CD5 expression identifies a subset of splenic marginal zone lymphomas with higher lymphocytosis: a clinico-pathological, cytogenetic and molecular study of 24 cases

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ABSTRACT

Background
Classically, splenic marginal zone B-cell lymphoma is characterized by the absence of CD5 expression. Cases of apparent splenic marginal zone B-cell lymphoma showing CD5 expression, as diagnosed by blood studies, have been described; however, in the absence of histological evidence, the correct diagnosis of these cases is controversial because of possible confusion with other CD5-positive small B-cell neoplasms.

Design and Methods
We report a series of 24 CD5-positive, t(11;14)-negative cases of splenic marginal zone B-cell lymphoma diagnosed by flow cytometry studies of blood and histologically proven on spleen sections. Clinical data as well as morphological, immunological, cytogenetic and molecular characteristics were assessed to evaluate the similarities and differences of these cases with those of classical CD5-negative splenic marginal zone B-cell lymphoma.

Results
The CD5 expression detected in blood by flow cytometry was confirmed in most cases by immunohistochemistry on spleen sections. In general, cases of CD5-positive and CD5-negative splenic marginal zone B-cell lymphoma did not appear different and, in particular, they showed similar karyotypic changes such as 7q deletion, trisomy 3, trisomy 18 and biased *IGHV* usage (i.e. VH1-2). The main differences were a higher lymphocyte count at diagnosis (8.15×10^9/L *versus* 3.90×10^9/L; P=0.005) and more frequent diffuse bone marrow infiltration (34% *versus* 8%; P=0.03) in the CD5-positive group. A tendency to a more mutated *IGHV* status in the CD5 positive cases was observed (80% *versus* 54.5%; (P=0.11). No significant differences in outcome were found in relation to CD5 expression.

Conclusions
This study confirms the existence of cases of CD5-positive splenic marginal zone B-cell lymphoma and shows that these cases are closely related to classical splenic marginal zone lymphoma. Whether or not CD5-positive splenic marginal zone B-cell lymphoma constitutes a true subset obviously requires the study of more cases.

Key words: splenic marginal zone B-cell lymphoma, CD5-positive B cells, chromosomal abnormalities, *IGHV* status.

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The online version of this article has a Supplementary Appendix

Introduction

Splenic marginal zone B-cell lymphoma (SMZL) is a B-cell lymphoma with an indolent clinical course that is characterized by a primary splenic infiltration. In the World Health Organization's (WHO) classification system,[1] SMZL is recognized as a true subtype distinct from extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) and nodal marginal zone B-cell lymphoma. The neoplastic cells of SMZL strongly express pan-B-cell markers, such as CD19, CD20, CD22 as well as surface immunoglobulin IgM and IgD, and are usually negative for CD5, CD23, CD43, CD10 and cyclin D1. Although CD5 expression has been reported in a few histologically proven cases of marginal zone lymphomas, including MALT lymphoma[2-4] and more recently SMZL,[5] it seemed rather incidental. As these CD5-positive lymphomas responded poorly to treatment, it was postulated that CD5 expression could be a marker of aggressiveness.[2,4-6] Besides the histologically proven cases of CD5-positive SMZL, frequent CD5 expression (nearly 20-25%) was reported in two large series of splenic lymphomas that were diagnosed from peripheral blood samples as splenic lymphoma with villous lymphocytes (SLVL), a term which is in fact used by many authors as a synonym for circulating SMZL.[7,8] However the diagnosis of CD5-positive SLVL/SMZL in these series was based only on cytological and immunological findings and their true diagnosis may be questioned in the absence of histological evidence; furthermore, a t(11;14) was reported afterwards in about 20% of the cases.[8,9] Another study which did not state whether CD5 positivity was found in blood, on spleen sections or both, reported a different cytogenetic pattern in CD5-positive and CD5-negative SMZL,[6,10] since 7q deletion, which is considered the most characteristic anomaly of SMZL when isolated, was not found in the first group.

In order to establish the reliability of blood studies for the diagnosis of CD5-positive SMZL, we report a series of 24 cases diagnosed in blood which were all splenectomized, either at the time of diagnosis or during the follow-up. Cytological and histological specimens, as well as immunophenotype, chromosomal anomalies, *IGHV* status and clinical characteristics were studied to determine similarities and differences between CD5-positive SMZL and classical CD5-negative SMZL, as well as to allow their distinction from other CD5-positive lymphoproliferative disorders, such as chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), mantle cell lymphoma (MCL), or lymphoplasmacytic lymphoma (LPL).

Design and Methods

Patients and samples

Twenty-four cases of SMZL expressing CD5, initially diagnosed by cytology, flow cytometry and cytogenetics of peripheral blood between 1995 and 2006, were selected from the files of the hematology laboratory on the basis of confirmatory splenic histology. The SMZL cases were considered to be CD5-positive when the percentage of CD19-positive B-cells co-expressing CD5 was greater than or equal to 20%, whatever the fluorescence intensity. A splenectomy was performed at the time of the diagnosis (made from blood studies) in nine patients and during the follow-up in 15 cases (median, 11 months; range, 3-64 months). Cases that were diagnosed as splenic red pulp lymphoma (SRPL) with numerous true villous lymphocytes[11] were excluded from this study. The group of 24 cases of CD5-positive SMZL was compared with a control group of 42 cases of well-defined CD5-negative SMZL from our department (histologically proven in all cases) which were diagnosed from peripheral blood studies during the same period. The majority of these CD5-negative cases have been previously reported.[12-16] Five CD5-positive cases and ten CD5-negative cases presented a SMZL signature in spleen and/or blood samples, according to previous micro-array gene expression analysis.[14]

Cytological review

Freshly made blood films were obtained at diagnosis, either manually or automatically prepared by Sysmex SP-100 or SP-1000 (Roche Diagnostics, Meylan, France), and stained with May-Grunwald-Giemsa stain). Smears were reviewed and evaluated for cytological features by expert hematologists (PF, DM): a manual 100-cell differential lymphocyte count was performed with special attention to monocytoid cells, lymphoplasmacytoid cells, plasmacytic cells, and cells with membrane irregularities. Among these, we distinguished villoid lymphocytes (with short and rare villi and clear scant cytoplasm) from true villous lymphocytes (with marked villi and relatively abundant basophilic cytoplasm, which define SRPL when numerous).[11]

Histological review and immunohistochemistry

Formalin- or Bouin-fixed, paraffin-embedded splenic tissue sections (stained with hematoxylin and eosin) were reviewed by expert hematopathologists (FB, ATG) according to the current criteria in 24 cases.[1] Standard immunohistochemical techniques were used, including heat-induced antigen retrieval and avidin biotin peroxidase detection on an automated immunostainer (Benchmark, Ventana Medical System) according to the manufacturer's instructions. Appropriate positive and negative controls were run for each antibody. The immunohistochemical panel included a B-cell marker (L26/CD20: Dako, Carpinteria, CA, USA), a T-cell marker (polyclonal CD3: Dako), and monoclonal antibodies against CD5 (clone 4C7, Novocastra, Lb Co. Ltd Newcastle, UK), CD23 (clone 1B12, Novocastra), CD43 (clone MT1, Novocastra) and cyclin D1 (clone SP4, Cliniscience). Expression of each marker was evaluated in the tumor lymphoid cells.

Resin-embedded bone marrow specimens, available in 15 cases, were reviewed by an expert hematopathologist (MF); immunohistochemical studies were performed, as previously described,[11] on one of these bone marrow resin-embedded core biopsy specimens from a patient for whom a discrepancy was found between CD5 expression in peripheral blood and in spleen sections.

Flow cytometry

All the peripheral blood samples (n=24) and some spleen cell suspensions (n=17) were stained using combinations of two or three monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or phycoerythrin-Texas Red® (ECD). The routine monoclonal antibodies used (CD classification, fluorochrome label and clone) included CD19-FITC (clone HD37), CD19-PE (clone HD37), CD5-PE (clone DK23), CD22-PE (clone 4KB128), CD23-FITC (clone MHM6), CD43-FITC (clone DF-T1), CD79b- PE (clone SN8), and FMC7-FITC (all from Dako), CD19-ECD (clone J4.119), CD11c-PE (clone BU15), CD25-PE (clone B1.49.9), and CD103-FITC (clone 2G5) (all from Beckman-Coulter, Hialeah, FL, USA);

and CD10-PE (clone HI10a), CD27-PE (clone L128), and CD38-PE (clone HIT2) (all from Becton Dickinson, San Jose, CA, USA). Methods for immunophenotypic analysis by flow cytometry have been reported previously.[11] Samples were analyzed on a Coulter EPICS XL flow cytometer (System II software or Expo32 ADC software, Beckman-Coulter). Cells were considered positive for a given marker if 20% or more of the cells expressed the marker. The fluorescence level for the different parameters was also measured in arbitrary units, such as the mean fluorescence intensity (MFI) and the ratio of fluorescence intensity (RFI), which corresponds to the normalized MFI over the MFI of the isotype negative controls. The RFI was calculated for peripheral blood specimens. All cases were scored using the scoring system established for the diagnosis of CLL.[17]

Cytogenetic analysis

Cytogenetic studies were performed on fresh samples used for morphological and immunological investigations [blood (n=7) and spleen (n=24)]. Methods for cytogenetic analysis have been previously reported.[18] Additional fluorescence *in situ* hybridization (FISH) experiments were performed on samples from each of the patients according to standard, previously reported methods.[19] Probes specific for the centromeric regions of chromosomes 3, 12 and 18 (Vysis, Downer's Grove, IL, USA) as well as the dual color IgH/CCND1-specific DNA probes (Vysis) were used. Six patients were investigated using different painting probes (Cambio, Cambridge, UK) and telomeric specific probes (Qbiogene, IllKrich, France) to confirm some abnormalities.

Molecular studies

Genomic DNA was extracted from the spleen samples and from the peripheral blood cells using High Pure PCR Template Preparation Kits (Roche, Mannheim, Germany). The mutational status of *IGHV* genes was determined by sequencing and analyzed as previously reported.[16] Cases were considered to be mutated when they displayed less than 98% homology with the closest germline *VH* gene.

Staging procedures and clinical data

Initial staging was based on age, stage and sites of involvement (peripheral blood count with cytological analysis and bone marrow biopsy), serum levels of lactate dehydrogenase (LDH) and β2–microglobulin. Serum protein electrophoresis, Coombs' test, and complete coagulation assays were performed systematically. Serum tests for human immunodeficiency virus and hepatitis B and C viruses were carried out after the patients' consent. Performance status was evaluated according to the Eastern Cooperative Oncology Group scale and the patients were staged according to the Ann Arbor system. Patients were treated according to standard clinical practice.

Statistical analysis

Overall survival was defined as the time between diagnosis and death or the last follow-up. Progression-free survival was defined as the time from initial diagnosis to the date of the first relapse or disease progression or last follow-up. Progression was defined as a relapse for patients in complete response, or as the appearance of a new lesion (in particular cytopenia) or an increase in the volume of spleen. Survival analysis of the patients was performed using the Kaplan-Meier method and significance was determined using the log-rank test. The Mann-Whitney U-test and χ^2 test were used for comparisons of the different parameters studied. A P value of less than 0.05 was considered to be statistically significant.

Results

Clinical data

The clinical and biological characteristics of the 24 CD5-positive patients at the time of diagnosis are summarized in Table 1. This CD5-positive group comprised 13 male and 11 female patients (sex ratio, 1.18) with a median age of 63 years (range, 36-84 years). Seven cases (29%) presented with anemia (hemoglobin level <100 g/L) and 13 (54%) with thrombocytopenia (platelet count $<150\times10^9$/L). The leukocyte and lymphocyte counts ranged from 5.43×10^9/L to 82.88×10^9/L (median, 12.44×10^9/L) and from 1.00×10^9/L to 81.22×10^9/L (median, 8.15×10^9/L), respectively.

High LDH and β2-microglobulin levels were observed in 47% and 81% of the patients, respectively. No patient had a poor performance status (i.e.≥2). According to the International Prognostic Index (IPI), 33% of the patients had a low risk, 52% had low-intermediate risk, and 15% had high-intermediate risk. Splenomegaly was present at diagnosis in 22 CD5-positive cases. The median weight of the spleen ranged from 650 to 4400 g (median, 1449 g).

Immunological events were observed in 5/24 (21%) cases: hemolytic anemia and/or positive Coombs' test (n=5) and immune thrombocytopenia (n=1). One patient had both immune anemia and thrombocytopenia. In this series, none of the patients was positive for human immunodeficiency, hepatitis B or hepatitis C virus. A monoclonal component was detected in 13/24 patients (54%). The monoclonal component was more often an IgM [11 cases (85%): 7 IgMκ, 2 IgMλ and 2 IgM] than an IgG [2 cases (15%): 2 IgGκ].

Patients were treated with the therapeutic protocols in use at the time of diagnosis (Table 1). Typically, elderly patients were not initially treated, except in cases of clinically aggressive disease. Patients with massive splenomegaly or marked cytopenia had a splenectomy at the time of diagnosis, and this was followed by chemotherapy in some cases. In fact, all the 24 patients were splenectomized: 7 patients were carefully followed without treatment before splenectomy, whereas 17 patients were treated directly by splenectomy, either alone or followed by chemotherapy.

The clinical comparison of the CD5-positive and CD5-negative cases of SMZL is presented in Table 1. The clinical features of the two groups appeared similar, with the exception of higher leukocyte and lymphocyte counts (P<0.05) in the CD5-positive cases, despite a similar tumor mass (spleen). Therapeutic options were identical in the two groups.

Cytological and histological results

The peripheral blood infiltrate presented some degree of heterogeneity in all cases but one. The most frequent cell population (23/24 cases) consisted of small lymphoid cells with compact chromatin, often condensed in small irregular clumps, which was predominant in 14 cases. Lymphoplasmacytoid cells were present in all cases but one, and accounted for more than 10% of the cells in 20 cases. Atypical lymphocytes (by size and/or dispersed chromatin and/or visibility of a nucleolus), which could not be classified in a well-defined category, were present in 20 cases and accounted for more than 10% of cells in 17 cases. Villoid cells were observed in 19 cases, at a level of more than 5% in 13 cases, but always less than 20%; vil-

lous lymphocytes were present in nine of these cases at a low percentage (less than 10% of the lymphocytes). Plasmacytic cells and medium-sized lymphoid cells with relatively abundant pale cytoplasm (labeled as monocytoid cells) were observed in four and five cases, respectively, at a low percentage (less than 10%). Centrocyte-like cells were rare. Medium to large cells with a prominent nucleolus were observed in three cases (Figure 1), and correlated with a higher number of large cells in the spleen. In addition, rouleaux formation was present in 14 cases. Smudge cells were absent or represented a small percentage of the cells (more than 20% of lymphocytes but less than 30% in 5/24 cases). Of note, one case showed a more polymorphous lymphoid population with rare bi-lobed nuclei simulating polyclonal B-cell lymphocytosis with binucleated lymphocytes. The cytological aspect of the blood cells was not different from that usually reported in classical SMZL/SLVL, and in our control group.[12]

In all cases, splenic sections stained with hematoxylin and eosin showed a micronodular pattern with a "marginal zone" of white pulp, consisting of larger cells with more abundant cytoplasm in the periphery. Invasion of the splenic red pulp was also seen in all cases with a micronodular and intrasinusoidal pattern (Figure 2). Splenic hilar lymph nodes, present in 14 cases, always showed effacement of the nodal architecture. In three cases we also observed a more aggressive morphology on spleen sections, with a high mitotic count and a higher level of large cells (<20 % in 2 cases and > 30% in 1 case). In all cases, the cell composition on paraffin-embedded tissue was heterogeneous including mainly small round cells with clumped chromatin, small- to medium-sized cells with clear cytoplasm (monocytoid B cells) and cells with plasmacytoid differentiation. Plasma cells were rare (5/24 cases). These morphological features were quite similar to those described in CD5-negative SMZL.[12] All cases were negative for cyclin-D1.

In bone marrow sections stained with hematoxylin and

Table 1. Clinico-biological data.

	CD5-positive SMZL		CD5-negative SMZL		*P* value
	Total patient	Data missing	Total patient	Data missing	
Number of patients	24	-	42	-	
Sex ratio (M/F)	1.18	-	0.75	-	
Age [range], years	63 [36-84]	-	62 [29-83]	-	ns
Performance status ≥2	0	0	1 (2%)	0	
IPI: Low risk	7 (33%)	3	15 (40%)	5	
Low-intermediate risk	11 (52%)		16 (43%)		
High intermediate risk	3 (15%)		6 (16%)		
High risk	0		0		
Anemia (Hb < 100 g/L)	7 (29%)	0	16 (38%)	0	
Hb [range], g/L	112 [68-160]		112 [65-150]		ns
Thrombocytopenia (platelet count < 150 ×10⁹/L	13 (54%)	0	24 (61%)	0	
platelet count [range], ×10⁹/L	135 [73-842]		134 [19-1 179]		ns
Lymphocyte count [range], ×10⁹/L	8.15 [1.00-81.22]	0	3.90 [0.40-102.70]	0	0.005
Clonal B cells [range], ×10⁹/L	5.33 [1.00-73.10]	0	2.50 [0.40-92.43]	0	0.02
LDH (> normal values)	10 (47%)	3	13 (35%)	5	
LDH [range], UI/L	391 [207-795]		376 [245-3 3714]		ns
β2-microglobulin (≥ 3 mg/L)	13 (81%)	8	18 (60%)	12	
β2-microglobulin [range], mg/L	3.3 [1.8-7.1]		3.5 [1.6-8.7]		ns
Peripheral lymphadenopathy	0	0	0	0	
Splenomegaly at diagnosis	22 (92%)	0	39 (93%)	0	
spleen weight [range], g	1 449 [650-4 400]		1 480 [140-4 300]*		ns
Monoclonal component	13 (54%)	0	17 (40%)	0	0.54
Immunological events	5 (21%)	0	18 (43%)	0	
Hemolytic anemia and/or positive Coombs'	5		13		0.12
Immune thrombocytopenia	1		6		
Bone marrow infiltration		9		16	
Interstitial and/or intrasinuoidal	10 (66%)		24 (92%)		
Diffuse	5 (34%)		2 (8%)		0.03
Treatment					
Watchful waiting before splenectomy	7	-	11	-	
Splenectomy alone	12	-	23	-	
Splenectomy + monochemotherapy	2	-	4	-	
Splenectomy + polychemotherapy	3	-	4	-	

*IPI: International Prognostic Index score; *In two cases of the CD5-negative group, the weight of the spleen was 140g and 180g : these two patients were splenectomized because of hemolytic anemia and thrombocytopenia.*

eosin, the infiltration was evident in all cases although highly variable [median=35%; (range, 15% to 90%)]. The pattern of infiltration was interstitial and intrasinusoidal in ten cases, with nodules in seven cases (located in the paratrabecular zones in 4 patients), whereas a diffuse involvement was present in five cases (34%). Fibrosis was absent in five cases and slightly increased in the others, without significant differences from the control group. Thus the bone marrow infiltration in CD5-positive cases was similar to that reported in classical SMZL, except that a higher proportion of CD5-positive cases had diffuse involvement, which is usually rare.[20] This was confirmed when we compared bone marrow infiltration in the CD5-positive groups with that in 26 patients of our control group, among whom only two cases (8%) had diffuse involvement; the difference between the two groups was statistically significant (P=0.03) (Table 1). Among the CD5-positive group, the diffuse pattern did not appear to correlate with either the level of lymphocytosis or the pattern of spleen involvement. Of note, among the patients with diffuse bone marrow infiltration, all cases displayed a typical splenic biphasic pattern.

Immunological results

Flow cytometry analysis

The proportion of clonal B lymphocytes varied from 35% to 95%. The restricted light chain of the surface immunoglobulin was patent (with a moderate to strong intensity) in all cases but one. The light chain κ was present in 16/23 cases (69%) and the light chain λ was present in 7/23 cases (31%). In one case, no light chain could be identified. Fourteen (58%) of the cases expressed both IgM and IgD, eight (34%) only IgM, while two cases (7%) expressed both IgM and IgG. Intracytoplasmic immunoglobulin was detected in 14/18 cases (78%). CD23 was dimly positive in 8/24 cases (33%), with a percentage of positive cells above 30% except in two cases. Lymphoma cells expressed CD43 dimly in 3/24 cases (12%). Two cases showed co-expression of CD23 and CD43. CD22 and CD20 were both strongly expressed in all the analyzed cases but one. In all the available cases, the neoplastic cells were CD27-positive. The neoplastic B cells expressed CD38 dimly in 6/20 cases (30%). No case was CD10-positive. The typical markers of hairy cell leukemia (i.e. CD103, CD25 and CD11c)[21] were tested in 23/24 cases and the profile obtained was clearly different from that in hairy cell leukemia: CD11c was positive in only two cases (8%) and CD103 and CD25 were not expressed in any case. According to the scoring system for CLL, cases scored 1 in 11 samples (46%) and 2 in 13 samples (54%). Except for CD5 expression, the immunological profile of the CD5-positive SMZL in blood was similar to that commonly described for CD5-negative SMZL and that observed in our control group,[11-13] especially with regards to the incidence of CD23/CD5 and CD43/CD5 co-expression by B cells (Table 2).

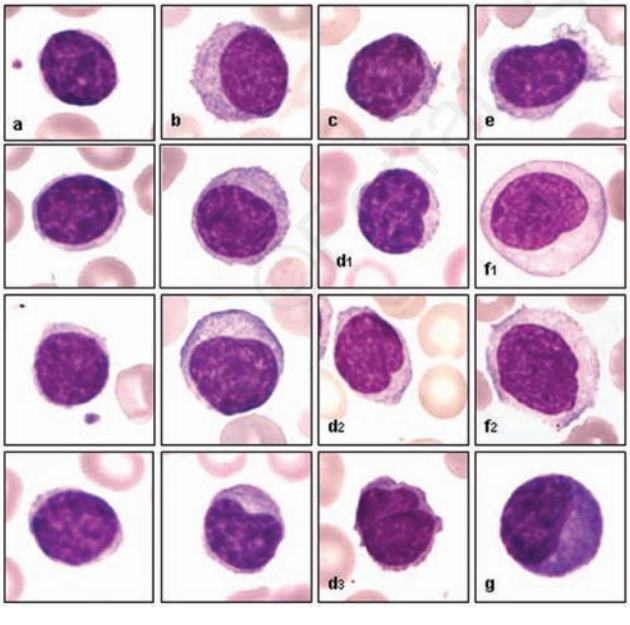

Figure 1. Cytology of the blood smears from CD5-positive SMZL patients (MGG staining). Tumor cells presented some degree of heterogeneity with a majority of small lymphoid cells with compact chromatin, which is rarely densely-packed in small irregular clumps (a), frequent lymphoplasmocytoid cells (b), villoid lymphocytes with short villi (c) and centrocytic-like cells (d1,d2,d3), rare true villous lymphocytes (e). Medium-sized lymphoid cells with a relatively abundant pale cytoplasm (resembling monocytoid cells)(f1,f2) and plasmacytic cells (g) were uncommon.

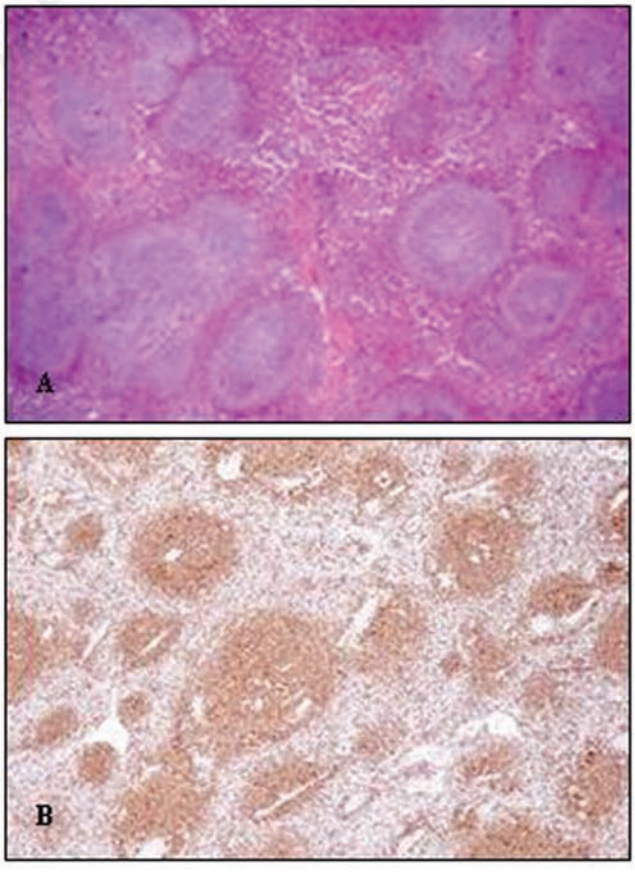

Figure 2. Morphology of spleen sections from patients with CD5-positive SMZL with a classic marginal-zone pattern (A) Hematoxylin eosin safran staining, low magnification (x4) and with strong CD5 immunostaining (B).

The median RFI of some markers was compared with that of a local series of 85 CLL and 42 MCL (with cytological, histological and cytogenetic features of CLL and MCL, respectively). The CD5-positive SMZL differed from CLL by a lower level of CD23 expression (median RFI, 4.2; range, 2.5 to 8.2 *versus* median RFI, 14.6; range, 1.9 to 75.0; $P<0.0001$), a higher level pf CD20 expression (median RFI, 35.0; range, 48.3 to 188.0 *versus* median RFI, 23.5; range, 6.6 to 60.5; $P<0.0001$) and a lower level of CD43 expression (median RFI, 3.4; range, 2.5 to 7.1 *versus* median RFI, 24; range, 6.7 to 53.7; statistical analysis not applicable). CD38 expression was lower among the CD5-positive SMZL cases than in the MCL cases (median RFI, 7.7; range, 1.5 to 12.5 *versus* median RFI, 29.3; range 7.5 to 155.0; $P<0.002$).

The immunological profile of all the cases but one, and the CD5 expression in particular, was identical when comparing blood and spleen samples. In one case, lymphoma cells analyzed by flow cytometry were CD5-positive in the blood and CD5-negative in the spleen. Interestingly, in this case, B cells were also CD5-positive in a bone marrow biopsy.

Comparison between flow cytometry and immunohistochemical analysis

On sections, 18/24 cases (75%) were CD5-positive with faint to strong staining of the majority of the lymphoma cells. The immunohistochemical staining correlated with the fluorescence intensity of flow cytometry staining, since the immunohistochemical-negative cases correlated with dim flow cytometry CD5 expression and the immunohistochemical-positive cases with stronger flow cytometry CD5 positivity (median RFI=6; range, 2 to 12 and median RFI=21.5; range, 8 to 63, respectively), although there was a small overlap. Despite the faintness of the expression (below 12), CD5 expression was present in more than 20% of B cells according to flow cytometry and was, therefore, considered as positive staining. In the case in which flow cytometry provided discordant results between blood and spleen samples (see above), CD5 was negative according to immunohistochemical staining. The CD5-negative cases on sections were fixed in Bouin liquid as well as in formalin liquid. Thus, the CD5 negativity could not be attributed to the fixative used.

Table 2. Immunological, cytogenetic and molecular results.

	CD5-positive SMZL	CD5-negative SMZL
Number of patients	24	42
Immunological profile		
CD23	8/24 (33%)	11/35 (30%)
CD43	3/24 (12%)	6/35 (16%)
Matutes score 0	0	12/35 (34%)
Matutes score 1	11/24 (46%)	10/35 (29%)
Matutes score 2	13/24 (54%)	13/35 (34%)
Main chromosomal abnormalities		
Clonal abnormalities	23/24 (96%)	31/38 (81%)
+3	8 (33%)	10 (32%)
7q deletion	5 (21%)	14 (45%)
+12	4 (17%)	5 (16%)
+18	7 (29%)	4 (13%)
13q deletion	2 (8%)	0
Mutated *IGHV*	12/15 (80.0%)	12/22 (54.5%)
VH1-2	3/15 (20%)	8/22 (36%)
VH1-18	0	2/22 (9%)
VH2-5*04	1/15 (5%)	0
VH3	8/15 (53%)	9/22 (40%)
VH4	2/15 (17%)	2/22 (9%)
VH5-51	1/15 (5%)	1/22 (4%)

Cytogenetic analysis

Clonal chromosomal changes were identified in 23 of the cases (96%) (Table 2). Karyotype results are detailed in the *Online Supplementary Appendix (Online Table S1)*. The patient with a normal karyotype was further studied by interphase FISH for the detection of +3, del11q22.3, +12, del13q14.3, del17p13.1 and +18. No chromosomal changes were identified. Trisomy 3 and trisomy 18 were observed in eight (33%) and seven patients (29%), respectively. Interstitial or terminal chromosome 7q deletions were present in five patients (21%). Trisomy 12 was observed in four cases (17%). Deletions involving the long arms of chromosome 13 were observed in two cases (8%), both in subclones. No chromosome 17p deletion was identified in this series. t(9;14) and t(14;19) were identified in one case each. No t(11;14)(q13;q32) was detected either by karyotype or FISH analysis. As a whole, the global distribution of chromosomal changes was not statistically different between the CD5-positive and CD5-negative groups (Table 2), although a 7q deletion occurred in 21% of the CD5-positive cases and in 45% of the control group, whereas trisomy 18 was identified more frequently in the CD5-positive group than in the CD5-negative group (29% *versus* 13%, respectively). Although the main associations of chromosomal changes, such as +3/+18, appeared to be more frequent in the CD5-positive group than in the CD5-negative one (5 cases *versus* 0 cases, respectively), the small number of cases precluded a meaningful statistical analysis.

Molecular results

The sequence of the *IGHV* gene was analyzed in 15 cases of CD5-positive SMZL and in 22 cases of CD5-negative SMZL; the sequences were found to be in frame and functional in each case (Table 2). Among the CD5-positive group, the *IGHV* gene was mutated in 12 cases (80.0%), with homology to germline sequence ranging from 85.40% to 97.20% (median, 93.01%). No ongoing mutation was observed and the length of the CDR3 was variable. The usage of the different VH families by each tumor is summarized in the *Online Supplementary Appendix (Online Table S2)*. The most frequent VH family used was VH3 (53%), with no selective VH gene usage, followed by VH1-2 (20%) and VH4 (17%). The CD5-positive cases appeared more often mutated than the control group (80% *versus* 54.5%), but the difference was not statistically significant ($P=0.11$). Although the same VH families were used in both groups, VH1-2 usage was less common in the CD5-positive cases than in the CD5 negative cases; this finding needs to confirmed in more cases.

Outcome

The median follow-up of the CD5-positive group and of the CD5-negative group was 59 months (range, 1-105 months) and 56 months (range, 2-212 months), respectively. During the follow-up, clinical progression was

observed in 10/24 patients with CD5-positive SMZL (41%) and in 24/39 patients with CD5-negative SMZL (61%). One patient in the CD5-positive group had disease progression to an extranodal site, while progression to a lymph node or extranodal site did not occur in any of the CD5-negative group. Six patients died in each group. No differences in outcome and overall survival were found between the two groups. In this series, no clinical or biological criteria (in particular, leukocyte count or the type of chromosomal anomaly) were found to influence the time to progression significantly or to be associated with a particular outcome.

Discussion

Although the incidence of CD5 expression has been reported to be approximately 20-25% in some large series of SLVL diagnosed on the basis of cytological and immunophenotypic studies of peripheral blood,[7,8] the diagnosis of CD5-positive SMZL, especially when based only on peripheral blood analyses, remains controversial in the absence of histological proof (although this requires at least the integration of clinical, morphological, immunological and cytogenetic data). In the few cases of SMZL with blood and spleen analyses previously reported, cells were CD5-positive in the blood and bone marrow but were CD5-negative on spleen sections.[5] A modulation of CD5 expression according to the involved compartment could account for these discordant results between blood and spleen, since CD5 expression can depend on the micro-environment, as a possible result of the response to the local cytokine network, especially T-cell-derived lymphokine, interleukin-4, chemokine and B-cell receptor signaling via the notch2 pathway.[22-24] However in the present series, CD5 appears to be almost always uniformly expressed whatever the anatomical site. Indeed a very good correlation of CD5 expression was observed in both compartments, given that all the cases studied by flow cytometry except one were CD5-positive in spleen suspensions as well as in blood. Thus, the under-representation of CD5-positive SMZL reported in the literature when the diagnosis is made only on sections is probably linked to a bias in the sensitivity of the diagnostic technique used. Flow analysis, especially when carried out using a strong fluorochrome, such as phycoerythin in our series, is a very sensitive technique allowing the detection of dim antigen expression that cannot always be detected by immunohistochemistry. In this series there was a global positive correlation between the staining intensities observed by flow cytometry and immunohistochemistry, and the negative cases on sections corresponded to the cases with dimmest CD5 expression by flow cytometry. Only one case which was CD5-positive in blood and bone marrow turned out to be negative in spleen by both techniques i.e. flow cytometry and immunohistochemistry. In addition, there were no cases of CD5-negative B cells in the blood with CD5-positive B cells in the spleen, which could have supported the hypothesis of preferential homing of CD5-positive and CD5-negative cells in a lymphoma presenting both distinct cell populations. Moreover, except for CD5 expression, the lymphoma cells had the same immunological profile in both tissue compartments, which rules out a biclonal proliferation or dual differentiation according to the site, as previously reported.[25]

The expression of CD5 in SMZL raises the question of the differential diagnosis with other CD5-positive small B-cell lymphomas including, in particular, CLL/SLL, MCL and LPL. Some of our cases could indeed have been confused cytologically with atypical CLL/SLL. However, the Matutes score was low (<3) in all our cases in contrast to the score in CLL/SLL, which is defined by a score 4 or 5.[7] CD23 and CD43 expression was dim when present, whereas CD20 was strongly expressed at difference with the situation in CLL.[26] Furthermore, all CD5- and CD23-positive cases had typical spleen histology. In addition, most cases showed trisomy 3, 7q deletion or trisomy 18 which are karyotypes known to be recurrent in SMZL. Only one case had a trisomy 12 as the main anomaly, which could suggest CLL, but neither the cytology simulating polyclonal B-cell lymphocytosis, nor the immunophenotype (CD5-positive, CD23-negative with a score of 2) nor the splenic histology (biphasic pattern) was consistent with a diagnosis of CLL. Furthermore, trisomy 12 has also been described as a recurrent anomaly in SMZL.[15] The observed *IGHV* mutation pattern was also distinct from that in CLL, with more cases mutated (80% *versus* 50%) and the VH1-2*04 segment, previously identified in SMZL[16,27,28] and present here in 20% of the cases, is very infrequent in CLL (4%).[27] Furthermore, no ongoing mutation was observed and the length of the CDR3 was variable, contrasting with the findings in CLL.[29] In the present series, typical MCL was never suggested by the findings in the blood films or splenic sections, the diagnosis of a possible small round cell variant was ruled out by a systematic search for t(11;14)(q13;q32) on blood films by FISH; moreover none of the cases expressed cyclin D1 in tissue sections. The diagnosis of MCL could, therefore, be definitively excluded, since the diagnosis of MCL without a t(11;14)(q13;q32) requires a typical morphology and phenotype.[30,31] On the other hand there were overlapping features between LPL, especially Waldenström's macroglobulinemia (WM), which may be CD5-positive in blood, and SMZL with a monoclonal component especially in the case of plasmacytic differentiation.[32] In the present series, while the presence of a monoclonal component (13/24 cases) could have suggested the diagnosis of LPL or WM, only five cases displayed true plasma cells in spleen, but showed an otherwise typical biphasic pattern. In addition, four of five cases showed trisomy 3, 7q deletion or trisomy 18, known to be recurrent in SMZL. Another case showed a trisomy 4, which has so far been considered specific to WM, but displayed a biphasic pattern in the spleen. Likewise, although five of our cases showed diffuse bone marrow involvement, which is rare in SMZL, four had a typical SMZL histology on spleen sections. However, the distinction between LPL and SMZL is not always clear-cut and further studies are need to clarify their relationships.

In fact, these CD5-positive SMZL cases have many similarities with classical CD5-negative SMZL cases, including their clinical presentation, cytological, morphological and immunological features, cytogenetics and molecular profile. This suggests that they could arise from a common B cell of the marginal compartment differing only by CD5 expression. While the tendency to a more frequently mutated *IGHV* status, as well as to less frequent 7q deletion and VH1-2 usage in CD5-positive cases could argue for a possible different lymphomagenesis pathway, these

differences were not statistically significant and could have been due simply to the small size of the series. The higher lymphocytosis observed at the time of diagnosis in the CD5-positive SMZL, which was not associated with greater tumor mass, was likely the result of a greater propensity of the CD5-positive cells to recirculate, as already proposed.[33] This is reminiscent of what is observed in other CD5-positive small B-cell lymphomas, such as CLL and MCL, illustrating the fact that CD5 expression by B cells promotes blood dissemination whatever the cell of origin. The CD5-positive B cells in SMZL may, therefore, represent a state of development and/or activation of the same B-cell lineage as that in CD5-negative SMZL.

In the present study, CD5 expression did not appear to influence the outcome or overall survival in contrast to the findings of other studies involving small numbers of cases.[4,5] We did not find any other feature correlated with prognosis. In particular, whereas we had shown in a previous study that a leukocytosis of more than 20×10^9/L was correlated with a shortened survival in SMZL,[34] this was not confirmed in the present series and could be explained by biases in the selection of cases (e.g. percentage of CD5-positive cases, treatment of the patients, median follow-up).

In conclusion, this study demonstrates that CD5 expression in SMZL (observed by flow cytometry as well as by immunohistochemical staining of spleen sections) is not an incidental finding, occurring in 25% of cases in our department) and that the CD5-positive SMZL cases are closely related to common CD5-negative SMZL cases. Whether or not they constitute a true subset of SMZL will, however, require the study of more cases with precise cytogenetic and molecular data.

Authorship and Disclosures

LB performed flow cytometry analyses, analyzed data and wrote the manuscript. ATG reviewed histological specimens, performed *IGHV* mutational pattern analysis and analyzed data. FP collected and analyzed data. ECB performed cytogenetic research and analyzed data. FB collected the cases, reviewed histological specimens and analyzed data. MF reviewed bone marrow biopsies and analyzed data. CMC performed statistical analyses. CT contributed patients' samples, performed molecular analyses and analyzed clinical data. DM reviewed cytological specimens. BC contributed patients' samples. GS contributed patients' samples, analyzed clinical data, performed statistical analyses and wrote the manuscript. PF designed the research, selected the cases, reviewed cytological specimens, analyzed data and wrote the manuscript.

The authors reported no potential conflicts of interest.

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