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ORIGINAL ARTICLE

Investigating the frequency of somatic *MYD88* L265P mutation in primary ocular adnexal B cell lymphoma

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Abstract

Background Ocular adnexal B cell lymphoma is the most common orbital malignancy in adults. Large chromosomal translocations and alterations in cell-signaling pathways were frequently reported in lymphomas. Among the altered pathways, perturbations of NFκB signaling play a significant role in lymphomagenesis. Specifically, the *MYD88* L265P mutation, an activator of NFκB signaling, is extensively studied in intraocular lymphoma but not at other sites. Therefore, this study aims to screen the *MYD88* L265P mutation in Ocular adnexal B cell lymphoma tumors and assess its clinical significance.

Methods and results Our study of twenty Ocular adnexal B cell lymphoma tumor samples by Allele-Specific Polymerase Chain Reaction identified two samples positive for the *MYD88* L265P mutation. Subsequent Sanger sequencing confirmed the presence of the heterozygous mutation in those two samples tested positive in Allele-Specific Polymerase Chain Reaction. A comprehensive review of *MYD88* L265P mutation in Ocular adnexal B cell lymphoma revealed variable frequencies, ranging from 0 to 36%. The clinical, pathological, and prognostic features showed no differences between patients with and without the *MYD88* L265P mutation.

Conclusion The present study indicates that the *MYD88* L265P mutation is relatively infrequent in our cohort, underscoring the need for further validation in additional cohorts.

Keywords Ocular adnexal lymphoma · *MYD88* L265P · Sanger sequencing · Allele Specific PCR · B cell Lymphoma · Orbital Malignancy

Introduction

Ocular Adnexal Lymphoma (OAL) is the common primary orbital malignancy in adults, comprising about 1% of all non-Hodgkin's Lymphoma (NHL) and 8% of extranodal lymphomas [1, 2]. Various studies emphasize the significant involvement of NFκB pathway activation in tumorigenesis. A prominent activator within this pathway is the Myeloid differentiation primary response gene 88 (*MYD88*), situated at chromosome 3p22. *MYD88* functions as an adapter protein, serving as a pivotal molecule in the activation of NF-κB signaling through both Bruton's tyrosine kinase (BTK) and Interleukin-1 Receptor Associated Kinases pathways [3]. Specifically, a hotspot gain of function mutation at amino acid position 265 (affecting Toll/interleukin-1receptor domain (TIR)), where a leucine to proline substitution (L265P) was shown to enhance NF-κB pathway activation, JAK-STAT signaling significantly and promotes lymphoma cell survival [4, 5]. Studies have highlighted the diagnostic and prognostic significance of *MYD88* in various B-cell malignancies. Particularly the *MYD88* L265P mutation is frequently reported in Waldenstrom macroglobulinemia /lymphoplasmacytic lymphoma patients. This mutation is also associated with other conditions, including primary central nervous system lymphoma,

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vitreoretinal lymphoma, chronic lymphocytic leukemia, splenic marginal zone lymphoma and immunoglobulin M (IgM) monoclonal gammopathy [6, 7]. Notably, the high prevalence of *MYD88* L265P mutation in primary cutaneous diffuse large cell lymphoma (DLBCL) shows its association with poor prognosis and shorter survival [8, 9]. Knowing its role in lymphomagenesis, Weber et al. suggested the oncogenic *MYD88* L265P mutation as a potential therapeutic target in B-cell NHLs [10]. Patients with *MYD88* L265P mutation showed a favourable clinical course with a higher response rate to BTK inhibitors [11]. However, the clinical impact and prognostic value of *MYD88* mutations vary significantly among different lymphoproliferative disorders and anatomical sites [3]. Thus, this study aims to assess the prevalence of *MYD88* L265P mutations in OABL and its clinical significance.

Materials and methods

Samples

Tumor samples were collected for analysis after obtaining informed consent from the patients. This work was done with approval of the Institutional Ethics Committee (IRB-2018014BAS), following the tenets of the Declaration of Helsinki. Clinical records were reviewed to retrieve the following data: age and sex of the patient, symptoms, laterality and extent of ocular adnexal involvement. Tumor sections were stained with Hematoxylin & Eosin and subjected to immunohistochemical analysis using CD45, CD20, and CD3 markers. Fresh frozen tumors diagnosed with OABL were included for genetic analysis.

Allele specific-PCR for *MYD88* L265P investigation

Genomic DNA was extracted from OABL tumor samples using Qiagen DNA mini-Kit (Qiagen, USA), following the manufacturer's protocols. The purity and yield of the extracted DNA was determined using a NanoDrop™ Lite Plus spectrophotometer (Thermo Scientific, USA). Allele specific PCR (AS-PCR) was carried out with primers specified in Table 1. The 10 ul reaction was set up with, 1X PCR buffer, 50 µM of dNTPs, 0.5 U Taq DNA polymerase, 4 µM of each primer and 20 ng of DNA. Thermal cycling conditions comprised an initial denaturation of 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 15 s at 58 °C, and 60 s at 72 °C followed by a final extension of 7 min at 72 °C. Appropriate positive and negative controls were included in each run. The amplified products were checked in 1.5% agarose gel using ethidium bromide under UV light. The sensitivity of the AS-PCR assay was assessed by serial dilution of DNA isolated from the mutation positive tumor which is heterozygous for *MYD88* L265P, with DNA from the SUDHL-6 cell line harboring wild-type *MYD88*.

Confirmatory sanger sequencing

PCR was conducted with all tumor samples to amplify the region flanking the *MYD88* L265P mutation following the reaction composition described above with primers detailed in Table 1. Thermal cycles were initial denaturation of 5 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 58 °C, and 60 s at 72 °C followed by a final extension of 7 min at 72 °C. The resulting amplicons were purified using ExoSAP-IT™. Cycle sequencing was performed with the amplified PCR products using Big Dye Terminator v3.1 cycle sequencing kit using the reverse PCR primer. Sequencing of the purified products was carried out using the Applied Biosystems 3500 Genetic Analyzer.

Results

Twenty histologically confirmed OABL tumor samples were analyzed, comprising 12 males (60%) and 8 females (40%). The mean age at diagnosis was 61.10 ± 5.83 years, with ages ranging from 30 to 85 years. Among the 20 OABL patients, 95% (n = 19) presented with unilateral disease, while 5% (n = 1) exhibited bilateral involvement. The predominant

Table 1 Primers used in the study

S. No	Method	Primer sequence	Product Length, bp
1	Allele Specific -PCR	MYD88-WT 5′-GTGCCCATCAGAAGCGCC T-3′ MYD88-MT 5′-GTGCCCATCAGAAGCGCC C-3′ MYD88-R 5′-GACGTGTCTGTGAAGTTG GCATCTC-3′ [30]	296
2	Sanger Sequencing	5′-GTTGAAGACTGGGCTTGTCC-3′ 5′-AGGAGGCAGGGCAGAAGTA-3′	292

WT wild type, *MT* mutant, *R* Common reverse

symptoms at presentation were proptosis and swelling. The predominant site of tumor was orbit (including lacrimal gland) (n = 16). Superior quadrant of the orbit was primarily involved (n = 9), followed by the inferior quadrant (n = 4) and both quadrants of the orbit (n = 3). Apart from the orbit, involvement of eyelid (n = 2) and conjunctiva (n = 2) was also observed. In all cases, the tumor lesions were localized, and all instances were identified as primary OABL.

Two out of twenty OABL (10%) were positive for *MYD88* L265P mutations in AS-PCR, whereas the remaining 18 (90%) cases were negative and showed wild-type *MYD88*. Subsequent Sanger sequencing of mutation flanking regions confirmed the presence of the heterozygous L265P mutation, characterized by a leucine-to-proline substitution at position 265, where T (wild type) was replaced by C (mutant) (Fig. 1). AS-PCR and Sanger sequencing showed a concordant *MYD88* profile across all 20 tumor samples (***ESM_1***). AS-PCR enables the reproducible detection of mutant DNA at concentrations as low as 1% (Fig. 2). Notably both the samples harbouring *MYD88* L265P mutation had tumor lesions located in the superior orbital quadrant. Other Clinical and histological features of OABL did not differ among the patients with and without *MYD88* L265P mutation. Furthermore, no correlation was observed between the presence of the mutation and patient prognosis or therapeutic response.

Discussion

Frequency of *MYD88 L265P* in ocular adnexal B cell lymphoma

The present study aimed to investigate the presence of *MYD88* L265P in OABL tumors and it is the first report from India. Intriguingly, our AS-PCR and Sanger sequencing analyses found positivity only in two (10%) samples. The mutational patterns of *MYD88* L265P in OABL showed their prevalence, ranging from 0 to 36% (Table 2). Our finding is concordant with Vela et al. and Zhao et al. who reported a comparable frequency of the *MYD88* L265P mutation at 9% using targeted high-throughput sequencing and 10% via whole exome sequencing, respectively. [12, 13].

However, few studies have reported a higher prevalence of *MYD88* L265P mutation in OABL. Behdad et al. employed allele-specific real-time PCR for *MYD88* L265P

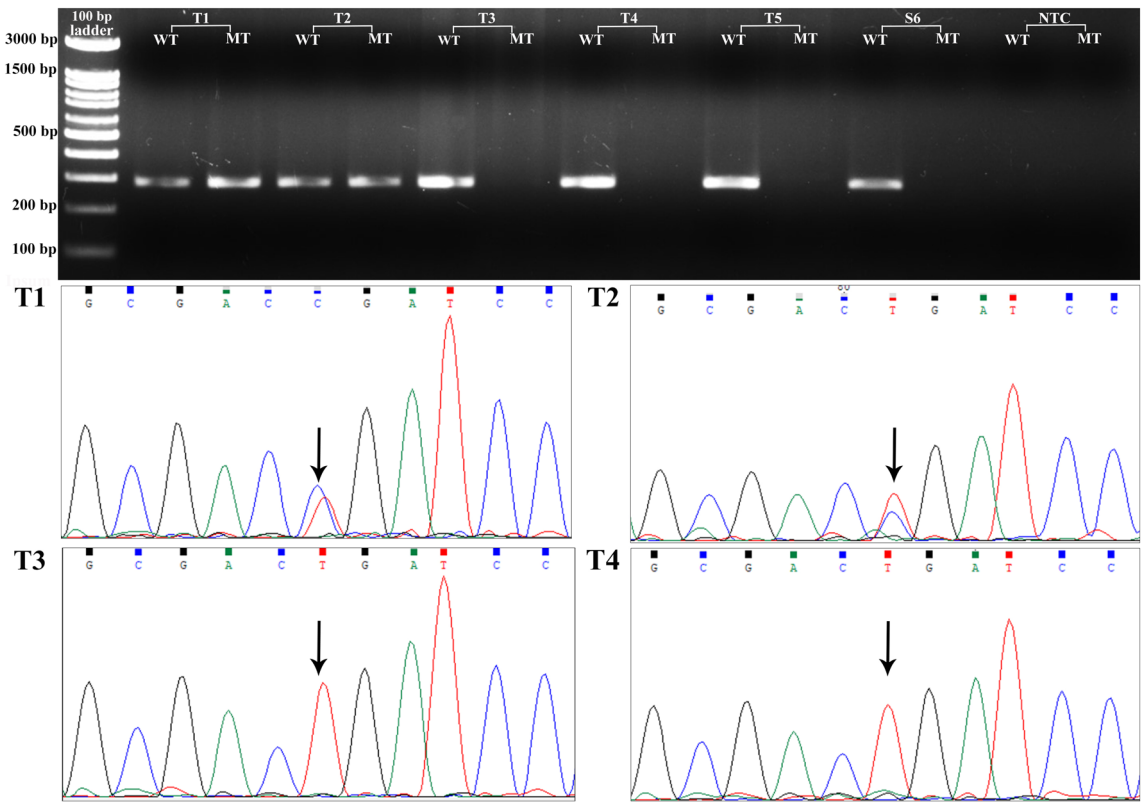

Fig. 1 An amplicon of 296 bp was observed in tumor samples T1 and T2, indicating the presence of the *MYD88* L265P mutation. Samples T3, T4, T5, and S6 showed amplification only in wild-type. Sanger sequencing confirms a heterozygous T > C transition in T1 and T2 samples. No mutation was observed in samples T3, and T4, which showed the wild-type sequence. (WT-Wild Type, MT-Mutant, S6-SUDHL-6 cell line, NTC- Non template control)

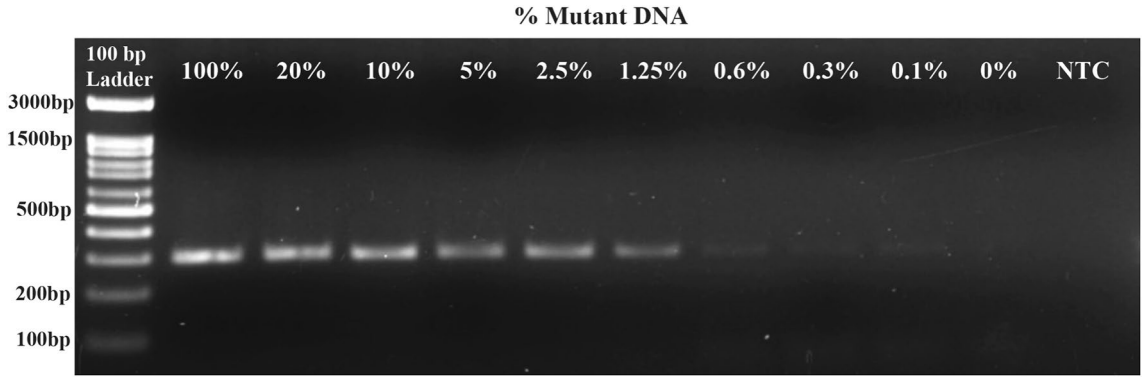

Fig. 2 Serial dilution of DNA from T1, containing a heterozygous *MYD88* L265P mutation, into DNA derived from the SUDHL-6 cell line, which harbors the wild-type *MYD88* allele. The mutant *MYD88* L265P allele was detected at dilutions as low as 1%

Table 2 Summary of various studies on *MYD88* L265P mutation in OABL

Author	Country, Year (No of cases)	Method	*Total MYD88 Mutation*	*MYD88 L265P*
Cani et al.,	USA, 2016 (n = 36)	Targeted Sequencing	10/36 (28%)	6/27 (22%)
Cascione et al.,	Switzerland & USA, 2019 (n = 20)	Targeted Sequencing	4/20 (20%)	2/20 (10%)
Vela et al.,	Switzerland, 2020 (n = 34)	Targeted HTS	3/34 (9%)	3/34 (9%)
Johansson et al.,	Germany, 2017 (n = 63)	Amplicon Sequencing	12/63(19%)	8/63 (13%)
Johansson et al.,	Germany, 2020 (n = 13)	WES/WGS	0	0
Jung et al.,	South Korea, 2017 (n = 48)	WES (n = 10)/ Targeted Sequencing (n = 38)	2/38 (4%)	0
Zhao et al.,	China 2021, (n = 21)	WES	2/21 (10%)	2/21 (10%)
Liu et al.,	Japan, 2012 (n = 24)	Sanger Sequencing	0	0
Kirkegaard et al.,	Denmark, 2021 (n = 34)	AS-PCR and Sanger sequencing	10/34 (29%)	8/34 (23%)
Behdad et al.,	Chicago, 2018 (n = 22)	AS-RT-PCR Assay	8/22 (36%)	8/22 (36%)
Magistri et al.,	Florida, 2021/2023 (n = 69)	WES	8/69 (12%)	1/69 (1.4%)
Zhu et al.,	USA, 2013 (n = 45)	Sanger Sequencing	3/45 (6.7%)	3/45 (6.7%)
Moody et al.,	China, 2018 (n = 115) (Multi-centric)	Targeted Sequencing	8/115 (7%)	4/115 (3.5%)
* Yan et al., Moody et al.,	China, 2012 (n = 105) China, 2017 (n = 101) (Multi-centric)	Targeted/Sanger Sequencing	6/105 (5.7%)	3/105 (3%)
Present Study	India, 2024 (n = 20)	AS-PCR and Sanger sequencing	2/20 (10%)	2/20 (10%)

AS-PCR Allele Specific-PCR, *WES* Whole exome sequencing, *AS-RT-PCR* Allele Specific-Real time PCR, *HTS* High throughput sequencing, *same samples subjected for the study

mutation and detected positivity in 8 out of 22 primary ocular adnexal marginal zone lymphoma (36%) [14]. Similarly, Kirkegaard et al. identified *MYD88* L265P mutations in 8 out of 34 lymphomas (29%) using AS-PCR and Sanger sequencing. The study also showed poorer progression-free survival in mutants compared to wild-type cases [15]. However, the present study did not observe any correlation between mutants and patient survival. *MYD88* TIR domain mutations were frequently reported in DLBCL (71%) and marginal zone lymphoma (MZL) (25%) subtypes of orbital and ocular adnexal lymphomas. Specifically, L265P mutations were identified in 43% of DLBCL and 15% of MZL

in OABL [16]. In our study cohort, the subtyping was not performed in these tumor samples due to inadequacy of sample.

Certain studies have also enunciated the *MYD88* mutation in Mucosa associated lymphoid tissue lymphoma (MALT) across different anatomical locations. A targeted next-generation study of MALT from different sites revealed *MYD88* mutation present only in 20% of Orbital/adnexal MALT and 7.9% of salivary gland tumors comparing other MALT sites. Notably, the L265P mutation accounts for approximately 10% of all MYD88 mutations identified. [17]. Similarly, a recent meta-analysis of 1663 marginal zone B-cell lymphomas from various origins revealed that *MYD88* mutations were specifically identified in ocular marginal zone lymphoma, constituting 10% of cases [18]. In an intriguing observation, among 53 cases of MALT lymphoma, only three instances exhibited *MYD88* mutation. Notably, two cases with L265P substitution manifested lymphoma involving the orbital adnexa [19]. Another comparative study by Moody et al., on MALT from different anatomical sites, highlighted that the *MYD88* mutation is notably distinct in ocular adnexa (7%) and salivary glands [20]. In the present study, *MYD88* L265P mutation was identified in patients with tumors located in the superior quadrant of the orbit.

Several groups utilized different approaches to investigate *MYD88* mutation in OABL. A large cohort study involving 105 cases of ocular adnexal MALT lymphoma, PCR and Sanger revealed *MYD88* mutation in 3 out of 105 (3%) cases [21]. Subsequently, the same group employed targeted sequencing on ocular adnexal cases, reaffirming the presence of the *MYD88* mutation in 3% of cases [22]. Another independent investigation focused on *MYD88* in *Chlamydia* negative MALT cases, revealed that 3 out of 45 (6.7%) harbored L265P mutations as detected by Sanger sequencing [23]. Magistri et al. conducted whole exome sequencing and found that 12% of ocular adnexal MALT exhibited *MYD88* mutations, with only one patient displaying L265P variant [24].

Johansson et al. conducted amplicon sequencing on genes recurrently mutated in the NFκB pathway across 63 formalin fixed tumor samples and revealed *MYD88* mutation in 19% of the samples, of which 13% are L265P variants [25]. Ironically, the same group reported no *MYD88* mutation in 13 fresh ocular adnexal MZL by whole genome and exome sequencing [26]. Similarly, Liu et al. showed no genetic alteration in *MYD88* L265P by Sanger sequencing in OABL [27]. Similarly, a comprehensive *MYD88* (L265P) somatic mutational profiling in 21 MALT samples including orbit showed no mutation [28]. Moreover, a whole genome study displayed only 4% of cases exhibited *MYD88* mutations, albeit not the L265P variant [29]. Despite the varied methodologies employed, the majority of the study outcomes align with our findings.

Conclusion

Molecular analyses showed that the *MYD88* L265P mutation was not highly frequent in our cohort. AS-PCR stands out as an efficient method for directly detecting point mutations by analysing the PCR product which detects mutants at relatively low tumor loads. A systemic review reveals that the *MYD88* L265P mutation ranges from 0 to 36%. We acknowledge that the present study is limited by its design, lack of lymphoma subtyping and relatively small sample size, given the rarity of the disease. A larger, multicenter cohort is required to validate this analysis.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-024-09903-w.

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Data availability The data supporting this study's findings are available on request from the corresponding author.

Declarations

Competing interest We declared that none of the authors has financial and personal competing interests.

Ethical approval This work was done with approval from the Institutional Ethics Committee (IRB2018014BAS) and followed the tenets of the Declaration of Helsinki.

Consent to participate All tumor samples were collected with the informed consent from patients.

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