Ann Hematol (2016) 95:201–210
DOI 10.1007/s00277-015-2539-0

ORIGINAL ARTICLE

Detection of *BCR-ABL1* mutations that confer tyrosine kinase inhibitor resistance using massively parallel, next generation sequencing

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Received: 11 June 2015 / Accepted: 25 October 2015 / Published online: 10 November 2015
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Abstract Detection of *BCR-ABL1* mutations that confer resistance to tyrosine kinase inhibitors is important for management of patients with t(9;22);*BCR-ABL1*-positive (Ph+) leukemias. Testing is often performed using Sanger sequencing (SS) which has relatively poor sensitivity. Given the widespread adoption of next generation sequencing (NGS), we sought to reevaluate the testing in the context of NGS methods. We developed an NGS-based *BCR-ABL1* mutation test on the Ion Torrent Personal Genome Machine (PGM) to test for resistance mutations, primarily in the kinase domain in *BCR-ABL1*. We analyzed 508 clinical samples from patients with Ph+ leukemias. In a subset of these samples (n=97), we conducted a comparison of the NGS results to a classical SS-based test. NGS facilitated detection of low-level mutations (<20 % allele frequency) that were not detectable by SS. In a subset of cases with multiple mutations, NGS was also able to determine if two mutations were on the same molecule (compound) or on separate molecules (polyclonal) but this was limited by the distance between mutated positions and by the effects of apparent distance-dependent PCR recombination. We found 22 compound mutations that centered on one or two key residues including two novel compound mutants: Q252H/Y253H and F311Y/F359I. The advantages of NGS make it a superior method for inventorying *BCR-ABL1* resistance mutations. However, data analysis may be complicated by short read lengths and the effects of PCR recombination.

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Keywords Chronic myelogenous leukemia · *BCR-ABL1* · Tyrosine kinase inhibitors · Molecular testing · Next generation sequencing · Lymphoblastic leukemia · Philadelphia chromosome

Introduction

Optimal clinical management of patients with chronic myeloid leukemia (CML) or Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL) requires the detection of acquired *BCR-ABL1* mutations that may impart resistance to tyrosine kinase inhibitors (TKIs). These mutations primarily arise in the kinase domain [1], with rare occurrences in upstream SH2 (src-homology 2) or SH3 (src-homology 3) signaling domains [2]. Clinical laboratories have traditionally relied on Sanger sequencing (SS) to identify mutations [3]. However, the nature of this method makes it unsuitable for identifying low-level variants (<20 % variant frequency). Also, it is generally not possible to unequivocally discern between multiple mutations occurring in the same clone (compound mutants, *in cis*) as opposed to separate clones (polyclonal mutants, *in trans*). The latter is true because SS yields a mixture of sequences, and an analysis of the underlying structure and relationship of potential subclones is not possible. Such analysis may be important in the context of heavily treated patients who have received multiple, sequential TKIs and who may have a complex underlying clonal structure, often with subclones that demonstrate a variety of patterns of mutations [4–6]. *BCR-ABL1* compound mutations can confer high-level resistance, even in the context of newer, highly active TKIs such as ponatinib [7]. For example, in vitro studies have demonstrated that E255V/T315I occurring as a compound mutation has a much higher ponatinib IC_{50} than either mutation occurring alone [8]. A recent clinical study supports

the problematic nature of E255V/T315I in the context of ponatinib treatment [9]. The TKI sensitivity of dozens of potential compound mutants remains to be addressed although progress has recently been made in this regard [9]. The importance of determining compound versus polyclonal mutation status is likely to increase as patients are sequentially exposed to an increasing number of TKIs and develop more complex underlying mutation patterns that may be recalcitrant to therapy.

Here, we describe a next generation sequencing (NGS)-based sequencing assay on the Ion Torrent Personal Genome Machine (PGM) that is suitable, in terms of both cost and performance, for deployment to a clinical hematology laboratory along with a comparison of the results to a SS-based test in a large number of clinical samples. Our data indicates that NGS is markedly superior to SS in terms of sensitivity with the added advantage, in certain circumstances, of determining the clonal configuration of multiple mutations.

Materials and methods

Patient samples

The use of surplus de-identified patient specimens processed in the ARUP Molecular Oncology laboratory was approved by the University of Utah Institutional Review Board.

NGS library construction

Eight microliters RNA (concentration range 30 to 300 ng/μL) were used to synthesize random-primed complementary DNA (cDNA) using the SuperScript III First-Strand Synthesis System (Life Technologies, Grand Island, NY). Two microliters cDNA were PCR amplified in 20 μL of 1X Phusion High-Fidelity PCR Mastermix with HF Buffer (New England Biolabs, Inc., Ipswich, MA) and 0.3 μM, each of multiplexed forward primers BCR.1-F, 5′-CT CGC AAC AGT CCT TCG AC and BCR.13-F, 5′-ACA GCA TTC CGC TGA CCA T and 0.3 μM of reverse primer ABL.10-R1, 5′-TGG AGT GAG GCA TCT CAG. The cycling conditions were 98° for 30 s followed by 40 cycles of 98° for 10 s, 60° for 10 s, 72° for 90 s, followed by a hold at 72° for 2 min and a cool down. This primary PCR product had a size between 1611 and 1742 base pairs, depending on the fusion breakpoint. One microliter of the PCR reaction was subjected to an additional amplification under the same conditions except with forward primer ABL.2-F, 5′-CTC AGG GTC TGA GTG AAG and reverse primer ABL.10-R2, 5′-TCA GGC ACG TCA GTG GTG for 30 cycles only. The 5′ ends of primers ABL.2-F and ABL.10-R2 were blocked with an amino modifier and C6 spacer (Integrated DNA Technologies, Inc., Coralville, IA) to prevent adaptor ligation to the unfragmented PCR product in subsequent steps. The secondary PCR product had a size of 1531 base pairs. The PCR product was purified using the MiniElute PCR Purification Kit (Qiagen, Inc., Valencia, CA), according to the manufacturer's instructions, with a final elution volume of 20 μL. The DNA concentration was determined on a NanoDrop instrument (Thermo Scientific, Wilmington, DE). One microgram of PCR product was used for enzymatic fragmentation, Ion Torrent adaptor ligation, and PCR amplification using the NEBNext Fast DNA Fragmentation and Library Prep Set for Ion Torrent (New England Biolabs, Inc.) according to the manufacturer's instruction. The universal library adaptors provided in the kit were substituted with 1 μL Ion Xpress Barcode adaptor plus 1 μL P1 adaptor (Life Technologies). The adaptor-ligated library was size fractionated according to the provided protocol for 100 base pair sequencing reads using AMPure XP Beads (Beckman Coulter, Indianapolis, IN). The libraries were amplified for 4 cycles. A 1000-fold dilution and a 20,000-fold dilution of the final library were quantified using the Ion Torrent/LightCycler 480 Library Quantification Kit (Kapa Biosystems, Inc., Woburn, MA).

Emulsion PCR and semiconductor sequencing

Equimolar amounts of sequencing libraries with different barcoded adaptors were mixed, and an absolute amount of 2.8×10^8 molecules was used for emulsion PCR using the Ion OneTouch 200 Template Kit v2DL on the Ion PGM OneTouch System (Life Technologies) according to the manufacturer's instructions. Up to five different libraries were pooled for downstream sequencing on an Ion 314 Chip (1.1×10^6 possible reads), and up to 15 libraries were pooled for sequencing on an Ion 316 Chip (5×10^6 possible reads, Life Technologies). The resulting template-positive Ion Spheres were then sequenced using the Ion PGM 200 Sequencing Kit with 500 nucleotide flows on the Ion PGM (Life Technologies) according to the manufacturer's instructions.

Data were analyzed using Torrent Server software v3.2 with default parameters, including a variant caller lower limit of 4 %. All variant calls were viewed with the Integrated Genomics Viewer (IGV) software. Low-level false variant calls that arise due to the presence of PCR duplicates were manually excluded by review in IGV if they were present in the context of identical read coordinates (i.e., same start position) and could thus be assumed to be false positives due to low-level sequence errors introduced during PCR. The assay covered *ABL1* codons 46–542 (Genbank accession NM_005157).

Sanger sequencing confirmation

For the NGS versus SS method comparison, most samples were analyzed using a clinically validated "standard" SS test used previously in the laboratory [10] that covered *ABL1*

codons 239–368. However, the NGS test covered a much wider region (*ABL1* codons 46–542). Therefore, in the rare instances when mutations were discovered by NGS that were outside the region covered by the standard SS test (ex. sample 50 and sample 59), they were subjected to custom SS as follows. The column-purified nested PCR product used for library construction was diluted 5-fold with water, and 6 μL were mixed with 8 μL 0.8 μM sequencing primer. Forward and reverse sequencing primers were chosen that hybridized at least 60 bases from the mutation site. The template/primer mixtures were subjected to dideoxy sequencing by BigDye technology (Life Technologies) and analyzed on an ABI 3730 instrument (Life Technologies). Sanger-generated sequence was analyzed and variant calls were made using SeqScape software (Life Technologies).

Assessment of *cis*/*trans* configuration of mutation pairs

An algorithm was written to assess the aligned NGS data (.bam files) for informative reads. These are reads spanning both positions of a mutation pair when multiple mutations were detected. The presence of a wild type or variant (mutated) nucleotide was then determined for each position (A and B) of an informative read. Using the information provided by all informative reads, the frequencies for all possible configurations were tabulated as follows: 1. variant A only (A_), 2. variant B only (_B), 3. both variants (AB). The reads with two wild type positions were not taken into consideration. The reads with only one variant present (A_ and _B) were considered *in trans* (polyclonal), and the reads with both variants present (AB) were considered *in cis* (compound). The fraction of reads with a compound configuration and the fraction with a polyclonal configuration were then calculated by dividing the number of *in cis* reads or *in trans* reads by the number of total reads (sum of *in cis* plus *in trans* reads). For scenarios one and two shown in Fig. 1, an *in trans* and an *in cis* configuration, respectively, can be derived by this method. For scenario 3, this method is not adequate. Cases with widely differing allele frequencies would be inappropriately classified as *in trans*. Therefore, the number of *in trans* reads for the more abundant variant (as in scenario 3 in Fig. 1) has to be adjusted to that of the less abundant variant, for example, 0.6 % _B in scenario 3 in Fig. 1. It is important to keep in mind that PCR recombination will result in artifactual *in cis* mutation configurations in scenarios where two mutations are actually *in trans* and artifactual *in trans* configurations in scenarios where two mutations are actually *in cis*.

Mutation pairs had to be at least 80 % *in cis* or *in trans* in order to be interpreted as having that configuration. This value was selected for two reasons. First, we noticed that when both mutations are at a low allele frequency and/or at an increasing distance, the distinction between an *in cis* and *in trans* configuration can become inconclusive due to the low number of informative reads. Second, the chosen cut-off also had to allow for a background of the alternate configuration (very likely due to PCR recombination) that generally did not reach more than 15 % (see Results). If mutation pairs were not at least 80 % *in cis* or 80 % *in trans*, then no interpretation of configuration was rendered. If informative sequencing reads were not available because of the length apart of two positions of a pair, then compound (*in cis*) status was inferred when one mutation was at ≥95 % frequency while the other was also highly frequent (i.e., ≥30 %). If informative reads were not available and if the above frequency criteria were not met then no interpretation with regard to mutation configuration was rendered.

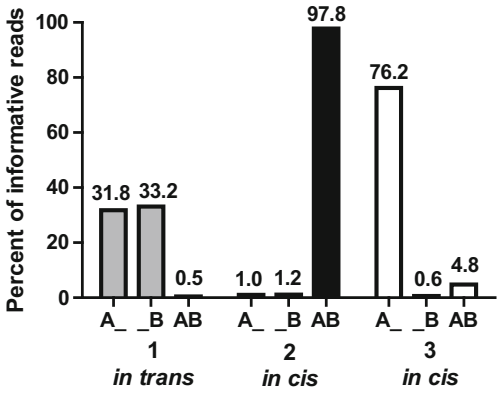

Fig. 1 Scenarios of double mutation configurations. Three classes of informative reads (covering both mutation sites) are shown with three different scenarios (*A_* variant A only, *_B* variant B only, *AB* variant A with variant B). The assumed true configuration is shown at the bottom. The percent of the informative reads with that configuration is shown above each bar

Results

We designed a next generation sequencing assay on the Ion Torrent PGM platform for analysis of *BCR-ABL1* mutations that may impart resistance to TKI therapy covering *ABL1* codons 46–542. In our assay, sequencing libraries were prepared from amplicons derived from nested PCR amplification of a 1531-base pair fragment containing the SH2, SH3, and kinase domains using first round PCR forward BCR primers complementary to *BCR* exon 1 and *BCR* exon 13 and a common reverse *ABL1* primer. This multiplex reaction is designed to amplify all of the common fusion forms including those that result in *BCR-ABL1* p190 (e1a2) and p210 (e13a2, e14a2) (Fig. 2a). Our strategy routinely yielded even sequence coverage across the region of interest (Fig. 2b), a characteristic that is important for detection of low-level mutations. Detection of low-level mutations, independent of their specific identities, has recently been demonstrated to have prognostic value [11]. Reproducibility studies were carried out on multiple samples with a wide range of mutant allele frequencies. Coefficients of variance ranged up to 26 % for allele frequencies

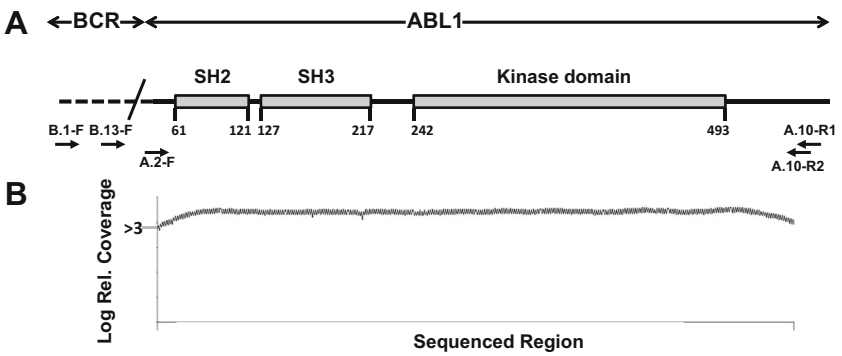

Fig. 2 *BCR-ABL1* PCR strategy. **a** Schematic of the strategy for nested amplification of a *BCR-ABL1* fragment containing the SH3, SH2, and kinase domains. The *ABL1* portion shown starts at exon2 (codon 28; *numbers* indicate codons). The *arrows* indicate primer binding sites; primer designations are shown. **b** Corresponding typical relative sequence coverage obtained. The coverage depth is shown schematically on a log scale with the value 3 (1000×) indicating the minimum coverage required. See text for a summary of coverage depths achieved

below 10 % and up to 10 % for allele frequencies above 10 % (data not shown). Mutations of unknown significance were found occasionally and at mostly low (<20 %) allele frequencies and were often not reproducible. In this study, we only describe mutations with a known association with TKI resistance. The accuracy of allele frequencies generated by the Ion Torrent software (default limit of detection is 4 %) was confirmed by comparisons to the values obtained from a quantitative pyrosequencing assay for the T315I mutation [10]. Samples with T315I mutant allele frequencies ranging from 4 to 78 % (by pyrosequencing) yielded an R^2 value of 0.9695 upon comparison between the two methods (data not shown). In addition, high quality sequence was obtained from samples with a tumor burden below 0.1 % on the international scale (MMR, major molecular response) as determined by a quantitative PCR test for the major breakpoint *BCR-ABL* transcript (data not shown).

Comparison of next generation sequencing to Sanger sequencing

We performed our NGS assay on a total of 508 clinical samples submitted to our reference laboratory for *BCR-ABL1* mutation analysis. To conduct a comparison of NGS to SS, 97 of 508 samples were included because they were previously characterized by a SS-based test. The remaining 411 consecutive clinical samples were analyzed by NGS alone. Twenty-one of the 97 samples were specifically chosen because they had multiple mutations in order to evaluate the ability to determine the *in cis* and *in trans* configuration of mutation pairs by NGS (see below). Twenty-two of the 97 samples were chosen because they had no mutations by SS, and the remaining 54 cases represent consecutively submitted cases that demonstrated mutations by SS.

For the 97 samples characterized by both methods, all mutations that were detected by SS were also detected by NGS. However, 12/75 Sanger sequencing-positive cases (16 %) demonstrated a total of 15 additional mutations by NGS with mutation frequencies ranging from 4–21 % (Table 1). Nine samples demonstrated one additional mutation by NGS and three samples demonstrated two additional mutations by NGS. Fourteen of the 15 additional mutations detected at a low level by NGS are known to be clinically significant including resistance mutations such as Y253F, E255K, V299L, F359V, and T315I. Only one of these 15 mutations (case 50; N49K) has not been reported in patients with TKI resistance and is thus of uncertain significance. One of 22 cases that were mutation-negative by SS demonstrated a mutation (Y253F at 5.5 %) by NGS that is known to confer TKI resistance. The high number of significant low level mutations detected in this subset of cases may be biased because of the sample selection process (samples with one or even multiple high level resistance mutations).

Compound *BCR-ABL1* mutations

Occasionally, multiple mutations may be detected by SS. It is often quite difficult to determine if multiple mutations in the same sequencing electropherogram are compound mutations or polyclonal mutations using SS. Compound mutational status may be definitively assigned if both mutations appear to represent 100 % of the signal at each position, but mutations are often present in a mixed background with wild-type sequence and in this context, configuration cannot be determined. Standard NGS is also limited in this regard unless the mutations are close enough in the sequence to provide informative sequencing reads spanning both mutated positions.

We typically achieve an average read length of approximately 120–140 base pairs that allow us to generate significant numbers of informative reads if two mutations are up to 50 codons apart. This number is an approximation as the number of informative reads that cover both mutation sites

Table 1 Sanger-positive cases that demonstrated additional mutations by NGS

Sample ID	Position	Reference	Variant	% Variant	SS detection
6	253	Y	*F*	8	No
	255	E	*K*	7	No
	255	E	V	39	
	315	T	I	43	
10	248	L	*V*	8	No
	250	G	E	25	
	351	M	T	72	
12	255	E	K	30	
	299	V	*L*	13	No
	317	F	L	50	
29	299	V	*L*	4	No
	315	T	I	36	
34	317	F	C	32	
	317	F	*L*	5	No
36	253	Y	*H*	6	No
	315	T	I	43	
	359	F	*V*	16	No
41	315	T	I	89	
	317	F	*I*	10	No
44	315	T	I	79	
	359	F	*V*	21	No
50	49	N	*K*	12	No
	317	F	L	100	
52	315	T	*I*	4	No
	317	F	L	95	
59	276	D	*A*	11	No
	397	A	P	85	
66	253	Y	*H*	5	No
	315	T	*I*	4	No
	317	F	L	91	

Italicized variants were detected by NGS but not Sanger sequencing

SS Sanger sequencing

also depends on each mutation's allele frequency. The data therefore allowed for at least a proof-of-principle study of compound mutation detection. Figure 3 shows examples of *in cis* and *in trans* double mutation conformations in NGS data visualized by Integrated Genomic Viewer software. For closely spaced mutation pairs as shown, the determination of the compound or polyclonal status is unequivocal since the vast majority of reads are in one of the configurations. In our data set, 45 mutation pairs were close enough to be designated *in cis* or *in trans* based on analysis of informative NGS reads using the algorithm described in Materials and Methods. Thirty-eight of those pairs were *in trans* and seven pairs (representing six patients and five unique mutation pairs) were identified as compound (*in cis*). An additional 23 mutation

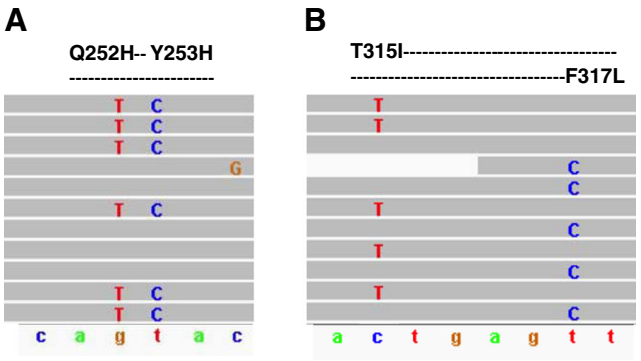

Fig. 3 Examples of raw NGS data showing *in cis* and *in trans* mutation configurations. Portions of the display of the Integrated Genomic Viewer program are shown for two cases, one with a Q252H/Y253H double mutation with an *in cis* configuration (**a**) and one with a T315I/F317L double mutation with an *in trans* configuration (**b**). The configuration of the resulting amino acid change is shown at the *top*. The reference sequence is shown at the *bottom*. Mutations are indicated in each read

pairs (representing 21 patients and 17 unique mutation pairs) could be classified as *in cis* (compound) because at least one mutation was at or near 100 % frequency while the other was also highly prevalent (i.e., >30 %), regardless of the distance between the mutation pairs. Table 2 lists the 22 different compound mutation pairs identified. T315I and V299L were most frequently identified as components of compound mutation pairs, appearing in 12/25 patients (48 %) and 5/25 patients (20 %), respectively, but they were never together as a compound mutation although two cases demonstrated both mutations as separate clones, *in trans*. The combinations of F317I/F359V, E255K/T315I, and E279K/T315I were each found in two patients. Compound mutations comprised of F317I/F359V, F317L/Y253H, and E279K/T315I were each identified twice at separate times in the same patients.

Evaluation of the effects of possible PCR recombination

PCR-mediated recombination can produce artificial sequences containing elements of two highly similar template sequences [12]. This results from extension of a previously incompletely extended PCR product, leading to the production and subsequent amplification of a chimeric sequence [12]. It has recently been suggested that this process results in artifactual PCR products with apparent *BCR-ABL1* compound mutations [13]. Thus, tests such as this one that rely on PCR amplification may detect compound mutations that have not actually arisen in vivo. When examining informative NGS reads that cover both mutation sites in a double mutant sample, the observed configuration was never 100 % *in cis* or 100 % *in trans*. We assumed that the "contaminating" configuration was likely due to PCR-mediated recombination during NGS library preparation. Since the likelihood of a PCR-mediated recombination event is thought to increase over distance, we attempted to test this hypothesis in our data set.

Table 2 Compound mutations

Mutation 1	Mutation 2	# Patients (# samples)	Sample ID	Mutation 1 and 2 Allele frequencies
Compound mutation involving T315I				
E255K	T315I	2	120, 214	65 % 100 %; 99 % 100%[c]
E255V	T315I	1	72	92 % 39 %
V279K	T315I[a]	2 (3)	102/172[b], 175	
L298V	T315I[a]	1	196	
F311L	T315I[a]	1	7	
T315I	M351T	1	18	99 % 99 %
T315I	E355G	1	157	61 % 99 %
T315I	F359C	1	15	97 % 99 %
T315I	F359V	1	19	99 % 99 %
T315I	A380S	1	61	100 % 93 %
Compound mutations involving V299L				
G250E	V299L	1	16	74 % 98 %
L298V	V299L[a]	1	14	
V299L	M351T	1	1	100 % 100 %
V299L	E355G	1	5	100 % 69 %
V299L	L384M	1	5	100 % 33 %
Other compound mutations				
Q252H	Y253H[a]	1	4	
Y253H	F317L	1 (2)	156/198	81 % 96 %/46 % 96 %
E255K	F359I	1	134	82 % 98 %
E255K	V379I	1	48	98 % 99 %
F311Y	F359I	1	3	100 % 99 %
F317I	F359V	2 (3)	2, 68/137	99 % 100 %, 98 % 98 %/100 % 100 %
F317L	E355G	1	45	74 % 99 %

[a] These mutation pairs were close enough to be called *in cis* by the NGS algorithm

[b] Sample IDs separated by a slash are from the same patient

[c] The mutant allele percentages used to infer an *in cis* configuration are listed in the same order as the mutations and where applicable in the same order as the sample IDs

Unless present in 100 % of NGS reads, most compound mutation pairs are expected to harbor some level of single mutant reads because the mutations are rarely present at exactly the same allele frequency. On the other hand, the presence of a compound configuration is never expected if the mutations occurred in distinct clones. We therefore assessed our data for a correlation between low levels of compound mutations in samples with multiple mutations classified as polyclonal by our criteria (see Materials and Methods) and distance apart. We examined 14 polyclonal (*in trans*) mutation pairs and found a positive correlation between the distance between mutated positions and the percentage of contaminating reads demonstrating a *cis* (compound) configuration (Fig. 4; R^2= 0.6785, p=0.0003 for a slope significantly non-zero). The mutation types, allele frequencies, and the number of available informative reads for the 14 mutation pairs are summarized in Table 3. However, the overall fractions of reads with compound mutations were relatively small (<20 %) in these cases

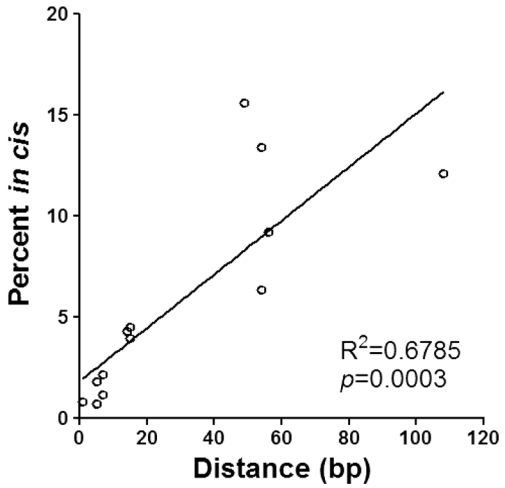

Fig. 4 Distance-dependent increase of detected reads with a compound mutation configuration. For double mutation pairs with at least 80 % *in trans* (polyclonal) reads, the proportion of co-occurring *in cis* (compound) reads is plotted against the distance between the two mutations

and were within our criteria (≥80 %) for determining the *cis*/*trans* configuration of mutation pairs (see Materials and Methods). The data clearly suggest though that a compound (*in cis*) configuration should not be inferred in the presence of only a minority of *in cis* reads if in vitro-generated compound reads cannot be excluded.

Results of sequencing of 411 consecutive clinical cases by NGS

We retrospectively analyzed NGS data on 411 consecutive clinical samples submitted to our laboratory for testing. Almost all samples (408/411; 99.3 %) yielded >1000× average coverage depth (range 616×–63,210×, average=23,500) with multiplexing of up to 15 samples on a single Ion Torrent 316 sequencing chip. A total of 89 samples (from 81 patients) out of 411 samples (21.7 %) demonstrated a total of 132 mutations that were previously associated with TKI resistance with allele frequencies ranging from 4–100 % (average=61.4 %, median=76 %). Of these mutations, 35 (26.5 %) had an allele frequency of <20 % and would not have been detected by SS. These low level mutations were detected in a total of 25 cases (out of 411 tested, 6.1 %). In eight of these cases (2 % of all cases tested), the low level mutation(s) was not associated with a high level mutation and these cases would have been termed negative by a SS test. No known or recurrent mutations were detected outside the kinase domain.

Overall, out of 411 samples, 57 (13.9 %) demonstrated a single mutation followed by a lesser number of samples with two (25; 6.1 %) and fewer still with 3–5 mutations identified (7 total; 1.7 %) (Fig. 5). No sample had more than five mutations.

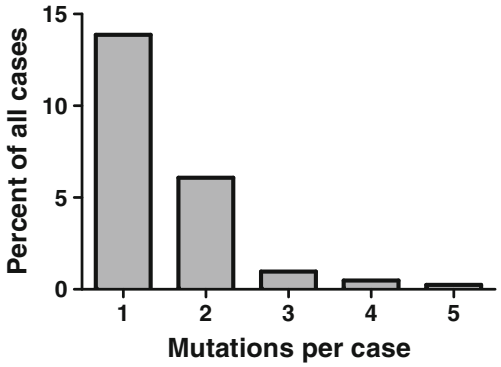

Fig. 5 Frequency of cases with one or multiple mutations

Discussion

BCR-ABL1 kinase domain sequence analysis has become a cornerstone of management of Ph+ leukemia patients on TKI therapy. Patients who demonstrate evidence of resistance manifested by rising *BCR-ABL1* transcript levels by quantitative RT-PCR should be tested for the presence of acquired mutations, primarily in the kinase domain, that impart TKI resistance [14]. The utility of the testing has become magnified in recent years with the approval of second generation TKIs dasatinib and nilotinib as alternatives to imatinib and by recent approval of third generation agents bosutinib [15, 16] and ponatinib [7, 17]. Clinical decision-making with respect to a TKI switch is facilitated by knowledge of the identity of a mutation or mutations and an understanding of the variable sensitivities of mutant *BCR-ABL1* forms to the available TKIs [14]. Furthermore, a prior study shows that the higher the number of low-level kinase domain mutations (as detected by mass spectrometry) present at the time of TKI

Table 3 Low level compound mutation reads likely to have arisen from PCR recombination in polyclonal cases

		Variant A		Variant B		
Distance in aa	% *in cis* reads	aa change	% var	aa change	% var	Informative reads
1	0.79	F359C	15	F359C	43	19,629
5	1.83	T315I	65	F317L	12	22,918
5	0.71	T315I	33	F317L	30	23,010
7	1.14	L248V	14	G250E	29	32,249
7	2.14	L248V	75	G250E	8	27,395
7	2.16	T315I	4	F317L	95	8194
14	4.3	G250E	14	E255K	85	15,568
15	4.51	G250E	74	E255V	22	7979
15	3.98	G250E	63	E255V	19	4710
49	15.62	V299L	76	T315I	16	29,808
54	6.34	V299L	17	F317L	25	23,638
54	13.41	V299L	11	F317L	76	23,173
56	9.23	V299L	41	F317L	16	19,944
108	12.1	E450G	51	F486S	44	2816

aa amino acid

switch, the worse the prognosis for CML patients, regardless of the degree of resistance associated with the mutations [11].

SS is commonly utilized for testing for *BCR-ABL1* kinase domain mutations. However, this technique suffers from a number of shortcomings that may potentially be eliminated by the use of NGS. In order to assess its effectiveness, we compared a laboratory developed NGS-based *BCR-ABL1* mutation test to a traditional SS-based method. Our findings demonstrate multiple advantages of NGS. We found that there is a relatively high rate of missed low-level mutations in cases positive for at least one high level mutation (defined here as >20 %) by SS. Our data also shows a lesser rate of finding undetected lower frequency mutations known to be associated with TKI resistance in cases that are negative by SS (or in random samples submitted for NGS analysis, see below). In total, our comparison of NGS to SS demonstrated mutations below the detection limit of SS in 16.5 % of samples, including mutations known to be clinically important. This number may be biased as the majority of samples examined by Sanger sequencing harbored at least one mutation by this method. Among random samples (sequenced by NGS only), 6.1 % showed low-level mutations. A recent study has shown that low-level mutations, beyond the detection limits of SS, may persist after a positive TKI response and cause resistance even after long periods of time [18]. The ability to routinely detect lower level mutations highlights one significant advantage of NGS over SS that may serve to justify the increased costs.

As patients are sequentially exposed to 2–3 or even more TKIs, the complexity of mutation patterns may increase [19]. This is demonstrated by our data set where 32 (7.8 %) of 411 sequential clinical samples (36 % of mutated samples) demonstrated more than one mutation by NGS. *BCR-ABL1* complexity may include both the number of mutations and the possibility that multiple mutations will be present in the same *BCR-ABL1* allele. These are commonly referred to as compound mutations, and they impart unique properties to the structure of the kinase domain and thus have sensitivity profiles that are unique when compared to the same mutations occurring alone. A recent report suggests that clinically important compound mutations center on 12 key positions within the kinase domain of *BCR-ABL1* including M244, G250, Q252, Y253, E255, V299, F311, T315, F317, M351, F359, and H396 [9]. In line with this study, we found that 22/22 (100 %) of the confirmed unique compound mutations include a key position, and 13/22 (59.1 %) are composed of two key positions. Among the key position pairings, ten have been clinically observed in prior studies and two, Q252H/Y253H and F311Y/F359I, are reported for the first time here. Our results lend further support to the notion that a limited set of kinase domain positions are heavily represented in clinically important compound mutants. The fact that the common key residues T315I or V299L never occurred together as a compound mutation, although they were detected as polyclonal mutations, suggests that there may be no selective advantage imparted by such a compound configuration or that kinase function is compromised. In support of this conclusion, clones with compound V299L/T315I mutations were not detected in other similar studies of cases with multiple mutations [4, 9]. We believe that the potential for detecting compound mutations in certain circumstances is an advantage of NGS, subject to the limitations discussed below.

The ability to identify compound mutations over longer distances between mutation sites than described here may be improved with longer read length options available for the Ion Torrent and other NGS platform but will always be hampered if the mutations occur at low allele frequencies. We identified a trend of distance dependence for the occurrence of low level compound reads among polyclonal reads. This is likely due to PCR-mediated recombination, as was also suggested by others [13]. We set our cutoff for calling a compound configuration at 80 % as a compromise between high stringency and tolerating a certain level of "noise" (up to 15 % in our study). This approach will not allow the identification of true low level compound mutations unless potential PCR recombination and related in vitro-generated artifacts can be excluded with certainty, possibly using PCR independent strategies. The correlation between apparent PCR recombination and distance between mutation pairs was not strict and may also be influenced by the position of the interval within the long range PCR product. In addition, the number of supporting NGS reads decreases with increasing distance between mutation pairs so an accurate estimate of the effect may not be possible. Results on clinical isolates obtained with the NGS technology described in this report can definitively identify a discrete group of compound mutations involving close-lying kinase domain positions such as the previously unreported Q252H/Y253H.

Considering the large knowledge base on TKI resistance mutations in *BCR-ABL1*, it is not surprising that no novel recurrent mutations were discovered. Our data set also indicates that sequencing outside the kinase domain is not warranted for the same reason. It is reasonable at this point to only report data on known ABL1 kinase domain mutations when in the context of managing TKI resistance.

In summary, we have shown that a significant number of *BCR-ABL1* resistance mutations below the detection threshold of routine SS are detectable by NGS. This can be achieved at approximately two times the cost of a SS test. As CML and Ph+ ALL therapies continue to improve and patients live longer with exposure to multiple TKIs [20], the detection of complex patterns of mutations including low-level and/or compound mutations will become increasingly important for proper clinical management. The advantages of NGS over SS for the reasons we have outlined make it the method of choice for routine clinical sequencing evaluation of *BCR-ABL1* mutations.

Compliance with ethical standards

Conflict of interest P.S. and J.A.S. are employed by ARUP Laboratories. T.W.K. is employed by the University of Utah, Department of Pathology.

Funding This study was funded by ARUP Laboratories (grant number not applicable), a reference laboratory owned and operated by the University of Utah, Department of Pathology.

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