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# Biased Immunoglobulin Light Chain Use in the *Chlamydophila psittaci* Negative Ocular Adnexal Marginal Zone Lymphomas

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## Abstract

Ocular adnexal mucosa associated lymphoid tissue lymphomas (OAMALTL) are the most common lymphomas of the eye. The potential roles for specific antigens in these lymphomas are still controversial. Previously we examined *IGHV* usage and mutations *IGHV*in *Chlamydophila (C) psittaci*-negative OAMALTL, demonstrating biased use of the *IGHV4* family and *IGHV4-34* gene and evidence for antigen selection. Herein, we examined the *IKGV/IGLV* gene usage and mutations in 34 *C. psittaci*-negative OAMALTL originating from the orbit (15), conjunctivae (14) and lacrimal gland (5). Clonal potentially functional *IGKV/IGLV* gene sequences were identified in 30 tumors (18 kappa and 12 lambda). An overrepresentation of the IGKV4 family (p<0.01) was observed. The IGKV3-20*01 allele was used at a greater frequency than in normal peripheral blood B-lymphocytes (p=0.02) and commonly paired with the IGHV4-34 allele. 27 of the 30 unique light chain sequences displayed mutations from germline and evidence for antigen selection. Overall our findings demonstrate that in *C. psittaci*-negative OAMALTL there is a biased usage of *IGKV* families and genes, which harbor somatic mutations. These findings and the specific paring between the IGKV3-20*01 and IGHV4-34 alleles suggest that specific antigens could play an important role in the pathogenesis of these lymphomas.

### Keywords

MALT lymphoma; ocular adnexa; IG light chain

## Introduction

Extranodal marginal zone lymphomas (also referred as mucosa-associated lymphoid tissue (MALT) lymphomas) are the most common lymphomas in the ocular adnexa, originating in the conjunctiva, orbit and lacrimal glands or lids. Similarly to extranodal MALT lymphomas in other tissues, ocular adnexa MALT lymphomas (OAMALTL) are frequently characterized by an indolent clinical course and often remain localized for many years [1]. Nevertheless, it is well recognized that MALT lymphomas at different anatomic sites have

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**Conflict of Interest**

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distinct etiologies and biological features. Specifically, the underlying inflammatory disorders leading to lymphomagenesis vary widely. The etiology and pathogenesis of OAMALTL are still controversial. Association with *Chlamydophila psittaci (C. psittaci*) has been shown in only some geographic regions, but the nature of the antigenic stimulus in *C. psittaci* negative OAMALTL is still unknown [2]. Important clues on the role of antigens in lymphomagenesis can be obtained by analyzing the clonal surface B-cell receptor containing the immunoglobulin (Ig) heavy and light chain variable regions (*IGHV* and *IGKV/IGLV,* respectively). While several groups, including ours, have analyzed the *IGHV* gene usage in OAMALTL tumors [3-10], to the best of our knowledge the *IGKV/IGLV* repertoire in OAMALTL has not been extensively characterized. Light chains may play an important role in the process of antigen recognition and/or selection. Consequently, in the present study we analyzed the *IGKV/IGLV* usage, somatic mutation patterns and heavy-light chain pairing in a series of 34 *C. psittaci*-negative, untreated OAMALTL.

## Materials and methods

### Patient materials and DNA extraction

DNA was previously extracted from a total of 67 fresh patient biopsy samples at the time of OAMALTL diagnosis (between 1991 and 2011) using a commercially available kit (QIAamp; Qiagen, Valencia, CA, USA), as previously reported by us [1, 3]. Clonality was confirmed by either Southern blot analysis or polymerase chain reaction (PCR) for immunoglobulin heavy or light chains in accordance with the BIOMED-2 recommendations and also as previously reported by our analysis of the *IGHV-IGHJ* PCR sequence [3]. DNA from 34 tumor biopsies was available for the analysis of light chain usage performed herein. No differences were present in the clinical characteristics and tumor locations between patients whose DNA was used in the analyses presented herein and those whose DNA was exhausted in our previous studies (not shown). All pathologic specimens were classified according to the WHO 2008 classification on the basis of the morphologic features observed on routinely prepared hematoxylin and eosin-stained slides of formalin-fixed, paraffin-embedded tissues along with immunophenotypic and genotypic result [11]. This study was approved by the University of Miami Institutional Review Board (IRB) and written informed consent was obtained according to the approved protocol.

### Polymerase chain reaction amplification and sequencing

The integrity of DNA from patient samples was verified by β-actin PCR amplification using specific primers yielding a 597 base pair (bp) amplicon as described previously [1, 3]. To amplify the *IGKV-IGKJ* and *IGLV-IGLJ* gene sequences, 10-200 ng of DNA were amplified by GoTaq Green Master Mix (Promega, Madison, WI) in a final volume of 50μl containing 10pmol of a specific 5′ primer corresponding to one of the 7 human variable kappa chain family leaders (*IGKV*1 through *IGKV*6b) or 12 human variable lambda chain family leaders (*IGLV*1 through *IGLV*10) and 10pmol of BIOMED-2 3′ antisense $J_{K1-4}$/ $J_{K5}$ or $J_{L123}$ consensus primers [12, 13]. The PCR conditions were: 96°C for 5 minutes, 65°C for 1 minute, 72°C for 3 minutes, 1 cycle; 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, 30 to 35 cycles; and 72°C for 7 minutes. For better visualization of the amplified bands on the agarose gel, heteroduplex analysis was performed by heating the PCR product to 95°C for 5 minutes and then cooling to 4°C for 1 hour, as previously reported [13]. For each PCR, a control with no added template was used to check for contamination. PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide. In the 10 cases in which a clonal amplicon was not observed with the above primers, an additional PCR reaction was performed using the modified BIOMED-2 FR1 primers as described by Payne et al [14].

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Bands of appropriate size were excised from the gels and purified by adsorption to a silica matrix (QIAquick Gel Extraction Kit, Qiagen). Direct DNA sequencing of PCR amplicons was performed on a 373 automatic DNA sequencer (Applied Biosystems, Foster City, CA) using the ABI Prism Big Dye Terminator Kit (Perkin Elmer, Foster City, CA) as recommended by the manufacturer. The same primers used for PCR were used for sequencing. The sequence was defined as clonal if identical CDR3 sequences were obtained from 2 independent PCR reactions. The sequences were checked using the Chromas Lite V2.01 (Technelysium Pty Ltd., Australia) software. OAMALTL Ig light chain sequences were analyzed using the IMGT databases and the IMGT/V-QUEST tool (http://www.imgt.org). All the clonal sequences were deposited in GenBank (JX489466-JX489495).

## Ig Gene Analysis

The IMGT/V-QUEST database was used to assign the *IGKV, IGLV, IGKJ* and *IGLJ* regions to the sequences from known germline components based on the highest percentage of sequence homology. These were used to analyze the gene usage and *IGKV, IGLV, IGKJ* and *IGLJ* region pairings in the light chains and between heavy and light chains from the same patient. Framework (FW) and Complementary Determining Regions (CDRs) were assigned based on the IMGT/V-QUEST numbering scheme. Mutations at the last nucleotide position of the sequenced fragment were excluded from the mutational analysis because they might result from nucleotide deletion at the joining sites [15]. Sequence homology was calculated as the % identity from the aligned sequences to corresponding germlines from the beginning of FR1 to the end of FR3. From this, the number of silent (S) and replacement (R) mutations was tabulated. To analyze the relative number of mutations per region, the total number of mutations per variable region (FR1-FR3) were added and normalized to the respective length of each region according to the IMGT numbering scheme. Extent of antigen selection analysis was analyzed using BASELINe [16]. For analysis of recurrent amino acid mutations across the FR and CDR regions, the ClustalW algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align sequences from which 3 or more specimens derived from the same germline *IGKV/IGLV* gene. The CDR3 isoelectric point was calculated using the Swiss-Prot/TrEMBL pI/MW application (http://web.expasy.org/compute_pi/).

## Statistical Analysis

The usage of the *IGKV/IGLV* gene segments and alleles in the OAMALTL specimens was compared to their utilization in normal peripheral blood lymphocytes by the $\chi^2$ test at the p=0.05 significance level.

# Results

## Light Chain Usage in OAMALTL

We have previously analyzed the Ig heavy chains of OAMALTL patients and demonstrated evidence of ongoing mutations and a bias in *IGHV* gene usage [3]. Herein we sought to extend our previous observations by analyzing the Ig light chain of OAMALTL patients and by determining whether correlates between light chain and heavy chain usage exist.

Previously extracted DNA from the tumors of 34 OAMALTL patients (median age 63, range 35-87; 18 females and 16 males) was used for PCR amplification of the *IGKV* and *IGLV* genes. The tumors originated in the orbit (15), conjunctiva (14) and lacrimal gland (5). All specimens were negative for *C. psittaci*, as determined in our previous studies [1, 3]. In 3 (9%) tumors, a clonal *IGKV-IGKJ* or *IGLV-IGLJ* PCR sequence could not be identified, despite successful identification of a clonal *IGHV-IGHJ* PCR sequence and actin

amplification, which served as a control for DNA integrity. In one tumor only a nonproductive *IGKV-IGKJ* sequence was amplified.

Overall, 30 clonal potentially productive *IGKV-IGKJ* and *IGLV-IGLJ* sequences derived from 30 tumors were obtained, with a corresponding heavy chain available for 26 of these patients from our previous work. In 6 of these tumors an additional nonproductive *IGKV-IGKJ* was also detected. Of the available 30 potentially productive light chains, 18 (60%) were kappa light chains while the remaining 12 (40%) were lambda (3:2 ratio). There was a good concordance between the molecularly identified *IGKV* and *IGLV* and surface expression of kappa and lambda light chains, as analyzed by flow cytometry, in all but 3 of the analyzed OAMALTL with potentially productive light chains. In these 3 cases we have confirmed the molecular identity of the clonal light chains in 3 independent PCR reactions. The observed discrepancy between the molecular and flow cytometry results are commonly observed in clinical practice and may be due to sampling of different tumor portions for distinct tests (e.g. flow cytometry and molecular clonality tests), disintegration of cells used for flow cytometry analysis or the presence of approximately equal proportions of malignant and nonmalignant lymphocytes in small OAMALTL biopsies. The latter may lead to incorrect determination of tumor light chain usage by flow cytometry.

Among the 7 kappa light chain families, only IGKV1 (n=5, 28%), IGKV2 (n=1, 6%), IGKV3 (n=8, 44%) and IGKV4 (n=4, 22%) were observed (Table 1). Meanwhile, 5 of the 8 lambda chain families (IGLV2 (n=5, 42%), IGLV3 (n=1, 8%), IGLV6 (n=1, 8%), IGLV7 (n=3, 25%) and IGLV8 (n=2, 17%)) encoded the lambda chains used by the OAMALTL tumors. In comparison with the IGKV and IGLV repertoire employed in normal peripheral blood B-lymphocytes [17-19], an overrepresentation of the IGKV4 family ($p<0.01$), which contains a single gene, was observed in the analyzed cohort of tumors. No statistically significant difference in the use of other kappa light chain families was observed between OAMALTL tumors and the repertoire employed in normal peripheral blood B-lymphocytes [17, 19, 20] (Table 1). Similarly, no biases in kappa J gene usage were observed, with the IGKJ1, IGKJ2 and IGKJ4 families being the most prevalent, as previously reported for normal peripheral blood lymphocytes [17, 19]. While the observed distribution of *IGLV* usage differed from normal peripheral blood, the small number of cases precluded conclusion about a biased usage of these genes. In tumors utilizing the IGKV3 family, the most commonly utilized family in our cases, only 2 (IGKV3-20*01 (n=6) and IGKV3-15*01 (n=2)) of the 7 functional alleles were observed. The IGKV3-20*01 kappa light chain sequence was used more frequently than IGKV3-15*01, similar to previous reports detailing *IGKV* usage in peripheral blood normal B lymphocytes, CLL and autoreactive antibodies [17, 21, 22]. Moreover, IGKV3-20*01 was used at a greater frequency than in normal peripheral blood B-lymphocytes ($p=0.02$) [17]. The usage of the IGKV3-20*01 allele was also elevated in non-orbital MALT lymphomas and was observed at a higher frequency than in other lymphoma types [22-36] (Table 2). No biases in V and J pairings were observed for both kappa and lambda chains.

Next, we sought to characterize the pairing of *IGHV* with *IGKV* and *IGLV* genes in OAMALTL. Of the six cases which utilized the IGKV3-20*01 allele, corresponding heavy chains were available for five cases. Moreover, of the eight IGHV4-34 cases we had previously characterized, corresponding light chains were available for 7 cases. Three of the IGKV3-20*01 alleles paired with the IGHV4-34 allele, one paired with the IGHV3-23 allele and one paired with the IGHV1-69 allele. Interestingly, each of these three alleles has been previously implicated in autoreactivity [36-40]. IGHV4-34 pairing with light chain kappa alleles was restricted to IGKV3-20*01, with other pairings (n=1 for each) corresponding to IGLV2-14*01, IGLV2-8*01, IGLV6-57*01 and IGLV3-25*03, respectively. The IGKV4-1*01 allele was observed to pair with IGHV3-74 (n=2), IGHV1-24 (n=1) and



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IGHV2-5 (n=1), respectively. There was no association between the usage of particular immunoglobulin light chain families or immunoglobulin light chain genes with the extent of plasma cell differentiation in the tumors, specific clinical presentation and outcome; however, the small number of cases prevents firm conclusions. Amyloid deposits were not observed in any of the analyzed OAMALTL biopsies, thus precluding analysis of association with lambda light chain expression.

### Mutation Pattern and CDR3 Analysis

Of the 30 unique light chain sequences derived from OAMALTL patients, 27 displayed mutations from germline. The average percent homology to germline was 96.35% (range: 81.8-100%). Most mutated cases (14 out of 27, 52%) exhibited less than a 2% difference from their original germline sequence. Four of the six tumors utilizing the IGKV3-20*01 allele exhibited more than 2% mutation away from germline, while the remaining two sequences were unmutated. Three of the four tumors employing IGKV4-1*01 exhibited greater than 2% mutation away from germline. No acquired N-glycosylation sites were observed in the OAMALTL cases, a phenomenon previously described for other lymphomas [23].

We next attempted to characterize the prevalence of mutations observed in the light chain V region. To do this, we characterized observed nucleotide differences from the corresponding germlines as either causing a change in amino acid (Replacement-R) or maintaining an identical protein sequence (Silent-S) and normalized the number of such mutations to the region length from which they derived. As expected, the CDR regions exhibited a greater prevalence of R than S mutations. However, the FR2 region also had a greater prevalence of R mutations, with the prevalence of these mutations in this region higher than that found in CDR2 (data now shown). In order to characterize the nature of the observed mutations in FR2, we performed an analysis of R mutations derived from the same germline genes in both CDR and FR regions to identify recurrent mutations. Interestingly, a recurrent mutation was observed in the FR2 region of sequences derived from IGKV3-20*01, with three of six cases demonstrating an acquisition of histidine in place of glutamine at position 39 (Table 3). No other recurrent mutations were found across different V-regions derived from the same germlines.

To analyze for potential selective pressure exerted by antigens, we utilized the BASELINe algorithm, which assesses for both the presence and strength of antigen selection. In order to establish the validity of the method as compared to the Focused Binomial [41] and Multinomial Tests [42], which we used for our analysis of the IGHV gene, we compared the results from these tests to BASELINe [16]. Of forty-four IGHV sequences analyzed with BASELINe, 11 sequences exhibited strong evidence of selection in the FR region while 2 sequences exhibited strong evidence of selection in the CDR region. These numbers were slightly lower than those derived from the Focused Binomial test (where 13 sequences exhibited selection in the FR and 4 in the CDR region) and much lower than the Multinomial Test (where 17 sequences exhibited selection in the FR and 15 sequences in the CDR region). However, because BASELINe quantifies the strength of selection using log-odds ratios, evidence of selection was observed in all but three heavy chain sequences based on acquired R and S mutations. Because sequences judged to be under selection by the Focused Binomial Test also showed strong evidence of selection by BASELINe, this suggests that BASELINe can reliably differentiate the strength of selective pressure based on observed mutation frequencies. When analyzing the OAMALTL light chains, 23 sequences exhibited evidence of selection in the CDR regions, while 26 sequences exhibited evidence of selection in the FR regions. Eighteen of the sequences exhibiting selection in the CDR and 10 of the sequences exhibiting selection in the FR showed evidence for positive selection. The observed positive selection in the FR region is in line with the observed

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higher prevalence of R mutations in FR2 (Table 4). Strong evidence of negative selection was observed in the FR of six sequences, with no sequences exhibiting evidence for strong selective pressures in the CDR region. Of the 26 sequence for which both heavy and light chains were available, 9 heavy chains exhibited strong evidence of selection (8 in FR, 1 in CDR) and 5 light chain sequences exhibited strong evidence of selection (all in FR). Two patients' tumors exhibited strong selection in both heavy and light chain FR regions. We next specifically focused on selection analysis in the observed IGKV3-20*01 sequences. Evidence of positive selection in the FR region was observed for the three sequences utilizing IGKV3-20*01 that exhibited the FR2 histidine mutation, while no evidence for positive selection was observed for the other three sequences which utilized this germline allele.

An analysis of the tumor derived CDR3 regions did not reveal stereotyped sequence determinants and showed no homology to other known bacterial binding antibodies. The average CDR3 isoelectric point was 6.37 ± 1.544 (SD). The average CDR3 length was 9.4 ± 0.73 (SD) (9.1 ± 0.47 for *IGKV-IGKJ* and 9.8±0.83 for *IGLV-IGLJ*), with all sequences harboring between 8-12 amino acids.

## Discussion

The observation that the surface Ig receptor is retained in OAMALTL indicates that stimulation via the B cell receptor may play an important role in the pathogenesis of the tumor. The study of Ig variable region genes is a key tool to provide circumstantial evidence for a potential role of an antigenic stimulus in clonal B cells selection and progression to lymphoproliferative disorders. For example, past studies have shown that a subset of CLL using the IGHV3-7 allele known to be over-represented in this disease [43], exhibits reactivity towards β-(1,6)-glucan. This binding depended on both the stereotypic Ig heavy and light chains, as well as on a distinct amino acid in the IGHV-CDR3 and induced CLL cell proliferation upon β-(1,6)-glucan stimulation [44]. Moreover, the IGKV3-20 allele, known to be over-represented in HCV-associated lymphomas, when paired with IGHV4-34 was shown to mediate binding of their B cell receptors to the HCV E2 protein [45]. Interestingly, seven of nine HCV-related splenic marginal zone lymphoma patients treated with interferon alpha exhibited complete remission of their disease [46]. These studies are indicative that antigens may play an important role in the pathogenesis of non-Hodgkin lymphomas and that studying the B cell receptor repertoire of these tumors may facilitate the discovery of antigen driven tumors and potentially the antigens themselves.

All previous studies in OAMALTL have focused on the analysis of *IGHV-IGHJ* sequences, demonstrating the presence of somatic mutations with intraclonal variation and evidence for antigen selection [3-9]. However, inconclusive data was obtained on the usage of *IGHV* families and specific gene segments. In our cohort of *C. psittaci*-negative OAMALTL patients there was an overrepresentation of the *IGHV4* family, specifically the *IGHV4-34* gene segment [3]. Similar biased usage of the *IGHV4-34* gene segment was previously suggested by Bahler et al. [4] and Mannami et al [7]. The *IGHV4* family was overrepresented in a study reported by Coupland et al [5]. In contrast, no overrepresentation of the *IGHV4* family and the *IGHV4-34* gene was observed in 4 other studies that analyzed mainly European and Japanese patients [6, 8, 9]. While information on association with *C. psittaci* infection was not available for all the analyzed patients reported in the previous studies, overrepresentation of the *IGHV4* family and the *IGHV4-34* gene was observed in the 2 studies characterizing *C. psittaci*-negative OAMALTL patients from the US [3, 4]. Furthermore, a recent study analyzing *C. psittaci*-negative OAMALTL patients from the Netherlands also reported biased overrepresentation of the *IGHV4-34* gene [10]. However,

overrepresentation of the *IGHV4* family was not observed in a recent study of *C. psittaci*-positive OAMALTL patients [9].

Herein we report the first analysis on Ig light chain usage in *C. psittaci*-negative OAMALTL. To the best of our knowledge, usage of the Ig light chain in *C. psittaci*-positive OAMALTL has not been previously analyzed. Similar to what was observed in heavy chains, OAMALTL light chains were frequently mutated and exhibited evidence of antigen selection. However, the extent of light chain mutations was smaller compared to the heavy chains, as was also previously observed in normal lymphocytes and various types of lymphomas with mutated Ig genes. Similarly to the Ig heavy chains, we did not observe stereotyped CDR3 sequence determinants, but detected a recurrent FR2 histidine mutation in the IGKV3-20*01 allele, suggesting that this mutation may be important for determining binding specificity to a presently unknown antigen. This recurrent histidine mutation was also observed in two IGKV3-20*01 allele sequences from HCV-related cryoglobulonemia, further implicating antigenic stimulation as driving the observed somatic mutation [47].

While no difference in the use of most kappa and lambda light chain families was observed between OAMALTL tumors and the repertoire employed in normal peripheral blood B-lymphocytes, we observed an overrepresentation of the IGKV4 family in the tumors. This family is comprised of a single gene, IGKV4-1*01, that was used in 22% of OAMALTL tumors with kappa light chain rearrangement in contrast to its use in 5% of normal peripheral IgM+ B cells expressing kappa light chain [17]. A previous study also reported that IGKV4-1*01 was overrepresented in in lymphocytes from systemic lupus erythematosus patients [48]. Nonetheless, our result should be interpreted with caution due to the relatively small number of analyzed cases, and therefore need to be verified in future studies. Further, we observed an overrepresentation of the IGKV3-20*01 allele in OAMALTL tumors compared to normal peripheral blood B-lymphocytes. It is well known that despite substantial germline-encoded diversity, specific light chain alleles, including IGKV3-20*01, are overrepresented in both neonatal and adult human peripheral blood lymphocytes [17]. In normal B lymphocytes IGKV3-20*01 is overrepresented in both the nonproductive and productive repertoires and its frequency is additionally increased in productive compared to nonproductive rearrangements, suggesting that it is overutilized in the recombination process and is further selected for expression [17]. Strikingly, even further overutilization was observed in the OAMALTL tumors. Review of IGKV3-20*01 usage (Table 2) revealed that it is also overused in non-orbital MALT and splenic marginal zone lymphomas, hepatitis C and Sjogren's syndrome related lymphoproliferative disorders, but not in other lymphoma subtypes [24, 34-36]. IGKV3-20*01 is frequently used by autoantibodies encoding rheumatoid factors and cold agglutinins, the latter binding to erythrocyte I/i antigens [37]. About 40% of cold agglutinins are comprised of paired IGKV3-20*01 with IGHV4-34 [37]. Notably, in OAMALTL both IGKV3-20*01 and IGHV4-34 alleles are overrepresented and in three tumor samples in our study the B-cell receptors were constructed from paired IGKV3-20*01 and IGHV4-34 alleles. These findings raise the possibility that B cell receptors in OAMALTL tumors may exhibit cold agglutinin or rheumatoid factor reactivity. Previous studies in gastric and salivary gland MALT lymphomas reported conflicting results on reactivity of tumor B cell receptors toward human IgG or exhibited polyreactivity to both human and *Helicobacter pilori* proteins [24, 25]. However, no studies on the reactivity of OAMALTL B-cell receptors were previously reported. Overrepresentation of specific heavy and light Ig genes and specific paring between them may indicate that particular antigens could play an important role in the pathogenesis of *C. psittaci*-negative OAMALTL. Further studies on binding of the B cell receptors cloned from the analyzed tumors are now necessary to identify potential antigens involved in OAMALTL pathogenesis.



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## Table 1

### Usage of the IGKV Family in OAMALTL

| IGKV Family | OAMALTL Present Study (%) | Relative IGKV Family Size (%) [17] | Adult PBL % (in situ hybridization) [20] | Adult PBL % (Phage display) [19] | Adult PBL % (PCR) [17] |
|---|---|---|---|---|---|
| IGKV1 | 28 | 47.5 | 43 | 33 | 44 |
| IGKV2 | 6 | 22.5 | 9 | 11 | 19 |
| IGKV3 | 44 | 17.5 | 29 | 46 | 29 |
| IGKV4 | 22 | 2.5 | 19 | 10 | 5 |
| IGKV5 | 0 | 2.5 | 0 | 0 | 2 |
| IGKV6 | 0 | 7.5 | 0 | 0 | 1 |

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## Table 2

**Usage of the IGKV3-20-01 Allele in B-cell Malignancies**

| B-Cell Malignancy | IGKV3-20 Observed (%) | Reference |
|---|---|---|
| OAMALTL | 6/30 (20%) | Present Study |
| Diffuse Large B-cell Lymphoma | 0/11 (0%) | Kuppers et al [26] |
| Chronic Lymphocytic Leukemia | 9/67 (13%) | Tobin et al [27] |
| Chronic Lymphocytic Leukemia | 27/179 (15%) | Stamatopoulos et al [21] |
| Chronic Lymphocytic Leukemia | 2/18 (11%) | Maloum et al [28] |
| MALT Lymphoma | 4/11 (36%) | Bende et al [24] |
| MALT Salivary * | 3/5 (60%) | Bahler et al [29] |
| Nodal MZL | 1/6 (17%) | Marasca et al [30] |
| Splenic MZL | 20/113 (18%) | Bikos et al [34] |
| Sjogren's Syndrome | 4/7 (57%) | De Re et al [35] |
| Follicular Lymphoma * | 3/47 (6%) | Smilevska et al [31] |
| Follicular Lymphoma * | 0/10 (0%) | Stamatopoulos et al [32] |
| HCV-Related NHL * | 5/16 (31%) | Ivanovski et al [36] |
| CNS-Related Lymphoma | 0/8 (0%) | Montesinos-Rongen et al [33] |

* Indicates studies in which only IGKV repertoire was analyzed

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## Table 3
### Recurrent Mutations in the FR2 region of the IGKV3-20 Allele in OAMALTL

| Germline | Case Number | Site | FR2 |
|---|---|---|---|
| IGKV3-20 | | | |
| | 3198 | Orbit | LAWYRQKPGQAPRLLIF |
| | 4438 | Lacrimal | LAWYQQKPGQAPRLLIY |
| | 4726 | Orbit | LAWYQ**H**KPGQAPRLLIS |
| | 5547 | Conjuctiva | LAWYQ**H**KPGQTPRLLIY |
| | 6239 | Conjuctiva | LAWYQ**H**KPGQAPRLLIH |
| | 11274 | Orbit | LAWYQQKPGQAPRLLIY |
| | Germline | | LAWYQQKPGQAPRLLIY |

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## Table 4

### Mutation Prevalence and Selection Extent of Ig Light Chains in OAMALTL

| Patient Number | Location | V-Gene | J-Gene | V Gene Homology (%) | FR R | FR S | CDR R | CDR S | BASELINe CDR Log Odds Ratio | BASELINe FR Log Odds Ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| 2491 | Conjuctiva | IGKV4-1*01 | IGKJ1*01 | 99.3 | 2 | 0 | 0 | 0 | NA | 1.6 |
| 3050 | Conjuctiva | IGLV2-23*02 | IGLJ2*01 | 100 | 0 | 0 | 0 | 0 | NA | NA |
| 3145 | Orbit | IGKV3-15*01 | IGKJ2*01 | 98.6 | 2 | 0 | 2 | 0 | 3.37 | 1.49 |
| 3198 | Orbit | IGKV3-20*01 | IGKJ2*01 | 91.8 | 8 | 3 | 6 | 1 | 0.959 | −0.543 |
| 3687 | Conjuctiva | IGKV2-28*01 | IGKJ4*01 | 99.1 | 1 | 0 | 0 | 0 | NA | 1.61 |
| 4277 | Orbit | IGLV2-14*01 | IGLJ3*02 | 90.6 | 9 | 5 | 9 | 0 | 1.15 | −0.386 |
| 4438 | Lacrimal | IGKV3-20*01 | IGKJ1*01 | 100 | 0 | 0 | 0 | 0 | NA | NA |
| 4465 | Conjuctiva | IGLV8-61*01 | IGLJ3*02 | 99.3 | 0 | 1 | 1 | 0 | 0.767 | −3.26 |
| 4672 | Orbit | IGLV8-61*01 | IGLJ3*02 | 95.5 | 4 | 4 | 2 | 0 | 0.14 | −0.809 |
| 4692 | Orbit | IGKV1-16*02 | IGKJ4*01 | 98.1 | 0 | 0 | 2 | 0 | 3.08 | NA |
| 4694 | Lacrimal | IGKV1-8*01 | IGKJ5*01 | 92.9 | 7 | 4 | 1 | 1 | −1 | −0.92 |
| 4726 | Orbit | IGKV3-20*01 | IGKJ1*01 | 95.7 | 7 | 1 | 2 | 0 | 1.4 | 0.835 |
| 4942 | Orbit | IGKV4-1*01 | IGKJ4*01 | 94.3 | 3 | 6 | 5 | 1 | 0.223 | −1.99 |
| 4968 | Conjuctiva | IGKV1-33*01 | IGKJ5*01 | 97.2 | 2 | 3 | 1 | 1 | −0.188 | −1.55 |
| 5101 | Orbit | IGLV7-46*01 | IGLJ3*02 | 96.9 | 2 | 3 | 2 | 0 | 0.3 | −1.21 |
| 5334 | Conjuctiva | IGKV3-15*01 | IGKJ1*01 | 98.9 | 2 | 1 | 0 | 0 | −1.52 | −0.344 |
| 5547 | Conjuctiva | IGKV3-20*01 | IGKJ2*01 | 97.2 | 6 | 2 | 0 | 0 | −1.66 | 0.138 |
| 5859 | Orbit | IGKV1-5*03 | IGKJ4*01 | 99.3 | 1 | 0 | 0 | 0 | NA | 1.54 |
| 5897 | Orbit | IGKV1-39*01 | IGKJ2*01 | 81.8 | 22 | 6 | 8 | 0 | 0.176 | −0.212 |
| 5984 | Lacrimal | IGLV2-8*01 | IGLJ1*01 | 94.4 | 6 | 3 | 3 | 1 | 0.104 | −0.898 |
| 6092 | Orbit | IGLV2-14*01 | IGLJ3*02 | 96.5 | 3 | 1 | 2 | 0 | 1.2 | 0.022 |
| 6239 | Conjuctiva | IGKV3-20*01 | IGKJ2*02 | 97.2 | 3 | 1 | 2 | 0 | 1.4 | 0.103 |
| 6901 | Conjuctiva | IGKV4-1*01 | IGKJ4*01 | 96.0 | 2 | 4 | 4 | 1 | 0.337 | −1.68 |
| 7126 | Conjuctiva | IGLV2-8*01 | IGLJ3*02 | 99.3 | 1 | 0 | 0 | 0 | NA | 1.55 |
| 11274 | Orbit | IGKV3-20*01 | IGKJ4*01 | 100 | 0 | 0 | 0 | 0 | NA | NA |

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| Patient Number | Location | V-Gene | J-Gene | V Gene Homology (%) | FR R | FR S | CDR R | CDR S | BASELINe CDR Log Odds Ratio | BASELINe FR Log Odds Ratio |
|---|---|---|---|---|---|---|---|---|---|---|
| 11652 | Orbit | IGLV7-43*01 | IGLJ3*02 | 99.0 | 1 | 1 | 0 | 0 | −1.87 | −0.796 |
| 13051 | Orbit | IGLV7-43*01 | IGLJ3*02 | 98.6 | 1 | 1 | 2 | 0 | 1.19 | −0.796 |
| 15137 | Conjuctiva | IGLV6-57*01 | IGLJ2*01 | 98.6 | 2 | 0 | 1 | 0 | 3.17 | 1.57 |
| 15721 | Orbit | IGLV3-25*03 | IGLJ2*01 | 93.6 | 5 | 6 | 2 | 0 | 0.124 | −0.981 |
| 18664 | Conjuctiva | IGKV4-1*01 | IGKJ2*01 | 90.8 | 7 | 1 | 6 | 2 | 0.252 | −1.26 |

Legend:

NA – Not available, since no mutations observed

Evidence of Negative Selection

Evidence of Strong Negative Selection

Evidence of Positive Selection