Secondary kinase domain (K2) mutations are the most well-recognized mechanisms of resistance to tyrosine kinase inhibitors (TKIs) in chronic myeloid leukemia (CML) and other cancers. In some cases, multiple drug resistant K2 mutations can coexist in an individual patient ("polyclonality"). Alternatively, more than one mutation can occur in a single allele ("compound mutations") following suboptimal and subclinical TKI therapy. Distinguishing between these two scenarios can allow for the clinical choice of subsequent TKI treatment. There is currently no clinically applicable methodology that offers the ability to distinguish polyclonal from compound mutations. Due to the size of the BCR-ABL KD when TKI-resistant mutations are detected, next-generation platforms are unable to generate reads of sufficient length to determine if two mutations separated by 50 or more nucleotides reside on the same allele. Pacific Biosciences (PacBio) Single Molecular Real Time (SMRT) circular consensus sequencing technology is a novel field generating deep sequencing technology capable of rapid and reliably achieving average read lengths of 1000bp and frequently beyond 1000bp, allowing sequencing of the entire 48K KD on single strands of DNA. We sought to address the ability of SMRT sequencing technology to distinguish polyclonality 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 SMRT sequencing to target an 863bp amplicon of the BCR-ABL KD. Following spatial amplification of the BCR-ABL KD, SMRT2D Libraries are generated and sequenced using circumin consensus sequencing technology (CCS) mode. The CCS mode generates reads by combining multiple independent single-pass sequencing reads of each individual molecule, and algorithmic error correction to generate a better accuracy consensus (Fig. 1). We report only the CCS reads which fail the quality control criteria for the same molecule in sequential 5 times, i.e., raw read length ~2000bp for the 863bp amplicon. With the CCS reads, we obtained ~1000 sequences containing the entire BCR-ABL KD with 95-98% average alignment identity and average read length of ~1500bp (Table 1). Single molecule sequencing coupled with long read lengths allows to capture the full complexity of mutation variation, underlie the characteristic individual mutation patterns, and quantify relative mutation abundances in individual patients. Although our assay is limited to the 1000bp reads, we found that the relative proportion of all mutations was significantly correlated with the relative proportion of 500bp reads (Fig. 3). For mutations with >1 mutation on a single allele, we were able to resolve polyclonal and compound mutations at a frequency of >1%. We resolved polyclonal and compound mutations at a single position, and provided no information about whether mutations occur cis or trans in frame (Fig. 3).

SMRT Sequencing Identifies Additional Mutations Present At Lower Abundance. SMRT sequencing to target an 863bp area from patient-derived cDNA and isolated by gel-purification. An 863bp fragment containing the ABL KD is generated from the BCR-ABL KD, which is amplified by PCR and sequenced using single-molecule real-time (SMRT) circular consensus sequencing technology to obtain high accuracy reads for the BCR-ABL KD by combining multiple independent single-pass sequencing reads for individual molecules to correct errors and generate a better accuracy consensus. Identifies Additional Mutations Present At Lower Abundance. We utilized PacBio SMRT sequencing to target an 863bp area from patient-derived cDNA and isolated by gel-purification. An 863bp fragment containing the ABL KD is generated from the BCR-ABL KD, which is amplified by PCR and sequenced using single-molecule real-time (SMRT) circular consensus sequencing technology to obtain high accuracy reads for the BCR-ABL KD by combining multiple independent single-pass sequencing reads for individual molecules to correct errors and generate a better accuracy consensus. 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.

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 Mutations Found by SMRT Sequencing at Frequency >1% Total Align Consensus CCS reads Average CCS Read Aligned Length (bp) Average Alignment Identity F359I Sample 1 F359I 47% 10149 786 94.05% Sample 2 F359I 47% 823 804 96.03% Sample 3 T315I T315I 47% 7987 762 94.73% Sample 4 F359V F359V 49% 9411 766 93.77% Sample 5 T315I T315I 99% 704 722 94.56% Sample 6 None None 41% 10075 785 94.73% Sample 7 F317L F317L 15% 10764 752 92.96% Sample 8 T315I T315I 96% 13444 644 94.31% Sample 9 T315I T315I 96% 11387 671 96.51% Sample 10 T315I V255H 7% 13172 798 95.93% E355G Sample 11 T315I T315I 99% 509 509 100% Sample 12 T315I T315I 99% 509 509 100%

Figure 4. Unsupervised Clustering Identifies Multiple Populations of Mutated BCR-ABL KD. Sequences were clustered using an unsupervised clustering approach to identify distinct mutation combinations within a single patient and evaluate the evolutionary diversity of the BCR-ABL KD. Mutations were clustered into 11 distinct mutation-bearing clusters with single or compound mutations, including multiple substitutions at T315 (Fig. 2, Sample 6).

Conclusions

• Pacific Biosciences’ Real-time sequencing technology can sensitively and accurately identify BCR-ABL K2 mutations in patients 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 a 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 be used to identify new biomarkers for disease aggressiveness and therapy resistance

References


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