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IgV$_H$ and *bcl6* somatic mutation analysis reveals the heterogeneity of cutaneous B-cell lymphoma, and indicates the presence of undisclosed local antigens

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Our understanding of the ontology of B-cell lymphomas (BCL) has been improved by the study of mutational status of *IgV$_H$* and *bcl6* genes, but only a few cases of cutaneous BCL have been examined for this status. We analyzed *IgV$_H$* and *bcl6* somatic mutations in 10 cutaneous BCL, classified as follicular (three primary and one secondary), primary marginal zone (two cases), and diffuse large BCL (three primary and one secondary). We observed a lower rate ($<$2%) of *IgV$_H$* mutation in all marginal zone lymphomas, and a preferential usage of *V$_H$2-70* (one primary follicular and two primary diffuse large BCL). Fewer than expected replacement mutations in framework regions (FR) were observed in three primary follicular lymphomas (FLs) and in all diffuse large BCL, indicating a negative antigen selection pressure. Ongoing mutations were observed in eight of 10 cases. Only two primary FLs and two diffuse large BCL showed bcl6 somatic mutation. These data support the heterogeneous nature of the different cutaneous BCL, and specifically the distinction between cutaneous follicular and marginal zone lymphomas. The biased usage of *V$_H$2-70*, the low rate of replacement mutation in the FR, and the presence of ongoing mutation imply that local antigens could modulate the growth of primary cutaneous BCL.
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Primary cutaneous B-cell lymphomas (BCLs) are conventionally defined as lymphomas arising in the skin, without evidence of extracutaneous disease, at least for a period of 6 months following initial diagnosis.[1–6] Although the clinical behavior of primary cutaneous BCL is dramatically different from those diagnosed in lymph nodes,[1,2,5,7–9] the morphology is strikingly similar. Until now no clear immunophenotypical or molecular differences have been known,[10–12] except that t(14;18) occurs rarely in skin follicular lymphomas (FLs), while it is characteristic of nodal FLs.[10,13–15] At the same time, it seems that primary cutaneous BCL constitutes a heterogeneous group encompassing tumors denominated as FL, marginal-zone BCL and diffuse large BCL, although the precise distinction of these different cutaneous BCLs is a matter of controversy.[1,2,5,7,16–18] Underlying all the uncertainties about the knowledge and classification of cutaneous BCLs is the relative paucity of available information about distinctive molecular events, with the exception of the identification of local phenomena such as *Borrelia burgdorferi* infection, and the expression of skin-related adhesion molecules[19–21] in a subset of cutaneous BCLs.

The study and comprehension of BCL has been notably facilitated by the finding that the acquisition of somatic *IgV$_H$* and *bcl6* mutations is the result of the exposure of B -cells to the germinal center microenvironment, which makes it possible to assign a pre or postgerminal center origin to the

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various types of BCL. Here, we have analyzed the presence of somatic IgV_H and *bcl6* mutations in a panel of the different BCL types that can be diagnosed in the skin. For comparative purposes, we have included two secondary BCLs diagnosed in the skin.

Materials and methods

Clinical Data

Only patients with complete staging at diagnosis were included in the study. In all cases, the staging procedure included physical examination, thoracic and abdominal X-ray and/or computer-assisted tomographic scan, peripheral blood-cell count, routine laboratory tests, and bone marrow aspirate and/or biopsy.

BCL diagnosed in the skin were considered as primary if the tumor remains localized on the skin for a period of at least 6 months following diagnosis. Cases included in this report were deemed secondary BCLs if the clinical staging at diagnosis showed disseminated disease.

Tissue Samples

Frozen samples belong to a larger collection of cutaneous BCLs obtained from the archives of the Spanish Tumor Bank Network. These cases were selected according to the availability of clinical information and DNA for mutational studies. Moreover, formalin-fixed, paraffin-embedded blocks were used for routine histological and immunohistochemical studies, and polymerase chain reaction (PCR) was used for *IgH* rearrangement and t (14; 18).

Immunohistochemistry

All cases were subjected to routine hematoxylin–eosin and immunohistochemical study, performed on formalin-fixed, paraffin-embedded tissue, using a prior heat-induced antigen-retrieval step for all antibodies. Thus, before incubation with the primary antibody, the slides were heated in a pressure cooker for 3 min in a solution of 0.01 mol/l sodium citrate. After incubation with the primary antibody, immunodetection was performed with biotinylated antimouse immunoglobulins, followed by peroxidase-labeled streptavidine (LSAB-DAKO, Glostrup, Denmark) with diaminobenzidine chromogen as substrate. All immunostaining was performed using the Techmate 500 (DAKO) automatic immunostaining device. The antibodies used for immunohistochemical study were the lymphoid differentiation markers CD20, CD79a, CD3, CD43, IgD, bcl6, CD21, CD23, p53 and bcl2, immunoglobulin light chains κ and λ (DAKO), CD10 and CD5 (Novocastra, Newcastle upon Tyne, UK) (Table 1). The proliferative index, measured as the Ki67 index, was studied using the MIB1 antibody (DAKO).

PCR Detection of t (14; 18)

DNA was isolated by conventional methods. PCR analysis of t(14;18) was performed using published primer sets to detect the Major Breakpoint region (MBR)[22] (Table 2). Amplification conditions have been described previously.[10]

IgV_H Study

Rearranged IgV_Hs were amplified using a seminested PCR method as previously described. In the first round of PCR, a mixture of six framework 1 (FR1) V_H family-specific primers and two consensus primers for J_H gene were used. The second round of PCR were performed in six separate reactions with one of the six V_H FR1 primers and J_H internal primers (Table 2).

Briefly, 200 ng of DNA were amplified in a volume of 50 μl with 1 × PCR buffer, 200 μmol/l dNTPs, 2.5 mmol/$MgCl_2$, 250 nmol/l of each primer and 1 U

Table 1 Characteristics of antibodies

Antigen	*Clone*	*Incubation time*	*Dilution*
CD20	L26 (DAKO)	40 min	1:50
CD79a	JCB117 (DAKO)	40 min	1:25
CD23	MHM6 (DAKO)	40 min	1:25
CD10	56C6 (Novocastra)	Overnight	1:25
CD3	Polyclonal (DAKO)	40 min	1:50
CD43	DFT1 (DAKO)	40 min	1:50
CD5	4C7 (Novocastra)	40 min	1:10
CiclD1	DC56 (DAKO)	40 min	1:100
P53	DO7 (DAKO)	40 min	1:50
Bcl2	124 (DAKO)	40 min	1:25
Bcl6	PG-B6P (DAKO)	40 min	1:10
MIB1	MIB1 (DAKO)	40 min	1:50

Table 2 Characteristics of primers

Gene	*Sense*	*Antisense*
IgH	JH:CACCTGAGGAGACGGTGACC	FRIII: ACACGGC(CT)(CG)TGTATTACTGT FRII: TGG(AG)TCCG(CA)CAG(GC)C(TC)(TC)CNGG
Bcl2	JH	GAGTTGCTTTACGTGGCCTG
V_H1	JH	AAATCGATACCACCATGGATGGACTGGACCTGGAGG
V_H2	JH	AAATCGATACCACCATGGACACACTTTGCT(A/C)AC
V_H3	JH	AAATGCATACCACCACCATGGAGTTTGGGCTGAGC
V_H4	JH	AAATCGATACCACCATGAAACACCTGTGGTTCTT
V_H5	JH	AAATCGATACCACCATGGGGTCAACCGCCATC
V_H6	JH	AAATCGATACCATGTCTGTCTCCTTCCTC
Bcl6	CCGCTGCTCATGATCATTATTT	TAGACACGATACTTCATCTCAT

of Ampli Taq Gold. In the second round of amplification, the same concentrations of reagent were used, except for $MgCl_2$, whose concentration was 1.5 mmol/l. A volume of 1 μl of the first-round PCR product was added to the seminested reaction as a template. The PCR conditions have been described previously.[23]

Each PCR product was subsequently cloned. Gel-purified PCR products from these cases were ligated into a PGEM-T easy vector (Promega). Plasmid with the inserts was transformed into JM109-competent cells (Promega). Plasmids were checked by PCR using primers of inserted sequences of the IgV_H family of its respective case. The PCR product of colonies with inserts was subsequently purified and directly sequenced in order to provide a minimum of four and a maximum of 10 colonies per case.[24]

Sequencing products were analyzed with the ABI PRISM 310 genetic analyzer (Applied Biosystems, Weiterstadt, Germany), following standard procedure. Mutations were identified by comparison with the germline sequence (Ig BLAST and V BASE directory).

To determine whether the number of replacement (R) and silent (S) aminoacid substitutions identified were indicative of antigen selection, the Chang and Casali method was used.[25]

For each case, the intraclonal variability was recorded, and the sequence of dominant clone, the *consensus sequence*, was considered the most frequent sequence among clones from each case. Only confirmed sequences were taken as evidence of clonal heterogeneity; the unconfirmed cases were disregarded because they could have been due to Taq polymerase error.

Bcl6 Study

For the *bcl6* gene study a unique segment product of 790 bp (+357–1142) was amplified (Table 2). This fragment is located downstream of the first *bcl6* noncoding exon, and has been shown to harbor >95% of the mutations in the 5′ noncoding region of *bcl6*.

The PCR reaction was carried out in a 100 μl volume containing 100 pmol of each primer, 800 μM of dNTPs, 10 mmol Tris-HCl, 50 mM KCl, 1.5 μM $MgCl_2$ and 2 U of Taq polymerase (TAQ Platinum, Gibco). Amplification conditions have been described previously.[26]

PCR product was then purified (Microcon PCR, Millipore) and was subjected to a cycle-sequencing reaction, using PCR producing primers and two previously described internal primers, E1.10 and E1.11.

Sequencing products were analyzed with the ABI PRISM 310 genetic analyzer and compared with the new *bcl6* gene germline sequence (z79581). When a suspected mutation was found, the sequencing reaction was repeated in a new PCR product from the same case.

Results

Clinical Characteristics

Our series included 10 patients, six males and four females, with a mean age of 55.3 years (range, 29–80). Two of these cases were disseminated at the time of diagnosis (stage IV). Localized skin lesions were observed in nine cases (six involving head, one trunk, one arm and one leg skin) and multiple lesions were observed in one case. The cases with systemic involvement received chemotherapy after the surgical excision. The other cases received exclusively local treatment (surgical excision and/or radiotherapy) (Table 3).

Histological and Immunohistochemical Features (Table 3)

All cases were classified according to the REAL/WHO classification. Four cases were classified as FLs, one as secondary FL and three as primary FLs, characterized by a nodular pattern of growth of malignant cells with centrocytic and centroblastic cytology, and CD20+, Bcl6+, CD10+ (2/3), Bcl2+ (2/4) immunophenotype. The presence of CD21+ follicular dendritic cells was noticed in all cases (Figure 1a–d).

Table 3 Clinico-pathological characteristics of patients

	Histology	*Sex; Age (years)*	*Site*	*Relapses*	*Follow-up (months)*	*CD10*	*CD43*	*Bcl-2*	*Light-chain restriction*	*MIB-1*	*t(14;18)*
1	PCFL	F; 59	Trunk	Skin	12	–	+	+	N	High	N
2	PCFL	M; 59	Head	Skin, LN	47	+	ND	–	N	Low	N
3	PCFL	M, 29	Arm	Skin	44	–	+	+	Y	Low	ND
4	sFL	F; 50	Head	Systemic	60	+	–	+	N	High	Y
5	PMZL	M; 50	Head and neck	Skin	12	–	+	–	ND	Low	N
6	PMZL	M; 54	Head	No	12	–	–	–	N		N
7	PDBCL	M;56	Head	No		–	–	–	N	High	ND
8	PDBCL	F; 55	Head	No	12	–	–	–	N	High	ND
9	PDBCL	M; 78	Leg	No		–	–	+	N	High	N
10	sDBCL	F; 80	Head	Systemic	24	–	–	-	N	High	ND

F, female; M, male; LN, lymph node; ND, not done; N, no; Y, yes.

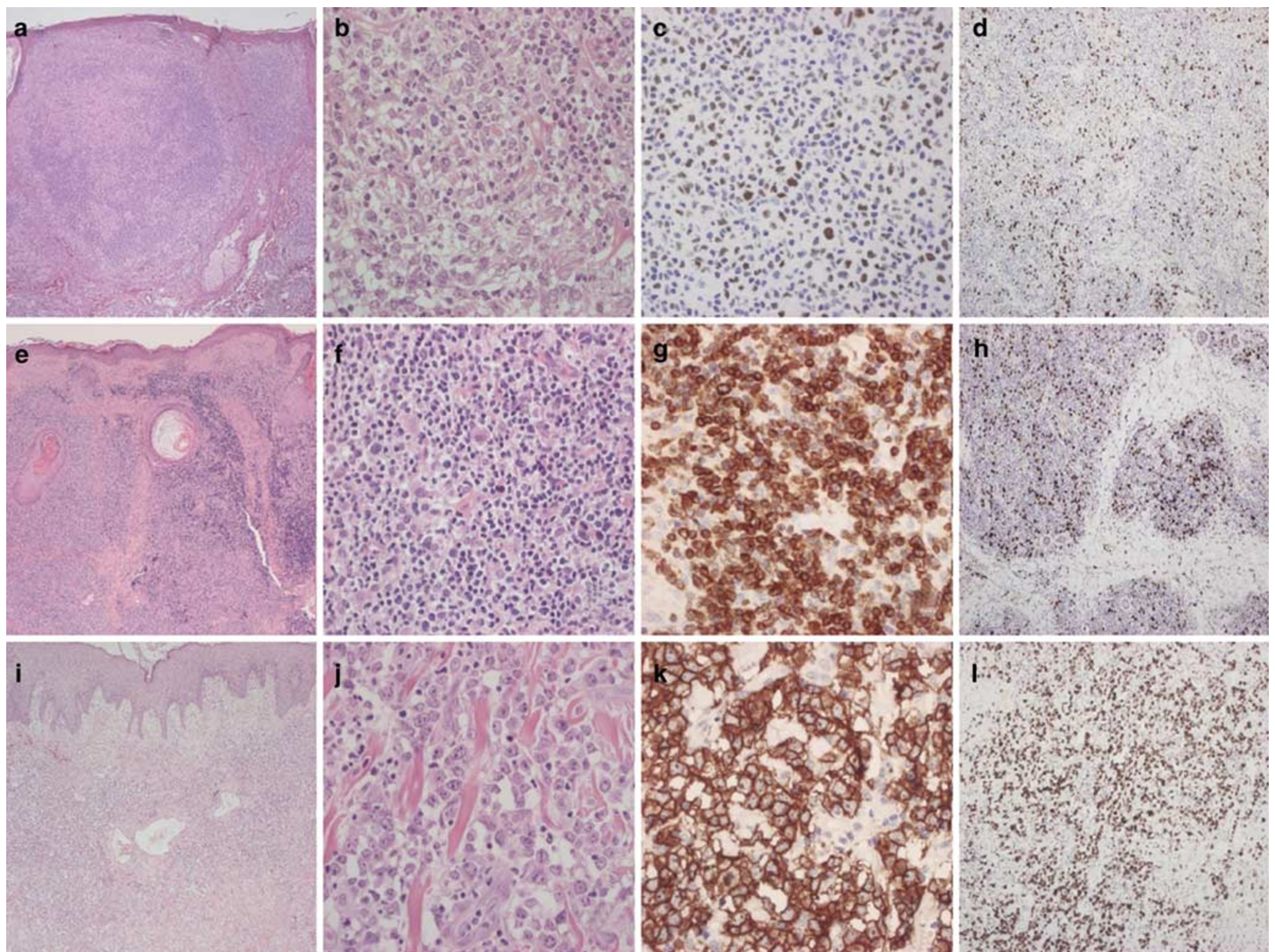

Figure 1 (**a**) Hematoxylin and eosin (H&E) of a primary FL case (original magnification ×4 and (**b**) detail of centrocytic and centroblastic cytology (×40). (**c**) Bcl6-positive expression in a primary FL case (×40). (**d**) Low proliferation index in a primary FL case (MIB1) (×10). (**e**) H&E of a MZL case (original magnification ×4) and (**f**) detail of marginal zone cytology (×40). (**g**) Bcl2-positive expression in a MZL case (×40). (**h**) Low proliferation index in a MZL case (MIB1) (×10). (**i**) H&E of a primary DBCL case (original magnification ×4) and (**j**) detail of large tumoral cells (×40). (**k**) CD20-positive expression in a primary DBCL case (×40). (**l**) High proliferation index in a primary DBCL case (MIB1) (×10).

Two cases were classified as primary MZL, characterized by a diffuse pattern of growth of cells with a marginal zone cytology; a variable number of monoclonal plasma cells and scattered reactive follicles. Neoplastic cells were Bcl2-positive, CD10- and Bcl6- negative, and CD43- and CD38-positive in varying proportions (Figure 1e–h).

Four cases were classified as DLBCLs (three primary and one secondary), characterized by a diffuse pattern of growth, and composed of large CD20+ cells with centroblastic and immunoblastic phenotype. Case 9 displayed strong Bcl2 overexpression and high MIB 1 proliferative index. No difference was observed between pDLBCLs and sDLBCL (Figure 1i–l).

V_H Gene Usage

We amplified and sequenced 54 clonal IgV_H rearrangements from 10 selected cases. All V_H rearrangements were potentially functional. Our data demonstrate a trend towards usage of the V_H3 family (cases 3, 4, 9 and 10) and V_H2 (cases 2, 5, 7 and 8), with four cases each, followed by V_H5 in case 1 and V_H6 in case 6. In particular, we observed more frequent use of the V_H2-*70* gene in one primary FL (case 2) and 2 primary DLBCLs (cases 7 and 8).

Mutational Analysis

With respect to consensus sequences, three of 10 cases displayed >98% sequence homology with the nearest germline V_H gene (one secondary FL and two cases of primary MZLs), while seven of 10 showed <98% sequence homology.

The majority of mutations were localized in FRIII and CDRII (complementarity-determining region) regions. The mutations were the result of single-nucleotide substitutions.

Analysis of the Mutational Pattern (Table 4)

We observed a range of number of mutations between–1 and 31 in the consensus sequences, representing a percentage of mutations of 6–100% of total mutations observed in colonies of each case.

The distribution of R and S mutations were calculated by considering all possible mutations, as described by Chang and Casali.[25] Significant evidence of positive selection was observed in one case (case 8, primary DBCL), where there were more replacement mutations in CDR regions than expected by comparison with FR regions.

In six cases (three pFLs, three pDLBCLs, and one sDLBCL) we observed fewer replacement mutations in FR regions than expected, indicating negative antigen selection, which points to the existence of pressure to maintain the germline configuration. The remaining three cases showed no significant evidence of antigen selection. Moreover, the analysis of the most representative clones apart the consensus sequence among colonies from pMZLs showed a trend towards negative antigen selection (data not shown).

Table 4 Mutational status

	Histology	*Colonies*	*VH family*	*ID%*	*Mutation number in consensus sequence*	*Presence of hypermutation in consensus sequence*	*Common mutations (%)*	*Presence of hypermutation in other sequence*	*Observed R/S*		*Expected R/S*		*pCDR*	*pFR*	*Ongoing mutation*	*Bcl6 mutations*
									CDR	*FR*	*CDR*	*FR*				
1	PCFL	6	VH5-51	96	10	Y	7/22(31)	Y	0	0.72	2.4	5.1	0.064	0.0082	Y	Y
2	PCFL	4	VH2-70	96	9	Y	9/16(56)	Y	0.5	2	2.16	4.59	0.42	0.059	Y	Y
3	PCFL	6	VH3-21	90	26	Y	26/26(100)	N	7	8	6.34	13.26	0.16	0.0190	N	N
4	sFL	4	VH3-21	99	2	N	2/4(50)	Y	0	0	0.48	1.02	0.36	0.24	Y	N
5	PMZL	9	VH2-5	99	1	N	1/15(6)	Y	0	1	0.24	0.51	0.76	0.71	Y	N
6	PMZL	6	VH6-1	99	1	N	2/4(50)	Y	0	0	0.24	0.51	0.76	0.49	Y	N
7	PDBCL	6	VH2-70	97	8	Y	8/24(30)	Y	3	2	1.92	4.08	0.16	0.027	Y	Y
8	PDBCL	6	VH2-70	94	15	Y	12/18(66)	Y	7	2	3.6	7.65	0.03	0.0026	Y	N
9	PDBCL	5	VH3-30	97	5	Y	5/19(26)	Y	0	0	1.2	2.55	0.25	0.028	Y	ND
10	sDBCL	4	VH3-23	87	31	Y	31/31(100)	Y	10	6	7.44	15.81	0.08	0.0002	N	Y

ID%, % of homology with germline sequence; R, replacement mutation; S, silent mutation; CDR, complementary-determining region; FR, framework region; Y, yes; N, no; ND, not done.

Ongoing Mutational Analysis

Information on intraclonal variations was obtained from analyzing the mutational status in all colonies arising from each case. Cases 1, 2, 3, 5, 6, 7, 8, and 9 showed intraclonal variation of 4.3, 2.5, 0.5, 3.0, 3.0, 2.8, 1.5 and 3.8 bases/clone, respectively. Thus, in general, three of four FLs (two pFLs and one sFL), three of four DLBCLs, and two of two MZLs showed intraclonal variability, compared with the two cases not showing ongoing mutation (one primary FL and one secondary DLBCL) (Table 4).

Analysis of *Bcl6* Gene Mutational Status

Bcl6 mutation was found in four cases, including two of three cases of primary FL (case 1 showed tandem duplication of the sequence +956–962; +599–+606 of our amplicon and case 2 had an A→G transition at position +862; +505 in our amplicon); and two of three primary DLBCLs, with T≡G at position +471 (+114 of our amplicon) and G≡C at position +793 (+436 of our amplicon) (cases 7 and 10) (Table 4).

Discussion

This study aimed to identify differences in the mutational status of the various types of cutaneous BCL. Particular attention has been paid to the mutational status of skin lymphomas of supposed origin in the lymphoid follicle.

The usage of *V_H2-70* gene in three cases (1 FL and 2 DLBCLs) seems to indicate a preferential usage of this gene in primary cutaneous BCL, hypothetically correlated with the local presence of specific antigens.[25]

Presence of IgV_H mutations was observed in all cases, with a lower rate (<2%) in primary MZLs and in the single case analyzed here of cutaneous involvement by disseminated FL. This seems to confirm the heterogeneity of cutaneous BCL.

The number and character of somatic mutations can also reveal the possible presence of local antigens promoting tumoral growth, since replacement mutations that affect residues in the hypervariable or CDR loops can be positively or negatively selected by antigen. Here, the analysis of mutations in V_H genes showed a significantly low number of R (replacement) mutations in framework regions in all primary FL and DLBCL cases. This implies the presence of a negative antigenic selective pressure. It has been proposed that selection against R mutations in FR regions acts to maintain immunoglobulin structure, because of its high affinity to the antigen involved in clonal selection.[27] This negative antigen selection is thus pointing towards the hypothetical existence of local antigens selectively pressuring to maintain the germline configuration. The role of hypothetical antigens in clonal selection is also suggested by the existence in one case, a significantly more R mutations in CDR regions than would be expected if mutations had occurred by chance alone, without the operation of selective forces. An interesting finding in the group of primary FLs was the presence of negative antigen selection, which parallels the observation by Aarts *et al*[28] in a large series of nodal FLs, where 26 of 30 cases exhibited negative antigen pressure and the results of previous work in primary cutaneous BCL, where the presence of negative antigen pressure was observed in three of four primary FLs and in two of three lymphoplasmacytic lymphomas.[24]

Ongoing IgV_H mutations were observed in all but two of these cases (cases 3 and 10). The presence of ongoing mutations has been claimed to be indicative of the existence of a germinal-center microenvironment within the tumor.[29] Moreover, it has been demonstrated that eradication of *Helicobacter pylori* reduces intraclonal variability in MALT lymphomas,[30] and proposed that ongoing mutation in MALT lymphoma IgV_H genes suggests that antigen stimulation plays a role in the clonal expansion.[31] Unlike primary DLBCL, the only secondary DLBCL examined here did not show intraclonal variability.

The study of *bcl6* mutation reveals the absence of somatic mutations in the group of primary MZLs, in contrast with the groups of pFLs and pDLBCLs. *Bcl6* gene, located in 3q27, encodes a POZ/Zinc-finger transcriptional repressor. Its expression is related with the formation of germinal center through an incompletely understood regulation of the balance between proliferation and apoptosis.[26,32] *Bcl6* gene is frequently involved in translocations in 30–40% of large cell lymphoma and in 6–10% of FLs.[33,34] *Bcl6* somatic mutations are described in germinal center (GC) and post-GC-derived lymphoma, probably caused by somatic mutation machinery acting on the 5′ noncoding region of the *bcl6* gene, due to its sequence homology with immunoglobulin gene.[27,35,36] Our analysis showed mutations of *bcl6* 5’ noncoding region in two of three pCFLs, and one of two pDBCLs and sDBCL. These mutational findings were observed in previous reports of nodal lymphomas, where no differences in *bcl6* mutation rate in FL and DBCL have been observed.[27] However, although *bcl6* mutations are absent from both cutaneous MZLs, they have been described in MZL–MALT types at other locations. In such cases, the *bcl6* gene mutation rate has been found to be similar to that observed in FL.[37] Nevertheless, it is consistent with the low IgV_H mutation rate observed here in cutaneous MZL, since, in general, mutation frequencies of the *bcl6* and IgV_H genes are similar.

In conclusion, our analysis showed that MZLs in the skin have low IgV_H and *bcl6* mutation rates, thus supporting the view of the heterogeneous nature of the different types of cutaneous BCL. The biased usage of *V_H2-70*, with the low rate of replacement mutations in the framework region, and the presence of ongoing IgV_H mutation suggest that the

growth of primary BCLs could be modulated by the presence of local antigens.

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