Cell-free DNA analysis for detection of *MYD88*^{L265P} and *CXCR4*^{S338X} mutations in Waldenström macroglobulinemia.

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Short Title: cfDNA for MYD88 and CXCR4 in WM
Abstract: 193
Text Word Count: 1219
Tables: 3
Figures: 0
References: 15
Keywords: Waldenström Macroglobulinemia, MYD88, CXCR4, cfDNA

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ajh.26184

Version: First Look Version 03/31/2021

Funding Statement: Supported by the Orszag Family Fund for WM Research, the D'Amato Family Fund for Genomic Discovery, the International Waldenström's Macroglobulinemia Foundation, the Leukemia and Lymphoma Society (Grant: R6507-18), the Kerry Robertson Fund for WM. SPT, ZRH and GY are supported by an NIH SPORE in Multiple Myeloma (Grant: 2P50CA100707-16A1).

Data Availability: Not applicable.

Abstract

MYD88^{L265P} and *CXCR4*^{S338X} variants are highly prevalent and impact disease presentation, prognosis, and/or treatment outcome in Waldenstrom's Macroglobulinemia (WM). The use of bone marrow (BM) aspirate represents the current "gold standard" for molecular testing in WM. Although these variants are identified in peripheral blood (PB), the diagnostic yield in PB is inferior to BM, particularly for previously treated patients. Tumor enrichment can significantly improve testing sensitivity, but is not feasible in most clinical laboratories. Recent studies have demonstrated the feasibility of identifying *MYD88* and *CXCR4* mutations using cell-free DNA (cfDNA) from the plasma of WM patients. We therefore prospectively collected matched BM and PB samples from 28 consecutive WM patients. Overall, five different tissue fractions were isolated for analysis: CD19-selected BM, unselected BM, CD19-selected PB, unselected PB, and cfDNA. Quantitative allele-specific polymerase chain reaction assays for *MYD88*^{L265P} and *CXCR4*^{S338X} mutations were performed for each tissue fraction, and findings benchmarked against CD19-selected BM. Both *MYD88*^{L265P} and *CXCR4*^{S338X} were identified with high sensitivity and specificity in cfDNA derived from the plasma of WM patients, including previously treated patients. The use of cfDNA represents a non-invasive, convenient, and potentially cost-effective method for genotyping patients with WM.

To the Editor:

Molecular testing for *MYD88* and *CXCR4* mutations is increasingly used in patients with Waldenström macroglobulinemia (WM). *MYD88*^{L265P} is the most frequent variant (93-95%) in WM patients, while non-L265P *MYD88* variants (1-2%) can rarely occur.[1, 2] The presence of mutated *MYD88* is associated with a decreased risk of histological transformation, longer overall survival, and sensitivity to ibrutinib.[3, 4] Over 40 nonsense and frameshift *CXCR4* variants have been identified in WM patients.[5, 6] Nonsense *CXCR4*^{S338X} variants are the most common, occurring in 50% of WM patients due to C>A or C>G nucleotide transversions.[5, 6] Among *CXCR4* mutations, *CXCR4*^{S338X} has the largest clinical impact with higher serum IgM levels, symptomatic hyperviscosity, earlier time to initiation of frontline therapy, and shorter progression-free survival with ibrutinib.[3] *MYD88* and *CXCR4* mutations can therefore provide important data regarding diagnosis, prognosis, and treatment response in WM.[3]

The use of bone marrow (BM) aspirate materials represents the current "gold standard" for molecular testing in WM.[1-3, 5, 6] Although *MYD88* and *CXCR4* mutations can also be identified in peripheral blood (PB), the diagnostic yield in PB is inferior to BM, particularly for previously treated patients.[7, 8] Tumor enrichment with B-cell selection can significantly improve testing sensitivity, but pre-sorting B-cells is time-consuming and not feasible in most clinical laboratories.[7, 9] Recent studies have demonstrated the feasibility of identifying *MYD88* and *CXCR4*

mutations by using cell-free DNA (cfDNA) from WM patients.[10-12] These findings prompted us to perform a comprehensive analysis comparing the use of cfDNA to matched BM and PB, with or without B-cell selection, for detection of the most common *MYD88* (L265P) and *CXCR4* (S338X) mutations in WM patients.

We prospectively collected matched BM and PB samples from 28 consecutive WM patients. PB was collected in Streck Cell-Free DNA tubes (Streck, La Vista, Nevada) to preserve cfDNA. Tubes were centrifuged and plasma was isolated within 30 hours of venipuncture. cfDNA was extracted using the QIAGEN Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) with all modifications employed by Kang et al.[13] Both CD19-selected and unselected BM and PB mononuclear cells were isolated as before.[2, 6, 7] Overall, five different tissue fractions were isolated for analysis: CD19-selected BM (BM19+), unselected BM (BMMC), CD19-selected PB (PB19+), unselected PB (PBMC), and cfDNA. Quantitative allele-specific polymerase chain reaction (AS-PCR) assays for *MYD88*^{L265P} and *CXCR4*^{S338X} mutations were performed for each tissue fraction using 2.5 ng of DNA as previously described.[2, 6, 7] To avoid a potential batch effect, tissue fractions from the same patient were run on the same plate. Calculations were performed with R (R Foundation for Statistical Computing, Vienna, Austria). P-values <0.05 were considered statistically significant. The Dana Farber/Harvard Cancer Center IRB approved this study, and all patients provided written consent for sample use.

Baseline patient characteristics are shown in **Table 1**. Mutation testing for *MYD88*^{L265P} and *CXCR4*^{S338X} was performed in 28 and 23 patients, respectively; limited tumor DNA precluded evaluation of *CXCR4*^{S338X} in five patients. Using BM19+ as the reference tissue,[1-3, 5, 6] we benchmarked the test performance of *MYD88*^{L265P} and *CXCR4*^{S338X} detection for BMMC, PB19+, PBMC, and cfDNA (**Table 2**). The rates of concordance between the different tissue fractions and BM19+ were consistent with previous studies.[7, 9-12]

We then compared the clinicopathological characteristics between patients with concordant and discordant results for *MYD88*^{L266P} by cfDNA. Discordant patients had a lower median BM involvement (9% vs. 45%; p=0.04) and serum IgM level (985 vs. 1597 mg/dL; p=0.02) as well as a higher hemoglobin level (12.3 vs. 10.7 g/dL; p=0.03) versus concordant patients. BM involvement significantly correlated with serum IgM (r=0.44, p=0.02) and hemoglobin (r=-0.39, p=0.04) levels. Consistent with these findings, the concordance for *MYD88*^{L265P} detection with cfDNA was significantly lower in those with a BM involvement <10% versus ≥10% (50% vs. 90%; p=0.046). Likewise, among those in whom *MYD88*^{L265P} was identified in BM19+, *MYD88*^{L265P} ΔCt was significantly higher in discordant versus concordant patients by cfDNA (6.1 vs. 2.9 cycles; p=0.02), indicating a lower BM19+ *MYD88*^{L265P} mutation burden in discordant patients. A prior study of cfDNA in WM patients did not identify an impact of BM involvement on test performance; however, 12 patients with a low BM involvement were excluded in that study due to insufficient DNA, which may have confounded the findings.[10, 11] Age, serum

albumin, serum IgM and IgG levels, and prior treatment status did not impact the concordance for *MYD88*^{L265P} detection with cfDNA ($p>0.05$ for all comparisons). Discordant patients by BMMC, PB19+, and PBMC tissue fractions similarly had a significantly lower BM involvement (data not shown), akin to previous studies.[7, 9] These findings collectively demonstrate that a low BM tumor burden adversely impacts *MYD88*^{L265P} detection with cfDNA in WM patients.

An unexpected observation was in one patient with a "false positive" result for *MYD88*^{L265P}. The patient had *MYD88*^{L265P} detected in BMMC and cfDNA, but not the BM19+ fraction following treatment with ixazomib, dexamethasone, and rituximab (IDR). Flow cytometric analysis identified clonal CD38+ plasma cells, but not CD19+ B-cells in this patient, suggesting that residual plasma cells unamenable to CD19-selection probably accounted for the detection of *MYD88*^{L265P} in BMMC and cfDNA but not the CD19+ fraction.[9] Indeed, *MYD88*^{L265P} is present in both clonal B and plasma cells derived from individual WM patients, and clonal plasma cells may persist long after B-cell depleting therapy.[1, 14] To account for this treatment effect, we re-calculated the test performance statistics for cfDNA using both the B and plasma cell compartments as reference tissue. The adjusted results yielded a concordance, sensitivity, and specificity of 82%, 80%, and 100%, respectively, for *MYD88*^{L265P} by cfDNA in WM patients (**Supplemental Table 1**).

Overall, our findings strengthen the available evidence that PB cfDNA can reliably be used to identify the most common *MYD88* (L265P) and *CXCR4* (S338X) variants in WM patients. Our data expand upon previous studies by validating cfDNA for *MYD88*^{L265P} and *CXCR4*^{S338X} against CD19-selected and unselected BM and PB tissue fractions.[10-12] This approach also revealed that cfDNA correctly identified the mutation status of a WM patient who underwent B-cell depleting therapy, a finding likely related to residual clonal plasma cells.[9] While a cfDNA "liquid biopsy" does not supplant a BM biopsy to diagnose WM, it can offer a non-invasive, convenient, and potentially cost-effective method to genotype WM patients. *MYD88*^{L265P} can help discriminate WM from other IgM-secreting malignancies, while both *MYD88* and *CXCR4* (particularly S338X) mutations may be useful in making treatment decisions, especially if considering therapy with BTK inhibitors.[2-4] Because of the high specificity, cfDNA could represent an initial testing modality for WM patients in whom an invasive BM biopsy might be needed solely for genotyping purposes.

Our study is not without limitations. The low number of patients with *CXCR4*^{S338X} precluded an assessment of clinicopathological characteristics and *CXCR4*^{S338X} concordance with cfDNA. Additionally, we focused our study on MYD88^{L265P} and *CXCR4*^{S338X} since these constitute the most common variants observed in WM.[5, 6] The use of cfDNA with targeted ultra-deep next-generation sequencing may lead to the reliable identification of other *MYD88* and *CXCR4* variants. Further investigation is also warranted to evaluate cfDNA as a treatment monitoring tool,

as acquired *BTK*, *CARD11*, and *PLCγ2* mutations are associated with disease progression on BTK inhibitors in WM patients.[15]

In summary, *MYD88*^{L265P} and *CXCR4*^{S338X} can be identified with high sensitivity and specificity in cfDNA derived from the plasma of WM patients. The use of cfDNA represents a non-invasive, convenient, and potentially cost-effective method for genotyping WM patients.

ACKNOWLEDGEMENTS

The authors would like to thank the Orszag Family Fund for WM Research, the D'Amato Family Fund for Genomic Discovery, the International Waldenström's Macroglobulinemia Foundation, the Leukemia and Lymphoma Society (Grant: R6507-18), the Kerry Robertson Fund for WM. SPT, ZRH and GY are supported by an NIH SPORE in Multiple Myeloma (Grant: 2P50CA100707-16A1).

AUTHORSHIP

MGD, JNG, ZRH, and SPT designed the study and performed the analysis. MGD, LX, GY, XL, AK, NT, MM, and MLG prepared and performed molecular testing on patient samples. KM, CJP, SS, CL, TW, CAF, ARB, JJC, and SPT provided clinical care for the patients and collected the samples. MGD, JNG and SPT drafted the manuscript. All authors critically reviewed and approved the manuscript.

DISCLOSURES

SPT, JJC, GY, ZRH have received research funding and/or consulting fees from Pharmacyclics Inc., Janssen Pharmaceuticals Inc., the manufacturer of ibrutinib. SPT has received research funding from Bristol Myers Squibb, X4 Pharmaceuticals, and Beigene. JJC received research funding and/or consulting fees from Abbvie, Beigene, Kymera, and TG Therapeutics.

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Characteristics	All Patients (N=28)
Median age, years (range)	72 (52-82)
Median hemoglobin level, g/dL (range)	10.9 (8.7-14.7)
Median albumin level, g/dL (range)	3.9 (3.4-4.5)
Median serum IgM level, mg/dL (range)	1303 (68-6956)
Median serum IgG level, mg/dL (range)	447 (184-1024)
Median serum IgA level, mg/dL (range)	52 (11-284)
Median bone marrow involvement, % (range)	25 (0-95)
Previously treated, N (%)	20/28 (71%)
MYD88^{L265P} mutation	
BM19+	24/28 (86%)
BMMC	15/28 (54%)
PB19+	19/28 (68%)
PBMC	9/27 (33%)
cfDNA	20/28 (71%)
CXCR4^{S338X} mutation	
BM19+	6/23 (26%)
BMMC	3/23 (13%)
PB19+	3/23 (13%)
PBMC	1/22 (5%)
cfDNA	4/23 (17%)

Table 1. Patient characteristics at the time of mutation assessment.Testing for *MYD88*^{L265P} and *CXCR4*^{S338X} mutations was evaluated in 28 and 23 patients, respectively; limited tumor DNA precluded evaluation of CXCR4^{S338X} in five patients. BM19+, CD19-selected bone marrow mononuclear cells; BMMC, unselected bone marrow mononuclear cells; PB19+, CD19-selected peripheral blood mononuclear cells; PBMC, unselected peripheral blood mononuclear cells; cfDNA, cell-free DNA fractions

Variable	MYD88 L265P					CXCR4 S338X				
	BM19+	BMMC	PB19+	PBMC	cfDNA	BM19+	BMMC	PB19+	PBMC	cfDNA
True positive – no.	24	14	19	9	19	6	3	3	1	4
True negative – no.	4	3	4	3	3	17	17	17	16	17
False positive – no.	0	1*	0	0	1*	0	0	0	0	0
False negative – no.	0	10	5	15	5	0	3	3	5	2
Concordance (Cohen κ) – %	Ref.	61 (0.17)	82 (0.52)	44 (0.12)	79 (0.38)	Ref.	87 (0.60)	87 (0.60)	77 (0.23)	91 (0.75)
Sensitivity (95% CI) – %	Ref.	58 (37-77)	79 (57-92)	38 (20-59)	79 (57-92)	Ref.	50 (14-86)	50 (14-86)	17 (1-64)	67 (24-94)
Specificity (95% CI) – %	Ref.	75 (22-99)	100 (40-100)	100 (31-100)	75 (22-99)	Ref.	100 (77-100)	100 (77-100)	100 (75-100)	100 (77-100)
PPV (95% CI) – %	Ref.	93 (55-100)	100 (79-100)	100 (63-100)	95 (73-100)	Ref.	100 (31-100)	100 (77-100)	100 (5-100)	100 (40-100)
NPV (95% CI) – %	Ref.	23 (6-54)	44 (15-77)	17 (4-42)	38 (10-74)	Ref.	85 (61-96)	85 (61-96)	76 (52-91)	89 (65-98)

Table 2. Test performance for *MYD88*^{L265P} and *CXCR4*^{S338X} mutations according to tissue fraction. *One patient was deemed a "false positive" because *MYD88*^{L265P} was detected in BMMC and cfDNA but not BM19+. The patient was previously treated with B-cell depleting therapy (IDR), and only had clonal plasma cells identified by flow cytometry in the bone marrow aspirate. Test performance data for cfDNA for *MYD88*^{L265P} using BMMC that encompass B- and plasma cell fractions is reported in **Supplemental Table 1**. Twenty-seven patients had PBMC available for *MYD88*^{L265P} testing; one patient was wild-type for *MYD88* in the BM19+ fraction. BM19+, CD19-selected bone marrow mononuclear cells; BMMC, unselected bone marrow mononuclear cells; PB19+, CD19-selected peripheral blood mononuclear cells; PBMC, unselected peripheral blood mononuclear cells; cfDNA, cell-free DNA; PPV, positive predictive value; NPV, negative predictive value; Ref, denotes reference tissue.