Somatic hypermutation of *IGVH* genes and aberrant somatic hypermutation in follicular lymphoma without *BCL-2* gene rearrangement and expression

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ABSTRACT

Background

Follicular lymphoma is characterized by the t(14;18) translocation resulting in constitutive expression of BCL-2 protein; however approximately 10-15% of follicular lymphomas do not express BCL-2 protein, and a small fraction of these cases does not exhibit translocation of the *BCL-2* gene either. It is highly debated whether cases of follicular lymphoma without *BCL-2* gene rearrangement and expression represent a separate lymphoma entity with distinct biological characteristics, different from the BCL-2-positive cases.

Design and Methods

To further characterize follicular lymphomas without *BCL-2* gene rearrangement and expression, we analyzed and compared the mutational status of *IGVH* genes as well as other genes (*C-MYC*, *PAX-5* and *RHOH*) frequently involved in the specific type of genomic instability called aberrant somatic hypermutation in 11 cases of BCL-2-negative and 7 cases of BCL-2-positive follicular lymphomas. We also determined the levels of expression of activation-induced cytidine deaminase in these cases.

Results

The analyzed cases were grade 2 and grade 3A follicular lymphomas. Our findings demonstrate that follicular lymphomas without *BCL-2* gene rearrangement and expression are associated with ongoing somatic hypermutation of the *IGVH* genes, low activity of aberrant somatic hypermutation and elevated activation-induced cytidine deaminase expression. These results were in concordance with the results found in the cases of BCL-2-positive follicular lymphoma.

Conclusions

Although, BCL-2 protein overexpression is considered to be a critical pathogenic event in the development of follicular lymphoma, our findings suggest that follicular lymphomas with the same morphology, immunophenotype, mutational pattern and activation-induced cytidine deaminase expression may develop without the involvement of *BCL-2* gene. The present data support the hypothesis that BCL-2-positive and BCL-2-negative follicular lymphomas (grades 1-3A) represent a homogenous group with different initial but several common additional molecular pathways.

Key words: follicular lymphoma, somatic hypermutation, aberrant somatic hypermutation, activation-induced cytidine deaminase.

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Introduction

Follicular lymphoma (FL) is the most prevalent form of indolent B-cell lymphomas, accounting for up to 20-35% of all non-Hodgkin's lymphomas, and is recognized as a distinct entity in the World Health Organization (WHO) classification of lymphoid tumors. Based on the proportion of centroblasts within the malignant follicles, FL are divided into grade 1 (predominantly centrocytes), grade 2 (centrocytes and centroblasts), grade 3A (predominantly centroblasts, but centrocytes are still present) and grade 3B (solid sheets of centroblasts) categories.[1] Approximately 85-90% of FL carry the t(14;18)(q32;q21) chromosomal translocation, juxtaposing the *BCL-2* gene with the immunoglobulin heavy chain (*IGH*) gene, resulting in the constitutive expression of BCL-2 protein, which induces prolonged cell survival by blocking programmed cell death.[2-4] FL are considered to derive from the germinal center (GC) B cells, based on the immunophenotype of tumor cells, accumulation of somatic hypermutation (SHM) in their immunoglobulin (*IG*) genes and expression of activation-induced cytidine deaminase (AID) required for SHM.[5,6] In FL, SHM may aberrantly target several proto-oncogenes relevant in lymphomagenesis including *BCL-6*, *PIM-1*, *PAX-5*, *RHOH* and *C-MYC*.[7,8]

Approximately 10-15% of FL do not express BCL-2 protein, and do not exhibit the t(14;18) chromosomal translocation.[9] Several reports suggest that FL without translocation and expression of *BCL-2* gene have distinct morphological, genetic and molecular characteristics that distinguish them from the BCL-2-positive FL. These BCL-2-negative FL are frequently grade 3 lymphomas, however BCL-2-negative cases were also observed in lower grade groups.[10-14] Furthermore, grade 3B cases frequently harbor translocation of the *BCL-6* gene,[15-17] carry trisomy of chromosome 3, and gain of chromosome 18 or 18q.[18] It is currently unclear whether this lymphoma derives from GC or post-GC B cells. Based on the immunophenotype ($CD10^-$, $MUM1^+$) and frequent *BCL-6* gene rearrangement, it has been suggested that these BCL-2-negative FL may have a *late-stage GC* or *post-GC* cell origin, and are probably more closely related to diffuse large B-cell lymphomas (DLBCL).[10,18] However, in a recently published study based on gene expression analysis, Piccaluga *et al.*[19] suggested that grade 3B FL does after all belong to the group of FL rather than DLBCL. The authors also propose a possible revision of the histological grading of FL, with their simple distinction into FL (grade 1-3A) and FL/large cell (grade 3B).

To provide further insight into the molecular pathways of lymphomagenesis and to reveal the cellular origin of FL without *BCL-2* involvement, we characterized the mutational pattern of *IGVH* genes and certain genes affected by aberrant SHM, as well as the expression level of AID in these lymphomas.

Design and Methods

Pathological samples

Lymph node biopsies from 18 patients with FL were selected for this study, based on the availability of frozen tissue for molecular analyses and formalin-fixed paraffin-embedded tissue for immunophenotyping and fluorescence *in situ* hybridization (FISH) analysis. Diagnoses were based on histopathology, immunophenotype, and result of FISH and molecular analyses, and classified according to the WHO classification of lymphoid tumors (1). The clinical, morphological and immunohistochemical data and the results of FISH analysis are summarized in Table 1.

The study was approved by the local Institutional Review Board and was conducted in accordance with the Helsinki Declaration. All patients provided informed consent to the analysis of their data.

Immunohistochemical analysis

The phenotype of lymphoma cells was characterized using the three-step avidin-biotin immunoperoxidase method with the following monoclonal antibodies: CD20, BCL-6, ki-67 (DAKO, Carpinteria, CA, USA), CD10, CD21, and CD23 (Novocastra Laboratories, Newcastle upon Tyne, England). Follicular dendritic cells were detected by CD21 (Novocastra) and CD23 (Novocastra) antibodies. Three monoclonal antibodies against different epitopes were used to study BCL-2 protein expression: bcl-2 Monoclonal Mouse Anti-Human Antibody (Clone 124, DAKO, Carpinteria, CA, USA) was used as the standard, whilst two additional antibodies bcl-2 Mab#c2 (Santa Cruz Biotechnology Inc, Santa Cruz, USA) and bcl-2 Mab#6C8 (Pharmingen, Franklin Lakes, USA) were used to confirm the results. In this way those cases in which BCL-2 protein expression was lost as a result of somatic mutations of the *BCL-2* gene were excluded. Furthermore, AID antibodies were used (anti-AID mouse monoclonal antibody clone 7E7, Cell Signaling Technology, Inc., USA) to detect the AID protein level in all 18 cases.

Fluorescence in situ *hybridization analysis*

FISH analysis was accomplished using a commercially available LSI *BCL-2* dual-color break-apart rearrangement probe (18q21), LSI *BCL-6* dual-color break-apart rearrangement probe (3p27) and LSI *IGH/BCL-2* dual-color, dual-fusion translocation probe set (14q32, 18q21) (Vysis, Downers Grove, IL, USA). At least 200 interphase nuclei per probe were evaluated in each reaction. Cases which lacked the t(14;18) translocation, but in which split signals were present involving the *BCL-2* gene, were excluded from further analysis.

Polymerase chian reaction amplification, cloning, sequencing and sequence analysis of IGVH *genes*

Genomic DNA isolation from tumor tissue specimens was performed according to the standard salting-out procedure.[20] DNA samples were amplified by PCR,

Table 1. Clinical, morphological and immunohistochemical data and results of FISH analysis of patients with and without *BCL-2* translocation and expression.

Case N.	Age (years)	Sex	Grade	Immunohistochemical analysis[a]							FISH		
				CD20(%)	CD10(%)	CD21/23	BCL-2(%)	BCL-6(%)	Ki-67(%)	AID(%)	t(14;18)	BCL-2 split	BCL-6 split
1	49	Male	3A	100	2[b]	+	–	90	25	15	-	-	–
2	46	Female	3A	100	100	+	–	30	15	60	-	-	not informative
3	60	Male	2	100	100	+	–	100	10	10	-	-	–
4	44	Male	3A	100	70	+	–	70	10	40	-	-	–
5	49	Female	3A	100	5[b]	+	–	80	15	10	-	-	–
6	74	Male	3A	100	100	+	–	60	10	15	-	-	–
7	33	Female	3A	100	100	+	–	80	25	40	-	-	40% - 1 split
8	48	Female	2	100	100	+	–	80	15	10	-	-	–
9	65	Male	3A	100	100	+	–	85	40	15	-	-	55%- 3-4 signals
10	73	Female	3A	100	70	+	–	60	35	50	-	-	–
11	71	Male	3A	100	100	+	–	40	70	10	-	-	60% - 1 split
12	57	Male	3A	100	100	+	100	100	25	50	+	ND	–
13	60	Female	3A	100	80	+	85	70	40	80	+	ND	60% - 3 signals
14	33	Female	3A	100	100	+	100	100	40	35	+	ND	ND
15	66	Female	3A	100	100	+	50	40	45	40	+	ND	–
16	78	Female	3A	100	80	+	95	70	20	30	+	ND	ND
17	50	Male	2	100	100	+	60	60	40	50	+	ND	–
18	60	Male	2	100	100	+	90	90	30	80	+	ND	–

ND: not determined. [a]CD21 and CD23 positivity was detected on the follicular dendritic cells, while the other markers stained the tumor cells. Number values indicate the percentage of cells expressing the marker in each sample. [b]Cases 1 and 5 were considered CD10 negative.

using sense *IGVH* gene family-specific (V_H1-V_H6) leader primers in conjunction with an antisense consensus J_H primer in independent reactions. Amplicons were cloned using the pCR 2.1-TOPO TA Cloning Kit (Invitrogen Corporation, San Diego, CA, USA). After the transformation of competent cells, colonies found to contain the appropriate insert of plasmid DNA were sequenced by an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). In all samples 20 sequences from independent bacterial isolates were analyzed. The corresponding germline *IGVH* gene sequences were determined, using the IMGT/V-QUEST (International ImMunoGeneTics Information System, *http://imgt.cines.fr*) and the NCBI GenBank databases. The binomial distribution model proposed by Chang and Casali[21] was used to determine whether the probability of the excess of replacement mutations in the complementary determining regions and the scarcity of silent mutations in the framework regions resulted from chance alone or were the consequence of antigenic selection. The results were then compared with results obtained by the multinomial distribution model suggested by Lossos *et al.*, using the JAVA applet available at *http://www-stat.stanford.edu/immunoglobin*.[22]

Mutational analysis of C-MYC, PAX-5 *and* RHOH *genes*

PCR amplification was performed in two independent reactions using the Phusion™ High-Fidelity DNA Polymerase system (Finnzymes, Finland) containing a low-error DNA polymerase. *C-MYC* (exon 1 and exon 2), *PAX-5* and *RHOH* genes were PCR-amplified as described previously.[23] Direct sequencing of purified amplicons from both sides was carried out with an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The obtained sequences were compared to the corresponding germline gene sequences available in the NCBI GenBank database. The accession numbers have already been published.[8] Previously reported polymorphisms and mutations appearing more than once in separate cases, thus considered as polymorphic variants, were excluded from further analyses.

Quantitative real-time polymerase chain reaction analysis of AID *mRNA expression*

Analysis of *AID* expression was carried out in eight cases of BCL-2-negative FL and seven cases of BCL-2-positive FL. Mononuclear cells from peripheral blood of eight healthy volunteers were used as negative controls and GC microdissected from five reactive lymph nodes, using a Leica laser microdissection system (Leica Microsystems, Wetzlar, Germany), were used as positive controls. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer. RNA (2.5 μg) was reverse transcribed to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The quantitative real-time PCR assay was performed on an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). For amplification of AID mRNA a TaqMan® based Gene Expression Assay (Applied Biosystems) was used. The *AID* primers chosen amplified the wild-type and several splice variants of *AID*. In order to normalize the *AID* values, *β-actin* was amplified using a pre-developed TaqMan® Control Reagent (Applied Biosystems). All samples were run in triplicate, in a 20 μL reaction volume that contained 100 ng of cDNA. Sequence Detection Software version 1.3 (Applied Biosystems) was used to analyze the data. Results were obtained as threshold cycle (C_T) values. C_T represents the cycle number at which fluorescence passes a fixed threshold. The levels of *AID* expression in the samples were normalized to *β-actin* expression.

Results

FISH analysis

In cases of BCL-2-negative FL the dual-fusion assay for the t(14;18) translocation showed no evidence of reciprocal translocations, and the absence of any translocations involving the *BCL-2* locus was confirmed by the lack of separated signals using the *BCL-2* break-apart probe. Using the *BCL-6* break-apart probe we detected two split signals in cases 7 and 11; in case 2 the result was not informative, and three or four signals were seen in case 9 and three signals in case 13.

Sequence analysis of IGVH genes

PCR analysis resulted in amplification in 13 cases; in five samples (cases 3, 4, 8, 13 and 18) *IGVH* gene mutations were not analyzed because of unsuccessful amplification. The closest germline *VH* gene, sequence homology and mutational frequency of *IGVH* genes are detailed in Table 2. To determine whether the tumor cells of FL had been under pressure for antigen selection, we analyzed the somatic mutations of all *IGVH* sequences amplified. In all analyzed sequences the binomial distribution model revealed more replacement mutations in the complementary determining regions, and in most cases fewer replacement mutations in the

Table 2. Analysis of the distribution of somatic mutations of *IGVH* genes in cases of follicular lymphoma with and without translocation and expression of *BCL-2*.

Case N.	Closest germline gene	Sequence homology (%)	Mutations /100 bp[4]	Range of mutation frequency (%)[b]	Intraclonal divergence N. of clones	CDR1 and CDR2 R	CDR1 and CDR2 S	CDR1 and CDR2 P_B-value	FR1, FR2 and FR3 R	FR1, FR2 and FR3 S	FR1, FR2 and FR3 p_B-value	P_M
1	VH3-30*03	90.73-91.12	9.44	8.46-10.48	6	7 (1.16)	2	<0.001[c]	10-11 (8.01-8.64)	3	0.101-0.127	0.011[c]-0.17
2	VH4-34*01	86.12-86.53	13.66	13.46-13.87	8	8 (1.06)	1	<0.001[c]	14 (15.23-15.87)	10-11	0.118-0.142	0.004[c]-0.028[c]
3	NA	-	-	-	-	-	-	-	-	-	-	-
4	NA	-	-	-	-	-	-	-	-	-	-	-
5	VH3-23*01	83.06-88.3	13.27	12.5-16.58	4	9-13 (1.51-1.68)	0-3	<0.001[c]	13-19 (10.39-18.35)	4-11	0.09-0.145	<0.001[c]-0.074
6	VH3-7*01	92.74-97.58	6.78	2.41-9.67	6	0-2 (0.69)	0-3	<0.001[c] -0.122	4-10 (3.73-8.11)	2-3	0.081-0.321	<0.001[c]-0.796
7	VH4-34*01	90.2-90.61	9.73	9.38-9.79	6	7-8 (0.87-0.98)	0	<0.001[c]	12 (10.1)	4	0.135	0.003[c]-0.143
8	NA	-	-	-	-	-	-	-		-	-	-
9	VH3-48*03	88.7-89.91	11.04	10.48-13.3	7	8 (1.33)	2	<0.001[c]	7-9 (9.33-11.28)	8-9	0.064-0.101	<0.001[c]-0.017[c]
10	VH3-33*01	89.87-90.68	9.76	9.27-10.08	7	4 (0.81-0.95)	2-3	0.003[c] -0.007[c]	9-10 (10.65-11.9)	8-9	0.101-0.154	0.003[c]-0.378
11	VH3-30*03	71.37-71.77	28.41	28.22-28.62	7	13 (2.49)	5	<0.001[c]	41-42 (31.99-32.61)	11	0.002[c]-0.003[c]	0.131-0.338
12	VH4-34*01	89.39-90.2	10.04	9.79-10.61	5	3 (3.1-4.95)	1-2	0.138-0.238	10-11 (13.46-14.95)	8-9	0.046[c]-0.059	0.013[c]-0.526
13	NA	-	-	-	-	-	-	-		-	-	-
14	VH3-48*02	89.11-89.92	10.8	10.08-10.88	3	11 (3.42-3.56)	2	<0.001[c]	7-9 (15.63-16.88)	5	<0.001[c]	<0.001[c]
15	VH3-48*02	86.27-87.12	12.41	12.09-13.33	7	9-10 (3.79-4.09)	2-3	0.003*-0.006[c]	16-17 (18.6-20.46)		3-50.065-0.09	0.003[c]-0.214
16	VH3-9*01	89.11-90.32	10.56	10.08-11.29	7	10 (3.2-3.59)	1	<0.001[c]	9-12 (15.47-17.32)	5	0.005[c]-0.019[c]	<0.001[c]-0.023[c]
17	VH3-9*01	83.87-84.68	15.69	15.32-16.12	4	14 (4.95-5.21)	1	<0.001[c]	17-18 (23.75-24.82)	6-7	0.011[c]	<0.001[c]-0.019[c]
18	NA	-	-	-	-	-	-	-	-	-	-	-

CDR: complementary determining region; FR: framework region; R: number of observed and (expected) replacement mutations; S: number of detected silent mutations, PB-value, the probability that this scarcity occurred by chance, according to the Chang-Casali binomial distribution model; P_M: probability according to the multinomial distribution model of Lossos et al.; [a]All sequence variants resulted from single nucleotide changes, except for case n. 15. in whom deletions were also detected, and all of these mutations seemed to represent functional rearrangements since no stop codons or crippling mutations were found; [b]The mutation frequency was obtained by dividing the number of mutations by the total number of nucleotides examined; [c]statistically significant (p<0.05); NA, not amplified.

framework regions than could be expected due to chance alone, with significant (p<0.05) clustering. The results obtained by the multinomial model revealed statistically significant p values, except in one case. Taken together, our results indicate the presence of antigen selection in these clones.

Mutational analysis of C-MYC, PAX-5 *and* RHOH *genes*

Eight cases of BCL-2-negative FL and seven cases of BCL-2-positive FL were subjected to mutational analysis. The position and type of mutations found are reported in Table 3, and the mutation frequencies are shown in Table 4.

Analysis of AID *mRNA and protein expression*

To compare the *AID* expression of BCL-2-negative and positive FL, we measured the levels of expression of *AID* mRNA in 15 cases of FL (8 BCL-2-negative and 7 BCL-2 positive) by quantitative real-time PCR assay. The data obtained as relative expression values are shown in Figure 1. These results were calculated as the mean of three independent measurements. The levels of *AID* mRNA were defined as a ratio of *AID* to *β-actin* expression. We also detected the AID protein levels in all 18 cases. The protein was predominantly localized in the cytoplasm. No correlation was observed between *AID* mRNA and protein expression levels, but due to the relative small number of examined cases we were not able to make major conclusions.

Discussion

The t(14;18) translocation and BCL-2 overexpression are believed to be strongly associated with the development of FL,[2,3,9] hence, the absence of these abnormalities in B-cell neoplasms may point to a substantially different transformational pathway and a separate entity. To characterize a possible homogenous subtype of FL in which overexpression of *BCL-2* gene is not involved, we selected FL cases in which neither translocation nor expression of *BCL-2* was detected. Using strict selection criteria, we excluded those cases in which tumor cells carried the t(14;18) translocation, but BCL-2 expression was lost as a result of somatic mutations of the translocated gene, or cases which lacked the t(14;18) translocation, but BCL-2 was overexpressed by different alternative mechanisms.[24,25] The GC origin of these lymphomas was supported by a follicular growth pattern, the BCL-6 and CD10 (except in cases 1 and 5) expression of the tumor cells, and by the presence of CD21 and CD23-positive follicular dendritic reticulum cells within the neoplastic follicles. Morphological analysis of these BCL-2-negative FL showed two cases of grade 2 and nine cases of grade 3A FL; there were no cases of grade 3B FL. In our series, only three out of 11 BCL-2-negative samples showed *BCL-6* gene alterations, in contrast with previous reports, in which a significant fraction of FL without the t(14;18) translocation harbored alterations of the *BCL-6* gene.[12,17] However, many *BCL-6* breakpoints are localized with several hundred kilobases upstream of the major breakpoint region, so they could still have been missed in the present series.[10] *BCL-6* alterations were also observed in one BCL-2-positive FL from the control group.

The SHM process of *IG* genes is a characteristic feature of FL.[26] *IGVH* sequence analysis of FL lacking *BCL-2* translocation and expression revealed ongoing somatic mutations generating intraclonal heterogeneity of the tumor clones. The average mutational frequency

Table 3. Distribution and type of mutations found in *C-MYC*, *PAX-5* and *RHOH* genes in follicular lymphoma cases with and without translocation and expression of *BCL-2*.

Case N.	C-MYC exon 1	C-MYC exon 2	PAX-5	RHOH
1	3397 (C>T)	–	1087 (C>A)	–
2	–	–	–	–
3	–	–	–	–
4	–	–	–	–
5	3442 (T>A)	–	–	–
6	3283 (C>T)	–	–	–
7	-	4658 (C>A)	ND	–
8	3542 (C ins)	–	1070 (G>A)	–
9	ND	ND	ND	ND
10	ND	ND	ND	ND
11	ND	ND	ND	ND
12	2406 (A ins)	–	1070 (G>C)	–
13	–	–	1267 (A ins)	–
14	–	–	1265 (G>A)	–
15	–	–	–	–
16	3259 (A>T)	–	–	–
17	–	–	1108 (C>G)	–
18	–	4539 (T>C)	–	–

ND: not determined.

Table 4. Mutational patterns of *C-MYC*, *PAX-5* and *RHOH* genes in cases of follicular lymphoma with and without translocation and expression of *BCL-2*.

Gene	Mutation frequency/ 100bp[a]	Single bp substitutions	Insertions or deletions	Transitions/ transversions	G+C/ A+T	RGYW[b]
			BCL-2 negative FL			
C-MYC exon1	0.038	3	1	2/1	2/1	0/3
C-MYC exon2	0.086	1	0	0/1	1/0	0/1
RHOH	0	0	0	0	0	0
PAX-5	0.062	2	0	1/1	2/0	1/2
All genes	–	6	1	3/3	5/1	1/6
			BCL-2 positive FL			
C-MYC exon1	0.038	1	1	0/1	0/1	0/1
C-MYC exon2	0.086	1	0	1/0	0/1	1/1
RHOH	0	0	0	0	0	0
PAX-5	0.062	3	1	1/2	3/0	2/3
All genes	–	5	2	2/3	3/2	3/5

[a]Mutation frequencies were calculated on the entire region analyzed and on mutated cases only, taking into account two alleles; [b]RGYW shows number of mutations embedded in this mutational motif (R= A, G; Y= C, T; W= A, T).

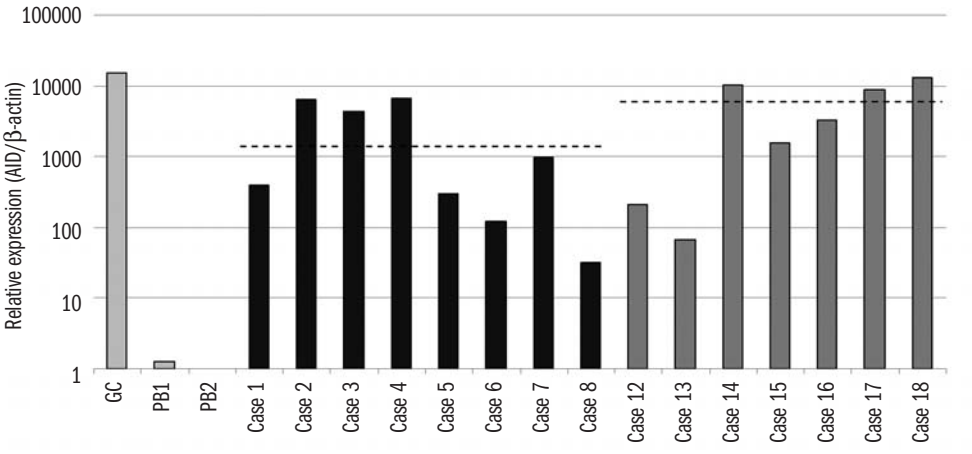

Figure 1. Activation-induced cytidine deaminase (AID) mRNA expression in follicular lymphoma with and without BCL-2 translocation and expression. Quantitative real-time PCR analysis of AID and β-actin was performed on germinal center B cells (GC; out of five analyzed GC, only one is represented), peripheral blood (PB) mononuclear cells (out of eight analyzed PB samples only PB1 and PB2 are shown), BCL-2-negative follicular lymphoma (Cases 1-8) and BCL-2-positive follicular lymphoma (Cases 12-18). Each column represents the average value of three independent AID/β-actin measurements. The dashed lines represent the average level of the relative expression values of follicular lymphoma without (Cases 1-8) and with (Cases 12-18) the presence of BCL-2. The difference is not statistically significant ($p>0.05$).

within the *IGVH* region of FL cases without *BCL-2* gene rearrangement and expression was 12.76% (range 2.41% to 28.62%) which is similar to the average 11.9% frequency (range 9.79% to 16.12%) found in FL harboring the t(14;18) translocation. The pattern and distribution of these mutations were highly consistent with antigen selection as calculated by the binomial and the multinomial distribution models.[21,22] Taken together, these results indicate that cases of FL without *BCL-2* gene rearrangement and expression are derived from GC B cells, and that this subtype of FL is not distinct from FL carrying the t(14;18) translocation, based on the mutational pattern of *IGVH*.

According to a widely accepted recent hypothesis a multistep model of tumorigenesis is instrumental in the pathogenesis of FL.[9] The initiating genetic event is the translocation of t(14;18), causing constitutive expression of the anti-apoptotic BCL-2, but antigenic stimulation and signaling through the antigen receptor is required for neoplastic transformation. Using protein microarray analysis Zha *et al.*[27] showed that *BCL-X*$_L$ or AKT/BAD pathways may provide an alternative anti-apoptotic signal in FL in the absence of BCL-2 protein, and our study may provide evidence that the GC microenvironment and antigenic selection are also instrumental in the development of BCL-2-negative FL. These data suggest that BCL-2-positive and BCL-2-negative FL may have different molecular alterations at the starting point of lymphomagenesis, but in both cases the immunoglobulin receptor complex provides additional signal(s) required for malignant transformation.

SHM occurs during the centroblast stage of B-cell maturation and requires the presence of AID, an enzyme that is expressed specifically in GC B cells,[6] while the constitutive expression of AID is associated with different GC-derived B-cell non-Hodgkin's lymphomas.[28] To analyze whether AID expression is associated with the pathogenesis of BCL-2-negative FL, we measured *AID* mRNA expression in eight cases of BCL-2-negative FL and compared the results to those of seven BCL-2-positive FL. *AID* mRNA was expressed at various levels in both BCL-2-positive and negative FL, but did not reach the level detected in normal GC cells. AID expression of FL may reflect the GC origin of the tumor cells as has also been suggested previously by the presence of somatic mutations of *IGVH* genes of the tumor cells. This is consistent with previous findings showing coincidence of AID expression with the ongoing type of SHM of *IG* genes in FL.[5] The fact that we did not find significant differences in *AID* mRNA expression levels between BCL-2-positive and negative FL adds further support to the concept that these two variants of FL belong to the same entity.

SHM and AID activity have also been implicated in the alterations of proto-oncogenes such as *PIM-1*, *PAX-5*, *RHOH* and *C-MYC*, which are involved in the pathogenesis of different types of B-cell lymphomas.[6,9] The rates of aberrant SHM we found in FL without *BCL-2* rearrangement and expression were lower than those previously reported in DLBCL,[8] but similar to those reported for BCL-2-positive FL[29,30] and similar to our findings in the BCL-2-positive FL, suggesting that both BCL-2-positive and negative FL are targeted by a low frequency of aberrant SHM, and BCL-2 expression does not influence the incidence of aberrant SHM in FL.

Although, BCL-2 protein overexpression is considered to be a critical pathogenic event in the development of FL,[2,4] our findings suggest that FL may develop even without the involvement of the *BCL-2* gene. The similarities of morphology, immunophenotype, mutational pattern and AID expression support the hypothesis that besides the initial step of tumorigenesis, BCL-2-positive and negative FL (grades 1-3A) represent the same entity with several molecular pathways in common.

Authorship and Disclosures

EG: designed and performed the research, collected and analyzed data and wrote the paper; ZB, CB, BT, and LR: designed and performed the research, analyzed and interpreted data; LD: performed the research; JC, BC, and AS: provided cases, interpreted results, revised the manuscript critically; AM: research conception and design, collected cases, analyzed and interpreted data, wrote the paper. All co-authors actively participated in the preparation of the manuscript. All authors gave their approval to the final version. The authors reported no potential conflicts of interest.

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