

Mutation Analysis of IgVH Genes in Splenic Marginal Zone Lymphomas: Correlation with Clinical Characteristics and Outcome

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Abstract. *Background:* To determine the immunoglobulin variable heavy chain (IgVH) gene usage and somatic mutation patterns in a series of SMZL patients and to correlate these findings with the clinical features and outcome. *Patients and Methods:* IgVH genes were amplified and sequenced from 22 SMZL cases. Clinical and laboratory data of these patients were recorded. *Results:* A biased usage of IgVH gene was found with overrepresentation of VH3 in 16/22 cases. A total of 13/22 (59%) of cases were found to have mutated IgVH genes, whereas 9/22 (41%) were unmutated. Positive antigen selection process was identified in two cases. Treatment was different between the cases with mutation and those without. No differences in clinical and laboratory characteristics, or survival were found between the mutated and unmutated cases. *Conclusion:* SMZL are characterized by marked molecular heterogeneity. A biased usage of certain sequences suggests antigen selection. Prognostic significance of mutational status was not confirmed in this study. However further studies are needed in order to confirm these results.

Splenic marginal zone lymphoma (SMZL) is a disease with an indolent clinical course, representing a distinct entity in the WHO classification (1). Diagnosis is based on a

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combined evaluation of clinical findings, morphology and immunophenotype. Clinically, SMZL is characterized by the presence of splenomegaly without lymphadenopathy, except for the splenic hilar nodes. Peripheral blood is often involved, while the bone marrow (BM) is invariably found to be infiltrated, especially when immunohistochemical and/or molecular studies are applied (2, 3). Lymphoma cells in the blood or the BM usually have a heterogeneous morphological appearance with villous projections, monocytoid and/or plasmacytoid features while small lymphocytes may also be present. The neoplastic lymphocytes express monoclonal surface immunoglobulin (Ig) mostly IgM and/or IgD, and panB-cell markers, while CD5, CD23, CD43 and CD10 antigens are usually negative (4). The histology of the spleen is also characteristic, with a micronodular pattern of infiltration and a biphasic cytology (5). The establishment of diagnosis is possible based on the blood and BM findings without the need for splenectomy (2, 6).

SMZL usually runs an indolent clinical course with prolonged survival. Median survival generally ranges from 9 to 13 years in various studies (7-9). However, there is a subgroup, comprising less than one third of the patients, in which the disease is characterized by a more aggressive clinical behaviour, frequent histological transformation to diffuse large B-cell lymphoma and a much shorter median survival of 1.7-3 years (8, 9). The early identification of this subgroup of patients is not possible due to the lack of generally accepted, even clinical, prognostic factors. At the biological level, various molecular and cytogenetic features such as absence of immunoglobulin variable heavy chain (IgVH) somatic mutations, 7q31 deletion, expression of genes involved in nuclear factor (NF)- κ B activation and p53 mutations have been implicated as adverse prognostic factors in some series (10-13). The precise prognostic significance of these biological markers, however, is not clear yet.

The aim of this study was to determine the IgVH gene usage and somatic mutation patterns in a series of SMZL cases and to correlate these findings with clinical and laboratory features and outcome of the patients.

Patients and Methods

Patients. Among a total of 80 SMZL patients diagnosed and followed up in our departments during the last 15 years, 22 were included in the present study, based on the availability of tissue specimens or frozen cell material. Diagnosis was based on standard criteria (1). In 12 patients, the diagnosis was based on peripheral blood and BM findings and in 10 patients by splenic histology after splenectomy. The baseline clinical and laboratory features as well as follow-up and outcome were recorded for every patient.

Analysis of the IgVH somatic mutations. Preparation of DNA: Genomic DNA was isolated from frozen tissue sections and frozen mononuclear cell suspension (from peripheral blood or BM) by proteinase K digestion and phenol/chloroform extraction.

Amplification of rearranged IgVH genes: Rearranged IgVH genes were amplified essentially in reactions that contained only one of the 5' leader region primers for the indicated 6 VH families and a 3' J primer (14). Individual primary polymerase chain reactions (PCR) were performed in 50 μ l total volumes and contained approximately 1 μ g of DNA and 5' V and 3' J primers each at a final concentration of 0.5 μ M. The DNA was added to the PCR buffer (50 mmol/l KCL, 10 mmol/l Tris-HCl (pH 8.4), gelatin 0.01%) containing 200 μ mol/l dNTPs, 1.5 mmol/l MgCl₂, 0.5 μ M of each primer and 1.5 U Taq Platinum Polymerase. The PCR conditions consisted of one cycle at 94°C for 5 minutes, followed by 36 cycles at 94°C for 20 seconds, 55°C for 30 seconds, 72°C for 30 seconds and one final cycle at 72°C for 5 minutes (14). An aliquot of 10 μ l of PCR product was size fractionated on a 3% agarose gel in 1x TBE buffer. All PCR reactions were performed using appropriate positive and negative controls.

Cloning and sequencing of the PCR products: Resultant PCR products were purified using NucleoSpin Extract II kit (Macherey-Nagel, Germany) and cloned using the TOPO TA Cloning Kit (Invitrogen, Paisley, UK) following the manufacturer's instructions. Plasmid DNA was isolated from overnight cultures of randomly selected colonies using Qiagen Plasmid Purification Kit (Hilden, Germany). Sanger's chain termination method and fluorescent dideoxynucleotide chain termination were performed using an ABI sequencer and all clones were sequenced in both directions by using the same primers as in the amplification. Only those cases in which identical sequences were obtained from the majority of clones were included in the study and further analyzed.

Sequence analysis. For each clone, the identification of the VH sequence from framework region (FR)1 to FR3 was performed by comparison with two databases: Ig BLAST (<http://www.ncbi.nlm.nih.gov/igblast/>) and IGMT (<http://igmt.cines.fr/>). The sequences were aligned with the human germline presenting the highest homology. Cases harboring <98% homology were considered as mutated. The ability to code for functional heavy chains was determined by translating DNA sequences into amino acids. Non-functional sequences were not further characterized.

Antigen selection analysis. For antigen selection analysis (in mutated cases), each single point mutation in 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 (silent) mutation. The R/S ratio was calculated from these data. The multinomial model described by Lossos *et al.* (15) was used to calculate the probability of antigen selection in the FR and in the CDR.

Clinical correlations and statistical analysis. The frequency of somatic mutations in the IgVH gene was tested for correlation with the following clinical and laboratory characteristics: sex, age, absolute lymphocyte count, anemia, thrombocytopenia, paraproteinemia, B-symptoms (fever, night sweats, weight loss), expression of CD25, CD5, CD38 and CD23 antigens, surface light chain, percentage of BM infiltration, type of treatment and histological transformation, along with disease outcome and survival.

The Chi square (χ^2) test (with continuity correction, as indicated) was used to compare baseline clinical characteristics of the patients by mutation status. Survival analysis was performed by the Kaplan-Meier method. Differences between survival curves were determined by the log-rank test. *P*-Values <0.05 were considered statistically significant.

Results

Clinical features. The studied population included 15 women and 7 men with a median age at diagnosis of 63 years (range, 43 to 91 years). Their main clinical characteristics are summarized on Table I. All patients had splenomegaly, with a median spleen size of 10 cm below the left costal margin. None of them had lymph node enlargement, except for the splenic hilar lymph nodes. BM infiltration was evident in all patients, either by conventional morphology and immunohistochemistry, or by flow cytometry. B-symptoms were present in two patients. Anemia (hemoglobin <12 g/dl), lymphocytosis (lymphocyte count >4 \times 10⁹/l) and thrombocytopenia (platelets <100 \times 10⁹/l) were recorded in 14, 13 and 5 patients respectively. Autoimmune hemolytic anemia was present in 4 patients, while paraproteinemia was detected in 9 patients, being of the IgM isotype in all cases except one. Serum lactate dehydrogenase (LDH) was elevated in 8, while serology for hepatitis C was negative in all patients. The distribution of our patients according to International Prognostic Index (IPI) is shown on Table I.

Histological and immunophenotypic findings. The histology of the spleen in the splenectomized patients was consistent with the diagnosis of SMZL (1).

Bone marrow infiltration was evident in all patients, although the extent of involvement was highly variable, ranging from 20% to 80% of nucleated bone marrow cells. Analysis of peripheral blood and BM mononuclear cells by flow cytometry revealed positivity of panB-cell markers in all cases and expression of monoclonal surface light-chain κ in 14 and λ in 8 cases. There was a weak expression of CD5

Table I. Clinical and laboratory features of 22 SMZL patients.

Feature	Patients	
	no.	%
Median age (range)	63 (43-91)	
Male gender	7	32
Palpable splenomegaly	22	100
Median spleen size*(cm) range	10 (2-15)	
B-symptoms	2	9
Anemia (Hb<12 g/dl)	14	64
Thrombocytopenia (platelets <100×10 ⁹ /l)	5	23
Lymphocytosis (>4×10 ⁹ /l)	12	55
Paraproteinaemia	9	41
AAA	4	18
LDH elevated	8	36
CD5 ⁺	1	4
CD23 ⁺	8	36
CD11c ⁺	7	32
CD25 ⁺	3	14
CD38 ⁺	2	9
sIgK ⁺	14	64
IPI		
Low	6	27
Low intermediate	12	55
High intermediate	4	18

*Below left costal margin; AAA, autoimmune haemolytic anemia; LDH, lactate dehydrogenase; IPI, International Prognostic Index.

in one case (which was cyclin-D1 negative), while CD23, CD11c, CD25 and CD38 were expressed at varying frequencies (Table I).

Treatment, follow up and survival. Ten patients underwent splenectomy as first-line therapy, 10 were treated with rituximab and 2 with chemotherapy (chlorambucil and cyclophosphamide, vincristine, prednisone, respectively). The overall response rate was 86%. Complete remission was achieved in 80% of the patients treated with rituximab (16), while the response to other treatment modalities was inferior. Among the responders, 6 patients relapsed at a median time of 22 months (range, 14-61 months). The median follow-up time was 29 months (range, 10-138 months). During the study period, 7 deaths were recorded: 3 were disease related, 2 due to toxicity, 1 of unrelated cause and 1 due to secondary neoplasia. Histological transformation into diffuse large B-cell lymphoma was reported in 3 patients.

Use of VH, D and JH gene segments and mutation status. The rearranged VH genes identified for each case seemed to represent functional rearrangements because no stop codons or crippling mutations were identified. A comparison of the VH genes to reported germline sequences revealed that 16 cases used the VH3 family VH gene segments, 4 the VH4 family and 2 the VH1 family segments. The VH3 family

Table II. VH gene usage in 22 SMZL cases.

Patient no.	VDJ gene	% Homology
1	VH3-11J4 D5-5	97.57
2	VH3-23J4D6-6	91.00
3	VH3-23J4D2-2	93.40
4	VH3-15J4D3-22	92.86
5	VH3-48J5D5-24	100.00
6	VH3-13J1D3-10	91.58
7	VH1-2J1D3-10	99.31
8	VH1-2J5D3-22	99.65
9	VH3-23J4D3-22	98.61
10	VH3-48J4D6-19	98.96
11	VH3-21J4D2-21	100.00
12	VH3-30J4D5-24	100.00
13	VH3-11J4D3-22	98.96
14	VH3-7J6D6-19	84.03
15	VH3-48J2D2-21	90.28
16	VH3-48J2D7-27	91.67
17	VH3-74J4D4-17	88.54
18	VH4-30J6D3-9	97.25
19	VH4-34J4D6-6	96.84
20	VH4-34J3D3-22	95.09
21	VH3-33J4D3-22	94.10
22	VH4-34J5D3-10	98.95

M, Mutated; UM, unmutated.

genes V3-48 were used in 4 cases, the V3-23 in 3 and the V3-11 in 2. The other 7 cases used the VH3 family genes V3-30, V3-13, V3-33, V3-74, V3-21, V3-15 and V3-7, respectively. The VH4 family genes VH4-34 were used in 3 cases and the VH4-30 in one case. The VH1 family genes V1-2 were used in 2 cases (Table II). Approximately half of the cases used J4 joining segments (12/22) and D3 families of D segments (10/22).

In 9 out of 22 cases (41%), IgVH genes were in germline or near germline configuration, whereas in 13 cases (59%), IgVH genes were somatically mutated (Table II).

Analysis of mutation pattern antigen selection analysis. In order to detect possible features of antigen selection, the mutation pattern of somatic hypermutations were further analyzed, based on the ratio of amino acid replacement (R) to silent mutations (S), which is higher in CDR regions and lower in the FR regions. The locations and type of VH gene mutations are given in Table III. In 2 out of the 13 mutated cases (case 1 and 3), the R/S ratio in CDRs (CDR1 and CDR2) was greater than that expected by chance only, suggesting antigen selection. In 4 mutated cases, (case 3, 4, 15, 17), the excess of R over S mutations targeted the FR regions rather than the CDRs. In the remaining 7 mutated cases, the pattern of R/S mutations in FR or CDR showed no evidence of antigen selection.

Table III. Analysis of IgVH gene mutations in the SMZL cases with mutation.

Patient no.	VDJ gene	FR		CDR	
		R:S	p-Value	R:S	p-Value
1	VH3-11J4 D5-5	3:0	0.28	3:0	0.022
2	VH3-23J4D6-6	5:1	0.55	5:1	0.19
3	VH3-23J4D2-2	3:2	0.01	5:1	0.004
4	VH3-15J4D3-22	4:5	0.008	3:2	0.23
6	VH3-13J1D3-10	6:1	0.97	0	0.93
14	VH3-7J6D6-19	18:0	0.99	2:0	0.65
15	VH3-48J2D2-21	15:0	0.00	3:0	0.34
16	VH3-48J2D7-27	7:1	0.52	3:0	0.12
17	VH3-74J4D4-17	14:0	0.00	0	0.94
18	VH4-30J6D3-9	5:1	0.53	2:0	0.19
19	VH4-34J4D6-6	3:1	0.57	1:0	0.51
20	VH4-34J3D3-22	3:4	0.08	1:0	0.46
21	VH3-33J4D3-22	6:5	0.06	2:1	0.38

FR, Framework region; R:S, replacement: silent; CDR, complementarity determining region.

Correlation between mutation status and clinical/laboratory findings and outcome. As shown on Table IV, no differences in clinical and laboratory characteristics, immunophenotype, outcome or overall survival were found between the mutated and unmutated cases. There was a trend for worse survival in unmutated *versus* mutated SMZL cases (median survival 70 vs. 109 months), but the difference did not reach statistical significance.

Discussion

SMZL usually runs an indolent clinical course. However the disease in a small fraction of patients displays a more aggressive clinical behavior, with histological progression and shorter survival (8, 9, 17, 18). This clinical heterogeneity may in fact reflect the molecular heterogeneity of these lymphomas.

The normal B-cell counterpart of SMZL was undefined until recently. Earlier studies suggested that SMZL originated from a postgerminal center memory B-cell, based on the findings that lymphoma cells carry mutated IgVH genes (19-21). Recent studies, however, revealed that in a proportion of SMZL cases at least, the IgVH genes were unmutated (13, 22-26). This is consistent with the apparent heterogeneity of normal splenic marginal zone B-cells (27, 28). The prognostic significance of these molecular findings is not clear yet, although in some series, unmutated IgVH genes were associated with a more aggressive form of SMZL (10, 13).

In the present study, we analyzed the mutation patterns of the IgVH genes in 22 SMZL cases. Diagnosis was based on clinical, morphological and immunophenotypic findings. As is currently accepted, a splenectomy specimen was not

Table IV. Correlation of disease features with IGVH mutation status in 22 SMZL patients.

Feature	Mutational status*	
	Mutated**	Unmutated**
No. of patients	13	9
Male/female	4/9	3/6
Median age (years)	63	63
Lymphocyte count (median)×10 ⁹ /l	4.68	10.00
Anemia	8	6
Thrombocytopenia	2	3
Paraproteinemia	7	2
B-symptoms	0	2
CD25+	2	1
CD5+	1	0
CD38+	1	1
Kappa+/Lambda+	9/4	5/4
BM infiltration % (median)	40	45
Rituximab as first-line treatment	4	6
Histological transformation	2	1
No. patients alive	8	7
Median survival (months)	109	70
Median follow-up (months)	25 (10-138)	50 (11-98)

*All p-values are non-significant. **Numbers represent number of pts unless otherwise indicated.

mandatory for the establishment of diagnosis (2, 6). Our results showed that 59% of the cases demonstrated mutated IgVH and 41% unmutated. These figures are in accordance with previously published data (13, 22-26).

Our analysis also showed the selective use of VH3 family genes in a high proportion of SMZL cases (73%), while VH4 and VH1 family genes were represented at a lower frequency (18% and 8%, respectively). Other published series have reported different frequencies in VH gene usage, with the VH1 family gene representing the most common in some reports (14, 21). The selective use of individual VH genes suggests that a common antigen may play a central role in pathogenesis of this lymphoma. For example, hepatitis C (HCV) has been implicated in the pathogenesis of SMZL, in a small number of cases (29). However, we did not confirm the involvement of HCV infection in any of the cases reported here. Antigen selection analysis in our study identified positive selection in 2 out of the 13 mutated cases analyzed. This is in accordance with other series which failed to show evidence of positive antigen selection in the majority of cases (10, 23). These results may either reflect the limited role of the antigen in the pathogenesis of these lymphomas, or may be due to the limitations of the methods applied.

Another interesting issue that emerges from the molecular studies on SMZL is the prognostic significance of the

mutation status of the IGVH genes. The extensive study of mutation status of IgVH genes in B-cell lymphocytic leukemia (B-CLL) has revealed the existence of a strong heterogeneity, with the unmutated cases showing a more aggressive clinical course (30,31). Similar studies in SMZL suggest that unmutated cases show a more aggressive clinical behaviour, with histological transformation and poorer outcome (10, 13). Other studies, however, including the present report, did not confirm these correlations (24, 25). In our series we did not find any significant difference regarding the clinical and laboratory features, nor survival in relation to mutational status. The heterogeneous treatment modalities applied in both groups may influence these correlations. Rituximab therapy may affect the prognostic implications of the absence of IgVH somatic mutations. Moreover our results are limited due to relatively small number of patients studied.

In conclusion, the present study revealed that a significant fraction of SMZL cases derive from naive B-cells with unmutated IgVH genes, thus indicating that this is a heterogeneous group of disease with respect to its cellular origin. A prognostic significance of mutation status was not observed in this study. In addition the biased usage of certain sequences suggests that lymphoma cells may be subjected to antigen selection. However, antigen selection analysis of mutated cases using the multinomial distribution did not clearly identify a positive antigen selection process in the majority of the analyzed cases.

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