Diagnostic Next-generation Sequencing Frequently Fails to Detect MYD88[L265P] in Waldenström Macroglobulinemia

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Mutations in MYD88 (MYD88[MUT]) are present in approximately 93%–97% of patients with Waldenström macroglobulinemia (WM), nearly all of which correspond to the c.978T>C transversion resulting in a p.Leu265Pro (L265P) substitution at the protein level.[1,2] MYD88[MUT] helps support the diagnosis of WM and differentiate from other IgM-secreting B-cell malignancies, such as marginal zone lymphoma and IgM myeloma, where it is absent or rarely expressed.[2] The presence of MYD88[MUT] is also associated with a better prognosis, lower risk of histological transformation, and predicts response to the BTK inhibitor ibrutinib in WM patients.[3–7] These findings prompted the World Health Organization, National Comprehensive Cancer Network, and WM Workshop guidelines to recommend MYD88[MUT] testing for all suspected WM cases.[8]

Despite the importance of MYD88[MUT], a uniform means for identifying them is currently lacking. The original studies that established the incidence of MYD88[L265P] used an allele-specific polymerase chain reaction (AS-PCR) with CD19-selected bone marrow (BM) aspirates to maximize sensitivity.[2] However, presorting B-cells before AS-PCR is not feasible for most clinical laboratories; hence, unselected BM aspirates are routinely used for the clinical detection of MYD88[L265P].[9] Additional testing with Sanger sequencing is recommended in patients with wild-type (WT) MYD88 by AS-PCR to evaluate for rare non-L265P MYD88[MUT].[9] Targeted next-generation sequencing (NGS) has emerged as an alternative to AS-PCR to identify MYD88[L265P], but the sensitivity of NGS for MYD88[L265P] detection in WM

patients is unknown. This prompted us to compare the results for MYD88[L265P] detection by NGS against AS-PCR in 414 consecutive WM patients who had both assays performed synchronously.

We used CD19-selected BM aspirate to detect MYD88[L265P] by quantitative AS-PCR, followed by Sanger sequencing to evaluate for non-L265P MYD88[MUT] in patients with MYD88[WT] by AS-PCR as previously described.[2,6,7] Qualitative AS-PCR (lower limit of detection ~1%) for MYD88[L265P] was also performed on unselected BM aspirate in the Molecular Diagnostics Laboratory, Brigham & Women's Hospital (Boston, MA). The findings for MYD88[L265P] were compared against a clinically validated and targeted NGS assay (Rapid Heme Panel) using unselected BM aspirate from the same patients.[10] The NGS assay has an average coverage of 1500X with <5% of the amplicons with 50X coverage, and reproducibly could detect single nucleotide variants at allele frequencies of ≥5%.[10] The median coverage of the MYD88 amplicon was 1521X (range 305–3707X). Calculations were performed with R (R Foundation for Statistical Computing, Vienna, Austria). The Dana-Farber/Harvard Cancer Center IRB approved this study, and all patients provided written consent for the use of their samples.

Clinical characteristics at the time of MYD88[MUT] testing are shown in Supplemental Digital Table 1, http://links.lww.com/HS/A180. Using AS-PCR with CD19-selected samples, 391 patients (94.4%) had MYD88[L265P] identified. Sanger sequencing of the 23 patients with MYD88[WT] by AS-PCR revealed that one patient had a dinucleotide substitution that resulted in MYD88[L265P], while another had a MYD88[S243N] mutation. Overall, the prevalence of MYD88[MUT] in this cohort was 393/414 (95%), and did not differ between treatment-naïve and previously treated WM patients (94% versus 96%, respectively; $P = 0.37$).

We compared the results for MYD88[L265P] by NGS with unselected BM aspirate against AS-PCR with CD19-selected BM. Among the 391 patients with MYD88[L265P] identified by AS-PCR, only 259 patients (66%) had MYD88[L265P] identified by the NGS method; the median variant allele fraction for MYD88[L265P] was 5.95% (range 0.5%–86.5%). No patient had MYD88[L265P] identified by NGS that AS-PCR did not also identify. The test performance statistics comparing the 2 methods are summarized in Table 1. We then evaluated factors that impacted the sensitivity of MYD88[L265P] detection by NGS. Modeling the false negative results by age, sex, hemoglobin level, platelet count, serum IgM

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Table 1.

Test Performance of Targeted Next-generation Sequencing and Allele-specific PCR for MYD88^{L265P} in WM Patients

	AS-PCR		NGS
	CD19-selected BM	Unselected BM	Unselected BM
True positive, no	391	377	259
True negative, no	24	24	24
False positive, no	0	0	0
False negative, no	0	14	132
Concordance (κ), %	Ref.	97 (0.76)	68 (0.19)
Sensitivity (95% CI), %	Ref.	96 (94–98)	66 (61–71)
Specificity (95% CI), %	Ref.	100 (83–100)	100 (83–100)
PPV (95% CI), %	Ref.	100 (99–100)	100 (98–100)
NPV (95% CI), %	Ref.	63 (46–78)	15 (10–22)

Results from both AS-PCR and targeted NGS with unselected bone marrow aspirate samples were compared against AS-PCR with CD19-selected bone marrow aspirate samples for MYD88^{L265P}. AS-PCR = allele-specific polymerase chain reaction; BM = bone marrow; CI = confidence interval; NGS = next-generation sequencing; NPV = negative predictive value; PPV = positive predictive value.

Figure 1. Impact of bone marrow involvement on false negative rates for MYD88L265P with targeted next-generation sequencing. AS-PCR with CD19-selected bone marrow aspirate samples for MYD88^{L265P} was used as the reference assay for this analysis. AS-PCR = allele-specific polymerase chain reaction.

level, BM involvement, prior treatment status, and MYD88 amplicon coverage revealed only BM involvement represented a significant predictor ($P < 0.0001$). Accordingly, the false negative rate for MYD88^{L265P} by NGS increased significantly with decreasing BM involvement (Figure 1).

To examine the relative importance of AS-PCR versus B-cell selection on NGS sensitivity for MYD88^{L265P}, we compared the AS-PCR results with both CD19-selected and unselected BM for all patients (Table 1). Among the 391 patients with MYD88^{L265P} identified by AS-PCR with CD19-selected BM, 377 (96%) had MYD88^{L265P} identified by AS-PCR when using unselected BM. This corresponds to a 45% increase in sensitivity with AS-PCR over NGS with unselected BM aspirate (96% versus 66%, respectively). These findings demonstrate that sequencing by NGS, rather than B-cell selection, accounts for most of the false negative results generated by NGS versus AS-PCR.

Overall, the findings demonstrate the use of unselected BM aspirate and NGS fails to detect MYD88^{L265P} in one-third of WM patients. We also show the false negative rate with NGS correlates with BM involvement, although false negative results still occurred with all levels of BM involvement. Sampling bias due to patchy BM involvement or hemodilution (ie, the admixture of peripheral blood during BM aspiration) of tumor samples below the NGS threshold may account for this observation.[11] Our findings should herald efforts to optimize MYD88^{MUT} testing in WM patients. Although tumor enrichment with B-cell selection can improve MYD88^{L265P} detection,[12] it is not feasible for most clinical laboratories. We show the use of AS-PCR for MYD88^{L265P} with unselected BM aspirate which produces a 45% increase in sensitivity over NGS, and may be sensitive enough to overcome the adverse impact of low BM involvement and/or hemodilution. Likewise, increased depth of NGS coverage could be considered to improve sensitivity, as NGS is ideally suited to simultaneously evaluate the TIR exon coding region of MYD88 for both L265P and non-L265P mutations. Evaluating alternative sensitive approaches adoptable to clinical practice to identify MYD88^{MUT} in WM patients also warrants consideration. The use of digital droplet PCR has shown high concordance with AS-PCR for the detection of MYD88^{L265P}.[13,14] Moreover, the detection of MYD88^{L265P} in cell-free DNA is feasible in WM patients and may represent a novel tissue for molecular testing.[15–18] These efforts to optimize molecular testing are collectively important to ensure the accurate identification of MYD88^{MUT}, which impact diagnosis, prognosis, and treatment selection in WM patients.[9]
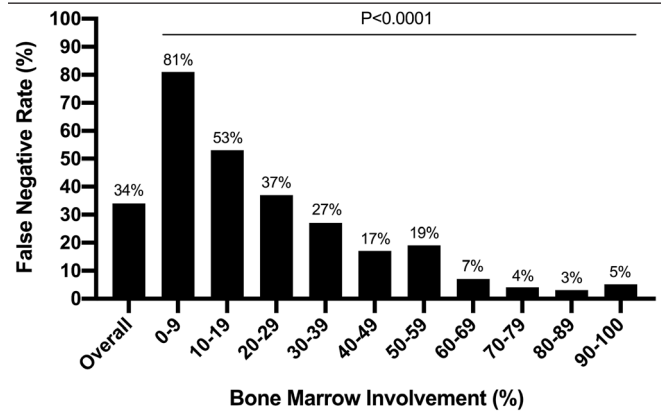
This study also provides important context for interpreting the iNNOVATE trial that evaluated ibrutinib plus rituximab in WM patients. Compared to ibrutinib monotherapy in the pivotal trial,[6,7] ibrutinib plus rituximab induced a higher major response rate (73% versus 0%) and median PFS (>4 versus 0.4 yrs) in MYD88^{WT} patients.[19,20] However, the iNNOVATE trial used an NGS assay (mean coverage >500X) with either unselected BM aspirate or formalin-fixed paraffin-embedded specimens to identify MYD88^{MUT},[19] whereas the pivotal ibrutinib trial utilized AS-PCR and Sanger sequencing with CD19-selected BM aspirate.[7,21] Given the high false negative rate with NGS, our findings suggest the iNNOVATE trial results may be confounded by the inclusion of patients with MYD88^{MUT} in the MYD88^{WT} cohort. Indeed, the prevalence of MYD88^{WT} was at least two-fold higher in iNNOVATE than the established prevalence in WM (16% versus 3%–7%), suggesting the presence of misclassified MYD88^{WT} patients.[1,2,9] Preclinical studies have also demonstrated MYD88^{WT} tumors have intrinsic ibrutinib resistance due to NF-kB activating mutations downstream of BTK.[22–24] It is therefore unlikely the addition of rituximab accounts for the high activity of ibrutinib reported with combination therapy, particularly since rituximab monotherapy only induced a major response rate of 22% and median PFS of 2 years in MYD88^{WT} patients.[19,20] Nevertheless, our findings highlight the importance of using highly sensitive approaches when investigating MYD88^{MUT} as a treatment biomarker. Such an approach is also critical given MYD88^{WT} WM patients typically have low BM tumor burden,[3] which adversely impacts the sensitivity of molecular testing. Our findings may also be important for molecular diagnostic testing in other B-cell malignancies, such as ABC DLBCL (30%–40%), primary Central Nervous System lymphoma (60%–80%), marginal zone lymphoma (5%–10%), and chronic lymphocytic leukemia (5%–10%), wherein MYD88^{MUT} is frequently observed.

In summary, our data show that targeted NGS frequently yields false negative results for MYD88^{L265P} in WM patients. Given the importance of MYD88^{MUT} status in the management of WM, our findings highlight the importance for standardized testing methods for MYD88^{MUT} in WM patients, as well as other diseases impacted by this mutation.

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