Immunoglobulin Heavy- And Light-chain Repertoire in Splenic Marginal Zone Lymphoma

KOSTAS STAMATOPOULOS,[1] CHRYSOULA BELESSI,[2] THEODORA PAPADAKI,[3] EVANGELIA KALAGIAKOU,[2] NIKI STAVROYIANNI,[1] VASSILIKI DOUKA,[1] STAVROULA AFENDAKI,[3] RIAD SALOUM,[1] AIKATERINI PARASI,[4] DIMITRA ANAGNOSTOU,[3] NIKOLAOS LAOUTARIS,[2] ATHANASIOS FASSAS,[1] AND ACHILLES ANAGNOSTOPOULOS[1]

The considerable heterogeneity in morphology, immunophenotype, genotype, and clinical behavior of splenic marginal zone lymphoma (SMZL) hinders firm conclusions on the origin and differentiation stage of the neoplastic cells. Immunoglobulin (IG) gene usage and somatic mutation patterns were studied in a series of 43 SMZL cases. Clonal IGHV-D-J rearrangements were amplified in 42/43 cases (4 cases carried double rearrangements). Among IGHV-D-J rearrangements, IGHV3 and IGHV4 subgroup genes were used with the highest frequency. Nineteen IGHV genes were unmutated (>98% homology to the closest germline IGHV gene), whereas 27/46 were mutated. Clonal IGKV-J and IGLV-J gene rearrangements were amplified in 36/43 cases, including 31 IGKV-J (8/31 in lambda light-chain expressing cases) and 12 IGLV-J rearrangements; 9/31 IGKV and 6/12 IGLV sequences were mutated. IGKV-J and IGLV-J rearrangements used 14 IGKV and 9 IGLV different germline genes. Significant evidence for positive selection by classical T-dependent antigen was found in only 5/27 IGHV and 6/15 IGKV+IGLV mutated genes. These results provide evidence for the diverse B-cell subpopulations residing in the SMZ, which could represent physiologic equivalents of distinct SMZL subtypes. Furthermore, they indicate that in SMZL, as in other B cell malignancies, a complementarity imprint of antigen selection might be witnessed either by IGHV, IGKV, or IGLV rearranged sequences.

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INTRODUCTION

The marginal zone of the human spleen (SMZ) is a microanatomical site at the border of the white and red pulp, mainly comprising of B cells, T cells, and macrophages. The SMZ appears especially well equipped for rapid humoral immune responses to blood-borne antigens. There is considerable evidence suggesting that responses to thymus independent type 2 antigens (for example, bacterial capsular polysaccharides) are dependent on the normal function of the splenic marginal zone (1–3). Human SMZ B cells are a heterogeneous population: evidence for this heterogeneity was provided by mutation analysis of rearranged immunoglobulin heavy (IGH) chain genes of microdissected SMZ cells as well as tonsillar marginal zone B cells (equivalent to SMZ B cells) (4–6), which has demonstrated that some cells carried mutated IGHV genes while other cells carried unmutated genes. Importantly, however, the distribution of mutations was not always suggestive of selection by conventional T-dependent antigen.

Splenic marginal zone lymphoma (SMZL) is a distinct clinical and pathological entity characterized by massive splenomegaly and very frequent (almost universal) involvement of bone marrow and peripheral blood (7). SMZL cells display a consistent, albeit not specific, phenotypic profile: pan B antigens +/CD5–/CD10–/CD23–/CD43–/BCL-6–/cyclin D1–/surface IgM+ /surface IgD+/- (8). This profile helps in distinguishing SMZL from other small B-cell lymphomas secondarily involving the spleen. In contrast to other B-cell lymphomas, no consistent or unique genetic lesion has been associated with SMZL. Furthermore, there is abundant cytogenetic and molecular evidence pointing to SMZL genetic heterogeneity and the existence of distinct subentities: with/without plasma cell differentiation, IG and BCL-6 somatic mutations (9-15), allelic loss at chromosome 7q21-32 (16), and hepatitis C infection (17).

The considerable heterogeneity of SMZL hinders firm conclusions concerning histogenetic origin and differentiation stage of the neoplastic cells. Important information pertaining to these issues can be provided by analysis of clonogenic IG gene mutations, which generally helps to trace the developmental stage at which neoplastic transformation had occurred and assign the neoplastic cells to their corresponding normal counterparts. In SMZL, the available data derive exclusively from analysis of IGH genes (mostly in small groups of patients [9–10, 12–13,15, with only 2 comprehensive series; 11,14]). These studies have provided evidence for the significant heterogeneity of SMZL with respect to IGH mutation load.

[1]Hematology Department, G. Papanicolaou Hospital, Thessaloniki, Greece; [2]Hematology Department, Nikea General Hospital, Piraeus, Greece; [3]Hemopathology Department, Evagelismos Hospital, Athens, Greece; and [4]Pathology Department, Nikea General Hospital, Piraeus, Greece.

Structural and functional data indicate that the IGH plays a more important role than immunoglobulin kappa (IGK) or lambda (IGL) light chain in the recognition mechanism of the IG. The diversity of the structural repertoire for immunoglobulin heavy chain variable (IGHV) genes is provided both by H1 and H2 loops whereas for immunoglobulin heavy chain variable (IGKV) genes or immunoglobulin heavy chain variable (IGLV) genes only L1 varies (18). Nevertheless, complementarity-determining regions (CDRs) from both IGHV and IGKV or IGLV contribute to the formation of the antigen-binding cleft of a B-cell sIg molecule. Thus, it is worth examining the effects of somatic hypermutation on IGKV or IGLV region genes in affinity maturation of the antibody response. In this context, a complementary impact of antigen selection on IGHV, and/or IGKV or IGLV genes has been demonstrated previously in studies of multiple myeloma (19) and follicular lymphoma (20).

In the present study, we examined IG gene repertoire in a series of 43 SMZL cases, diagnosed after established WHO criteria (8). To avoid selection and/or classification uncertainties, only splenectomy specimens were evaluated. The analysis included both IG heavy- and light-chain genes, so as to gain a more accurate insight into the molecular organization of the IG repertoire in SMZL and assess the impact of selective (antigenic) influences.

MATERIALS AND METHODS

Tissue Samples

Forty-three cases of SMZL diagnosed since 1989 from which formalin-fixed, paraffin-embedded, and/or snap-frozen material was available were taken from the files of the Hematopathology Department of the Evangelismos Hospital, Athens, and the Pathology Department of the General Hospital, Piraeus. Our series included 18 males and 25 females with a median age of 68.6 y (range: 45 to 91). A monoclonal component was detected in the serum in 13/32 cases (40.6%), anti-HCV antibodies were positive in 3/31 cases, and 41/43 cases (95%) had stage IV disease. The study was approved by the local Ethics Review Committee of each institution.

All cases were evaluated morphologically (routine hematoxylin/eosin stain) and immunohistochemically. Antibodies against the following antigens were routinely applied: CD20, CD22, CD79a, CD5, CD23, CD43, CD10, CD3, BCL-6, BCL-2, CCND1 (cyclin D1), kappa, and lambda.

DNA Extraction and PCR Amplification of Clonal Heavy-/Light-chain Rearrangements

DNA was isolated from 10 μm sections by proteinase K digestion and phenol/chlorophorm extraction. PCR was performed as described in Marks et al. (21). Rearrangements in the IGK locus to the kappa-deleting element (KDE) were also analyzed. Both IGKV to KDE as well as IGKJ-C-INTRON (JKI) to KDE rearrangements were studied with appropriate primers (22).

DNA Sequence Analysis of Heavy- and Light-chain Variable Region Genes

PCR products were directly sequenced on the Applied Biosystems ABI 3730 sequencer, using version 1 "Big Dye" dye-terminator chemistry. The reaction was performed using (a) for antisense strand analysis, consensus oligonucleotides complementary to the IGHJ, IGKJ, and IGLJ genes; and (b) for sense strand analysis, clone-specific primers whose sequences were determined on the basis of antisense strand sequence results. Each sequencing reaction was repeated 3 times on polymerase chain reaction (PCR) products of 3 independent amplification reactions performed at different times.

Sequence data were analyzed using the international ImMunoGeneTics information system (23–27) (IMGT, http://imgt.cines.fr; initiator and coordinator: Marie-Paule Lefranc, Montpellier, France) and more particularly, the IMGT/V-QUEST (24) and IMGT/Junction Analysis (24) and the Basic Local Alignment Search Tool database (http://www.ncbi.nlm.nih.gov/igblast/ National Center for Biotechnology Information, Bethesda, MD, USA) to determine the respective homology of each gene with the closest germline counterpart. Assessment of the distribution of mutations (for cases with >2% deviation from the closest germline V gene) was performed by the multinomial model (28). To facilitate direct reference and comparison with other studies, in the case of IGKV and IGLV genes, we also provide in parentheses gene names after the Zachau (IGKV) and Kawasaki (IGLV) nomenclatures.

RESULTS

Histology and Immunohistochemistry

In most cases, low magnification examination of the spleen showed increased white pulp nodules with expansion of the marginal zone (MZ) and concomitant, although variable, neoplastic lymphocytic infiltration of the red pulp (cords and/or sinuses). In a few cases, the red pulp infiltration predominated with minimal tumor load in the white pulp. Finally, in 2 cases, there was complete effacement of both the white and the red pulp by the neoplastic lymphocytic infiltration. In most cases, the neoplastic white pulp nodules showed the "classical" biphasic appearance and were composed of an inner zone of small B lymphocytes surrounded by an outer zone of larger pale cells admixed with a variable amount of B immunoblasts, growing in an MZ pattern with disappearance of the mantle zone. Plasmacytic differentiation was observed in 5 cases.

Neoplastic lymphocytes exhibited a phenotypic profile, which is considered consistent, albeit not specific, with SMZL: CD20+ /CD22+ /CD79a+ /CD5– /CD23– /CD43– /CD10– /CD3– /BCL-6–/BCL-2–/ CCND1 (cyclin D1)– /kappa+ or lambda+. The use of a broad spectrum of monoclonal antibodies against the above-mentioned markers permitted the exclusion of other B-cell lymphoproliferative disorders mimicking SMZL in the spleen, such as B chronic lymphocytic leukemia (CLL), hairy cell leukemia, mantle cell lymphoma, follicular lymphoma, and lymphoplasmacytic lymphoma. All cases tested negative by PCR for either BCL-2/IGH or BCL-1/IGH chimeric genes.

IGHV, IGKV, and IGLV Gene Usage in Individual SMZL Clones

Clonal IGHV-D-J rearrangements were amplified by PCR in 42/43 cases. Four cases had 2 different rearrangements involving a different IGHV gene. The 46 amplified rearrangements used

Table 1. Immunoglobulin rearranged IGHV genes in SMZL cases of the present study

Gene	Number	Gene	Number
IGHV1-18	2	IGHV3-66	2
IGHV1-2	3	IGHV3-7	1
IGHV1-69	2	IGHV3-72	1
IGHV3-15	2	IGHV4-34	8
IGHV3-21	1	IGHV4-39	3
IGHV3-23	3	IGHV4-b	1
IGHV3-30	4	IGHV4-59	3
IGHV3-33	2	IGHV4-61	4
IGHV3-43	1	IGHV5-51	3

germline IGHV genes of the IGHV1 (7 cases), IGHV3 (17 cases), IGHV4 (19 cases), and IGHV5 subgroup (3 cases). Two out of 44 rearrangements for which complete CDR3 sequence data were available had a stop codon or an out-of-frame rearrangement and, thus, were unproductive; 1/2 unproductive sequences belonged to a case with double rearrangements. Among IGHV3 subgroup genes, no bias was observed in favor of a particular gene. In contrast, IGHV4-34 was detected in 8/19 rearrangements using IGHV4 genes (Table 1). Other frequent genes were IGHV4-61 (4/19 rearrangements), IGHV4-59, and IGHVH4-39 (3/19 rearrangements each).

Information on clonotypic light-chain expression was available in 35/43 cases: 20/43 expressed kappa chain, whereas 15/43 cases expressed lambda chain. Clonal IGKV-J rearrangements were amplified in 17/20 kappa-SMZL cases, 7/15 lambda-SMZL cases (one case with double rearrangements, each with a different IGKV gene; IGKV4-1 [B3], IGKV1-33/1D-33 [O18,O8]), and 6/8 cases for which information on light-chain expression was missing. Nine unproductive IGKV-J sequences were amplified in 1 kappa-SMZL case utilizing the IGKV2-29 (A18) gene with a stop codon instead of cysteine at position 104 in FR3-IMGT), 4/15 lambda cases (one case with a double rearrangement), and 3/7 cases for which information on light-chain expression was missing. The 31 IGKV-J rearrangements analyzed used 14 different germline IGKV genes (Table 2); IGKV4-1 (B3) was the most frequently used IGKV gene (8/31 IGKV sequences), always with a different CDR3 region.

IGKV-KDE rearrangements were amplified in 9/15 cases; assignment of IGKV gene was possible for 6/10 IGKV-KDE rearrangements and revealed usage of the following genes: IGKV1-16 (L1) [2 cases], IGKV2D-28 (A3) [2 cases], IGKV2-30 (A17), IGKV1D-16 (L15) [1 case each]. JKI-KDE rearrangements that would delete the IGKV-J rearrangement (if present) were detected in 6/9 cases analyzed. Taking IGKV-J, IGKV-KDE, and JKI-KDE rearrangements together, 13/15 SMZL cases with clonotypic lambda chain had at least one rearranged IGK locus.

Clonal IGLV-J sequences were amplified by the PCR in 12/15 cases expressing clonotypic lambda chain; one rearrangement was out-of-frame. Nine different germline IGLV genes were recognized in the twelve rearrangements analyzed (24). IGLV1 subgroup genes were used with the highest frequency (5/12 cases) (Table 2).

CDR3 in heavy- and light-chain rearrangements of individual SMZL clones

Heavy-chain genes. Analysis of the HCDR3 region was possible in 44/46 rearrangements and revealed that SMZL-HCDR3 composition was comparable to normal PB IgM+ B cells in length (median: 17 aminoacids, range: 8-35), number of N-nucleotides and 5′ and 3′ exonuclease activity at, respectively, the V and J genes. Similar to normal B cells (29), longer HCDR3 regions were observed in SMZL cases with unmutated sequences. The distribution of IGHJ genes was similar to that reported for normal B cells (30): IGHJ4 was the most frequent gene followed by IGHJ6 (23 and 11 sequences, respectively). Most cases used IGHD genes from the IGHD2, IGHD3, and IGHD6 subgroups (6, 19, and 8 sequences, respectively) (31),

Table 2. Immunoglobulin IGKV and IGLV rearranged genes in SMZL cases of the present study

IGKV gene	Number	IGLV gene	Number
IGKV4-1 (B3)[a]	8	IGLV3-1 (VL2-1)[b]	1
IGKV1-5 (L12, L12a)[a]	3	IGLV3-19 (VL2-13)[b]	1
IGKV1-8 (L9)[a]	1	IGLV3-21 (VL2-14)[b]	1
IGKV3-11 (L6, L6a)[a]	1	IGLV2-23 (VL1-7)[b]	1
IGKV1-12 (L5)[a]	1	IGLV1-40 (VL1-13)[b]	2
IGKV3-15 (L2)[a]	1	IGLV1-44 (VL1-16)[b]	1
IGKV1-17 (A30)[a]	1	IGLV5-45 (VL4-2)[b]	1
IGKV3-20 (A27, A27a)[a]	3	IGLV1-51 (VL1-19)[b]	2
IGKV2-28/ IGKV2D-28 (A19, A3)[a]	1	IGLV6-57 (VL1-22)[b]	2
IGKV2-29 (A18a, A18b)[a]	1		
IGKV2-30 (A17)[b]	2		
IGKV1-33IGKV1D-33 (O18, O18a, O8)[a]	3		
IGKV1-37/IGKV1D-37 (O14, O4)[a]	1		
IGKV1-39/IGKV1D-39 (O12, O12a, O2)[a]	4		

[a]Gene names in parentheses: Zachau nomenclature.
[b]Gene names in parentheses: Kawasaki nomenclature.

which are also frequently used by normal B cells (32). Similar to normal peripheral blood B cells (30) as well as tonsillar subepithelial B cells (6), the hydrophilic IGHD gene reading frame (RF) predominated in CDR3 sequences for which IGHD segment assignments could be made. Among unmutated sequences, a more even distribution of the hydrophilic and hydrophobic RFs was observed. No subgroups of cases with homologous HCDR3 sequences were identified.

Light-chain genes. Complete analysis of the CDR3 region was possible in 28/31 IGK and 12/12 IGL rearrangements. The KCDR3 composition of the analyzed SMZL sequences was comparable to normal PB IgM+ B cells in length (median: 9 aminoacids, range: 7-11), number of N-nucleotides, and 5′ and 3′ exonuclease activity at, respectively, the V and J genes. All possible IGKJ gene segments were recognized. Similar to normal peripheral blood IgM+ B cells (33), IGKJ2 was the most frequent gene (10 cases), followed in order by IGKJ4 (8 cases), IGKJ1 (6 cases), IGKJ3 (3 cases), and IGKJ5 (1 case).

LCDR3 had a median length of 11 aminoacids (range, 9 to 12); N-nucleotide insertion and 5′ and 3′ exonuclease activity at, respectively, the V and J genes, was similar to normal B cells. Similar to the normal repertoire (34–36), the IGLJ2/3 genes were used in most (9/12) IGLV-J rearrangements with complete LCDR3 sequence data.

Somatic Hypermutation Analysis

Twenty-seven IGHV-D-J rearrangements carried mutated IGHV genes (less than 98% homology with the closest germline gene), while the remainder (19/46) had unmutated IGHV genes. IGH-mutated rearrangements most often utilized IGHV3 subgroup genes (14/27 cases). A strong association with IGHV4 subgroup usage was identified in unmutated rearrangements (11/19 cases). Mutations were exclusively single nucleotide substitutions and localized mainly in CDR1, CDR2, and FR3-IMGT (23). Similar to normal peripheral blood B cells (37), follicular lymphoma, and multiple myeloma (38), a higher incidence of mutations in RGYW/WRCY motifs were observed in CDRs rather than in framework regions (FRs)

Among IGHV1 genes, replacement mutations were frequent at IMGT-CDR1 positions 32 (frequency of sequences carrying a replacement mutation at that position: 43%) and 33 (28%) and at IMGT-FR3 positions 87 and 101 (25% each). Nevertheless, mutations at the last 2 positions led to replacement by amino acids of similar charge and size. Among IGHV3 genes, replacement mutations were frequent at IMGT-CDR1 position 32 (70%), IMGT-FR2 position 55 (30%), IMGT-CDR2 positions 56 (30%) and 62 (42%), and at IMGT-FR3 positions 92 and 96 (32% each). Finally, among IGHV4 genes, replacement mutations were frequent at IMGT-FR2 positions 45 (40%) and 48 (30%; all mutations leading to replacement by aminoacids of similar charge and size), IMGT-CDR2 positions 59 (29%; in sharp contrast to IGHV1 and IGHV3 genes with 0% and 10% sequences mutated at that position) and 61 (36%), and IMGT-FR3 positions 78 (29%) and 92 (43%). Compared with IGHV1 and IGHV3 genes, IGHV4 genes had a lower incidence of replacement mutations in IMGT-CDR1. The invariant serine (S)-92 at IMGT-FR3 was mutated in 43/32/0% of IGHV4/3/1 sequences. Frequently mutated serines in IGHV4 genes were also S-59 and S-61 in IMGT-CDR2 (see above). In contrast, other invariant serines among IGHV4 genes were either rarely mutated (for example, S-29, S-31, S-32 in IMGT-CDR1) or never mutated at all (for example, S-16, S-20, S-26 in IMGT-FR1, S-70, S-74, S-79, S-83, S-88, and S-93 in IMGT-FR3). Positions with conserved properties (cysteines at IMGT positions 23 and 104, tryptophane-41, aliphatics at positions 21 and 89, amide-44, proline-46, glycine-47, basic-75, acidic-98 and tyrosine-102 [39]) as well as tryptophane-52, involved in the VH-VL domain interaction (36), were never found to carry a replacement mutation in the present series. Among IGKV + IGLV genes, only 1 replacement mutation was observed in one of the string of 5 glycines at positions 16, 47, 70, 78, and 84 (39). Mutation analysis after the multinomial distribution model disclosed statistically significant evidence for positive selection by antigen in 5/27 mutated IGHV genes.

In the case of IG light chains, 9/31 IGKV-J and 6/12 IGLV-J rearrangements carried mutated V genes. As in IGHV genes, mutation targeting to the RGYW/WRCY motifs was observed in CDRs rather than FRs. Statistically significant evidence for positive selection by antigen was obtained in 4/9 mutated IGKV and 1/6 mutated IGLV genes.

DISCUSSION

The expressed immunoglobulin repertoire is shaped by molecular processes (for example, cleavage efficiency of individual genes by RAG proteins, physical distance between recombining genes and pairing of heavy and light chains) as well as positive/negative selection by antigen and affinity maturation of immune responses (30,33–35,40–41). Our study confirms in a large cohort of strictly defined SMZL patients predominant rearrangements of IGHV3 and IGHV4 subgroup genes. Preferential rearrangement of IGHV3 subgroup genes and, to a lesser extent, IGHV4 subgroup genes has also been reported for microdissected normal SMZ B cells (5). The frequent usage of IGHV3 in the present series is similar to what has been observed among both CD5+ and CD5– IgM+ peripheral blood (PB) normal B cells (30) and reflects its germline complexity. Importantly, as in both CD5+ and CD5– IgM+ normal PB B cells (30), overrepresentation of the IGHV3 subgroup in the productive rearranged repertoire was noted only in cases with mutated IGHV-D-J rearrangements (14/27 rearrangements with < 98% homology). This implies that IGHV3 bias might result from positive selection of IGHV3-expressing B cells by antigen. Because no preference in IGHD or IGHJ expression was associated with the bias in the IGHV3 subgroup in general, it is likely that positive selective influences were related to the IGHV region and not to the antigen binding CDR3. A similar observation has been made for normal PB IgM+ B cells.

The high frequency of IGHV4 genes in the present series contrasts normal PB CD5– IgM+ B cells (30), where the frequency of the IGHV4 subgroup in productive rearrangements was found significantly diminished compared with that in nonproductive rearrangements, strongly implying that this subgroup was negatively selected in the expressed repertoire of CD5– B cells. This might be related to the fact that IGHV4 subgroup genes are

known to encode several autoantibodies (42). Similar to the IGHV repertoire of normal PB IgM+ B cells, in the present series a small number of IGHV4 subgroup genes were detected more frequently than expected, including IGHV4-59/IGHV4-61, IGHV4-39, and IGHV4-34. The IGHV4-34 gene predominated in an analysis of IGHV4+ cDNA clones from resting IgM only, resting IgD^{low} as well as activated tonsillar subepithelial (SE) B cells (6). In that analysis, IGHV4-61 and IGHV4-39 were also frequently found among activated SE B cells. As in both tonsillar SE B cells (6) and normal PB IgM+ cells (30), in our study the IGHV4-34 gene was found more frequently in unmutated sequences and was consistently underrepresented in mutated productive rearrangements.

The overrepresentation of IGHV4-34 in our series prompts speculations about the possible role of specific antigens in SMZL pathogenesis or evolution; similar arguments have been proposed by Algara et al. (11) to account for the overrepresentation of the IGHV1-2 gene in their series. In the analysis of microdissected MZ normal B cells by Dunn-Walters et al. (4), mutation distribution in IGHV4-34 (+) rearrangements was not suggestive of a "classical" hypermutation process in the context of affinity maturation within the germinal center. A similarly "unorthodox" replacement (R) mutation distribution was observed in our IGHV4-34 sequences as well, with a higher incidence of R mutations in FRs than in CDRs and a remarkable paucity of R mutations in CDR1. Finally, the bias in favor of individual genes, and in particular IGHV4-34, could reflect molecular events of recombination unaffected by subsequent selective influences dependent on IG expression: IGHV4-34 along with IGHV4-39, IGHV4-59, IGHV3-23, and IGHV3-30 predominated in an analysis of out-of-frame rearrangements in human pro-B cells, suggesting that their expression in peripheral B cells might be determined significantly by preferential rearrangement rather than selection (43). Furthermore, a recent study (44) showed that IGHV4-34 is 1 of only 5 out of the 39 functional IGHV genes with a heptamer/nonamer signal conforming to the CACAGTG/ACAAAAACC consensus (the others being IGHV3-9, IGHV3-43, IGHV4-39, and IGHV4-59) and has the highest cleavage efficiency by RAG proteins. In the aforementioned study, a striking similarity was noted between RAG cleavage data and the observed peripheral unselected repertoire; thus in vivo frequencies (at least for unselected rearrangements) may reflect how well individual IGHV genes are cleaved by the RAG complex (44).

The present study differs significantly with regard to IGHV gene usage from the 2 previous large studies on IGH genes in SMZL (11,14): if preferential use of certain IGHV genes is indicative of recognition of particular antigenic epitopes, then the observed differences may reflect different antigenic (environmental?) stimuli that could have played a role in lymphomatogenesis (45). In this context, it is perhaps relevant that in CLL the IGHV3-21 gene seems to be used much more frequently in Northern Europe compared with other large series from different geographical regions (46–47). Alternatively, the observed differences might be related to differences in the way the lymphomas have been defined. According to the World Health Organization classification, SMZL should be defined on the basis of splenic histology. This might be particularly relevant for the study of Tierens et al. (14), who studied not only spleen tissues but also PB or bone marrow samples of 23 patients with villous lymphocytes on PB smear and, furthermore, included in the analysis 7 CD5+ as well as 2 CD23+ cases.

In IGKV-J rearrangements, the frequent appearance of the IGKV4-1 (B3), 2-30 (A17), and 1-39/1D-39 (O2/O12) genes (14/31 rearrangements; 45%) is reminiscent of the normal repertoire and has been shown to be related to their over-utilization in the rearrangement process and not to selection. This is further supported by the detection of IGKV4-1 (B3) in 3/9 out-of-frame rearrangements of the present series. In IGLV-J rearrangements, no bias was observed in favor of a particular V gene.

In B-cell lymphomas, accurate assessment of somatic mutational status has implications for defining tumor origin and, in some categories, for predicting clinical outcome. In 1999, it was found that CLL patients (CLL) that express V genes with > 98% homology to the closest germline gene follow a more aggressive clinical course and have strikingly shorter survival than patients with significant V gene mutations (48–49). Initially, the 98% cutoff was used to exclude potential polymorphic variant sequences. It was a short cut to avoid analyzing the corresponding germline gene in each patient, and is now used for assignment of mutational status in other B cell malignancies, including SMZL (11, 14). The most integrated IGV database (http://imgt.cines.fr) describes polymorphisms in several functional IGV genes, with nucleotide differences from germline generally of less than 1%, and only 5 IGHV genes (IGHV1-03, 1-69, 3-13, 4-28, and 4-b) and 1 IGKV gene (IGKV1-5 [L12]) with > 2% variation. Aligned IGV genes readily map to the correct allele and, therefore, the 98% cutoff point seems reasonable (50). Nevertheless, to ensure that the mutations described herein were not germline encoded, we sequenced the corresponding germline gene in 4 patients carrying mutated IGHV4-34/IGHV4-39/IGHV1-69 rearrangements. This approach always yielded negative results, thus confirming the somatic origin of the identified mutations.

The present study confirms the conclusions of Algara et al. (11) regarding the existence of at least 2 SMZL subtypes (mutated vs. unmutated) based on IGHV mutation status and a 98% homology cutoff in the study of Algara et al. (11), the group of unmutated cases was associated with chromosome 7q deletion and had an adverse clinical course. In the present series, follow-up data were available for 30/43 cases. Seven cases have been diagnosed since December 2002. All are alive and only 1 has progressed. Statistical analysis failed to disclose any significant association between IGHV mutation status and outcome, most probably due to small numbers and relatively short median follow-up.

Our parallel analysis of IG heavy- and light-chain genes demonstrates considerable heterogeneity regarding mutation "load" in both IGHV and IGKV/IGLV genes, with very few or even no mutations in a substantial proportion of cases. Nevertheless, a low level of mutations (as observed in some IG sequences of the present study) could be functionally relevant: as previously shown, even single-base changes (0.33% to 0.35% from germline) may be important for improving antigen recognition (for example, high-affinity anti-DNA antibodies in systemic lupus erythematosus with minimal somatic hypermutation) (51). Thus, a low muta-

tion load, while unlikely to affect prognostic power, may have implications for understanding the behavior of B-cell malignancies. One problem with assessing low levels of mutation is that of the approaching Taq error rate. An indicator of the reality of base changes is identification of the same change in IG sequences from the same patient derived from separate amplification reactions. In this context, all experiments of the present study were repeated at least 3 times and always gave identical results.

Mutated IGKV or IGLV genes of the present series exhibited a more even distribution of mutations within the CDRs and FRs. In general, somatic hypermutation seems to have affected IGHV, IGKV, and IGLV genes of SMZL patients to a similar extent. Mutation distribution analysis showed that 5 SMZL cases had evidence for selection by antigen exclusively for IGHV genes, 4 different cases had similar evidence exclusively for IGKV genes and, finally, one different case had evidence for selection for the IGLV gene. Similar observations on the complementary impact of antigen selection on IGHV, and/or IGKV or IGLV genes have been made in studies of multiple myeloma and follicular lymphoma (19–20). Importantly, as in almost all studies on IGH genes in SMZL, the distribution of mutations in the IGHV region was suggestive of selection by classical T-dependent antigen only in a minority of cases. Based on the above results, it is reasonable to argue that a complementary imprint of antigen selection witnessed either by IGHV, IGKV, or IGLV sequences might constitute an important event in the pathogenesis of a proportion of SMZL cases.

In conclusion, the present study confirms the existence of 2 subtypes of SMZL, which can be discriminated on the basis of IGHV mutational status. Furthermore, it provides evidence for the considerable molecular and biological heterogeneity of SMZL neoplastic cells, which probably reflects the multifaceted nature of their normal counterparts (1–6,52–53). SMZL cases frequently use V genes that encode for autoantibodies, a common finding among normal MZ B cells as well (1,3). Similar to normal SMZ B cells (4–6), they carry both mutated and unmutated IG genes with mutation patterns not always indicative of conventional germinal center responses to T-cell dependent antigens. This is in keeping with results in normal microdissected SMZ cells as well as tonsillar marginal zone B cells (5–6) and with admittedly indirect evidence that normal MZ B cells may accumulate Ig mutations outside classical GC, perhaps within the MZ itself, while the cells are responding to T-cell–independent antigens (54). Thus, SMZL may be considered as a broad spectrum of entities deriving from neoplastic transformation of cells with distinct phenotypic status and important developmental "plasticity." In this context, the functional heterogeneity of MZ B cells was strikingly revealed recently by Song and Cerny (55), who demonstrated that upon stimulation with a T-cell–dependent antigen, SMZ cells rapidly produced many antibody-forming cells with distinct clonotypic repertoire and later also gave rise to GCs with characteristic somatic hypermutation and generated immunological memory. Thus, the nature (quality and strength) of signaling through the B-cell receptor might be the primary determinant of the ultimate lineage choice made by every B-cell clone (53), and also drive the phenotype upon malignant transformation of B cells.

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Address correspondence and reprint requests to *Kostas Stamatopoulos, P.O. Box 51, 57010 Asvestohori, Thessaloniki, Greece. Phone and fax: 30-2310-359172; e-mail: stavstam@otenet.gr.*

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