

# Clonal architecture of CXCR4 WHIM-like mutations in Waldenström Macroglobulinaemia

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We recently described the existence of somatic mutations in the C-terminal domain of CXCR4 in patients with Waldenström Macroglobulinaemia (WM) (Treon *et al*, 2012;

## Summary

CXCR4<sup>WHIM</sup> somatic mutations are distinctive to Waldenström Macroglobulinaemia (WM), and impact disease presentation and treatment outcome. The clonal architecture of CXCR4<sup>WHIM</sup> mutations remains to be delineated. We developed highly sensitive allele-specific polymerase chain reaction (AS-PCR) assays for detecting the most common CXCR4<sup>WHIM</sup> mutations (CXCR4<sup>S338X C>A</sup> and C>G) in WM. The AS-PCR assays detected CXCR4<sup>S338X</sup> mutations in WM and IgM monoclonal gammopathy of unknown significance (MGUS) patients not revealed by Sanger sequencing. By combined AS-PCR and Sanger sequencing, CXCR4<sup>WHIM</sup> mutations were identified in 44/102 (43%), 21/62 (34%), 2/12 (17%) and 1/20 (5%) untreated WM, previously treated WM, IgM MGUS and marginal zone lymphoma patients, respectively, but no chronic lymphocytic leukaemia, multiple myeloma, non-IgM MGUS patients or healthy donors. Cancer cell fraction analysis in WM and IgM MGUS patients showed CXCR4<sup>S338X</sup> mutations were primarily subclonal, with highly variable clonal distribution (median 35.1%, range 1.2–97.5%). Combined AS-PCR and Sanger sequencing revealed multiple CXCR4<sup>WHIM</sup> mutations in many individual WM patients, including homozygous and compound heterozygous mutations validated by deep RNA sequencing. The findings show that CXCR4<sup>WHIM</sup> mutations are more common in WM than previously revealed, and are primarily subclonal, supporting their acquisition after MYD88<sup>L265P</sup> in WM oncogenesis. The presence of multiple CXCR4<sup>WHIM</sup> mutations within individual WM patients may be indicative of targeted CXCR4 genomic instability.

**Keywords:** Waldenström macroglobulinaemia, IgM MGUS, Marginal Zone Lymphoma, CXCR4, WHIM, MYD88 L265P.

Hunter *et al*, 2014). Mutations in CXCR4 were the second most common somatic variant identified after MYD88<sup>L265P</sup>, and are distinctive for WM. By whole genome sequencing,

somatic mutations in *CXCR4* were detected in 27% of WM patients, and confirmed by Sanger sequencing (Hunter *et al*, 2014). The location of somatic mutations in the C-terminal domain in WM patients is similar to that observed in the germline of patients with WHIM (Warts, Hypogammaglobulinaemia, Infections and Myelokathexis) syndrome, a congenital immunodeficiency disorder characterized by chronic noncyclic neutropenia (Dotta *et al*, 2011).

*CXCR4* plays a critical role in WM cell trafficking to the bone marrow (BM) stroma, wherein protection is afforded to tumour cells against many anti-neoplastic agents (Ngo *et al*, 2008). Over 30 different types of somatic C-terminal mutations in *CXCR4* have been described in WM patients, including frameshift and nonsense variants (Hunter *et al*, 2014; Treon *et al*, 2014). The most common variant is *CXCR4*<sup>S338X</sup>, which represents over 50% of *CXCR4* mutations found in WM patients. A C>A or C>G transversion at nucleotide position 1013 in the *CXCR4* gene results in the generation of a stop codon which leads to truncation of protein at amino acid position 338, and loss of the terminal 15 amino acids of the C-regulatory domain. Both *CXCR4*<sup>S338X</sup> C>A and C>G nonsense mutations are associated with more aggressive disease presentation at diagnosis, including higher BM involvement and serum IgM levels, as well as symptomatic hyperviscosity (Treon *et al*, 2014; Schmidt *et al*, 2015). *In vitro* modelling has shown that, in response to CXCL12 (SDF-1a), *CXCR4*<sup>S338X</sup> engineered cells show muted *CXCR4* receptor internalization, which results in enhanced and prolonged AKT and ERK activation and confers resistance to many anti-neoplastic agents used to treat WM patients, including BTK, PI3Kδ, BCL2 and proteasome inhibitors (Roccaro *et al*, 2014; Cao *et al*, 2015). *CXCR4*<sup>WHIM</sup> mutations are also associated with clinical resistance to the BTK inhibitor ibrutinib in patients with previously treated WM (Treon *et al*, 2015). The detection of *CXCR4*<sup>WHIM</sup> mutations therefore has both diagnostic and therapeutic implications in WM.

The Sanger technique is commonly used to sequence genes although is not very sensitive and often requires a minimum mutation burden of 15–20%. In contrast, allele-specific polymerase chain reaction (AS-PCR) is considerably more sensitive, with a range of detection down to 0.1%, and is easier to adopt and provide interpretive results in a clinical diagnostic setting. We therefore developed quantitative AS-PCR assays to detect the most common *CXCR4*<sup>WHIM</sup> mutation, S338X, which results from C>G and C>A nucleotide substitutions. We then investigated its use in a separate cohort of patients with untreated and previously treated WM, IgM monoclonal gammopathy of unknown significance (MGUS), marginal zone lymphoma (MZL), chronic lymphocytic leukaemia (CLL), multiple myeloma (MM), and non-IgM MGUS and compared these findings against those derived from Sanger sequencing. Finally, we determined the cancer cell fraction (CCF) of *CXCR4* mutations in WM by synchronous quantitative AS-PCR analysis against *MYD88*<sup>L265P</sup>. The findings from these studies detail a highly complex clonal architecture for *CXCR4* mutations, and are indicative of targeted genomic instability for the C-terminal region of this gene in WM patients.

## Patients and methods

### Patient samples

CD19-selected cells derived from BM aspirates of 13 WM patients, and peripheral blood of 12 healthy donors was used in the development of *CXCR4*<sup>S338X</sup> AS-PCR assays. Tumour samples from a separate cohort of WM, IgM and non-IgM MGUS, MZL, CLL, MM (including IgM MM), and healthy donors were then assayed. Consensus criteria were used for WM and IgM MGUS determinations, and their baseline characteristics are depicted in Table I (Owen *et al*, 2003). CD19-selected cells from BM aspirates were isolated, and both DNA and RNA extracted as previously described (Xu

**Table I.** Baseline characteristics for WM and IgM MGUS patients whose samples were evaluated for *CXCR4* mutations by AS-PCR and Sanger sequencing.

	Untreated WM	Previously Treated WM	IgM MGUS
N	102	62	12
Age (years)	62 (33–88)	63 (44–86)	69 (56–82)
Gender (Male/Female)	58/44	48/15	6/6
Serum IgM (g/l)	26.70 (2.70–86.30)	36.10 (7.35–83.90)	3.97 (1.42–16.40)
Haemoglobin (g/l)	117 (48–155)	105 (82–138)	134 (119–163)
Serum β <sub>2</sub> -microglobulin (mg/l)	2.9 (1.0–9.5)	3.9 (1.3–14.2)	1.9 (1.7–3.4)
Adenopathy (≥ 1.5 cm)	34 (33.3%)	37 (58.7%)	0 (0%)
Splenomegaly (≥15 cm)	15 (14.7%)	7 (11.1%)	0 (0%)
Bone marrow involvement (%) by IHC	40 (5–95)	60 (3–95)	0 (0%) (0–0)
<i>MYD88</i> <sup>L265P</sup> positive	97 (95.1%)	55 (89%)	6 (50%)

WM, Waldenström Macroglobulinaemia; IgM, immunoglobulin M; MGUS, monoclonal gammopathy of unknown significance; AS-PCR, allele-specific polymerase chain reaction; IHC, immunohistochemistry.

Values are shown as median (range) unless otherwise indicated.

*et al*, 2013, 2014). MYD88 mutation status was determined by quantitative AS-PCR and Sanger sequencing (Xu *et al*, 2013). Subject participation was approved by the Harvard Cancer Center/Dana-Farber Cancer Institute Institutional Review Board. All participants provided written consent.

#### *Development of quantitative AS-PCR assays for CXCR4<sup>S338X</sup> mutations*

Since CXCR4<sup>S338X</sup> mutations occur due to either C>G or C>A transversions at nucleotide position 1013, we developed two AS-PCR assays to permit their detection. The details for the development of these assays are presented in the online supplement.

#### *Sanger sequencing of CXCR4 C-terminal domain*

The forward PCR primer 5'- ATG GGG AGG AGA GTT GTA GGA TTC TAC -3' and reverse PCR primer 5'- TTG GCC ACA GGT CCT GCC TAG ACA-3' were designed to amplify the CXCR4 open reading frame. Amplified PCR products were isolated by QIA quick gel extraction kit (Qiagen, Valencia, CA) and sequenced using both forward and reverse PCR primers and an additional sequencing primer 5'- GCTGCCTTACTACATTGGGATCAGC-3'.

#### *Cloning and sequencing*

The CXCR4 forward PCR primer 5'- ATG GGG AGG AGA GTT GTA GGA TTC TAC -3' and reverse PCR primer 5'- TTG GCC ACA GGT CCT GCC TAG ACA-3' were used to amplify the gene fragment. TOPO Cloning Kits were used as per manufacturer's protocol (Thermo Fisher Scientific Inc., Grand Island, NY).

#### *Confirmatory deep RNA sequencing*

Deep RNA sequencing of CD19-selected cells from BM aspirates was performed to validate findings in five patients with multiple CXCR4<sup>WHIM</sup> mutations. RNA sequencing data was generated from 50 cycle HiSeq paired-end sequences (Illumina Inc., San Diego, CA) and aligned to HG19/GRCh37 ensemble genome reference using STAR (<https://github.com/alexdobin/STAR/>). Reads supporting each call were calculated using Integrative Genomics Viewer (IGV; Broad Institute, Cambridge, MA, USA). The median number of reads mapping to the CXCR4 C-terminal domain per patient was 8208 (range 5316–12 235).

#### *Cancer cell fraction analysis for CXCR4 mutations in WM and IgM MGUS patients*

Cancer cell fraction analysis for CXCR4<sup>S338X</sup> mutations was performed using synchronous, parallel quantitative AS-PCR analyses for MYD88<sup>L265P</sup> and CXCR4<sup>S338X C>A or C>G</sup> in

sorted CD19-cells derived from BM aspirates from 21 untreated WM and IgM MGUS patients expressing MYD88<sup>L265P</sup> and CXCR4<sup>S338X C>A and/or C>G</sup> mutations. A highly sensitive quantitative MYD88<sup>L265P</sup> AS-PCR was used (Xu *et al*, 2013). Standard curves were established for each AS-PCR assay that was run on the same plate for each sample, and cell count expressive of these mutations was determined by standard curves and  $\Delta C_T$ . The CCF was determined as the ratio of cells expressing CXCR4<sup>S338X C>A or C>G</sup>/MYD88<sup>L265P</sup>. MYD88 and CXCR4 copy number was determined using TaqMan Copy Number Assays (Applied Biosystems, Grand Island, NY, USA).

#### *Statistical analysis*

Estimates of sensitivity, specificity and predictive values were performed using VassarStats (Poughkeepsie, NY, USA).

## Results

#### *Development of AS-PCR assays for CXCR4<sup>S338X C>A</sup> and CXCR4<sup>S338X C>G</sup> detection*

Given that CXCR4<sup>S338X</sup> mutations can result from either C>A or C>G transversions at nucleotide position 1013, and are the most common WHIM-like mutations in WM, we developed and validated sensitive real-time AS-PCR assays for detecting these mutations in samples obtained from WM patients and healthy donors. The details for the development of these assays are presented in the online supplement. In a separate cohort of 102 untreated WM patients whose mutation status was established by Sanger sequencing, the sensitivity for the CXCR4<sup>S338X C>A</sup> AS-PCR assay was 100% (95% confidence interval [CI] 39.5–100%) and specificity was 100% (95% CI 95.2–100%). For the CXCR4<sup>S338X C>G</sup> AS-PCR assay, the sensitivity was 100% (95% CI 67.8–100%) and specificity was 100% (95% CI 94.9–100%).

#### *Detection of CXCR4<sup>WHIM</sup> mutations, including multiple mutations within individual patients using combined AS-PCR assays and Sanger sequencing*

We next applied the CXCR4<sup>S338X C>A</sup> and CXCR4<sup>S338X C>G</sup> AS-PCR assays using the standard curve established in tumour-containing samples from a separate cohort of 164 WM (102 untreated, 62 previously treated), 12 IgM MGUS, 20 MZL, 32 CLL, 14 MM (including 2 IgM MM), and 7 non-IgM MGUS patients, as well as 32 healthy donors. All samples underwent Sanger sequencing. Sanger sequencing of BM CD19-selected samples from WM patients revealed CXCR4<sup>WHIM</sup> mutations that included nonsense or frameshift mutations in 37/102 (36.3%) of untreated and 17/62 (27.4%) previously treated WM patients (Table II). Sanger tracings showed the presence of multiple CXCR4<sup>WHIM</sup> mutations within each of three untreated patients (WM1-WM3)

**Table II.** *CXCR4* C-terminal domain mutations detected by Sanger sequencing in 164 WM patients.

N	Treatment status	<i>CXCR4</i> Mutation status	Nucleotide change	Amino acid change	Other nucleotide change	Other amino acid change
65	Untreated	WT	None	None		
1	Untreated	FS	r.931_933insT	T311 fs		
1	Untreated	FS	r.951_953delACCTC	T318 fs		
3	Untreated	FS	r.952_954insA	T318 fs		
1	Untreated	FS	r.954_956insC	S319 fs		
1	Untreated	FS	r.978_980insT	K327 fs		
1	Untreated	FS	r.984_986insT	L329 fs		
1	Untreated	NS	r.993 G>T	G332X		
1	Untreated	FS/NS	r.993_994insA	G332 fs	r.1013C>G	S338X
1	Untreated	NS	r.997 A>T	K333X	r.1013C>G	S338X
1	Untreated	NS	r.1000C>T	R334X		
1	Untreated	FS	r.1005_1007insT	G336 fs		
1	Untreated	FS	r.1012_1014insT	S338 fs		
3	Untreated	NS	r.1013C>A	S338X		
6	Untreated	NS	r.1013C>G	S338X		
3	Untreated	NS	r.1013C>G	S338X	r.1013C>A	S338X
1	Untreated	FS/NS	r.1013C>G	S338X	r.1012_1014insT	S338 fs
2	Untreated	FS	r.1013_1015delATCT	S338 fs		
1	Untreated	FS	r.1013_1015delATCTGTTTCCACTGAGT	S338 fs		
1	Untreated	FS	r.1016_1017insT	S339 fs		
1	Untreated	FS	r.1016_1017insC	S339 fs		
2	Untreated	FS	r.1020_1022delT	S341 fs		
1	Untreated	FS	r.1020_1023delTC	S341 fs		
1	Untreated	FS	r.1033_1035delAG	E345 fs		
1	Untreated	FS	r.1034_1035insG	E345 fs		
45	Treated	WT	None	None		
1	Treated	FS	r.969_971insG	S324 fs		
2	Treated	NS	r.1000C>T	S334X		
2	Treated	FS	r.1012_1014insT	S338 fs		
5	Treated	NS	r.1013C>A	S338X		
7	Treated	NS	r.1013C>G	S338X		

WM, Waldenström Macroglobulinaemia; WT, Wild-type; NS, nonsense mutation; FS, frameshift mutation.

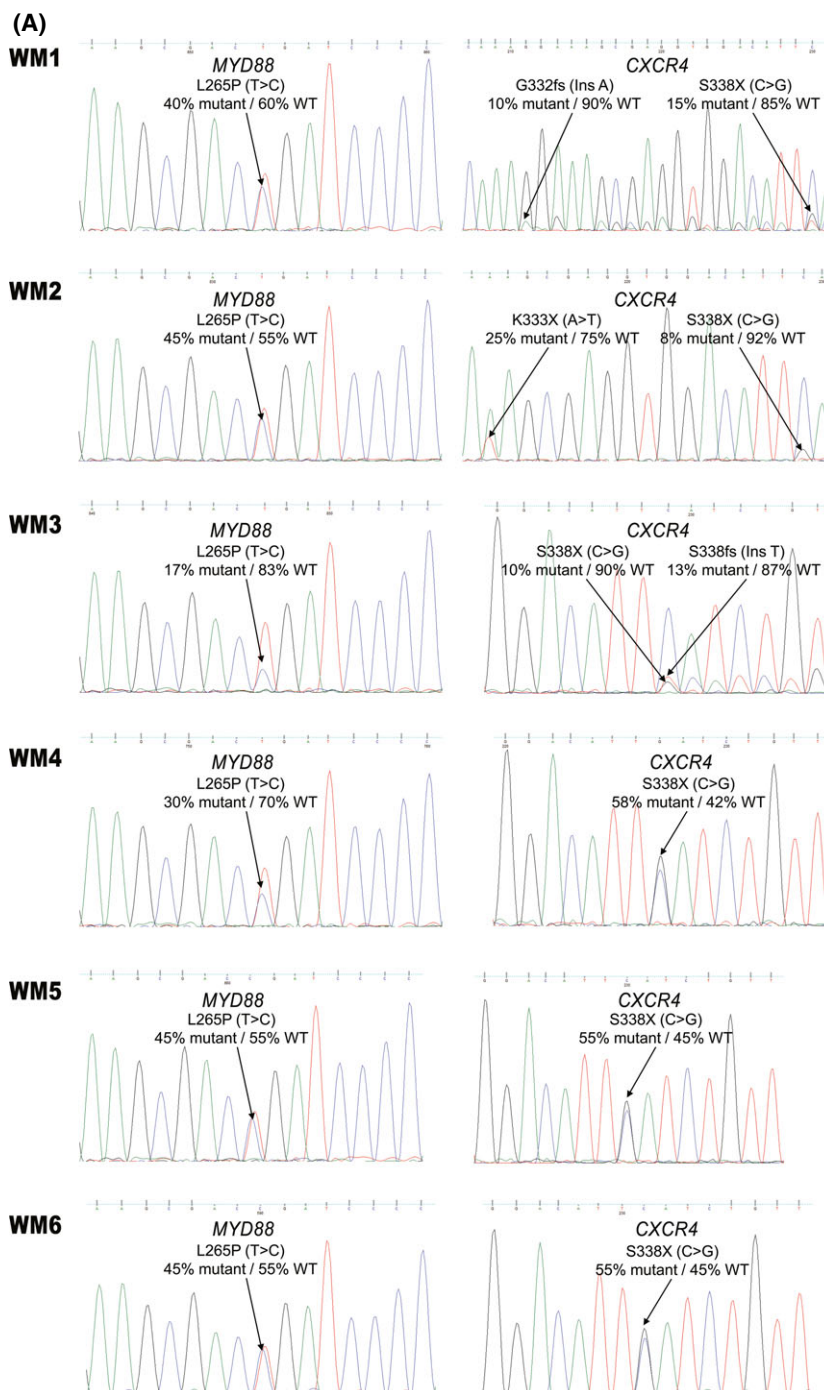
(Fig 1A). Cloning and sequencing studies indicated that the *CXCR4*<sup>WHIM</sup> mutations were compound heterozygous for all three of these patients, with 3/44 and 12/44 clones expressing *CXCR4*<sup>G332 fs</sup> and *CXCR4*<sup>S338X</sup>, respectively for WM1; 12/43 and 6/43 clones expressing *CXCR4*<sup>K333X</sup> and *CXCR4*<sup>S338X</sup>, respectively, for WM2; and 2/40 and 8/40 clones expressing *CXCR4*<sup>S338X</sup> and *CXCR4*<sup>S338Xfs</sup>, respectively, for WM3 (Fig 1B). Deep RNA sequencing performed in one of these patients (WM3) also showed map reads consistent with a compound heterozygous mutation (Table III). Sanger tracings in three other untreated patients (WM4-WM6) showed an allele burden for *CXCR4*<sup>S338X</sup> that exceeded that of the wild-type *CXCR4* allele. The *MYD88*<sup>L265P</sup> allele burden for these individuals was less than wild-type *MYD88*, a finding highly suggestive of homozygous *CXCR4*<sup>S338X</sup> expression. Deep RNA sequencing performed in one of these patients (WM4) also showed map readings supporting the existence of a homozygous *CXCR4*<sup>S338X</sup> subclone (Table III). No copy number variant was detected in the *CXCR4* region for any of the above patients (data not shown).

Among the 102 untreated WM patients, the AS-PCR assay for *CXCR4*<sup>S338X C>A</sup> identified 11 patients with this mutation (Fig 2). This included 4 patients previously identified as having this variant by Sanger sequencing, as well as 7 patients who were wild-type by Sanger sequencing (Fig 3). Among the same cohort of 102 untreated patients, the AS-PCR assay for *CXCR4*<sup>S338X C>G</sup> identified 20 patients with this mutation (Fig 2). This included 11 patients previously identified as having this variant by Sanger sequencing, as well as 9 patients who were wild-type by Sanger sequencing (Fig 3). Five of these patients had both *CXCR4*<sup>S338X C>A</sup> and *CXCR4*<sup>S338X C>G</sup> mutations detected by the AS-PCR assays. In total, 7 individuals among the 65 untreated patients whose Sanger sequencing showed wild-type *CXCR4* were identified as having a *CXCR4*<sup>WHIM</sup> mutation by AS-PCR. Taken together, the results of AS-PCR and Sanger sequencing revealed that 44/102 (43%) of untreated patients had *CXCR4*<sup>WHIM</sup> mutated disease (Table IV), with multiple mutations within 13/44 (31%) of these patients. Deep RNA sequencing confirmed the presence of compound heterozygous mutations in 3 patients (WM7-WM9)

whose samples were used for validation (Table III). Forty-three of the 44 (97.7%) untreated *CXCR4*<sup>WHIM</sup> WM patients were also positive for the *MYD88*<sup>L265P</sup> mutation by AS-PCR. Sanger sequencing did not reveal any other *MYD88* mutations in the sole patient who did not express the *MYD88*<sup>L265P</sup> mutation by AS-PCR.

Among the 62 previously treated WM patients, the AS-PCR assay for *CXCR4*<sup>S338X C>A</sup> identified seven patients with this mutation (Fig 2). This included five patients who had this variant by Sanger sequencing, as well as two patients

who were wild-type by Sanger sequencing (Fig 3). Among the same cohort of 62 previously treated WM patients, the AS-PCR assay for *CXCR4*<sup>S338X C>G</sup> identified 9 patients with this mutation (Fig 2). This included seven patients who had this variant by Sanger sequencing, as well as two patients who were wild-type by Sanger sequencing (Fig 3). In total, four additional individuals were identified as having a *CXCR4*<sup>WHIM</sup> mutation among 45 previously treated WM patients who were wild-type by Sanger sequencing. Combining the results of AS-PCR and Sanger sequencing revealed



**Fig 1.** Sanger tracings from CD19-selected cells derived from bone marrow aspirates of untreated WM patients showing compound heterozygous and homozygous *CXCR4*<sup>WHIM</sup> mutations. (A) Sanger tracings showed the presence of multiple *CXCR4*<sup>WHIM</sup> mutations in 3 Waldenström Macroglobulinaemia (WM) patients (WM1, WM2, WM3), and suggestive of homozygous *CXCR4*<sup>WHIM</sup> mutations in another 3 WM patients (WM4, WM5, WM6). The estimated percentage of mutant and wild-type (WT) allele burden for *MYD88* and *CXCR4* are shown. (B) Representative Sanger tracings from cloning and sequencing studies for the 3 WM patients (WM1, WM2, WM3) with multiple *CXCR4*<sup>WHIM</sup> mutations are shown, with mutant alleles shown in shaded areas. TA cloning and sequencing studies showed 3/44 and 12/44 clones to express *CXCR4*<sup>G332fs</sup> and *CXCR4*<sup>S338X</sup>, respectively for WM1; 12/43 and 6/43 clones to express *CXCR4*<sup>K333X</sup> and *CXCR4*<sup>S338X</sup>, respectively for WM2; and 2/40 and 8/40 clones to express *CXCR4*<sup>S338X</sup> and *CXCR4*<sup>S338fs</sup>, in WM3, respectively.

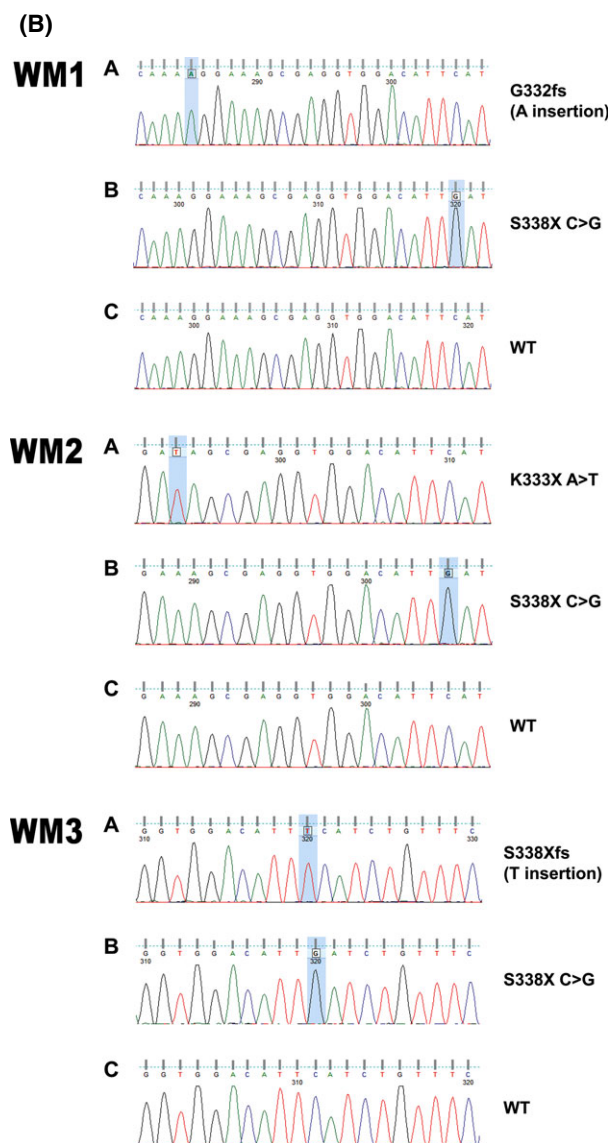


Fig 1. Continued.

that 21/62 (34%) of untreated patients had  $CXCR4^{WHIM}$  mutated disease (Table IV), with multiple mutations present in 4/21 (19%) of these patients. All 21 of the previously treated  $CXCR4^{WHIM}$  mutated patients were also positive for the  $MYD88^{L265P}$  mutation by AS-PCR.

In 12 patients with IgM MGUS, the  $CXCR4^{S338X C>A}$  AS-PCR assay did not detect any mutations (Fig 2). However, the  $CXCR4^{S338X C>G}$  AS-PCR assay detected this mutation in 2 of 12 (17%) patients with IgM MGUS (Fig 2). All 12 of these IgM MGUS patients were negative for  $CXCR4^{WHIM}$  mutations by Sanger sequencing. Both IgM MGUS patients who were found to have the  $CXCR4^{S338X C>G}$  mutation by AS-PCR were also positive for  $MYD88^{L265P}$  by AS-PCR.

Sample analysis using both AS-PCR assays failed to detect the  $CXCR4^{S338X C>A}$  or  $C>G$  variants in any of the tumour samples taken from the 20 MZL, 32 CLL and 14 MM patients, nor in any of 32 healthy donors (Fig 2). Sanger sequencing was also performed in these samples, and was remarkable for one (5%) MZL patient having a  $CXCR4^{S344 fs}$  mutation that resulted from r.1030\_1031insT (Table IV). This patient was wild-type for  $MYD88$  by both AS-PCR assay for  $MYD88^{L265P}$ , and also by Sanger sequencing of the entire  $MYD88$  gene.

*$CXCR4^{S338X}$  mutations are primarily subclonal with highly variable clonal distribution in WM and IgM MGUS patients*

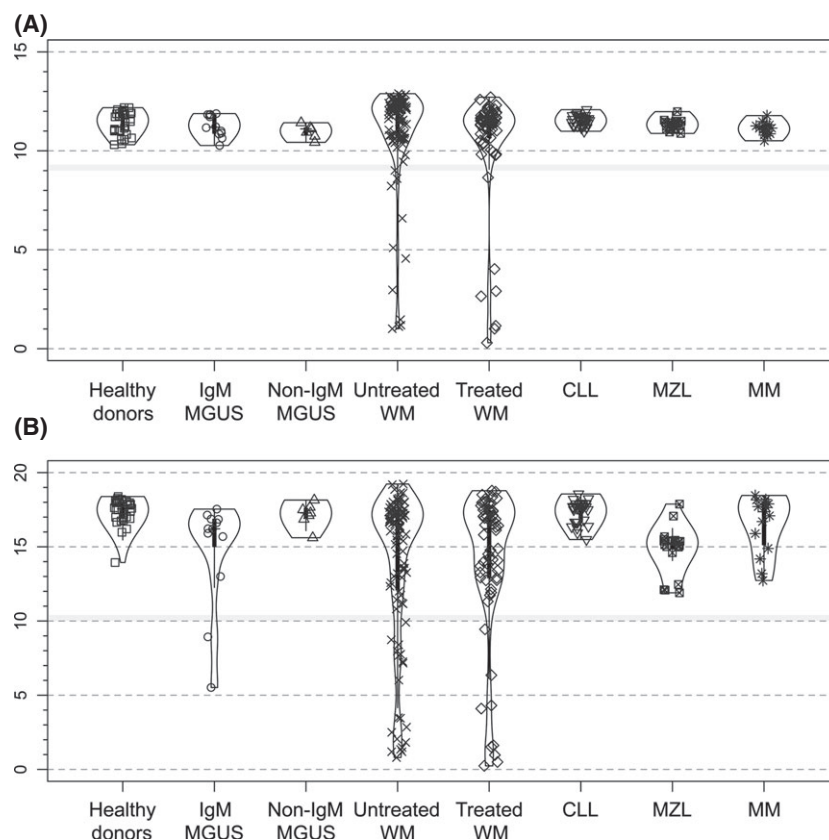
$MYD88^{L265P}$  expression levels, as determined by AS-PCR analysis, show strong correlation with tumour cell content in BM specimens from WM patients (Xu et al, 2013, 2014). To clarify the CCF of  $CXCR4$  mutations in WM, parallel quantitative AS-PCR analyses for  $MYD88^{L265P}$  and  $CXCR4^{S338X C>A}$  or  $C>G$  were performed using tumour samples from 21 untreated WM and 2 IgM MGUS patients who were known to express the  $MYD88^{L265P}$  and  $CXCR4^{S338X C>A}$  and/or  $C>G$  mutations. The cell fraction expressive of these mutations

Table III. Validation studies using deep RNA sequencing in CD19-selected cells from bone marrow aspirates of 5 WM patients with multiple  $CXCR4$  mutations identified by AS-PCR and Sanger sequencing.

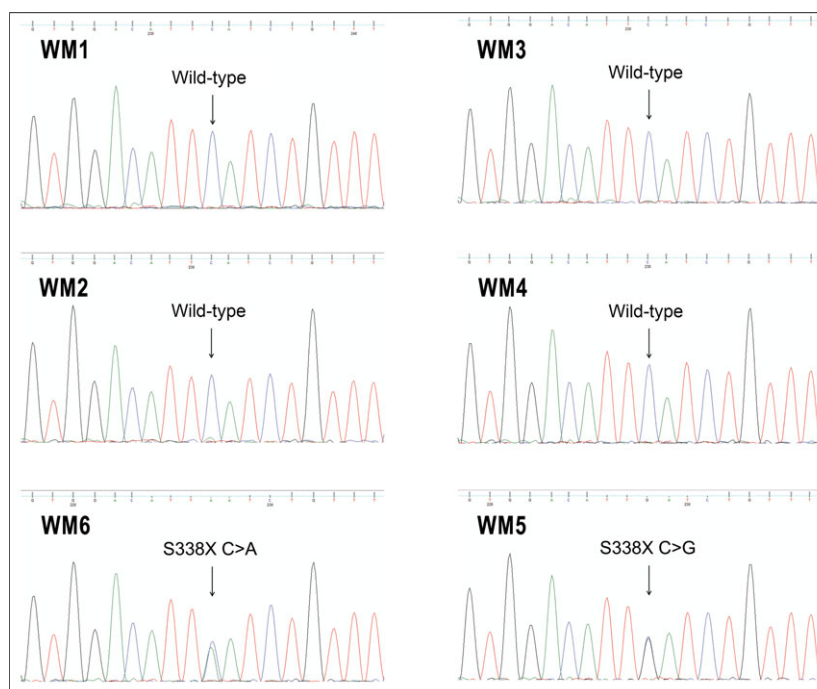
	$\Delta C_T$ $CXCR4^{S338X C>A}$	AS-PCR $CXCR4^{S338X C>A}$	$\Delta C_T$ $CXCR4^{S338X C>G}$	AS-PCR $CXCR4^{S338X C>G}$	Sanger Sequencing	RNASeq Reads	RNASeq Findings
WM3	10-48	Negative	3-45	Positive	$CXCR4^{S338X C>G}$ $CXCR4^{S338 fs}$	5316 5217	r.1013 C>G 10% r.1012_1014 insT 5%
WM4	10-83	Negative	1-19	Positive	$CXCR4^{S338X C>G}$	12235	r.1013 C>G 56%
WM7	5-10	Positive	8-73	Positive	$CXCR4^{WT}$	8208	r.1013 C>A 6% r.1013 C>G 1%
WM8	4-56	Positive	7-16	Positive	$CXCR4^{WT}$	8766	r.1013 C>A 5% r.1013 C>G 1%
WM9	2-97	False Positive	7-26	Positive	$CXCR4^{S339 fs}$	5494 5819	r.1013 C>G 3% r.1016_1017 insT 18%

WM, Waldenström Macroglobulinaemia; AS-PCR, allele-specific polymerase chain reaction.

**Fig 2.** Real-time AS-PCR results for  $CXCR4^{S338X\ C>A}$  or  $CXCR4^{S338X\ C>G}$  variants in samples from healthy donors, IgM MGUS, non-IgM MGUS, untreated and previously treated WM, CLL, MZL and MM patients. Violin plot representing allele-specific polymerase chain reaction (AS-PCR) differences in cycle threshold ( $\Delta C_T$ ) for samples evaluated for  $CXCR4^{S338X\ C>A}$  (A) and  $CXCR4^{S338X\ C>G}$  (B) variants. The span of grey 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 health donors ( $n = 32$ ), as well as immunoglobulin M monoclonal gammopathy of unknown significance (IgM MGUS;  $n = 12$ ), non-IgM MGUS ( $n = 7$ ), untreated Waldenström Macroglobulinaemia (WM) ( $n = 102$ ), previously treated WM ( $N = 62$ ), marginal zone lymphoma (MZL;  $n = 20$ ), chronic lymphocytic leukaemia (CLL;  $n = 32$ ) and multiple myeloma (MM;  $n = 14$ ) patients. Gray lines denote  $\Delta c_t$  cut-off values established for each AS-PCR assay.



**Fig 3.** Sanger tracings in CD19-selected cells from bone marrow aspirates of patients with  $CXCR4^{WT}$  and who demonstrated  $CXCR4^{S338X}$  mutations by allele-specific polymerase chain reaction assays. Sanger tracings show four patients with  $CXCR4^{WT}$  who demonstrated  $CXCR4^{S338X\ C>A}$  (WM1, WM2) and  $CXCR4^{S338X\ C>G}$  (WM3, WM4). Sanger tracings for two patients with  $CXCR4^{S338X\ C>A}$  (WM5) and  $CXCR4^{S338X\ C>G}$  (WM6) are shown for comparison.



was determined by  $\Delta C_T$  and standard curves for  $MYD88^{L265P}$  and  $CXCR4^{S338X\ C>A}$  or  $C>G$  that were run on the same plate for each sample. The CCF was determined as the ratio of cells expressing  $CXCR4^{S338X\ C>A}$  or  $C>G$ / $MYD88^{L265P}$ . No copy

number alterations for  $MYD88$  and  $CXCR4$  were found in this cohort by TaqMan Copy Number Assays.

Cancer cell fraction analysis for all (WM and IgM MGUS) patients showed  $CXCR4^{S338X}$  mutations were primarily

**Table IV.** *MYD88* and *CXCR4* mutation status in patients with WM, IgM MGUS, MZL, CLL, MM and non-IgM MGUS patients.

	N	<i>MYD88</i> <sup>L265P</sup>	<i>CXCR4</i> <sup>WHIM</sup>
Healthy Donors	32	0 (0%)	0 (0%)
IgM MGUS	12	6 (50%)	2 (17%)
Non-IgM MGUS	7	0 (0%)	0 (0%)
Untreated WM	102	97 (95%)	44 (43%)
Treated WM	62	57 (92%)	21 (34%)
MZL	20	2 (10%)	1 (5%)
CLL	32	1 (3%)	0 (0%)
MM	14	0 (0%)	0 (0%)

WM, Waldenström Macroglobulinaemia; IgM, immunoglobulin M; MGUS, monoclonal gammopathy of unknown significance; MZL, marginal zone lymphoma; CLL, chronic lymphocytic leukaemia; MM, multiple myeloma; WHIM, Warts, Hypogammaglobulinemia, Infections and Myelokathexis; AS-PCR, allele-specific polymerase chain reaction.

*CXCR4* mutation status includes all WHIM mutations identified by *CXCR4*<sup>S338X C>A</sup> and *CXCR4*<sup>S338X C>G</sup> AS-PCR and Sanger sequencing.

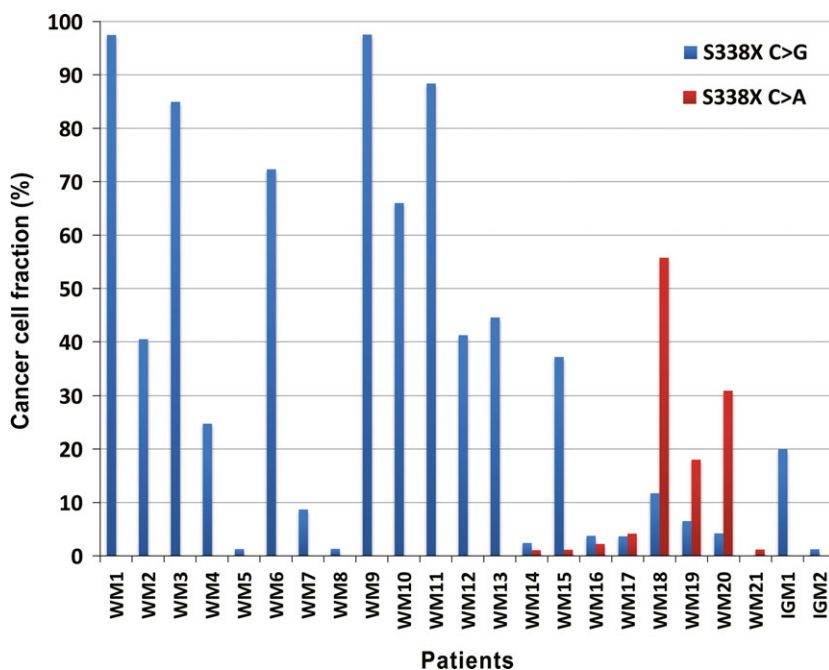
subclonal, with highly variable clonal distribution (median 35.1%, range 1.2–97.5%) (Fig 4). For the 13 WM patients who expressed *CXCR4*<sup>S338X C>G</sup> only, the fraction of cells expressing *CXCR4*<sup>S338X C>G</sup> relative to *MYD88*<sup>L265P</sup> was 44.5% (range 1.2–97.5%). For 7 WM patients who expressed both *CXCR4*<sup>S338X C>G</sup> and *CXCR4*<sup>S338X C>A</sup>, the fraction of cells expressing *CXCR4*<sup>S338X C>G</sup> relative to *MYD88*<sup>L265P</sup> was 4.2% (range 2.4–37.2%) and for *CXCR4*<sup>S338X C>A</sup> to *MYD88*<sup>L265P</sup>, it was 4.1% (range 1.1–55.8%). In one patient who expressed only *CXCR4*<sup>S338X C>A</sup>, the fraction of cells expressing *CXCR4*<sup>S338X C>A</sup> relative to *MYD88*<sup>L265P</sup> was 1.2%. For the two IgM MGUS patients who expressed

*CXCR4*<sup>S338X C>G</sup>, the fraction of cells expressing *CXCR4*<sup>S338X C>G</sup> relative to *MYD88*<sup>L265P</sup> was 21.8% and 1.2%.

## Discussion

Despite the common dysregulation of *CXCR4* in many solid and haematological malignancies, somatic mutations in this gene remain largely distinctive of WM. In WM, *CXCR4*<sup>WHIM</sup> somatic mutations are important determinants of WM disease presentation, as well as treatment outcome (Treon *et al*, 2014, 2015; Schmidt *et al*, 2015). We therefore investigated the clonal architecture of *CXCR4*<sup>WHIM</sup> mutations in WM patients using Sanger sequencing and quantitative AS-PCR assays, which detect the most common *CXCR4*<sup>WHIM</sup> mutation variants (*CXCR4*<sup>S338X C>A</sup> and *CXCR4*<sup>S338X C>G</sup>), as well as by deep RNA sequencing.

The AS-PCR assays developed for these studies showed high levels of specificity and sensitivity for the *CXCR4*<sup>S338X C>A</sup> and *CXCR4*<sup>S338X C>G</sup> detection, and discriminated samples bearing their target variants from those samples with *CXCR4*<sup>WT</sup>, *CXCR4*<sup>WHIM</sup> frameshift and other nonsense mutations. Importantly, the AS-PCR assays identified *CXCR4*<sup>WHIM</sup> mutations in many WM and IgM MGUS patients who were identified as *CXCR4*<sup>WT</sup> by Sanger sequencing. At first glance, the findings might suggest that the tumour burden for these patients was under the detection limit for *CXCR4*<sup>S338X</sup> by Sanger sequencing. However, the BM burden for these 11 individuals with WM who were identified as having *CXCR4*<sup>S338X</sup> by AS-PCR (but not Sanger sequencing) was 40% (range 5–70%), and the median CD19<sup>+</sup> cell clonality by light chain restriction analysis was 89.8% (range 4.2–100%). These findings would suggest that *CXCR4*<sup>S338X</sup> mutations in these patients are likely



**Fig 4.** Cancer cell fraction analysis for *CXCR4*<sup>S338X</sup> expression in CD19-selected cells from bone marrow aspirates of WM patients. The clonal penetrance of *CXCR4*<sup>S338X</sup> mutations in Waldenström Macroglobulinaemia (WM) was determined in 21 untreated WM and 2 immunoglobulin M monoclonal gammopathy of unknown significance (IgM MGUS) patients with known *CXCR4*<sup>S338X</sup> and *MYD88*<sup>L265P</sup> mutations. The cancer cell fraction for *CXCR4*<sup>S338X C>A</sup> and/or *CXCR4*<sup>S338X C>G</sup> expression relative to *MYD88*<sup>L265P</sup> is shown. *CXCR4*<sup>S338X C>G</sup> was expressed in patients WM1–WM20, and IgM MGUS 1 and 2; *CXCR4*<sup>S338X C>A</sup> was expressed in patients WM14–21, with both *CXCR4*<sup>S338X C>G</sup> and *CXCR4*<sup>S338X C>A</sup> present in WM14–20.



to be subclonal. To affirm these findings, a CCF analysis was performed by synchronous AS-PCR analysis of *MYD88*<sup>L265P</sup> and *CXCR4*<sup>S338X</sup> that included untreated WM and IgM MGUS patients. The results from this study showed that for most untreated WM, as well as IgM MGUS patients, *CXCR4*<sup>S338X</sup> mutations are subclonal with highly variable clonal distribution. The subclonal existence of *CXCR4*<sup>S338X</sup>, as well as other *CXCR4*<sup>WHIM</sup> mutations identified by Sanger, as well as deep RNA sequencing studies in WM and IgM MGUS patients supports that *CXCR4*<sup>WHIM</sup> mutations are probably an early oncogenic event that follows acquisition of *MYD88*<sup>L265P</sup>. The finding of *CXCR4*<sup>S338X</sup> mutations in IgM MGUS has also been shown by another group, though *MYD88* mutation status for these patients was not reported (Roccaro *et al*, 2014). In our series, both *MYD88*<sup>L265P</sup> and *CXCR4*<sup>WHIM</sup> mutation status was determined for all patients. Between 50% and 80% of IgM MGUS patients harbour the *MYD88*<sup>L265P</sup> somatic mutation, and the presence of *MYD88*<sup>L265P</sup> is associated with a higher rate of evolution to malignancy, including WM and MZL (Landgren & Staudt, 2012; Jiménez *et al*, 2013; Varettoni *et al*, 2013; Xu *et al*, 2013). Both IgM MGUS patients with the *CXCR4*<sup>S338X</sup> mutation in our series also expressed the *MYD88*<sup>L265P</sup> mutation, and co-expression of both *CXCR4*<sup>WHIM</sup> and *MYD88*<sup>L265P</sup> mutations is nearly universal in WM (Hunter *et al*, 2014; Treon *et al*, 2014). It is therefore plausible that co-expression of *CXCR4*<sup>WHIM</sup> and *MYD88*<sup>L265P</sup> mutations may facilitate progression of IgM MGUS to WM in a subset of patients. Further studies are needed to help expand on these findings and hypotheses. It is interesting that the one MZL patient had a *CXCR4*<sup>S344 fs</sup> mutation but was *MYD88*<sup>WT</sup>. Similarly, Martínez *et al* (2014) identified a *CXCR4*<sup>R334X</sup> nonsense mutation in one of 15 MZL patients, who was also wild-type for *MYD88*. *MYD88*<sup>L265P</sup> is uncommon in MZL patients with an estimated frequency of 6–10% (Ngo *et al*, 2011; Trøen *et al*, 2013). Additionally, determination of both *MYD88* and *CXCR4* mutation status may help in further discriminating WM from MZL and other overlapping B-cell malignancies, which often share similar morphological, immunophenotypic, cytogenetic and clinical findings (Swerdlow *et al*, 2008; Arcaini *et al*, 2011; Kyrtsolis *et al*, 2011).

A remarkable but surprising finding when using the AS-PCR assays with Sanger sequencing was the common occurrence of multiple *CXCR4* mutation types within individual patients. A third of WM patients harboured multiple mutations at initial presentation that included both nonsense and frameshift mutations, findings that were supported by next generation sequencing. The clinical significance for these findings remains to be clarified, and larger studies with longitudinal follow-up will invariably be required to delineate their importance. However, the common presence of multiple *CXCR4*<sup>WHIM</sup> mutation types in many WM patients is suggestive of targeted genomic instability within the C-terminal regulatory domain and warrants further investigation.

The findings of this study may also have implications for the management of WM patients given the importance

of *CXCR4* mutations in disease presentation and treatment response. The use of targeted deep next generation sequencing may provide the most comprehensive assessment of nonsense and frameshift mutations, including the presence of multiple *CXCR4* mutations in WM patients. Longitudinal studies to address clonal evolution in patients with single and multiple *CXCR4* mutations will also be interesting, particularly with targeted and highly selective agents, such as ibrutinib. It is interesting that fewer previously treated versus untreated patients harboured *CXCR4*<sup>WHIM</sup> mutations in our study, a finding that will invariably need further validation in a larger study cohort. However, these findings may allude to therapies that differentially impact *CXCR4*<sup>WHIM</sup> expressing clones, and longitudinal studies examining evolution of *CXCR4*<sup>WHIM</sup> clones across various WM therapies may be illuminating. The use of *CXCR4* antagonists has also been proposed for *CXCR4*<sup>WHIM</sup> WM patients following encouraging preclinical data, and a clinical trial combining the anti-*CXCR4* antibody ulocuplomb with ibrutinib is being initiated in WM patients (Roccaro *et al*, 2014; Cao *et al*, 2015). The development of assays that comprehensively evaluate *CXCR4*<sup>WHIM</sup> mutations could also help identify candidates for *CXCR4* antagonist therapy.

In summary, our findings show that *CXCR4*<sup>WHIM</sup> mutations are more common in WM patients than previously revealed by whole genome sequencing or Sanger sequencing. Moreover, *CXCR4* mutations are primarily subclonal, with highly variable clonal distribution among *CXCR4*<sup>WHIM</sup> mutated WM patients. The subclonal existence of *CXCR4*<sup>WHIM</sup> mutations in WM, as well as IgM MGUS patients, supports that the acquisition of *CXCR4*<sup>WHIM</sup> mutations is likely to be an early oncogenic event, but follows acquisition of *MYD88*<sup>L265P</sup>. Lastly, the presence of multiple *CXCR4*<sup>WHIM</sup> mutations in many WM patients may be indicative of targeted *CXCR4* genomic instability and warrant further study.

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## Author contributions

SPT, LX and ZRH designed the study. MLP, RA, SK, JJC, AT, AT, AG, EM, MV, LA, JB, YT, JS, RC, NM, KCA and SPT collected study samples and data. LX, NT, YC, GY, JC, XL, JC, JZ and KA processed tumour samples and performed *CXCR4* genotyping studies. LX, ZRH and SPT

analysed the study data. SPT, LX and ZRH wrote the manuscript.

## Conflicts of interest

No conflicts of interest are identified by the investigators for this work.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Development of quantitative AS-PCR assays for CXCR<sup>S338X</sup> mutations.