Molecular Characterization of Waldenstrom's Macroglobulinemia Reveals Frequent Occurrence of Two B-Cell Clones Having Distinct IgH VDJ Sequences

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Abstract

Purpose: Malignant B lineage cells in Waldenstrom's macroglobulinemia (WM) express a unique clonotypic IgM VDJ. The occurrence of biclonal B cells and their clonal relationships were characterized.

Experimental Design: Bone marrow and blood from 20 WM patients were analyzed for clonotypic VDJ sequences, clonal B-cell frequencies, and the complementary determining region 3 profile.

Results: Two different clonotypic VDJ sequences were identified in 4 of 20 WM. In two cases, partner clones had different VDJ rearrangements, with one clonotypic signature in bone marrow and a second in blood. For both cases, the bone marrow clone was hypermutated, whereas the blood clone was germ line or minimally mutated. In two other cases, partner clones shared a common VDJ rearrangement but had different patterns of somatic mutations. They lacked intraclonal diversity and were more abundant in bone marrow than in blood. VDJ mutation profiles suggested they arose from a common IgM progenitor. Single-cell analysis in one case indicated the partner clones were reciprocally expressed, following rules of allelic exclusion.

Conclusions: The existence of two B-cell clones having distinct VDJ sequences is common in WM, suggesting that frequent transformation events may occur. In two cases, the partner clones had distinct tissue distributions in either blood or bone marrow, were of different immunoglobulin isotypes, and in one case exhibited differential response to therapy. The contributions of each clone are unknown. Their presence suggests that WM may involve a background of molecular and cellular events leading to emergence of one or more malignant clones.

Waldenstrom's macroglobulinemia (WM) is a lymphoproliferative disorder characterized by pleomorphic B cells with IgM monoclonal gammopathy (1). Immunophenotypic analysis showed that 90% of WM cases express $CD19^+CD20^+CD5^-CD10^-CD23^-$ surface antigens (2). A higher incidence of CD5, CD10, and CD23 expression has also been reported (3, 4). Our previous studies of WM B cells showed that clonotypic B cells are $CD20^+$, surface IgM $(sIgM)^+$, $sIgD^+$ B cells, but expression of the CD27 memory marker was heterogeneous (5, 6). Clonotypic VDJ preferentially uses V_H3/J_H4 gene families, mostly hypermutated, lacks intraclonal heterogeneity, and does not undergo class switch recombination (5–11). Clonality of WM was confirmed at the single-cell level by the presence of the clonotypic VDJ sequence in individual bone marrow and blood B cells (5). However, a decreased frequency of circulating clonotypic WM cells during treatment has been shown to correlate well with reduced serum IgM levels, even when the aggregate B-cell population, as defined by CD20 positivity, remains elevated (5). This suggests that aggregate polyclonal B-cell abnormalities may accompany the monoclonal B-cell expansion characteristic of WM.

B-cell lymphomas, including WM, are believed to arise from monoclonal proliferation of neoplastic lymphocytes derived from a single transformed progenitor (12). In various lymphoproliferative disorders, this has been supported by the demonstration of light chain restricted tumor cell surface immunoglobulin, detection of monoclonal immunoglobulin gene rearrangements, and the presence of common cytogenetic abnormalities (12–14).The coexistence of two or more different B-cell clones has been occasionally reported in different types of B lymphoma, with variable clinical significance (15–17). Biclonality has been described in B-chronic lymphoproliferative disorders and WM in several studies. In B-chronic lymphoproliferative disorders, 4.8% of 477 patients exhibited biclonality or oligoclonality (17). The incidence is

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higher among patients with hairy cell leukemia, large-cell lymphoma, and atypical chronic lymphocytic leukemia. Chronic lymphocytic leukemia patients with two or more clones required early treatment when compared with cases having only one clone. Biclonal gammopathies characterized by the presence of two different M-proteins occur in 5% to 8% of patients with monoclonal gammopathy (18). Among these, 65% are considered biclonal gammopathy of undetermined significance (18). In multiple myeloma, biclonal gammopathy defined by analysis of serum proteins has a frequency of ~1% to 2% (19), whereas biclonality identified by DNA studies was shown to be ~7% (20).

Biclonality in WM is considered a rare entity, with only a few single case reports (21–24). These include one case with two IgM κ paraproteins having distinct biochemical and physicochemical properties (21); another case having IgM κ and IgM λ paraproteins confirmed by immunofixation, immunohistologic staining, flow analysis, and immunoglobulin gene rearrangement analysis (24); and a patient with IgM κ and IgG λ corresponding to the simultaneous occurrence of clinically manifest WM and multiple myeloma (22). In these reports, however, the origins and genealogic relationships of the two coexisting clones were not addressed. In a more recent study, two clonally related IgM-producing clones were found in a WM patient, postulated to result from clonal diversification occurring after the original transformation event (23).

In the cohort reported here, the incidence of biclonality in WM has been evaluated by analysis of the complementary determining region 3 (CDR3) expression profile, clonotypic VDJ sequence, and clonal B-cell frequency. Our results show that WM involving two clonotypic sequences are identified in 4 of 20 patients studied. In two cases, they are derived from distinct parent B cells having different VDJ rearrangements, and different tissue localization in bone marrow or blood. In two other cases, partner clones share identical VDJ rearrangements with mutational profiles suggestive of distantly related clones, and a preference for the bone marrow. These studies highlight the importance of detailed molecular analysis in WM.

Patients and Materials and Methods

Patients. A total of 20 WM patients were included in this study. Among these, 19 of them are from the Cross Cancer Institute and the University of Alberta Hospital (Edmonton, AB, Canada). A biclonal gammopathy patient (WM10) from Dana-Farber Cancer Institute (Boston, MA) is also included in the study. WM was identified based on consensus criteria established at the Second International Workshop on Waldenstrom's Macroglobulinemia (1). The study was approved by the Ethics Committee of each institute and informed consent was provided in accordance with the Declaration of Helsinki.

Molecular identification of clonotypic IgM VDJ sequences. Identification of clonotypic IgM VDJ sequences has been previously described (5). Clonotypic sequences were validated by amplification of patient-specific CDR2/CDR3 from bone marrow–sorted $CD20^+$ cells, using reverse transcription-PCR (RT-PCR). For 18 WM patients who had a single B-cell clone in their bone marrow, IgM VDJ sequences defined as clonotypic were shown to be expressed in 56 ± 9.2% of total bone marrow $CD20^+$ cells.

RT-PCR. RT-PCR has been previously described (5). Unless otherwise stated, PCR was run using Platinum *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada) at 94°C for 2 min, 30 cycles of amplification at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and final extension at 72°C for 7 min. PCR products were analyzed on 2% agarose gel electrophoresis.

Antibodies, cell sorting, and clonal frequency analysis. Flow cytometric analysis, sorting of $CD20^+$ cells, clonal frequency analysis by single-cell hemi-nested RT-PCR using patient-specific CDR2 and CDR3 primers, and related PCR primer sequences have been previously described (5). Clonal frequency is defined as the percentage of the total number of cells scoring as positive in β2 microglobulin amplification that also scored as positive in CDR2/CDR3 amplification.

DNA sequence analysis. DNA sequencing was done using Big Dye 1.1 reagent (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The sequencing reaction was run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using ABI Prism DNA Sequencing Analysis version 5.1 and Seqscape software version 2.1 for data analysis. Clonotypic variable region sequences were compared with the closest germ line sequence using the International ImMunoGeneTics database.[4] Base differences between the immunoglobulin genes sequenced and the corresponding germ line genes were scored as mutations. The name of the *V*- and *J*-gene segments, location of CDR and framework regions, and the numbering system are according to International ImMunoGeneTics system (25).

Analysis of CDR3 by capillary electrophoresis. The profile of CDR3 expression by populations of cells was analyzed by DNA fragment analysis, using RT-PCR with 5′-hexachloro-fluorescein phosphoramidite–labeled FR3 [sense 5′-CCGAGGACACGGC(T/C)(C/G)TGTATTACTG-3′] and J_Hc [antisense 5′-ACCTGAGGAGACGGTGACC(A/G)(G/T)(G/T)GT-3′] or C_H1 primers (antisense; Cμ, 5′-CCAATTCTCACAGGAGAC-3′; Cγ, 5′-GGGGAAGACCGATGGGCCCT-3′; Cα, 5′-GAGGCTCAGCGGGAAGACCTT-3′) following the PCR protocol described in the RT-PCR section. Various amounts of PCR products were mixed with formamide and size standard [GeneScan-500 (ROX), Applied Biosystems] and analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Data analysis was done using GeneMapper software version 3.5. CDR3 is defined at VDJ junction between V-CYS105 and J-TRP118 based on International ImMunoGeneTics numbering system (25).

Results

Typical WM is a monoclonal proliferation arising from a single progenitor. Monoclonality of tumor B cells could be readily identified in bone marrow and quite often in blood of WM patients by PCR using fluorescent labeled universal immunoglobulin primers that bind to FR3 and J_H regions. The length of these PCR products, which include the CDR3 region, can be precisely measured by capillary electrophoresis (Fig. 1A), and the profile of fragment lengths is a rough measure of polyclonal diversity within the B-cell population analyzed. For the normal immunoglobulin repertoire, CDR3 fragment analysis typically results in a multiple peak profile, which reflects a diverse population of B-cell receptors. A normal distribution of CDR3 ranges between 24 and 72 bp. A single-peak profile generally results from a monoclonal proliferation, which in WM reflects expansion of a malignant B-cell clone. Representative results of this type of CDR3 analysis in normal and WM patients are shown in Fig. 1B. In 19 WM patients studied, a monoclonal peak corresponding to IgM is consistently identified in bone marrow, but the CDR3 profile in blood can be either single (monoclonal) or multiple-peak (diverse), depending on clonal B-cell frequency. In all patients studied, the fragment size of the product peak observed using DNA fragment analysis was

[4] http://imgt.cines.fr:8104/cgi-bin/IMGTdnap.jv

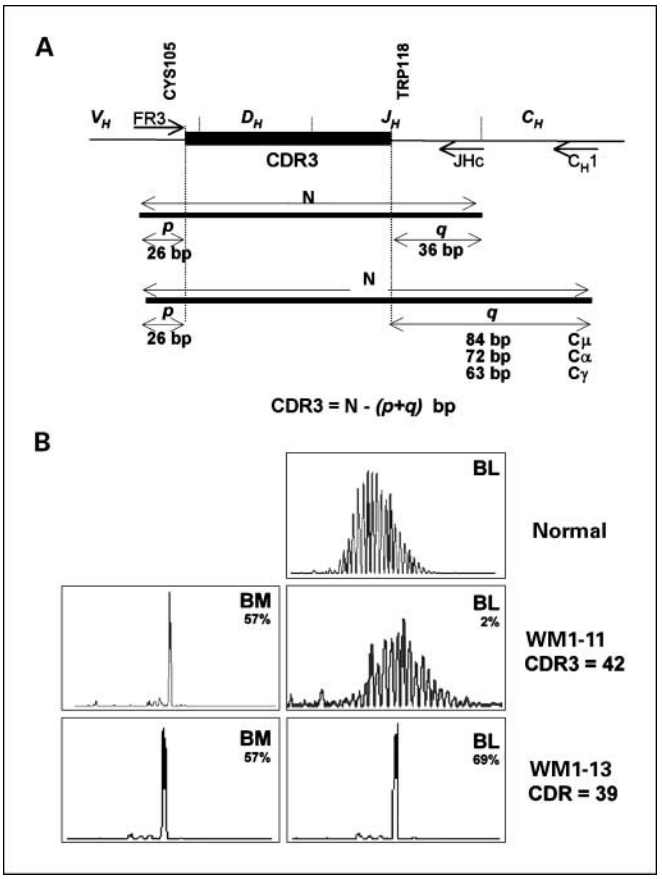

Fig. 1. Monoclonality of WM B cells can be shown by a CDR3 expression profile. *A,* length of CDR3. FR3/J_Hc or FR3/C_H1 fragments were amplified by PCR, and the length of each fragment was determined by capillary electrophoresis. CDR3 is defined as the region between CYS105 and TRP118, which can vary in length due to recombinatorial and junctional diversity. N, length of PCR product; p, length of FR3 primer; q, length from J-TRP118 to the end of PCR fragment. *B,* representative results of CDR3 profile in a normal donor and two WM patients. RT-PCR of unfractionated mononuclear cells using fluorescent-labeled FR3 and JHc primers are subjected to DNA fragment analysis. *X* axis, length of DNA fragment; *Y* axis, peak intensity. Each peak is separated by 3 bp (one amino acid). Percentage at the top right corner, clonal frequency within the population of $CD20^+$ cells as analyzed by single-cell hemi-nested RT-PCR using primers to the clone-specific CDR2/CDR3, with primers to β2 microglobulin as an internal control. BM, bone marrow; BL, blood.

identical to the length determined by the clonotypic VDJ sequence analysis.

Frequent incidence of biclonotypic VDJ sequences is observed in WM. In the present study, we analyzed 20 WM, four of whom carry two clonotypic VDJ sequences. In three of four cases, they were identified by molecular means from among 19 WM patients seen at the Cross Cancer Institute and the University of Alberta Hospital. An additional WM case seen at the Dana-Farber Cancer Institute exhibited biclonal gammopathy as measured by serum proteins. As might be predicted, molecular analysis reveals clonal expansions that would remain cryptic in conventional analyses of serum immunoglobulin. The characteristics of B-cell partner clones, including the clinical data of these four WM patients, are summarized in Table 1. The clinical data of biclonal patients are within the same range as monoclonal WM patients reported previously (7).

WM1-09: biclonal IgM/IgM with differing tissue localizations. Biclonality in WM1-09 is primarily identified by the CDR3 expression profile. Monoclonal IgM peaks (FR3/Cμ) amplified from parallel bone marrow and blood samples taken at the same point in time yielded two different lengths of CDR3, 39 and 66 bp, respectively (Fig. 2A, bone marrow and BL-2). Clonotypic VDJ sequence analysis confirmed that these represent two B-cell populations with clonally distinct rearrangements (Fig. 2B). The bone marrow dominant clone, 109.39, uses the V_H3/J_H4 gene family, is hypermutated (6.2%), and is expressed in 61% of bone marrow $CD20^+CD138^-$ cells (5). In addition, $CD20^+138^+$ cells comprise 4% of total bone marrow B cells. This rare subpopulation has been shown in our previous report to be exclusively antibody secretors of the 109.39 species (5). Clone 109.39 exhibits intraclonal homogeneity and is infrequently detected in the blood.

The second IgM clone, 109.66, uses V_H1/J_H6 gene family, retains the germ line sequence, has a long CDR3, and is predominant in blood. The usage of the V_H1 gene family has not been previously observed in our cohort of WM patients (20 of 21 clones used V_H3; 1 of 21 clones used V_H5) but has been reported by others (9, 11). Lack of mutation in the VDJ sequences has been shown in a small subset of our WM patients (5, 6). Clone 109.66 shares characteristics common to WM clones, including lack of intraclonal heterogeneity, lack of isotype switching (Table 1), and expression of membranous and secreted form of IgM (Fig. 2C). The clonal 109.66 transcript is detectable in the bone marrow using CDR2/CDR3 amplification (Fig. 2C), but its absolute amount is very small when compared with 109.39 in the CDR3 analysis (Fig. 2A, FR3/J_Hc, bone marrow). It is possible that 109.66 may be a B-cell chronic lymphocytic leukemia clone, as VH1-69 usage is frequently observed in unmutated B-cell chronic lymphocytic leukemia cases (26). This was inconsistent with the clinical data and the peripheral blood B-cell count; however, it could not be completely ruled out. Longitudinal studies of WM1-09 indicated that clone 109.66 predominated in the circulation and despite therapy persisted over a 17-month period (Fig. 2A, FR3/JHc, BL-2, at relapse and matched with bone marrow; BL-4, after a cycle of chlorambucil, then treatment discontinued; BL-7, another relapse). Single-cell analysis of sample BL-4 showed that clone 109.66 was expressed in 13% of blood B cells (Fig. 2D), confirming the CDR3 analysis and indicating that circulating clonal B cells remained frequent even after a cycle of chlorambucil treatment. In our previous report, for the cohort as a whole, the frequency of circulating clonal B cells dropped from 20% to 60% to as low as 1:4000 (0.025%) after successive treatments (5). In comparison, the efficacy of treatment for WM1-09 seems to be somewhat compromised. However, bone marrow samples were not taken at the same time point so the response of the partner clone 109.39 in the bone marrow is unknown. Longer-term monitoring of the B-cell clones in blood and bone marrow may facilitate understanding their relationship and their roles in the clinical manifestations and progression of WM. In a previous report (7), we showed that stimulation of bone marrow or blood cells with CD40 ligand plus IL-4 failed to induce class switch recombination in either of the IgM partner clones. This, together with the lack of intraclonal heterogeneity for the two partner clones, implies that both have properties expected of WM cells. The coexistence in parallel of one hypermutated and one germ line clone suggests that malignant transformation events may occur at different stages of B lineage differentiation in WM.

Table 1. Summary of WM patients and characteristics of biclonal B cells

Patient	Clinical status at time BM samples were taken	Shared VDJ rearrangement	VDJ gene segments	Clonal isotype (ID)	% Mutation	CDR3 nt (aa)	Distribution* (clonal frequency)		CD40L/IL-4 induced † clonal CSR
							BM	Blood	
WM1-09	Female, 58 y WBC, 3.6 × 10^9/L HB, 88 g/L HCT, 25% RBC, 2.67 × 10^{12}/L PLT, 260 × 10^9/L	No	*V_H3-15*02* *D_H3-10*01* *J_H4*02*	IgM (109.39)	6.2	39 (13)	++++ (61% $CD20^+138^-$; 92% $CD20^+138^+$)	+	No
	Alb, 35.2 g/L M peak, 4.5 g/dL Treatment, chlorambucil		*V_H1-69*01* *D_H2-2*03* *J_H6*02*	IgM (109.66)	0.0	66 (22)	+	++++ (BL-4:13% $CD20^+138^-$)	No
WM1-18	Male, 77 y WBC, 4.6 × 10^9/L HB, 134 g/L HCT, 40% RBC, 4.15 × 10^{12}/L PLT, 318 × 10^9/L M peak, 5.8 g/dL Alb, 40 g/L Treatment, rituximab, cyclophosphamide, vincristine, and prednisone	No	*V_H3-74*01* *D_H2-2*01* *J_H4*02*	IgM (118.51)	3.8	51 (17)	++++	+	Yes‡
			*V_H3-30*18* *D_H2-8*02* *J_H6*02*	IgA (118.60)	1.0	60 (20)	+	++++	ND
WM1-19	Male, 77 y WBC, 8.2 × 10^9/L HB, 98 g/L HCT, 0.29 L/L RBC, 3.18 × 10^{12}/L Alb, 25.76 g/L PLT, 323 × 10^9/L M peak, 5.3 g/dL Treatment, rituximab, cyclophosphamide, vincristine, and prednisone	Yes	*V_H3-30*02* *D_H2-15*01* *J_H4*02*	IgM	8.7	39 (13)	++++ (42% $CD20^+138^-$)	++	ND
				IgM	9.3	39 (13)	++++ (21% $CD20^+138^-$)	++	ND
WM10	Male, 65 y WBC, 4.9 × 10^9/L HCT, 36.3% PLT, 209 × 10^9/L M peaks, IgM, 0.24 g/dL IgG, 1.52 g/dL Treatment, none	Yes	*V_H3-74*01* *D_H2-15*01* *J_H6*03*	IgM	6.1	24 (8)	++++	±	ND
				IgG	9.7	24 (8)	++++	+	ND

Abbreviations: HB, hemoglobin; HCT, hematocrit; PLT, platelet; Alb, albumin; aa, amino acid; ND, not done; CSR, class switch recombination.
*Relative distribution was graded based on the CDR3 peak profile and relative band intensity analyzed by bulk RT-PCR. Clonal frequency was analyzed by single-cell hemi-nested RT-PCR.
† Clonal class switch recombination was analyzed in WM1-09 BL-7 and WM1-18 bone marrow. BL-1 and has been reported elsewhere (7).
‡ Clonal class switch recombination was detected but clonal IgG B cells were very infrequent based on a requirement for 35 cycles of PCR to detect them (7).

WM1-18: biclonal IgM/IgA in two different anatomic sites. Monoclonal IgM and IgA peaks were identified in both bone marrow and blood of WM1-18 by FR3/Cμ and FR3/Cα analysis (Fig. 3A, bone marrow and BL-1). FR3/J_Hc analysis, which amplifies all heavy chain isotypes, gave us a better idea of the relative amounts of each clone in the two compartments: cells with a CDR3 fragment of 51 bp (IgM clone) predominate in the bone marrow, whereas cells with a CDR3 fragment of 60 bp predominate in the blood. Clonotypic VDJ-C_H1 sequence analysis of the IgM and IgA PCR fragments confirmed the existence of two B-cell clones with distinct VDJ rearrangements (Fig. 3B). Clonotypic IgM, designated 118.51, uses V_H3/J_H4 gene families, is hypermutated (3.8%), and has characteristics typical of WM B cells. Previous work showed that postswitch isotypes of clone 118.51 were detectable but very infrequent (7). Clonotypic IgA, designated 118.60, uses V_H3/J_H6 gene families (Fig. 3B) and has very few mutations (1%). The clinical effect of the circulating monoclonal IgA B cells is unknown.

Longitudinal studies of WM1-18 blood samples (Fig. 3A; Table 2) showed that during the course of treatment, the monoclonal IgA peak originally identified in bone marrow and BL-1 became undetectable (sample BL-4), suggesting that the

IgA clone was extensively depleted by combined treatment of rituximab, cyclophosphamide, vincristine, and prednisone. As a result, the repertoire of IgA returned to a multiple-peak CDR3 fragment profile, indicative of restored clonal diversity (Fig. 3A, BL-4, FR3/Cα). The M protein concentration in sample BL-4 when compared with the diagnostic sample BL-1 was decreased from 6.0 to 4.7 g/dL. However, molecular analysis showed that the monoclonal IgM peak (BL-4, FR3/Cμ) was retained and became more prominent in the blood, presumably due to the loss of the IgA clone (BL-4, FR3/J_HC), suggesting that the IgM clone was less susceptible to treatment. At the completion of the treatment (BL-9), the M protein concentration further dropped to 1.9 g/dL, and the CDR3 repertoire for IgM, IgA, IgG, and total immunoglobulin indicated diverse CDR3 fragment sizes and a return to polyclonal profiles. This shows that analysis of the CDR3 fragment profile is informative and provides a simple method to monitor B-cell populations as well as to track changes in the B-cell repertoire occurring in response to treatment and throughout the course of disease.

WM1-19: two IgM subclones sharing a common rearranged VDJ gene. Initial analysis of clonotypic IgM in WM1-19 using CDR3 expression profiling showed the single-peak profile predicted for a monoclonal proliferation of WM B cells (Fig. 4A). Detailed analysis of clonotypic VDJ sequences, however, indicated that the monoclonal peak is made up of two IgM subpopulations, both of which carry CDR3 segments of equal length (39 bp). Sequence analysis showed that both use V_H3 and J_H4 gene families and are hypermutated (9.3% and 8.7%; Fig. 4B). Sequence homology in the D region suggests that these two IgM sequences are derived from a single progenitor. Within 12 bp, they shared five common unmutated bases and three common point mutations. The rest of the four bases are mutated in one clone but remain germ line in another. Despite having a common VDJ rearrangement, each clone has its own distinct profile of V gene mutations, suggestive of early divergence. Both clones have in-frame rearrangement and exhibit intraclonal homogeneity. Using clone-specific CDR2/CDR3 amplification in a single B-cell hemi-nested RT-PCR, we analyzed $CD20^+$ cells from this patient to determine whether the two transcripts are coexpressed. Figure 4C shows that each clonotypic IgM sequence is independently expressed in individual B cells, indicating that two related but diversified IgM B-cell clonal expansions coexist in the bone marrow of this patient. The frequencies of these B cells are 42% and 21% of bone marrow B cells, respectively, a 2:1 ratio. Colocalization at high frequency in the bone marrow suggests their mutual contribution to paraproteinemia and disease development. Both clonal transcripts are also detectable in the blood but at lower

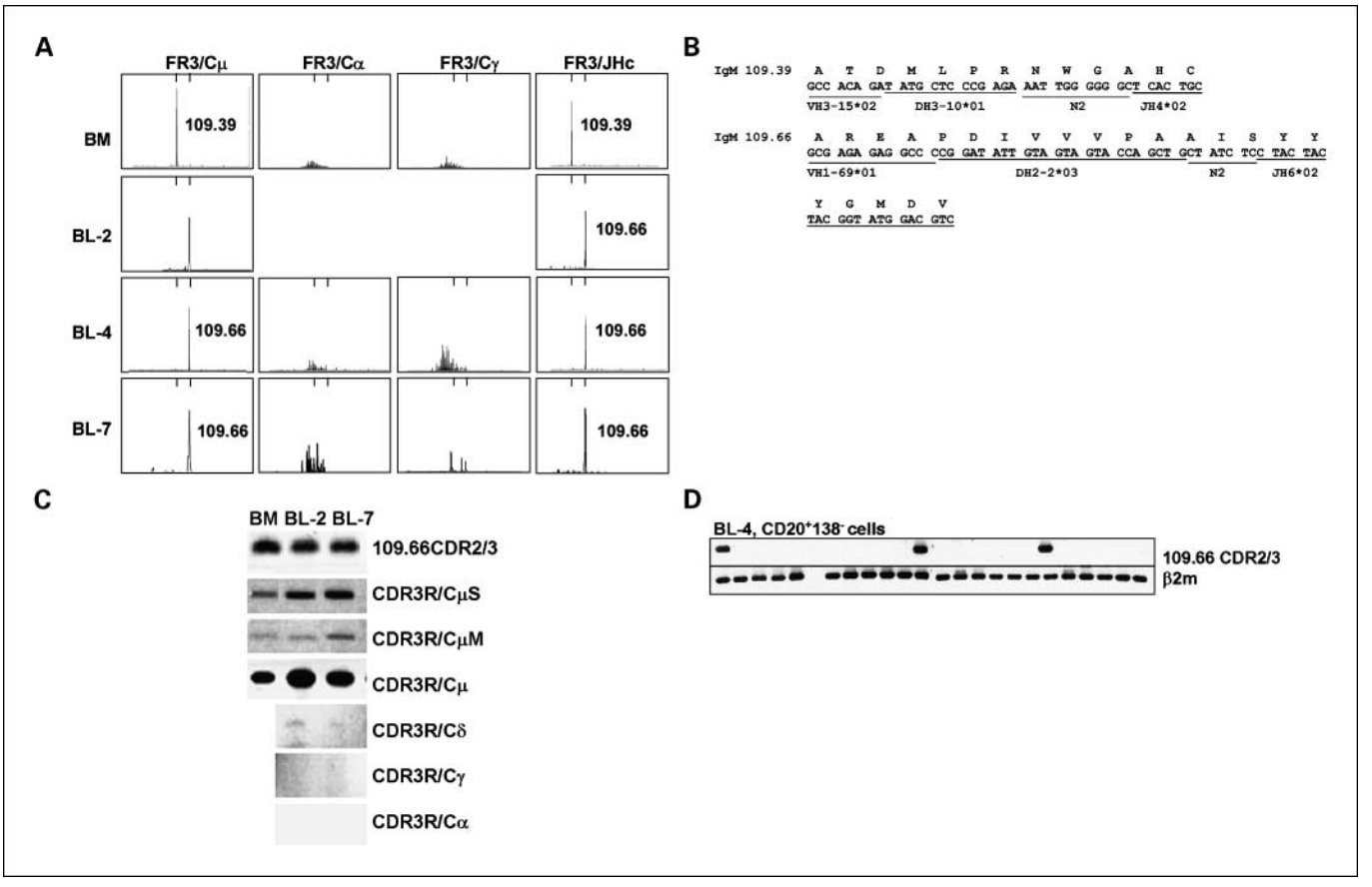

Fig. 2. Biclonal IgM B cells of WM1-09 carry different rearranged VDJ genes and are unequally distributed in bone marrow and blood. *A,* CDR3 analysis of bone marrow and blood showed monoclonal IgM peaks of different CDR3 lengths. Blood samples were collected at different time points: BL-2, at relapse, matching with bone marrow; BL-4, after completing the first cycle of chlorambucil treatment; BL-7, at another relapse. *B,* CDR3 sequences of both clones. *C,* bulk RT-PCR using various combination of clone 109.66–specific primers (CDR2, CDR3, or CDR3R) and immunoglobulin universal primers were analyzed. CμS, secreted IgM; CμM, membrane-bound IgM; Cμ, Cδ, Cγ, and Cα, CH1 primers for IgM, IgD, IgG, and IgA, respectively. *D,* clonal frequency of clone 109.66 was analyzed in BL-4 $CD20^+138^-$ cells by single-cell hemi-nested RT-PCR. Each column represents the PCR results of a single cell amplified by clone-specific CDR2/CDR3 primers or β2 microglobulin (*β2m*) as internal control.

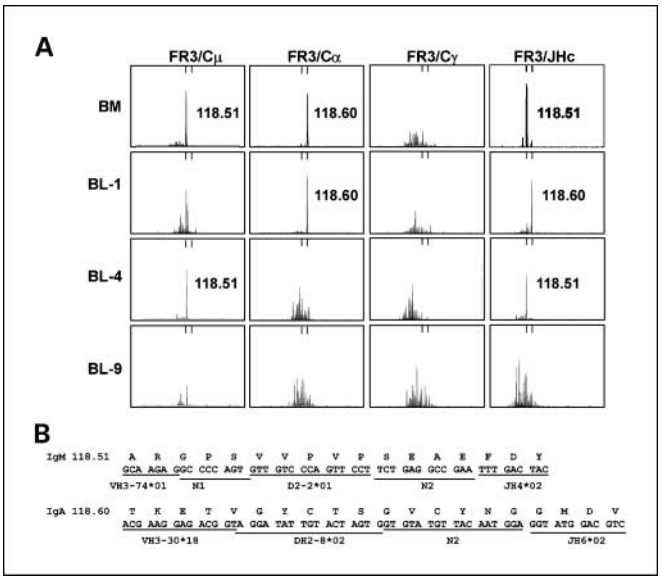

Fig. 3. Biclonal IgM/IgA B cells of WM1-18 are derived from different VDJ gene rearrangement events and are unequally distributed in bone marrow and blood. *A,* the CDR3 expression profile is determined in bone marrow and blood of WM1-18. Monoclonal IgM and IgA were shown to carry different lengths of CDR3. BL-1, BL-4, and BL-9, blood samples collected at different time points. *B,* the CDR3 sequences of IgM and IgA clones.

frequencies (Fig. 4D). This is the first reported case of dual clones in WM to be characterized by analysis of individual B cells.

WM10: IgM/IgG subclones sharing a common rearranged VDJ gene. A WM patient with biclonal IgM/IgG gammopathy was initially identified by serum protein electrophoresis and immunofixation. The biclonal relationship of these two paraproteins was determined by molecular studies of clonotypic immunoglobulin heavy chain genes. The CDR3 fragment profile as determined by FR3/C_H1 and FR3/J_Hc amplification revealed a monoclonal peak of IgM and one of IgG, which shared an equal length of the CDR3 segment (24 bp; Fig. 5A). Clonotypic VDJ sequence analysis showed that both clones used V_H3 and J_H6 gene families and shared common VDJ rearrangement (Fig. 5B). Within the 12 bp of the D region, they shared six common unmutated bases and four common point mutations. The rest of the two bases are mutated in IgG clone but remain germ line in IgM clone. In the V gene segment, the IgG clone displayed a higher mutation rate than did the IgM clone (9.7% versus 6.1%). Increased somatic hypermutation in IgG consists mostly of replacement mutations that are clustered within the CDR regions, strongly supporting a contention that the IgG clone has undergone affinity maturation. Despite a lower mutation rate in the IgM clone, there are 11 point mutations found in the IgM sequence that are not present in the IgG sequence. Thus, although these two clones are derived from a common precursor, a divergence seems to have occurred early in their development.

In an attempt to understand the distribution and the history of biclonal IgM/IgG B-cell development, we conducted PCR experiments using clonotypic CDR1 primers and CH1 primers (Cμ or Cγ), which together amplify the VDJ sequences of the IgM and the IgG clones. Our analysis revealed that both clonotypic IgM and IgG partner clones are abundant in bone marrow, but infrequent in blood (Fig. 5C, bone marrow and blood, *lanes 2* and *3*). Clonotypic IgG transcripts can be detected in blood if amplification is increased to 35 cycles; however, even at 35 cycles, the clonotypic IgM remains undetectable. We also looked for the postswitch counterpart to the IgM clone and found that only trace amounts of product could be detected at 35 cycles of amplification ($CDR1_{IgM}$/Cγ; Fig. 5C, bone marrow, *lane 4*), in agreement with our previous observation that the majority of WM B cells do not undergo isotype switching even when stimulated *in vitro* (7). It was also found that the preswitch counterpart to the IgG clone cannot be detected under these experimental conditions ($CDR1_{IgG}$/Cμ; Fig. 5C, bone marrow, *lane 1*), suggesting that the IgM precursor to the IgG clone is below the limits of detection. Clonal expansion within the population of IgG B cells does not seem to include its preswitch precursor. Taken together with the difference in their VDJ mutational profiles, our results suggest that transformation of the clonotypic IgG parent B cell may occur after the isotype switching and is distinct from the transformation event giving rise to the clonotypic IgM B cell in this WM patient.

Discussion

WM tumor cells are typically monoclonal IgM B cells defined by a specific VDJ rearrangement. In this study, we observed that some WM patients have two predominant clones: 2 of 20 WM patients are biclonal, defined by the presence of two distinct clonotypic VDJ sequences and two additional WM patients carry two related B-cell clones. Although biclonality has been identified in several independent WM case studies (21–24), to our knowledge this is the first report to establish a frequency of biclonality in WM defined in molecular terms that permit resolution of clonal populations not readily detectable using serum protein analysis. DNA fragment analysis by capillary electrophoresis enabled sensitive detection of the coexisting VDJ rearrangements, and sequence analysis was used to further characterize these rearrangements. In addition, this is the first study to use single-cell RT-PCR on flow cytometrically sorted B cells to measure the frequencies of the two clonal populations. This approach shows that individual B cells expressed only one VDJ rearrangement per cell, excluding the possibility that aberrant expression of biallelic IgH rearrangements occurs, thereby validating the distinct identity of the two clonal populations.

Table 2. Clinical data of WM1-18 corresponding to BL-1, BL-4, and BL-9

Sample	Date	M protein (g/dL)	Hb (g/L)	RBC ($\times10^{12}$/L)	WBC ($\times10$/L)	Platelet ($\times10^{9}$/L)	Total protein (g/L)
BM, BL-1	Oct 2004	6.0	130	4.15	5.7	240	107
BL-4	Mar 2005	4.7	127	4.11	4.4	291	101
BL-9	Sep 2005	1.9	151	5.14	5.4	387	82

Two biclonal cases reported here (WM1-09 and WM1-18) contain clonal subpopulations with CDR3 fragments of distinct size and DNA sequence. WM 1-18 has two clones expressing different heavy chain isotypes (IgM and IgA), whereas WM 1-09 has distinct clones that both express IgM. In Table 1 and previously (7), we have shown that WM can undergo class switching at a reduced frequency, which may explain the existence of the WM IgA clone in WM1-18 and the WM IgG clone in WM10 discussed below. For both WM1-09 and WM1-18, one clone is enriched in the bone marrow whereas the other clone is enriched in the blood. The bone marrow–enriched clones express IgM and have more mutations than the blood clones. The characteristics of the blood-localized clone in WM1-09, namely V_H1-69 usage and unmutated VDJ, leave open the possibility that it may be a chronic lymphocytic leukemia–like clone. However, the clinical data do support its classification as a WM case, and both unmutated VDJ and V_H1 usage has been reported previously in WM (26). Longitudinal studies of WM 1-18 (Fig. 3) and previous studies (5) suggested that the repertoire of blood B cells may recover some level of diversity after successive cycles of treatment, as evidenced by a polyclonal pattern of CDR3 fragment analysis, whereas the persistence of the monoclonal peak, as for WM1-09 (Fig. 2), likely reflects a clone that does not respond to treatment. However, the clinical significance of this observation is unknown. Although it is possible that those clones resident in the bone marrow may be of greater clinical relevance, this is not necessarily true because a circulating population may have considerably greater potential for dissemination of progeny through the body. The greater numbers of clonal cells in bone marrow compared with blood may also be indicative of clinical effect and disease pathology. In the end, however, the clone to which patients eventually succumb remains unidentified. The possibility exists that both bone marrow and blood-localized clones contribute to the pathology and the increasingly aggressive character of the disease as time progresses. Overall, the distinct nature of these partner clones emphasizes the importance of clonotypic VDJ sequence analysis and CDR3 fragment analysis in WM.

These aspects of the partner clones, distinct VDJ, distinct predominant site, and distinct response to treatment, suggest that the clones have either arisen independently, or, in addition to sharing a common set of mutations, have accumulated independent distinct mutations. In follicular lymphoma, biclonal B cells with distinct VDJ rearrangements but identical translocation breakpoints have been identified, suggesting that clones with distinct VDJ can be related by earlier events (27). In WM, this hypothesis is harder to address because genetic markers other than clonotypic VDJ, such as translocations, are rare events (28). Nevertheless, the partner clones in these WM

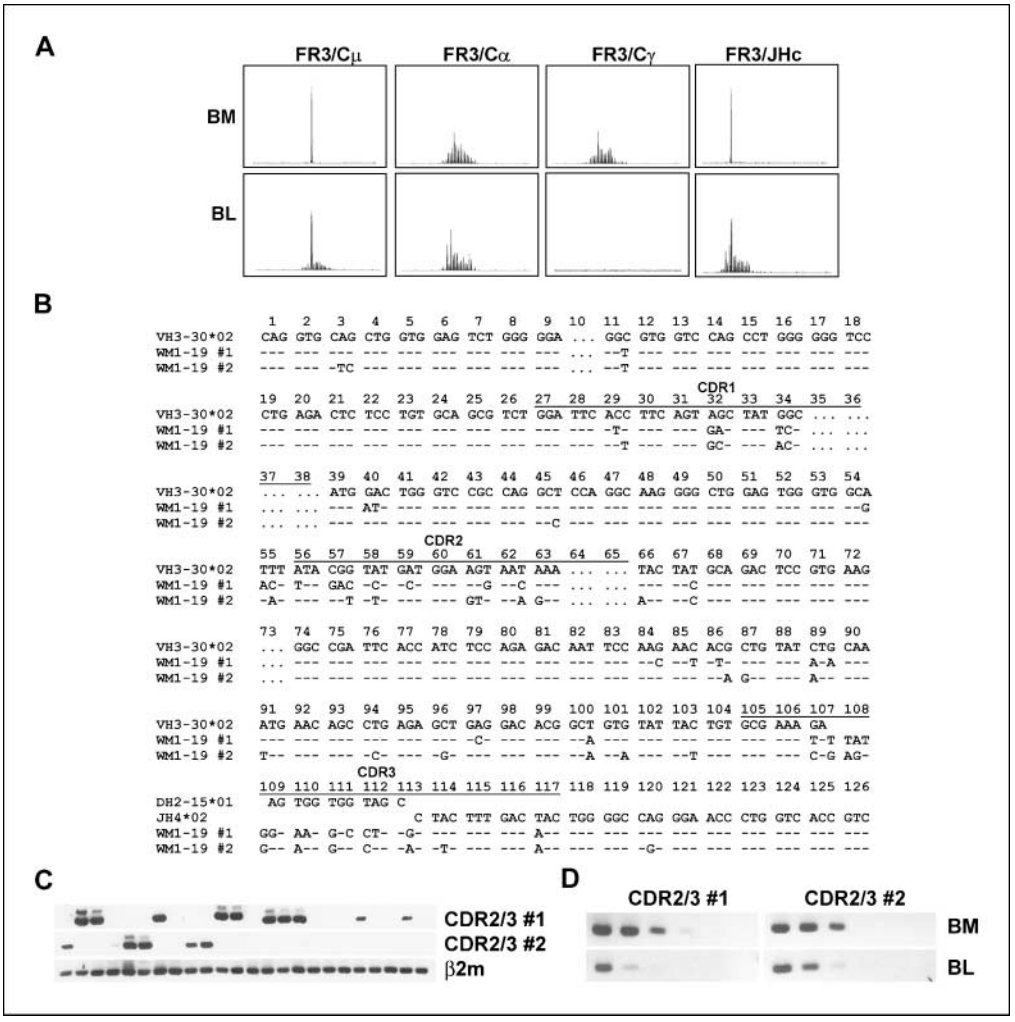

Fig. 4. Biclonal IgM B cells of WM1-19 are derived from a common B-cell progenitor with nonoverlapping expression in individual B cells. *A,* the CDR3 expression profiles of WM1-19 bone marrow and blood. Clonotypic VDJ sequences are compared with the closest germ line sequences as shown in (*B*). *C,* clonal distribution of each clone was determined by single-cell analysis in bone marrow B cells. Each column represents PCR results from a single B cell. *D,* relative clonal transcript distribution between the two IgM clones was also analyzed by semiquantitative RT-PCR. An equal amount of cDNA from each sample was serially diluted and amplified by CDR2/CDR3 primers specific to clone 1 or 2.

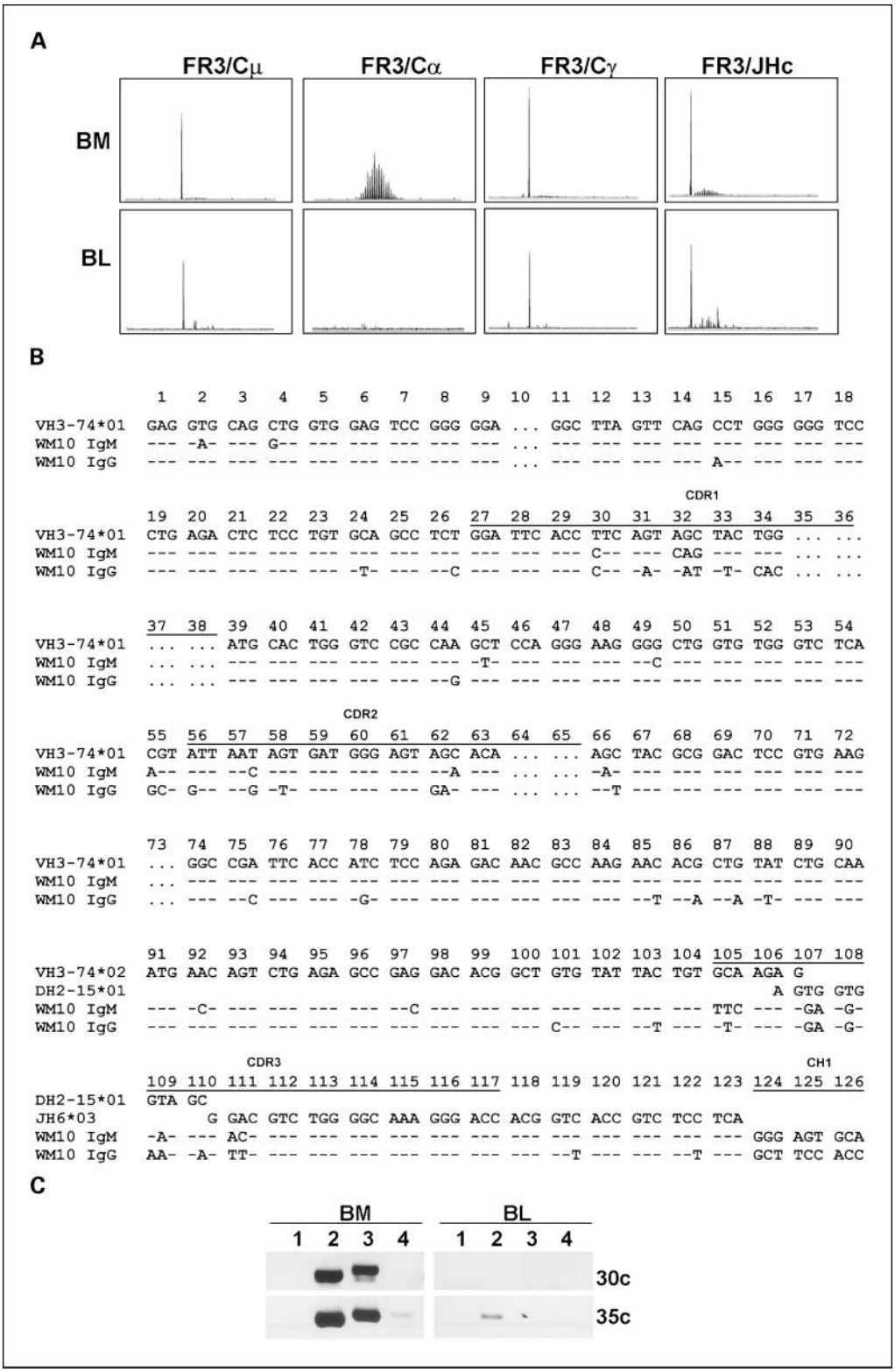

Fig. 5. Clonotypic IgM and IgG B cells in WM10 are derived from a common B-cell progenitor. *A,* the CDR3 expression profiles of bone marrow and blood. All monoclonal peaks share equal lengths of CDR3 (24 bp). *B,* clonotypic IgM and IgG sequences are compared with the closest germ line sequences. *C,* bulk RT-PCR using clone-specific CDR1 primers and CH1 primers were analyzed to determine each clonal isotype expression. An equal amount of cDNA was used in all reactions. Amplification was done for 30 or 35 cycles (*30c* and *35c*). Lane 1, $CDR1_{IgG}/C\mu$; lane 2, $CDR1_{IgG}/C\gamma$; lane 3, $CDR1_{IgM}/C\mu$; lane 4, $CDR1_{IgM}/C\gamma$. The expected size of PCR products: lanes 1 and 3, 318 bp; lanes 2 and 4, 297 bp.

cases have distinct phenotypes with potentially important clinical implications.

In the second set of cases, the partner clones are distantly related, and share a common tissue localization. In WM1-19 and WM10, the partner clones have identical VDJ rearrangements. In WM1-19, both clones express IgM, whereas in WM10, one clone expresses IgM whereas the other clone expresses IgG. The VDJ mutation profiles of the partner clones suggest that they are distantly, rather than closely, related (Figs. 4B and 5B). In WM10, the V region of the IgM clone has incurred several mutations, whereas the IgG clone shares some of these mutations and has incurred new mutations. Furthermore, the

IgM clone contains point mutations not present in the IgG clone, suggesting that the IgG clone is not simply a descendent of the IgM clone but that both clones are descendents from an earlier common IgM progenitor. Similar to WM10, the mutational profiles of the two related IgM clones of WM1-19 suggest an early divergence and an independent development of the two subclones. Despite these differences, the IgM clones of WM1-19 share similar mutation frequencies and a preference for the bone marrow. In WM10, the IgG clone has a higher mutation frequency than the IgM clone, possibly reflecting a continued exposure to somatic hypermutation. This is unusual in that our studies monitoring WM clonotypic VDJ sequences in bone marrow samples (taken 3 years apart in one patient and 9 years apart in another patient; data not shown), and several other reports (5–7, 10, 11), suggest that WM clones do not undergo diversification. Rather, clonal evolution is more commonly reported in follicular lymphoma (29, 30).

In conclusion, analysis of clonotypic VDJ sequences reveals a frequent incidence of WM cases exhibiting two B-cell clones. They may be derived from distinct B cells harboring different VDJ rearrangement or they may be clonally related. For those deriving from different VDJ rearrangement, it remains to be determined whether these populations share other relevant genetic alterations that may predispose them to transformation. The implication of the skewed distribution between two clonal B cells in some patients and their clinical manifestation remains to be understood. Biclonality and differential distribution in blood and bone marrow may contribute to the relatively frequent discordant response to therapy as measured by serum IgM and bone marrow involvement and/or adenopathy (31).[5] Overall, our results suggest that for the four WM cases evaluated here, the partner B-cell clones seem to have undergone separate transformation events. The extent to which each partner clone contributes to disease progression and death, whether separately or in synergy, is as yet unknown. This work suggests that WM may be characterized by multiple transformation events that usually lead to the emergence of one dominant clone but occasionally involve two clones. This raises the possibility that multiple cryptic clones may participate in the disease process as WM progresses. Future work will determine the mechanisms that contribute to multiple transformation events in WM and the significance of the partner clones.

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