. 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) [8], 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 [23, 25]. In this line, our results showed an upregulation of *ADARB1* (alternative splicing), *CCND3*, *GPSM2* (cell cycle), and *LEF1* (transcription regulation), but not of *BACH2* in AWM [25]. 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 [45]. 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 [21].

In summary, our data reveal a higher incidence of mutations during the different stages of evolution (from IgM-MGUS to symptomatic WM), meaning that, in contrast to

MYD88 p.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. This could represent a diagnostic tool for a better assessment of IgM monoclonal gammopathies and be the basis for an extended study with more cases, maybe including more genes. The expression study also highlighted significant differences between indolent and symptomatic patients with respect to genes involved in WM pathogenic mechanisms, such as *MYD88*. Finally, TLR, CXCR4, and BCR pathways were confirmed as playing an important role in the biology and pathogenesis of WM. This prompts us to evaluate in detail these pathways including more cases and with protein analysis approaches. In the end, this could help to develop new therapeutic strategies that block these routes and result in a clinical benefit for the patients.

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.**Financial support** This work was supported by research grants from the Gerencia Regional de Salud (GRS 847/A/13), the Asociación Castellano-Leonesa de Hematología y Hemoterapia (FUCALHH 2015), and the Gilead Sciences (GILEAD) Fellowship Program (GLD16/00162), as well as funds from the Instituto de Salud Carlos III (ISCIII), Spanish Ministry of Economy and Competitiveness, CIBERONC-CB16/12/00233.

Compliance with ethical standards All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008, and informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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