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Follicular lymphoma without t(14;18) and with *BCL-6* rearrangement: a lymphoma subtype with distinct pathological, molecular and clinical characteristics

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Translocations involving the *BCL-6* gene are frequently observed in diffuse large B cell lymphoma, but have rarely been reported in follicular lymphoma (FL). We studied a distinct cohort of FLs with a 3q27/*BCL-6* gene rearrangement, but lacking the t(14;18) translocation. In 13/15 cases, translocations involved the 3q27 and the 14q32 regions. All cases displayed a marked follicular growth pattern and, in some instances, a monocytoid component. Tumor cells were CD5$^-$ CD20$^+$ CD23$^-$ CD43$^-$ BCL-6$^+$, and in the main CD10 negative (*n* = 10, 71%) and BCL-2 negative (*n* = 11, 78%). When compared to 20 typical t(14;18)$^+$ FLs, the presence of large follicles (*P* = 0.01) and a CD10$^-$/BCL-2$^-$ phenotype were more frequently observed (*P* = 0.001) in our cohort. Clonal mutations arising in the *BCL-6* first intron were observed in 5/7 cases with evidence of intraclonal heterogeneity, consistent with a germinal center origin. No significant difference was found in comparison to t(14;18)$^+$ FL regarding age, sex, performance status, bone marrow involvement or overall survival. However, in the 3q27$^+$ FL group, a stage III/IV disease and a bulky mass were less frequently observed. This study indicates that 3q27$^+$ FL without t(14;18) translocation have peculiar clinico-pathologic features and may correspond to a rare and distinct subtype of lymphoma originating from the germinal center.
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Introduction

Follicular lymphoma (FL) is one of the most common subtypes of non-Hodgkin's lymphoma (NHL) in the western world. FL tumor cells reside in the germinal center (GC), and are characterized by the t(14;18)(q32;q21) translocation which occurs in 80–90% of cases.[1] A consequence of the translocation is a functional overexpression of the anti-apoptotic BCL-2 protein,[2–6] which is usually not expressed in normal GC centroblastes and centrocytes.[7,8] In contrast, the product of the *BCL-6* gene on chromosome 3q27, a transcriptional repressor[9] which regulates normal GC B-cell function,[10,11] is highly expressed in these cells. High levels of BCL-6 protein are also observed in FL tumors, irrespective of 3q27 translocations[7,12] which occur in 9–14% of FL cases as detected by conventional cytogenetics or Southern blotting.[13–15] By comparison, *BCL-6* expression levels are variable in diffuse large B-cell lymphomas (DLBCL),[7,16] which carry a higher frequency of translocation into the 3q27 locus.[13,17,18] In about half FLs, *BCL-6* rearrangements, when present, occur together with the t(14;18) event, considered as the primary genetic event.[13,17,19]

FL with *BCL-6* rearrangements in the absence of any evidence for t(14;18) translocation have rarely been reported in the literature.[13,14,20] We report 15 such cases and compare their characteristics with those of FL bearing the hallmark t(14;18) translocation. Using immunophenotype and mutational analysis of the first *BCL-6* intron, a marker of GC transit,[21–26] we identified a subtype of indolent lymphoma which is closely related to typical FL, but is characterized by distinctive pathological features.

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Materials and methods

Patient selection

Patients diagnosed between 1984 and 2001 with FL were selected from the cytogenetic data base of the Centre Henri Becquerel (Rouen, France). Selection criteria for this study were: (1) a diagnosis of follicular lymphoma confirmed by at least two pathologists; (2) the absence of t(14;18) translocation by standard cytogenetic and/or molecular analysis; and (3) the presence of a 3q27 rearrangement. Fifteen FL cases fulfilled these criteria. Twelve of these were found in a series of 225 FL (5.3%) in which cytogenetic analysis were available and performed at the time of diagnosis (Table 1), including 149 t(14;18)$^+$/3q27$^-$ cases (66%) and 21 t(14;18)$^+$/3q27$^+$ cases (9%). In one additional case, cytogenetic analyses was obtained only at the time of relapse (case 4) and in two cases (cases 14 and 15), initial diagnosis and treatment were performed in a distinct institution (H Mondor Hospital, Créteil, France). A group of 88 t(14;18)$^+$/3q27$^-$ FL cases were available as a control group.

Follow-up and clinical information taken at the time of initial diagnosis, including a complete medical history and physical examination, CT scan of the chest, abdomen and pelvis, and a bone marrow trephine biopsy were available in all patients.

Cytogenetic analysis

Cytogenetic analyses were performed as previously reported.[13] R-banded metaphases were karyotyped and chromosomal abnormalities were evaluated according to the International System for Human Cytogenetic nomenclature.[27]

Histology

Lymph node biopsies were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin, silver impregnation, periodic acid-Schiff, and May–Grünwald-Giemsa. Lymphomas were classified and graded according to the WHO classification.[28] Morphological features were retrospectively reviewed, particularly the architecture tumor proliferation, the cytological grading and the size

Table 1 Cytogenetic and molecular features of 15 follicular lymphomas with 3q27 rearrangement and absence of t(14;18) translocation

Case No.	Karyotype	*BCL-6* rearrangement	*BCL-2* rearrangement
1	46,xx,**t(2;3)(q22;q27)**[1] / 47,xx,**t(2;3)(q22;q27)**,+8 [5] / 47,xx,**t(2;3)(q22;q28)**,t(4;11)(q22;q24),+8 [6] / 46,xx [6]	ND	neg MBR mcr
2	47xx,**t(3;14)(q27;q32)**,del(6)(q12q16),dup(12)(q15q24),-15,add(16)(q22),add(18)(q21),+r,+mar, +min [17]/46,xx [1]	positive	neg MBR mcr
3	47,X,t(x;1)(p21;p21),del(2)(q31),**t(3;14)(q27;q32)**,add(12)(p12),+15,der(17)t(1;17)(q12;p11) [12]	positive	neg MBR mcr
4	46,XY,**t(3;14)(q27;q32)**,der(22)t(1;22)(q12;p11)[19]	positive	neg MBR mcr
5	96-100,<4n>,xxxxxx,i(1)(q10),-2,-2,**add(3)(q27)**,i(6)(p10),add(7)(q21),+9,del(9)(q13),**add(14)(q32)**, -15,-16,del(17)(p13),+18,+18,del(18)(q21),+22,+mar [7] / 46,xx [9]	negative	neg MBR mcr
6	46,xx/46,xx,**t(3;14)(q27;q32)**,del(4)(q32),del(17)(q25),add(18)(q21) [17]/ 46,xx[1]	positive	neg MBR mcr
7	47,xx,+3,**t(3;14)(q27;q32)**,dup(10)(q22q25) [14]/ 46, xx[4]	positive	neg MBR mcr
8	46,xy,dup(1)(q12q23),**t(3;14)(q27;q32)**,der(9)t(9;9)(p24;q23) [10]	positive	neg MBR mcr
9	49,xx,+x,+1,i(1q),**t(3;14)(q27;q32)**,+5,iso(5p),-6,der(10)t(8;10)(q13;q25),dup(12)(q13q22),del(13) (q12q31),+mar [15]/ 46,xx[1]	positive	neg MBR mcr
10	47,xx,+x,add(1)(q21),**t(3;14)(q27;q32)**,del(6)(q12q26),t(9;21)(p24;q11),add(11)(q13)[9]/49,xxx, id,+5,+12[3]/46,xx[3]	positive	neg MBR mcr
11	95,xxxyy,<4n>,-1,**t(3;14)(q27;q32)**,-4,del(6)(q15q26),del(6),+del(6),+del(6),add(7)(q21),add(7), +add(7),+add(7),-8,-9, add(9)(p13),+11,+11,-13,-13,+add(14)(q23),+add(14),-15,add(16)(p13),add (16),-17,i(17)(q10),-18,+19,-21,-21,+22,+mars[8] / 46,xy [4]	ND	ND
12	46,xx,dup(1)(q12q25),**t(3;14)(q27;q32)**,del(6)(q12q27),der(12)t(12;12)(p13;q12),add(14)(q31) [16]	positive	neg MBR mcr
13	55,xx, +2,der(2)t(2;11)(p24;q11), +3, **t(3;14)(q27;q32)**,+5,del(5)(q11q23),+6,del(6)(q15q27), +7, der(16)t(1;16)(q21;q24),+18,+19, der(19)t(1;19)(q24;p13), +21 +r [5]/ 46,xx[12]	positive	neg MBR mcr
14	47,x,-y,dup(1)(q21q32),+3,**t(3;14)(q27;q32)**,del(4)(q11q22),del(5)(q15q31),add(9)(p24),del(15) (q12q15),add(15)(q25),+mar [cp18]	ND	neg MBR mcr
15	47,xy,dup(1)(q21q41),+3,**t(3;14)(q27;q32)**,i(6p)[4]/46,xy[2]	positive	neg MBR mcr

of follicles. The latter was determined by the mean diameter obtained from the measurement of 10 to 25 follicles per case using a graded reticule at a low magnification (×40).

Immunohistochemistry

Immunohistochemistry was performed on deparaffinized tissue sections using an indirect immunoperoxidase automated method with Nexes (Ventana Medical systems, Tucson, AZ, USA) according to the manufacturer's instructions. Paraffin-embedded tissue sections were evaluated with a panel of antibodies including CD3, CD20, CD23, BCL-2, BCL-6 (Dako, Glostrup, Denmark), CD5, CD10 (Nova Castra Labs, Newcastle, UK), and CD43 (Bioscience Products, Emmenbrüke, Switzerland). A semi-quantitative evaluation of p53 (Dako, clone DO-7), Ki67 antigen (Dako), CD10 and BCL-6 expression was performed by assigning to each case a score according to the percentage of positive tumors cells: −, 0%, to 10%; +, 10 to 25%, ++, 25 to 50%; +++, 50 to 100%. In order to assess distinctive histopathological and immunophenotypical characteristics, we compared the $3q27^{+}/t(14;18)^{-}$ FLs with 20 $3q27^{-}/t(14,18)^{+}$ FLs from the control group.

PCR amplification of t(14;18) translocation

DNA was extracted from tumor biopsies by standard methods including digestion with proteinase K followed by a 'salting out' procedure and ethanol precipitation. PCR detection of *BCL-2-JH* rearrangement was performed using specific primers for the major break point region (MBR) and minor breakpoint cluster region (mcr), in combination with a *JH* consensus primer, as previously described[2,29] (all primers were supplied by GensetOligos, Genset, Paris, France).

Analysis of the BCL-6 first intron

Southern blotting: DNA was digested using at least two of four restriction enzymes (*Bam*HI, *Hin*dIII, *Eco*RI or *Xba*I). Rearrangement of *BCL-6* gene was studied using the genomic probes F370, F372 or F381 overlapping the major translocation cluster (MTC) as previously described.[13]

Analysis of the 5′ end of the BCL-6 first intron and cloning procedure: Genomic DNA from seven tumoral lymph nodes was obtained. In order to preferentially amplify the untranslocated allele, primers amplifying the 5′ region of the first intron were designed to span the most frequent 3q27 breakpoints.[9,30] A PCR product of 2.1 kb was obtained using the following primers: gg_1F,5′CTCTTACTCGCCTCTCTAACCC TACTATATATAT3′ and gg_1R,5′GCAAAATCACTCACAAAGA TCTCCCT3′. DNA (800 ng) was amplified in a 50 μl volume and the PCR conditions were as follows: 94°C for 3′ (one cycle), followed by 94°C for 45 s, 58°C for 30 s and 72°C for 2 min (35 cycles). Contamination was checked in control reactions with no added template. PCR products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. The PCR products were cloned into a pGEM-T cloning vector (Promega, Madison, WI, USA) and used to transform competent *Escherichia coli* DH5α cells.

DNA sequence analysis: At least four clones from each patient were sequenced with appropriate internal primers using a commercially available kit based on the dideoxy chain termination method (Thermosequenase; Amersham Life Science, Buckinghamsire, UK). A region of 1100 bases was sequenced and aligned with the germline sequence derived from monocyte DNA using the BLAST 2.0 software.

Germline nucleotide sequence of the BCL-6 first intron region: Circulating monocytes from three healthy donors were isolated by Ficoll–Isopaque density centrifugation using anti-CD13 coated microbeads and a magnetic cell separation system (MidiMacs system; Miltenyi Biotec, Auburn, CA, USA). An identical sequence was obtained from eight clones and was considered as the reference sequence. This sequence of 1100 nucleotides included the total sequence of the major mutation cluster sequence previously described (Genbank accession number AF 191831)[31] and approximately an additional 300 bases.

Taq polymerase error rate: The *Taq* polymerase rate error was estimated from monocyte *BCL-6* DNA template under identical conditions to analysis of tumoral cells: six mutations were detected in six clones obtained from cloned monocyte DNA, giving an error rate of 1 in 1101 bases (0.1%).

Statistical analysis: Overall survival was calculated using the method of Kaplan and Meier and compared by a log-rank test. Distinct clinical and pathological features in the $3q27^+$ group and in the control t(14;18) group were compared using the Fisher's exact test. The mean diameters of follicle between the $3q27^+$ group and the control group were compared using the Mann and Whitney test. The observed distribution of mutations of the first *BCL-6* intron was compared to the theoretic distribution on the basis of the composition of the sequence using the χ^2 test.

Results

Clinical features of t(14;18)$^-$/3q27$^+$ FL cases

Clinical features of the 15 patients in this category are summarized in Table 2. The median age was 56 years. There was no difference with the group of 88 $t(14;18)^+/3q27^-$ FLs with regard to age, sex, performance status, bone marrow involvement and albumin or LDH level (Table 3). However, a stage III/IV disease ($P = 0.06$) or a bulky mass ($P = 0.06$) were less frequently observed in the $3q27^+$ FL group. In these 15 patients, four patients developed a high grade lymphoma. Seven are currently alive and three are still in complete remission. A survival curve was established for 14 $t(14;18)^-/3q27^+$ patients and compared with 88 $t(14;18)^+$ patients (Figure 1). The median survival was 42 months and 69 months respectively ($P = 0.43$). Event-free survival was similar in the two groups.

Cytogenetic findings and Southern blot analysis

In 13/15 cases, translocations involved both 3q27 and the 14q32 regions. In 11/12 patients, the *BCL-6* rearrangement was detected by Southern blotting. Other abnormalities associated with *BCL-6* rearrangement at the cytogenetic level included structural abnormalities of chromosome 1 ($n = 7$), trisomy 3 ($n = 4$), break at 6q23-26 ($n = 4$) or at the short arm of chromosome 17 ($n = 2$) (Table 1).

Histological features

All cases presented a prominent follicular growth pattern. In eight cases, follicles were particularly large (Figure 2a), and areas of diffuse infiltration were present in six cases (Table 4). A well-formed mantle zone surrounding most follicles was present in four cases and was reduced or nearly absent in the 11 remaining cases.

In all cases, neoplastic follicles were composed of a variable proportion of centrocytes and centroblasts. The 15 FLs were classified as grade I ($n = 10$; 67%), grade II ($n = 3$; 20%) and grade III ($n = 2$; 13%) according to the WHO scheme (Table 4). In case 12, an area of DLBCL was also observed.

In five cases (cases 1, 6, 8, 9 and 14) however, cytological features of tumor follicle cells were unusual (Figure 2b), with follicles showing a prominent population of medium-sized cells with a central non-cleaved nucleus and moderate amounts of pale cytoplasm, consistent with a monocytoid dif-

Table 2 Clinical characteristics of 15 follicular lymphomas with 3q27 rearrangement and absence of t(14;18) translocation

Case number	*Sex/Age (years)*	*Clinical stage*	*Performance status*	*Bone marrow involvement*	*Other extranodal sites*	*Elevated LDH level*	*Initial treatment*	*Transformation (time in months)*
1	F/ 61	IV	2	positive	1	—	chlorambucil	—
2	F/ 40	I	0	negative	0	ND	radiotherapy	—
3	F/ 64	III	2	negative	1	+	CHOP-like	—
4	M/ 39	II	0	negative	0	ND	radiotherapy	—
5	M/ 59	IV	0	positive	0	—	chlorambucil	+ (49)
6	F/ 78	IV	0	positive	2	+	CHOP-like	+ (59)
7	F/ 47	III	0	negative	0	+	CHOP	—
8	M/ 42	III	0	negative	0	—	CHOP-like + Interferon	—
9	F/ 71	I	0	negative	0	—	radiotherapy	—
10	F/ 54	IV	0	positive	0	+	CHOP	—
11	M/ 53	III	0	negative	0	+	CHOP-like	—
12	F/ 64	II	2	negative	0	+	CHOP	+ (10)
13	F/ 38	I	0	negative	0	+	radiotherapy	—
14	M/ 64	II	0	negative	0	—	abstention	—
15	M/ 72	IV	2	positive	1	—	CHOP-like + Interferon	+ (23)

Table 3 Comparison of the main clinical and pathological features of follicular lymphomas associated either with 3q27 rearrangement or t(14;18) translocation

Cases	Pathological features			Phenotype					Cytogenetic and molecular features		
	Grade	Cytology	Mean diameter of the follicules (mm ± SD)	Presence of a diffuse area	CD10[a]	BCL2[b]	BCL6[a]	3q27 rearrangement	t(14;18) translocation karyotype/PCR	Number of BCL6 clonal mutations at the time of diagnosis/bp[c]	Intraclonal heterogeneity
1	1	cc,cb,ic	1.24 ± 0.27	–	–	–	+	t(2;3)	–/–	ND	ND
2	1	cc,cb	1.38 ± 0.78	+	–	–	++	t(3;14)	–/–	ND	ND
3	3	cc,cb	1.23 ± 0.61	+	–	–	++	t(3;14)	–/–	5.4×10^{-3}	+
4	1	cc,cb	0.66 ± 0.22	–	+++	–	+	t(3;14)	–/–	ND	ND
5	1	cc,cb	0.63 ± 0.21	–	+++	+	+	add 3q27	–/–	unmutated	–
6	1	cc,cb,ic	0.71 ± 0.27	+	–	–	–	t(3;14)	–/–	ND	ND
7	1	cc,cb	0.36 ± 0.14	–	+++	–	++	t(3;14)	–/–	9.0×10^{-4}	+
8	1	cc,cb,ic	1.15 ± 0.48	+	+++	+	++	t(3;14)	–/–	2.7×10^{-3}	+
9	2	cc,cb,ic	1.08 ± 0.34	–	–	–	++	t(3;14)	–/–	ND	ND
10	1	cc,cb	0.60 ± 0.19	–	–	–	+	t(3;14)	–/–	unmutated	–
11	2	cc,cb	1.40 ± 0.46	–	–	–	++	t(3;14)	–/–	9.0×10^{-4}	+
12	2	cc,cb	1.20 ± 0.69	+	–	+	–	t(3;14)	–/–	5.4×10^{-2}	+
13	3	cc,cb	1.35 ± 0.38	–	ND	ND	ND	t(3;14)	–/ND	ND	ND
14	1	cc,cb,ic	1.71 ± 1.38	–	–	–	+	t(3;14)	–/–	ND	ND
15	1	cc,cb	0.78 ± 0.28	+	–	–	+	t(3;14)	–/–	ND	ND

cc, centrocytic; cb, centroblastic; ic, with monocytoid component.
[a]CD10 and BCL-6 tumoral cells expression score: –, 0% to 10%; +, 10 to 25%; ++, 25 to 50%; +++, 50 to 100%.
[b]BCL-2 expression was diffuse in case 5 and remained negative in the center of follicles in cases 8 and 12.
[c]Clonal mutations observed in a sequence of 1100 nucleotides including the total Major Mutation cluster sequence previously described,[31] and approximately an additional 300 bases.

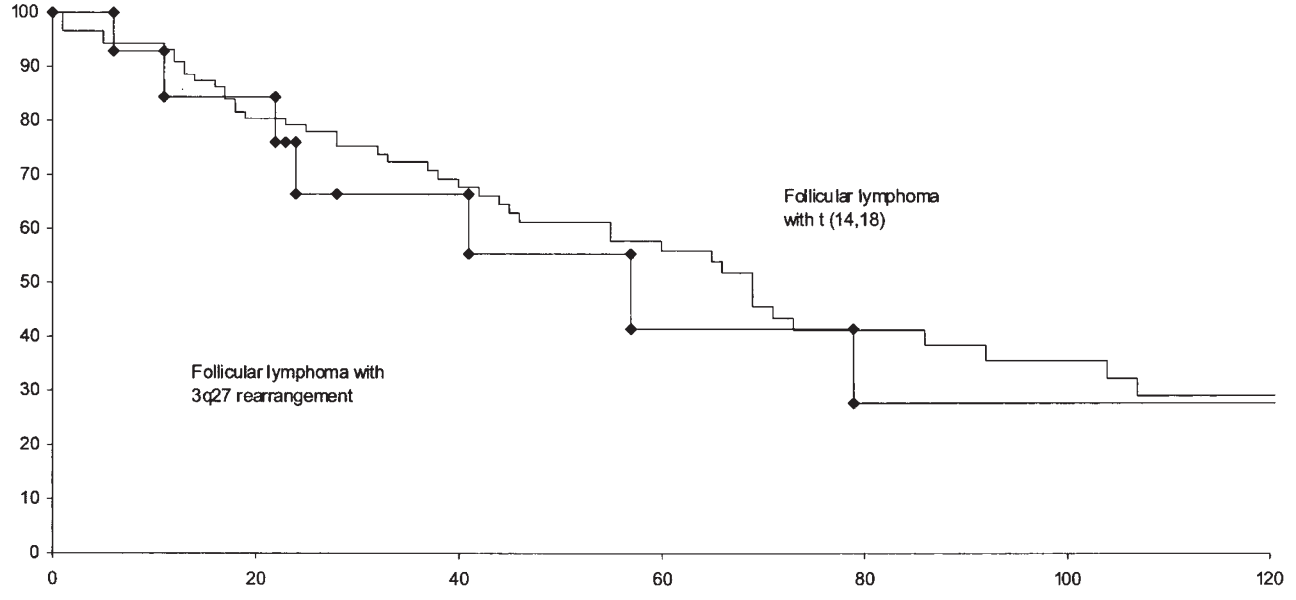

Figure 1 Overall survival curves of patients with follicular lymphomas and 3q27 rearrangement ($n = 14$) or follicular lymphoma and t(14;18) translocation ($n = 88$).

ferentiation. These cells frequently had a single central nucleolus and disclosed an intermediate mitotic index (11 to 25 mitosis per 10 hpf). A variable content of centrocytic and centroblastic-like cells were admixed with this population. Numerous tangible body macrophages were detected within follicles in four patients and a high apoptotic activity with a 'starry sky' appearance was observed in cases 2, 3 and 14 (Figure 2c).

Phenotypic features

In all cases, tumor cells had a $CD20^+$, $CD5^-$, $CD43^-$ phenotype. They were admixed with a variable proportion of $CD3^+/CD5^+$ reactive T cells. In 13 cases, immunostaining for CD23 showed a tight meshwork of follicular dendritic cells. Negative immunoreactivity for CD10 antigen was observed in 10/14 cases (71%), although granulocytes were positive (Table

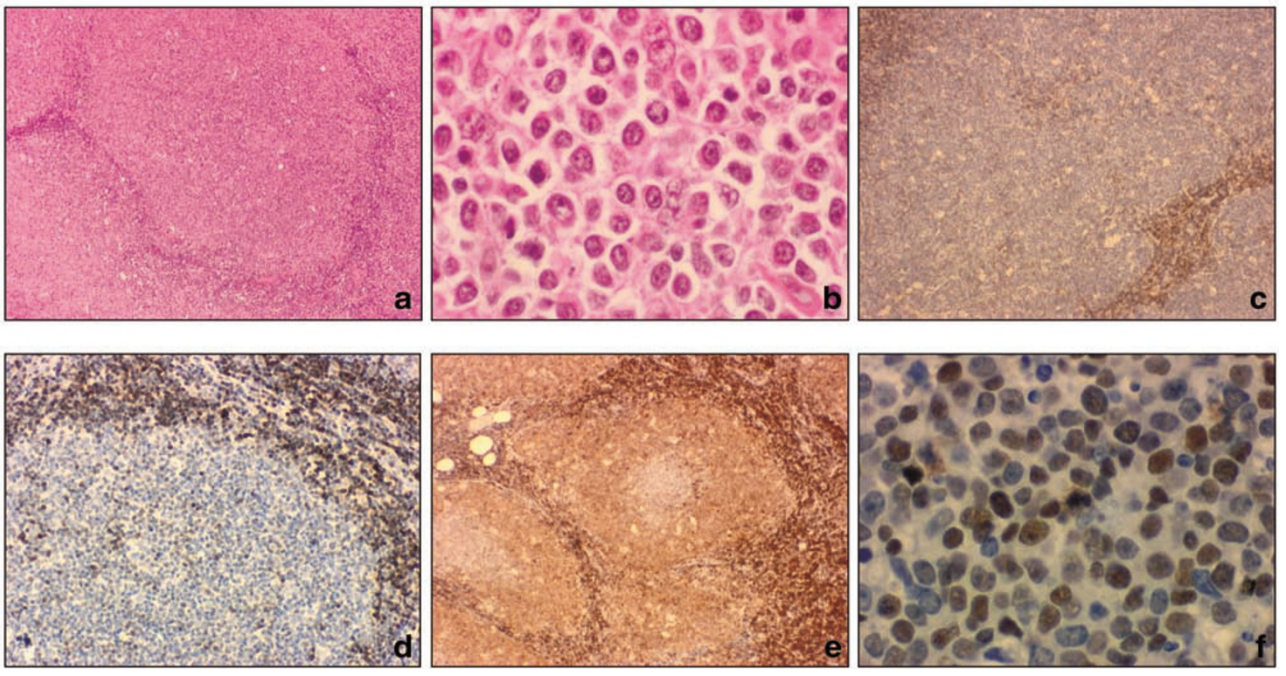

Figure 2 Histology and immunochemistery of 15 follicular lymphomas with a chromosome 3q27 rearrangement and without t(14;18) translocation. (a) In eight cases, the nodal architecture was characterized by large follicles (case 9). (b) A monocytoid cell component admixed with centrocytic-like cells was observed in five cases (case 14). (c) In three cases, a high rate of apoptotic activity was observed in BCL-2$^-$ follicles (case 2). (d) In 11 cases, follicle cells were BCL-2$^-$(case 5). (e) BCL-2 expression was absent in the center of tumoral follicle, contrasting with the positive inter-follicular area and the periphery of follicle in two cases (case 12). (f) In 12/14 cases, centroblastic and centrocytic-like cells showed a nuclear BCL-6$^+$ immunostaining (case 7).

Table 4 Pathological and molecular features of 15 follicular lymphomas with chromosome 3q27 rearrangement

Clinical features	*Follicular lymphoma with 3q27 rearrangement n = 15 (%)*	*Follicular lymphoma with t(14;18) n = 88 (%)*	*P*
Sex male	6 (40)	45 (51)	NS
Age >60 years	7 (47)	31 (35)	NS
Performance status >1	4 (27)	13 (14)	NS
Ann Arbor stage III–IV	9 (60)	70 (80)	NS
Largest mass >7 cm	3 (20)	44 (50)	NS
Bone marrow involvement	5 (30)	40 (45)	NS
Extra nodal site >1	4 (27)	15 (17)	NS
Elevated LDH level	7 (47)	33 (38)	NS
Histological and immunohistochemical feature	*n = 15*	*n = 20*	*P*
Mean diameter of follicules (mm) ± s.d.	1.03 ± 0.45	0.66 ± 0.24	0.006
Persistence of mantle zone	4	8	NS
Presence of diffuse area	6	7	NS
Presence of high apoptotic activity (starry sky appearance)	3	0	NS
Grade 3 staging	2	2	NS
BCL-2 staining positive cases	3	19	<0.001
BCL-6 staining positive cases	11	16	NS
CD10 positive cases	4	18	<0.001
CD23 residual meshwork staining	13	17	NS
p53 >50%	4	1	NS
Ki67 >50%	3	7	NS

4). Immunostaining for BCL-2 was carried out in 14 patients; virtually all tumor cells were BCL-2$^-$ in 11 cases (78%), whereas three cases revealed BCL-2 expression (Figure 1d). In two of these three BCL-2-postive cases, BCL-2 staining was heterogeneous, being mainly confined to the periphery of large follicles and mainly negative in the center of the follicle (Figure 2e). In the third positive case, the expression showed a diffuse pattern.

Despite variation in intensity, BCL-6 protein expression by tumor cells was scored as positive in 12 cases (86%) (Figure

2f). In case 12 with features of transformation into DLBCL, a composite pattern of expression was present, comprising BCL-6$^+$ positive follicles and BCL-6 negativity in the DLBCL area.

p53 protein expression was scored as negative (<10%) in 10 cases (67%), and positive in four cases (cases 3, 8, 11, 12), which were also associated with a high degree of Ki67 expression.

Comparative pathological features of 3q27$^+$ FL vs t(14;18)$^+$ FL

The main histopathological features of FL associated either with *BCL-6* or *BCL-2* rearrangements are presented in Table 3. Follicles were significantly larger in the 3q27$^+$FL group than in the t(14,18) group ($P = 0.006$), whereas there were no significant differences between the two groups regarding the presence of diffuse areas or well-formed mantle zones, the presence of tangible body macrophages and the number of large non cleaved cells. 3q27$^+$FL were more frequently BCL-2$^-$ ($P < 0.0001$) and CD10$^-$ ($P < 0.001$). They also showed a trend toward a higher rate of p53 expression ($P = 0.16$). No significant difference was found with respect to BCL-6, CD23 or Ki67 immunostaining.

Analysis of somatic mutations of the BCL-6 first intron

Single mutational changes (observed only in one BCL-6 clone) from the germ line BCL-6 sequence were present in the analyzed DNA of all cases, but were indistinguishable from *Taq* error or polyclonal mutations of reactive cells, and were therefore disregarded. In five cases, however, a total of 12 clonal and non-polymorphic mutations were observed, with a mutation frequency ranging from 9.0×10^{-4} to 5.4×10^{-2} bp^{-1} (Table 4).

In four cases, the observed transition/transversion ratio was higher (2.3) than expected and the T→C substitution was the most frequently observed mutation. Among the five mutated cases, four showed evidence of intraclonal heterogeneity consistent with an ongoing mutation process (Figure 3). In case 3, a deletion of a 174 bp region was observed. In the other cases, the incidence of single substitutions was higher than the *Taq* error rate suggesting that some of these mutations may be real. Case 12 showed a more complex pattern, including a high mutation rate, together with small insertions and deletions. The observed transition/transversion ratio was high (1.6) and mutations were more frequently located in RGYW/WRCY motifs (R = purine, Y = pyrimidine, W = A or T) than expected ($P < 0.05$).

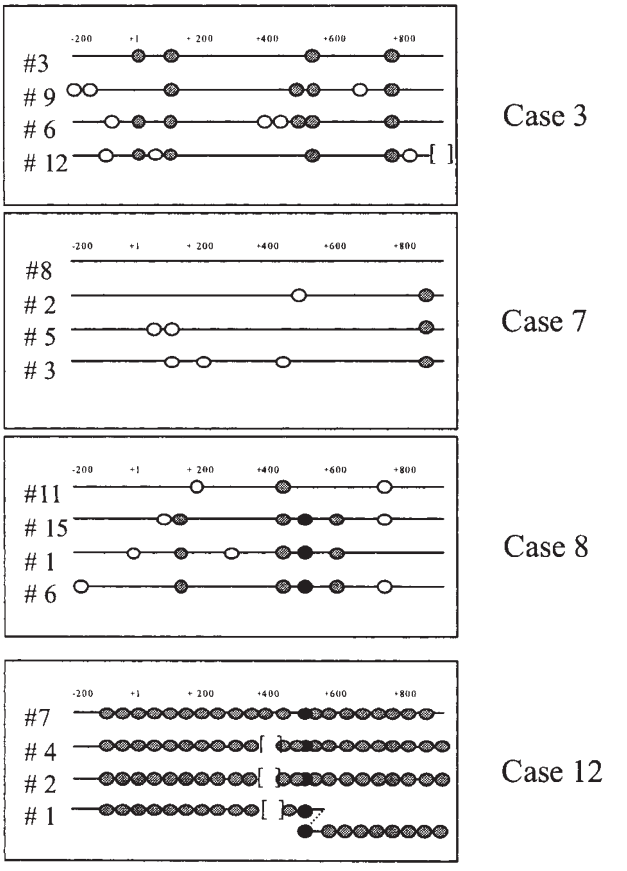

Figure 3 Intraclonal heterogenity and polymorphic variation of the *BCL-6* first intron in four cases of follicular lymphomas with 3q27 rearrangement. In order to facilitate comparison of observed mutations with previously published data, the *BCL-6* region is numbered as described by Lossos and Levy.[31] Due to differences in primer design we used a negative numbering to describe the first 200 bp. Horizontal lines represent sequenced clones derived from lymphoma samples. Mutations are indicated as circles (grey circle = observed >1 in *BCL-6* clones; white circle = observed in only one *BCL-6* clone; black circle = deletion T520, polymorphic variation) and mutational deletions as square brackets. In case 12, partial mutations are shown. Case 3, T18C; T95A; T542C; C548T; A782T; deletion [835–1009]; Case 7, T902C; Case 8, T118C; G448A; deletion T520; A601C; Case 12, C(-20)T; T(-7)C; CT29C; A58C; C68G; T75C; G82A; A86C; T87G; A90C; A121G; T131A; G132A; T133C; A135T; C145T; A149G; A151G; A174C; A176T; G184A; T189G; A191C; T194A; A208G; G234T; A283G; G299A; G300T; deletion [391–393]; deletion [401–410]; C411A; C415T; C419T; G422T; C423T; C435G; T436C; G437A; T460A; T492C; A505C; deletion T520; duplication [505–536]; G539A; G597A; G598A; A600G; A601C; T625C; G647A; C662A; A702G; T737A; T740C; G780C; C792T; C819G; T823C; C829T; G850A.

Discussion

In the present study, we show that a subtype of FL associated with a *BCL-6* rearrangement, but lacking t(14;18), has distinct pathological features in comparison to classical FL. A peculiar histological feature of these lymphomas is a prominent nodal architecture, frequently constituted by significantly larger follicles than in t(14;18)$^+$ FL. It is known from studies in knockout mice that BCL-6 protein expression is required for germinal center formation.[11] The mechanism by which *BCL-6* regulates GC development and influence its morphology is as yet not established. It is unclear whether prolonged *BCL-6* expression, potentially induced by translocation, could lead to an exuberant follicle growth pattern. The capacity of BCL-6 to block terminal B-cell differentiation, most likely by inhibition of the *Blimp-1* gene,[32] and the description of peripheral T-cell lymphoma cases expressing BCL-6 protein with a follicular growth pattern,[33] may implicate a function for BCL-6 in follicular architecture.

The second remarkable feature is the BCL-2 negative phenotype observed in the majority of cases. BCL-2 protein is expressed in most FL, including cases lacking the t(14;18) translocation.[7] However, BCL-2 expression by itself is not sufficient to induce FL, and it is established that additional genetic abnormalities are required.[5] Our study also suggests that the t(14;18) translocation is not strictly required in the patho-

genesis of at least a subset of FL. This raises the question whether an anti-apoptotic function of BCL-6 could act as a surrogate for BCL-2 defects in this group of FL. BCL-6 is known to inhibit myocyte apoptosis[34] and to repress expression of the cycline-dependent-kinase inhibitor p27/kip1,[32] which controls G1/S transition. However, in contrast, studies in different cell models, including osteosarcoma cells, macrophages or myocytes have shown that BCL-6 can act as a pro-apoptotic protein.[35–37] These observations show that many aspects of *BCL-6* activity are as yet incompletely understood, and that BCL-6 function may depend on the cellular subtype in which the gene is expressed.

The third remarkable feature observed in our series was the unusual CD10$^-$ phenotype. Although CD10 expression is observed in lymphomas which are thought to arise from the GC cells, including FL and some DLBCL or Burkitt's disease,[8] a CD10$^-$ BCL-6$^+$ phenotype has also been reported in some FLs, particularly with splenic involvement[38] and in L&H cells.[39] This phenotype suggests that expression of both GC markers can be dissociated, but it is unknown whether it corresponds to a normal stage of GC B cell development or to a tumor specific antigen loss.

Finally, it appears that FLs associated with 3q27 rearrangement, but lacking the t(14;18) translocation, share a common and unusual CD10$^-$ BCL-2$^-$ BCL-6$^+$ phenotype and frequently disclose a monocytoid component. Interestingly, Horsman and colleagues[20] observed a monocytoid differentiation in 2/7 3q27$^+$/t(14;18)$^-$ FL cases, as well as a CD10$^-$ immunophenotype in 7/7 cases. Nevertheless, it remains to be determined if the 3q27$^+$ FL cases represent a homogeneous subtype of lymphoma. In our series, three cases with small follicles composed of CD10$^+$, BCL-6$^+$ and BCL-2$^+$ cells were indistinguishable from 'true' FLs with t(14;18) translocation. Concerning the other cases, nodal marginal zone lymphoma (MZL) in which *BCL-6* rearrangements may also occur[40,41] certainly represents the most difficult differential diagnosis. Indeed, the presence of a monocytoid component in five cases, a CD5$^-$/CD10$^-$ phenotype and a trisomy 3 in four cases are consistent with this diagnosis.[8] However, BCL-6 protein expression, useful to discriminate subtypes of lymphomas with a follicular growth pattern and consistently negative in MZL,[38] was found in 12/14 cases. Furthermore, foci of monocytoid B cells, which are clonally identical to follicular cells,[42] are observed in about 10% of FL[43] and such cases are regarded as FLs with marginal zone differentiation.[42]

Mutations of the first *BCL-6* intron are acquired during B cell transit through the GC[23,24] and their presence provides additional information concerning the tumor cell of origin.[21,22,44] Five of seven cases had mutations in *BCL-6*, similar to those previously described, consisting of single substitutions, excess of transitions and hot spots in RGYW motifs, consistent with features of the somatic hypermutation machinery.[21,25,26] In four cases, an ongoing mutation process was observed, suggesting that these lymphomas appear and remain in the germinal center environment.[45] PCR primers, used in our study, widely flanked a 120 bp region, described by Akasaka and colleagues,[30] in which the majority of breakpoints cluster, especially when *IgH* genes are involved. Therefore, it is likely that we preferentially amplified the untranslocated allele, excluding effects of translocated *IgH* regulatory regions, as described in *c-MYC* or *BCL-2* genes when they are juxtaposed to *IgH* loci.[46,47]

There is evidence to indicate that the t(14;18) translocation arises at the pre-B stage of development, during activation of the enzymatic mechanisms governing the V(D)J recombination process.[48] However, *IgH/BCL-6* translocations, invariably involving switch regions of *IgH*,[30] are most likely to occur during isotype class switch process, also occurring in the GC. Thus, the t(3;14) translocation observed in 13/15 of our cases, which can be regarded as the primary event, most likely occurs at a later stage of B-cell differentiation than in t(14;18)$^+$ FL. At this stage, *BCL-6* mutations and isotype switch mechanisms are both activated. It is of note that in recent reports, FL cells have been shown to engage the switch process, suggesting that in rare FL cases, the primary genetic event could occur at a later stage of maturation.[49,50]

Although the t(3;14)(q27;q32) translocation is mainly associated with *de novo* DLBCL,[14,17] our findings clearly indicate that at least some of them could have an initial follicular growth development. Indeed, four patients developed a DLBCL during the course of the disease and one initially presented with features of both FL and DLBCL. However 3q27$^+$ FL has an overall survival curve similar to t(14;18)$^+$ FL cases and does not mimic that of aggressive lymphomas. The clinical signification of the lack of t(14;18) translocation in FL is a controversial issue.[51–53] Shen and colleagues[53] showed that t(14;18)$^-$ FL had distinct clinical characteristics including a higher rate of localized stage (I, II), a lower incidence of bone marrow involvement, a higher incidence of extra-nodal involvement and a higher incidence of trisomy 3 (25%). All of these trends are present in our series but have also been described in DLBCL with 3q27 rearrangement.[18] Nevertheless, survival was similar to indolent t(14;18)$^+$ FL cases in our study, and this result contradicts that obtained by Lopez-Guillermo and colleagues,[52] showing that 'germline' FLs had a worse prognosis, similar to DLBCL. However, in the latter series, FLs were not characterized by cytogenetics, a method known to be more reliable than the molecular approach which gives false negative results, specially due to alternative breakpoints.[54,55] The prognostic value of *BCL-6* translocation in DLBCL and in FL is also a controversial issue[13,18,56,57] and most likely appears less crucial than *BCL-6* mRNA expression, which is strongly associated with a better prognosis in DLBCL.[58,59] Whether *BCL-6* mRNA level of expression in 3q27$^+$ FL is higher than in standard FL cases and could have a prognostic significance or could explain the distinctive pathological features reported in this study remains to be determined.

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