Single Molecule Real Time (SMRT™) Sequencing Sensitively Detects Polyclonal and Compound BCR-ABL in Patients Who Relapse on Kinase Inhibitor Therapy

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Introduction

Secondary kinase domain (KD) mutations are the most well-recognized mechanism of resistance to tyrosine kinase inhibitors (TKIs) in chronic myeloid leukemia (CML) and other cancers. In some cases, multiple drug resistant KD mutations can coexist in an individual patient ("polyclonality")[1]. Alternatively, more than one mutation can occur in tandem on a single allele ("compound mutations") following response and relapse to sequentially administered TKI therapy. Distinguishing between these two scenarios can inform the clinical choice of subsequent TKI treatment. There is currently no clinically adaptable methodology that offers the ability to distinguish polyclonal from compound mutations. Due to the size of the BCR-ABL KD where TKI-resistant mutations are detected, next-generation platforms are unable to generate reads of sufficient length to determine if two mutations separated by 500 nucleotides reside on the same allele. Pacific Biosciences *RS* Single Molecule Real Time (SMRT) circular consensus sequencing technology is a novel third generation deep sequencing technology capable of rapidly and reliably achieving average read lengths of ~1000bp[2] and frequently beyond 3000bp, allowing sequencing of the entire *ABL* KD on single strand of DNA. We sought to address the ability of SMRT sequencing technology to distinguish polyclonal from compound mutations using clinical samples obtained from patients who have relapsed on BCR-ABL TKI treatment.

Results

SMRT Sequencing Allows Sequencing of the Entire BCR-ABL KD With Accuracy and Precision. We utilized PacBio *RS* sequencing to target an 863bp area of the *BCR-ABL* KD. Following nested amplification of the *BCR-ABL* KD, SMRTbell libraries are prepared[2] and sequenced using circular consensus sequencing (CCS) mode. The CCS mode generates reads by combining multiple independent single-pass sequencing reads for each individual molecule to correct raw errors and generate a better accuracy consensus (Fig. 1). We report only the CCS reads where the same molecule is sequenced ≥3 times, i.e., raw read length > ~2600bp for the 863bp amplicon. With the CCS reads, we obtained >7000 sequences containing the *BCR-ABL* KD with ~93-96% average alignment identity and average read length of 700-800bp (Table 1). Single molecule sequencing coupled with long read lengths allowed us to capture the full spectrum of mutations across the length of the KD, characterize individual mutation patterns, and quantify relative mutation abundances. In comparison, direct sequencing can identify mutations at individual amino acid (AA) positions but is not quantitative, is unable to resolve multiple nucleotide substitutions at a single position, and provides no information about whether mutations occur in *cis* or in *trans* (Fig. 1).

SMRT Sequencing Identifies All Mutations Found By Direct Sequencing and Identifies Additional Mutations Present At Lower Abundance. We sequenced the *BCR-ABL* KD in 11 samples from Ph+ patients clinically relapsed on ABL kinase inhibitor therapy and the *ABL* KD from 3 normal controls. Previous direct sequencing in CLIA-certified laboratories reported KD mutations in 9 of 11 Ph+ samples. SMRT sequencing detected all the mutations found by direct sequencing (Table 1). In 2 samples, SMRT sequencing detected additional mutations present at a frequency of >1% and detected lower abundance mutations (≤1%) in a total of 9 samples (Fig. 2). All mutations reported in this analysis were filtered for local quality at the codons of interest and mutation frequencies were significant when compared to normal control (p-value range: $1x10^{-7}$ to $1x10^{-21}$). The clinical significance of mutations found at a frequency of ≤1% remains to be determined.

SMRT Sequencing Identifies Polyclonal and Compound Mutations in Individual Patients. We tallied the number of times different mutations were observed together in a single-molecule read and calculated the simple fractional abundances of each of the mutation combination found at a frequency of >1%. We resolved polyclonal and compound (>1 mutation on a single allele) in 3 cases (Fig. 3). In the most complex case (Sample 10), 12 distinct mutation-bearing alleles were detected in an individual patient after sequential relapse on imatinib and dasatinib. Mutant clones contained single and compound mutations combining 7 distinct mutations (Y253H, T315F, T315A, T315I, T315V, T319A, E355G). Four distinct substitutions at residue T315 were detected in Sample 10: T315A, T315I, T315V and T315F. Clustering analysis reveals multiple distinct populations of sequences in this single sample (Fig. 4).

Polyclonal KD Mutations Observed in A Single Patient Are Associated with Polyclonal Drug Resistant Cell Populations. Phospho-flow analysis for p-Crkl, a direct substrate of BCR-ABL, was conducted following *ex vivo* exposure of patient cells from Sample 10 to BCR-ABL inhibitors imatinib, dasatinib, nilotinib and aurora kinase/ABL inhibitor VX680. This analysis demonstrated the existence of distinct populations of cells with varying sensitivity to each drug (i.e. polyclonal drug sensitivity), underscoring the potential clinical importance of distinguishing polyclonal and compound mutations (Fig. 5). The larger persistent p-Crkl high peak in the dasatinib-treated sample is likely is due to the T315A mutation-containing population which confers resistance to dasatinib but not imatinib or nilotinib, while the smaller p-Crkl high peaks in the imatinib and nilotinib-treated samples likely represent T315F and T315I mutation-containing populations resistant to all three BCR/ABL inhibitors. The lack of p-Crkl high population in the VX680-treated sample is consistent with retained sensitivity of these variants to VX680.

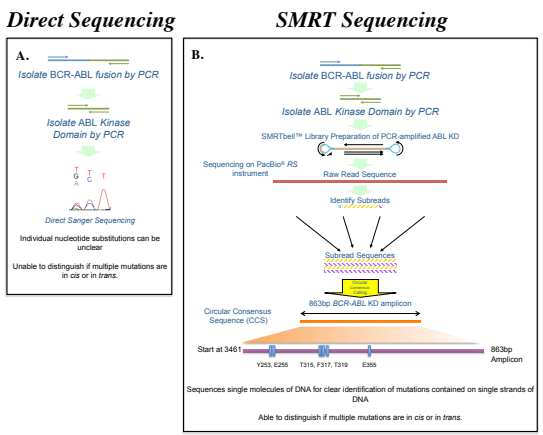

Figure 1. Description of Direct Sequencing and SMRT Sequencing Workflows. A. Typical strategy for direct sequencing of the BCR-ABL KD in patients: ~1400bp fragment of the BCR-ABL fusion is amplified from patient-derived cDNA and isolated by gel-purification. An 863bp fragment containing the ABL KD is further amplified by nested PCR and sequenced using Sanger sequencing. B. Strategy for SMRT sequencing of the BCR-ABL KD: ~1400bp fragment of the BCR-ABL fusion is amplified from patient-derived cDNA and isolated by gel-purification. An 863bp fragment containing the ABL KD is amplified by nested PCR. SMRTbell libraries are prepared as previously described[2] and sequenced on a PacBio *RS* instrument using circular consensus sequencing mode to obtain high accuracy reads for the 863bp amplicon by combining multiple independent single-pass sequencing reads for individual molecules to correct raw errors and generate a better accuracy consensus.

Table 1. *ABL* KD Mutations Identified by SMRT Sequencing in Ph+ Patients and Normal Controls at a Frequency of >1%

Sample	Mutations Found by Direct Sequencing	Mutations Found by SMRT Sequencing At Frequency >1%	Frequency of Mutations Found by SMRT Sequencing	Total Aligned CCS reads	Average CCS Read Aligned Length (bp)	Average Alignment Identity
Sample 1	F359I	F359I	47%	10149	786	94.05%
		F359V	6%			
Sample 2	None	None	-	8237	804	96.03%
Sample 3	T315I	T315I	47%	7878	762	92.78%
	F359V	F359V	49%			
Sample 4	T315I	T315I	99%	9411	766	93.77%
Sample 5	T315I	T315I	99%	7094	722	94.56%
Sample 6	None	None	-	10075	785	94.73%
Sample 7	F317L	F317L	15%	10754	762	92.96%
Sample 8	T315I	T315I	96%	13444	644	94.31%
Sample 9	F317L	F317L	96%	13870	674	95.81%
Sample 10	T315?	Y253H	7%	13172	798	95.93%
	E355G	T315A	59%			
		T315F	20%			
		T315I	10%			
		T315V	3%			
		T319A	7%			
		E355G	82%			
Sample 11	E255V	E255V	47%	14689	769	95.80%
		T315I	45%			
Normal 1	None	None	-	11123	810	93.98%
Normal 2	None	None	-	14510	752	95.22%
Normal 3	None	None	-	12482	787	93.29%

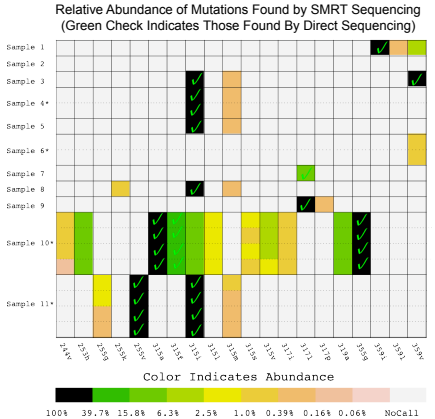

Figure 2. SMRT Sequencing Identifies All Mutations Identified by Direct Sequencing And Additionally Identifies Lower Abundance Mutations. All mutations identified by direct sequencing were found at ≥15% frequency by SMRT sequencing.

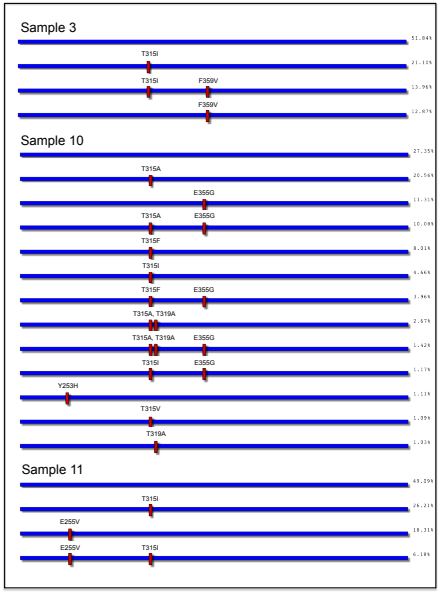

Figure 3. SMRT Sequencing Identifies Polyclonal and Compound Mutations in Patients. Multiple *BCR-ABL* KD mutations at distinct AA residues were observed in 3 samples. For each sample, the number of times different mutations were observed together in a single-molecule read were tallied and the simple fractional abundances of each of the mutation combination was calculated.

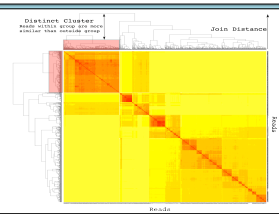

Figure 4. Unsupervised Clustering Identifies Multiple Populations of Mutational Clones in An Individual Patient at a Single Timepoint. For a single run, ~1000 reads from Sample 10 were used to construct a multiple alignment. Pairs of aligned reads were used to construct an agglomerative clustering of the reads. Each cluster represents a distinct population of reads.

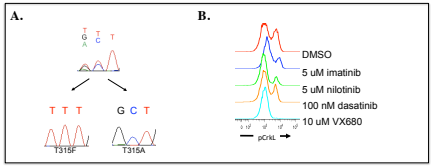

Figure 5. Polyclonal KD Mutations Observed in a Single Patient Are Associated with Polyclonal Drug Resistant Cell Populations. A. Multiple peaks seen by direct sequencing of codon 315 suggests several nucleotide substitutions and PCR sub-cloning reveals an ACT→TTT substitution (T315F) and an ACT→GCT substitution (T315A). SMRT sequencing reveals 12 distinct mutation-bearing alleles with single and compound mutations, including multiple substitutions at T315 (Figure 2, Sample 10). B. Phospho-flow analysis for p-Crkl, a direct substrate of BCR-ABL, conducted after an *ex vivo* exposure of patient cells to a panel of inhibitors reveals distinct populations of cells with varying sensitivity to each inhibitor (i.e. polyclonal drug sensitivity).

Conclusions

- Pacific Biosciences *RS* SMRT sequencing sensitively and accurately identifies *BCR-ABL* KD mutations in patient samples
- SMRT sequencing identifies the same mutations found in CLIA-certified direct sequencing assays with equal or improved sensitivity
- SMRT sequencing can distinguish polyclonal and compound mutations in individual patient samples not detectable by direct sequencing
- The complex mutational landscape in an single patient revealed by SMRT sequencing can inform clinical decision-making and provide insight into the evolution of drug resistance in an individual tumor
- SMRT sequencing of the BCR-ABL KD can feasibly be developed into a rapid and economical clinical test and given the growing numbers of patients exposed to multiple TKIs in a sequential manner, has the promise to further facilitate a personalized approach to patient management.

References

1. Shah, N.P., *et al.* Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* **2**, 117-125 (2002).
2. Travers, K.J., Chin, C.S., Rank, D.R., Eid, J.S. & Turner, S.W. A flexible and efficient template format for circular consensus sequencing and SNP detection. *Nucleic Acids Res* **38**, e159.

Acknowledgements

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Disclosures

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