Ultra-sensitive *KIT* Testing Uncovers Previously Undetected KIT Mutations in Patients With ISM: Results From the PIONEER Trial

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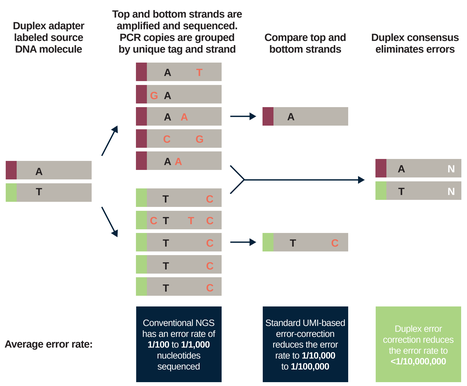
Introduction

- Indolent systemic mastocytosis (ISM) is a clonal mast cell disease driven by the *KIT* D816V mutation in 95% of cases[1–3]
- Presence of a *KIT*-activating point mutation, most commonly at codon 816 (e.g., D816V), is one of four minor diagnostic criteria for SM[4–6]
- Low levels of *KIT* mutations in peripheral blood (PB) can lead to a false negative due to the limit of detection (LOD) of the assay used
- Droplet digital polymerase chain reaction (ddPCR) is one of the most sensitive tests in clinical use and has a LOD ranging from 0.022–0.03% variant allele frequency (VAF)[7]
  - ddPCR has demonstrated a 30-fold greater sensitivity for the detection of *KIT* D816V VAF compared to next-generation sequencing (NGS)[8]
- Serum tryptase level >20 ng/mL, another minor diagnostic criterion, may be absent in up to 30% of patients with ISM and may lead to missed diagnoses[4,5,9]
- To facilitate the use of PB to identify SM diagnostic criteria, we explored two methodologies that putatively have a higher sensitivity than ddPCR for the detection of rare mutations: duplex sequencing and rolling circle amplification assay (RCA)
- PIONEER (NCT03731260), a randomized, double-blind study, demonstrated the efficacy of avapritinib, a potent, highly selective oral therapy targeting *KIT* D816V and other pathogenic *KIT* mutations, compared with placebo and best supportive care in patients with symptomatic ISM[10]
- To further understand the value of ultra-sensitive testing in the clinical setting, we developed two custom assays for detection of *KIT* D816V mutations, duplex sequencing, and RCA. The duplex sequencing assay was further tested retrospectively in PIONEER patients who were undetectable for *KIT* D816V at baseline by ddPCR

Duplex sequencing

- The TwinStrand Duplex Sequencing® technology is a biochemical and informatics-based error correction approach that facilitates detection of genomic variants at ultra-low VAF
- In the duplex sequencing assay, both complimentary DNA strands of each DNA duplex are uniquely labelled, and then amplified and sequenced (**Figure 1**)
- Post-sequencing, computational analysis maps the detected alterations to each original DNA duplex. By retaining only the alterations identified in both strands (and discarding the alterations in only one of the strands of the duplex), nearly all background errors are eliminated. This results in a >10,000-fold increase in accuracy over standard NGS and revealing ultra-low frequency variants[11]
- Ultra-sensitive duplex sequencing can detect rare mutations close to a VAF of ~0.001% and also has the potential to detect a broader spectrum of *KIT* mutations than ddPCR

Figure 1. The process of duplex sequencing



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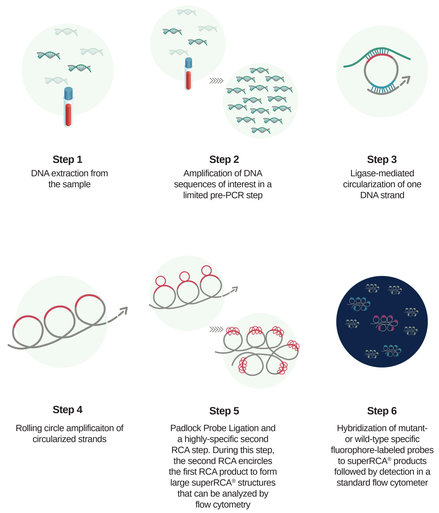
NGS, next-generation sequencing; PCR, polymerase chain reaction; UMI, unique molecular identifier.

RCA assay

- The overview of the superRCA® assay, developed in collaboration with Rarity Bioscience, is shown in **Figure 2**
- The target DNA sequence is ligated, circularized, and amplified such that multiple repeats of the target sequence are concatenated together
  - These repeated DNA chains can then be probed with a fluorescently labelled oligonucleotide sequence specific to the wild-type or the mutant sequence
  - Fluorescently labelled probes bound to tandem DNA repeats form a large structure with high molecular weight detectable by flow cytometry
- The RCA assay is reported to detect one mutant copy out of 100,000 wild-type DNA copies[12]

Figure 2. Steps of the RCA assay



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RCA, rolling circle amplification.

- Details on common testing methodologies currently available and in development are shown in **Table 1**

Table 1. Testing modalities for *KIT* mutations

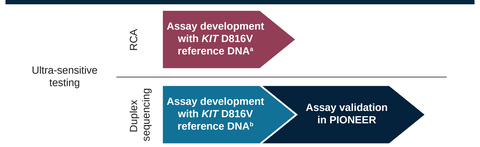
Assay status	Technology	LOD for *KIT* D816V mutations	*KIT* mutations that can be detected	Sample input	*Number of patients undetectable (<LOD)*
Commercial use	NGS (TruSight™ Myeloid NGS Panel)	5%[13]	Multiple exon 17 mutations	Isolated DNA, frozen/fresh blood	209/250 (estimated)
	ddPCR/ dPCR	0.022-0.03%[7,10]	D816V only		37/250
	ASO-PCR	0.1% (clinically available)[14]	D816V only		86/250 (estimated)
Research use	Duplex sequencing	0.0013% (this poster)	Multiple exon 17 mutations		11/250
	RCA	0.001% (this poster)	D816V only		ND

ASO-PCR, allele-specific oligonucleotide polymerase chain reaction; dPCR, digital polymerase chain reaction; ddPCR, droplet digital polymerase chain reaction; LOD, limit of detection; ND, not determined.

Methods

- Both custom assays were initially developed with commercially available *KIT* D816V–mutant DNA serially diluted in wild-type DNA
- Duplex sequencing custom assay was then further validated within the PIONEER study via retrospective patient sample testing (**Figure 3**)
- In PIONEER, testing for samples that did not have detectable *KIT* D816V by ddPCR was done at ICON plc with the TwinStrand Duplex Sequencing® custom panel for human *KIT* exon 17 mutations, including *KIT* D816V, with the assay performed per manufacturer's instructions:
  - Briefly, enzymatically fragmented DNAs were ligated to DuplexSeq adapters, and the tagged libraries were PCR amplified with sample-specific barcode
  - Target capture was achieved with probe-based hybridization and PCR amplification
  - The final library was sequenced on a NextSeq 550Dx (Illumina, San Diego, CA)
  - The variant call was made by the TwinStrand DuplexSeq software on the DNAnexus® portal (Mountain View, CA)

Figure 3 Validation and assessment of the duplex sequencing and RCA assays



[a]Reference DNA obtained from GeneCopoeia, Rockville, MD. [b]Reference DNA obtained from SeraCare Life Sciences, Gaithersburg, MD.

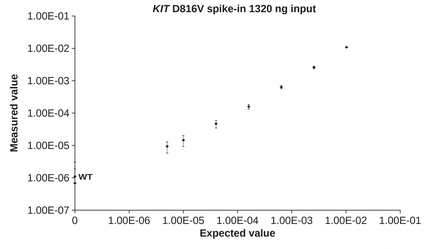
Results

Ultra-sensitive assay proof of concept

RCA assay

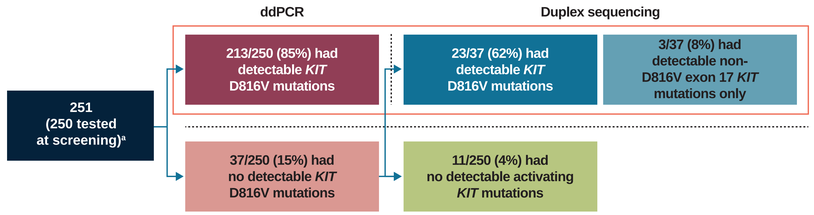
- We conducted a proof-of-concept RCA run to detect spiked-in *KIT* D816V mutation at ≤0.001% VAF (**Figure 4**)

Figure 4. RCA detected *KIT* D816V at a VAF of 0.001% in a proof-of-concept study



WT DNA: Roche Human Genomic DNA from human blood (buffy coat; Sigma-Aldrich), pooled. *KIT* D816V DNA: GeneCopoeia *KIT* D816V 100% VAF positive genomic DNA. VAF, variant allele fraction; WT, wild type.

Figure 6. Mutations detected in patients enrolled in PIONEER



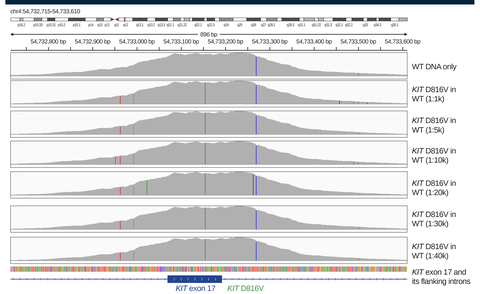
[a]A total of 251 patients were enrolled in the PIONEER trial, but only 250 samples were available at screening.

Ultra-sensitive assay proof of concept

Duplex sequencing

- As shown in **Figure 5**, the duplex sequencing *KIT* exon-17 custom assay was able to detect the *KIT* D816V mutation with ultra-low background noise at a VAF cutoff of 0.0013%

Figure 5. Duplex sequencing showed ultra-low noise level in detecting *KIT* D816V and no noise on exon-17 with a VAF cutoff at 0.0013%



The IGV image with VAF cutoff at 0.001% shows the *KIT* exon-17 (bold blue bar at the bottom) and its flanking introns from the proof-of-concept duplex sequencing with serially diluted *KIT* D816V DNA into WT DNA. The green vertical bars are the *KIT* D816V mutation, c.2447A>T. All other bars are limited sequencing artifacts (background noise) and are only shown in the flanking introns.
IGV, integrative genomics viewer.

Validation of duplex sequencing in PIONEER patient samples

- A total of 250 patients were tested for *KIT* mutations in PIONEER, and based on PB *KIT* mutation testing at screening, were divided into three mutually exclusive groups (**Table 2**):
  - Patients with detectable *KIT* D816V by ddPCR (n=213/250, 85%)
  - Patients without detectable *KIT* D816V by ddPCR in PB but with detectable mutations by duplex sequencing (26/250, 10%)
  - Patients without detectable *KIT* mutations by both ddPCR and duplex sequencing (n=11/250, 4%)

Table 2. Baseline demographics and disease burden markers

	Detectable by ddPCR[a] (n=213)	Undetectable by ddPCR[a] and detectable by duplex sequencing[b] (n=26)	Undetectable by ddPCR[a] and duplex sequencing[b] (n=11)
Age, years (range)	51 (18–79)	48 (31–64)	50 (21–58)
Female, %	158 (74%)	20 (77)	6 (54)
Median baseline serum tryptase, ng/ml (range)	44.95 (4.2–501.6)	23.4 (3.6–200.0)	20.8 (4.5–288.0)
Median BM MC, % (range)	10 (1.0–70.0)	5.0 (1.0–40.0)	3.0 (2.0–30.0)
Median *KIT* D816V VAF, % (range)	0.49 (0.02–41.3)	0.0068 (0.0013–0.0261)	NA

[a]*KIT* D816V detection by ddPCR; [b]Detection of activating *KIT* mutations by duplex sequencing.
BM, bone marrow; MC, mast cell; NA, not applicable.

- By aggregating PB testing results from ddPCR and duplex sequencing, the total number of PIONEER patients that had detectable *KIT* exon 17 activating mutations was 239/250 (96%), including 70% of those patients who had tested negative by ddPCR (**Figure 6**)

Patient characteristics grouped by KIT mutation detection

- Baseline median serum tryptase (range) was 45.0 ng/mL (4.2–501.6) in patients with detectable *KIT* mutations by ddPCR, 23.4 ng/mL (3.6–200.0) in patients with undetectable *KIT* D816V by ddPCR but detectable *KIT* mutations by duplex sequencing, and 20.8 ng/mL (4.5–288.0) in patients without detectable mutations by both ddPCR and duplex sequencing (**Table 2**)
- In patients who had detectable *KIT* mutation by duplex sequencing, 10/26 had serum tryptase levels <20 ng/mL; thus, on clinically available PB testing, these patients were negative for two minor SM diagnostic criteria, despite harboring a *KIT* mutation and carrying the diagnosis of ISM
- Baseline median bone marrow mast cells (range) were 10% (1.0–70.0), 5.0% (1.0–40.0), and 3.0% (2.0–30.0) for patients who had detectable *KIT* D816V by ddPCR, no detectable *KIT* D816V by ddPCR but detectable *KIT* mutation by duplex sequencing, and no detectable *KIT* mutation by both ddPCR and duplex sequencing, respectively
- The median *KIT* D816V VAF (range) for patients with detectable *KIT* D816V by ddPCR was 0.49% (0.02–41.3) and the median *KIT* D816V VAF for patients with detectable mutations by duplex sequencing and no detectable mutations by ddPCR was 0.0068% (0.0013–0.0261)
- Patients who had detectable *KIT* mutations by duplex sequencing included two patients with dual mutations in *KIT* (D816I+D816V, C788Y+D816V) and three patients with lone non-D816V *KIT* exon 17 activating mutations (D816I, D816Y; VAF 0.0075–4.5%; **Table 3**)

Table 3. Characteristics of patients with dual or non-D816V mutations

Age, years	Gender	Mutations detected	Median *KIT* mutation VAF, % by duplex sequencing	Avapritinib sensitivity *in vitro* (IC_{50} <1 nm)[15]
63	Female	D816I/D816V	0.0013/0.0026	Yes/Yes
33	Female	D816I	0.7820	Yes
52	Male	D816Y	4.4781	Yes
51	Female	D816Y	0.0075	Yes
31	Female	C788Y/D816V	0.0041/0.0037	ND/Yes

Conclusions

- Due to the rarity of circulating mast cells in PB in ISM, more sensitive assays are needed to aid clinicians in making diagnoses of SM
- While PB tryptase and ddPCR testing for *KIT* D816V are important steps in the work-up of suspected SM, the possibility of SM cannot be ruled out when these tests are negative
- Duplex sequencing and RCA are examples of assays which can detect individual genetic variants, including *KIT* D816V, with a sensitivity at least an order of magnitude beyond that of currently available clinical tests
- Application of ultra-sensitive duplex sequencing to samples from the 15% (n=37) of patients with ISM from PIONEER that had undetected *KIT* D816V by ddPCR identified activating *KIT* mutations in 26 (70%) out of these 37 patients
- Bone marrow biopsy, including ddPCR of the bone marrow aspirate sample for *KIT* D816V, should still be performed if SM is suspected and remains the standard-of-care for evaluating SM until more sensitive tests are clinically available

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Conflicts of interest/disclosures

Dr Radia has been a clinical advisory board/study steering group member (EXPLORER) for Blueprint Medicines Corporation; and was involved with educational events and advisory boards for Novartis. For all author disclosures, please contact medinfo@blueprintmedicines.com.

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