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ORIGINAL ARTICLE

Immunoglobulin gene repertoire in ocular adnexal lymphomas: hints on the nature of the antigenic stimulation

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Evidence from certain geographical areas links lymphomas of the ocular adnexa marginal zone B-cell lymphomas (OAMZL) with *Chlamydophila psittaci* (Cp) infection, suggesting that lymphoma development is dependent upon chronic stimulation by persistent infections. Notwithstanding that, the actual immunopathogenetical mechanisms have not yet been elucidated. As in other B-cell lymphomas, insight into this issue, especially with regard to potential selecting ligands, could be provided by analysis of the immunoglobulin (IG) receptors of the malignant clones. To this end, we studied the molecular features of IGs in 44 patients with OAMZL (40% Cp-positive), identifying features suggestive of a pathogenic mechanism of autoreactivity. Herein, we show that lymphoma cells express a distinctive IG repertoire, with electropositive antigen (Ag)-binding sites, reminiscent of autoantibodies (auto-Abs) recognizing DNA. Additionally, five (11%) cases of OAMZL expressed IGs homologous with autoreactive Abs or IGs of patients with chronic lymphocytic leukemia, a disease known for the expression of autoreactive IGs by neoplastic cells. In contrast, no similarity with known anti-Chlamydophila Abs was found. Taken together, these results strongly indicate that OAMZL may originate from B cells selected for their capability to bind Ags and, in particular, auto-Ags. In OAMZL associated with Cp infection, the pathogen likely acts indirectly on the malignant B cells, promoting the development of an inflammatory milieu, where auto-Ags could be exposed and presented, driving proliferation and expansion of self-reactive B cells.
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Introduction

Lymphomas arising in the ocular adnexa (conjunctiva, orbit, eyelid, and lachrymal gland and sac) account for 15% of extranodal lymphomas.[1] Almost all lymphoma categories may occur in these anatomical structures, but up to 80% of ocular adnexa lymphomas are represented by extranodal ocular adnexa marginal zone B-cell lymphomas (OAMZL).[2]

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Extranodal marginal zone lymphomas (EMZL) provide a valuable *'in vivo'* model for assessing the actual impact of the microenvironment in lymphomagenesis. Several lines of evidence indicate that the development of neoplastic B lymphocytes is dependent upon chronic stimulation operated either by autoimmune processes[3,4] or persistent infections.[5–7] OAMZL exemplifies these concepts, as it has been reported to be significantly associated, although with variable geographic prevalence, with *Chlamydophila psittaci* (Cp) infection.[8] Cp is the etiological agent of human psittacosis, a disease caused by exposure to infected animals (e.g. birds, cats and other pets). The key role had by Cp infection in OAMZL pathogenesis has been further underscored by immunohistochemistry, immunofluorescence, electron microscopy, PCR, direct sequencing, *in vitro* cultures, and the demonstration of an objective clinical response following Cp eradication with doxycycline treatment in half of patients with OAMZL.[9,10]

The actual mechanisms linking Chlamydiae to OAMZL development are likely multifaceted,[1] though they have not yet been elucidated. The evaluation of immunoglobulin (*IG*) gene repertoire appears an appealing approach to address this question. Previous analysis of the IG heavy variable (*IGHV*) genes in OAMZL demonstrated a restricted repertoire[2,11,12] with mutated genes and ongoing somatic mutations in most cases,[11,13,14] thereby alluding to an origin from an antigen (Ag)-experienced B lymphocyte, which has undergone Ag selection. However, all these studies, but one, included $\leqslant 10$ investigated cases; in addition, no information on correlation with infectious agents, Chlamydiae in particular, was provided.

Information about the potential antigenic elements stimulating neoplastic B-lymphocytes in OAMZL is not available. Theoretically, antigenic stimulation might be provided either directly by the bacteria, or indirectly through cellular and humoral interactions resulting from the inflammatory milieu that may precede and permeate OAMZL. This speculation is corroborated by the paradigm of gastric EMZL, where malignant B lymphocytes do not directly interact with *Helicobacter pylori* (*Hp*),[15] but are inappropriately stimulated, via CD40–CD40L interactions, by activated helper T cells present in the inflammatory background.[16] In addition, neoplastic B cells are likely to be stimulated by tissue auto-Ags exposed during the inflammatory process.[17]

Molecular analysis of the Ag-binding site of the monoclonal IG in gastric EMZL confirmed the hypothesis of auto-Ag engagement, showing a strong similarity of the Ag-binding sites (heavy complementarity-determining region 3, VH CDR3) of the expressed B-cell receptors, with those of rheumatoid factors.[18] This similarity was also observed in salivary gland EMZL, which is known to be associated to primary Sjögren's syndrome, but not to pulmonary EMZL,[18] suggesting that the actual antigenic stimulation operating in each tissue may differ, despite similar histological features shared by these clinico-pathological entities. Accordingly, the immune-mediated interactions occurring in gastric EMZL cannot be *a priori* transferred to OAMZL and mechanisms engaged by Cp infection should be elucidated, as they may not necessarily overlap with those of *Hp*.[19]

In the present study, we analyzed the primary IG structure, and, in particular, the Ag-binding sites (i.e., VH CDR3) of a large series of OAMZL cases coming from four western countries, aiming at investigating distinctive features that may be helpful to get hints about the potential selecting ligands. Our data show that OAMZL express a distinctive IG repertoire, with VH CDR3 molecular features suggestive of a pathogenic mechanism of autoreactivity. Interestingly, some cases of OAMZL express IGs displaying high similarity to that of autoreactive antibodies (Abs). Taken together, these findings suggest that OAMZL may arise from B cells selected for their propensity to bind self-Ags, supporting the hypothesis that stimulation by auto-Ags, likely provided in the context of an inflammatory reaction triggered by infectious agents such as Cp, may have a major role in the process of lymphomagenesis in the ocular adnexa.

Materials and methods

Study group

Forty-four patients with OAMZL, diagnosed according to WHO classification,[20] from France ($n=6$), Greece ($n=9$), Italy ($n=23$) and USA ($n=6$) were included in the study. Median age was 69.5 years old (range 24–90), with a male/female ratio of 0.83. Chronic infections consisted of gastric *Hp* infection in 3 of 22 (14%) patients, hepatitis B or C virus in 3 of 29 (10%). Thirty-two percent of patients (11/34) complained a history of chronic conjunctivitis. One patient (number 59808000) reported an overt autoimmune disorder (anti-phospholipid syndrome). Sites of involvement included conjunctiva in 14 patients (32%), lachrymal gland in 6 (14%) and other soft tissues of the orbit in the remaining 24 (54%) patients. All tissues were taken before first-line treatment.

Cp DNA was tested by touchdown enzyme time release–PCR analysis, as previously reported,[21] and confirmed by an additional PCR protocol amplifying Cp HSP60 (*GroEl*) gene sequences.[22] The analysis was performed in 38 of 44 OAMZL patients and showed the presence of the bacteria in 16 of 38 (42%) patients. In the remaining six cases, Cp analysis was not possible due to lack of material. This study was performed according to the institutional ethics committee guidelines and the Declaration of Helsinki.

DNA extraction and PCR amplification of IGHV-D-J rearrangements

Sections of 5 μm thick obtained from formalin-fixed, paraffin-embedded diagnostic samples underwent DNA extraction using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

PCR amplification of *IGHV–D–J* genes was performed as previously described,[23,24] using a seminested method. Briefly, all PCR reactions were carried out first with VH family-specific FR1 5′-primers, together with an external IGHJ consensus 3′-primer, for the first amplification round, followed by a seminested PCR using as template 1 μl of the first amplification round and substituting the IGHJ 3′-primer with a more internal one. In those cases where the amplification of the IGHV–IGHD–IGHJ rearrangement was not achieved by the above approach (35/44), a PCR protocol with an FR2 consensus 5′-primer (instead of FR1 primer) was performed.[24] PCR products were run onto 6% polyacrylamide gels, allowing the detection of monoclonal rearrangements. To separate the monoclonal rearrangement from the background of polyclonal rearrangements originating from residual non-malignant B cells, all PCR products positive for monoclonal *IGHV–IGHD–IGHJ* gene rearrangements (as evidenced by the polyacrylamide gel electrophoresis) were run overnight onto 3% low-melting agarose gels. The monoclonal bands were identified and excised with a scalpel under UV lamp, and the DNA was extracted from the agarose by using QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions. The recovered DNA was directly sequenced on both strands using an automated DNA sequencer.

Finally, to evaluate the presence of intraclonal diversity, PCR products from four cases, amplified with the FR2 primer, were ligated and transferred into TOP10′ bacteria according to manufacturer's instructions (original TA-cloning kit, Invitrogen, Stockholm, Sweden). A colony-direct PCR was performed (between 11 and 20 reactions per sample) and then directly sequenced.

Intraclonal diversity among subcloned sequences obtained from the same sample was assessed by examination of the sequence variation in the V domain. All 'non-ubiquitous' sequence changes from the germline were evaluated and further characterized as follows: (i) unconfirmed mutation—a mutation observed in a single subcloned sequence ('unique'); (ii) confirmed mutation—a mutation observed in two or more subcloned sequences ('partially shared') The presence of ongoing mutations was calculated as percentage using the following formula '(confirmed + unconfirmed mutations × 100)/(IG length × number of clones)' as previously described.[25] Cases with a mutational rate lower than the expected PCR error rate due to misincorporation by theTaq DNA polymerase (2×10^5 per base pair per cycle) were considered as lacking intraclonal diversity.

IG sequence analysis and interpretation

The quality of the sequences was checked using the VectorNTI software (Invitrogen, Carlsbad, CA, USA) that allowed confirming the presence of single nucleotide peaks and the absence of relevant polyclonal background. Sequence data were analyzed using the IMGT databases and the IMGT/V-QUEST tool (http://www.imgt.org). Codons and amino acid positions are defined according to the IMGT unique numbering for V domain. To avoid misidentification of mutations when IGHV FR1 or FR2 consensus primers were used in the amplification reactions, nucleotide substitutions in the obtained sequences were evaluated from codon 9 in FR1–IMGT or, for the sequences obtained with a VH FR2 primer, from codon 48 in FR2–IMGT, respectively. The downstream end of the analyzed V region corresponds to the 5′-end of the germline CDR3–IMGT as defined by IMGT/Junction Analysis.

OAMZL IGHV–D–J sequences were aligned to a panel of 13461 unique IGHV–D–J sequences from various types of normal, autoreactive and malignant B-cell clones, available to our groups or previously reported in the literature and/or

retrieved from the IMGT/LIGM-DB sequence database.[26] For such sequences, the accession numbers shared by GenBank, EMBL-Bank, DNA Data Bank of Japan and IMGT/LIGM-DB are designated by the 'GEDI' abbreviation. This panel included the following: (i) 7967 sequences from chronic lymphocytic leukemia (CLL; 7596 from our 'internal' database and 371 from other groups), (ii) 604 sequences from miscellaneous B-cell lymphoproliferative disorders, (iii) 3326 sequences from normal B cells, (iv) 364 sequences from 'immune dysregulation' disorders (allergy, asthma, various types of immunodeficiency), and (v) 1200 sequences from autoreactive cells.[26] We also utilized IGHV–D–J sequences of previously reported anti-Chlamydia Abs.[27,28]

To comprehensively identify possible restrictions in the VH CDR3 amino acid composition of the complete data set (OAMZL + other entities), we used the TEIRESIAS algorithm,[29] a computational tool developed by the Bioinformatics and Pattern Discovery group at the IBM Computational Biology Center, freely available from http://cbcsrv.watson.ibm.com/download.phtml.html. The parameters used in this analysis are as previously reported.[29] The adopted parameters ensured an extremely high level of sensitivity of the procedure, enabling the connection of all pairs of sequences that shared at least 50% amino acid identity and 70% similarity.

Evolutionary history of sets of subcloned sequences

The evolutionary history of the sets of subcloned sequences was inferred using the maximum parsimony method.[30] The consistency index is 1.000 000 (1.000000), the retention index is 1.000 000 (1.000 000), and the composite index is 1.000 000 (1.000 000) for all sites and parsimony-informative sites (in parentheses). The maximum parsimony tree was obtained using the close-neighbor-interchange algorithm with search level 0, in which the initial trees were obtained with the random addition of sequences (10 replicates). Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated.

Statistical methods

Clinical and epidemiological characteristics of studied subgroups and distribution of Cp infection were compared using Fisher's exact test. Independent associations among studied variables and Cp infection were assessed by logistic regression. All statistical tests were two-sided, with a significant '*P*-value' of 0.05. Analyses were carried out using the Statistica 4.0 statistical package for Windows (Statsoft Inc, Tulsa, OK, USA).

Results

Biased IGHV *gene usage in OAMZL*

Forty-six IGHV–IGHD–IGHJ rearrangements were amplified in 44 OAMZL cases, as 2 cases carried double-productive rearrangements. Clonal PCR products were obtained with FR1 or FR2 primers in 9 and 35 cases, respectively. *IGHV3* subgroup genes predominated (36/46, 78.3%), followed by *IGHV4* (8/46, 17.4%) and *IGHV1* (2/46, 4.3%) subgroup genes (Table 1). At the individual gene level, 19 different *IGHV* genes were utilized. Of note, only four *IGHV3* subgroup genes cumulatively accounted for 45.7% of the entire series. The most frequent *IGHV* gene was *IGHV3–23* (8/46, 17.4%), followed by *IGHV3–30* (5/46 10.9%), *IGHV3–11* and *IGHV3–7* (4/46 cases, 8.7%). Comparison of the *IGHV* gene repertoire between Cp-positive and Cp-negative cases did not show significant differences, likely due to the relatively small numbers of cases in each subgroup. Similarly, a comparison of the *IGHV* gene usage between individual institutions was also not possible.

On the basis of somatic hypermutation status, the rearrangements under study were classified into three different subgroups as follows: (i) truly unmutated (100% germline identity): 3 of 46 samples (6.5%); (ii) minimally/borderline mutated (97–99.9% germline identity): 7 of 46 samples (15.2%); and (iii) significantly mutated (<97% identity): 36 of 46 samples (78.3%). The majority of mutations occurred in CDRs rather than FRs; in addition, most CDR mutations were R rather than S, leading to high R/S ratios (5.22) in CDR2 and, vice versa, low R/S ratios (1.88) in FR3, in keeping with a canonical SHM process.

Intraclonal diversity in rearranged *IGHV* genes was analyzed in four cases (11–20 subcloned sequences per sample). All nucleotide mutations from the germline were taken into account and were subdivided into confirmed or unconfirmed (following the definitions provided in the Materials and methods section). Confirmed mutations leading to intraclonal diversity were detected in two of four cases (cases number JN182372 and number JN182360 in Table 2 and Figure 1), with an overall nucleotide substitution rate of 0.39 and 0.61%, respectively. In both cases, the sequences obtained from cloning differed by up to three nucleotides from the sequence obtained with direct sequencing.

Hints for autoreactivity from the analysis of VH CDR3 sequences

A reliable VH CDR3 sequence was obtained in 44 of 46 IGHV-D-J rearrangements from OAMZL. *IGHD* genes were identified in 43 of 44 cases; the most frequent IGHD subgroup was IGHD3 (19/43, 44.2%). Seventeen *IGHD* genes were identified; the *IGHD3–3* gene predominated (10/43 cases, 23.3%). In keeping with the normal adult B-cell repertoire, the *IGHJ4* and *IGHJ6* genes predominated (16 and 11 cases, respectively).

VH CDR3 length ranged from 8 to 30 amino acids (median, 15). Interestingly, 30 of 44 HCDR3 sequences had a predicted isoelectric point (pI) value of greater than 6.0, (18/44 with pI values >8.0), a feature shared also by Abs with anti-DNA activity.[31]

Cluster analysis of the VH CDR3 amino-acid sequences within the OAMZL data set did not reveal sequence restrictions. In contrast, when the same approach included sequences from other entities, we observed that OAMZL rearrangements clustered against some other sequences on the basis of a high VH-CDR3 similarity (Table 3). In particular, one Cp-positive OAMZL case carried VH CDR3 homologous to that of a B-cell clone derived from a patient affected by rheumatoid arthritis; another OAMZL case, again Cp-positive, carried VH CDR3 homologous to the IG expressed by a B-cell clone from a patient with primary Sjögren's syndrome; two additional OAMZL cases both Cp-negative, showed VH CDR3 homology to CLL mAbs.

Finally, one Cp-positive OAMZL case (number JN182384) showed homology to multiple sequences, creating a distinctive cluster. This case carried a IGHV3–7/IGHD3–22/IGHJ3 rearrangement that, based on shared VH CDR3 patterns, was similar to six rearrangements utilizing the same *IG* genes and expressed by the following B-cell clones from: (i) a gastric EMZL (GEDI/AY281326); (ii) a salivary gland EMZL (GEDI/AF216825); (iii) a hepatitis C virus-associated diffuse large B-cell lymphoma (GEDI/AF303909); (iv) a rheumatoid factor from a healthy individual immunized with mismatched red blood cells (GEDI/U85242); (iv) two CLL cases (NL-01-0121 and NL-01-0591).

Table 1 Patient characteristics, Cp infection status and *IGHV* gene usage in OAMZL

GenBank accession number	*Gender*	*Age*	*Clinical stage*[a]	*Site*	*Cp infection*	*IGHV gene*	*IMGT (%)*[b]
JN182346	M	74	I	Lachrymal gland	NT	IGHV3–66*02	99.23
JN182347	F	58	I	Conjuctiva	NT	IGHV3–9*01	91.29
JN182348	M	85	I	Orbit	NT	IGHV4–4*02	93.18
JN190950	M	64	I	Orbit	NT	IGHV4–59*08	94.97
JN182349	F	24	I	Conjuctiva	NT	IGHV3–11*01	95.71
JN182350	F	63	I	Orbit	NT	IGHV3–30*04	97.55
JN182351	F	66	I	Orbit	NEG	IGHV4–59*07	95.57
JN182352	M	47	I	Orbit	NEG	IGHV3–73*01	88.02
JN182353	M	74	I	Orbit	NEG	IGHV4–34*01	93.75
JN182354	F	79	I	Lachrymal gland	NEG	IGHV3–23*03	91.88
JN182355	M	88	I	Orbit	NEG	IGHV3–48*01	92.73
JN182356	M	78	I	Orbit	NEG	IGHV3–23*01	93.94
JN182357	F	75	I	Orbit	NEG	IGHV3–11*01	96.86
JN182358	F	64	IV	Lachrymal gland	POS	IGHV3–23*01	93.12
JN182359	F	65	I	Orbit–conjuctiva	POS	IGHV1–69*01	91.41
JN182360	F	80	I	Orbit	POS	IGHV3–7*01	93.87
JN182361	F	82	IV	Orbit	POS	IGHV4–31*03	95.62
JN182362	M	39	I	Conjunctiva	NEG	IGHV3–64*05	98.16
JN182363	F	78	I	Conjunctiva	NEG	IGHV3–23*01	89.57
JN182364	M	48	I	Lachrymal gland	NEG	IGHV4–34*01	100
JN182365	M	48	IV	Orbit	NEG	IGHV3–23*01	88.34
JN182366	F	86	I	Orbit	NT	IGHV3–21*01	98.77
JN182367	M	51	I	Orbit	NEG	IGHV3–43*01	94.48
JN182368	F	78	I	Orbit	POS	IGHV3–23*01	93.87
JN182369	F	25	I	Conjunctiva	POS	IGHV3–11*03	95.09
JN182370	F	74	I	Orbit	POS	IGHV3–43*01	94.48
JN182371	F	68	I	Conjunctiva	NEG	IGHV3–7*01	93.87
JN182372	F	50	I	Conjunctiva	POS	IGHV3–7*01	94.48
JN182373	M	74	IV	Conjunctiva	NEG	IGHV3–48*03	93.25
JN182374	M	56	I	Orbit	POS	IGHV3–48*01	98.77
JN182375	M	43	I	Orbit	POS	IGHV3–30*06	93.25
JN182376	F	55	I	Conjunctiva	POS	IGHV3–15*01	91.72
JN182377	F	68	I	Orbit	POS	IGHV3–33*01	89.57
JN182378	M	75	I	Orbit	NEG	IGHV3–30*03	95.76
JN182379	M	42	I	Conjunctiva	POS	IGHV4–59*02	100
JN182380						IGHV3–15*01	97.04
JN182381	M	80	I	Orbit	NEG	IGHV3–23*01	94.48
JN182382						IGHV3–43*01	91.41
JN182383	F	61	I	Lachrymal gland	POS	IGHV3–30*03	95.09
JN182384	F	55	I	Orbit	POS	IGHV3–7*01	98.79
JN182385	M	90	I	Lachrymal gland	NEG	IGHV3–11*01	100
JN182386	F	72	I	Eyelid	NEG	IGHV4–34*02	87.79
JN182387	M	74	I	Conjunctiva	NEG	IGHV3–74*01	94.34
JN182388	F	75	IV	Conjunctiva	NEG	IGHV1–69*01	96.98
JN182389	M	71	IV	Conjunctiva	NEG	IGHV3–23*01	92.38
JN182390	F	82	I	Orbit	NEG	IGHV3–30*03	88.68

Abbreviations: Cp, *Chlamydophila psittaci*; F, female; *IGHV*, immunoglobulin heavy variable gene; IMGT, ImMunoGeneTics; M, male; NEG, negative; NT, not tested; OAMZL, ocular adnexa marginal zone B-cell lymphomas; POS, positive.
[a]Ann Arbor.
[b]Percentage of identity with the closest germline *IGHV* gene.

Table 2 Intraclonal diversity in rearranged *IGHV* genes in OAMZL

Sample code	*No. of subcloned sequences*	*No. of nucleotides*	*No. of confirmed mutations*	*No. of unconfirmed mutations*	*Intraclonal diversity %*
JN182372	11	1793	4	3	0.39
JN182360	15	2445	11	4	0.61
JN182370[a]	20	3260	0	19	NA
JN182376[b]	13	2119	0	2	0.09

Abbreviations: *IGHV*, immunoglobulin heavy variable gene; NA, not applicable; OAMZL, ocular adnexa marginal zone B-cell lymphomas.
[a]In this case, all mutations were present in a single clone, and the possibility exists that they are due to a PCR artifact. Thus, they were considered 'unconfirmed' and not included in the analysis.
[b]This case had a mutational rate lower than the expected experimental error frequency due to Taq DNA polymerase (2×10^5 per base pair per cycle); thus, it was considered as if it did not show intraclonal diversity.

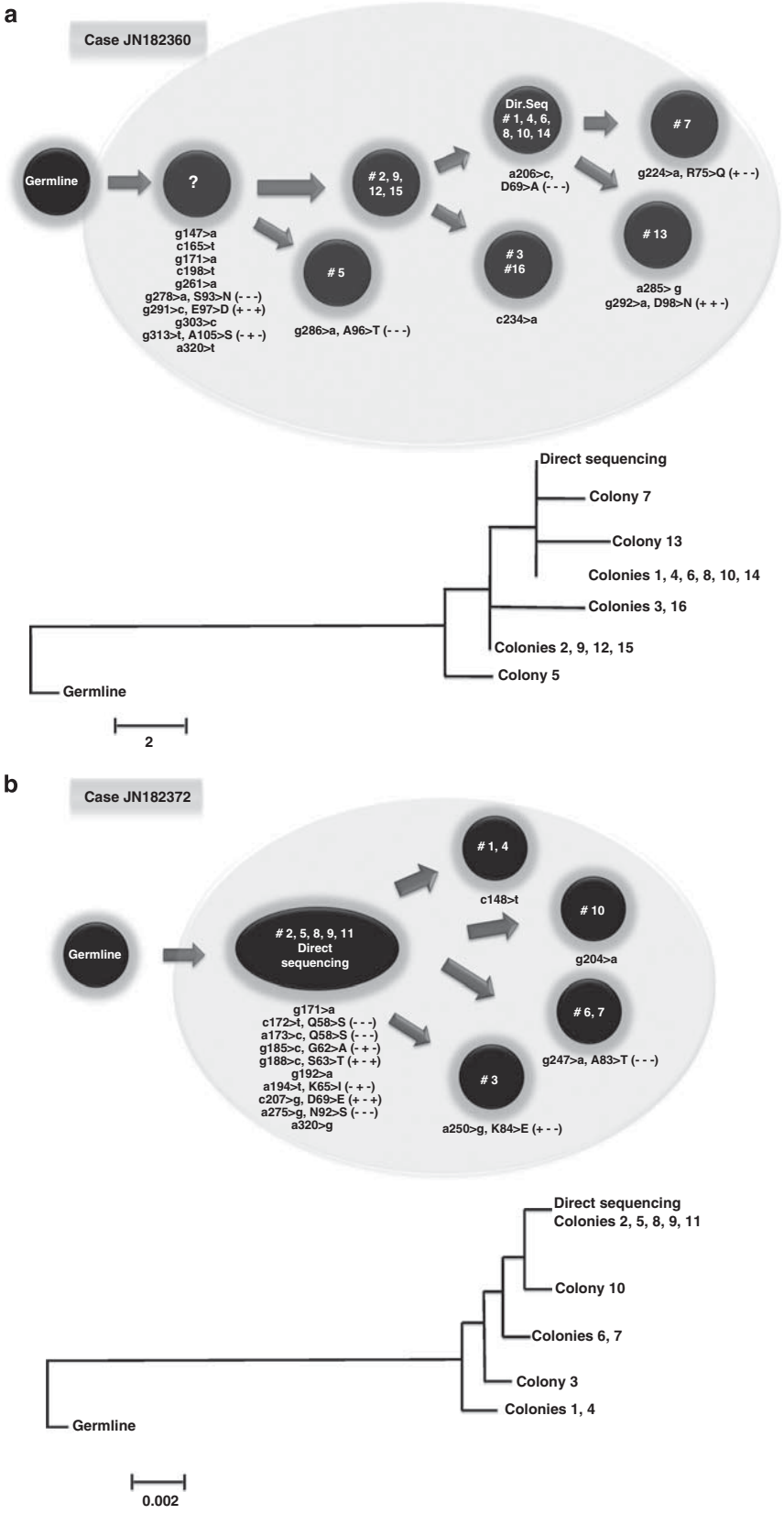

Figure 1 Clonal evolution trees. The evolutionary history of both OAMZL cases with clear evidence for intraclonal diversification (i.e., carrying confirmed mutations) was inferred drawing clonal evolution trees that were constructed by MEGA5 algorithms and tools. (**a**) Case JN182360; (**b**) Case JN182372.

Table 3 VH CDR3 similarity between OAMZL and unrelated sequences

Case	*IGHV*	*VH CDR3*	*Diagnosis*
JN182365	IGHV3–23	ANRNGSSPSYYFDY	OAMZL
AY393382	IGHV2–5	–H–Q––––––GL–V	Rheumatoid arthritis
JN182359	IGHV1–69	ARGPGDTNTYYYFY	OAMZL
AF038446	IGHV1–69	–––––––NSG–––––	Primary Sjogren's syndrome
JN182384	IGHV3–7	ARGDYYDSSGYYHDAFDI	OAMZL
AY281326	IGHV3–7	–––––F––––––SF I –––––	Gastric malt lymphoma
AF216825	IGHV3–7	––––––––––––FS––––––T	Salivary gland malt lymphoma
AF303909	IGHV3–7	––––––––T–––FS–––––––	DLBCL, HCV-related
U85242	IGHV3–7	––––––––––––N–––––––V	Rheumatoid factor
NL-01-0121	IGHV3–7	–––––F–E––––N–––––––V	CLL
NL-01-0591	IGHV3–7	–––––F–DT––FN–––––––	CLL
JN182388	IGHV1–69	AREFASDSSGYYYYY	OAMZL
UK-02-0059	IGHV1–69	–––G–G––––––––FF–	CLL
NY-01-0831	IGHV1–69	––G–DYE–––––––––	CLL
JN182354	IGHV3–23	AKPYYDFWSGYSTSWFDP	OAMZL
UK-01-0470	IGHV3–23	––E––––––––––GRL––Y	CLL

Abbreviations: CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; *IGHV*, immunoglobulin heavy variable gene; HCV, hepatitis C virus; OAMZL, ocular adnexa marginal zone B-cell lymphomas; VH CDR 3, heavy complementarity determining region 3. Functionally equivalent amino acids are highlighted in grey. Dashes indicate identities.

Interestingly, no structural similarities between IGs expressed by OAMZL in the present study and the structure of Abs specific for Chlamydiae[27,28] could be demonstrated.

Discussion

This molecular study of the IGs expressed by neoplastic B cells in OAMZL, so far the largest available, provides new information on distinctive features that may be helpful for understanding the role of antigenic stimulation in this malignancy. The main findings of this study embrace a restriction of *IGHV* gene usage, likely due to an antigenic exposure, with a strong bias in the usage of *IGHV3–23, IGHV3–30*, *IGHV3–11* and *IGHV3–7* genes, and a high homology between IG sequences (mainly, but not exclusively from Cp-positive OAML) and previously reported autoreactive Abs. This suggests a scenario where the B-cell receptors carried by OAMZL neoplastic B cells is not directed against Cp, but rather against auto-Ags exposed in non-neoplastic, reactive milieu accompanying the lymphoma.

OAMZL, in particular those associated with Cp infection, represent an intriguing model for studying the process of lymphomagenesis.[1] Further support for this concept is provided by the fact that many OAMZL cases have a previous history of conjunctivitis,[32] indicating that an inflammatory/immune process taking place in the ocular adnexa is likely to have a critical role in the onset and maintenance of the disease by stimulating responsive B cells. In line with these observations: (i) Chlamydiae have been directly visualized within monocytes/macrophages present in lymphomatous lesions;[33] (ii) viable and infectious Cp have been isolated in conjunctival swabs from OAMZL patients[32] and, (iii) clinical responses have been obtained with Cp-eradicating antibiotic therapy.[9] Notwithstanding these achievements, the precise mechanisms of antigenic stimulation are not yet fully elucidated and could theoretically evoke a direct role of still unidentified bacterial Ags and/or the indirect triggering by unrelated antigenic element(s) exposed within the context of the tissue inflammation.[34]

In the present study, the predominant usage of *IGHV3* subgroup genes (78.3%) and, mostly, the preferential usage of four *IGHV* genes, accounting for almost half of cases, allude to selection of IGHV specificities by antigenic exposure.[2,11–14] Previous studies on more limited series[2,11–14] indicated that the *IGHV3–23* and *IGHV3–7* genes were the predominant *IGHV* genes in ocular adnexal lymphomas, albeit their overall combined frequency was rather low (10.6% in total). At a variance with these figures, we herein demonstrate a strong bias in the usage of these two genes that cumulatively account for 26% of the series. This finding, together with the presence of somatic mutations and the observed somatic hypermutation patterns, allows to conceive that the cell of origin may be an Ag-experienced B cell, which has undergone an Ag-based selection process, as also suggested by previous publications.[2,11–14]

In addition to the high mutational load, intraclonal diversity in rearranged *IGHV* genes was observed in two of four cases analyzed, suggesting that the malignant clones may still be under the influence of hypermutational mechanism, very likely within the context of Ag-driven stimulation. These data are in concordance with previous studies[11–14] showing that also EMZLs derived from post-GC B cells can show ongoing mutations.

The present study provides for the first time molecular data concerning the relationship between *IGHV* gene repertoire and Cp infection in OAMZL. Due to the relatively small number of clones utilizing each particular *IGHV* gene, we could not reach definitive conclusions on potential repertoire differences between Cp-positive versus Cp-negative cases that would offer insight into the role had by this infection in shaping the IG receptors in ocular adnexal lymphomas. Several additional factors should also be kept into consideration, as potential confounding elements in this analysis, including the possibility that other, not yet identified, microorganisms may be also involved, the presence of samples originating from different geographical regions and the reduction of bacterial load in lymphoma samples due to use of local antibiotics therapy before diagnostic biopsy.

Prompted by the observed *IGHV* gene restrictions, we also sought to analyze in detail, through a bioinformatics approach, the molecular structure of the Ag-binding sites (VH CDR3s) of the IG receptors expressed by the OAMZL in our series. By this approach, we obtained significant, though admittedly indirect, molecular evidence, suggesting that, at least in a proportion of

cases, the antigenic elements recognized by the malignant clones may potentially be auto-Ags.

Though it is known that IG specificity cannot be predicted by sequence analysis alone, the comparison of IG sequences with other Abs of known specificity could provide useful hints. We took advantage of a large available panel of unique IGHV-D-J rearrangements from neoplastic and non-neoplastic B cells, including autoreactive clones,[26] and compared them with the IG sequences, in particular, the VH CDR3 sequences utilized by our OAMZL cases. Our intentional focus on VH CDR3 sequences is justified by its impact on Ag recognition, with transgenesis,[35] structural[36] and functional[37] studies, indicating that VH CDR3 sequences act often as the major factor of specificity in Ag recognition.

No IG sequence 'stereotypy'[38] (i.e., sequence identity or similarity) could be found within the OAMZL *IG* gene sequences. However, 11.3% (5/44) of cases carried IGs that were found to be homologous with Abs from other entities. Interestingly, three of these Abs were autoreactive, and all were similar to the IGs obtained from Cp-positive OAMZL. Interestingly, case number JN182365 (Table 3) utilized a different *IGHV* gene from the homologous Ab, though sharing a similar VH CDR3 sequence. This finding is not unexpected, due to the fact that the contribution of the *IGHV* gene to CDR3 is much less extensive (2–3 aminoacids) when compared with that of the *IGHD* and *IGHJ* genes. At this point it is also worth underscoring the fact that heavy CDR1/CDR2 loops adopt a small number of main chain conformations,[39] meaning that different germline *IGHV* genes may in fact adopt a similar structure. Furthermore, our finding is also not unprecedented, as exemplified by CLL, where different cases utilizing distinct *IGHV* genes may show stereotyped VH CDR3s, especially so when they are phylogenetically related.[29] Overall, homology in VH CDR3 sequences implies selection by a similar, if not identical, antigenic element rather than a random association of a particular *IGHV* gene, simply occurring because of the usage of identical *IGHV* gene sequences.

One of these OAMZL cases carried a highly similar VH CDR3 with Abs amplified in a case of rheumatoid arthritis, and one each gastric EMZL and hepatitis C virus-related lymphoma, respectively, suggesting that common patterns of stimulation by unrelated or cross-reactive pathogens or auto-Ags can lead to strikingly different B-cell disorders, likely depending on the stage of lymphocyte differentiation or the anatomical site of antigenic exposure. Moreover, two Cp-positive OAMZL cases carried a similar VH CDR3 with IGs expressed in CLL, a disease where leukemic cells often express autoreactive IGs.[40,41] Along the same lines, it is worth underscoring the fact that at least two-thirds of investigated OAMZL cases carried electropositive VH CDR3 sequences, similar to Abs with anti-DNA activity,[31] thus alluding to hitherto unknown auto-Ag recognition for the majority of OAMZLs.

Our present findings are highly reminiscent of those obtained in gastric EMZL[18] and CLL.[41] In the former case, similarities between the monoclonal IG and Abs with rheumatoid factor activity were observed in 18% of cases,[18] whereas in the case of CLL, several clonotypic IG receptors have been proven similar to Abs reactive toward different auto-Ags, including IgG, cardiolipin and DNA.[41]

Taken together, the molecular findings reported herein strongly support, though indirectly, the hypothesis that OAMZL may originate from B cells selected for their capability to bind Ags, in particular auto-Ags. In addition, they justify drawing for OAMZL a scenario that closely recapitulates what happens in gastric EMZL, where the B-cell receptors carried by neoplastic B cells is not directed against *Hp*, but rather against auto-Ags.[17,18] In this model, Cp likely acts indirectly on the malignant B cells, promoting the development of an inflammatory milieu, where auto-Ags could be exposed and presented, driving proliferation and expansion of self-reactive B cells.[15] This hypothesis is further supported by the lack of structural similarities between IGs expressed by investigated cases of OAMZL and the structure of Abs specific for Chlamydiae,[28] suggesting that antigenic elements differing from bacterial epitopes may shape the *IG* gene repertoire.

Conflict of interest

The authors declare no conflict of interest.

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