OPEN

Citation: Blood Cancer Journal (2013) 3, e112; doi:10.1038/bcj.2013.12

www.nature.com/bcj

ORIGINAL ARTICLE

Molecular analysis of immunoglobulin genes reveals frequent clonal relatedness in double monoclonal gammopathies

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Monoclonal gammopathies (MGs) are hematological diseases characterized by high levels of a monoclonal immunoglobulin (Ig) or M-protein. Within this group are patients with more than one M-protein, referred to as double MGs (DMGs). The M-proteins in DMG patients may have different heavy chain (HC) isotypes that are associated with different light chains (LCs), or different HCs that are LC matched. In this study, we examined the clonal relatedness of the M-proteins in the latter type in a cohort of 14 DMG patients. By using PCR, we identified 7/14 DMG patients that expressed two Ig HC isotypes with identical Ig HC variable (IGHV), diversity (IGHD), joining (IGHJ), and complementarity determining region (HCDR3) sequences. Two additional DMG patients had two Ig transcripts using the same *IGHV*, *IGHD* and *IGHJ* genes but with slight differences in variable region or HCDR3 mutations. LC analysis confirmed that a single LC was expressed in 3/7 DMG patients with identical HC transcripts and in the two DMGs with highly similar transcripts. The PCR findings were confirmed by immunofluorescence for HC and LC expression. Clonally related HC-dissimilar/LC-matched DMGs may occur often and defines a new subtype of MG that may serve as a tool for studies of disease pathogenesis.

Blood Cancer Journal (2013) **3,** e112; doi:10.1038/bcj.2013.12; published online 19 April 2013

Keywords: monoclonal gammopathy; IGHV; biclonal; clonality

INTRODUCTION

Monoclonal gammopathies (MGs) encompass a number of disorders characterized by the presence of a clonal expansion of malignant plasma cells (PCs) typically located within the bone marrow (BM). In addition, these patients generally have high levels of monoclonal serum immunoglobulin (Ig) of a single isotype, which is referred to as the M-protein. As with normal PCs, MG PCs are derived from B cells that have undergone rearrangement of the Ig heavy chain (HC) and Ig light chain (LC) variable (V) region genes.

Further diversification arising from somatic hypermutation (SHM) of the Ig V regions and class switch recombination (CSR) during germinal center (GC) reactions results in a unique Ig clonotypic signature.[1–6] Indeed, in post-follicular malignancies including the MGs multiple myeloma (MM), MG of undetermined significance (MGUS), LC amyloidosis (AL) and Waldenstrom's macroglobulinemia (WM), the malignant PCs have acquired Ig HC and LC V region somatic mutations. In addition, MM, MGUS and AL have typically undergone IGH isotype switch to IgG, IgA or IgE, and studies in MM suggest that the malignant PCs no longer undergo SHM as evidenced by lack of intraclonal diversity.[7–10]

Despite being considered a clonal malignancy expressing single rearranged Ig HC and LC V regions, 3–4% of all patients with MGs express more than one M-protein in serum and urine, a condition termed biclonal gammopathy or double MG (DMG).[11–14] These patients can express M-proteins with different isotypes of HCs that are LC isotype-matched, the same HC isotypes with different LC isotypes, or different HC isotypes with different LC isotypes. The latter situation would signify true biclonal or polyclonal gammopathy, most likely arising from two or more distinct cell populations. Within DMGs, the most frequently observed combination of HCs is IgA and IgG followed by IgG and IgM.[14,15] However, when LC expression was considered, 73% of those patients that expressed IgG/IgA also expressed the same LC compared with 60% of IgG/IgM combinations.[14] These results suggest that DMGs may include patients with independent malignant PC clones, as well as those that have clonally related malignant PCs resulting from CSR that has occurred at some point during disease pathogenesis. However, thorough molecular studies of the Ig HC and LC V region genes in patients with DMGs are extremely limited and published reports in this area are largely restricted to analysis of single patients. Thus, detailed analysis of the clonal relationship between these cell types is lacking. Indeed, Fair and Krueger[15] reviewed several early studies of individual DMG cases analyzed with the techniques available at the time, but concluded that more detailed and sensitive methods were needed to determine the clonal relationships (if any) and whether the clones are from a common precursor cell or distinct clones arising from different malignant PCs. In a more recent study, Bakkus *et al.*[11] used PCR to analyze an $IgA_1\kappa$/IgEκ DMG MM patient and found that the nucleotide sequence of the Ig HC V (*IGHV*) genes in the IgA and IgE clones were identical, including somatic mutations and the clone-identifying third HC complementarity determining region (HCDR3). They could find no evidence of pre-switched clonally related B cells, and therefore suggested that a malignant transformation most likely occurred in an IgA precursor cell at the post-switch stage allowing for further CSR to IgE. Because this analysis was restricted to a single patient, it currently remains unknown how frequently LC-matched DMGs indeed reflect clonal disorders that include class switch variants.

In this study, therefore, our goal was to exploit our extensive tissue bank of MGs and examine a larger cohort of HC-dissimilar/

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Received 27 February 2013; accepted 8 March 2013

LC-matched DMGs for clonal relationships. Using molecular and immunofluorescence (IF) methods, we present evidence suggesting that class switch variants in the DMGs may be more frequent than anticipated. Our observations provide further insight into the clonal origins of DMG and possible mechanisms of disease progression.

MATERIALS AND METHODS

Ethics statement

Mayo Clinic Institutional Review Board approval was granted to the Mayo Clinic Dysproteinemia Cell Bank for collection of BM from Mayo Clinic patients with malignant PC disorders. A BM specimen was collected from the iliac crest only after the patients provided written informed consent in accordance with the Declaration of Helsinki. Review of the clinical records identified a cohort of 14 MG patients for whom cryopreserved cells were available for analysis with a hematological diagnosis, and for which immunofixation electrophoresis[16] showed more than one HC of different isotypes and a monoclonal LC. Patients with these criteria that had not undergone transplant or other current treatment were considered for further study.

Cell isolation

BM mononuclear cells (BM MNCs) obtained from donors were isolated by Ficoll Paque (GE Healthcare, Piscataway, NJ, USA) density centrifugation or by ACK lysis (Life Technologies, Grand Island, NY, USA). For most patients, PCs were enriched by magnetic bead sorting using Human CD138-Positive Selection kits (StemCell Technologies, Vancouver, BC, Canada) and a Robosep instrument (StemCell Technologies). The purity of the PC samples was $>98\%$ as assessed by morphology.

RNA isolation, cDNA synthesis, Ig HC/LC V region PCR and sequence analysis

RNA was isolated by the TRIzol method (Life Technologies) and 2 μg of RNA was converted to cDNA using the GE Healthcare First Strand Synthesis kit. To determine the *IGHV*, Ig HC diversity (*IGHD*), and Ig HC joining (*IGHJ*) gene usage, 2 μl of cDNA was amplified using the Qiagen HotStarTaq MasterMix kit (Qiagen, Valencia, CA, USA) in a multiplex PCR reaction using 0.5 μM of each of seven sense primers, representing the seven IGHV families in conjunction with 0.5 μM of antisense primer to either the IgG, IgA or IgM constant region.[17,18] An additional reaction for β-actin was included for each cDNA. For most patient samples, individual PCR reactions were also performed using IGHV family-specific primers to detect any secondary clones and to ensure that the resulting products were not a result of PCR recombination events (hybrids).[19]

To determine Ig LC V region gene usage, individual PCR reactions for each Ig LC family using 0.5 μM of the sense primer in conjunction with 0.5 μM antisense primer to the κ or λ constant region were performed.[20] Amplification was carried out in a Perkin Elmer 9700 thermocycler (Perkin Elmer, Waltham, MA, USA) using the following conditions: 95 °C for 15 min; 35 cycles of 95 °C for 30 s, 60 °C for 60 s, 72 °C for 60 s and a final cycle of 72 °C for 10 min. Amplified products were visualized on a 1.5% agarose TAE gel with ethidium bromide, excised and purified with the Purelink Gel Extraction kit (Life Technologies). Purified products were directly sequenced on an ABI PRISM 3730 × l DNA Analyzer (Applied Biosystems/Life Technologies, Grand Island, NY, USA). Resulting sequences were aligned to germline (GL) Ig HC and LC V region gene sequences using ImMunoGeneTics Information (IMGT) System reference sets and IMGT/V-Quest software (http://imgt.cines.fr).[21]

Immunofluorescence

Total BM MNCs were collected onto glass slides using a Cytospin 2 (Thermo Shandon, Waltham, MA, USA) and stored at −20 °C until needed. For cytoplasmic Ig staining, the slides were fixed in 95% ethanol for 5 min, incubated in PBS + 0.1% Tween 80 (Sigma-Aldrich; St Louis, MO, USA) for 1 min and then rinsed in PBS alone. For staining, antibodies were diluted 1:100 or 1:200 in antibody diluent with background reducing components (Dako, Carpinteria, CA, USA). The following antibodies that were used in combination are as follows: anti-IgM FITC, anti-IgA TRITC, anti-λ FITC (all from Southern Biotech, Birmingham, AL, USA), anti-κ AMCA (Vector Labs, Burlingame, CA, USA) and an anti-IgG antibody (Southern Biotech) that was labeled with CF350 using a Sigma Mix-n-Stain kit. Slides were incubated with appropriate antibodies at 37 °C for 45 min under humidified conditions. After incubation, the slides were rinsed in PBS + 0.1% Tween 80 for 5 min, rinsed in PBS for 3 min, and then air dried. A coverslip was mounted using Vectashield (Vector Labs) mounting medium with or without propidium iodide depending upon the combination of antibodies used. Slides were visualized using an Olympic Provus AX79 microscope (Olympus, Center Valley, PA, USA), equipped with necessary fluorescent filters, and images were acquired with an Olympus DP71 digital camera with Olympus DP Manager software.

RESULTS

DMG patient cohort

Review of clinical data for patient samples in our MG tissue bank identified 14 patients with DMGs characterized by two HCs and a single monoclonal LC upon immunofixation electrophoresis (example shown in Supplementary Figure 1). Importantly, none of these patients had undergone prior transplant or had received treatment within 6 months of BM collection. Of the 14 patients, nine were diagnosed with MM or its precursor disease MGUS, three with AL, one with WM and one with WM/MGUS (Table 1). The percent BM PCs ranged from 2 to 74%, with 9/14 patients having $\geqslant 10\%$ PCs and the PC labeling index ranged from 0 to 7.7% (when data was available). The M-protein (M-spike) for IgA,

Table 1. Characteristics of DMG patient cohort

Patient	*Diagnosis*	*Immunofixation*	*% PC*	*PCLI*	*IgA*[a]	*IgG*[a]	*IgM*[a]
1	Relapsed MM	IgGκ/IgAκ	50	1.8	1.4	3.3	—
2	Relapsed MM	IgGλ/IgAλ	34	2.7	2.0	0.5	—
3	MM Untxt	IgGκ/IgAκ	74	7.7	2.8	0.3	—
4	AL Txt	IgGλ/IgAλ	38	0.2	NQ	0.8	—
5	AL Untxt	IgGλ/IgAλ	5	0	NQ	0.3	—
6	MM Untxt	IgGκ/IgAκ	54	2.4	3.2	SV	—
7	Relapsed MM	IgGλ/IgAλ	11	1.4	NQ	NQ	—
8	MGUS	IgGλ/IgMλ	2	ND	—	2.7	SV
9	WM/MGUS Txt	IgGκ/IgMκ	12	0.4	—	3.0	3.1
10	AL Untxt	IgGλ/IgAλ	10	0.4	0.4	1.0	—
11	Relapsed MM	IgGλ/IgAλ	13	4.2	0.5	SV	—
12	WM	IgGκ/IgMκ	7	2.8	—	0.2	2.1
13	MGUS	IgGλ/IgMλ	5	0	—	0.2	1.6
14	MGUS	IgGκ/IgMκ	2	0	—	0.9	SV

Abbreviations: AL, primary light chain amyloidosis; Ig, immunoglobulin; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; ND, not determined; NQ, the M-spike was present but too small to be quantified; PC, plasma cells; PCLI, plasma cell labeling index; SV, the M-spikes were recorded as a single value; Txt, treated; Untxt, untreated; WM, Waldenstrom's macroglobulinemia. [a]M-protein immunoglobulin on immunofixation (g/dl).

IgG or IgM ranged from 0.2 to 3.2 g/dl when able to quantify. In three cases, the M-spikes were evident, but one or both were too small to quantify, and in four others, the two M-spikes was recorded as a single value with no distinction between the two Igs.

Ig HC V region gene PCR and isotype variant comparisons in DMG patients

The Ig HC V region gene expression was determined by multiplex PCR with sense primers to the seven *IGHV* gene families in conjunction with antisense primers specific for IgA, IgG or IgM isotypes (Supplementary Figure 2). Some DMG samples were also amplified using individual PCR reactions for each of the seven IGHV families. In 13 of the 14 DMG samples, a rearranged Ig HC V region gene was amplified using antisense primers to the two HCs identified by immunofixation (Table 2). One sample (patient 5) did not result in a clonal PCR transcript using either a multiplex or individual *IGHV* family-specific PCR reactions for one of the HCs determined by immunofixation. Of the 13 DMG patients with amplified Ig HC V region genes using antisense primers to two different HC isotypes, four patients (patients 9, 12, 13, 14) had two or more distinct *IGHV* genes and different HCDR3s for each HC amplified. This analysis suggests that the DMGs in these patients most likely reflect the presence of two or more divergent malignant (or normal) PCs that are not clonally related at least at the level of detection by Ig HC V region PCR (Table 2).

Of great interest, molecular analysis revealed that the IgG and IgA transcripts from 9/14 (64%) DMG samples used the same *IGHV* gene as well as the same *IGHD* and *IGHJ* genes when aligned to GL reference sequences using IMGT/V-Quest (Table 2). In 7/9 patients, the entire IGHV region and HCDR3 (including deviations from the GL sequence) were identical at the nucleotide and amino acid (AA) levels, suggesting a clonal relationship despite different HC usage. Patient 2 is shown as an example of this pattern of identity in Figure 1a. In the remaining two patients (patients 6 and 11), although the Ig HC V region sequences were not identical, the IgG and IgA transcripts were highly homologous. Patient 11 had an identical HCDR3 at both the nucleotide and AA levels in the IgG

Table 2. *IGHV*, *IGHD*, and *IGHJ* gene usage for DMG isotypes

Patient	*Ig*	*IGHV*	*IGHD*	*IGHJ*	*% Mut*[a]	*HCDR3*
1	IgG	1–46	1–26	J4*02	13.9	TFLGGGGDF
	IgA	1–46	1–26	J4*02	13.9	TFLGGGGDF
2	IgG	2–5	6–19	J5*02	8.3	AGTRLGMAVSGKEKSAWFDP
	IgA	2–5	6–19	J5*02	8.3	AGTRLGMAVSGKEKSAWFDP
3	IgG	4–39	5–5	J6*02	10	ASQSAYSSSLPLSV
	IgA	4–39	5–5	J6*02	10	ASQSAYSSSLPLSV
4	IgG	3–48	6–19	J6*03	7.1	ARDNGWSKSYYYFHMDV
	IgA	3–48	6–19	J6*03	7.1	ARDNGWSKSYYYFHMDV
5	IgG	3–73	2–21	J6*03	11	TVPMDYRAFDGYYYMDV
	IgA					No clonal sequence determined
6	IgG	3–48	2–15	J4*02	6.7	AREAP**V**IA**S**TPFD**S**
	IgA	3–48	2–15	J4*02	7.9	AREAP**I**IA**A**TPFD**Y**
7	IgG	4–39	6–6	J5*02	13.6	ARDGLTARALEVKNLFDP
	IgA	4–39	6–6	J5*02	13.6	ARDGLTARALEVKNLFDP
8	IgG	5–51	3–22	J5*02	0.8	ARRGRYDSSGYQT
	IgM	5–51	3–22	J5*02	0.8	ARRGRYDSSGYQT
9[b]	IgG	3–30	2–8	J5*02	11.7	ATGGFDS
	IgM	3–7	5–12	J4*02	10.8	VRLGSGYPDI
10	IgG	1–2	2–15	J2*01	4.7	ARDAGWGGLYWHFDL
	IgA	1–2	2–15	J2*01	4.7	ARDAGWGGLYWHFDL
11	IgG	3–23	2–8	J4*02	8.6	ASSLGVYSITGPFDI
	IgA	3–23	2–8	J4*02	10.0	ASSLGVYSITGPFDI
12[b]	IgG	4–31	3–22	J6*01	15.6	ARVVRPEAAVLVTFPLRRGGMDV
	IgM	4–61	4–23	J4*02	0.4	AREATVVPYFNY
13[b]	IgG	4–39	3–22	J4*02	5.8	ARHRSGYYDTSGYYQFDS
	IgM	3–7	6–19	J4*02	2.5	ASNMAVPGD
	IgM	4–61	6–13	J4*02	6.6	ARVGRQLVDS
14[b]	IgG	3-30-3	4–17	J4*02	6.3	ARDAGYDTLTGYYLALDYW
	IgM	3–7	2–8	J5*02	12.1	ARGNGGVL
	IgM	4-30-4	5–18	J5*02	3.3	AREPWGYGSGNI
	IgM	5–51	6–19	J4*02	5.2	ARSAFEGWRDGWYSVDY

Abbreviations: Ig, immunoglobulin; IGHV, Ig HC variable; IGHD, Ig HC diversity; IGHJ, Ig HC joining. Changes in HCDR3 amino acid sequence in Patient 6 are in bold. Patient numbers are identical to numbers shown in Table 1. [a]% Mut = percent mutation from GL sequence in the IGHV region. [b]True bi- or polyclonal.

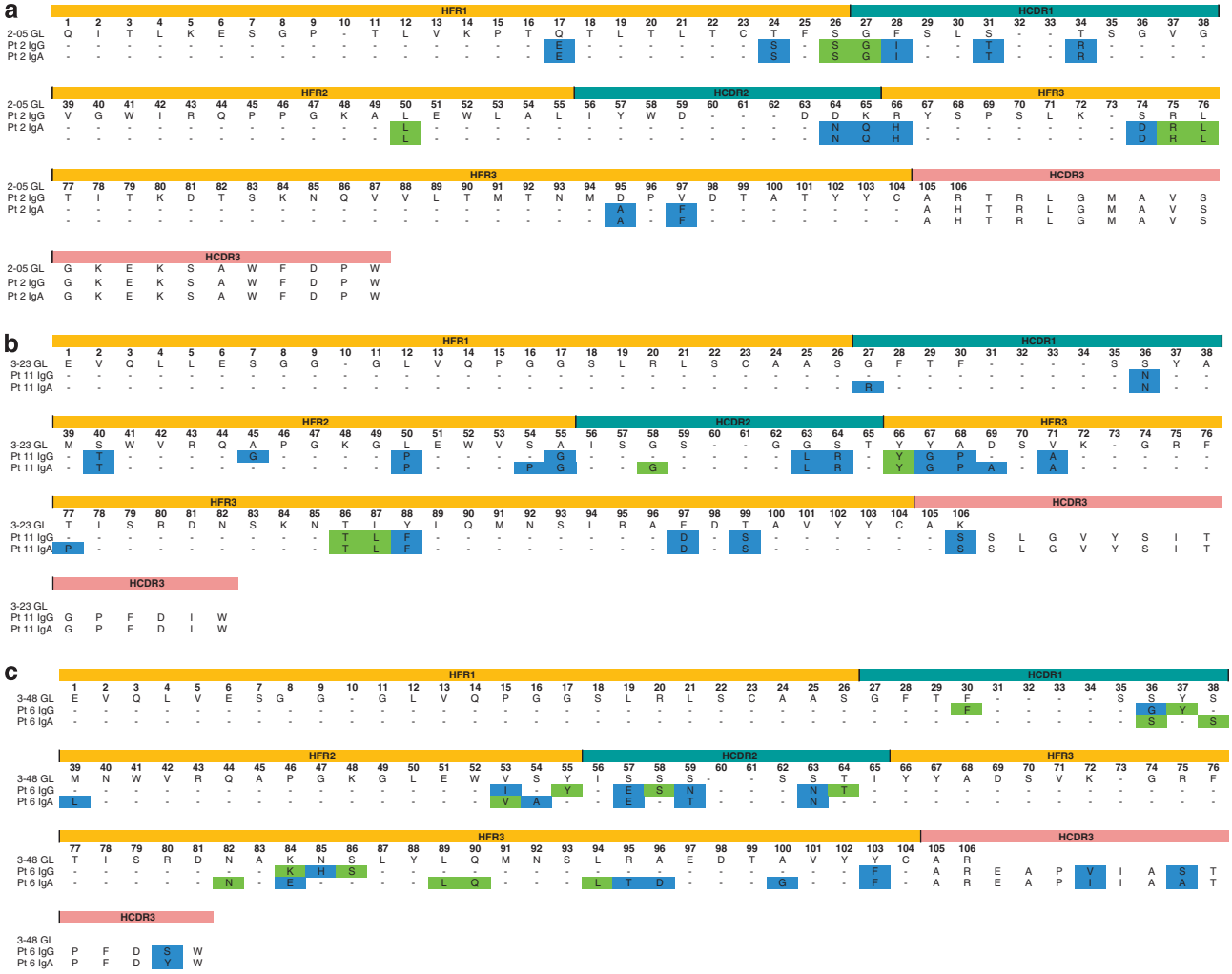

Figure 1. DMG sequence alignments. (**a**) Sequence alignment of patient 2 representing those patient samples that had identical sequences in the variable and HCDR3s in two different HC transcripts. (**b**) Sequence alignment of patient 11 showing shared and distinct somatic mutations in the Ig HC V region, but identical HCDR3s in IgG and IgA transcripts. (**c**) Sequence alignment of patient 6 showing shared and distinct somatic mutations, and highly homologous but not identical HCDR3s in IgG and IgA transcripts. Blue denotes replacement mutations resulting in AA change; green denotes silent mutations resulting in AA preservation; HFR, heavy chain framework regions; HCDR, heavy chain complementarity determining regions.

and IgA transcripts, and the majority of the V region mutations were shared. However, the IgA transcript included five additional somatic mutations, four of which resulted in an AA replacement, whereas the IgG transcript had one additional mutation resulting in an AA replacement (Figure 1b). In patient 6, the IgG and IgA Ig HC V region transcripts were also very similar. As shown in Figure 1c, the same *IGHV* gene was used in both transcripts (IGHV3-48). Similar to patient 11, the pattern and number of mutations in the variable region gene differed between the IgG and IgA transcripts (Figure 1c). However, unlike the identical HCDR3s observed in patient 11, the HCDR3s in the IgG and IgA transcripts of patient 6 were not identical but remarkably homologous. Specifically, both transcripts: (1) used the same D (*IGHD2-15*01*) and the J region gene (*IGHJ4*02*); (2) have the same number and similar pattern of nontemplated (N) and palindromic (P) nucleotides and (3) share the same CDR3 AA length. Each of these features has a crucial role in defining the HCDR3 and clonality.[22–25] Thus, these transcripts may also be clonally related but may have diverged from each other as a result of exposure to antigen and transit through the GC.

Further PCR confirmation of identical Ig isotype transcripts in DMG patients

The multiplex PCR method for Ig HC V region analysis lends itself to possible PCR recombination events (hybrids), which may result from amplification of multiple but similar *IGHV* genes.[19] To strengthen the PCR results suggesting clonal relatedness, alternative PCR strategies were used. Specifically, patients 1 and 4 were further evaluated using a single-tube PCR reaction for each *IGHV* gene family in conjunction with IgG or IgA antisense primers. In patient 1, the IgA reactions resulted in 1 amplified product (IGHV 1) and the IgG reactions amplified two products (IGHV 1 and 7; Supplementary Figure 3). When aligned to GL reference sequences using IMGT/V-Quest, the three amplicons had identical homology to each other and to the product amplified in the multiplex reaction. In patient 4, both the IgG and the IgA reactions resulted in multiple amplification products. However, only one IgA and one IgG sequence was readable, and both were homologous to each other and the sequence generated by the multiplex PCR. Therefore, while normal PCs or a second malignant clone may be

Table 3. *LC* gene usage for κ (IGKV) and λ (IGLV) isotypes in DMG patients

Patient	*Clinical LC*	*IGKV*	*Mutation*	*IGLV*	*Mutation*
1[a]	κ	IGKV3-11	M	ND	NA
2[a]	λ	ND	NA	IGLV1-47	M
3[a]	κ	IGKV1-27	UM	IGLV4-69	M
4[a]	λ	Multiple	M/UM	Multiple	M/UM
5	λ	IGKV1-39	M	Multiple	M/UM
6[b]	κ	IGKV1-33	UM	ND	NA
7[a]	λ	IGKV2-28	UM	Multiple	M
8[a]	λ	IGKV4-1	UM	IGLV6-57	UM
9	κ	Multiple	M	ND	NA
10[a]	λ	ND	NA	IGLV2-23	M
11[b]	λ	ND	NA	IGLV3-25	M
12	κ	IGKV1-5	M	IGLV6-57	M
13	λ	Multiple	M/UM	Multiple	M
14	κ	IGKV1-16	M	ND	NA

Abbreviations: IGKV, Ig Kappa variable; IGLV, Ig lambda variable; LC, light chain; M, mutated (⩾2% deviation from germline (GL) sequence); ND, no readable sequence detected; NA, not applicable; UM, unmutated (<2% deviation from GL sequence); . [a]HC transcripts were identical for these patients. [b]HC transcripts were not identical, but clonally related for these patients.

present in these samples, they do not represent a clonal population that can be detected by Ig HC V region PCR. Moreover, the identical amplicons generated by two different methods supports the results as being authentic and not due to PCR hybrids.

To further support the PCR results, we also included a PCR to amplify *IGHV* genes using an Ig isotype not detected by immunofixation electrophoresis. Supplementary Figure 4 shows that in patients 1–6, who expressed IgG or IgA on immunofixation electrophoresis, a multiplex Ig HC V region PCR with IgM either yielded no amplification products (patients 1–3 and 5), or the amplified products did not result in a readable sequence (patients 4 and 6), indicating that a PCR hybrid with the prominent Ig HC V region and IgM constant region did not occur. Rather any amplicons represent nonspecific amplification or polyclonal background of normal PCs.

Ig LC region gene expression in DMG patients with different HCs

The Ig LC V region results were more complex, but nonetheless revealing. Of the nine patient samples using the same *IGHV* gene with two different HCs, we could clearly detect a single LC transcript corresponding to the LC detected by immunofixation in five of the patients, thereby providing further evidence that these transcripts are from clonally related cells (Table 3). Within this group were patients 6 and 11 that were highly homologous by HC analysis but not identical (Figures 1b and c), again further demonstrating a clonal link between the cells expressing those two transcripts. Patients 3, 4, 7 and 8 had identical transcripts with two different HCs, but we detected more than one LC. Patient 14 had multiple HCs, but only a single LC was identified.

Immunofluorescence of Ig HC and LC in DMGs

To further validate the presence of clonally related cells with different HCs, we utilized multi-color IF to stain BM MNCs for cytoplasmic HC and LC. Indeed, we found expression of more than one HC in all the cases stained simultaneously with anti-IgA TRITC, anti-IgM FITC and anti-IgG CF350. In Figure 2, BM PCs from patient 2 expressed IgG and IgA, but no IgM. Staining for LC with anti-κ AMCA and anti-λ FITC showed that when we detected a single LC by PCR, we found only a single LC isotype by IF (Figure 3a). Conversely, in cases where more than one LC was detected by PCR, we did find both Ig κ and λ expression by IF (Figure 3b).

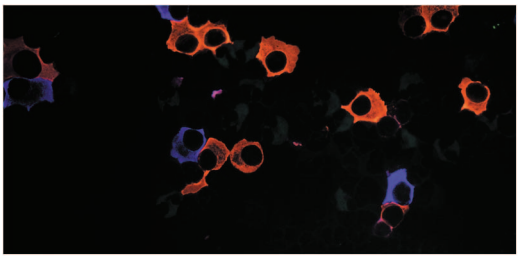

Figure 2. HC staining of DMG patient. BM MNCs from patient 2 were collected onto glass slides and stained with anti-IgM FITC (green), anti-IgA TRITC (red) and anti-IgG labeled with CF350 (blue). The image is over saturated for the FITC channel to show that no IgM FITC-labeled PCs were found. (× 60 magnification).

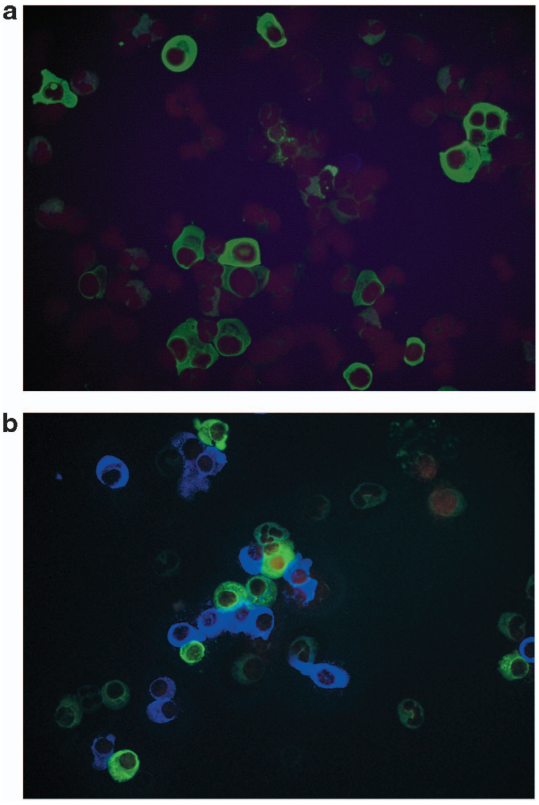

Figure 3. LC staining of DMG patients. (**a**) BM MNCs from patient 2 were collected onto glass slides and stained with anti-κ AMCA (blue), anti-λ FITC (green) and counterstained with PI (red). A single-λ LC was found by PCR in patient 2. (**b**) BM MNCs from patient 4 were stained as in **a**. Both κ and λ LCs were found by PCR in patient 4. (× 60 magnification).

DISCUSSION

The presence of two or more M-proteins in a MG is an infrequent occurrence. However, the existence of such a phenomenon affords an opportunity to examine DMG disease pathogenesis and possible origins of the two clones. Although DMGs may be present in different combinations with respect to HCs and LCs, in this study we chose to focus on those DMGs that exhibited HC-dissimilar/LC-matched M-proteins. Given that there is no known mechanism for LC switch, HC-dissimilar/LC-matched DMGs have a greater probability of being clonally related than DMGs with dissimilar LCs, hence the rationale for our focus on this subtype of DMGs. Of importance, we selected only those patients that had

not undergone stem cell transplant to eliminate oligoclonality that can arise after such a procedure.[26,27] Using a sizable cohort in this disease context, we observed that the HC-dissimilar M-proteins in 9/14 DMG patients were encoded by the same *IGHV*, *IGHD* and *IGHJ* genes, suggesting a clonal relationship (Table 1). Of those nine patients, seven had identical Ig HC V region nucleotide sequences (and thus AA sequences), thereby confirming the clonal relatedness of the two M-proteins (Figure 1a). Early studies of DMGs that looked at clonality by AA identity with techniques of the day were limited to a single patient.[28–32] Even recent studies of DMGs that included Ig HC V region PCR molecular analysis are rare and include only a single patient case study.[11,33] Our study is the first to look at a larger cohort of DMG patients with detailed molecular analysis, and we have found that more than half of these DMGs are clonally related. However, two of our 14 DMGS were WM, a MG in which the PCs always secrete IgM.[34] In both cases of WM, the IgG and IgM HC isotypes were not clonally related and served somewhat as an internal control for the detection of nonrelated clones. Therefore, we demonstrate that 9/12 non-WM DMGs are clonally related. Indeed, the low incidence of the MG MM, in general, suggests that malignant transformation of two or more B cells resulting in two unrelated M-proteins in the same MM patient would be very rare, and that instead, it would be more likely that two M-proteins within the same MG patient would be clonally related.[35]

Of interest, we also identified two patients whose M-proteins were encoded by the same *IGHV*, *IGHD* and *IGHJ* genes but with differences in the number and pattern of somatic mutations. Thus, patient 11 expressed two transcripts with the same *IGHV*, *IGHD* and *IGHJ* genes and therefore identical HCDR3s, but had differences in the Ig HC V region somatic mutations. Patient 6 is newly diagnosed and untreated with MM, where the IgA and IgG clones used the same *IGHV* gene but differed by a few mutations in the variable region. In addition, in this patient the HCDR3s in the two clones were highly homologous and were of the same length but could be distinguished by four nucleotide changes resulting in three different AAs (one resulted in a silent mutation). Despite these differences, these two cases are also highly suggestive of a clonal relationship between the two transcripts, and are particularly interesting because they suggest the possibilities that either some of the malignant cells have had additional transits through the GC resulting in CSR and further diversification; or, the malignant B-lineage cell acquired limited Ig HC V region somatic mutations and underwent CSR after the transforming event. Although we were not able to perform analysis on memory B cells to evaluate either of these scenarios, it is of interest that Bakkus *et al.*[11] were not able to detect clonally related B cells in their study of an $IgA_1\kappa$/IgEκ DMG MM. A third possibility is that the mutations in patients 6 and 11 may reflect random somatic mutations independent of GC reactions. This is of importance as MM has recently been shown to undergo considerable genetic evolution at the genome level[36] and intraclonal heterogeneity due to SHM[37] or DNA double-strand breaks.[38] Thus, the key question remains whether the molecular diversification occurs early in the disease or is acquired post-transformation and reflects genetic evolution of the tumor and disease progression.

Less clear was the LC analysis of the patient samples. Of interest, we detected a single LC gene in five patients using the same *IGHV*, *IGHD* and *IGHJ* genes in two HC transcripts. Included in this group were patients 6 and 11 noted above for having highly homologous but slightly different HC sequences. Thus, our detection of only one LC provides further evidence that the clones are related. In the remaining four patients with matched or highly homologous *IGHV* genes we detected more than one apparent rearranged Ig LC V region. We speculate that this may reflect an increased number of normal PCs in these patient samples. In addition, of these four samples, two had $\leqslant$20% PCs, suggesting a low abundance of clonal cells for LC amplification. Finally, we have some evidence that the LC PCR is more sensitive in detecting transcripts from normal PCs or nonexpressed LC transcripts (RCT and DFJ, unpublished observations).

It remains possible that the PCs in these DMG patients expressed both M-proteins as a result of long nuclear transcripts, a feature described in both mouse and human B cells.[39,40] Alternatively, the PCs in these patients may be able to synthesize multiple isotypes as shown in studies of hairy cell leukemia.[41] However, by IF we were able to demonstrate that individual PCs only expressed a single HC in all samples (representative results shown in Figures 2 and 3), thus proving that individual PCs were not producing both M-proteins. Most importantly in those samples with only a single LC, we were able to find only a single corresponding LC by IF (patients 1, 2, 6, 10, 11 and 14).

In summary, we have shown clonally related HC-dissimilar/LC-matched DMGs occur more frequently than believed, and that the two M-proteins are produced from independent HC isotype switch variant PCs. We believe the frequent occurrence of clonal relatedness in this subset of MG patients with two or more M-proteins provides new insight into disease pathogenesis. To understand in greater detail the clinical significance of our observations, future studies of DMG patients should include long-term tracking of M-proteins to identify new DMG patients at the earliest stage possible, and then analysis of serial samples to determine if this state is transitory or if it truly defines a unique phenotype of MGs. In addition, parallel cytogenetic studies would uncover any genetic transformational events that may provoke the manifestation of two M-proteins, as well as determining if the two clonally related class switch variants exhibit similar biological properties. Careful molecular analysis may reveal if additional Ig Hc and/or LC V region mutations in highly similar transcripts are continually occurring or are a result of an initial deviation. Although a previous study did not find evidence of a clonally related memory B-cell component,[11] our studies provide the rationale for extensive single-cell analysis of more patients to uncover these possible precursors. Finally, it would be of interest to study DMGs post-treatment to see if both clones recur.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Ms Linda Tostrud for her assistance in collecting and verifying clinical information.

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