Molecular characterization of heavy chain immunoglobulin gene rearrangements in Waldenström's macroglobulinemia and IgM monoclonal gammopathy of undetermined significance

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ABSTRACT

Background and Objectives

Waldenström macroglobulinemia (WM) and monoclonal gammopathy of undetermined significance (MGUS) are IgM-related disorders in which monoclonal B cells harbor a unique clonotypic rearrangement of the immunoglobulin heavy chain gene (*IgH*). The aim of this study was to characterize *IgH* rearrangements in a larger series of IgM-related disorders than any previously described.

Design and Methods

Seventy-two patients with monoclonal IgM disorders (64 with WM and eight with IgM-MGUS) were studied to amplify and sequence both VDJ_H and DJ_H rearrangements. Twenty-nine of them were also tested for the existence of class switch recombination (CSR).

Results

VDJ_H and DJ_H rearrangements were detected in 91% and 42% of WM patients and in 100% and 13% of IgM-MGUS patients, respectively. In WM, the most frequently observed V_H family and single segment were V_H3 and V_H3-23 (76% and 29%, respectively), with their frequencies differing markedly from those that would occur if the rearrangements were random. The V_H3-23 segment was never selected in IgM-MGUS. The distribution of both D_H and J_H families in WM did not differ from that in normal B-lymphocytes. Somatic hypermutation with >2% deviation was seen in 90% of cases of WM and in 71% of IgM-MGUS. DJ_H rearrangements were more frequent in WM than in MGUS (42% and 13%, respectively). All DJ_H rearrangements were unmutated, which makes them an attractive target for minimal residual disease investigation. IgM clonotypic transcripts were observed in all cases and IgD in 83%. IgA and/or IgG monoclonal isotypes were seen in three WM cases (14%) but in none of the IgM-MGUS patients.

Interpretation and Conclusions

WM and IgM-MGUS exhibit dissimilarities in VDJ_H and DJ_H rearrangements that could suggest different differentiation processes. There is evidence that WM cells are able to undergo CSR *in vivo*, a fact that was initially thought to be impossible in this disease.

Key words: Waldenström's macroglobulinemia, monoclonal gammopathy of undetermined significance, immunoglobulin rearrangements, class switch recombination.

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Waldenström's macroglobulinemia (WM) is an uncommon lymphoproliferative disorder primarily characterized by the presence of an immunoglobulin M (IgM), monoclonal protein and unequivocal evidence of bone marrow infiltration by lymphoplasmacytic lymphoma.[1] It is conceivable that WM evolves from an IgM monoclonal gammopathy of undetermined significance (IgM-MGUS), although this has only been demonstrated in approximately 8% of all WM cases.[2] Both conditions typically have a monoclonal component (M-component) produced by monoclonal B-cells harboring a unique clonotypic rearrangement of immunoglobulin heavy chain gene (*IgH*), the VDJ_H rearrangement, associated with a specific constant region IgM. Although clearly defined and reproducible criteria distinguish between IgM-MGUS and WM, the precise cells from which these two entities originate remains unclear.[1] The characterization of VDJ_H rearrangements as well as related processes such as somatic hypermutation (SHM) and class switch recombination (CSR) may help to shed light on this area because the differentiation process follows a strict hierarchical order in generating the Ig repertoire. Therefore, studies could indicate the B-cell differentiation stage at which the oncogenic event occurs. Early in B-cell development, rearrangement starts with D_H joining to J_H. This can produce a complete functional VDJ_H rearrangement but can also remain as an incomplete non-functional rearrangement that forces the second allele to be rearranged. Incomplete rearrangements are frequently found in precursor B-cell acute lymphoblastic leukemia,[3] but may also occur in mature B-cell lymphoproliferative disorders, such as multiple myeloma,[4] and hairy cell leukemia.[5] It is unknown whether incomplete DJ_H rearrangements exist in WM. Incomplete DJ_H rearrangements may be an interesting alternative target for polymerase chain reaction (PCR)-based clonality assessment because they lack SHM. Finally, in contrast to other B-cell lymphoproliferative disorders,[6-9] in WM information on the composition of the third complementary determining region (CDR3) and frequency of SHM is limited.

The normal counterpart of the WM malignant cell is believed to be a post germinal center IgM B cell that transforms once somatic mutation has ended. Traditionally, it has been assumed that WM cells are constitutively unable to or are being prevented from carrying out isotype CSR.[10,11] It was initially suggested that this was due to the presence of genetic abnormalities involving the CSR machinery, but this possibility has been excluded since the machinery seems to be intact.[12] Another explanation could be the absence of mutations at the switch region.[12] Despite this presumed lack of CSR, *ex-vivo* clonotypic transcripts encoding post-switch isotypes have recently been detected in WM and IgM-MGUS cells cultured with CD40L/interleukin-4.[12] This would indicate that CSR is possible in WM cells, but up until now, this has not been shown to occur *in vivo*. In this study, we characterized complete VDJ_H and incomplete DJ_H rearrangements in 64 patients with untreated WM and eight with IgM-MGUS, and documented the existence of CSR in 29 of them.

Design and Methods

Patients and diagnosis

Seventy-two patients with monoclonal IgM disorders were included in the study. According to the consensus criteria from the 2nd International Workshop of WM,[1] 64 of them had a diagnosis of WM and eight were considered to have an IgM-MGUS. Although the existence of bone marrow infiltration by lymphoplasmacytic lymphoma was established by bone marrow biopsy, the presence of tumor B cells was always confirmed by immunophenotypic analysis with flow cytometry, including evaluation of surface and cytoplasmic IgM expression.[13]

Genomic DNA preparation, mRNA extraction and cDNA synthesis

High molecular weight DNA was isolated from bone marrow samples from all patients according to standard methods.[4] In addition, total RNA was isolated from samples with representative tumor cells from 21 WM patients as well as in all the eight IgM-MGUS patients, using the guanidium thiocyanate/phenol chloroform method. Reverse transcription was performed on 1 μg of total RNA, according to the rules and protocols approved in the *Europe Against Cancer Program* (EAC).[14]

PCR amplification of the VDJ_H, DJ_H and VDJ-C of rearranged Ig_H genes

For amplification of complete VDJ_H rearrangements, a set of family-specific primers of the framework 1 (FR1) region and one J_H consensus primer were used in a multiplexed PCR reaction. When no amplification was detected from FR1, PCR was carried out from FR3, in which case analysis of SHM was impossible. Amplification of incomplete DJ_H rearrangements was performed in two different reactions using family-specific primers for D_H1 to D_H6 and D_H7 families, respectively, together with the consensus J_H primer. All primers were designed and tested during the BIOMED-2 Concerted Action: *PCR based clonality studies for early diagnosis of lymphoproliferative disorders* (BMH4-CT98-3936), in which our group participated actively in standardization.[15] All reactions were carried out in a 50 μL mixture containing 0.1 μg DNA and 10 pmoL of each primer. All these amplifications were carried out using genomic DNA.[15]

Pre- and post-switch clonal isotype expression was analyzed from RNA using a slight modification of the method described by Billadeau *et al.*[16] First-round cDNA amplification was carried out in 50 μL reactions using

the family-specific VH primer as the forward primer and the constant region primer as the reverse primer. The second-round amplification was carried out in 50 μL reactions with 5 μL of the first-round amplification product, family-specific VH primers and an internal constant region primer. Amplified products were denatured for 5 min at 95°C and renatured for 60 min at 4°C to allow heteroduplex and/or homoduplex formation. The final product was then electrophoresed on an 8% polyacrylamide gel and visualized under UV light after ethidium bromide staining.[4,15]

Sequencing and analysis of IgH genes

Monoclonal PCR products were purified from polyacrylamide gels for direct sequencing in an automated ABI 377 DNA sequencer using BigDye® Terminator with the v1.1 Cycle Sequencing kit (Applied Biosystems).

Germline VH, DH and JH segments from complete VDJH rearrangements were identified by comparison with the V BASE.[17] An IgH diversity gene segment (DH) was identified in most cases (DH1, DH4, DH5, DH6 and DH7) by homology of a minimum of six successive nucleotides (nt) or seven successive nt interrupted by no more than two mismatches with the germline sequence.[18] In the remaining cases, involving the longest DH families (DH2 and DH3), the DH gene was identified using a stricter criterion: at least ten consecutive nt.[19] The ability to code for functional Ig heavy chains was determined by translating VDJH DNA sequences into amino acids.

DH and JH germline segments from incomplete DJH rearrangements were identified using the BLAST search (accession number X97051).

In the same way, all PCR products obtained by RT-PCR with VH family-specific primers and CH-specific primers (CSR amplifications) were also sequenced and compared with the original VDJH clonotypic sequence as well as with the BLAST sequences.

The VH, DH and JH distribution obtained in functional VDJH rearrangements was compared with that occurring randomly in normal B-cell populations,[17,18,20] as well as with that observed in DJH, non-functional rearrangements.

Composition of the third complementary-determining region

The length of the CDR3 was determined by counting the number of amino acids between the last amino acid of FR3 and the first amino acid of the JH (FR4).[21] To detect whether a particular antigen might have been responsible for the antigen-selective pressure observed in those cases showing features of antigen selection, we evaluated nucleotide and derived amino acid sequence similarities of the CDR3 regions.

Somatic hypermutation

To confirm base changes in the germline IgH sequence, two independent PCR amplifications for each sample were sequenced in both the forward and reverse amplified fragment in order to observe the same change in separate reactions. VH sequences containing ≥2% deviation from the germline sequence were considered to be somatically mutated.[22] The nucleotide sequences were translated to amino acids in order to determine whether a mutation was a replacement (R) or silent (S). In such cases, the binomial algorithm described by Chang and Casali[23] was used to discriminate between R and S mutations in CDR and FR derived from antigen selection or acquired randomly. This analysis is based on the fact that as a result of antigen-selective pressure, the R/S ratio of amino acid mutations is higher than expected in the CDR regions, which is consistent with the need to provide the best fit for the antigen. In contrast, the R/S ratio is lower in the FR regions in order to conserve the antibody structure. p values ≤0.05 according to the binomial distribution model were considered statistically significant indicators of antigen-selective pressure. We also analyzed mutations in DH and JH regions, considering that these regions were mutated when they contained one or more mutations.

Results

Detection of monoclonal rearrangements by PCR

All patients (100%) showed monoclonal rearrangement when complete functional VDJH and/or incomplete non-productive DJH were amplified. VDJH rearrangements were detected in 58 out of 64 WM patients (91%) and in all eight Ig-MGUS patients (100%). DJH rearrangements were observed in 27 of the 64 WM patients (42%) and in one of the eight patients with IgM-MGUS (13%).

VDJH rearrangements: VH, DH and JH gene segment usage

VH, DH and JH gene segment usage, as well as the rate of SHM for all WM and IgM-MGUS patients are shown in Table 1.

Waldenström's macroglobulinemia

Table 2 shows the expected and observed frequencies of VH gene segments per family. Patients with WM displayed an under-representation of the VH1 family (4/58; 7%) and a marked over-representation of the VH3 family (44/58; 76%). In addition, the VH3-23 gene segment was very highly used, since it was present in 17 out of the 58 identified functional rearrangements (29%), a frequency much higher than would be expected from random occurrence (1.96%; p<0.001). In contrast, several VH segments frequently found in normal early and/or

Table 1. V_H, D_H and J_H gene segment usage in complete and incomplete Ig_H rearrangements, as well as somatic hypermutation and antigen selection of VJD_H rearrangements in WM and IgM MGUS.

Rearrangement												Rearrangement										
VDJ_H									DJ_H*			VDJ_H									DJ_H*	
ID	D	V_H	SM	D_H	J_H	CDR3 Length	pCDR	pFR	AS	D_H	J_H	ID	D	V_H	SM	D_H	J_H	CDR3 Length	CDR	FR	AS	D_H	J_H
3413	WM	1-2	8.99	–	5-b	21	0,080	0,221	R	4-23	6-b	5582	WM	3-7	7,45	–	6-b	15	0,420	0,17	R	2-2	ND
4335	WM	1-2	5.71	1-7	1-1	24	0.03	0.2	AS	ND	ND	4565	WM	3-7	5.26	–	3-a	33	0.001	0.16	AS	ND	ND
3388	WM	1-2	0	–	NSO	NA	NA	NA	NA	ND	ND	5578	WM	3-7	6.17	–	NSO	NA	0.003	0.1	AS	ND	ND
3936	WM	1-3	12.5	–	6-2	9	0.000	0.173	AS	ND	ND	2106	WM	3-72	5.64	–	4-b	30	0.080	0.16	R	2-2	4-d
5579	WM	3-1	9.82	–	3-2	27	0.000	0.04	AS	2-15	5-b	2326	WM	3-72	6.83	6-19	2	30	0.320	0.088	R	3-9	ND
3167	WM	3-15	11.11	6-6	3-b	9	0.001	0.213	AS	ND	ND	5444	WM	3-72	8.19	–	4-b	21	0.004	0.24	AS	ND	ND
4070	WM	3-15	7.01	–	5-2	9	0.29	0.27	R	ND	ND	709	WM	3-73	5.55	–	4-b	36	0.000	0.3	AS	ND	ND
5581	WM	3-21	9.77	–	6	21	0.000	0.3	AS	ND	ND	4268	WM	3-73	5.9	–	NSO	NA	0.022	0.226	AS	ND	ND
8079	WM	3-21	8.18	3-22	6-c	51	0.129	0.2264	R	ND	ND	3168	WM	3-74	10.08	–	4-d	18	0.000	0.035	AS	2-15	4-b
1758	WM	3-21	0	4-23	6-b	24	NA	NA	NA	ND	ND	1991	WM	3-74	6.39	1-26	6-a	30	0.011	0.317	AS	ND	ND
125	WM	3-23	13.06	6-25	4-b	15	0.000	0.1	AS	4-23	ND	447	WM	3-74	12.44	–	5-b	33	0.000	0.16	AS	ND	ND
532	WM	3-23	11.4	6-1	4-b	48	0.000	0.17	AS	2-2	6-b	4864	WM	3-9	5.33	5-12	1	42	0.011	0.317	AS	ND	ND
3665	WM	3-23	7.35	–	2-1	12	0.0001	0.17	AS	2-2	6-b	1937	WM	4-28	7.45	4-11	2	30	0.000	0.17	AS	2-2	6-b
4057	WM	3-23	8.77	–	6-b	9	0.000	0.225	AS	4-23	ND	18423	WM	4-30	8.65	–	3-b	18	0.19	0.01	AS	6-19	4-b
4035	WM	3-23	NA	2-15	2	39	NA	NA	NA	5-12	4-b	4056	WM	4-39	5.37	4-22	4-a	27	0.3	0.2	R	2-21	ND
50	WM	3-23	16.19	–	6-b	30	0.000	0.112	AS	5-12	ND	5584	WM	4-39	10.45	3-22	4-b	33	0.001	0.25	AS	ND	ND
5586	WM	3-23	12.12	–	NSO	NA	0.000	0.25	AS	6-13	4-d	5756	WM	4-39	4.32	–	NSO	NA	0.015	0.374	AS	ND	ND
1956	WM	3-23	7.11	3-10	4-a	33	0.2053	0.3121	R	ND	ND	13426	WM	4-59	10.22	–	6-c	27	0.12	0.02	AS	ND	ND
5320	WM	3-23	10.38	6-13	5-b	27	0.0003	0.13	AS	ND	ND	5775	WM	4-61	6.92	6-6	4-b	27	0.000	0.03	AS	2-2	5-b
2385	WM	3-23	7.01	–	NSO	NA	0.0012	0.3179	AS	ND	ND	5216	WM	4-61	0.86	3-3	3-b	51	NA	NA	NA	6-6	5-b
3166	WM	3-23	7.89	–	4-b	27	0.003	0.16	AS	ND	ND	1062	WM	5-51	0.45	1-26	4-b	18	NA	NA	NA	ND	ND
3414	WM	3-23	9.21	–	5	27	0.02	0.3	AS	ND	ND	5589	WM	6-01	1.61	5-5	6	21	NA	NA	NA	ND	ND
2661	WM	3-23	11.71	–	4-b	36	0.000	0.186	AS	ND	ND	2776	WM	ND	NA	–	ND	NA	NA	NA	NA	1-26	6-c
4269	WM	3-23	15.5	–	NSO	NA	0.000	0.11	AS	ND	ND	1999	WM	ND	NA	–	ND	NA	NA	NA	NA	2-15	ND
5588	WM	3-23	NA	–	NSO	NA	NA	NA	NA	ND	ND	3260	WM	ND	NA	–	ND	NA	NA	NA	NA	2-2	5-b
923	WM	3-23	NA	–	NSO	NA	NA	NA	NA	ND	ND	2311	WM	ND	NA	–	ND	NA	NA	NA	NA	2-2	6-b
5208	WM	3-23	NA	–	4-b	36	NA	NA	NA	ND	ND	17665	WM	ND	NA	–	ND	NA	NA	NA	NA	5-5	4
2040	WM	3-30	9.33	1-26	3-a	30	0.000	0.1729	AS	ND	ND	1053	WM	ND	NA	–	ND	NA	NA	NA	NA	5-5	4-b
2550	WM	3-30	14.22	1-26	4-b	27	0.000	0.02	AS	ND	ND	5302	MGUS	3-11	0	3-10	5	30	NA	NA	NA	ND	ND
1079	WM	3-30	9.21	–	NSO	NA	0.000	0.05	AS	ND	ND	4233	MGUS	3-30	5.71	1-1	4-a	30	0.045	0.035	AS	ND	ND
4587	WM	3-30	8.62	–	NSO	NA	0.056	0.59	R	ND	ND	1860	MGUS	3-30	NA	–	NSO	NA	NA	NA	NA	ND	ND
5587	WM	3-30	NA	–	NSO	NA	NA	NA	NA	ND	ND	3894	MGUS	3-73	0.85	6-25	6-2	18	NA	NA	NA	4-23	3-b
4349	WM	3-33	9.09	1-26	4-b	39	0.032	0.21	AS	4-23	5-b	4631	MGUS	3-74	10.66	–	4-3	3	0.000	0.25	AS	ND	ND
5591	WM	3-53	NA	–	NSO	NA	NA	NA	NA	ND	ND	2261	MGUS	3-74	6.57	1-26	5-a	27	0.080	0.24	R	ND	ND
5626	WM	3-7	6.57	–	5-b	15	0.17	0.259	R	2-8	5-b	1218	MGUS	4-34	4.44	1-26	3-b	39	0.138	0.100	R	ND	ND
5592	WM	3-7	4.25	–	6-b	15	0.372	0.259	R	4-17	6-b	407	MGUS	4-39	8.21	–	3-a	24	0.056	0.225	R	ND	ND

ID: identification; D: diagnosis; SM: somatic mutation; CDR3: third complementary-determining region; pCDR and pFR: p values according to the binomial distribution model for the complementary-determining region and framework region, respectively; p<0.005 was considered a statistically significant indicator of antigen-selective pressure.[23]
AS: antigenic selection; S: selection; R: random choice; WM: Waldenström's macroglobulinemia; MGUS: IgM monoclonal gammopathy of undetermined significance;
NA: not analyzed; ND: not detected; NSO: no sequence was obtained; -- The DH segment could not be identified with the criteria used.[18,19]

Table 2. Distribution of V_H families in VDJ_H rearrangements from WM and IgM MGUS patients.

	Known functional V_H segments in V-BASE[a] (n=51)	*VDJ_H rearrangements in WM (n=58)*	*VDJ_H rearrangements in IgM-MGUS (n=8)*
V_H1	11 (22%)	4 (7%)*	0
V_H2	3 (6%)	0 (0%)	0
V_H3	22 (43%)	44 (76%)**	6 (75%)
V_H4	11 (22%)	8 (13%)	2 (25%)
V_H5	2 (4%)	1 (2%)	0
V_H6	1 (2%)	1 (2%)	0
V_H7	1 (2%)	0 (0%)	0

*[a]The number of V_H genes per family in normal B lymphocytes was obtained from the VDJ_H recombination data contained in the V-BASE Sequence Directory[18] WM: Waldenström's macroglobulinemia; IgM-MGUS: monoclonal gammopathy of undetermined significance. *p<0.05 between the distribution of V_H in WM and V_H in normal cells; **p<0.001 between the distribution of V_H in WM and V_H in normal cells .*

Table 3. Distribution of D_H families in VDJ_H and DJ_H rearrangements from WM and IgM MGUS patients.

	D_H genes per family[a] (n=27)	*VDJ_H rearrangements in WM (n=22)*	*DJ_H rearrangements in WM (n=27)*	*VDJ_H rearrangements in IgM-MGUS (n=5)*
D_H1	5 (19%)	6 (27%)	1 (4%)*	3 (60%)
D_H2	4 (15%)	1 (4.5%)	13 (48%)*	0 (0%)
D_H3	5 (19%)	4 (18%)	1 (4%)	1 (20%)
D_H4	4 (15%)	3 (14%)	5 (19%)	0 (0%)
D_H5	4 (15%)	2 (9%)	4 (15%)	0 (0%)
D_H6	4 (15%)	6 (27%)	3 (11%)	1 (20%)
D_H7	1 (4%)	0 (0%)	0 (0%)	0 (0%)

*[a]The number of D_H genes per family in normal B lymphocytes was obtained from Corbett et al.[18] WM: Waldenström's macroglobulinemia. IgM-MGUS: monoclonal gammopathy of undetermined significance. *p<0.05 between the frequency of D_H in VDJ_H and that in DJ_H rearrangements in WM .*

Table 4. Distribution of J_H gene segments in VDJ_H and DJ_H rearrangements from WM and IgM MGUS patients.

	J_H in normal B lymphocytes[a] (n=100)	*VDJ_H rearrangements in WM (n=45)*	*DJ_H rearrangements in WM (n=20)*	*VDJ_H rearrangements in IgM-MGUS (n=7)*
J_H1	2%	2 (5%)	0 (0%)	0 (0%)
J_H2	2%	4 (9%)	0 (0%)	0 (0%)
J_H3	9%	6 (14%)	0 (0%)	2 (28%)
J_H4	51%	16 (35%)	7 (35%)	2 (28%)
J_H5	14%	6 (14%)	6 (30%)	2 (28%)
J_H6	22%	11 (24%)	7 (35%)	1 (14%)

[a]The number of J_H genes per family in normal B lymphocytes was obtained from Wasserman R;[20] WM: Waldenström's macroglobulinemia; IgM-MGUS: monoclonal gammopathy of undetermined significance.

circulating B cells, such us V_H3-20 and V_H3-49, which were completely absent in this series (Table 1).

D_H segments could be identified in only 22 of the 58 VDJ_H rearrangements detected (38%), according to stringent criteria.[18,19] The most frequently involved families were D_H1 and D_H6, each present in six of 22 cases (27%), while the least frequent was D_H2 (4.5%) (Table 3). Finally, the most commonly represented J_H families were J_H4 (16/45; 35%) and J_H6 (11/45; 24%) (Table 4). The distribution of both D_H and J_H families in WM did not differ significantly from the distribution in normal B lymphocytes.

IgM monoclonal gammopathy of undetermined significance

Only the V_H3 and V_H4 families were represented within complete VDJ_H rearrangements in patients with IgM-MGUS (6/8 and 2/8, respectively) (Table 2). The D_H element was identified in five cases, and D_H1 was the most represented family (3/5; 60%) (Table 3). The J_H selection was not specifically different from the pattern observed in other B-cell malignances or normal B cells.

CDR3 analysis

The CDR3 sequence could be analyzed in detail in 45 WM patients. The average lenght was 26.6 nt±10.7nt. As mentioned above, the D_H element was identified in 22 patients (49%), in whom the average length of the CDR3 region was 30 nt±11.4. The remaining 23 cases contained an unidentifiable D_H element according to conventional criteria,[18,19] probably because the CDR3 length was shorter (23 nt±9.12) (Table 1). No consensus sequence showing evidence of selection by the same antigen could be demonstrated between the CDR3 regions of the rearranged Ig genes.

The CDR3 was analyzed in seven of the eight cases of IgM-MGUS and had a mean length of 24 nt±11.4. In the five cases in which the D_H segment was identified, the mean length of 28.8 nt±7.5 was longer than in the two cases without an identified D_H segment in whom the mean length was 13.5 nt±14.8.

DJ_H rearrangements: D_H and J_H family usage

DJ_H rearrangements were detected in 27 WM cases (42%), with a preferential usage of the D_H2 family (13/27; 48%) (Table 3). In almost half of these rearrangements, only two segments were selected (D_H2-02 in eight cases and D_H4-23 in five) (Table 2). Regarding J_H segments, only J_H4, J_H5 and J_H6 were selected in these rearrangements (Table 4). Finally, incomplete rearrangements were detected in only one IgM-MGUS patient (13%) in whom D_H4 and J_H3 gene segments were used (Table 1).

Somatic mutation analysis

The complete sequence was available for somatic mutation analysis for 52 of the 58 patients with WM and seven out of the eight with IgM-MGUS. The

remaining cases could only be amplified from FR3, which prevents such analysis. Using the 2% deviation from the germline sequence as a cut-off value for SHM, 90% of WM and 71% of IgM-MGUS cases had SHM. Within these cases, the mean deviation from the germline was 8.7% (range 4.3-16.2) in WM and 7.1% (range 4.4-10.6) in IgM-MGUS. This means that there were seven cases lacking SHM (five WM and two IgM-MGUS). Interestingly, three of them (two WM, one MGUS) had a complete match with the germline configuration (0% deviation). The J_H segment contained occasional mutations in 71% of the VDJ_H rearrangements.

The SHM pattern was investigated to ascertain whether WM and IgM-MGUS cases had different features concerning random or antigen-driven selection. The distribution of R and S mutations in the FR and CDR regions is summarized in Table 1. A random distribution of SHM was observed in 23% of WM patients and in 60% in IgM-MGUS patients.

The vast majority of the incomplete DJ_H rearrangements were unmutated, since we observed only occasional single point mutations in three cases.

Class switch recombination

Twenty-one WM patients and eight IgM-MGUS patients had representative mRNA available to test the presence of pre-switch (IgM, IgD) and post-switch (IgA, IgG) isotype expression. As expected, IgM clonotypic transcript isotypes were present in all samples and IgD in most of them (83%), with exactly the same clonotypic sequence as that obtained from genomic DNA. Interestingly, hemi-nested RT-PCR revealed the presence of post-switch clonotypic isotypes in three WM patients: two IgA and one IgG. In these three cases, sequencing of CSR amplified products showed the same CDR3 (V_H-D_H-J_H) sequence and the same somatic hypermutation pattern, which demonstrates that all isotypes were derived from the same clone.

Discussion

In this study we characterized molecular IgH rearrangements in 72 patients, 64 of whom had WM and eight of whom had IgM-MGUS. This constitutes the largest series of patients with IgM-related disorders studied up to now; indeed, combining previous literature data, only 59 cases have so far been reported.[11,12,24,25] Our results confirm previous estimations about Ig_H repertoire usage and show that incomplete rearrangements are frequent in WM. In addition, we also confirm that isotype switching is possible *in vivo* in WM.

Previous studies on mutation analysis of antigen receptor genes revealed that most cases of WM presumably derive from a post-germinal center cell, a cell with somatic hypermutation, but without evidence of CSR.[10,11] However, the low number of cases previously analyzed made it impossible to ascertain whether there is a bias in VDJ_H repertoire selection. Our study definitively demonstrated a preferential usage of V_H3 family segments, with a >75% representation, which is much higher than that observed in normal B cells[17] or in other lymphoproliferative disorders such as multiple myeloma,[26] hairy cell leukemia[5] or chronic lymphocytic leukemia.[19] These results concur with those previously reported by Kriangkum *et al.*,[10,12] who described a 93% usage in 15 patients, although our study demonstrates that the use of other segments is not an exception. The V_H3-23 segment was selected in nearly one-third of all WM cases, which contrasts with the complete absence of this segment in IgM-MGUS cases. Another interesting finding in our series is the usage of the J_H segment, which is relatively more diverse than that described by Kriangkum *et al.*,[12] who found that the frequency of usage of J_H4 was as high as 73%. Other B-cell lymphoproliferative disorders such as multiple myeloma and B-cell chronic lymphocytic leukemia have also shown, as in our series of WM patients, a diverse J_H selection.[19,26]

DJ_H rearrangements were observed in 42% of WM patients, a frequency similar to that observed in other mature B-cell lymphoproliferative disorders.[4,5] In contrast, these incomplete rearrangements were less frequent (13%) in IgM-MGUS cases, which suggests some differences in the neoplastic origin of the two entities. The use of D_H and J_H segments differed between cases with complete and incomplete rearrangements. This could indicate that D_H1, D_H3 and D_H6 family selection (very frequent in complete rearrangements and almost absent in incomplete rearrangements) would favor a functional result, while the use of D_H2, and especially the D_H2-02 gene segment (never present in complete VDJ_H functional rearrangements but frequent in incomplete rearrangements), could lead to non-productive rearrangements, similar to pseudo-genes.

The average length of the CDR3 was 26.6 nt, which is shorter than previously reported.[27] This shorter CDR3 is typical of B cells selected for binding to antigens.[28] The CDR3 is the region where the greatest evidence of positive and negative selection was observed, especially for the use of different D_H families between complete and incomplete rearrangements. This is in line with the key role that the CDR3 plays in antigen recognition.[28,29] In the present series, the percentage of cases with somatic hypermutation according to conventional criteria was very high: 90% for WM and 71% for IgM-MGUS. Nevertheless, five out of the 52 WM cases tested (9.6%) showed a naïve B-cell origin, which concurs with other published data.[10-12,24,30] This would indicate that the transformation event that leads to WM or IgM-MGUS does not necessarily target somatically mutated B cells.

Antigenic selection was detected in 77% of the cases of WM, a figure more than twice that previously reported.[10,12,31] This difference could be due to variations in ref-

erence databases,[32] but some differences could also be caused by the multimodal[33] or binomial[23] models used in the analyses. Interestingly, antigenic selection was found in only 40% of cases of IgM-MGUS, again suggesting some possible differences between WM and IgM-MGUS.

Considering all these data together, we can hypothesize that WM and IgM MGUS may develop differently, at least in a subgroup of IgM MGUS patients whose disease will never evolve into WM. This hypothesis is compatible with the results of mutational analysis of the upstream Sμ region recently published by another group.[12] Therefore, molecular analysis could be an interesting tool for a better diagnostic definition of IgM MGUS, identifying patients at a high risk of malignant evolution. However, it must be appreciated that this hypothesis is based on a very small group of IgM MGUS patients (four in Kriangkum's study and eight in the present one), so additional studies are required to confirm this possibility.

Although several studies found only clonotypic IgM transcripts in WM patients,[10,11,34] a recent study demonstrated that WM cells are capable of undergoing CSR *in vitro*.[12] In the present series, we found and sequenced post-switch clonotypic isotypes in three out of 21 cases with representative RNA available, demonstrating that WM cells are capable of undergoing CSR *in vivo,* albeit rarely. Furthermore, we recently reported a case showing that not only are WM cells capable of undergoing CSR *in vivo*, but that the CSR can be functional, since an additional IgG serum M-component was observed five years after diagnosis.[35] Therefore, the fact that WM cells do not usually carry out CSR can no longer be explained by the presence of irreversible genetic lesions, but is more probably due to a response to inhibitory regulation. Accordingly, such negative regulation could eventually be overcome depending on the microenvironmental conditions, as occured in three of our cases.

In conclusion, in this study we characterized IgH rearrangements in the most extensive series of IgM-related disorders reported up until now. We have documented some dissimilarities in VDJ_H and DJ_H rearrangements between WM and IgM-MGUS, which could suggest a distinct differentiation process between the two disorders. In addition, we found evidence demonstrating that WM cells are capable of undergoing CSR *in vivo*, which was initially thought to be impossible in this disease.

Authors' Contributions

RG-S and PMJ were the initial designers of the study. PMJ carried out all molecular estudies and prepared the database for the final analysis. She prepared the initial version of the paper. RG-S. made the database and supervised the statisticall analysis. He rewrote the paper and provided the pre-approval of the final version. AB helped in the molecular analysis and data collection; EO was the clinician responsible for the patients who took care of the protocol, sampling and collecting the clinical data; MLS: carried out flow cytometry studies; MG reviewed the conception and design of the work; JFSM was responsible for the group and the final revision of the draft and gave final approval of the version to be published.

Conflict of Interest

The authors reported no potential conflicts of interest.

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