Analysis of V_H genes in marginal zone lymphoma reveals marked heterogeneity between splenic and nodal tumors and suggests the existence of clonal selection

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Background and Objectives. To clarify the relationship between splenic (SMZL) and nodal marginal zone (NMZL) lymphomas, we analyzed immunoglobulin variable heavy chain (V_H) gene usage and mutation patterns in these tumors.

Design and Methods. V_H genes were cloned and sequenced from 49 lymphoma samples (35 SMZL and 14 NMZL).

Results. A biased usage of V_H gene was found with overrepresentation of V_H1 in SMZL cases (13/35) and V_H4 in NMZL cases (7/14). Evidence for antigen driven mutations was identified in 8 SMZL and 4 NMZL cases. Three cases out of 18 with clones analyzed from spleen and peripheral blood demonstrated intra-clonal diversity, with evidence of clonal selection in one case, indicating the possibility of antigen-driven clonal expansion. Eleven SMZL cases (31%) but only 2 NMZL (14%) cases were unmutated. No differences in clinical outcome and overall survival were found between the unmutated and mutated cases.

Interpretations and Conclusions. The pattern of somatic mutation and the V_H gene segment usage appear to differ between SMZL and NMZL, suggesting that these are distinct pathological entities. Moreover, a biased usage of certain sequences suggests that tumor cells in SMZL may be subjected to antigen selection.

Key words: somatic mutation, marginal zone, B cell lymphoma, immunoglobulin

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The germinal center reaction in T-cell -dependent antibody responses is crucial for the generation of B-cell memory and plays a critical role in B-cell lymphomagenesis. During this process, random mutations are introduced into immunoglobulin V_H genes to increase the antibody molecule's affinity and to further diversify the B-cell repertoire. Despite the possibility of a somatic hypermutation pathway outside of the germinal center, somatically mutated variable-region genes are currently considered a faithful marker for the transit of B cells into the germinal center.[1] Analysis of V_H gene sequences in B-cell lymphoma provides an estimate of the B-cell developmental stage at which malignant transformation occurs.[2] Furthermore the pattern of somatic mutation allows the origin of malignant B cells to be distinguished according to the germinal center trafficking.[3-8]

Three lymphoma types linked to the B-cell marginal zone have been recently recognized as distinct clinico-pathological entities in the World Health Organization (WHO) Classification[9] extranodal mucosa-associated lymphoid tissue (MALT) lymphoma, splenic marginal zone B-cell lymphoma (SMZL) with or without villous lymphocytes and nodal marginal zone B-cell lymphoma (NMZL) with or without monocytoid B-cells. These three lymphoma entities may have a common origin in the marginal B-cell compartment of the lymphoid organs. However, the precise cell of origin, its status in the B-cell differentiation pathway and the mechanisms involved in lymphomagenesis remain unclear.[9,10] In contrast with MALT lymphoma, in which lymphomagenesis is better understood, analysis of immunoglobulin heavy-chain variable region genes in SMZL and NMZL has been limited to a few series of patients.[11-17] The first report showed that all cases of marginal zone lymphomas had somatic mutations, suggesting that these tumors arise from memory B cells, without any significant pattern resulting from antigen selection.[12] However, recent studies have indicated a more important molecular heterogeneity than expected before,[11,13,14] including cases harboring germline V_H genes with higher clinical aggressiveness and shorter overall survival. There has, however, been no direct

comparison between SMZL and NMZL with respect to somatic mutations of the rearranged V_H gene.

In the present study we investigated V_H gene usage and mutation pattern in 49 lymphoma cases (35 SMZL and 14 NMZL) in order to clarify the relationship between splenic and nodal marginal zone B-cell lymphomas, to further examine the cell of origin of these lymphomas and to correlate the somatic mutation pattern with disease characteristics.

Design and Methods

Patients and tissues samples

Thirty-five SMZL cases and 14 NMZL were selected from the files of the Pathology Department of the Hospital Lyon Sud (France), including cases already described[15] and some new cases extensively characterized. The selection was based on the availability of clinical information and fresh-frozen biopsy specimens of lymph nodes or spleen before treatment. In addition, for 18 of the 35 splenic cases with significant bone marrow or peripheral blood infiltration (>20% tumor cells), an analysis of a paired sample of distinct origin (peripheral blood in 15 cases and bone marrow in 3 cases) was also performed. A paired peripheral blood sample was also examined for 1 of the 14 nodal cases. These paired samples were obtained during routine diagnostic procedures, at the same time as tissue biopsies and before any specific treatment. All cases met the diagnosis criteria of SMZL or NMZL in the WHO classification of lymphoid neoplasms.[9] The patients' medical records were reviewed to determine age, gender, sites of involvement, stage of disease, International Prognosis Index (IPI) parameters at diagnosis and disease course.

Cytogenetic studies were performed in 43 patients of the present series, on the samples used for histological and immunologic investigations as previously described.[18] In 32 patients, chromosomal analysis was performed on spleen samples. In 9 patients, chromosomal analysis was performed on lymph node biopsy material and in 2 patients on peripheral blood only.

Immunohistochemistry and pathology review

Routine hematoxylin-eosin and immunohistochemical studies on sections were available for all cases and were histologically reviewed by expert hematopathologists (FB, PF, ATG) according to the current criteria, as previously described.[19]

Polymerase chain reaction (PCR) amplification of the rearranged immunoglobulin (Ig) heavy chain genes

Genomic DNA was extracted from frozen tissue sections and frozen cellular suspension (peripheral blood or bone marrow) using the *High pure PCR template preparation kit* (Roche, France) according to the manufacturer's recommendations. The rearranged Ig heavy chain genes were amplified from genomic DNA (1 μg) using a mixture of 6 forward primers annealing in the framework 1 region[20] in combination with a joining heavy chain consensus primer [J_HC: TCGCAGACCCTCTCACTCACCTGT]. The PCR mixture contained 1×Taq buffer, 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 1 U of Taq polymerase (Ampli-Taq Gold, Applied, France), and 0.2 μM of each primer in a final volume of 50 μL. PCR amplification consisted of an initial denaturation step at 94°C for 10 minutes, followed by 35 cycles at 94°C for 30 seconds, 64°C for 1 minute, and 72°C for 1 minute, with a final extension step at 72°C for 10 minutes. All PCR reactions were performed using appropriate positive and negative controls. PCR products were denatured at 95°C for 5 minutes followed by rapid random renaturation at 4°C for 1 hour (to enhance heteroduplex formation)[20] and then migrated on a 6% polyacrylamide gel. All patient's samples were evaluated by at least 2 independent PCR reactions (with similar conditions and using a V_H family specific FRI primer in the second test). Moreover, we were able to perform an additional analysis on reverse-transcribed cDNA obtained from the same samples[21] in 6 cases: 2 splenic cases and 4 nodal cases.

Cloning and sequencing of Ig heavy chain sequences

Monoclonal bands corresponding to V_H gene rearrangements were excised from 1,5% low-melting agarose gels and the PCR products were purified from the gel with QIAEX II kit (Qiagen, France) and subcloned in the pGEM T-easy II vector (Promega, France) following the manufacturer's recommendations. Sanger's chain termination method and fluorescent dideoxynucleotide chain termination[22] was performed using an ABI sequencer and all clones were sequenced in both directions.

Each of the purified and cloned PCR products was submitted to sequencing from 2 to 4 times. Any case showing variation in 2 sequences was submitted to sequence confirmation with at least 2 other independent PCR, cloning and sequencing reactions.

Analysis of Ig heavy chain sequences

For each clone, the identification of the V_H sequence from FR1 to FR3 was performed by comparison with two databases: IgBlast (*http://www.ncbi.nlm.nih.gov/igblast/*) and IGMT (*http://igmt.cines.fr/*).[23] The sequences were aligned with the human germline sequences presenting the highest homology. The ability to code for functional heavy chains was determined by translating DNA sequences into amino acids. Non-functional sequences were not further characterized. Somatic mutations were defined as nucleotide substitutions

after elimination of the known polymorphisms described in the IGMT and IgBlast databases. Isolated single nucleotide discrepancy between 2 sequences obtained from the same sample was always submitted to verification as described above. When this single nucleotide change was found in only one sequence reaction, it was arbitrarily ascribed as a Taq polymerase error. Confirmed mutations found in multiple sequencing reactions in the same sample or in distinct specimens from the same patient were considered to represent intraclonal diversity. Percent of sequence identity was calculated for the aligned sequences from the beginning of FR1 until the end of FR3. Cases harboring < 98% homology were considered as mutated. D_H gene segments were identified by alignment to human germline D_H gene segments and were considered homologous when a minimum of ten consecutive matches was observed.

For antigen selection analysis, each single point mutation in framework regions (FR) and complementarity-determining regions (CDR) of potentially functional sequences was compared with the respective germline sequence and classified as an R (replacement) or S (silence) mutation. The R/S ratio was calculated from these data. The binomial distribution model was used to calculate the probability that the number of R and S mutations in the FR and CDR sequences occurred by chance only.[24] In addition a multinomial model, described by Lossos *et al.*[25] and available at *http://www-stat.stanford.edu/immunoglobin*, was used to assess the probability of antigen selection in the FR and in the CDR.

Statistical analysis

Associations between variables were tested using the Yates' corrected χ^2 test. Survival analyses were performed using the Kaplan-Meier method.[26] The statistical significance of associations between individual variables and progression-free or overall survival was determined using the log-rank test.[19] Values of $p < 0.05$ were considered statistically significant.

Results

Clinical and histological data

This population included 22 males and 27 females with a median age of 62 years [extremes 27–82 years] (Table 1). Patients were treated according to current guidelines at our institution.[19] At the time of analysis, with a median follow-up of 71 months for patients remaining alive, 27 patients had experienced disease progression and 11 patients had died. Most cases had typical morphological, immunological and cytogenetic features of SMZL or NMZL. Cases of splenic lymphoma with more than 20% of villous lymphocyte in peripheral blood were not included in this study. Expression of CD5 was observed in 7 of our cases (6 splenic and 1 nodal) in association with t(11;14)(q13;q32) in 5 cases (4 splenic and 1 nodal), an unusual feature already described in marginal zone lymphoma.[19,21,27,28] Recent gene expression profiling studies confirmed that these features are also encountered in marginal zone lymphoma.[21,29] These cases are further referred to here as *borderline cases* between marginal zone and mantle cell lymphoma.

Table 1. Characteristics of the 49 patients.

	Splenic type	*Nodal type*	*All patients*
Number of patients	35	14	49
Mean age (years)	62	52	
Stage			
I	1	2	3
II	0	0	0
III	0	3	3
V	31	8	39
Bone marrow involvement	27	4	31
Performance status (ECOG scale)			
0-1	27	11	38
2-4	5	1	6

Analysis of VDJ_H

We amplified and sequenced 55 clonal IgV_H rearrangements in 49 MZL patients (35 SMZL, 14 NMZL) since 6 cases displayed two distinct rearrangements, one isolated from the primary tumor and the other one from peripheral blood or bone marrow. Since we could not further demonstrate whether these cases represented bi-allelic rearrangement or detection of an unrelated B-cell clone in peripheral blood or bone marrow,[13,30] these circulating clones were not further considered in the study. All cases described here, including cases with 2 distinct rearrangements, lacked stop codons in the V_H-J_H segments and were therefore potentially functional. Cases with no functional sequence were not included in this study

Table 2 shows the V_H gene used by each tumor and its homology with the germline counterpart. The most frequent V_H family encountered was V_H3, followed by V_H1, V_H4 and V_H5 (Table 3). In contrast, the V_H2 and V_H6 families were not represented. The frequencies of the V_H families differed from those usually present in peripheral and lymph node lymphocytes in normal individuals (Table 3). We identified a preferential usage of V_H gene in SMZL with overrep-

Table 2. VDJ_H families and number of mutations in the VDJ_H sequence observed in the series

n°	specimen	VH GENE	allele	ID (%)	DH	JH	N
1*	spleen	1-8	1	100	ND	JH6	0
	blood	1-8	1	100	ND	JH6	0
2*	spleen	3-23	1	100	D2-15	JH5	0
	blood	3-23	1	100	D2-15	JH5	0
3	spleen	1-2	4	97	D3-3	JH4	8
	blood	1-2	4	96	D3-3	JH4	9
4	spleen	1-2	4	98	D3-9	JH6	4
	blood	1-2	4	98	D3-9	JH6	5
5	spleen	3-48	ND	92	D2-2	JH4	18
	bone marrow	3-48	ND	92	D2-2	JH4	18
6	spleen	3-7	1	99	D3-22	JH4	2
	bone marrow	3-15	7	91	ND	JH5	25
7	spleen	1-2	4	98	D3-3	JH4	3
	blood	1-2	4	98	D3-3	JH4	3
8	spleen	3-23	ND	100	D4-17	JH6	0
	blood	3-20	1	99	D3-22	JH6	2
9	spleen	3-23	3	89	ND	JH1	29
	bone marrow	3-7	1	92	ND	JH5	21
10	spleen	1-18	ND	99	D3-3	JH4	2
	blood	1-18	ND	99	D3-3	JH4	2
11	spleen	3-7	1	93	ND	JH1	19
	blood	3-7	1	93	ND	JH1	19
12	spleen	1-2	4	98	D3-9	JH5	4
	blood	1-2	4	97	D3-10	JH3	6
13	spleen	3-15	1	91	D1-7	JH5	22
	blood	3-15	1	91	D1-7	JH5	22
14	spleen	3-30	ND	95	D2-21	JH2	13
	blood	3-30	ND	95	D2-21	JH2	12
15	spleen	1-2	2	87	D1-26	JH5	32
	blood	3-30	ND	96	D2-21	JH2	10
16	spleen	1-2	4	96	D3-3	JH4	11
	blood	1-2	4	96	D3-3	JH4	11
17*	spleen	3-7	1	99	D3-22	JH4	2
	blood	3-7	1	99	D3-22	JH4	2
18	spleen	3-33	3	91	D3-10	JH3	21
	blood	3-33	3	91	D3-10	JH3	21
19	spleen	1-3	1	93	D3-10	JH1	17
20	spleen	4-39	1	91	D6-13	JH5	22
21	spleen	1-2	4	96	D3-9	JH2	8
22	spleen	1-2	4	97	D3-10	JH3	5
23	spleen	4-34	ND	96	D3-3	JH4	11
24	spleen	4-61	1	88	D3-22	JH4	28
25	spleen	1-2	4	96	D3-3	JH4	7
26	spleen	3-33	1	94	D4-17	JH6	18
27	spleen	3-23	1	97	D6-13	JH4	8
28	spleen	3-30	ND	92	D3-16	JH5	17
29	spleen	3-7	1	92	ND	JH4	19
30*	spleen	5-51	1	100	ND	JH4	0
31	spleen	1-8	1	98	D6-6	JH6	4
32	spleen	4-39	1	90	D2-2	JH4	24
33	spleen	4-34	2	96	D5-12	JH6	10
34	spleen	3-15	7	91	ND	JH5	24
35	spleen	3-23	1	93	D3-10	JH6	18
36	node	3-23	1	93	ND	JH6	17
	blood	1-2	ND	99	D3-3	JH4	2
37	node	4-34	2	96	D3-22	JH4	8
38	node	4-61	1	89	D5-24	JH4	27
39	node	4-59	1	97	ND	JH6	6
40	node	3-7	ND	96	D3-22	JH3	11
41*	node	1-3	1	93	D3-10	JH4	14
42	node	3-64	1	99	D3-10	JH5	1
43	node	4-34	10	95	D3-22	JH4	10
44	node	4-34	2	92	D3-22	JH4	19
45	node	1-2	4	96	D3-3	JH4	10
46	node	3-11	3	92	ND	JH6	16
47	node	4-34	2	94	ND	JH6	16
48	node	3-53	1	98	D3-3	JH6	7
49	node	4-34	11	93	ND	JH5	17

*The similarity of the V_H gene to the closest germline segment is shown according to the IgBlast (http://www.ncbi.nlm.nih.gov/igblast/) and IGMT (http://igmt.cines.fr/) databases. The number of mutations in each V_H segment is shown (N). Allele indicates the allele polymorphisms reported in the IGMT V-Quest database. In total, 35 splenic (cases 1 to 35) and 14 nodal cases (36 to 49) are included. The borderline cases between mantle cell lymphomas and marginal zone lymphomas (4 splenic and 1 nodal cases) are indicated with a " * ".*

Table 3. Distribution of V_H gene usage in splenic and nodal marginal zone lymphoma.

V_H Family	*Physiologic distribution*[57]	*All (n=49)*	*Samples splenic (n=35)*	*nodal (n=14)*
V_H1	21.6%	15 (30.6%)	13 (37.1%)	2 (14.3%)
V_H2	5.9%	0 (0%)	0%	0%
V_H3	43.1%	21 (42.9%)	16 (45.7%)	5 (35.7%)
V_H4	21.6%	12 (24.5%)	5 (14.3%)	7 (50%)
V_H5	3.9%	1 (2%)	1 (2.9%)	0%
V_H6	1%	0 (0%)	0%	0%

The frequency of the VH family distribution is compared with that described in normal peripheral blood lymphocytes.[57]

resentation of the V_H1 gene family, especially V_H1-2 allele 04 segment (8/35)[31] while the V_H1-69 segment commonly found in chronic lymphocytic leukemia, was never identified. In contrast, NMZL demonstrated a selective usage of V_H4 family segments (50%) with overrepresentation of V_H4-34 (5/14)474. Interestingly 5 cases (4 SMZL and 1 NMZL) displayed a common use (with a distinct mutation pattern) of V_H1-2 04 D_H3-3 and J_H4 with few or no mutation (96% to 100%) (Table 2). The alignment of the heavy chain third complementary-determining region 3 (HCDR3) amino acid sequences in these cases and 23 showed 2 similar sequences (Figure 1) indicating the possibility of selection by a common antigen. Although serological tests for hepatitis C virus (HCV) had been done for all patients, no case with HCV infection was found in this series.

Analysis of mutation status

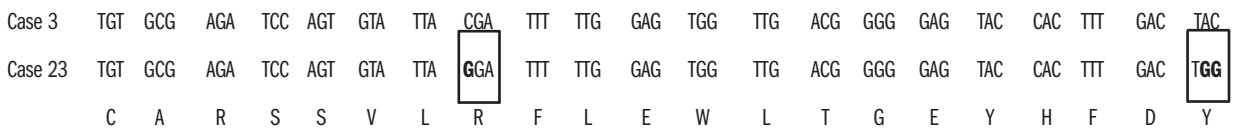
In total, 13 cases (26%) were considered as unmutated (homology > 98%) whereas 36 cases (74%) were mutated. Out of 35 SMZL, 11 cases (31%) were unmutated whereas 2 single cases (14%) out of 14 NMZL were unmutated (Table 3).

Most of the mutations were single nucleotide substitutions but the substitution of 2 or 3 adjacent nucleotides was frequent within the same codon. No deletion was observed. One insertion (3 nucleotides) was observed. Transitions were more frequent (n=314) than transversions (n=254).

Intraclonal diversity

Three cases out of 18 for which clones were analyzed in spleen and peripheral blood demonstrated intraclonal-diversity. For each case, the same related sequence was repeatedly found (Table 5) with strong evidence of clonal selection in one case (LS-3). The other 15 cases were strictly identical in both tissues in multiple PCR amplicons (see Material and Methods)

Figure 1. Alignment of amino acid and nucleotide sequences of CDR3 in cases 3 and 23.



Out of the 5 cases using the same VH1-2 04/DH3-3/ JH4, with a unique sequence and junctional motif (Table 3), cases 3 and 23 also showed identical amino-acid CDR3. The sequences of CDR3 were determined by counting the number of amino acids between position H94 at the 3' end of FR3 (usually two amino acids downstream of the conserved cysteine) and position H102 at the beginning of FR4 (a conserved tryptophan in all JH sequences). As indicated in the boxes, distinct nucleotide sequences result in an identical amino-acid sequence of the CDR3, suggestive of the recognition of a common antigen.

therefore confirming that the VH sequence (spleen, node, peripheral blood or bone marrow) was clearly that of the tumor clone.

Antigen selection

A typical pattern of antigen selection, as tested by the binomial and multinomial models, was found in 12 cases (8 SMZL and 4 NMZL) with increased replacement mutations in the CDR and decreased mutations in the FR (Table 4). The number of cases with significant antigen selection was found to be higher using the multinomial model rather than the binomial models (12 versus 6 cases, respectively).

Hypermutation status, cytogenetic findings and clinical outcome

The 4 splenic cases that were borderline between marginal and mantle cell lymphoma with a translocation t(11;14) were characterized by the lack of mutation identified in the VDJH sequence. The pattern of VH usage in these borderline cases did not display any specific characteristics. The absence of mutation was not associated with deletion 7q or any other cytogenetic abnormality. With a median follow-up of 71 months, no differences in progression-free or overall survival were found between the unmutated and mutated cases (log-rank, p=0.58 and p=0.14, respectively) (*not shown*). These findings were identical even when only the 35 SMZL cases were considered or when the t(11,14) positive cases were excluded (*not shown*).

Discussion

The pathogenesis of SMZL and NMZL and the relationship between the two entities are still poorly understood. Whereas SMZL and NMZL share many morphological, immunophenotypic and cytogenetic features, their clinical outcome is distinct[19] and some karyotypic aberrations are more common in one or the other entity, suggesting the possible existence of a different pathogenesis. The present study was undertaken to clarify the issues of VH gene use and somatic mutations in SMZL and NMZL. For this purpose, molecular analysis of the immunoglobulin VH region was performed in 49 untreated patients (35 SMZL and 14 NMZL). Clinical, morphological, cytological, immunophenotypic and cytogenetic data were available for all the patients. In addition, peripheral blood or bone marrow samples from 18 splenic cases and 1 nodal case were analyzed. The proportion of cases with immunoglobulin gene hypermutation and the VH gene segment usage appear to differ markedly between SMZL and NMZL, suggesting distinct pathological entities that may arise from different B-cell subsets. In addition, the pattern of VH gene usage and somatic mutation observed in some cases is suggestive of antigen-driven lymphomagenesis.

Our analyses confirm the molecular heterogeneity previously reported in SMZL and NMZL with unmutated and mutated cases.[13-15] Out of 35 SMZL, 13 cases were unmutated whereas 2 single cases out of 14 NMZL were unmutated. In addition, this study demonstrated a biased VH gene use in both lymphoma entities, with overrepresentation of VH1, especially VH1-2, in SMZL cases and over-representation of VH4-34 in NMZL cases. A biased VH gene family usage with respect to the peripheral blood cell repertoire has previously been reported in SMZL (VH1-2),[13,32] but not in NMZL.[14,15] When borderline cases with mantle cell lymphoma were not taken into account, this biased usage of VH gene appeared even more striking (8 SMZL cases with VH1-2 out of 32). Interestingly within the 9 cases which harbored the VH1-2 segment, 5 of them (4 SMZL, 1 NMZL) had a common use of VH1-2 allele 04, DH3-3 and JH4, with no or few distinct mutations (100% to 96% homology). Of note, 2 of these 5 cases had an amazing conservation of the idiotype sequence. This feature may imply the recognition of a common antigen. Overall, this pattern of biased VH gene usage is suggestive of an antigen selection process. It is reminiscent of chronic lymphocytic leukemia, in which cases with canonical CDR3 have been described[33] and with the restricted VH1-69 DH3-22 JH4 usage and similar CDR3 in cases of nodal marginal zone lymphomas associated with HCV.[34] In addition a biased usage of the VH1-69/VκA27 combination has also been described in HCV-associated lymphomas.[35]

Table 4. Distribution of mutations in V_H sequences and antigen selection analysis with the binomial (Chang and Casali) and multinomial (Lossos) method.

Case	Gene	Identity(%)	N	Observed R/S CDR	Observed R/S FR	Expected R/S CDR	Expected R/S FR	Lossos p CDR	Lossos p FR	Chang and Casali p CDR	Chang and Casali p FR
SMZL											
3	VH1-2	97	6	0/1	3/2	1/0	03/01	0.83722	0.16013	0	0.3
5	VH3-48	92	12	7/0	4/1	3/1	7/2	**0.00784**	**0.00150**	**0.0043**	**0.0011**
9	VH3-23	89	21	5/3	9/4	4/1	12/4	0.66095	0.19851	0.18	0.43
11	VH3-7	93	13	4/2	2/5	3/0	7/2	0.06042	0.00042	0.17	0.004
13	VH3-15	91	21	5/3	8/5	4/1	11/4	0.12245	0.01941	0.7	0.07
14	VH3-30	95	11	2/1	6/2	0/5	4/2	0.30732	0.30787	0.29	0.22
15	VH1-2	87	26	9/2	8/5	7/1	14/5	**0.00109**	**0.0176**	0.46	0
16	VH1-2	96	12	7/0	4/1	3/1	7/2	**0.00012**	**0.02126**	**0.0043**	**0.0011**
17	VH1-3	93	14	4/2	4/4	3/1	8/2	0.44069	0.00038	0.18	0.03
18	VH4-39	91	18	5/1	7/5	4/1	9/4	0.06377	0.03459	0.17	0.09
19	VH1-2	96	8	4/1	2/1	1/0	8/3	**0.00652**	**0.01868**	**0.043**	0.081
20	VH1-2	97	4	1/0	3/0	1/0	2/1	0.70894	0.91993	0.41	0.29
21	VH4-34	96	8	3/1	2/2	2/0	4/2	0.35592	0.55828	0.15	0.07
22	VH4-61	88	24	5/2	9/9	5/1	12/5	0.18288	0.00704	0.19	0.052
23	VH1-2	96	5	1/2	2/0	1/0	3/1	0.30741	0.16355	0.4	0.27
24	VH3-33	94	14	3/0	1/10	3/1	8/3	0.20651	0.00002	0.25	0.0003
25	VH3-23	96	6	5/0	1/0	1/0	3/1	**0.00010**	**0.01765**	**0.0019**	0.063
26	VH3-30	92	14	4/1	5/3	3/1	8/3	0.19474	0.07891	0.18	0.073
27	VH3-33	91	18	6/2	10/0	4/1	10/3	**0.00652**	0.11920	0.096	0.18
28	VH3-7	92	16	8/1	6/1	3/1	9/3	**0.01018**	0.16530	**0.0083**	0.079
31	VH4-39	90	20	5/3	6/6	4/1	10/4	**0.01724**	**0.00082**	0.19	0.09
32	VH4-34	96	7	1/2	3/1	1/0	4/1	0.40770	0.15400	0.35	0.24
34	VH3-15	91	20	5/1	7/7	4/1	10/0	0.10331	0.0111	0.15	0.17
35	VH3-23	93	15	4/2	6/3	3/1	8/3	0.07832	0.04636	0.152	0.105
NMZL											
36	VH4-34	96	7	2/1	1/3	1/0	4/1	0.12843	0.00676	0.28	0.0333
37	VH4-61	89	25	7/3	8/8	6/1	13/5	**0.03709**	**0.00119**	0.13	0.09
38	VH4-59	97	6	0/3	2/1	1/0	3/1	0.77456	0.09219	0	0.17
39	VH3-7	96	8	1/1	3/3	2/0	4/1	0.50387	0.08983	0.31	0.18
40	VH1-3	93	11	4/2	4/1	2/0	6/2	**0.0245**	**0.03747**	**0.047**	**0.045**
41	VH3-64	95	9	4/2	6/3	3/1	8/3	0.08271	0.04473	0.19	0.01
43	VH3-23	95	9	4/1	3/1	2/0	5/2	**0.01194**	**0.04829**	0.072	0.12
44	VH4-34	92	16	3/3	5/5	3/1	9/3	0.22238	0.0635	0.25	0.034
45	VH1-2	96	6	1/1	1/3	1/0	3/1	0.36287	0.01516	0.382	0.06
46	VH3-11	92	16	5/2	5/3	3/1	8/3	**0.02632**	**0.0000**	0.12	0.054
47	VH4-34	94	14	2/1	7/4	3/1	8/3	0.38943	0.16832	0.24	0.19
49	VH4-34	93	14	4/0	5/5	3/1	8/3	0.05364	0.02301	0.171	0.076

The distribution of replacement (R) and silent (S) mutations was analyzed by considering all possible mutations, as described by Chang and Casali[24] or by Lossos in cases with mutations superior to 2%. Statistical evidence (with both P for CDR and Fr regions < 0.05) for antigen driven mutations are indicated in bold. When considering the multinomial method, antigen selection was identified in 8/24 SMZL and 4/12 NMZL. R denotes a replacement mutation, S a silent mutation, and R/S the ratio; N is the total number of mutations observed. CDR = complementary-determining region; FR: framework region; VH: variable region of the heavy chain .

The possibility of antigen-driven lymphomagenesis has already been demonstrated in some subsets of marginal zone lymphomas. For instance, MALT lymphoma of the stomach is acquired in the context of chronic lymphoid proliferation due to the presence of *Helicobacter pylori* and can be cured with antibiotics.[36-40] There is some evidence indicating that *Borrelia burgdorferi* may be implicated in the pathogenesis of certain cutaneous marginal zone lymphomas.[41,42] Several recent studies, but not the present series, have reported a high rate of HCV infection in patients with marginal zone lymphomas.[43,44] Furthermore, the treatment of HCV infection in HCV-infected patients with SMZL has been shown to be associated with an anti-lymphoma effect.[45] The findings that an antigen-driven selection process may occur in other marginal zone lymphoma types is therefore of interest. In the present study, evidence for antigen-driven mutations was identified in 8/35 SMZL and 4/14 NMZL with characteristics of antigen-selected antibodies that are under positive pressure to provide the *best fit* for antigen while retaining the framework structure to provide the scaffolding for the antigen-contacting CDR.[24]

An ongoing somatic mutation process usually indicates that the mutation machinery is active in the tumor cells after malignant transformation, suggesting

Table 5. Intraclonal diversity.

Case no.	Specimen	Mutation number	Variations number	Subclones number
3	spleen	8	0	6
3	blood	8 or 9	1	6 (2 with 8 mutations and 4 with 9 mutations)
4	spleen	3 or 4	0	6 (3 with 3 and 3 with 4)
4	blood	4 or 5	1	7 (3 with 4 mutations and 4 with 5 mutations)
14	Spleen	12 or 13	1	6 (4 with 12 mutations and 2 with 13)
14	blood	12	0	6

Cases in which sequence variations in a related clone were identified between different clones in the same sample (case 4 and 14) or in distinct specimens obtained from the spleen and from the peripheral blood (cases 3, 4 and 14).

of a germinal center (GC) origin.[1] As opposed to the common finding in cases of follicular lymphoma,[46] the low number of cases with intraclonal heterogeneity described here could also suggest that the tumor cells went through the germinal center but are no longer submitted to the somatic hypermutation mechanism. Of note, the average mutation frequency was rather low. This low frequency of somatic mutation in the V_H genes indicates that the tumor cells may derive from cells different from usual GC cells.[47] Indeed, GC cells are considered to acquire their hypermutation profile during prolonged interaction of the tumor cell with the GC microenvironment. In the present series, evidence of antigen encounter (as shown by the presence of somatic mutation) but accompanied by a marked proportion of clones without any affinity maturation characteristics may suggest that the cell of origin of these lymphomas could be a B-cell implicated in T-cell-independent immune responses. Recent studies in rodents demonstrated that marginal zone B cells play a role in immunologic response to T-independent type 2 antigens, such as bacterial capsular polysaccharide, indicating that they are the first line of the immune reaction against blood-borne antigen.[48] All together and emphasized by the lymphoma localization in the spleen, these findings allow us to hypothesize that SMZL cells may share several characteristics with superantigen T-independent selected B cells.

Our group and others have reported some cases of marginal zone lymphomas associated with the translocation t(11;14)[21,27,19] that do not have the characteristics of mantle cell lymphomas. The lack of somatic mutation in the 4 splenic borderline cases supports the individualization of this subgroup but does not allow these cases to be classified as mantle cell lymphomas which also harbor mutations in about 30% of the cases.[49]

The mutational pattern distinguishes the cases in this series from other B-cell lymphomas. Other B-cell lymphoproliferations, in particular chronic lymphocytic leukemia have more unmutated cases (50%) with a distinct V_H usage: V_H1-69 (20%), V_H4-34, and sometimes V_H3-7,[50,51] 3-21 and 3-23. V_H1-69 gene usage has also been described in MALT lymphoma and in HCV-associated lymphomas,[34] whereas it was not observed in this series. Over-representation of V_H4-34 was reported in diffuse large B-cell lymphoma, high grade MALT, mantle cell and Burkitt's lymphomas[52-55] and primary central nervous system lymphomas.[56] The V_H family usage and mutational pattern of NMZL is therefore closer to that observed in diffuse large B-cell lymphoma and follicular lymphomas (V_H4-34) but these cases are usually characterized by a higher number of mutations, marked antigen selection and more intraclonal diversity.[46]

In contrast to previous reports on a similar number of SMZL patients or to classical findings in patients with chronic lymphocytic leukemia, in whom unmutated cases behave more aggressively, the prognosis of marginal zone lymphoma patients in this series does not appear to be influenced by the immunoglobulin mutation status. Whether this reflects some heterogeneity in SMZL patient populations or the lack of prognostic significance of immunoglobulin hypermutation status in marginal zone lymphoma patients, as also reported recently in mantle cell lymphoma patients,[49] remains to be determined.

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