bjh short report

Bone marrow involvement and subclonal diversity impairs detection of mutated *CXCR4* by diagnostic next-generation sequencing in Waldenström macroglobulinaemia

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Summary

CXCR4 mutations impact disease presentation and treatment outcomes in Waldenström macroglobulinaemia (WM). Non-uniform testing for *CXCR4* mutations may account for discordant findings in WM clinical trials. We compared two approaches used in these trials for detection of the most common *CXCR4* (S338X) variant: targeted next-generation sequencing (NGS) using unselected bone marrow (BM) samples, and combined allele-specific polymerase chain reaction (AS-PCR) and Sanger sequencing with unselected and CD19-selected BM samples. Our findings showed that targeted NGS frequently yielded false-negative results. Both CD19 selection and AS-PCR markedly improved detection of $CXCR4^{S338X}$ mutations. Sensitivity was adversely impacted by low BM involvement and *CXCR4* mutation clonality.

Keywords: Waldenström macroglobulinemia, CXCR4, ibrutinib, zanubrutinib, next generation sequencing.

Activating somatic mutations in *CXCR4* are present in 30–40% of patients with Waldenström macroglobulinaemia (WM), and represent the second most prevalent somatic variant after *MYD88*.[1,2] *CXCR4* mutations ($CXCR4^{MUT}$) are typically subclonal to mutated *MYD88* with highly variable clonality, and more than 40 different $CXCR4^{MUT}$ have been reported, including nonsense and frameshift variants.[3,4] In WM patients, the $CXCR4^{MUT}$ subtype can differentially impact disease presentation and treatment outcome. Patients with nonsense $CXCR4^{MUT}$ have high serum IgM levels and an increased risk of symptomatic hyperviscosity.[4] The presence of $CXCR4^{MUT}$, particularly nonsense variants, can also impact depth of response and progression-free survival with Bruton tyrosine kinase (BTK) inhibitor-based therapy.[5-8] Therefore, identifying $CXCR4^{MUT}$ has diagnostic, predictive and treatment selection roles in WM.

Non-uniform testing for $CXCR4^{MUT}$ has complicated the interpretation of findings from clinical trials evaluating BTK inhibitors in WM patients, wherein variable $CXCR4^{MUT}$ detection rates and treatment impact have been reported.[5-8] The original studies that established the incidence of $CXCR4^{MUT}$ in WM used CD19-selected BM samples to maximize sensitivity. A combination of allele-specific polymerase chain reaction (AS-PCR) and Sanger sequencing was then used for detection of nonsense and frameshift $CXCR4^{MUT}$, respectively.[2-4] This same approach was also used to identify $CXCR4^{MUT}$ in the pivotal trial that led to the regulatory approval of ibrutinib for WM.[5] However, many subsequent clinical trials have used unselected BM samples and targeted NGS to permit efficient evaluation of both nonsense and frameshift $CXCR4^{MUT}$.[7,9] As such, we sought to directly compare outcomes for these different testing approaches in 107 consecutive WM patients.

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We used CD19-selected BM samples and AS-PCR to detect *CXCR4*^{S338X} and MYD88^{L265P}, and Sanger sequencing for frameshift *CXCR4*^{MUT} as employed in the pivotal ibrutinib trial.[5] AS-PCR for the *CXCR4*^{S338X} and *MYD88*^{L265P} variants was also performed on unselected BM samples.[3,10] The findings were compared against a clinically validated and targeted NGS assay (Rapid Heme Panel; RHP) using unselected BM aspirate.[11] The RHP assay has an average coverage of 1500× with <5% of the amplicons with <50× coverage, and reproducibly could detect single nucleotide variants and small insertions/deletions at allele frequencies of ≥5%. For *CXCR4*, two amplicons were used with a median coverage of 589× and 1311×, respectively. Patients provided written consent for use of their samples, and the study was approved by our institutional review board.

Using CD19-selected BM samples and combined AS-PCR and Sanger sequencing, 43/107 (40%) patients had a *CXCR4*^{MUT} identified (Table I). For patients with *CXCR4*^{S338X} identified by AS-PCR, the median ΔC_t was 4·51 cycles (range 1·79–7·45). Only 16/107 (15%) patients had *CXCR4*^{MUT} identified by use of unselected BM samples and targeted NGS; the median variant allele fraction (VAF) for the *CXCR4*^{MUT} identified by NGS was 5·2% (range 2·6–31·8%). No *CXCR4*^{MUT} was identified by NGS that was not identified by combined AS-PCR and Sanger sequencing. Comparison between the two approaches showed concordance for *CXCR4* mutational status (wild-type or mutated) in most patients (80/107, 75%; $\kappa = 0{\cdot}42$). The 27 patients with discordant results had *CXCR4*^{MUT} detected by combined Sanger sequencing and AS-PCR but missed by NGS. These cases represent false-negative results in 27/43 (63%) of true cases with *CXCR4*^{MUT}. Collectively, the results yield a sensitivity and specificity of 37% (95% CI 23–53%) and 100% (95% CI 93–100%), respectively, for the detection of *CXCR4*^{MUT} by NGS; and positive and negative predictive values of 100% (95% CI 76–100%) and 70% (95% CI 60–79%), respectively.

We next evaluated factors that impacted the sensitivity of *CXCR4*^{MUT} detection by NGS. The median BM involvement was significantly lower in patients with false-negative results by NGS for *CXCR4*^{MUT} compared to those without (15% vs 55%; $P < 0{\cdot}001$). Accordingly, the sensitivity of *CXCR4*^{MUT} detection increased significantly with increasing BM involvement: 0–20% (3/22; 14%); 21–40% (2/7; 29%); 41–60% (4/7; 57%); 61–80% (5/5; 100%); and 81–100% (2/2; 100%) ($P = 0{\cdot}001$; Fig 1). Sensitivity was also higher for *CXCR4*^{S338X} *versus* *CXCR4*^{R334X} (100% vs 16%; $P = 0{\cdot}004$); however, the four patients with *CXCR4*^{R334X} all had substantial BM involvement (median 60%; range 40–70%), which likely accounts for this observation. There were no significant differences in age, haemoglobin level, platelet count, serum IgM level or prior treatment status between *CXCR4*^{MUT} patients with and without false-negative results by NGS. Likewise, sensitivity was not significantly associated with nonsense *versus* frameshift mutations, insertion *versus* deletion frameshift mutations, indels with >1 vs ≤1 nucleotide, and *CXCR4* amplicon coverage ($P > 0{\cdot}05$ for all comparisons).

The relative importance of B-cell selection *versus* AS-PCR on the sensitivity of *CXCR4*^{S338X} detection by NGS was also examined. Sixteen patients with *CXCR4*^{S338X} had both CD19-selected and unselected BM samples tested with AS-PCR.

Table I. Test performance of targeted next-generation sequencing for *CXCR4* mutations identified in patients with Waldenström macroglobulinaemia.

Mutation type	*CXCR4* nucleotide change	Amino acid change	Identified by NGS (*n*/*N*)	NGS sensitivity
FS	r.931_933insT	T311fs	0/1	0%
FS	r.952_954insA	T318fs	0/2	0%
FS	r.954_956insC	S319fs	1/1	100%
FS	r.974_976delGC	S325fs	1/1	100%
FS	r.978_980insT	K237fs	1/1	100%
FS	r.984_986insT	L329fs	0/1	0%
NS	r.1000C>T	R334X	4/4	100%
FS	r.1005_1007insT	G336fs	1/2	50%
FS	r.1012_1014insT	S338fs	1/4	25%
NS	r.1013C>A	S338X	1/8	12·5%
NS	r.1013C>G	S338X	2/11	18%
FS	r.1016_1017insT	S339fs	1/2	50%
FS	r.1020_1022delT	S341fs	0/1	0%
FS	r.1025_1026delCT	T342fs	1/2	50%
FS	r.1031_1037delTTCAAGT	S344fs	1/1	100%
FS	r.1033_1035delAG	E345fs	1/1	100%

Combined Sanger sequencing and allele-specific polymerase chain reaction with CD19-selected bone marrow aspirate samples was used as the reference assay for the analysis. NS, nonsense; FS, frameshift; NGS, next-generation sequencing.

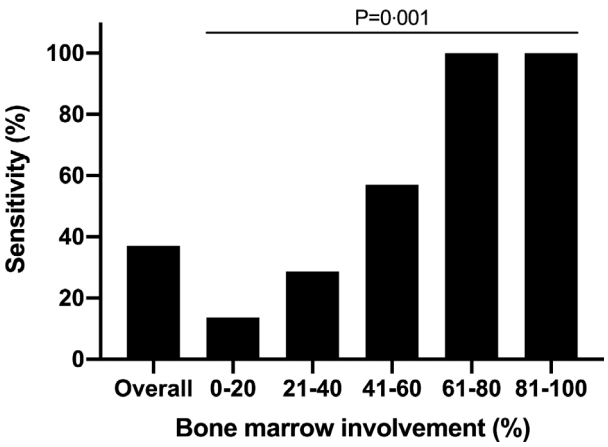

Fig 1. Sensitivity of targeted next-generation sequencing for *CXCR4* mutations in Waldenström macroglobulinaemia. Impact of bone marrow disease involvement on the sensitivity of *CXCR4* mutation detection with targeted NGS. Combined Sanger sequencing and allele-specific polymerase chain reaction with CD19-selected bone marrow aspirate samples was used as the reference assay for the analysis.

Using unselected BM samples, 10 of 16 patients (63%) had $CXCR4^{S338X}$ detected by AS-PCR, which corresponds to a fourfold higher sensitivity compared to NGS for $CXCR4^{S338X}$ (16%; Table I). These results indicate that the use of B-cell selection and AS-PCR both contribute to the lower sensitivity of $CXCR4^{S338X}$ detection observed with NGS.

We next sought to understand the impact of $CXCR4^{S338X}$ clonality on NGS sensitivity. As part of these efforts, we first performed a sensitivity analysis for $MYD88^{L265P}$ in the same sample set. Compared to AS-PCR with CD19-selected BM, the use of AS-PCR and NGS on unselected BM had a sensitivity of 98% and 69%, respectively, for $MYD88^{L265P}$. These results suggest that the subclonal nature of $CXCR4^{S338X}$ adversely impacts NGS performance, as evidenced by the fourfold difference (69% vs 16%) in sensitivity between $MYD88^{L265P}$ and $CXCR4^{S338X}$ when using NGS with unselected BM samples. Cancer cell fraction (CCF) analysis was then performed for 14 patients with $CXCR4^{S338X}$ using synchronous, parallel quantitative AS-PCR analysis for $CXCR4^{S338X}$ and $MYD88^{L265P}$ as previously described.[3] The median BM involvement for these patients was 20% (range 5–70%), and the median CCF for $CXCR4^{S338X}$ was 9% (range 0·8–31%). The $CXCR4^{S338X}$ allele burden was subsequently approximated by calculating the product of CCF by BM involvement which was 1·8% (range 0·05–13%). Only the patient with the highest $CXCR4^{S338X}$ allele burden (13%) had the mutation detected by NGS. Likewise, patients with false-negative results for $CXCR4^{S338X}$ by AS-PCR with unselected BM samples had a significantly lower $CXCR4^{S338X}$ clonality (1·1% vs 17·9%; $P = 0·009$) and allele burden (0·6% vs 3·1%; $P = 0·008$). Taken together, these findings show that in addition to BM involvement per se, the extent of subclonal involvement of $CXCR4^{MUT}$ can further compound identification of $CXCR4^{MUT}$ by use of unselected tissue with both NGS and AS-PCR.

Overall, the findings demonstrate that the use of unselected tissue and targeted NGS frequently fails to detect $CXCR4^{MUT}$. Indeed, nearly two-thirds of WM patients with $CXCR4^{MUT}$ had a false-negative result by the NGS approach, and the sensitivity correlated with the extent of BM involvement. Our findings also highlight the adverse impact of low *CXCR4* clonality on both NGS and AS-PCR performance. Efforts to establish standardized approaches for detecting $CXCR4^{MUT}$ in WM patients are urgently needed. The use of AS-PCR for nonsense variants such as $CXCR4^{S338X}$ could produce a fourfold increase in sensitivity over NGS, thereby increasing detectability in patients with lower BM and clonal involvement. Likewise, increased depth of coverage with NGS might help improve the sensitivity of detecting $CXCR4^{MUT}$. Consistent with previous work for $MYD88^{L265P}$, we showed that tumour enrichment with B-cell selection improved the sensitivity of $CXCR4^{S338X}$ detection by AS-PCR, but this approach may not be feasible in most clinical laboratories.[10,12] The use of tumour-derived cell-free DNA may represent an alternative testing modality given encouraging initial results in WM patients.[13,14] The evaluation of other sensitive approaches (e.g., digital droplet PCR, error-corrected NGS) also warrants evaluation to detect $CXCR4^{MUT}$. These collective efforts are particularly important since $CXCR4^{MUT}$ represents an emerging therapeutic target in WM with ongoing clinical trials investigating *CXCR4* antagonists (NCT03225716, NCT04274738). The ability to reliably detect $CXCR4^{MUT}$ will be essential to identify WM patients appropriate for *CXCR4*-targeted therapy.

Our study provides an important context for interpreting recent randomized trials such as the INNOVATE and ASPEN studies that evaluated BTK inhibitors in WM patients. To identify $CXCR4^{MUT}$, these trials utilized unselected tissue and targeted NGS assays, though specifications including coverage depth and sensitivity for these assays remain to be reported.[7,9,15] The findings herein suggest these trial results could be confounded in part by inclusion of patients with $CXCR4^{MUT}$ into the wild-type *CXCR4* cohort. Nevertheless, future efforts at evaluating the impact of $CXCR4^{MUT}$ in clinical trials with BTK inhibitors may well focus on the $CXCR4^{MUT}$ with a more virulent phenotype. Indeed, patients with nonsense variants such as $CXCR4^{S338X}$ and those with higher $CXCR4^{S338X}$ clonality (>25%) have been shown to have poorer long-term outcomes with ibrutinib.[8,16,17] Patients with higher $CXCR4^{S338X}$ clonality also appear more likely to have a higher disease burden at ibrutinib initiation.[17] Additional studies are needed to further clarify the clinical impact of $CXCR4^{MUT}$ subtype (nonsense *versus* frameshift) and clonality, which may help inform testing priorities in WM patients.

In summary, our findings demonstrate that use of unselected BM aspirate and targeted NGS frequently yielded false-

negative results for *CXCR4*^{MUT} in WM patients, especially those with low BM involvement and *CXCR4* clonality. Efforts are urgently needed to establish accurate and standardized testing methods adaptable to clinical practice for *CXCR4*^{MUT} in WM patients.

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Author contributions

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

Conflicts of interest

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