Use of Similar Immunoglobulin *VH* Gene Segments by MALT Lymphomas of the Ocular Adnexa

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Abstract

Extranodal marginal zone lymphomas of mucosa associated lymphoid tissue type (MALT lymphomas) develop from acquired reactive infiltrates directed against external or auto-antigens. Although some European cases of ocular adnexal MALT lymphoma have been associated with *Chlamydia psittaci* infections, *C. psittaci* has not been detected in large studies of U.S. based cases. To evaluate whether the growth of U.S. based ocular adnexal MALT lymphomas may be promoted by a similar antigen, the expressed immunoglobulin *VH* genes were identified and analyzed in 10 cases. Interestingly, the *VH* genes in 2 cases used the same *VH1* family *V1-2* gene segment, and 3 cases used the same *VH4* family *V4-34* gene segment. The other five cases all used different gene segments *V4-31*, *V5-51, V3-23, V3-30*, and *V3-7.* All of the *VH* genes were mutated from germline, with percent homologies ranging between 96.9% to 89.0%. The distribution of replacement and silent mutations within the *VH* genes was non-random consistent with the maintenance of immunoglobulin function and also strongly suggestive of antigen selection in the 6 *VH* genes with highest mutation loads. The CDR3 sequences in 2 of 3 *VH-34* cases were the same size (15 amino acids), and had similar sizes in the 2 VH1-2 cases (18 & 16 amino acids). In conclusion, U.S. based MALT lymphomas of the ocular adnexa preferentially express a limited set of *VH* gene segments not frequently used by other MALT lymphomas and consistent with some recognizing similar antigens. Analysis of somatic mutations present within the *VH* genes is also consistent with antigen binding stimulating the growth of these lymphomas.

Keywords

MALT lymphoma; ocular adnexa; immunoglobulin *VH* gene

Introduction

Extranodal marginal zone lymphomas of mucosa associated lymphoid tissue type (MALT lymphomas) are typically indolent B-cell neoplasms that develop out of acquired reactive

infiltrates associated with localized long standing autoimmune diseases or chronic infections [1,2]. The growth of early MALT lymphomas is thought to be dependent on antigen stimulation but may become antigen independent over time with the acquisition of additional genetic abnormalities [1,3]. Antigen stimulation of MALT lymphoma growth is strongly supported by studies of gastric MALT lymphomas which develop out of infiltrates associated with chronic *H. pylori* associated infections and can often be cured with *H. pylori* eradicating antibiotics [4–6]. Studies by an Italian group have suggested *Chlamydia psittaci* may play a similar role in ocular adnexal MALT lymphoma and reported tumor regressions in patients receiving *C psittaci* eradicating antibiotic treatment [7,8]. However, studies of U.S. based cases have not found evidence for *C psittaci* in ocular adnexal MALT lymphomas using sensitive PCR techniques [9,10].

Analysis of expressed immunoglobulin heavy chain variable region (*VH*) genes can provide strong support for surface immunoglobulin mediated direct antigen stimulation of lymphoma growth without prior identification of the reactive MALT antigenic trigger [11–15]. For example, studies of salivary gland MALT lymphoma *VH* genes have demonstrated that approximately 60% of cases use a single *VH* gene segment, *V1–69*, which is suggestive of antigen selection since there are approximately 40 different functional *VH* gene segments that can potentially be used[16]. In addition, antigen selection of salivary gland MALT lymphoma growth is further supported from finding non-random distributions of point mutations typically present in the expressed *VH* genes and stretches of similar CDR3 amino acid sequences in *VH* genes from different patients [12,16]. Evidence supporting antigen stimulation of low grade B-cell neoplasm growth is not limited to MALT lymphomas as recent studies of chronic lymphocytic leukemias have documented nearly identical expressed heavy and light chain variable genes from different patients suggesting that some of these cases recognize the same antigens or epitopes[14,17–19].

In this study, we identified and analyzed the *VH* genes used by 10 U.S. based ocular adnexal MALT lymphoma cases to look for evidence of antigen stimulation. Our findings provide support that direct antigen stimulation by a common antigen is playing a role in the growth of these neoplasms that are not associated with *C psittaci*.

Materials and Methods

Cases

Cases of MALT lymphoma involving the ocular adnexa as defined by the WHO[2] with available frozen tissue were identified by searching the pathology files of the University of Pittsburgh Medical Center and the Cleveland Clinic. Ten of the identified 13 cases were included in our previous study of ocular MALT lymphoma [10], and three cases are new to this study. The hematoxylin and eosin stained slides were reviewed on all cases. Immunohistochemistry and flow cytometry studies (done on all cases) preformed as described [10] were also reviewed. Two independent real-time nested PCR assays for detection of *C psittaci* were previously performed on 7 of the cases as described [10]. The research use of these specimens was approved by the Institutional Review Boards of the University of Pittsburgh (no. 0505102), the Cleveland Clinic (no. 81008), and the University of Utah (no. 13172).

VH Gene Analysis

Genomic DNA was prepared from frozen tissues using the Gentra Puregene kit (Qiagen Inc., Valencia, CA) or the Genomic DNA Purification kit (Gentra Systems, Minneapolis, MN) following the manufacture's directions. Rearranged immunoglobulin heavy chain variable regions were amplified from the isolated DNA using a reverse 3' *JH* primer and seven different 5' *VH* leader region primers, 6 of which were previously described[20], with an additional primer that perfectly matches the leader sequence of the *VH3–21* gene segment, 5'-

CCATGGAACTGGGGCTCCGC . Genomic DNA (20 ng) was amplified in 1x GoTaq flexi PCR buffer (Promega Corp., Madison, WI), 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µM leader prime, 0.2 µM J primer, and 1 unit GoTaq Flexi DNA polymerase in a final volume of 20 µL. Cycling conditions were 2 minutes at 94°C followed by 35 cycles of 20 seconds at 94 °C, 10 seconds at 55°C, 30 seconds at 72°C followed by a hold 2 minutes at 72°C and cool down. The PCR reactions were run on a 2% agarose gel and the DNA was visualized with ethidium bromide (0.5 µg/mL). Prior to sequencing, primers and dNTP were removed from the reactions with the most prominent bands by mixing 10 µL of reaction with 2 µL ExoSAP-IT (USB Corp., Cleveland, OH) and incubating at 37°C for 45 minutes followed by 15 minutes at 85°C. The treated samples were appropriately diluted in water and 6 µL was mixed with 8 µL of the appropriate leader primer (0.8 µM) and subjected to DNA sequence analysis using BigDye Terminator chemistry (Applied Biosystems Inc., Foster City, CA). The readable DNA sequences were searched against the IMGT database (http://imgt.cines.fr/) to identify the most closely related *VH, DH* and *JH* germline segments and to confirm a productive *VDJ* rearrangement.

Results

Of the 13 ocular adnexal MALT lymphoma cases initially identified with available frozen tissue, the expressed VH genes were successfully obtained from the 10 cases further described in Table 1. The patients mean age was 60 years old, range 43 years to 82 years, and included 6 males and 4 females. Evaluated biopsies were from orbital soft tissue in 9 cases, the lacrimal gland in 2 cases, with one case (#9) having both orbit and lacrimal gland biopsies. All showed morphological features typical of orbital MALT lymphoma, namely a predominance of small lymphocytes admixed with variable numbers of monocytoid appearing cells and sometimes smaller numbers of plasma cells (Figure 1). Flow cytometry which was performed in all cases revealed populations of CD5 negative, CD10 negative B-cells with monotypic expression of kappa (8/10 cases) or lambda (2/10 cases) light chains. Sensitive PCR testing for *C. psittaci* was negative in all 7 cases evaluated. Only one patient (# 4) had a prior history of lymphoma, reportedly a diffuse large cell lymphoma of the small bowel treated with R-CHOP 2 years prior to the orbit biopsy, and 9 months later a cutaneous low grade lymphoma with a follicular growth pattern treated with radiation. Slides were not available for review from either biopsy, but the patient was clinically thought to represent a stage 1 orbital MALT lymphoma and received only radiation treatment.

A single rearranged functional *VH* gene without stop codons was identified in the 10 cases by directly sequencing PCR products generated by amplifying lymphoma DNA with *VH* leader and *JH* primers. Comparing the lymphoma *VH* genes to germline genes in the IMGT database revealed that 2 used *VH* gene segments from the *VH1* family, 4 used *VH4* family gene segments, 3 used *VH3* family gene segments, and one used a *VH5* family segment (Table 2). Interestingly, the 2 *VH1* family cases used the same *V1–2* gene segment, while 3 of the 4 *VH4* family cases used the same *V4–34* gene segment. The 3 *VH3* family cases all used different *V3* gene segments.

All of the lymphoma *VH* gene segments showed significant numbers of point mutations from the most closely related germline counterparts, with percent homologies ranging between 96.9% to 89.0% homology, mean percent homology 93.4% +/− 2.4%. The mutation locations, complementarity determining regions (CDRs) or framework regions (FWRs), and type, those causing an amino acid replacement (R) or not, i.e. silent (S) are listed in Table 3. All of the lymphoma VH genes segments had fewer replacement mutations in the FWRs than would be expected by chance alone[21], i.e. random mutation, consistent with the maintenance of immunoglobulin functionality [22]. The 5 cases with higher numbers of mutations (#2,4,5,8, & 10) also had fewer replacement mutations than would be expected by chance alone in the CDRs,

suggestive of negative selection or selection against replacement mutations in these areas. In only one case (#1) were mutations above what would be expected by chance alone in the CDRs that could be suggestive of positive selection.

The CDR3 regions of the lymphoma *VH* genes appeared to be encoded by a variety of different *D* segments and to have variable sizes (Table 4). Most used either *J4* or *J6* joining segments, which are also employed the majority of normal B-cells[23]. No conserved amino acid motifs in the CDR3 regions were appreciated among the different cases.

Discussion

The development and early growth of MALT lymphomas appears to be dependent on antigen stimulation, which can be direct, mediated by surface immunoglobulin, and/or indirect and mediated by T-cells. The antigens playing key roles in MALT lymphomagenesis are mostly unknown, but are likely dependent on the type of tissue the lymphomas arise from and could also vary with different geographical areas. We analyzed the *VH* genes expressed by 10 cases of ocular adnexal MALT lymphoma to look for evidence of direct antigen stimulation. An important finding along this line was observing preferential use of certain *VH* gene segments with 2 cases using the same *V1–2* gene segment and 3 other cases using the same *V4–34 VH* gene segment. Since there are approximately 40 different functional *VH* gene segments that can potentially be used [23,24], the probability that 2 or 3 of the 10 cases evaluated would use the same *VH* gene segments by chance alone is 0.023 and 0.002, respectively. By contrast, approximately 3% of normal peripheral blood B-cells use *V1–2* and *V4–34*, which is the expected frequency if use is random[23]. One of the other 10 cases expressed a *VH4* family gene segment different from *V4–34*, *V4–31*, which further suggests orbital MALT lymphomas may also display preferential use of *VH4* family gene segments.

Non-random or preferential use of certain *VH* gene segments suggests that these encode important determinants that have been selected by an antigen binding to surface immunoglobulin leading to stimulation of B cell growth. Besides our study, preferential use of *V4–34* and *V1–2* has been reported for other types of low grade B-cell neoplasms. The *V4–34* gene segment is used by approximately 20% of chronic lymphocytic leukemia (CLL) cases with mutated *VH* genes [25], and by approximately 60% of primary central nervous system (CNS) lymphoma cases [26,27] (Table 5). Preferential use of *V1–2* is seen in splenic marginal zone lymphomas and used by approximately 40% of cases [28,29]. It is interesting that the expression of *V1–2* and *V4–34* does not appear to be increased above random use levels in MALT lymphomas that occur at other sites such as the stomach, lung, or salivary gland [12,30,31]. This is consistent with the concept that antigens stimulating MALT lymphoma growth in the ocular adnexal will be different than those at other MALT lymphoma sites. It may be significant regarding the antigen specificity of ocular adnexal MALT lymphomas that both *V1–2* and *V4–34* are used by auto-antibodies, and almost all cold agglutinin auto-antibodies employ *V4–34* [32].

Our findings of preferential use of *V1–2* and *V4–34* differ from the 2 earlier studies of ocular adnexal MALT lymphoma *VH* genes. In the study by Coupland et al. [33] that involved 26 cases, 2 (8%) used *V4–34* and only 1 (4%) used *V1–2*, while none of the 12 cases analyzed by Hara et al. [34] used *V1–2* or *V4–34*. Since the cases analyzed in Coupland's study were European and those in Hara's study were Japanese, it is possible that antigens driving ocular adnexal MALT lymphomagenesis vary depending on geographical location. This possibility is also supported by reports showing an association of C. psittaci with mostly Italian orbital MALT lymphoma cases [7,8,35], while U.S. based orbital MALT cases using sensitive PCR techniques appear to be *C. psittaci* negative [9,10,35]. Technical considerations could also have complicated positive lymphoma *VH* gene identification in the study by Coupland et al. [33] since paraffin extracted

DNA was used in nested PCR reactions involving consensus FW1 and FW2 primers and 65 total cycles of amplification.

All of the lymphoma *VH* genes identified in this study were mutated relative to their most closely related germline counterparts consistent with the proposed post-germinal center B-cell origin. In addition, the level of mutation we observed in the orbital MALT lymphoma *VH* gene segments (mean percent homology to germline of 93% +/- 2.4%) is also close to the mutation load values reported for MALT lymphomas that occur at other locations [12,16]. Analysis of the point mutation types (replacement or silent) and distribution (FWRs or CDRs) within many of the *VH* gene segments suggested there is selection against mutations occurring in CDR1 and CDR2, or negative selection. Finding evidence of negative selection in these CDRs was not an artifact related to analyzing small numbers of mutations since the 5 cases showing clear negative selection also had some of the highest mutation loads. Negative selection observed in the CDRs of these cases is similar to the negative selection that was observed in the FWRs of all these cases which is an indicator of functionality in that many FWR residues cannot withstand replacement mutations if immunoglobulin function is to be maintained [22]. Selection against replacement mutations in the CDRs is also a feature of *VH* genes expressed by MALT lymphomas that occur at other sites such as the salivary gland [36,37], and could function in preventing the disruption of antigen mediated signaling which may be detrimental to lymphoma cell survival [38,39]. Negative selection is also seen with antibodies that display reactivity towards autoantigens and may play a role in preventing the emergence of high affinity variants [40].

The CDR3 nucleotide sequence, made up of randomly templated nucleotides and diversity and joining segments that have been shortened by nuclease trimming, is the most variable part of a *VH* gene and serves as a clonal marker [41]. Because of the tremendous variability, antibodies that recognize the same antigens typically do not have similar CDR3 sequences [42–44]. As such, not finding similarities of CDR3 sequences among our MALT lymphoma cases does not alter the possibility that some may recognize similar or common antigens. Since the size of the CDR3 sequence also plays an important role in antigen binding [45], it may be significant that the CDR3 of 2 cases using *V4–34* are the same size, while the CDR3 sequences for the 2 *V1–2* cases differ by only 2 amino acids.

In summary, our study suggests that U.S. based ocular adnexal MALT lymphomas that are not associated with *C. psittaci* preferentially express a limited set of *VH* gene segments not frequently used by MALT lymphomas that occur at other sites. This finding is consistent with these cases recognizing a common antigen that is responsible for selecting and promoting the transformation of B-cells with the frequently expressed *VH* gene segments via immunoglobulin mediated growth stimulation. Although the nature of the antigen is unknown, frequent use of the *V4–34* and *V1–2* gene segments as well as selection against replacement point mutations occurring in the CDRs, favors these cases recognizing a self or auto-antigen.

Acknowledgements

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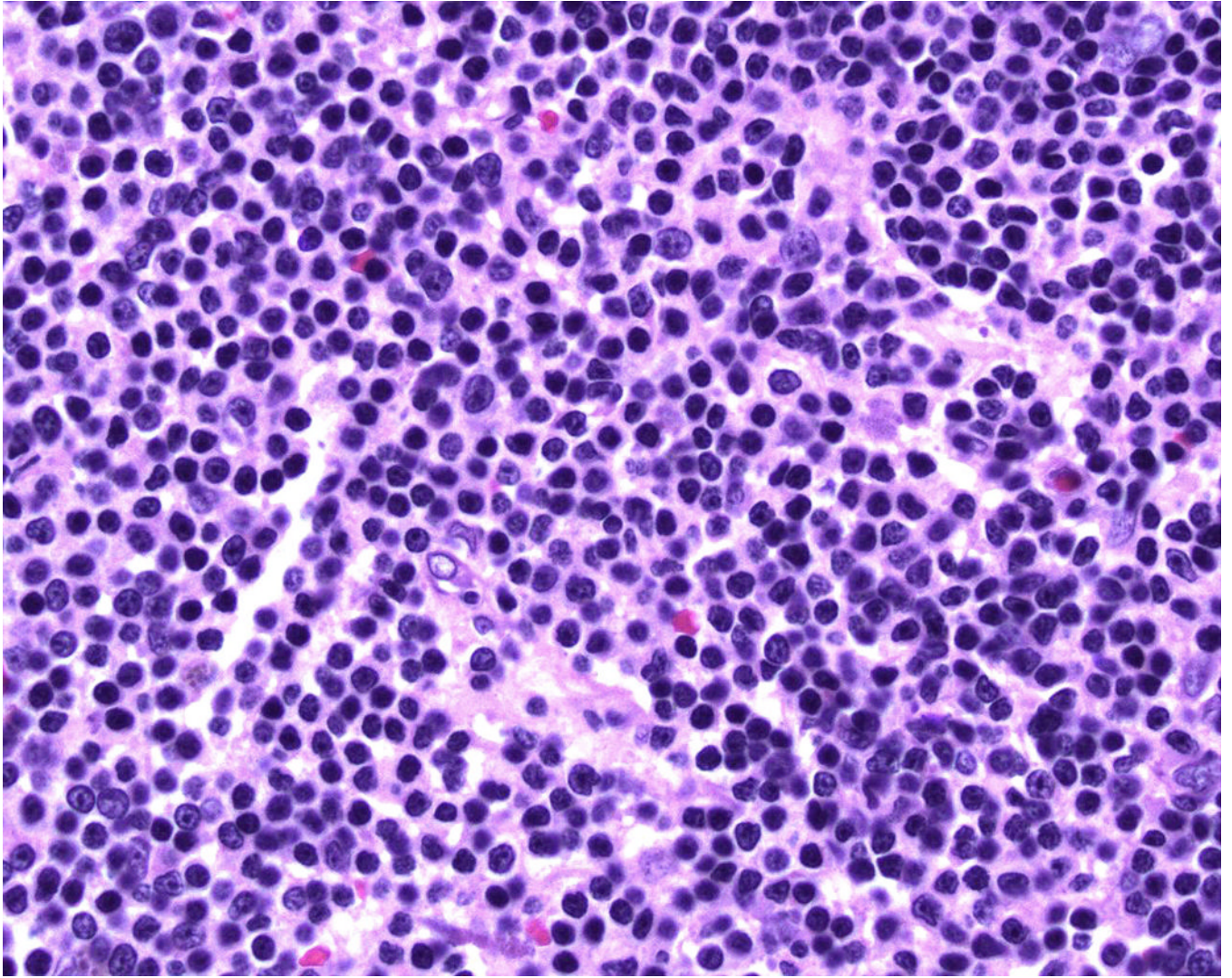
Figure 1.
Hematoxylin and eosin stained section of a representative case (#4) showing morphologic features typical of ocular adnexal MALT lymphoma.

Table 1

Ocular MALT lymphoma case information

Case #	Age/Sex	Biopsy Site	Light Chain	C. Psittaci Status
1	54 / M	R Lacrimal	Kappa	Neg
2	74 / F	R Orbit	Kappa	Neg
3	55 / F	L Orbit	Lambda	Neg
4	81 / M	L Orbit	Kappa	ND[*]
5	82 / M	L Orbit	Kappa	ND
6	76 / M	L Orbit	Kappa	Neg
7	43 / F	R Orbit	Kappa	Neg
8	68 / M	L Orbit	Lambda	Neg
9	58 / F	R Orbit/Lacrimal	Kappa	ND
10	80 / M	L Orbit	Kappa	Neg

[*] ND , Not determined

Table 2

Ocular MALT Lymphomas use of *VH* gene segments

Case	Family	Name	# Mutations	% Homology[*]
1	VH1	V1-2	19	93.5
2	VH1	V1-2	20	93.1
3	VH4	V4-34	17	94.2
4	VH4	V4-34	32	89.0
5	VH4	V4-34	19	93.5
6	VH4	V4-31	9	96.9
7	VH3	V3-30	21	92.9
8	VH3	V3-74	24	91.8
9	VH3	V3-23	10	96.9
10	VH5	V5-51	23	91.8

[*] Relative to the most closely related germline gene

Table 3

Distribution of mutations in lymphoma *VH* segments

Case #	CDR 1&2				FWR 1,2&3			
	R*	S	R/S	Random R/S	R	S	R/S	Random R/S
1	8	1	8.0	4.3	6	4	1.5	3.0
2	5	3	1.7	4.3	5	7	0.7	3.0
3	3	1	3.0	4.5	9	11	0.8	2.7
4	4	4	1.0	4.5	13	11	1.2	2.7
5	4	3	1.3	4.5	6	6	1.0	2.7
6	3	0	3	4.1	3	3	1	2.7
7	9	3	3	3.9	6	3	2	2.9
8	5	3	1.7	4.2	11	5	2.2	2.8
9	4	1	4	3.6	3	2	1.5	2.9
10	6	5	1.2	3.5	5	7	0.7	3.3

*
Columns headed by "R" or "S" give the number of replacement or silent mutations, respectively, relative to the most closely related germline VH gene segment. "Random R/S" refers to the ratio of all possible replacement to silent mutations.

Table 4

Ocular lymphoma *VH* gene CDR3 sequences

Case #	D_H*	J_H	Amino Acid Sequence
1	2-2	4b	GPRQCSSITCLSYFHY
2	3–16	6b	AARVVRLFNPDRSSGMDV
3	6–13	6b	GEAVTGPPRSDDMDV
4	5-5	4b	TGGPHLGNSDGVTHS
5	2–15	4b	ITEIIDVHYCSGGDCNEGGFDY
6	4-4	6b	VHSNYYYRMDV
7	3–9	4b	QYDILTDYYKWGVFDY
8	1-1	4b	GNGY
9	5–24	4b	AWAYIAGNYFDY
10	2–21	3b	PYCGGDCYSGDAPFHI

*D_H and J_H refer to the germline heavy chain diversity and joining segments used in the CDR3 , respectively

Table 5

Differential use of *V1-2* and *V4-34* segments

Cell Types	*V1-2*	*V4-34*
Peripheral blood B-cells	3% [**]	3% [**]
Mutated CLL	7% (3/46) [*]	20% (9/46) [*]
Unmutated CLL	8% (3/38) [*]	3% (1/38) [*]
Splenic Marginal Zone Lymphoma	42% (20/48) [§]	15% (7/48) [§]
1° CNS Lymphoma	0% (0/15) [¶]	60% (9/15) [¶]
Other MALT Lymphomas (gastric, salivary gland, lung)	1% (1/76) [¥]	4% (3/76) [¥]

[**] Brezinschek et al. [23]

[*] Hamblin et al. [25]

[§] Bahler et al. [29], & Algara et al. [28]

[¶] Thompsett et al. [27], & Montesions-Rongen et al. [26]

[¥] Bende et al. [12], Sakuma et al. [31], & Lenze et al. [30]