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Original contribution

The BCL2 E17 and SP66 antibodies discriminate 2 immunophenotypically and genetically distinct subgroups of conventionally BCL2-“negative” grade 1/2 follicular lymphomas☆

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Summary Follicular lymphoma (FL) is characterized by the translocation t(14;18)(q32;q21) resulting in constitutive overexpression of BCL2. However, in 10% to 15% of FL grade 1/2, immunohistochemical staining for BCL2 remains negative. To analyze the incidence of BCL2 negativity and the underlying molecular mechanisms in FL grade 1/2, BCL2 expression was investigated with 3 antibodies (clones 100D5, E17, SP66). The presence of a break in the *BCL2* locus was determined by fluorescence in situ hybridization. The region of the *BCL2* gene where the epitope of the standard BCL2 antibody resides was sequenced. Twenty-two (9.2%) of 240 identified cases of FL grade 1/2 were negative with the standard BCL2 antibody. Of these, 12 cases (55%) carried a break in the *BCL2* gene locus, which, in all but one case, correlated with BCL2 expression using the alternative antibodies E17 and SP66 and with missense mutations of *BCL2*. Ten (45%) of the 22 cases had an intact *BCL2* gene locus; 2 cases carried a *BCL6/IGH* translocation. All 10 cases were negative for the E17/SP66 antibodies and showed a wild-type sequence of *BCL2*. Six of these showed an aberrant phenotype, with CD10 negativity (30%) or CD23 expression (30%). In summary, the alternative E17/SP66 antibodies identify 2 immunohistochemically and genetically distinct subgroups of BCL2-“negative” FL grade 1/2.
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1. Introduction

Follicular lymphoma (FL) is characterized by the chromosomal translocation t(14;18)(q32;q21), resulting in constitutive overexpression of the antiapoptotic protein BCL2 [1]. However, in 10% to 15% of FL grade 1/2,

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immunohistochemical staining for BCL2 remains negative [1]. Increasing evidence suggests that the pathogenesis of BCL2-negative FL grade 1/2 without a t(14;18) translocation is different from those FL cases carrying a t(14;18) [2,3]. The t(14;18)-negative FLs are often characterized by lack of CD10 expression and increased proliferation rate [2]. In addition, a recent study demonstrated that FL grade 1/2 with and without t(14;18) also differ in their miRNA profiles [3]. FL without t(14;18) revealed a characteristic miRNA expression profile indicating a late germinal center B-cell phenotype.

Interestingly, in the last years, it has been reported that in some immunohistochemically BCL2-negative FL, a t(14;18)(q32;q21) can, nevertheless, be demonstrated by interphase fluorescence in situ hybridization (FISH) [4]. In addition, some of these t(14;18)-positive cases were shown to carry missense mutations of the *BCL2* gene, suggesting that the BCL2 protein pseudonegativity in these cases is the result of structural protein modifications that interfere with the binding of the standard BCL2 antibody (clone 100/D5) [5,6]. Recently, 2 novel BCL2 antibodies—clones E17 and SP66—recognizing different parts of the BCL2 protein have become available. Therefore, the aims of this study were (1) to determine the incidence of BCL2 "negativity" in our series of FL grade 1/2 using the standard antibody (clone 100/D5), (2) to analyze the presence of breaks in the *BCL2* locus in the BCL2-"negative" FL grade 1/2 by FISH, (3) to correlate the presence of *BCL2* breaks with the expression of BCL2 using 2 alternative BCL2 antibodies (clones E17 and SP66), and (4) to determine the mutation status of the *BCL2* gene in the cases with and without detectable alterations of the *BCL2* locus.

2. Materials and methods

2.1. Case selection

All FL grade 1/2 cases diagnosed between January 2005 and August 2011 at the Institute of Pathology of the University of Tübingen, Germany, from which sufficient tissue was available, were included in the study. Cases were reevaluated using hematoxylin and eosin (H&E) and Giemsa stains and standard diagnostic immunohistochemistry. The grading of FL was performed following the recommendations of the 2008 World Health Organization classification of tumors of hematopoietic and lymphoid tissues [1]. FL cases with predominantly grade 3A and 3B morphology were excluded from the study. The study was approved by the local ethics committee.

2.2. Immunohistochemical analysis

All FL cases were stained for CD20 (DAKO, Hamburg, Germany; dilution 1:500), CD10 and CD23 (both from Novocastra, Berlin, Germany; both dilution 1:30), CD3 (DCS, Hamburg, Germany; dilution 1:100), BCL6 (Zytomed, Berlin, Germany; dilution 1:25), MIB1 (Ki-67; DAKO; dilution 1:200), and BCL2 (clone 100/D5 against residues amino acid [aa] 41-54; DAKO; dilution 1:50). Cases that were negative in the staining with BCL2 (100/D5) were additionally stained with the 2 alternative BCL2 antibodies, clone E17 (Zytomed; dilution 1:50, against residues aa 61-76) and SP66 (Cell Marque, Rocklin, CA; dilution 1:100, against the N-terminal portion of the protein). FL cases that remained negative with all 3 BCL2 antibodies were additionally stained for the germinal center–associated markers LIM domain only 2 (LMO2; clone 1A9-1; Ventana Roche, Tucson AZ, ready to use) and human germinal center–associated lymphoma (HGAL; Zytomed; dilution 1:400) as well as IRF4/MUM1 (DAKO; dilution 1:200). Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections on an automated immunostainer (Ventana Medical Systems, Tucson AZ), following the manufacturer's protocols.

Statistical significance of the differences in MIB1 levels between cases with and without *BCL2* break was evaluated using the Wilcoxon rank sum test.

2.3. Fluorescence in situ hybridization

In all BCL2 (100/D5)-negative FL cases, FISH analysis was performed using a break-apart probe for the *BCL2* gene locus (Vysis LSI *BCL2* Dual Color Break Apart Rearrangement Probe; Abbott Molecular, Wiesbaden, Germany), suggestive of the chromosomal translocation t(14;18)(q32;q21). In all cases negative for *BCL2* breaks, a FISH-based analysis of the *IGH* gene locus was performed (Vysis LSI *IGH* Dual Color Break Apart Rearrangement Probe; Abbott Molecular). In the cases with a break detected in the *IGH* gene locus, subsequently, the status of the *BCL6* gene locus (Vysis LSI *BCL6* Dual Color Break Apart Rearrangement Probe; Abbott Molecular) was analyzed.

2.4. Clonality analysis

DNA used for polymerase chain reaction (PCR) was extracted from 10-μm paraffin sections after dewaxing and proteinase K digestion, applying standard phenol-chloroform purification procedures. The PCR for the detection of *IGH* gene rearrangements using framework 2 and 3 primers was performed as previously described, using 0.5 U Phusion Hot Start DNA Polymerase (Finnzymes, Woburn, MA) and 200 ng of genomic DNA [5,7]. Modified amplification conditions were performed with an initial denaturation step of 98°C (30 seconds), 40 cycles (98°C 10 seconds, 60°C 30 seconds, 72°C 30 seconds), and a final step of 10 minutes. The JH primer was modified with D4 fluorescent dyes (Sigma-Aldrich, St Louis, MO). For GeneScan analysis, 0.5 μL of the PCR products was mixed with sample loading solution containing 0.24 μL DNA Size Standard 400 (Beckman Coulter, Brea, CA). The products were separated

by capillary electrophoresis on the GenomeLab GeXP Genetic Analysis System and analyzed by the GenomeLab GeXP software 10.2 (Beckman Coulter).

2.5. Sequence analysis of *BCL2*

Assignment of *BCL2* exon numbers was done according to the Transcript ID ENST00000398117 using the Ensembl Genome Browser (http://www.ensembl.org).

A specific *BCL2* gene product mainly corresponding to the flexible loop domain (FLD) of 204 base pairs was generated using a forward primer 5′-GAGTGGGATGCGGGAGAT-3′ and a reverse primer 5′-GGTCAGGTGGACCACAGGT-3′. This transcript includes part of the FLD (aa 32-87). PCR was performed using 100 ng DNA template in a final volume of 25 μL with 1 μM of each primer, 0.4 mM dNTPs, 1.5 mM $MgCl_2$, and 1.25 U Taq polymerase (AmpliTaq Gold DNA Polymerase; Applied Biosystems, Foster City, CA).

PCR products were purified (AMPure, Beckman Coulter), and aliquots of 2 to 7 μL were used for the sequencing reaction with 1 μM of the universal sequencing primer and 2 μL of GenomeLab DTCS-Quick Start Kit (Beckman Coulter) in a final volume of 10 μL according to the manufacturer's protocol. Sequencing reactions were purified (CleanSEQ; Beckman Coulter) and analyzed in a GenomeLab GeXP Genetic Analysis System and evaluated by the GenomeLab GeXP software 10.2 (Beckman Coulter) to determine the mutation status of *BCL2* in the range between nucleotides c.121G and c.249G (coding sequence of the DNA) corresponding to aa 41 to aa 83.

2.6. Protein structure prediction

The 3-dimensional protein structure prediction analysis of wild-type and mutated BCL2 protein was performed using the m4t server [8,9]. Structural representations were drawn using RasMol 2.7.5.2 (net freeware, written by Roger Sayle, supported by Glaxo Wellcome).

2.7. RNA extraction and real-time quantitative PCR

Total RNA was isolated from formalin-fixed, paraffin-embedded tissues after proteinase K digestion with a standard phenol-chloroform-extraction protocol. Complementary DNA synthesis and real-time quantitative PCR analyses for the quantification of BCL2 were performed as previously described using the LightCycler 480 Probes Master (Roche Applied Science, Penzberg, Germany) [10]. Sequences for primers and probes for *TBP* (TATA box binding protein) as housekeeping gene control have been published [11]. Data were analyzed using the $2^{-\Delta\Delta Ct}$, method as previously described [12]. *BCL2* messenger RNA (mRNA) levels of FL cases were calculated as *x*-fold expression in comparison with the mean value of normal lymph nodes. All reactions were performed in duplicate.

3. Results

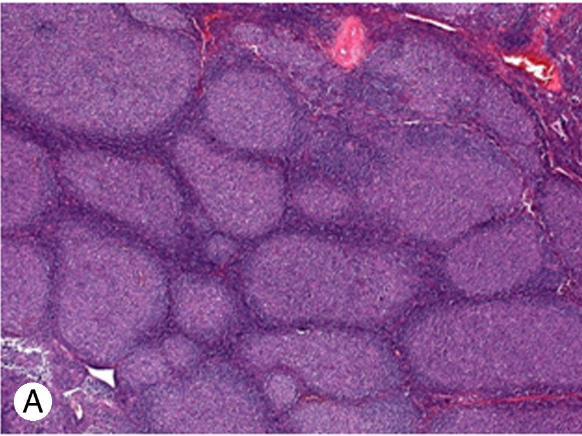
3.1. Clinical, morphological, and FISH analyses

A total of 240 cases of FL grade 1/2 with exclusively or predominantly follicular growth pattern were included in the analysis. Of the 240 cases, 22 (9%) cases were negative with the standard BCL2 antibody (clone 100/D5). These 22 cases are the group of interest in this study. The clinical data of the BCL2-negative cases are shown in Table 1. There were 13 male and 9 female patients, with a mean age of 64 years (range, 39-83 years). The diagnostic biopsy was in most cases a lymph node, either cervical or axillary. Most patients were stage III at diagnosis. FISH analysis using a *BCL2* break-apart probe demonstrated a *BCL2* break in 12 cases (55%), whereas 10 cases (45%) showed no alterations in the *BCL2* gene locus. Taken together, only 4% (10/240) FL grade 1/2 cases lacked *BCL2* breaks and thus were considered t(14;18) negative. The 10 cases without *BCL2* alterations were further analyzed with *IGH* and *BCL6* break-apart probes. Two of them carried a break both in the *IGH* and in the *BCL6* gene loci, indicating a translocation t(3;14)(q27;q32). Eight cases remained negative for all 3 probes.

Table 1 Clinical data of 22 patients with BCL2-negative FL grade 1/2

Case	Age (y)	Sex	Localization	Stage at diagnosis
FL with *BCL2* break				
1	63	m	Tonsil	NA
2	48	m	LN	NA
3	64	m	Axillary LN	IIIA
4	39	f	Cervical LN	III/IV
5	55	m	Mesentery (extranodal)	IIB
6	58	m	Cervical LN	NA
7	68	f	Cervical LN	IIIA
8	51	m	Inguinal LN	IIIA
9	65	m	Cervical LN	IIIA
10	67	m	Retroperitoneal LN	IIIA
11	68	m	Inguinal LN	IIA
12	76	m	Supraclavicular LN	NA
FL without *BCL2* break				
13	60	f	Axillary LN	III
14	65	f	Axillary LN	NA
15	72	f	Axillary LN	IIIA
16	53	f	Cervical LN	NA
17	83	f	LN	NA
18	48	m	Submandibular LN	I
19	70	f	Axillary LN	III
20	72	f	Axillary LN	IIIA
21	72	m	Cervical LN	IIB
22	70	m	Axillary LN	IIIA

Abbreviations: m, male; f, female; LN, lymph node; NA, not available.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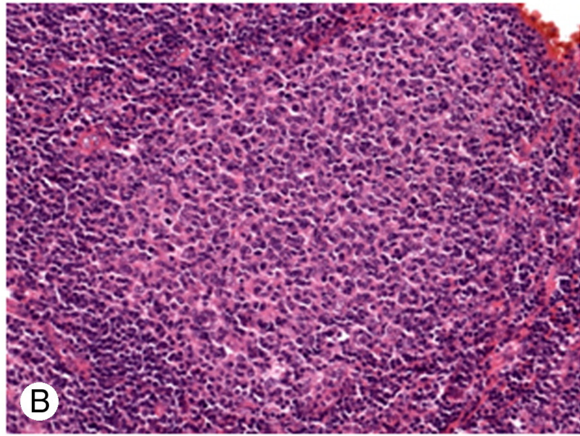
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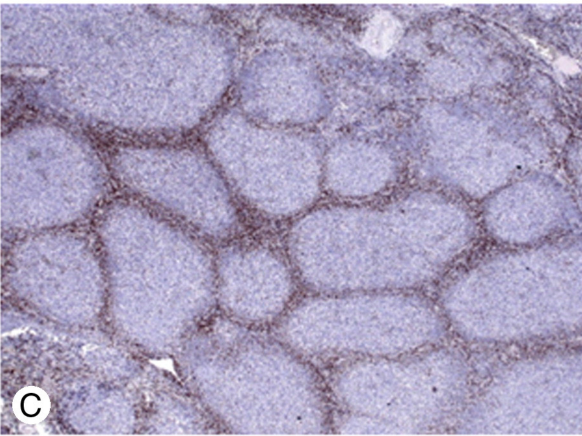
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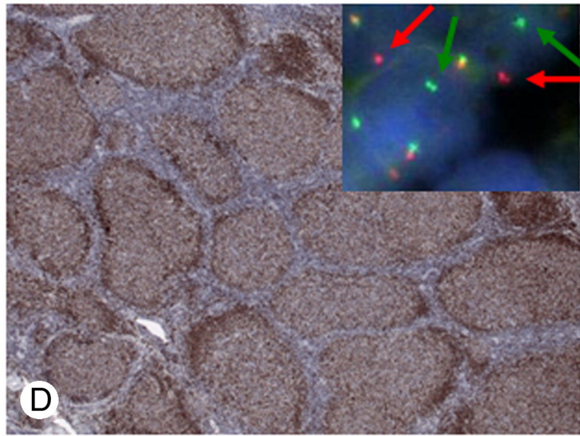
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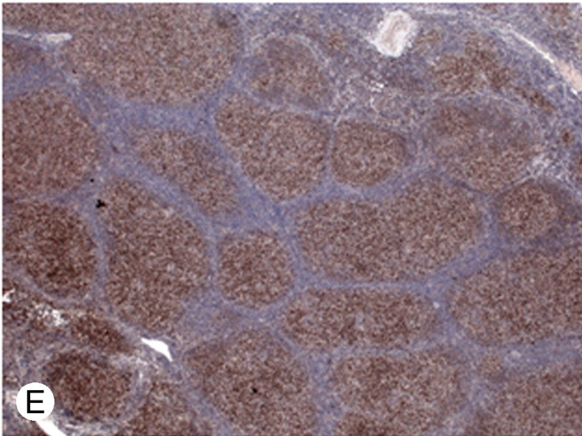
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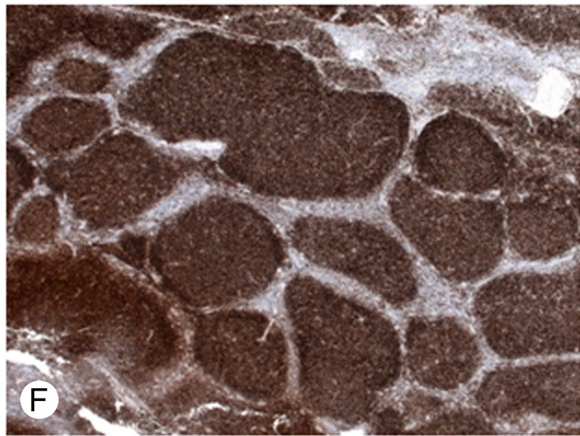
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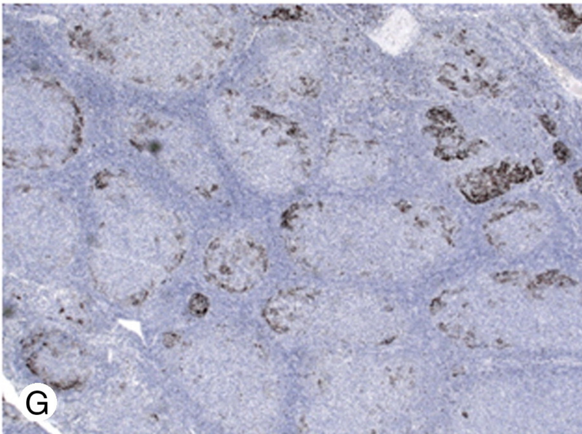
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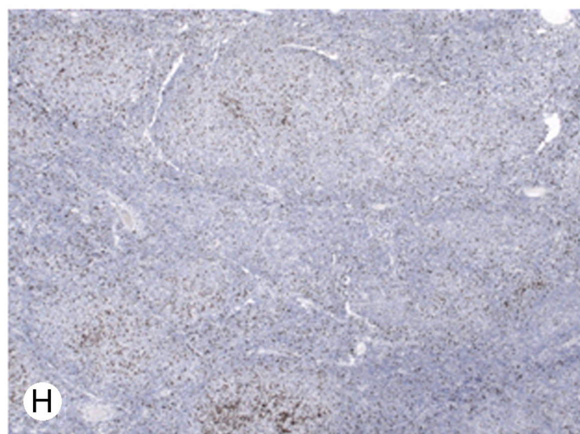
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3.2. BCL2-negative FLs grade 1/2 with break in the *BCL2* gene locus

The 12 patients in this group corresponded to 10 men and 2 women (male-to-female [M/F] ratio, 5:1), with a median age of 63 years (rate, 39-76). All cases featured a characteristic H&E morphology with effacement of the normal lymph node architecture by a follicular lymphoid infiltrate consisting mainly of centrocytes with some admixed centroblasts (Fig. 1A and B). The partially confluent follicles often had a blurred mantle zone. The results of the immunohistochemical and molecular analyses are summarized in Table 2. Using the alternative BCL2 antibodies (clone E17 and SP66), 11 of the 12 cases of FL grade 1/2 with *BCL2* break showed a strong BCL2 positivity (Fig. 1C and D), whereas only 1 case remained negative for all 3 BCL2 antibodies. These 12 cases had the characteristic FL phenotype with positivity for CD20, CD10, and BCL6. (Fig. 1E and F). CD23 staining only showed the disrupted networks of follicular dendritic cells but was negative in the tumor cells (Fig. 1G). The MIB1 staining showed a mean proliferation rate of 23% (range, 5%-80%) (Fig. 1H). In 66% of the cases (8/12), the proliferation rate was below 25%. Of interest, the only case with high proliferation rate (80%) in this group corresponded to case 2, with lack of BCL2 protein expression.

Table 2 Immunohistochemical data of 22 cases of BCL2-negative FL grade 1/2

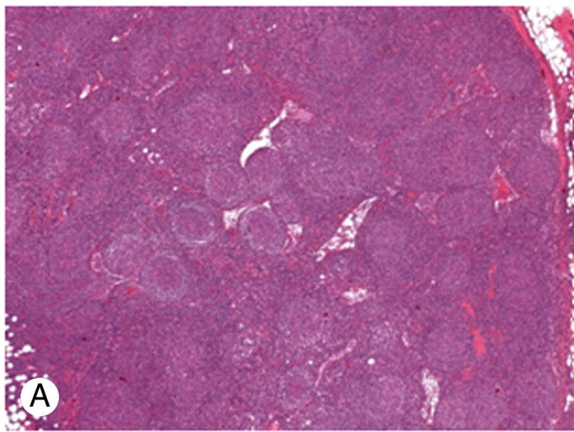
Case	BCL2 100/D5	BCL2 E17/SP66	CD10	BCL6	CD23	MIB1 (%)	*BCL2*-BAP
FL with *BCL2* break							
1	−	+	++	+	−	5	+
2	−	−	++	+	−	80	+
3	−	+	++	+	−	20	+
4	−	+	++	+	−	30	+
5	−	+	++	+	−	20	+
6	−	+	++	+	−	5	+
7	−	+	++	+	−	40	+
8	−	+	++	+	−	15	+
9	−	+	++	++	−	30	+
10	−	+	+	+	−	5	+
11	−	+	++	+	−	20	+
12	−	+	++	+	−	20	+
FL without *BCL2* break							
13	−	−	+	++	−	15	−/*BCL6*+
14	−	−	++	++	−	15	−
15	−	−	++	+	+	10	−
16	−	−	++	+	+	10	−
17	−	−	−	+	−	10	−
18	−	−	+	+	−	25	−/*BCL6*+
19	−	−	−	+	−	80	−
20	−	−	−	+	−	70	−
21	−	−	+	+	−	30	−
22	−	−	+	+	+	30	−

NOTE. +, positive; ++, strongly positive; −, negative.

3.3. BCL2-negative FLs grade 1/2 with intact *BCL2* gene locus

The 10 patients in this group were 3 men and 7 women (M/F ratio, 1:2.5), with a median age of 70 years (range, 48-83 years). These cases also revealed the typical morphology of FL grade 1/2 with effacement of the lymph node architecture and a follicular lymphoid infiltrate with predominantly centrocytes and few scattered centroblasts (Fig. 2A and B). Morphologically, there was no difference between the cases with and without *BCL2* break. The results of the immunohistochemical and molecular analyses are summarized in Table 2. All cases were negative for the conventional (Fig. 2C) as well as the alternative BCL2 E17/SP66 antibodies (Fig. 2D). The immunophenotype of this group was more heterogeneous. BCL6 staining was positive in all cases (Fig. 2E). Staining for CD10 tended to be weaker and more heterogeneous than in the E17/SP66-positive cases. Three (30%) of the 10 cases were completely negative for CD10 (Fig. 2F), and 3 additional cases expressed CD23 (30%) (Fig. 2G). In addition, CD23 showed disrupted networks of follicular dendritic cells in the tumor infiltrates, underlining the follicular growth pattern. The mean proliferation rate in these cases was 30% (range, 10%-80%) (Fig. 2H). Two cases in this group showed very high proliferation rates (70% and 80%). However, no statistically significant differences in the proliferation rates of the 2 subgroups with and without *BCL2* break were found ($P = .79$). To confirm the diagnosis of FL in these cases, immunohistochemical analysis with the germinal center–associated markers LMO2 and HGAL was performed (Supplementary Fig. 1A and B). All cases strongly reacted with both antibodies, further supporting the diagnosis of

Fig. 1 A, Lymph node with effaced normal architecture by a lymphoid infiltrate with follicular growth pattern and blurred mantle zones (H&E, original magnification ×25). B, Higher magnification shows that the infiltrate in the follicles consist mainly of centrocytes with some admixed centroblasts (H&E, original magnification ×200). C, Immunohistochemical staining for the standard BCL2 antibody remains negative in the tumor cells (immunoperoxidase BCL2 clone 100/D5, original magnification ×25). D, The alternative BCL2 antibody E17 is positive in the tumor cells (immunoperoxidase BCL2 clone E17, original magnification ×25). FISH analysis using a *BCL2* gene break-apart assay (inset) confirmed the presence of a break in the *BCL2* locus (arrows). E and F, Note the characteristic BCL6 (E) and CD10 (F) expression in the tumor cells (immunoperoxidase BCL6 and CD10, original magnification ×25). G, The tumor cells are negative for CD23. Note the disrupted networks of follicular dendritic cells (immunoperoxidase CD23, original magnification ×25). H, MIB1 staining demonstrates a proliferation rate of 5% in the neoplastic follicles (immunoperoxidase MIB1, original magnification ×25).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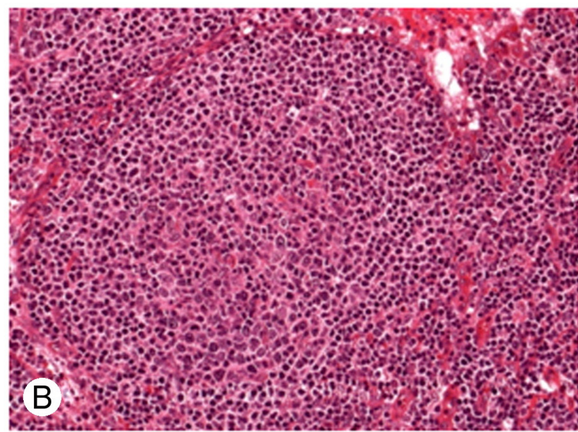
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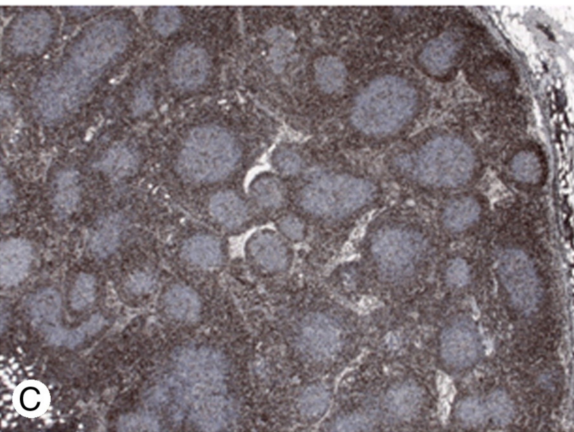
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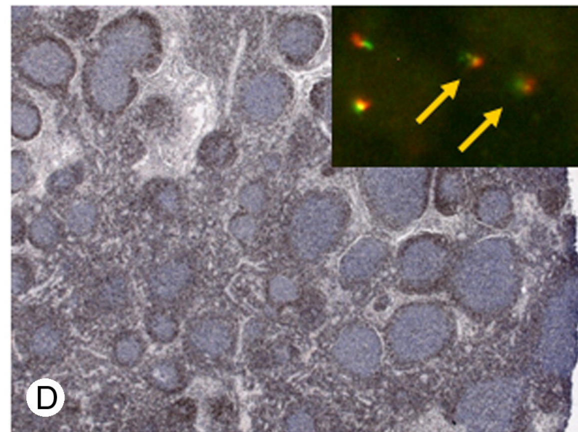
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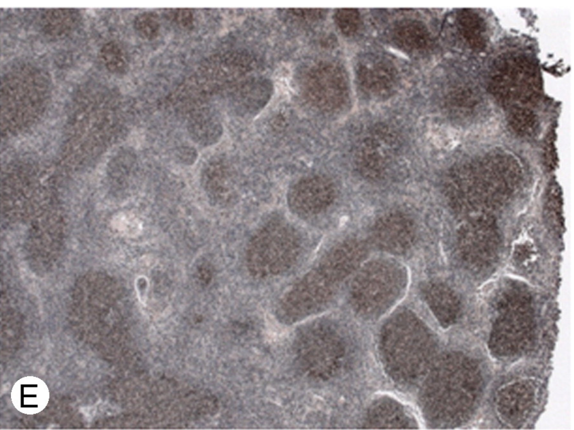
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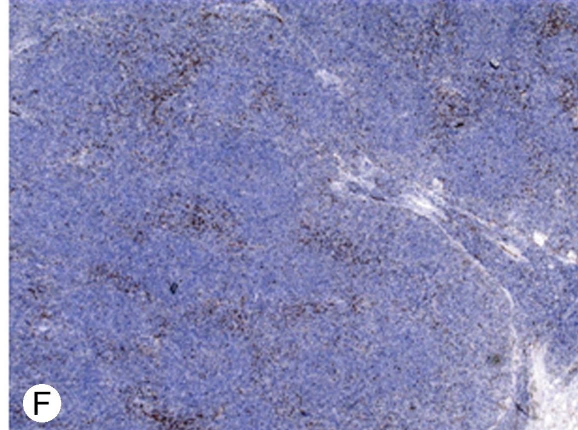
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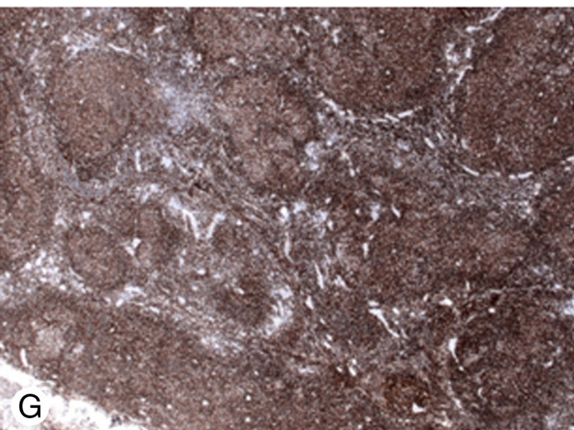
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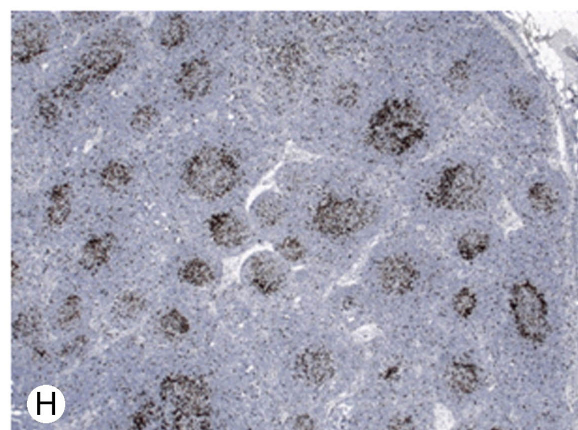
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BCL2-negative FL. Staining for IRF4/MUM1 remained widely negative in the tumor cells.

3.4. Clonality and *BCL2* mutational analyses

Clonality analysis of *IGH* framework regions 2 and 3 was performed in 9 of the 10 BCL2 E17/SP66-negative FL without *BCL2* translocation from which DNA was available for analysis (Table 3). A monoclonal population was demonstrated in all cases analyzed, confirming the diagnosis of malignant lymphoma.

Mutational analysis of *BCL2* mainly corresponding to the FLD was performed in 16 of 22 cases (Table 3). The fragment analyzed included the part encoding aa 41 to aa 83 where the epitopes of the 100/D5 and E17 clones reside (Fig. 3). Two cases from the first group and 4 cases from the second group could not be analyzed because of either poor DNA quality or lack of material. In 9 of 10 analyzable BCL2 E17/SP66-positive cases, 15 mutations resulting in aa substitutions with a hot spot around codon 144 (aa 48) were detected (Fig. 3A and B). These mutations occurred in the FLD in sites predicted to affect the BCL2-p53 binding domain. Two cases showed 3 mutations, 2 cases 2 mutations and 4 cases 1 mutation each. Case 2, the only case with a translocated *BCL2* gene and negativity for BCL2 E17/SP66, showed no mutation in the complete coding region of the *BCL2* gene. The 6 analyzable cases without *BCL2* translocation and BCL2 E17/SP66 negativity showed a *BCL2* wild-type sequence in the analyzed region. Mutational analysis of the *BCL2* gene region mainly corresponding to the FLD was also performed in 10 typical cases of BCL2+ FL, used as controls. One case showed a silent mutation p.T74T (c.222C>T, ACC-ACT), and 1 case showed a missense mutation p.A60V (c.179C>T, GCC-GTC). This latter mutation occurred outside the binding epitopes of all 3 antibodies. The remaining 8 cases revealed a wild-type *BCL2* gene sequence. Protein structure prediction analysis using the most frequently found missense mutation p.I48M (c.144C>G, ATC-ATG) alone and in combination with other mutations as present in case 11 showed a clear structural change in the FLD where the binding site of the BCL2 antibody clone 100/D5 resides, indicating a structural inhibition of antibody binding (Fig. 3C). These mutations did not affect the binding site of the E17/SP66 clones. Rare missense mutations (p.A76T; c.226G>A, GCT-ACT) were identified in the predicted binding site of the E17 antibody (Fig. 3B and C); however, these mutations obviously did not preclude the binding of the E17 antibody. The reason for this might be that this mutation was found at the end of the

Table 3 Mutation data of the *BCL2* gene region mainly corresponding to the FLD of 22 patients with BCL2-negative FL grade 1/2 and 10 control cases of FL with *BCL2* break

Case	Mutated aa	Mutated complementary DNA	Mutated base triplet
FL with *BCL2* break			
1	p.I48M	c.144C > G	ATC → ATG
	p.P53T	c.157C > A	CCC → ACC
	p.A76T	c.226G > A	GCT → ACT
2	WT	WT	–
3	p.G47D	c.140 G > A	GGC → GAC
	p.I48M	c.144 C > G	ATC → ATG
4	p.F49S	c.146 T > C	TTC → TCC
5	NE	NE	NE
6	p.G47A	c.140G > C	GGC → GCC
	p.Q52H	c.156G > C	CAG → CAC
7	p.P46S	c.136C > T	CCG → TCG
8	p.P46A	c.136C > G	CCG → GCG
9	p.P46S	c.136C > T	CCG → TCG
	p.T69T (silent)	c.207C > G	ACC → ACG
10	NE	NE	NE
11	p.I48M	c.144C > G	ATC → ATG
	p.P53T	c.157C > A	CCC → ACC
	p.A76T	c.226G > A	GTC → ATC
12	p.I48M	c.144C > G	ATC → ATG
FL without *BCL2* break			
13	NE	NE	–
14	WT	WT	–
15	NE	NE	–
16	WT	WT	–
17	WT	WT	–
18	WT	WT	–
19	NE	NE	–
20	NE	NE	–
21	WT	WT	–
22	WT	WT	–
FL control cases with *BCL2* break			
1	p.T74T (silent)	c.222C > T	ACC → ACT
2	p.A60V	c.179C > T	GCC → GTC
3-10	WT	WT	–

Abbreviations: NE, not evaluable; WT, wild type.

Fig. 2 A, The tumor shows the characteristic follicular growth pattern of FL (H&E, original magnification ×25). B, The tumor cells consist mainly of centrocytes with some admixed centroblasts typical of FL grade 1/2 (H&E, original magnification ×200). C, Immunohistochemical staining for the standard BCL2 antibody (clone 100/D5) remains negative in the tumor cells with good internal control in the reactive T lymphocytes (immunoperoxidase, original magnification ×25). D, The BCL2 antibody clone E17 also remains negative in the tumor cells (immunoperoxidase, original magnification ×25). FISH analysis using a *BCL2* gene break-apart assay shows colocalized signals (arrows), indicative of an intact *BCL2* gene locus (inset). E, The tumor cells express BCL6 characteristic of FL (immunoperoxidase, original magnification ×25). F, Note the lack of CD10 expression in the tumor cells in this case (immunoperoxidase, original magnification ×25). G, Example of an FL with CD23 expression in the tumor cells (immunoperoxidase CD23, original magnification ×25). H, The MIB1 staining shows a proliferation rate of 30% in the tumor cells within the neoplastic follicles (immunoperoxidase MIB1, original magnification ×25).

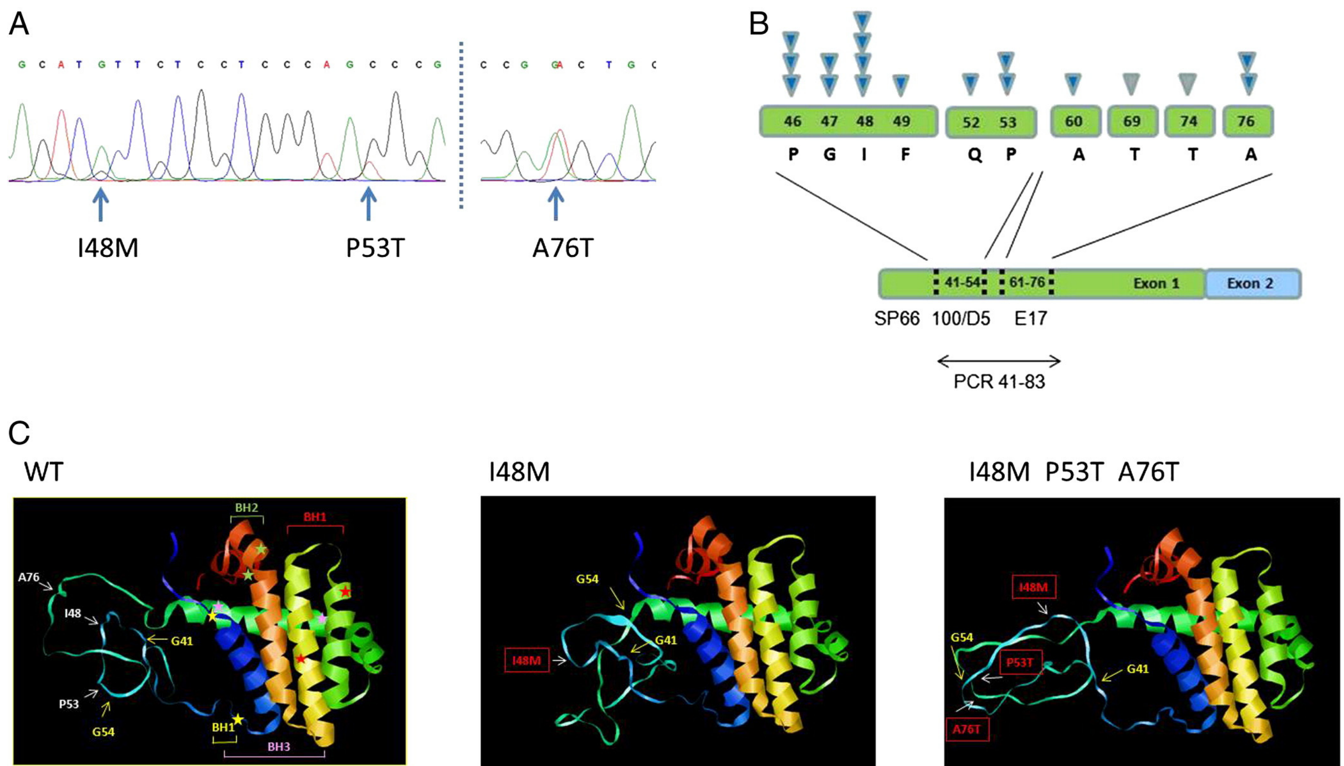

Fig. 3 A, Representative sequence of an FL case with point mutations in residues 48, 53, and 76 resulting in aa substitution (c.144C>G, p.I48M ATC-ATG; c.157C>A, p.P53T CCC-ACC; c.226G>A, p.A76T GCT-ACT). B, Schematic representation of the different mutations found in the analyzed cases of FL. Fifteen missense mutations were detected in the analyzed sequence of cases 1 to 12 (blue arrowheads) with a hot spot around codon 144, aa 48. Mutations between aa 41 and aa 54 affect the binding site of the standard BCL2 antibody 100/D5. Mutations between aa 61 and aa 76 affect the binding site of the E17 antibody. Mutations at the N-terminal affect the binding site of the SP66 antibody. Some cases harbored more than 1 point mutation. One missense mutation was found in a control case at codon 179, aa 60. Two silent mutations were also detected (gray arrowheads). C, Comparative representation of the normal 3-dimensional BCL2 protein structure (left) versus the BCL2 protein structure with the most frequent mutation p.I48M (middle) and the BCL2 structure with 3 different mutations. Note that the change in the protein structure does not involve the BH domains. G41 to G54 correspond to the residue recognized by the standard BCL2 antibody 100/D5.

peptide sequence against which the E17 antibody was raised and not in the middle as in the 100/D5 clone.

3.5. Quantitation of BCL2 mRNA

To establish and validate the real-time BCL2 mRNA expression analysis, normal lymph nodes were first analyzed and used as control for relative quantification (BCL2/TBP 1-fold expression) (Supplementary Fig. 2). Increased expression of BCL2 mRNA levels correlated with both *BCL2* amplification in Granta 519 mantle cell lymphoma cell line (2.74-fold expression) and with FL cases carrying t(14;18) (2.54-fold expression ± 0.33). BCL2 mRNA transcript levels were not increased in FL cases with intact *BCL2* gene locus (0.88-fold expression ± 0.44), compared with normal lymph nodes. Case 2 with a detected break in the *BCL2* gene locus without BCL2 protein expression showed BCL2 mRNA expression (1.26-fold expression) comparable with normal lymph nodes and FL cases without t(14;18), suggesting deletion or transcriptional inactivity of the translocated *BCL2* allele.

4. Discussion

Lack of immunohistochemically detectable BCL2 reactivity has been described in approximately 10% to 15% of FL grade 1/2 [1]. In this study, in good correlation with previous data, 22 (9%) of 240 cased of FL grade 1/2 were negative with the standard BCL2 antibody (clone 100/D5). However, using FISH analysis and 2 alternative BCL2 antibodies (clones E17 and SP66), the truly BCL2-negative FL grade 1/2 cases were found to represent only 4% of all cases (10/240 cases). Most importantly, the FISH analysis and/or the E17/SP66 antibodies were able to clearly subdivide the BCL2-negative cases into 2 immunohistochemically and genetically distinct subgroups. The first group (12/22; 55%) was characterized by the presence of a

break in the *BCL2* gene locus, BCL2 mRNA overexpression, E17/SP66 positivity, and point mutations in the FLD of *BCL2* gene with a hot spot around codon 144. The second group of the truly BCL2-negative cases (10/22; 45%) was characterized by an intact *BCL2* gene locus, lack of reactivity to the E17/SP66 antibodies, and a wild-type *BCL2* sequence. Morphologically, the 2 groups were very similar; however, the phenotype of the truly BCL2-negative FL grade 1/2 was more heterogeneous with often weak or loss of CD10 staining and occasional expression of CD23 in the tumor cells.

Mutations in the *BCL2* gene as explanation for the lack of BCL2 protein staining in FL with a t(14;18) were first reported by Schraders et al [6]. Subsequently, Masir et al [13] reported 7 cases of BCL2 pseudonegative FL with t(14;18), 4 of these cases also showed a mutation within the peptide sequence against which the standard antibody was raised. However, no mutations could be found within the region studied in the 3 residual cases. The authors suggested that mutations in *BCL2* distant from the target epitope might alter the conformation of the binding site to such a degree that it is no longer recognized by the antibody. Our data do not support this contention because in case 2, the only case where the E17/SP66 antibodies remained negative despite the presence of a *BCL2* translocation, no mutation of the *BCL2* gene was found. Furthermore, missense mutations at the end of the peptide sequence of the E17 epitope (aa 76 c.226G>A GCT-ACT) and outside the epitope of all 3 BCL2 clones (aa 60 C.179C>T GCC-GTC) did not interfere with antibody binding. The reason why rare cases of FL with translocated and wild-type *BCL2* gene do not express BCL2 protein is not clear and warrants further study. A possible explanation might be either loss or transcriptional silence of the translocated *BCL2* allele as a consequence of genetic evolution of the malignant clone, as suggested by the lack of increased BCL2 mRNA expression in case 2, in addition to the absence of the protein. That mutations of the *BCL2* gene were found only in cases with translocated *BCL2* gene suggests that these mutations are acquired after the translocation; are likely caused by somatic hypermutation, which affects only the translocated allele; and, most probably, are irrelevant for the initial clonal expansion of FL. Accordingly, we recently demonstrated in a synchronous case of FL in situ and manifest FL that the *BCL2* gene mutation was a secondary event and most probably represents clonal evolution [5].

An important question is whether *BCL2* gene mutations interfere with the ability of the BCL2 protein to suppress apoptosis. The replacement mutations observed in our study are outside the BH domains, which mediate interactions with proapoptotic proteins and which are critical for the function of the BCL2 protein, indicating that these mutations do not impair the tumorigenic role of BCL2 in FL. Of note, in 2 very recent studies of diffuse large B-cell lymphoma, it was shown that replacement mutations in the *BCL2* gene occurred predominantly in cases carrying a t(14;18) translocation and were usually located outside the BH2 regions, indicative of a strong negative selection against mutations in these functionally important domains [14,15]. Furthermore, we found that *BCL2* mutations were enriched in the FLD in sites that would affect the putative BCL2-p53 binding domain (aa 32-68) believed to have a negative regulatory function [16]. If these mutations interfere with p53 binding, enhanced BCL2 antiapoptotic function would be expected, thus promoting survival of the tumor cells.

To rule out the possibility of misclassifying atypical reactive follicular hyperplasia or nodular growth pattern of a marginal zone B-cell lymphoma as BCL2-negative FL, we performed *IGH* gene rearrangement studies and IHC analysis for the newly described germinal center–associated markers HGAL [17] and LMO2 [18]. In addition to the classical morphology of FL, all cases analyzed were monoclonal and strongly expressed HGAL and/or LMO2, arguing in favor of the diagnosis of a germinal center–derived B-cell lymphoma. However, the immunohistochemical phenotype of this subgroup was more heterogeneous. In accordance with published data [2], the FL without evidence of a *BCL2* translocation in this study showed a weaker, rather heterogeneous CD10 expression, with 3 cases being completely negative. In addition, 3 cases were CD23 positive. The expression of CD23 in FL has been described to be more common in inguinal lymph nodes than in other anatomical sites [19]. However, none of the 3 cases with CD23 expression in this study were in an inguinal lymph node. Two of our 10 t(14;18)-negative cases had a disruption of the *IGH* and *BCL6* gene loci in the FISH analysis, indicative of a translocation t(3;14)(q27;q32). Although this translocation is characteristically present in a significant number of t(14;18)-negative FL with grade 3B morphology, our study shows that rare cases of FL grade 1/2 can also carry this translocation [20,21].

In addition to the genetic and phenotypic differences, the 2 groups also showed a striking difference in the sex distribution with the t(14;18)+ group consisting of 10 male and 2 female patients (M/F ratio, 5), whereas the second group consisted of 3 male and 7 female patients (M/F ratio, 0.43). Although the number of cases in this study is too small for definite conclusions, this difference warrants further studies.

In summary, our data clearly show that the BCL2 E17/SP66 antibodies discriminate 2 immunohistochemically and genetically distinct subgroups of BCL2-negative FL grade 1/2. Positivity with the BCL2 E17/SP66 antibodies is a good surrogate marker for the presence of a *BCL2* translocation and in the case of negativity with the standard BCL2 antibody for point mutations in the FLD of the *BCL2* gene. The molecular pathogenesis of the BCL2 (E17/SP66) and t(14;18)-negative FL grade 1/2 remains to be determined.

Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.humpath.2013.02.004.

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