High frequency of MYD88 mutations in vitreoretinal B-cell lymphoma: a valuable tool to improve diagnostic yield of vitreous aspirates

Irina Bonzheim¹, Sabrina Giese¹, Christoph Deuter², Daniela Süsskind², Manfred Zierhut², Maria Waizel³, Peter Szurman³, Birgit Federmann¹, Janine Schmidt¹, Leticia Quintanilla-Martinez¹, Sarah E. Coupland⁴, Karl Ulrich Bartz-Schmidt², Falko Fend¹

¹Institute of Pathology and Neuropathology, University Hospital Tübingen, Tübingen, Germany
²Centre of Ophthalmology, University Hospital Tübingen, Tübingen, Germany, ³Department of Ophthalmology, Knappschaftskrankenhaus Saar, Sulzbach, Germany, ⁴Department of Molecular & Clinical Cancer Medicine, Institute of Translational Medicine, University of Liverpool, Liverpool, UK

Short title: MYD88 mutations in vitreoretinal B-cell lymphoma

Corresponding author:
Falko Fend, M.D.
Institute of Pathology and Neuropathology
University Hospital Tübingen
Liebermeisterstr. 8
72076 Tubingen
Phone: 0049-7071-2982266
Fax: 0049-7071-29-2258
falko.fend@med.uni-tuebingen.de

Key points:
MYD88 mutation analysis significantly improves the detection rate of vitreoretinal B-cell lymphoma (VRL)
The high frequency of MYD88 mutations in primary VRL provides further evidence that VRL and primary CNS lymphoma represent the same entity.
Abstract

Vitreoretinal diffuse large B-cell lymphoma (VRL) is a rare disorder, occurring as primary ocular disease or as secondary involvement by primary central nervous system lymphoma (PCNSL). It is usually diagnosed by cytological, immunocytochemical and molecular examination of vitreous aspirates. However, distinguishing VRL from uveitis remains difficult, and clonality analysis may either be unsuccessful or misleading. DLBCL arising in immune-privileged sites, e.g. the CNS, shows a high frequency of MYD88 mutations. Therefore, we assessed retrospectively the frequency of MYD88 mutations in VRL and their diagnostic potential in 75 vitrectomy samples of 69 patients, and validated our results in a separate cohort (n=21). MYD88 mutations were identified in 20/29 (69%) clinically, histologically and molecularly confirmed VRL, including 6 cases of the test cohort initially diagnosed as reactive (3/6) or suspicious (3/6) for lymphoma. MYD88 mutations, especially L265P, are very frequent in VRL and their detection significantly improves the diagnostic yield of vitrectomy specimens.
Introduction

Vitreoretinal lymphoma (VRL), known previously as ‘primary intraocular lymphoma’, is a rare ocular malignancy with overall poor prognosis, manifesting most commonly in retina and vitreous body. Most cases are classified as diffuse large B-cell lymphomas (DLBCL) and represent either primary VRL or secondary ocular involvement by primary central nervous system lymphoma (PCNSL).\(^1\)\(^-\)\(^5\) VRL are highly aggressive tumors that need to be distinguished from the second most common intraocular lymphoma, low-grade choroidal extranodal marginal zone B-cell lymphomas, which are not associated with CNS disease but which do occasionally extend extracocularly.\(^2\)

VRL is usually diagnosed by cytological, immunocytochemical and molecular examination of vitreous body aspirates. However, diagnosis is often challenging due to the limited material sent for analysis. Clinically, VRL frequently resembles uveitis\(^6\) and cytological diagnosis is difficult because of low numbers of neoplastic cells, a commonly high admixture of non-neoplastic lymphocytes, and profound degenerative changes.\(^2\)

Detection of monoclonal rearrangements of the immunoglobulin heavy chain (\(\text{IGH}\)) genes provides support for a diagnosis of VRL,\(^7\) but clonality analysis may be misleading due to pseudoclonal/oligoclonal patterns resulting from low cellularity or benign B-cell clones in immunological disorders.\(^8\)\(^9\) Additional helpful diagnostic procedures include flow cytometry and the identification of an elevated IL-10/IL-6 ratio, but the small amount of vitreous fluid remains the limiting factor.\(^10\)\(^-\)\(^15\) PCNSL, which is considered as closely related to primary VRL, is usually of post-germinal center origin (activated B-cell type) as defined by gene expression and mutational profile.\(^1,\)\(^16\)

A feature of DLBCL arising in immune-privileged sites e.g. the CNS is a high frequency of \(\text{MYD88}\) mutations (mostly L265P, rarely codon 103 and 143), found in up to 75% of PCNSL.\(^17\)\(^-\)\(^19\) Given the lack of mutational data and the diagnostic difficulties in VRL, the aim of our study was to assess the frequency of \(\text{MYD88}\) mutations in VRL and to investigate their diagnostic potential in a large series of vitrectomy specimens.

Materials and methods

Patients

Seventy-five diagnostic vitrectomy samples of 69 patients with available archival DNA obtained consecutively between 01/2008 and 08/2014 and analysed at the Institute of Pathology, University Hospital Tübingen were included in this study. All samples had been examined by standard cytology and immunocytochemistry for B- and T-cell markers, as well as clonality analysis for the presence of immunoglobulin heavy chain (\(\text{IGH}\)) rearrangements, unless cytomorphology had revealed evidence for infectious endophthalmitis. As validation cohort, 21 archival DNA samples derived from
vitrectomy specimens previously diagnosed at the Department of Molecular & Clinical Cancer Medicine, University of Liverpool, Liverpool, UK were analysed in comparison. All available data including clinical charts, ophthalmic findings, cytology and clonality analyses, as well as follow-up information were jointly reviewed for all patients. After review, a joint consensus diagnosis was rendered and correlated with MYD88 mutation status.

**Cytology and Immunohistochemistry**

Native or HOPE-fixed vitrectomy specimens were rapidly transported to the Institute of Pathology and immediately processed. Cytospins were prepared by centrifugation at 1100 rpm for 4 min. Cytological evaluation was performed on May-Grünwald-Giemsa stains. Immunocytochemical stains for CD20 and CD3 (Dako, Glostrup, Denmark, 1:250) were performed on an automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA) after 30 min of parafomaldehyde fixation. Additional stains were added depending on initial findings and available material.

**DNA isolation and clonality analysis of IGH rearrangements**

DNA was extracted from the remaining vitrectomy material according to previously published standard procedures. After assessment of DNA quality by control PCR using the BIOMED-2 ladder, PCR for the detection of IGH gene rearrangements was performed in duplicate as previously described using BIOMED-2 FR2 and FR3 primers, sometimes complemented by additional analyses with BIOMED-2 IGH FR1 and kappa primers (Supplemental methods).

**MYD88 mutation analysis**

Detection of the MYD88<sup>L265P</sup> (c.728G>A) mutation was performed by melting curve analysis of amplification products with and without LNA for wild type suppression, using the Light Cycler 480 II System (Roche Applied Science, Mannheim, Germany), as recently described. All VRL cases with MYD88<sup>L265</sup> wild type and selected VRL with MYD88<sup>L265P</sup> were subjected to Sanger sequencing of MYD88 exons 3 and 4 (Supplemental methods). The study was approved by the local ethics committee (105/2013B02).

**Results and Discussion**

Details of VRL patients are listed in Table 1. The test cohort included 31 males and 38 females (median age 73 years, range 23-94) who had undergone diagnostic vitrectomy for a suspected
diagnosis of VRL. Based on cytological findings and IGH PCR results, a diagnosis of VRL was rendered in 14/69 patients (20%). Clonal IGH rearrangements were found in 12/14 patients. In the remaining two cases (4 and 11) with negative or inadequate clonality results, VRL diagnosis was based on increased numbers of large atypical B-cells. A diagnosis of inflammation or insufficient evidence for VRL was made in 55 patients (80%), including 5 cases with clonal IGH rearrangements, which showed low cellularity and/or predominance of small lymphocytes and were considered suspicious, but not diagnostic for VRL.

Melting curve analysis detected the MYD88<sup>L265P</sup> mutation in 13/69 patients (18.8%), including 7/14 patients (50%) with a diagnosis of VRL, and 6/55 patients (10.9%) diagnosed as negative (3 cases) or suspicious (3 cases). Sequencing confirmed the presence of MYD88<sup>L265P</sup> in all tested positive cases and additionally identified a p.P258L in a MYD88<sup>L265</sup> wild type VRL sample. Review of clinical data and follow-up confirmed a diagnosis of VRL in 21/69 (30%) patients, including 13/14 with an initial VRL diagnosis and all 6 MYD88<sup>L265P</sup> patients originally considered negative or suspicious for VRL. MYD88 analysis increased the sensitivity of vitreous fluid analysis from 62% to 90.5% and the negative predictive value from 85.5% to 96%, without change in specificity. Only for two patients with MYD88<sup>WT</sup> VRL and suspicious vitrectomy results and B-cell clonality, chorioretinal biopsy was required to confirm the diagnosis. A single MYD88<sup>WT</sup> patient, a 23-year-old HIV+ male, initially diagnosed as VRL based on numerous atypical cells and B-cell clonality, was reclassified as viral retinitis, demonstrating the difficult differential diagnosis between viral retinitis and VRL. 22

In the validation cohort, MYD88<sup>L265P</sup> was identified in 6/8 VRL cases (75%) and 0/13 reactive samples. In total, MYD88 mutations were identified in 20/29 (69%) VRL with the canonical L265P in 19/20 patients. There was no significant association of MYD88 mutation status with age, sex, CNS involvement or other clinicopathological parameters. However, two mutated cases with secondary involvement by systemic DLBCL had a history of MYD88<sup>L265P</sup> positive testicular lymphoma, another immune-privileged site with dominance of DLBCL of ABC type, common MYD88 mutations and increased risk for CNS relapse. 23-25 Case 20 represented intraocular involvement by marginal zone B-cell lymphoma, which is usually negative for MYD88 mutations.

The high frequency of MYD88 mutations in VRL observed in this large series mirrors published data on PCNSL and provides further evidence that primary VRL and PCNSL represent the same entity. Most importantly, mutational analysis, especially detection of p.L265P with a allele-specific PCR technique 21, significantly increases the sensitivity of vitreous body analysis (Table 1). Poor cytological preservation, low cellularity and spurious occurrence of clonal or pseudoclonal PCR result in an unsatisfactory high frequency of false negative and occasional false positives in vitreous body aspirates, often leading to significant delays in diagnosis and commencement of therapy. 8,9 The
presence of \textit{MYD88}^{L265P} in the majority of VRL allows a definitive lymphoma diagnosis even in poor quality samples, representing a significant diagnostic advance in this difficult and rare entity. Detection of \textit{MYD88}^{L265P} will enable the more timely commencement of treatment in mutation-positive cases, and might be used for monitoring treatment response, e.g. using small volume aqueous taps, thus potentially contributing to an improved outcome in VRL/PCNSL patients.

\textbf{Acknowledgements}

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\textbf{Authorship and Conflict of Interest Statements}

IB and FF designed the study, IB, SG and JS performed the experiments, CD, MZ, MW, PS, DS, BF, SC and KUB-S contributed samples, evaluated data and performed clinico-pathological correlations, IB, SC, LQ-M, KUB-S and FF interpreted the results and IB and FF wrote the manuscript. All authors critically reviewed the manuscript. The authors have no conflicts of interest to declare.
References


Table 1. Clinicopathological data of VRL patients from test cohort

<table>
<thead>
<tr>
<th>VRL with CNS involvement</th>
<th>Age</th>
<th>Sex</th>
<th>Initial Diagnosis</th>
<th>Comments</th>
<th>MYD88 status</th>
<th>B-cell clonality</th>
<th>Cellularity</th>
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<tr>
<td>1A</td>
<td>71</td>
<td>f</td>
<td>DLBCL</td>
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<td>p.265P</td>
<td>mono</td>
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<tr>
<td>3B</td>
<td></td>
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<td>WT</td>
<td>mono</td>
<td>mono</td>
<td>-</td>
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<td>WT</td>
<td>mono</td>
<td>mono</td>
<td>2</td>
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<tr>
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<td>82</td>
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<td>mono</td>
<td>2</td>
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<td></td>
<td>DLBCL</td>
<td>DLBCL diagnosed in CNS biopsy, history of breast cancer</td>
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<td>mono</td>
<td>2</td>
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<tr>
<td>6A</td>
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<td>f</td>
<td>reactive</td>
<td></td>
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<td>poly</td>
<td>2</td>
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<tr>
<td>6B</td>
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<tr>
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<td>History of Hodgkin lymphoma</td>
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<td>poly</td>
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<tr>
<td>8</td>
<td>45</td>
<td>f</td>
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<td></td>
<td>p.265P</td>
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<td>Primary VRL without CNS involvement</td>
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<td></td>
<td>p.265P</td>
<td>mono</td>
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<tr>
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<td>15</td>
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<td>DLBCL</td>
<td>DLBCL diagnosed in choroid biopsy</td>
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<td>DLBCL</td>
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<td>18</td>
<td>86</td>
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<td>19</td>
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<td>m</td>
<td>DLBCL</td>
<td>Testicular DLBCL 10 years previously</td>
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<td>mono</td>
<td>1</td>
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<td>20</td>
<td>73</td>
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<td>MZL of the conjunctiva</td>
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<td>21</td>
<td>53</td>
<td>m</td>
<td>Suspected for lymphoma</td>
<td>Testicular DLBCL 2 years previously</td>
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<td>mono</td>
<td>1</td>
</tr>
</tbody>
</table>

VRL: vitreoretinal lymphoma; CNS central nervous system
A, B denote vitreous samples obtained at different time points
*: this sample was WT for the L265P mutation and also showed WT sequence for MYD88 exons 3 and 4, probably due to low tumor cell content. * The primary testicular lymphomas of both patients showed presence of MYD88 L265P mutation.
f, female; m, male; mono, monoclonal; poly, polyclonal; oligo, oligoclonal, NE not evaluable; DLBCL; diffuse large B-cell lymphoma; WT, wild type; MZL, extranodal marginal zone B-cell lymphoma
Cellularity: 1, low; 2, medium; 3, high cellularity
**Figure Legends**

**Figure 1:** Clinical, cytological immunohistochemical and molecular findings in VRL.

**A**) Representative fundus photographs of three VRL patients: VRL with retinal and choroidal infiltration (left), with massive infiltration at rear pole (middle) and with vitreous infiltration only (right).

**B**) Upper left: PVRL, 49-year-old female, **MYD88**<sup>L265F</sup>. Highly cellular vitreous body aspirate with numerous atypical lymphocytes (May-Grünwald-Giemsa staining, left). Virtually all cells stain for CD20 (immunoperoxidase, right). Lower left: PVRL, 63-year-old female, **MYD88**<sup>L265P</sup>. The aspirate shows rare atypical cells with basophilic cytoplasm (arrowheads, May-Grünwald-Giemsa). Clonality analysis was non-contributory, and the case was considered as non-diagnostic for PVRL. Lower right: PVRL, 70-year-old female. **MYD88**<sup>L265P</sup>, monoclonal **IGH** rearrangement. CD20 stain. All images taken at 400x original magnification.

**C**) Representative example of **MYD88** codon 265 LNA clamped allele-specific PCR and melting point analysis. One case with final diagnosis uveitis shows the wild type curve with the melting peak at 62.7°C, whereas case 6B, which initially was considered reactive turned out to be **MYD88**<sup>L265P</sup> mutated, melting at 53.1°C.
Melting temperature

WT: 53.1 °C
MYD88<sup>1265P</sup>. 62.7 °C

Legend:
- Uveitis - LNA oligo
- Uveitis + LNA oligo
- Positive control MYD88<sup>1265P</sup> - LNA oligo
- Positive control MYD88<sup>1265P</sup> + LNA oligo
- Case 6B - LNA oligo
- Case 6B + LNA oligo
- Negative control
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