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ORIGINAL ARTICLE

Detection of MYD88 L265P in peripheral blood of patients with Waldenström's Macroglobulinemia and IgM monoclonal gammopathy of undetermined significance

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MYD88 L265P is highly prevalent in Waldenstrom's Macroglobulinemia (WM) and IgM monoclonal gammopathy of unknown significance (MGUS). We investigated whether MYD88 L265P could be identified by peripheral blood (PB) allele-specific PCR. MYD88 L265P was detected in untreated WM (114/118; 96.6%); previously treated WM (63/102; 61.8%); and IgM MGUS (5/12; 41.7%) but in none of 3 hyper-IgM or 40 healthy individuals. Median PB MYD88 L265P ΔCt was 3.77, 7.24, 10.89, 12.33 and 14.07 for untreated WM, previously treated WM, IgM MGUS, hyper-IgM and healthy individuals, respectively ($P<0.0001$). For the 232 IgM MGUS and WM patients, PB MYD88 L265P ΔCt moderately correlated to bone marrow (BM) disease ($r=-0.3553$; $P<0.0001$), serum IgM ($r=-0.3262$; $P<0.0001$) and hemoglobin ($r=0.3005$; $P<0.0001$) levels. PB MYD88 L265P ΔCt and serum IgM correlated similarly with BM disease burden. For positive patients, PB MYD88 L265P ΔCt was <6.5 in 100/114 (88%) untreated WM, and >6.5 in 4/5 (80%) IgM MGUS patients ($P=0.0034$). Attainment of a negative PB MYD88 L265P mutation status was associated with lower BM disease ($P=0.001$), serum IgM ($P=0.019$) and higher hemoglobin ($P=0.004$) levels in treated patients. These studies show the feasibility for detecting MYD88 L265P by PB examination, and the potential for PB MYD88 L265P ΔCt use in the diagnosis and management of WM patients.

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INTRODUCTION

Waldenström's Macroglobulinemia (WM) is a B-cell neoplasm categorized as an IgM-secreting lymphoplasmacytic lymphoma (LPL) by the World Health Organization classification system.[1,2] The disease is primarily characterized by bone marrow (BM) infiltration with lymphoplasmacytic cells (LPC), although up to 20% of patients can exhibit extramedullary disease.[3] Circulating WM cells identified by flow cytometry or clonotypic IgM V/D/J rearrangements are present in WM patients, and parallel disease burden.[4,5] At present, the diagnosis of WM is contingent on demonstrating a LPC infiltrate, most typically by BM biopsy, which can produce patient discomfort, result in unforeseen complications, represent a significant burden of cost and delay the diagnosis of WM.

MYD88 L265P is a somatic mutation initially described by Ngo *et al.*[6] in 29% of patients with activated B-cell subtype of diffuse large B-cell lymphoma and 9% of gastric mucosa-associated lymphoid tissue lymphomas. By whole-genome sequencing, we identified MYD88 L265P in CD19-selected B cells as the most prevalent somatic gene mutation in WM (91% of patients), and confirmed these findings by Sanger sequencing and allele-specific PCR (AS–PCR) in BM samples from patients with WM, IgM MGUS and non-IgM-secreting LPL.[7–9] The presence of MYD88-L265P in WM and IgM MGUS has also been described by others, with 80–100% of WM, and 50–80% of IgM MGUS patients found to express MYD88 L265P using both CD19-selected and unselected BM samples.[10–17] The presence of MYD88 L265P in IgM MGUS suggests that this somatic mutation is likely to represent an early oncogenic event in WM/LPL pathogenesis. In contrast to WM and IgM MGUS, MYD88 L265P is absent or rarely expressed in other IgM-secreting disorders including splenic marginal zone, nodal marginal zone, extranodal marginal zone (mucosa-associated lymphoid tissue) lymphoma and IgM-secreting multiple myeloma. Few patients (3%) with chronic lymphocytic leukemia also express MYD88 L265P.[18] MYD88 L265P therefore represents a novel somatic mutation for use in discriminating WM/LPL from other related B-cell disorders.[7,9,12–16]

MYD88 is an adaptor molecule that has an important role in modulating Toll-like receptor and interleukin-1 receptor signaling through interactions with interleukin-1 receptor-associated kinase 4 (IRAK4) followed by activation of IRAK1 and IRAK2.[19–21] Inhibition of MYD88 signaling in L265P-mutated WM cells blocks IRAK-driven IκBa and nuclear factor-κB p65 phosphorylation, and/or nuclear translocation of p65 nuclear factor-κB.[8,14,22] In addition, MYD88 L265P triggers Bruton tyrosine kinase and downstream nuclear factor-κB signaling independent of IRAK1 and 4.[22] In relapsed/refractory WM patients, treatment with the Bruton tyrosine kinase inhibitor ibrutinib leads to high rates of response supporting a role for MYD88 pathway-targeted therapy in WM.[23] Determination of MYD88 L265P mutation status may therefore help identify those patients who might benefit from targeted therapies of the MYD88 pathway.

We therefore investigated the potential use of a quantitative AS–PCR assay for MYD88-L265P using the peripheral blood (PB) of

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patients with WM. As part of these efforts, we also examined paired PB and BM samples from previously untreated WM patients with smoldering and symptomatic disease, previously treated WM patients as well as individuals with IgM MGUS. AS–PCR was used in both unselected and CD19-selected PB samples using an AS–PCR assay for MYD88 L265P that we previously validated against a large cohort of healthy donors and WM patients.[9] The results of this study support the feasibility for using an AS–PCR assay for MYD88-L265P detection by PB examination, thereby providing a convenient, less invasive and potentially less costly method to support the diagnosis of WM and IgM MGUS.

MATERIALS AND METHODS

PB was collected from 220 patients with WM, including 118 untreated patients with smoldering ($n=51$) and symptomatic ($n=67$) disease; 102 with previously treated disease, 12 individuals with IgM MGUS, 3 with polyclonal hyper-IgM, 1 with IgG LPL and 40 healthy donors. WM and IgM MGUS patients met consensus criteria for diagnosis and symptomatic patients met consensus criteria for initiation of therapy.[1,24] Among untreated WM patients, 88 had unselected PB mononuclear cells that were used in initial studies aimed at determining the sensitivity and specificity of PB MYD88 L265P testing in unselected PB mononuclear cells. Subsequently, we used PB CD19-selected tissues from both PB and BM samples. For these experiments, samples were obtained from 61 untreated WM patients that included 13 and 48 patients with smoldering and symptomatic disease, respectively, as well as 66 previously treated WM and 12 IgM MGUS patients (Figure 1). The clinical characteristics for all WM and IgM MGUS patients, and those with paired CD19-selected PB and BM samples are provided in Table 1. BM disease assessment was performed by direct morphological assessment following hematoxalin and eosin staining, and enumeration of lymphoplasmacytic cells occupying the intertrabecular space of complete representative slide sections were performed.

CD19-selected cells from BM aspirates were isolated as previously reported.[7,9] CD19-cells from PB samples were isolated using CD19 Dynabeads Pan B Kit (Life Technologies, Carlsbad, CA, USA). For this assay, 8 ml of PB and 200 μl of Dynabeads magnetic beads were mixed and incubated for 20 min at 4 °C with gentle rotation. The beads were magnetically collected and washed thrice with isolation buffer. Three hundred and fifty microliters of RLTplus cell lysis buffer (AllPrep DNA/RNA Mini Kit; Qiagen, Valencia, CA, USA) was added to the beads and DNA was extracted according to the manufacturer's protocol (Qiagen). All samples were obtained after informed consent was approved by the Harvard Cancer Center/Dana Farber Cancer Institute Institutional Review Board. Quantitative AS–PCR for MYD88-L265P using unselected or CD19-selected cells was performed as previously described.[9] The previously established sensitivity and specificity for MYD88 L265P detection by this assay against CD19-selected cells from 104 WM patients and 40 healthy donors was 100% and 92.1%, respectively, with positive predictive and negative predictive values of 95.9% and 100%, respectively, and a $\Delta Ct < 9.6$ defining presence of MYD88 L265P.[9] Statistical analysis was conducted using the Mann–Whitney *U*-test. Linear correlation and regression analyses were performed with Spearman's rank correlation. Calculations were performed using R (R Foundation for Statistical Computing, Vienna, Austria). Fisher's exact probability testing and estimates of sensitivity, specificity and predictive values were performed using VassarStats (Poughkeepsie, NY, USA).

RESULTS

MYD88 L265P detection in paired CD19-selected BM and unselected PB mononuclear cells from WM patients

We first sought to clarify the feasibility of using real-time AS–PCR assay to detect MYD88-L265P in PB using unselected PB mononuclear cells taken from 88 of the untreated WM patients who also had paired CD19-selected cells derived from BM aspirates. Eighty-one (92%) of these patients expressed the MYD88-L265P mutation in CD19-selected B cells derived from BM aspirations. Of the 81 patients who expressed MYD88-L265P, 32 (40%) were positive for MYD88-L265P using unselected PB mononuclear cells (data not shown). Taken together, these findings yielded a sensitivity of 39.5%, specificity of 100%, and positive and negative predictive values of 100% and 12.5%, respectively, for determination of MYD88 L265P by AS–PCR assay using unselected PB mononuclear cells in untreated WM patients.

MYD88 L265P detection in paired CD19-selected BM and PB cells from WM and IgM MGUS patients

To improve the sensitivity for detecting MYD88-L265P by PB AS–PCR examination, we next used CD19-selected cells from WM and IgM MGUS patients that were isolated with a magnetic bead kit (Dynabeads). The purity of PB $CD19^+$ cells isolated with magnetic beads ($91.07 + 7.36$ s.d.) was on par with that achieved by with MACS Microbeads ($95.13 + 1.14$ s.d.) that relies on column separation for six paired test cases ($P=0.2$). From the 220 WM patients, paired CD19-selected samples from the PB and BM were available for 61 untreated and 66 previously treated WM patients (Figure 1), as well as for 12 IgM MGUS patients. The baseline clinical parameters for paired patients did not significantly differ when compared with unpaired patients' cohorts (Table 1). In addition, no significant differences in median prior therapies, time from last therapy, on versus off active therapy and prior treatment with a rituximab-containing regimen were observed for paired versus unpaired treated patients (data not shown).

Analysis of paired BM and PB samples from the untreated WM cohort that included 13 smoldering and 48 symptomatic patients is shown in Figure 1. As shown in Table 2, MYD88 L265P was detected in 12 (92.3%) and 46 (95.7%) CD19-selected BM samples from smoldering and symptomatic untreated WM patients, respectively. In the same patients, MYD88 L265P was positive in CD19-selected PB samples for 11 (84.6%) and 46 (95.7%) of the smoldering and symptomatic untreated patients, respectively. Therefore, among the positive MYD88 L265P patients defined by BM sampling, MYD88 L265P was detected in their paired PB CD19-selected samples for 11/12 (92%) and 46/46 (100%) smoldering and symptomatic patients, respectively. In one of the smoldering patients with negative PB MYD88 L265P, expression for MYD88 L265P in the corresponding BM sample was weakly positive with a ΔCt close to the cutoff for positivity. Taken together, 57/58 (98.3%) of all untreated (smoldering and symptomatic combined) WM patients who were positive for MYD88 L265P by PB examination were also positive by BM examination, and the ΔCt for their samples is shown in Table 2. These findings therefore yield a sensitivity of 98.2%, specificity of 100%, and positive and negative predictive values of 100 and 75%, respectively, for determination of MYD88 L265P by AS–PCR assay using CD19-selected PB cells in untreated WM patients.

Analysis of paired BM and PB samples from the 12 IgM MGUS patients is shown in Figure 2. MYD88 L265P was present in six (50%) and five (41.7%) BM and PB CD19-selected samples for these patients, respectively. All five IgM MGUS patients who were

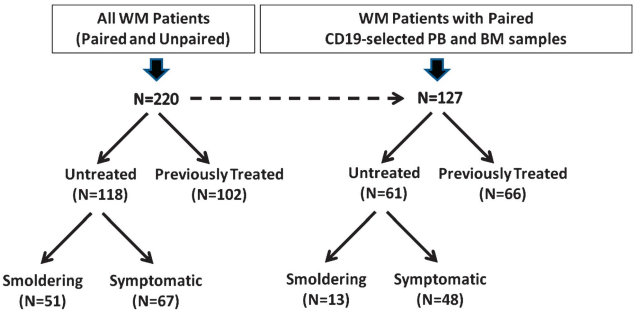

Figure 1. Distribution of WM patients who underwent PB MYD88 L265P testing by AS–PCR. The distribution of all WM patients and those with paired BM and PB samples is shown.

Table 1. Baseline characteristics of patients with WM and IgM MGUS

	N	*Gender*	*Age*	*BM*	*WBC*	*HGB*	*HCT*	*PLT*	*ANEUT*	*ALYMP*	*IgG*	*IgA*	*IgM*
All patients													
IgM MGUS	12	6M/6F	62.5	0	5.45	13.35	38.35	248.5	3.78	1.25	937	139.5	437
Smoldering WM	51	31M/20F	61	20	7.2	13	38	247	4.43	1.78	658.5	72	1900
Symptomatic WM	67	33M/34F	60	50	6	11.4	33	247	3.53	1.7	639.5	52	3320
Treated WM	102	72M/30F	57.5	40	5.3	11.8	34.8	205	3.175	1.04	402	27	1700
ANOVA *P*-value			0.611	<0.0001	0.0006	<0.0001	<0.0001	0.0282	0.0016	0.225	<0.0001	<0.0001	<0.0001
Paired patients													
IgM MGUS	12	6M/6F	62.5	0	5.45	13.35	38.35	248.5	3.78	1.25	937	139.5	437
Smoldering WM	13	9M/4F	63	15	6.9	12.4	37.1	208	3.655	1.89	651	81	1240
Symptomatic WM	48	24M/24F	58.5	50	6.3	11.45	33	253	3.71	1.73	610	53	3285
Treated WM	66	49M/17F	56	32.5	5.35	11.95	35	216	2.97	1.035	353.5	26	1540
ANOVA *P*-value			0.213	<0.0001	0.0513	0.0006	0.0015	0.0456	0.126	<0.0001	0.0062	<0.0001	0.0007

Abbreviations: ALYMP, absolute lymphocyte count; ANEUT, absolute neutrophil count; ANOVA, analysis of variance; BM, bone marrow; F, female; HCT, hematocrit; HGB, hemoglobin; M, male; MGUS, monoclonal gammopathy of unknown significance; PB, peripheral blood; PLT, platelets; WBC, white blood cell; WM, Waldenstrom's Macroglobulinemia. Median values provided. *P*-values denote significance by ANOVA for comparisons within WM and IgM MGUS cohorts for all patients, and for BM- and PB-paired patients.

Table 2. Detection of MYD88 L265P in bone marrow and peripheral blood samples from WM, IgM MGUS, other B-cell lymphoproliferative disorders, and healthy donors

A

Paired patients		*MYD88 L265P positive*		
	N	*BM*	*PB*	*% Of patients positive by PB with positive BM*
Untreated (all)	61	58 (95.1%)	57 (93.4%)	98.20%
Smoldering	13	12 (92.3%)	11 (84.6%)	91.60%
Symptomatic	48	46 (95.8%)	46 (95.8%)	100%
Previously treated (all)	66	61 (92.4%)	45 (68.2%)	73.70%
IgM MGUS	12	6 (50%)	5 (41.7%)	83.30%

B

Paired patients		*MYD88 L265P ΔCt for positive patients (median and ranges)*		
	N	*BM*	N	*PB*
Untreated (all)	58	2.06 (0.21–8.10)	57	3.27 (1.39–9.27)
Smoldering	12	3.00 (1.23–8.09)	11	4.75 (1.67–7.38)
Symptomatic	46	1.99 (0.21–8.10)	46	2.91 (1.39–9.27)
Previously treated (all)	61	2.32 (0.54–9.32)	45	4.68 (0.92–9.24)
IgM MGUS	6	6.29 (2.89–8.30)	5	7.96 (3.12–9.02)

C

All patients		*MYD88 L265P positive*	
	N	*PB*	*Median and range for PB MYD88 L265P ΔCt for positive patients*
Untreated (all)	118	114 (96.6%)	3.58 (1.27–9.27)
Smoldering	51	49 (96.1%)	4.46 (1.63–8.95)
Symptomatic	67	65 (97.0%)	3.08 (1.27–9.27)
Previously treated (all)	102	63 (61.8%)	4.99 (0.92–9.34)
IgM MGUS	12	5 (41.7%)	7.96 (3.12–9.02)
Polyclonal hyper-IgM	3	0 (0%)	NA
IgG LPL	1	1 (100%)	2.47 (NA)
Healthy donors	40	0 (0%)	NA

Abbreviations: BM, bone marrow; LPL, lymphoplasmacytic lymphoma; MGUS, monoclonal gammopathy of unknown significance; NA, not applicable; PB, peripheral blood; WM, Waldenstrom's Macroglobulinemia. Denotes results for patients with paired BM and PB samples (A, B) and from all patients including those with and without paired BM and PB samples (C).

positive for MYD88 L265P by PB examination also expressed this mutation by BM examination; therefore, MYD88 L265P was detected in PB samples from five out of six (83.3%) individuals who were positive by BM examination, and the ΔCt for their samples is reported in Table 2. In one of the IgM MGUS patient with negative PB but positive BM results, MYD88 L265P expression in the corresponding BM sample was weakly positive with a ΔCt close to the cutoff for positivity. In comparison with untreated WM patients, fewer IgM MGUS patients were MYD88 L265P positive by either BM or PB examination ($P<0.0001$).

We next performed an analysis of paired BM and PB samples from 66 previously treated WM patients (Figure 2) that included 44 patients off therapy and 22 on continued active therapy including ibrutinib ($n=11$), everolimus ($n=5$), rituximab-based maintenance ($n=5$) and chlorambucil ($n=1$). Analysis of CD19-selected BM samples from these patients showed expression of MYD88 L265P in 61/66 (92.4%) and 45/66 (68.2%) BM and PB CD19-selected samples, respectively (Table 2). There was no significant difference in presence of MYD88 L265P in either BM or PB samples based on 'off' versus 'on continued active' treatment status, time from prior therapy, number of prior therapies, including prior rituximab treatment. All 45 previously treated WM patients who were positive for MYD88 L265P by PB examination also expressed this mutation in their BM samples. Therefore, MYD88 L265P was detected in the PB from 45 of the 61 (73.7%) patients who tested positive in their BM, and the ΔCt for their samples is reported in Table 2. These findings therefore yield a sensitivity of 73.7%, specificity of 100%, and positive and negative predictive values of 100% and 23.8%, respectively, for PB MYD88 L265P testing using CD19-selected cells in previously treated WM patients. Finally, we correlated PB MYD88 L265P status with disease-related manifestations in the 66 previously treated WM patients with paired samples. Patients with absence of PB MYD88 L265P but who were positive by BM examination showed lower BM disease burden ($P=0.001$) and serum IgM levels ($P=0.019$), as well as higher hemoglobin levels ($P=0.004$) versus those patients who were positive for MYD88 L265P in both their PB and BM samples.

MYD88 L265P in CD19-selected PB cells from WM, IgM MGUS, other B-cell disorder patients and healthy donors

Having established parameters for sensitivity and specificity of PB MYD88 L265P testing in paired patients, we next analyzed CD19-selected PB cells using magnetic beads from all patients (paired and unpaired) from cohorts that included 118 untreated WM, 102 previously treated WM, 12 IgM MGUS, 3 hyper-IGM and 1 IgG LPL patients, and 40 healthy donors (Figure 3). The median PB MYD88 L265P ΔCt for all patients regardless of their MYD88 L265P status was 3.77, 7.24, 2.47, 10.89, 12.33 and 14.07 for patients with untreated WM, previously treated WM, IgG LPL (for one patient), IgM MGUS, hyper-IgM syndrome and healthy donors, respectively ($P<0.0001$ by analysis of variance). Among untreated WM patients, the median PB MYD88 L265P ΔCt was 4.55 and 3.27 for all smoldering and symptomatic patients, respectively ($P=0.098$).

Using a ΔCt of 9.6, 114/118 (96.6%) untreated WM patients, including 49/51 (96.1%) smoldering and 65/67 (97.0%) symptomatic patients were positive for MYD88 L265P, and the ΔCt for these patients is shown in Table 2. MYD88 L265P was also detected in CD19-selected PB cells from previously treated WM patients, although a lower fraction of patients, that is, 63/102 (61.8%) demonstrated positivity ($P<0.0001$) and a higher ΔCt ($P=0.0073$) for these patients was observed versus untreated WM patients who were positive for PB MYD88 L265P (Table 2). MYD88

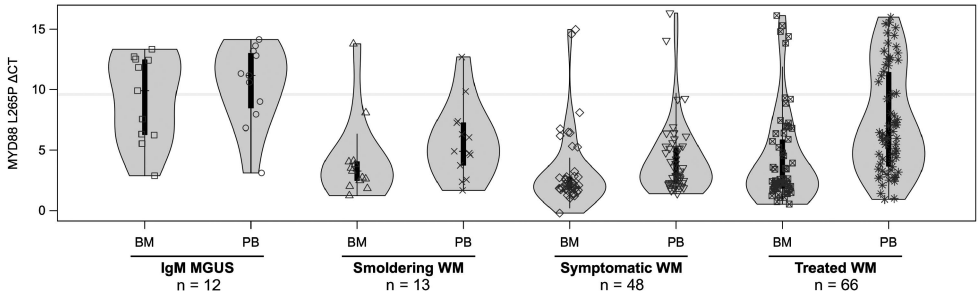

Figure 2. Real-time AS–PCR results for paired BM and PB MYD88 L265P in patients with WM and IgM MGUS. Violin plots representing AS–PCR differences in cycle threshold (ΔCt). The span of gray area for each cohort represents the kernel density estimation of the sample distribution and highlights the bimodal nature of the data. Box plots with interquartile ranges are shown in black with an overlay of the individual data points. Paired PM and PB samples evaluated were from patients with IgM MGUS ($n=12$); smoldering WM ($n=13$); symptomatic WM ($n=48$); and previously treated WM ($n=66$). The light gray bar represents the distance between the highest positive and lowest negative ΔCt values.

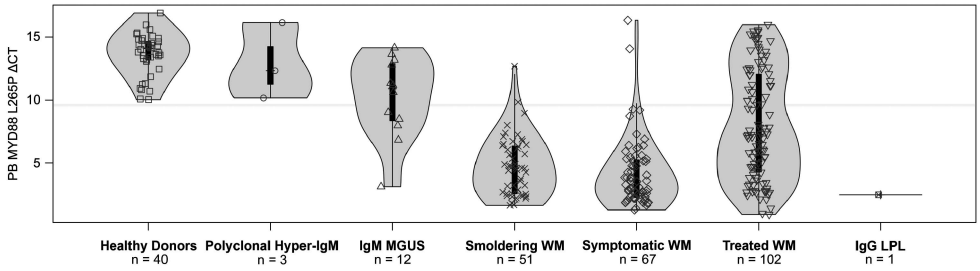

Figure 3. Real-time AS–PCR results for PB MYD88 L265P in all (paired and nonpaired) patients with WM, IgM MGUS, other B-cell disorders and healthy donors. Violin plots representing AS–PCR differences in cycle threshold (ΔCt). The span of gray area for each cohort represents the kernel density estimation of the sample distribution and highlights the bimodal nature of the data. Box plots with interquartile ranges are shown in black with an overlay of the individual data points. Samples evaluated were from healthy donors ($n=40$) or patients with polyclonal hyper-IgM ($n=3$); IgM MGUS ($n=12$); smoldering WM ($n=51$); symptomatic WM ($n=67$); previously treated WM ($n=102$); and IgG LPL ($n=1$). The light gray bar represents the distance between the highest positive and lowest negative ΔCt values.

L265P was also positive in a lower fraction of IgM MGUS patients, that is, 5/12 (41.7%; $P<0.0001$) who had a significantly higher ΔCt ($P=0.0073$) versus untreated WM patients ($P=0.046$). No difference in ΔCt for MYD88 L265P was observed between positive IgM MGUS and previously treated WM patients ($P=0.124$). PB MYD88 L265P was also detected in one untreated IgG LPL patient. All 3 hyper-IgM syndrome patients and 40 healthy donors were negative for MYD88 L265P by AS–PCR examination of PB CD19-selected cells (Table 2).

PB MYD88 L265P ΔCt correlates with BM disease burden, serum IgM and hemoglobin levels

We next sought to clarify whether PB MYD88 L265P ΔCt correlated with important disease parameters in WM. Among all 139 paired IgM MGUS and WM patients, PB MYD88 L265P ΔCt correlated to BM MYD88 L265P ΔCt ($r=0.835$; $P<0.0001$), BM disease burden assessed by histological determination of intertrabecular space involvement by lymphoplasmacytic cells ($r=-0.4398$; $P<0.0001$), as well as more moderately with serum IgM ($r=-0.3173$; $P=0.0001$) and hemoglobin ($r=0.3343$; $P<0.0001$) levels. Among all 232 IgM MGUS and WM patients, PB MYD88 L265P ΔCt moderately correlated to BM disease burden assessed by histological determination of intertrabecular space involvement by lymphoplasmacytic cells ($r=-0.3553$; $P<0.0001$), serum IgM ($r=-0.3262$; $P<0.0001$) and hemoglobin ($r=0.3005$; $P<0.0001$) levels.

As serum IgM is typically used to monitor IgM MGUS and WM patients for changes in underlying BM disease burden, we next compared the relative strengths of correlation for serum IgM levels and PB MYD88 L265P ΔCt with underlying BM disease burden in all 232 IgM MGUS and WM patients by performing a Fisher's *r–z* transformation test to assess the significance of the difference between their respective correlations. Both serum IgM ($r=0.3503$; $P<0.0001$) and PB MYD88 L265P ΔCt ($r=-0.3553$; $P<0.0001$) demonstrated similar strengths of correlation to underlying BM disease involvement, and were not significantly different ($P=0.8887$) by Fisher's *r–z* transformation testing.

PB MYD88 L265P ΔCt may help distinguish WM from IgM MGUS patients

As both IgM MGUS and WM patients express the MYD88 L265P mutation by PB AS–PCR examination, and as PB MYD88 L265P ΔCt strongly correlated with underlying BM disease involvement, we next sought to determine whether ΔCt could discriminate IgM MGUS from untreated WM patients who were positive for PB MYD88 L265P. One hundred and fourteen untreated WM patients, including 49 smoldering and 65 symptomatic patients, and 5 IgM MGUS patients were included in this analysis, all of whom were positive for PB MYD88 L265P (Table 2). This analysis included both paired and unpaired patients. Of these 119 patients, 101 (84.8%) with a PB MYD88 L265P ΔCt of 6.5 or lower had the diagnosis of WM. This included 40/49 (81.6%) smoldering and 60/65 (92.3%) symptomatic untreated WM patients. In contrast, four of the five patients with IgM MGUS had a PB MYD88 L265P ΔCt greater than 6.5 ($P=0.0034$). One IgM MGUS patient with a PB MYD88 L265P ΔCt of 3.12 was also strongly positive in the BM for MYD88 L265P with a ΔCt of 2.89 and subsequently progressed to WM.

DISCUSSION

The high prevalence of MYD88 L265P in LPC from BM samples of IgM MGUS and WM patients prompted us to examine whether PB cells could be used as a source for demonstrating presence of this somatic mutation. We used a highly sensitive and specific AS–PCR assay that we previously established for determining MYD88 L265P status in BM CD19-selected cells.[9] We first examined unselected PB cells from untreated WM patients. The low sensitivity (39.5%) for detecting MYD88 L265P by this method prompted us to examine the use of CD19-selected PB cells using magnetic bead isolation for AS–PCR testing. Magnetic beads represent a convenient and affordable means for CD19 selection, which may be easily adopted for use in clinical pathology laboratories where flow-based sorting is time and cost prohibitive. Using PB CD19-selected cells for AS–PCR testing, we demonstrated that PB MYD88 L265P testing was associated with high rates of sensitivity (98.1%) and specificity (100%) in untreated WM patients. These findings contributed to high positive and negative predictor values for determining MYD88 L265P status in untreated WM and IgM MGUS patients, although a few patients who were negative by examination of PB CD19-selected cells were positive by BM examination. These findings suggest that for the vast majority of untreated WM (98%) and IgM MGUS (83%) patients, AS–PCR examination of CD19-selected PB samples should be able to determine MYD88 L265P status. Although in some patients whose findings are negative, a BM biopsy could be considered for establishing mutation status. In contrast to the high sensitivity for PB MYD88 L265P testing observed in untreated patients, MYD88 L265P detection in previously treated patients was associated with a lower sensitivity of 73.7% and a negative predictive value of 23.8%. The specificity (100%) and positive predictive value (100%) in previously treated patients still remained high and coincided with values observed in untreated patients. These findings would affirm that while MYD88 L265P is likely to be detected by AS–PCR in most (74%) previously treated patients in whom the mutation is present, absence of its recognition by PB CD19-selected examination should not reflect a negative finding and a BM examination should be considered in these patients to establish mutation status. By use of AS–PCR, most untreated (96.6%) and previously treated (61.8%) WM patients, and many IgM MGUS (41.7%) patients were positive for MYD88 L265P by PB examination in these studies. It is also interesting that one IgG LPL patient was also positive by PB examination. In our previous studies, three of three non-IgM LPL patients were MYD88 L265P positive by BM examination. Further testing of this uncommon patient population will be needed to fully clarify the utility of PB examination for determining MYD88 L265P status.

The above findings suggest that PB AS–PCR testing for MYD88 L265P may in the appropriate clinical context be used to establish the diagnosis of WM. Appropriate clinical context could include demonstration of cytopenias in the absence of other medical etiologies, and/or presence of morbidities attributable to WM such as hyperviscosity, adenopathy or splenomegaly in the presence of elevated serum IgM levels and the presence of an IgM monoclonal protein. Use of PB MYD88 L265P ΔCt could also help distinguish WM from IgM MGUS patients who are typically asymptomatic, have low serum IgM levels and lack cytopenias and/or extramedullary disease. Further studies with larger numbers of IgM MGUS patients will invariably be needed to confirm these findings and help establish PB MYD88 L265P ΔCt cutoffs that might distinguish WM from IgM MGUS patients. In patients who are negative by PB AS–PCR testing for MYD88 L265P, a BM biopsy may then be considered in order to clarify the underlying diagnosis. Similar molecular-based testing has obviated the need for routine BM examination in patients with other hematological conditions including chronic myelogenous leukemia (*BCR-ABL*), polycythemia vera (*JAK2 V617F*) and hairy cell leukemia (*BRAF V600E*).[25–27] The use of PB MYD88 L265P testing for establishing the diagnosis of WM could potentially save time, reduce costs and alleviate pain and patient anxiety associated with a BM biopsy.

It is interesting that the absence of PB MYD88 L265P expression in previously treated patients was associated with lower but detectable levels of BM disease burden. From a biological point of view, the absence of PB circulating disease in these patients is intriguing and consistent with prior studies that

showed decreased circulating disease in responding patients.[4,5] These findings may be indicative of tumor cell sparing in BM relative to the peripheral circulation that may be afforded by a protective microenvironment.[28,29] The application of PB MYD88 L265P testing, including serial assessment of MYD88 L265P ΔCt values in patients undergoing therapy could be useful in assessing not only treatment response but also the differential impact of various treatments on PB and BM compartments. The use of BM MYD88 L265P ΔCt to assess changes in BM tumor burden following therapy has previously been demonstrated by us and others,[9,13] and the high degree of correlation between PB and BM MYD88 L265P ΔCt as shown in these studies supports the investigation of PB MYD88 L265P ΔCt in prospective therapeutic trials.

The recognition that serum IgM and PB MYD88 L265P ΔCt values showed similar strengths of correlation with underlying disease burden is also noteworthy. Frequent discordance between serum IgM and underlying BM disease has frequently been reported with agents used in the treatment of WM including rituximab, bortezomib, everolimus and ibrutinib.[3,23,30,31] Rituximab induces an IgM flare in about half of WM patients, which is more pronounced with concurrent administration of an immunomodulatory agent.[3] Increased serum IgM can be mistaken for disease progression leading to change in drug therapy. Conversely, bortezomib, everolimus and ibrutinib can block IgM secretion out of proportion to tumor load, thereby lending to underestimations of post-treatment disease burden, and in some instances missing WM disease progression.[3,23,30,31] The use of PB MYD88 L265P ΔCt to estimate underlying disease burden in patients undergoing treatments that differentially affect serum IgM levels could help guide clinical management and avoid repetition of BM biopsies to clarify IgM discordance as is the current standard of care.[32] Prospective studies are therefore warranted to help validate the use of PB MYD88 L265P ΔCt in monitoring disease response, particularly with therapies affected by IgM discordance.

In summary, we demonstrate the feasibility of detecting MYD88 L265P by use of PB AS–PCR testing, with high rates of sensitivity and specificity particularly for untreated WM and IgM MGUS patients. In the appropriate clinical context, and supported by PB MYD88 L265P ΔCt, the use of PB MYD88 L265P testing may help provide a convenient, noninvasive and inexpensive method to establish the diagnosis of WM, and follow changes in underlying disease burden.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

LX and SPT conceived and designed the experiments, and wrote the manuscript. LX, ZRH and SPT performed the data analysis. LX, YC, XL and JC procured and/or prepared samples, and LX designed and performed PCR-based sequencing studies. LX, GY, YC and XL performed validation studies. SPT, CJP, SK and CT provided patient care, obtained consent and samples. RJM collected patient data. NL and MK provided input for development and validation of AS–PCR assay.

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