Phasing Analysis of TKI Resistance Mutations in the BCR-ABL1 Kinase Domain and Neighboring Domains Using Next-Generation Sequencing

Abstract #: 3817

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Introduction

Many CML patients treated with tyrosine kinase inhibitors (TKIs) eventually develop resistance as a result of ABL1 kinase domain (KD) mutations, and sequential treatment with different TKIs may select for multiple BCR-ABL1 mutations. Whether multiple mutations arise in distinct clones (in *trans*, or polyclonal mutations) or instead are present within the same BCR-ABL1 molecule (in *cis*, or compound mutations), has been shown to have important implications with respect to TKI sensitivities (ref. 1). Distinguishing between polyclonal and compound mutations, or mutation phasing, for the ABL1 KD has not been clinically practical with standard mutation detection methods. Here we have developed a highly sensitive next-generation sequencing (NGS) assay on the Ion Torrent PGM along with a proprietary data analysis pipeline that together enable deep sequencing of the BCR-ABL1 KD and neighboring domains with a 1% limit of detection and quantitative reporting of mutation phasing.

Materials and Methods

Workflow: As shown in **Fig.1**, RT and long range PCR was performed to amplify BCR-ABL1 e1a2/3, e13a2/3, and e14a2/3 fusion transcripts, and the PCR products were enzymatically randomly fragmented and ligated with Ion Torrent sequencing adaptors. Size-selected libraries were quantified, pooled, and amplified with the OneTouch system and sequenced with the Ion Torrent PGM using 400 bp sequencing chemistry. Sequencing data were analyzed with Torrent Suite 3.4.2 with variant frequency cutoff adjusted to 1%. Variants were further annotated with a proprietary analysis pipeline and the variant report was produced following manually review of variants by Integrative Genomics Viewer. If more than one non-synonymous variant was reported in a sample, a proprietary phasing analysis pipeline was applied to report the mutation spectrum of all of the combinations of multiple mutations in the sample.

Algorithm: Here as we employed a random fragmentation based sequencing strategy, multiple variants, such as A, B and C shown in **Fig.2**, will be covered by a variety of reads starting and ending at different nucleotide positions. To determine the phasing of the mutations, theoretically only reads covering all of the three variant positions can be used. However, the limited number of reads covering all variant positions, especially for the more widely separated variants (>300 bp), may result in lower accuracy. To maximize the reads to be used for phasing computation and further increase the accuracy, we developed an algorithm to compute the phasing status by using all of the reads. As shown in **Fig.1**, if more than one positive variant is reported after variant calling and IGV review, we calculated the possible combinations of these variants. For example, three variants will have the four possible compound mutations, A-B, B-C, A-C, and A-B-C. The relative frequency of each potential compound mutation was calculated by using the number of reads containing the compound mutation divided by the total number of reads covering all of the positions of the compound mutation. Mutation spectrum, or real mutation frequency, is derived based on the relative frequency of the longest compound mutation, i.e. A-B-C, as shown in **Table 1**, because relative mutation frequencies of A-B, B-C, or A-C will also reflect the contribution of A-B-C. The computation workflow was established as a pipeline analysis by integrating samtools pileup and custom scripts.

Figure 1. Workflow of ABL1 Phasing Test

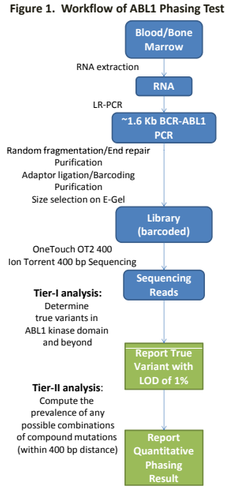


Figure 2. Scheme of phasing status of three variants

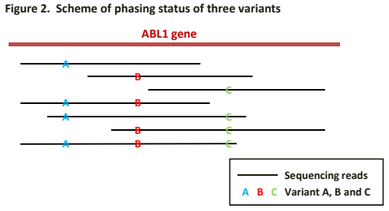


Table 1. Mutation spectrum frequency calculation (using 3 mutations, i.e. A, B, and C as an example)

Mutation Phasing	Relative Frequency	Spectrum Frequency
A-B-C	r1	r1
A-B	r2	r2-r1
A-C	r3	r3-r1
B-C	r4	r4-r1
A	r5	r5-r2-r3+r1
B	r6	r6-r2-r4+r1
C	r7	r7-r3-r4+r1
WT	r8	100 – sum (all variant spectrum frequency)

Results

Verification of the accuracy of Ion Torrent 400 bp sequencing chemistry

To validate the accuracy of the sequencing method, which employs 400 bp sequencing chemistry, we compared this assay with our previously-validated BCR-ABL1 NGS assay based on Ion Torrent 200 bp sequencing chemistry for a set of clinical specimens from CML patients previously treated with TKI (ref. 2). Results as shown in **Table 2** were highly concordant and similarly sensitive, with 11/11 variants (frequencies ranging from 2% to 100%) identified with comparable frequencies by both methods. For example, NGS analysis of sample b2_6 using 400 bp and 200 bp sequencing chemistries both identified 4 mutations, i.e. L248V, G250E, T315I and M351T with mutation frequency at 32.9%, 51.2%, 15.4%, and 3.5%, respectively.

Table 2. Comparison of accuracy of 400 bp chemistry with that of 200 bp chemistry

Sample	CDS Change	Amino Acid Change	Mutation Frequency (200 bp chemistry)	Mutation Frequency (400 bp chemistry)
b2_6	c.742C>G	L248V	35.95%	32.88%
	c.749G>A	G250E	47.02%	51.16%
	c.944C>T	T315I	14.31%	15.43%
	c.1052T>C	M351T	4.23%	3.53%
b2_10	c.951C>G	F317L	99.95%	99.46%
	c.1375G>A	E459K	98.02%	97.52%
b2_13	c.742C>T	P158S	3.73%	3.82%
	c.487A>G	I163V	2.15%	2.09%
	c.778G>T	V260L	3.79%	4.00%
	c.835G>T	E279X	4.34%	3.95%
	c.1245G>T	K415N	3.05%	3.21%
b2_15	No variant			
5% b2_10 in b2_6	c.742C>G	L248V	Sample not tested by 200 bp seq	31.01%
	c.749G>A	G250E		48.83%
	c.944C>T	T315I		13.81%
	c.1052T>C	M351T		3.62%
	c.951C>G	F317L		6.72%
	c.1375G>A	E459K		6.38%

Verification of the specificity of phasing analysis

To validate the specificity of our phasing analysis, an artificial sample was created by mixing sample b2_10 (containing F317L at 100%, and E459K at 98%) with b2_6 at ratio of 1:19. Since variants in b2_6 and b2_10 are unique in each sample, any compound mutation identified containing a variant from b2_6 and a variant from b2_10 will be false positive. **Table 3** shows that the background error rate (percentage of b2_6 variant as compound mutation with b2_10 variant and percentage of b2_10 variant as compound mutation with b2_6 variant) ranged from 0 to 0.6%, which was close to the sequencing error rate. We established a conservative baseline determination of a compound mutation as true if it is present in at least 5% of any one of the component variants in the compound mutation.

Table 3. Specificity of phasing analysis using mixture of sample b2_6 and b2_10

		% of b2_6 variant as compound mutation with b2_10 variant		% of b2_10 variant as compound mutation with b2_6 variant	
Variant in b2_6	Variant in b2_10	Expected	Observed	Expected	Observed
L248V	F317L	0%	0.14%	0%	0.60%
G250E	F317L	0%	0.05%	0%	0.29%
T315I	F317L	0%	0%	0%	0%
M351T	F317L	0%	0.13%	0%	0.07%
M351T	E459K	0%	0%	0%	0%

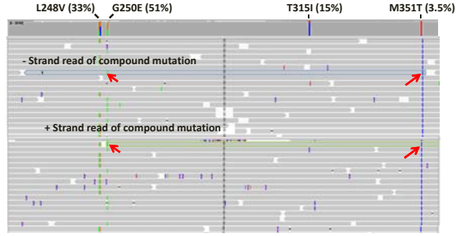
Phasing analysis of clinical samples from CML patients

After verifying the accuracy and specificity of the assay, we performed phasing analysis on the set of samples. **Table 4** shows an example of mutation spectrum results from sample b2_6. No compound mutations containing 3 or 4 variants were observed. Among the 6 combinations of compound mutation with two variants, L248V and G250E were in *trans*, while the other 5 compound mutations existed at variable frequencies. Clearly, the 4 single mutations were still dominant in this sample. The compound mutation status was further confirmed with IGV review as shown in **Fig. 3**.

Table 4. Quantitative phasing analysis of sample b2_6

L248	G250	T315	M351	Spectrum Frequency
L248V	WT	T315I	WT	2.38%
L248V	WT	WT	M351T	0.84%
L248V	WT	WT	WT	32.78%
WT	G250E	T315I	WT	2.80%
WT	G250E	WT	M351T	1.20%
WT	G250E	WT	WT	43.00%
WT	WT	T315I	M351T	0.16%
WT	WT	T315I	WT	8.7%
WT	WT	WT	M351T	1.80%
WT	WT	WT	WT	6.34%

Figure 3. IGV view of compound mutation in sample b2_6



Phasing status of double mutations can be variable among patients

A recent study has indicated that a compound mutation may confer stronger resistance to TKI than mutations in *trans* (**ref. 3**). In a second set of CML patient samples, we identified 7 samples with both T315I and F317L mutations present (data not shown). Interestingly, our phasing analysis showed that in 6 of the 7 samples containing these two mutations, they are in *trans* as shown in **Fig. 4**, which is consistent with the findings from Khorashad's recent study (ref. 3). However, one sample shown in **Fig. 5** clearly harbored a compound mutation of T315I and F317L. This result indicates the complexity of the phasing status of double mutations, and suggests distinct mutation evolution paths are possible in patients taking TKIs.

Figure 4. IGV view of T315I and F317L in *trans*

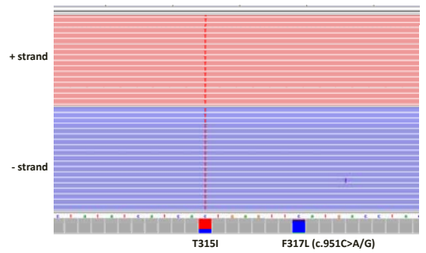
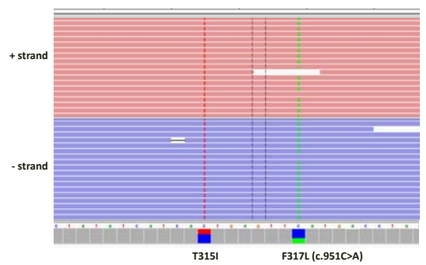


Figure 5. IGV view of T315I and F317L in *cis*



Conclusions

We have developed and validated a sensitive NGS assay that enables deep sequencing of the BCR-ABL1 KD and neighboring domains along with quantitative mutational phasing. This method has been applied in evaluating more than 250 clinical specimens for a clinical trial of a third-generation TKI (results reported separately). The ability to readily determine the mutation phasing of a CML patient's mutation profile using this assay will allow for investigations into compound mutation-based resistance mechanisms and may be used to better guide treatment decisions.

References

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