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Development and Evaluation of a Multiplex Digital PCR Assay for Detecting CXCR4 S338X Mutations in Waldenstrom's Macroglobulinemia.

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Background

CXCR4 mutations are found in 30-40% of Waldenstrom's Macroglobulinemia (WM) patients and impact bone marrow (BM) diseased involvement; serum IgM levels, time to initial therapy; and outcomes to BTK-inhibitors. Next generation sequencing (NGS) is most often used to detect mutated CXCR4 but shows low sensitivity in WM patients with <20% (Gustine et al, BJH 2021). The CXCR4 S338X ($CXCR4^{S338X}$) mutation is the most common and impactful variant. We previously developed quantitative allele-specific PCR (qPCR) assays that offered sensitivities to 0.4% for the $CXCR4^{S338X}$ C>A variant and 0.16% for the $CXCR4^{S338X}$ C>G variant (Xu et al, BJH 2016) but required a standard curve for each plate, and both mutations had to be assessed in separate reactions. Multiplex digital PCR may offer greater sensitivity, as well as simultaneous detection and quantification of both variants. We therefore sought to develop a dPCR assay for both $CXCR4^{S338X}$ variants for clinical utilization.

Methods

A TaqMan SNP genotyping assay was designed, consisting of a set of amplification primers, a VIC-labeled probe for detecting the wild-type allele, a FAM-labeled probe for mutant allele G, and an ABY-labeled probe for mutant allele A. DNA from CD19+ selected BM mononuclear cells (BM19+) and unselected BM mononuclear cells (BMMC) from previously identified $CXCR4^{S338X}$mutated patients was used that consisted of 24 with $CXCR4^{S338X}$ C>A mutation and 45 with $CXCR4^{S338X}$ C>G mutation. Samples were evaluated by both qPCR and dPCR. The cutoffs used for determining positivity by qPCR were 0.5% for C>A and 0.2% for C>G, in line with our previously published data. For dPCR, cutoffs of 0.05% for both the C>A and C>G assays were established, representing a 10x and 4x increase in sensitivity versus qPCR, respectively.

Results

For both the C>A and C>G assays, detection was improved in samples with increased disease burden (>20% BM involvement) by either qPCR and dPCR. Estimates of mutant allele burden were highly correlated between qPCR and dPCR for both the C>A ($r = 0.99$) and C>G (0.96) assay (**Fig. 1**). In BM19+ samples, dPCR detected $CXCR4^{S338X}$ C>A mutations in 23/24 (96%) versus 17/24 (71%) WM patients by qPCR ($P<0.0001$). The one wild-type sample by dPCR showed a $CXCR4^{S338X}$ C>G mutation. Conversely, $CXCR4^{S338X}$ C>G mutations were detected in all 45 BM19+ samples by both assays. Among BMMC samples, $CXCR4^{S338X}$ C>A mutation was detected in 17/24 (71%) and 13/24 (54%) by dPCR and qPCR, respectively ($p=0.37$). In BMMC, the $CXCR4^{S338X}$ C>G mutation was identified in 33/44 (75%) and 27/45 (60%) of samples evaluated by dPCR and qPCR, respectively ($p=0.56$). When restricted to BM samples with <20% WM disease burden, $CXCR4^{S338X}$ C>A was detected in 7/7 (100%) by dPCR and 4/7 (57%) by qPCR

(p=0.19) in CD19+ samples; and 4/7 (57%) and 3/7 (43%) in BMMC samples (p=1.0). CXCR4^{S338X} C>G was detected in all 17 BM19+ samples by either dPCR or qPCR, and 11/17 (65%) and 9/17 (53%) of BMMC samples when restricted to patients with <20% BM disease involvement.

Conclusion

dPCR can simultaneously detect both the CXCR4^{S338X} C>A and C>G mutations without the need for running standard curves, while showing enhanced performance for identifying CXCR4^{S338X} C>A mutations versus qPCR including in WM patients with <20% BM disease burden.

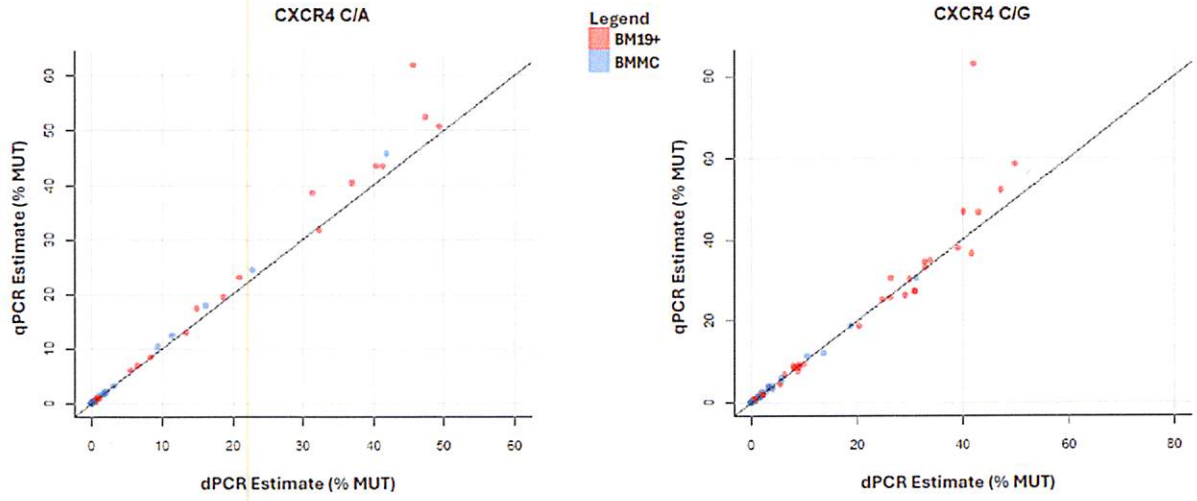

Fig.1. Correlation between mutant allele burden estimated by quantitative PCR (y-axis) and digital PCR (x-axis) for both CXCR4 assays.