High Frequency of MYD88 L265P Mutation in Primary Ocular Adnexal Marginal Zone Lymphoma and Its Clinicopathologic Correlation

A Study From a Single Institution

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Context.—The pathogenesis of primary ocular adnexal marginal zone lymphoma (POAMZL) remains unclear. The reported associations with Chlamydia psittaci infection and MYD88 mutations are highly variable.

Objective.—To examine MYD88 L265P mutation in ocular marginal zone lymphomas and correlate with clinicopathologic features and Chlamydia infection.

Design.—Presence of MYD88 L265P mutation and Chlamydia infection in lymphoma was analyzed by using sensitive polymerase chain reaction (PCR) methods.

Results.—The MYD88 L265P mutation was identified in 8 of 22 POAMZLs (36%), including 2 of 3 cases in which PCR failed to detect clonal IGH gene rearrangement; none of the 4 secondary marginal zone lymphomas were positive. Test results for Chlamydia were negative in all cases. Patients with and without the MYD88 mutation had similar clinicopathologic features.

Conclusions.—The MYD88 mutational analysis provides important information in diagnostic workup of POAMZL. The frequent MYD88 mutation suggests a critical role of this aberration in the pathogenesis of POAMZL and may serve as a therapeutic target for patients with progressive disease.

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Lymphomas of ocular adnexa comprise 8% of extranodal lymphomas.1 Primary ocular adnexal marginal zone lymphoma (POAMZL) is the most common orbital tumor, comprising more than half of all orbital tumors.2–4 Most patients with POAMZL present with Ann Arbor stage I disease, and more than 80% achieve complete remission with first-line therapy.5 Radiation therapy is the primary treatment modality, but single-agent or combination chemotherapy and immunotherapy with rituximab are also available.

The pathogenesis of extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) appears to be associated with environmental factors, such as bacterial infections, that drive inflammatory response that may evoke acquisition of oncogenic mutations. Chronic antigenic stimulation by infections or other etiologies is presumed to induce genetic instability with subsequent chromosomal abnormalities or gene mutations. Among infectious causes, Chlamydia psittaci has been most implicated in the development of POAMZL. However, this association appears to be dependent on the geographic regions.6 Some studies originating from North American institutions have shown no association with bacterial infections.7,8

The most commonly described genomic alterations in MALT lymphomas are balanced translocations that appear to be site specific.9 The most common cytogenetic alterations in POAMZL are trisomies of chromosomes 3 and 18.10,11 Rearrangements of the MALT1 gene are frequently seen in MALT lymphomas of other anatomic sites, such as t(14;18)(q32;q21) IGH-MALT1 and t(11;18)(q21;q21) API2-MALT1, but have not been reported in POAMZL studied in North America.11,12 The findings suggest that the underlying molecular mechanisms in the pathogenesis of POAMZL are different from marginal zone lymphoma (MZL) arising from other sites. Two recent studies investigating the genomic landscape in ocular MZL showed different results.13,14 One study from South Korea by Jung et al14 reported that...
TNFAIP3 is the most frequently mutated gene followed by TBL1XR1 and CREBBP, and the MYD88 mutation was detected only in 4% of patients. The other study from the United States by Cani et al.\(^1\) found high frequency (25%) of MYD88 mutations in their cohort of patients, and no other recurrent mutations were identified. Another study from Germany by Johansson et al.\(^1\) reported frequent mutations in both MYD88 (19%) and TNFAIP3 genes in ocular MZL in addition to other recurrent mutations.

The goal of this study was to evaluate MYD88 L265P mutations in our cohort of patients with POAMZL and correlate the mutational status with clinicopathologic features and Chlamydia infection.

**MATERIALS AND METHODS**

**Case Selection**

This study was approved by the Institutional Review Board of Northwestern University, Chicago, Illinois. Cases diagnosed with MZL involving the ocular adnexal tissue between January 2003 and June 2017 were searched from the database in the Department of Pathology at Northwestern Memorial Hospital, Chicago, Illinois. The hematoxylin–eosin stains and immunohistochemical-stained sections and the results of ancillary studies, including flow cytometric immunophenotyping, and molecular and fluorescence in situ hybridization analysis, were reviewed. A total of 28 cases of ocular adnexal lymphoma that fulfilled the diagnostic criteria for MZL, based on the 2008 World Health Organization classification,\(^1\) were included in this study.

**Immunohistochemistry**

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded (FFPE) biopsy tissue. An automated Ventana Benchmark XT platform (Ventana System, Tucson, Arizona) with the IView Detection Kit was used for the staining with CD3 (predilute; clone LN10, Leica, Wetzlar, Germany), CD5 (1:40; clone 4C7, Leica), CD20 (1:800; clone L26, Dako, Carpinteria, California), immunoglobulin (Ig) M (1:1000; rabbit polyclonal, Dako), IgG (1:10,000; rabbit polyclonal, Dako), IgA (1:1000; rabbit polyclonal, Dako), and Ki-67 (prediluted; clone 30-9, Ventana, Oro Valley, Arizona). An automated Leica Bond III platform with the Bond Polymer Refine Detection Kit was used for the staining with CD10 (1:25; clone 56C6, Leica) and CD138 (prediluted; clone M115, Leica).

**Flow Cytometric Immunophenotyping**

Flow cytometric analysis was performed on 23 of 28 cases. Data collection was performed on a Becton Dickinson Bioscences (BD; Franklin Lakes, New Jersey) LSR II flow cytometer. Data analysis was performed by using FACS Diva software (BD). Antibodies used for flow cytometric analysis included the following: \(\kappa\) FITC, \(\gamma\) PE (Kappa/Lambda dual cocktail, F(ab')2 fragments of affinity-isolated polyclonal rabbit anti-human antibodies; Dako/Agilent), CD5 Pe-Cy5.5 (clone BL1a, Immunotech/Beckman Coulter, Brea, California), CD3 Pe-Cy7 (clone UCHT1, Immunotech/Beckman Coulter), CD10 APC (clone HIT10a, BD), CD20 APC-H7 (clone L27, BD), CD19 BV421 (clone HIB19, BD), and CD45 Krome Orange (clone J3.33, Immunotech/Beckman Coulter).

**DNA Extraction**

DNA was extracted from FFPE tissue by using the automated QiAsymphony system and QiAsonomy DSP DNA Mini Kit according to the Tissue_HC_200_V7_DSP protocol (Qiagen Germantown, Maryland). Before the DNA extraction, tissue was deparaffinized by using Hemo D, 100% ethanol, and digested overnight at 37°C in Qiagen Tissue Lysis and protease K. DNA was quantified by spectrophotometry with NanoDrop 1000 (Thermo Fisher Scientific, Waltham, Massachusetts). The DNA samples were used for molecular analysis for immunoglobulin heavy chain (IGH) and immunoglobulin \(\kappa\) light chain (IGK) gene rearrangements, MYD88 mutational analysis, and detection of Chlamydia organisms.

**Clonality Assessment by Polymerase Chain Reaction**

The polymerase chain reaction (PCR) was performed to assess IGH gene rearrangement by using Invivoscribe IGH chain clonality assay (Invivoscribe Technologies, San Diego, California) according to the manufacturer’s instruction. Three master mixes targeted framework 1, 2, and 3 regions within the variable region, and the joining region of the Ig heavy-chain locus. The last master mix, the specimen Size Ladder, targets multiple genes and generates a series of amplicons to ensure quality and quantity of DNA. Light-chain analysis was performed with Invivoscribe IGV V-J primer sets. IGK clonality testing used 2 master mixes, Tube A and Tube B, to target conserved regions within variable (VK1-7) and joining regions (JK1-5). The PCR products were analyzed with capillary electrophoresis (ABI 3130xl-1, Applied Biosystems, Carlsbad, California). The PCR products from a clonal B-cell population produce a narrow spike on capillary electrophoresis. A population is considered clonal if the height of the spike is more than 3 times the height of the third highest peak in a given range of DNA sizes. Samples were run in duplicate to ensure the reproducibility of the clonal peaks.

**Allele-Specific Real-Time PCR Assay for Analysis of MYD88 L265P Mutation**

Two forward primers were designed to differentiate the mutant and wild-type allele of MYD88 L265P, which resulted from a T→C transversion at position 38182641 on chromosome 3p22.2.

To optimize the specificity, an internal mismatch in the third position from the 3’-end was introduced. The mutant-specific forward primer was 5’-GTGCCCATCAGAAGGCT–3’ and the wild-type-specific forward primer was 5’-GTGCCCATCA-GAACGGCT–3’. The common reverse primer was 5’-GGCAGTCCCCAGAACTTGGT–3’. The common TaqMan minor groove–binding probe (MGB) probe was 5’-6FAM-TGCGACTACCAACC-MGB-3’.

For each sample, 2 reactions were run including one as a control of the DNA quality (using wild-type forward primer, common reverse primer, and probe) and one for the detection of the mutation (mutant forward primer, common reverse primer, and probe). The assay was run on a 96-well plate. Each patient sample was run in duplicate for each allele analyzed (T/wild-type allele and C/mutant allele) as well as 3 controls: positive (2% mutant allele), negative, and blank (H2O) control. Each reaction was carried out in a final volume of 20 μL, containing 300 nM of each primer (forward and reverse wild-type or mutant), 125 nM of the probe, 1X of the TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, California), and 20 ng of genomic DNA. Experiments were performed on the Quant Studio 6 Flex (Applied Biosystems) and consisted of an initial denaturation step of 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Data were analyzed with the Quant Studio Real-time PCR software v1.1 (Applied Biosystems). The cycle in which fluorescent emission reaches 10-fold the basal emission was known as the cycle threshold (CT), a value that was proportional to the copy number of the target gene. A positive versus negative result was determined by the delta cycle threshold (ΔCT) of the wild-type, and the mutant reactions (ΔCT) were defined by Ct of wild-type allele minus Ct of mutant allele. A positive result was defined by (ΔCT) values less than 13, and a negative result was defined by (ΔCT) greater than 13. This assay was validated by using several positive and negative samples including FFPE, blood, and bone marrow samples. The limit of detection (for the variant allele) is estimated to be 0.1%; false-positive and false-negative rates were zero.

**PCR for Detection of Chlamydia Species**

Genomic DNA extracted from the FFPE tissue was used for PCR analysis using primers targeting Chlamydia species including C
Table 1. Summary of the Clinicopathologic Characteristics of 24 Patients With Primary Ocular Adnexal Marginal Zone Lymphoma

| Clinical features                          |        |
|-------------------------------------------|--------|
| Age, y                                    | 29–86  |
| Sex, M:F                                  | 11:13  |
| History of autoimmune diseases            | 4/24 (17%) |
| Site of involvement                       |        |
| Orbital soft tissue                       | 12/24 (50%) |
| Lacrimal gland                            | 4/24 (17%) |
| Conjunctiva                               | 8/24 (33%) |
| Lesion size, cm                           | 1–5.1  |
| Stage                                     |        |
| Stage 1E                                  | 19/22 (86%) |
| Stage 4                                   | 3/22 (14%) |
| Primary treatment*                        |        |
| Observation                               | 3/22 (14%) |
| Radiation                                 | 10/22 (46%) |
| Rituximab                                 | 5/22 (22%) |
| Rituximab + chemotherapy                  | 4/22 (18%) |
| Recurrence                                | 4/18 (33%) |
| Pathologic features                       |        |
| FC positive for monotypic B cells         | 17/19 (90%) |
| Immunophenotype (FC and immunohistochemistry) |        |
| CD5+/CD10−                                 | 20/24 (83%) |
| CD5+                                      | 2/24 (8%) |
| CD10+                                     | 2/24 (8%) |
| Plasmacytic differentiation               | 9/19 (47%) |
| IgM                                       | 6/9 (67%) |
| IgG                                       | 1/9 (11%) |
| IgA                                       | 2/9 (22%) |
| Proliferation index Ki-67, %              | <10 to 30 (mean: 7) |
| Clonal IgH gene rearrangement             | 18/21 (86%) |
| MYD88 L265P mutation                      | 8/22 (36%) |

Abbreviations: FC, flow cytometry; Ig, immunoglobulin; IgH, immunoglobulin heavy chain.

* One patient received radiation followed by rituximab, cyclophosphamide, vincristine, and prednisone, and another patient received radiation followed by rituximab.

psittaci and C trachomatis, as described previously. The original primer and probe sets were developed for quantitative PCR from genomic DNA, but the primers without the probe were used for this study in a standard PCR protocol to generate amplicons for next-generation sequencing. The previously designed primers were modified by the addition of contained 5’ common sequence tags known as common sequence 1 and 2, CS1 and CS2, as described previously, to allow for later incorporation of sample-specific barcodes and Illumina sequencing adapters, using the Fluidigm Access/Array primer set (eg, as described previously in Green et al18). The primers used were named CS1_TQF (ACA CTTG AGC ACA TGG TTC TAC AGA AAA GAA CCC TTM TTA AGG GAG) and CS2_TQR (TAC GGT AGC AGA GAC TGT GTC TCT TAA CTC CCT GCC TCA TGA T), and the underlined regions represent the CS1 and CS2 linker sequences. Amplifications were performed with the following thermocycling conditions: 95°C for 5 minutes followed by 28 cycles of 95°C for 30 seconds, 57°C for 45 seconds, 72°C for 30 seconds, and a final elongation at 72°C for 8 minutes. Each reaction was performed in 10 μL volumes, using MyTaq HS 2X mastermix (Bioline, Taunton, Massachusetts), with final primer concentrations of 200 nM, and DNA concentrations of approximately 20 to 50 ng per reaction. The reactions generated an amplicon of approximately 171 bp, of which 44 bases represented the linker sequences. For all samples, a second set of reactions was performed in which a synthetic DNA standard was spiked into the mastermix to verify that the samples did not contain PCR inhibitors. The PCR yield was visualized by using agarose gel electrophoresis. A dilution series was also performed to determine the limit of detection. A synthetic DNA template (see below) was synthesized and quantitated by using a Qubit 3.0 fluorometer (Life Technologies, Grand Island, New York). This standard was serially diluted (1:10) over 7 orders of magnitude, and each dilution used as template for PCR amplification using the conditions described above. All reactions were performed in triplicate, and agarose gel electrophoresis was performed to verify amplification (E-Gel Precast Agarose Gels, Thermo Fisher Scientific). The undiluted stock was quantitated at 1.35e-9 copies per microliter, and amplification was visible at a one-millionth dilution in all 3 replicates. Thus, the limit of detection for agarose gel electrophoresis was set at 1.35e3 copies per microliter.

A synthetic template containing the primer sites was synthesized by using the gBlocks gene fragments service (Integrated DNA Technologies, Skokie, Illinois). The sequence of the gBlock is shown below, with forward and reverse primer sites underlined and bolded. The overall sequence is identical to the 23S rRNA genes of several Chlamydia sequences, including the C suis isolate SWA-2 (GenBank reference: IILT821323), with the exception of most of the sequence between the forward and reverse reads. The region between the primers (underlined, below) was derived from a eukaryotic mRNA transcript (KC775387) to allow the synthetic DNA to be identified when using next-generation sequencing approaches:

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AGAAAGGAGAGAAAGACTGACCTCAATACCTGAGTAGGACT
CTTTGAAAAAGGTTTGGATGAGTTGT
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**RESULTS**

Clinical Characteristics of the Cohort

Our cohort of 28 patients included 24 patients with POAMZLs (86%) and 4 patients with secondary orbital involvement (14%) by previously diagnosed MZLs at other sites. The clinicopathologic features of patients with POAMZL are summarized in Table 1. The 24 patients included 13 women (54%) and 11 men (46%), and the median age at diagnosis was 64 years (range, 29–86 years). Four patients (17%) had history of autoimmune diseases including 3 patients with rheumatoid arthritis and 1 patient with Sjögren disease. Sites of lymphoma involvement included orbital soft tissue (n = 12, 50%), lacrimal gland (n = 4, 17%), and conjunctiva (n = 8, 33%). Bilateral orbital involvement was noted in 2 patients. Imaging studies or bone marrow biopsy for staging was available for 22 and 12 patients, respectively. Among these, 19 (86%) had stage 1E disease, and 3 had stage 4 disease (14%).

Information regarding treatment was available for 22 patients. The first-line treatment included observation for 3 patients (14%), radiation therapy alone for 10 patients (45%), rituximab alone for 5 patients (23%), and chemotherapy plus rituximab for 4 patients (18%) (R-CVP: rituximab, cyclophosphamide, vincristine, and prednisone for 3 patients and rituximab + bendamustine for 1 patient).
Of note, 1 patient received antibiotic therapy at the time of diagnosis of lymphoma, but the lymphoma progressed and was then treated with rituximab with good response.

Follow-up information was available for 18 of 24 patients with POAMZL (75%). Fifteen patients (83%) went into complete remission with first-line treatment or remained at stable condition with observation. Two patients who initially received radiation therapy had incomplete response and subsequently were treated with rituximab (1 patient) and R-CVP (the other patient). The third patient showed no response to rituximab and therapy was switched to ibrutinib. One patient was under observation for 11 years and later treated with rituximab owing to disease progression. Four patients (22%) had relapse, including 3 patients with stage IE disease and 1 patient with stage 4 disease. The interval between first remission to relapse ranged from 24 to 92 months.

In 4 of 28 patients (14%), the ocular MZL likely represented secondary disease, as 3 patients had prior history of MZL (2 with MALT lymphoma at other sites and 1 with nodal MZL) and 1 patient’s orbital lymphoma represented an extension from parotid MALT lymphoma. All 4 patients were female with a median age of 60 years. Treatment information was available for 3 patients; 2 were treated with R-CVP and achieved remission, and 1 was treated with rituximab only and her condition progressed to diffuse large B-cell lymphoma in 4 years.

Pathologic Characteristics of the Cohort

Figures 1, A through H; 2, A through H; 3, A through F; and 4, A through H, demonstrate representative histologic findings, immunohistochemical stains, and MYD88 PCR results of 4 cases from our cohort. The histologic sections of all cases were reviewed and showed a dense lymphoid infiltrate composed of predominantly small lymphocytes with occasional scattered larger lymphoid cells (Figures 1, A; 2, A, 3, A, and 4 A). The lymphoepithelial lesions and/or complete destruction of the underlying glandular structures were seen in all cases with lacrimal gland involvement (Figure 3, A).

Flow cytometric analysis was performed for 19 of 24 patients with POAMZL, and a monotypic B-cell population was identified in 17 of 19 cases (90%). Flow cytometric analysis and/or immunohistochemistry showed that 20 of 24 cases (83%) were CD5+/CD10+, while 2 cases (8%) were CD5+ and 2 cases were CD10+ (8%). The possibility of small lymphocytic lymphoma or follicular lymphoma was excluded in these 4 cases by the morphologic features, immunophenotypic profile assessed by flow cytometry, and a battery of immunohistochemistry tests. All cases showed sheets of CD20+ B cells (Figures 1, B; 2, B; 3, B, and 4, B). Plasmacytic differentiation was evaluated from the presence of light-chain restriction in the plasma cells or plasmacytoid lymphocytes. Immunostains for κ and λ were available in 19 of 24 POAMZL cases, 9 (47%) of which showed evidence of plasmacytic differentiation; however, the plasmacytic differentiation was focal or subtle and best appreciated with immunostaining for CD138, κ (Figures 1, C; 2, C, and 4, C), λ (Figures 1, D; 2, D, and 4, D), and immunoglobulin heavy chains (Figures 1, E through G; 2, E through G; and 3, C through E). The plasma cells or plasmacytoid cells were intimately associated with the neoplastic infiltrate. The heavy chains were determined in these 9 cases with plasmacytic differentiation and showed predominantly IgM+ cells (Figures 1, E; and 2, E) in 6 cases, IgG+ in 1 case, and IgA+ (Figure 3, E) with scattered IgM+ cells (Figure 3, C) in 2 cases. The proliferative index assessed by Ki-67 staining ranged from less than 10% to 30%.

Molecular analysis was positive for clonal IGH gene rearrangement in 18 of 21 POAMZL cases (86%). The IGH clonality testing was performed on 2 of the 3 cases (inadequate DNA for the analysis on the third case) that were negative for clonal IGH gene rearrangement, and both were also negative for clonal IGK rearrangement. Among 3 cases with negative IGH clonality, flow cytometry results were available in 2 cases, one of which showed monotypic B-cell population, while the other showed polytypic B cells. Figure 2 demonstrates a case in which PCR failed to detect clonal IGH and IGK rearrangement but MYD88 mutation testing was positive.

The 4 cases of secondary ocular MZL were all CD5+/CD10+, and 1 case showed plasmacytic differentiation with predominantly IgM+ plasma cells. Ki-67 ranged from less than 10% to 15%. All 4 cases were positive for clonal IgH gene rearrangement.

Chlamydia-Specific PCR Assay

Using Chlamydia-specific PCR primers, no amplification was observed in any of the 26 cases examined, including 22 POAMZLs and 4 secondary ocular MZLs. These samples were then verified to be inhibitor-free through spike-in of synthetic DNA containing Chlamydia-specific primer sites. All samples generated robust PCR amplification after spike-in of the synthetic template, indicating that there were no inhibitors in the samples preventing amplification of Chlamydia genomic DNA. As Chlamydia-specific PCR findings were negative in all samples, no further next-generation sequencing for speciation was performed.

Prevalence of MYD88 L265P Mutation and Its Clinicopathologic Correlation

The MYD88 mutational analysis by allele-specific real-time PCR was performed on 22 of 24 POAMZLs with sufficient quantity and quality of DNA samples available. Eight of 22 POAMZLs (36%) were positive for MYD88 mutations; representative cases are shown in Figures 1, H; 2, H; and 3, F, and a MYD88-negative case is shown in Figure 4, E.

The clinicopathologic features of the patients with and without MYD88 mutation are summarized in Table 2. The clinical features, including age, sex distribution, history of autoimmune diseases, and lymphoma stage and recurrence, were similar between MYD88-mutated and nonmutated groups. Similarly, the pathologic features, including plasmacytic differentiation and proliferative index, were also similar between the 2 groups. All of the 8 cases with MYD88 mutations were CD5+/CD10+. Three of the 8 cases showed subtle plasmacytic differentiation; interestingly, 2 of the 3 cases were IgA+. In 3 cases with convincing morphologic and immunophenotypic evidence of POAMZL, molecular analysis failed to detect clonal IGH and IGK gene rearrangements; however, a robust positivity for MYD88 L265P mutation was identified in 2 of the 3 cases (Figure 2, H).

DISCUSSION

Lymphomas of the ocular adnexa represent approximately 1% of all non-Hodgkin lymphomas and 8% of extranodal lymphomas. However, lymphoma is the most common
Figure 1. A representative case of primary ocular adnexal marginal zone lymphoma with MYD88 L265P mutation and IgM+ plasma cells or plasmacytoid lymphocytes. A, The biopsy specimen shows diffuse proliferation of small lymphocytes with dense chromatin and scant cytoplasm. B, The neoplastic cells are CD20+. C, Most plasma cells or plasmacytoid lymphocytes are κ-positive. D, The plasma cells and plasmacytoid lymphocytes are λ-negative. E, The plasma cells or plasmacytoid lymphocytes are positive for IgM. Only rare IgG+ (F) or IgA+ (G) cells are seen. Flow cytometric analysis and additional immunohistochemical stains showed that the neoplastic lymphocytes are surface κ-restricted and negative for CD5, CD10, CD23, and BCL-1 (not shown). H, The allele-specific real-time polymerase chain reaction analysis was positive for MYD88 L265P mutation. Abbreviations: MUT, mutant; WT, wild type (hematoxylin-eosin, original magnification ×400 [A]; original magnification ×400 [B through G]).
Figure 2. A case of primary ocular adnexal marginal zone lymphoma for which polymerase chain reaction (PCR) failed to detect a clonal IgH gene rearrangement but which was positive for MYD88 L265P mutation. A, The biopsy shows diffuse proliferation of small to intermediate-sized lymphocytes with dense chromatin and scant cytoplasm; admixed scattered larger atypical cells with irregular nuclear contours and conspicuous nucleoli are also seen. B, Focal sheets of CD20+ B cells are seen. Abundant CD3+ T cells are also present (not shown). C, The scattered larger cells within the abnormal B-cell infiltrate are negative for \( \kappa \). D, These larger cells are weakly positive for \( \lambda \), consistent with \( \lambda \) restriction. E, Focal area shows aggregates of IgM+ cells. Only rare IgG+ (F) or IgA+ (G) cells are seen. H, The allele-specific real-time PCR analysis was positive for MYD88 L265P mutation. Abbreviations: MUT, mutant; WT, wild type (hematoxylin-eosin, original magnification \( \times 600 \) [A]; original magnifications \( \times 600 \) [B through D] and \( \times 400 \) [E through G]).
Figure 3. A representative case of primary ocular adnexal marginal zone lymphoma with MYD88 L265P mutation and IgA+ plasma cells or plasmacytoid lymphocytes. A. The biopsy specimen shows diffuse infiltration of small to intermediate-sized lymphocytes within the lacrimal glands, and the neoplastic lymphocytes invade into or destroy the underlying glandular structure. B. The neoplastic lymphocytes are CD20+. The admixed plasma cells or plasmacytoid lymphocytes are predominantly negative for IgM (C) and IgG (D), but positive for IgA (E). F. The allele-specific real-time polymerase chain reaction analysis was positive for MYD88 L265P mutation. Abbreviations: MUT, mutant; WT, wild type (hematoxylin-eosin, original magnification ×400 [A]; original magnification ×400 [B through E]).
Figure 4. A representative case of primary ocular adnexal marginal zone lymphoma that was negative for MYD88 L265P mutation. A, The biopsy specimen shows diffuse infiltration of small lymphocytes with a monotonous appearance. B, The abnormal lymphocytes are CD20⁺. Focal aggregates of plasmacytoid lymphocytes are seen, and these cells are positive for κ (C) and negative for λ (D), consistent with κ restriction. E, The allele-specific real-time polymerase chain reaction analysis was negative for MYD88 L265P mutation. Abbreviations: MUT, mutant; WT, wild type (hematoxylin-eosin, original magnification ×400 [A]; original magnification ×400 [B through D]).
primary neoplasm in the orbital region\textsuperscript{20,21} and is an important consideration in workup of orbital lesions. POAMZL is the most common type of lymphoma in orbit, representing more than half of all cases in most series.\textsuperscript{2,20,22} Our study included a total of 28 ocular adnexal MZLs (24 primary; 4 secondary). The clinical characteristics of our patients are similar to previous reports, including a slight female predominance, median age of onset at the seventh decade of life, presenting at low stage, and good response to radiation therapy.\textsuperscript{5,11,22,23}

POAMZL often arises in a chronic inflammatory background and is thought to be associated with infectious agents or autoimmune diseases. Among infectious causes, \textit{C. psittaci} has been most associated with POAMZL, but this association shows geographic variability.\textsuperscript{6} The variations have also been attributed to the different testing methodology.\textsuperscript{8} Recurring cytogenetic abnormalities are thought to contribute to the pathogenesis of MALT lymphomas, and these abnormalities also demonstrate anatomic site and geographic variability.\textsuperscript{9,24} In POAMZL, chromosomal translocations are rare but gains of chromosome 3 or 18q are most common.\textsuperscript{11} These 2 abnormalities represent some of the most common cytogenetic aberrations in all MZLs, as well as in other B-cell lymphomas.\textsuperscript{24–26} Unlike chromosomal translocations that explain the initiating event in pathogenesis of lymphomas, the aneuploidies in lymphomagenesis are less clear and may represent secondary genetic events. Lack of specific chromosomal abnormalities in POAMZL is suggestive of other underlying genetic alterations such as gene mutations.

Two recent studies on the mutational landscape of ocular MZL showed variable results.\textsuperscript{13,14} A South Korean study by Jung et al\textsuperscript{14} reported that \textit{TNFAIP3} was the most frequently mutated gene in ocular MZL followed by \textit{TBL1XR1} and \textit{CREBBP}, and MYD88 mutation was identified in only 4% of patients. However, a study from the United States by Cani et al\textsuperscript{13} reported a high frequency (25%) of \textit{MYD88} mutations in their cohort with no other recurrent mutations identified. Another recent study from Germany by Johansson et al\textsuperscript{15} found frequent mutations in both \textit{TNFAIP3} and \textit{MYD88} (19%) and frequent mutations in \textit{KMT2D} and \textit{NOTCH1/2}.

Several other earlier studies investigating the mutations in genes associated with the NF-\textit{kB} (nuclear factor \textit{kB}-light-chain-enhancer of activated B cells) pathway in ocular MZL demonstrated low frequency (0%–6%) of \textit{MYD88} mutations.\textsuperscript{12,27,28} The variability in the \textit{MYD88} mutations in ocular MZL could be attributed to the variable sensitivities of the tests used in different studies. However, another possible explanation could be geographic variability, similar to the infectious causes and chromosomal abnormalities.

The goal of our study was to assess the prevalence of \textit{MYD88} L265P mutation in our cohort of patients and investigate whether \textit{MYD88} mutation was correlated with \textit{Chlamydia} infection or associated with certain clinicopathologic features. We used a highly sensitive, allele-specific real-time PCR assay to detect \textit{MYD88} L265P mutation, and

| Clinical Features | MYD88– (n = 14) | MYD88+ (n = 8) |
|-------------------|----------------|---------------|
| Age, y            | 29–86 (median: 62) | 30–78 (median: 65) |
| Sex, M:F          | 6:8 | 3:5 |
| History of autoimmune diseases | 2/14 (14%) | 2/8 (25%) |
| Site of involvement | | |
| Orbit soft tissue | 8/14 (57%) | 3/8 (37.5%) |
| Lacrimal gland    | 3/14 (21.5%) | 1/8 (12.5%) |
| Conjunctiva       | 3/14 (21.5%) | 4/8 (50%) |
| Lesion size, mean, cm | 2.6 | 2.5 |
| Stage             | | |
| Stage 1E          | 10/11 (91%) | 7/8 (88%) |
| Stage 4           | 1/11 (9%) | 1/8 (12%) |
| Recurrence        | 2/10 (20%) | 3/8 (38%) |
| Treated with chemotherapy | 2/13 (15%) | 2/8 (25%) |
| Pathologic features | | |
| Immunophenotype   | | |
| CD5–/CD10\textsuperscript{+} | 11/14 (79%) | 8/8 (100%) |
| CD5\textsuperscript{+} | 1/14 (7%) | 0 |
| CD10\textsuperscript{+} | 2/14 (14%) | 0 |
| Plasmacytic differentiation | | |
| IgM               | 5/6 (87%) | 1/3 (33%) |
| IgG               | 1/6 (13%) | 0 |
| IgA               | 0 | 2/3* (66%) |
| Proliferation index Ki-67, % | <10 to 30 (mean: 5) | <10 to 30 (mean: 7) |
| Clonal IgH gene rearrangement | 12/13 (92%) | 6/8 (75%) |
| PCR positive for \textit{Chlamydia} species | 0/14 | 0/8 |

Abbreviations: Ig, immunoglobulin; PCR, polymerase chain reaction.

\textsuperscript{a} In these 2 cases most plasma cells were IgA\textsuperscript{+} with some scattered IgM\textsuperscript{+} plasma cells.
demonstrated a high prevalence (8 of 22; 36%) of MYD88 mutation in our cohort of POAMZL.

The activating mutation of MYD88 enhances tumor survival through IRAK1 (interleukin-1 receptor-associated kinase)/IRAK4, and NF-κB signaling pathways. The MYD88 mutations are frequent and occur in more than 95% of patients with lymphoplasmacytic lymphoma/Waldenstrom macroglobulinemia, but are uncommon in MZL and other low-grade B-cell lymphomas. Our study, similar to 2 other recent studies, demonstrated a high frequency of MYD88 mutations in POAMZL, which is in contrast to MZL occurring at other anatomic sites. Interestingly, there is a high frequency of MYD88 mutations among diffuse large B-cell lymphomas (DLBCLs) of the orbit. Similar findings were also reported in DLBCLs occurring in other immune-privileged sites. This intriguing observation suggests that mutations that activate MYD88 signaling may provide a growth advantage to lymphoma-initiating cells in these sites.

One question that we attempted to address in this study was whether the presence or absence of MYD88 mutation was related to the Chlamydia infection. We further investigated Chlamydia infection by using a highly sensitive Chlamydia-specific PCR assay on the DNA samples extracted from the lymphoma tissue, and the results showed that none of the 26 cases tested were positive. A consideration is that prior antibiotic treatment may affect detection of Chlamydia infection, but to our best knowledge none of our patients was treated with antibiotics before the lymphoma diagnosis. Large studies using standard testing method are needed in order to address whether MYD88 mutations are more prevalent in C. psittaci-negative POAMZLs, hence suggesting different underlying molecular mechanisms in lymphomagenesis in C. psittaci-negative versus C. psittaci-positive patients.

Our study also correlated the MYD88 mutational status to various histologic and clinical features. To further investigate the possible resemblance of MYD88-mutated POAMZL to lymphoplasmacytic lymphoma, we evaluated the plasmacytic differentiation in our cohort and showed that 47% of POAMZLs exhibited subtle plasmacytic differentiation, and the features were similar between the MYD88-mutated and nonmutated cases. Immunohistochemical stains for immunoglobulin heavy chains in cases with plasmacytic differentiation showed IgM predominance (6 of 9 cases). Interestingly, in the MYD88-mutated group, 2 of 3 cases with plasmacytic differentiation were positive for IgA with only scattered IgM plasma cells. The significance of the finding is not clear given the small sample size. Additionally, the proliferation index was also similar between the mutated and nonmutated groups. Another important question that we sought to address in this study was whether the presence of MYD88 mutation predicts the clinical behavior of the disease. In our series, however, the clinical features are similar in patients with and without MYD88 mutation, including lesion size, lymphoma stage, recurrence, and response to treatment.

A potential utility for the detection of MYD88 mutation is to provide additional evidence for the diagnosis of lymphoma. Distinction between reactive lymphoid proliferation versus low-grade B-cell lymphoma can be challenging in some cases. As orbital biopsy specimens are often small and may not be adequate for flow cytometric analysis, molecular analysis for immunoglobulin gene rearrangement is often performed to prove clonality. In our series, molecular analysis failed to detect clonal IGH gene rearrangement in 3 cases of POAMZL; however, 2 of these cases showed robust positivity for MYD88 L265P mutation. It should be noted that the sensitivity of the IGH assay alone may not be adequately high to detect all clonal cases. To increase the sensitivity we also performed the IGK clonality test in 2 of the 3 IGH-negative cases, which were also negative for IGK.

The MYD88 L265P mutation triggers lymphomagenesis through the activation of the NF-κB pathway by Bruton tyrosine kinase (BTK). BTK can be targeted by ibrutinib, a drug that is widely used in the treatment of B-cell lymphomas. The efficiency of the drug may depend on the presence of MYD88 mutations. Although POAMZL is generally an indolent disease and responds to radiation or rituximab therapy alone, some patients do have disease progression or recurrence. In our series, 4 of 22 patients required chemotherapy in the disease course. Given the frequent MYD88 mutations in POAMZL, targeted therapy, such as BTK inhibitors, could be an alternative therapeutic option for selected patients with recurrent or progressive diseases.

In summary, we demonstrate a high frequency of MYD88 L265P mutation in our cohort of POAMZL. MYD88 mutations were not associated with any distinguishing pathologic or clinical feature or Chlamydia infection. The MYD88 mutational analysis provides important information in diagnostic workup of patients with possible POAMZL. The frequent MYD88 mutation suggests an important role of this genetic aberration in the pathogenesis of POAMZL. The MYD88 mutation may also serve as a therapeutic target for patients with progressive disease.

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