

Utilization of Next-Generation Deep Sequencing to Analyze BCR-ABL1 Kinase Domain Mutation for Imatinib-Resistant Chronic Myeloid Leukemia Patients in Vietnam

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Received date: May 10, 2017; Accepted date: May 27, 2017; Published date: June 05, 2017

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Abstract

Background: Chronic myeloid leukemia is a clonal myeloproliferative neoplasm, characterized by the presence of chromosomal translocation t(9; 22)(q34; q11). This is found in over 95% of the cases and results in the BCR-ABL1 fusion protein with high tyrosine kinase activity. During the last decades, imatinib and other generations of tyrosine kinase inhibitor have been used effectively for target therapy of the disease. However, many of the drug resistant cases have been reported recently, due to the mutation within kinase domain of the BCR-ABL1 fusion gene. In order to provide further information about this incidence, we performed a retrospective study of 141 imatinib-resistance chronic myeloid leukemia patients to analyze kinase domain mutation by deep sequencing. Another group of 20 untreated patients were added as control.

Methods: RNA from bone marrow cells were extracted and followed by cDNA synthesis. Nested polymerase chain reaction was performed to amplify kinase domain of the BCR-ABL1 fusion gene. The amplified products were monitored size, concentration and prepared DNA sequencing library. Sequence analysis was performed using Illumina MiSeq sequencer and Sequence Pilot software. The sequencing results were randomly chose for Sanger sequencing.

Results: None of the control group was positive with kinase domain mutation. There were 47 out of 141 patients (33%) detected with at least one nucleotide substitution. The sequencing results were also confirmed by Sanger sequencing. Of those 47 samples, 72 nucleotide substitutions of 28 types, altered 24 codons were identified. Among those, Y253F/H, M351T, G250E, F359V/I and M244V were the most frequent mutations, while T315I took only 4.1%. There were also a number of samples harboring multiple substitutions and new variations. Conclusion: Next-generation deep sequencing is a sensitive and effective method to detect kinase domain mutation and our results could provide further information about the drug-resistance mutation in chronic myeloid leukemia.

Keywords: Chronic myeloid leukemia (CML); BCR-ABL1; Imatinib resistance; Tyrosine kinase inhibitor (TKI); Kinase domain mutation; Next-generation sequencing (NGS); Deep sequencing

Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm, which is characterized at molecular level by the presence of chromosomal translocation t(9;22)(q34;q11) [1]. This namely Philadelphia (Ph) chromosome, found in more than 95% of CML patients, results in the fusion gene BCR-ABL1 and the fusion protein that having constitutive tyrosine kinase activity [2]. The progression of CML consists of three phases which differs in time and cellular characteristics. The chronic phase (CP) marks with slightly elevated number of blast cells and is the least aggressive period. The more aggressive accelerated phase (AP) is defined as increasing number of immature cells and additional chromosomal abnormalities. The last and most aggressive phase is blast crisis (BC), which characterized by a

large number of blast cells that populated in bone marrow and peripheral blood [3].

Since 2001, imatinib mesylate (Gleevec) has been using as front-line targeted therapy for CML patients [4]. This tyrosine kinase inhibitor (TKI) and other generations of TKI, including dasatinib and nilotinib, have been effectively used over the last decade. Despite the high response rate, treatment resistance to TKIs within CML patients have been observed elsewhere, which count for 20%-40% [5]. Primary (intrinsic) resistance is defined as lack of initial response and secondary (acquired) resistance is defined as loss of achieved response during TKI treatment [6,7]. According to the National Comprehensive Cancer Network (NCCN) guidelines version 4, 2016, resistance is defined as the patients who gained no achieved response, or failed to achieve partial cytogenetic response (PCyR) at 3 months, or failed to achieve complete cytogenetic response at 12 months and 18 months. At the molecular level, the resistance is defined as the ratio of BCR-ABL1/ABL1 mRNA transcripts is above 10% of international scale (IS) [8]. So far, several mechanisms of TKI resistance have been clarified. These

include drug intolerance, enhance of BCR-ABL1 transcription level and most importantly, BCR-ABL1 kinase domain mutation [5]. A large number of mutations (mainly nucleotide substitution) have been observed, by Soverini and later by other groups, within approximately 600 base pairs of the kinase domain [9-12]. However, these mutations are showing different resistant properties to TKIs. To date, several of these mutations have been well characterized and led to reselection or change of TKI. Of those, T315I is known to drive drug resistance to all three generations of TKI, while other mutations conferring clinical resistance to either nilotinib (Y253H, E255K/V, F359V/C/I) or dasatinib (V299L, T315A, F317L/I/V/C) [13]. Recently, it has been described that patients already carried a mutation tend to develop additional mutations, associated with poor prognosis [14].

Mutation analysis has been much benefited by the development of sequencing technologies, especially next-generation sequencing (NGS). Unlike Sanger-based sequencing, NGS provides more advanced mutation analysis with larger parallel, deeper target resequencing and, therefore, able to detect lower frequency and earlier existence of mutations. As the national diagnostic and treatment center for CML, we aimed to utilize NGS system (MiSeq sequencer, Illumina, USA) to investigate the occurrence and ratio of kinase domain mutations for a group of our CML patients in Vietnam. The results could add some more information to previous serial studies of kinase domain mutation of CML [8,11].

Pat. No.	Sex	YoB	Phase	BCR-ABL	Karyotyping	Response	Nucleotide substitution	AA change
1	M	1980	AP	B2A2	46,XY,t(9;22)[20]	No response	T --> C (62%)	M351T
2	M	1974	CP	B3A2	46,XY,t(9;22)[20]	No response	G --> A (22%)	G250E
							T --> C (15%)	M351T
3	F	1988	CP	B3A2	46,XX[10]/46,XX,t(9;22)[10]	Minimal response	C --> T (44%)	E505STOP
4	M	1978	CP	B3A2	46,XY,t(9;22)[20]	No response	T --> C (71%)	Y253H
5	M	1961	CP	B2A2	46,XY,t(9;22)[20]	No response	T --> A (10%)	F359I
							A --> G (37%)	D276G
6	F	1982	BP	B3A2	46,XX,t(9;22)[20]	No response	A --> G (69%)	M244V
7	M	1954	CP	B2A2	46,XY,t(1,9;22)[20]	No response	G --> A (75%)	G250E
							T --> C (20%)	M351T
8	F	1988	CP	B3A2	46,XX,add(7p),t(9;22)[20]	No response	T --> C (45%)	M351T
9	M	1989	CP	B3A2	46,XY,t(9;22)[20]	No response	C --> G (41%)	L248V
							C --> A (10%)	T272N
							C --> A (15%)	F317L
10	F	1971	BP	B3A2	46,XX,t(9;22)[20]	No response	G --> A (80%)	E450K
11	F	1959	CP	B3A2	46,XX,t(9;22)[20]	No response	A --> G (46%)	I418V
12	M	1981	CP	B3A2	46,XY[8]/46,XY,t(9;22)[12]	Relapsed	G --> A (49%)	E453K
13	M	1945	CP	B2A2	46,XY,t(9;22)[20]	No response	A --> T (49%)	M388L
14	F	1950	CP	B3A2	46,XX,t(9;22)[20]	No response	C --> A (80%)	L364I
15	F	1956	CP	B2A2	46,XX,t(9;22)[20]	No response	T --> C (39%)	Y253H
							A --> G (3%)	M244V
							G --> A (10%)	G250E
16	F	1994	CP	B3A2	46,XX,t(9;22)[20]	No response	G --> A (56%)	G250E
17	M	1972	CP	B3A2	46,XY,t(9;22)[20]	No response	T --> C (25%)	M351T
							G --> A (34%)	E459K
18	M	1973	CP	B3A2	46,XY,t(9;22)[20]	No response	G --> A (20%)	G250E
							T --> C (25%)	M351T

							T --> G (35%)	F359V
19	M	1957	AP	B3A2	46,XY,t(9;22)[20]	No response	G --> T (44%)	Q252H
							A --> T (45%)	Y253F
20	F	1978	CP	B3A2	46,XX,t(9;22)[20]	No response	T --> C (54%)	Y253H
							C --> T (12%)	T315I
21	F	1968	CP	B3A2	46,XX,t(9;22)[20]	No response	T --> C (8%)	M351T
22	M	1974	CP	B3A2	46,XY,t(9;22)[20]	No response	G --> T (79%)	Q252H
23	M	1973	CP	B3A2	46,XY,t(9;22)[20]	No response	T --> C (48%)	Y253H
							T --> C (21%)	F311L
24	F	1995	CP	B3A2	46,XX,t(9;22)[20]	Relapsed	A --> T (40%)	Y253F
25	M	1953	CP	B3A2	46,XY,t(9;22)[20]	No response	T --> C (69%)	Y253H
26	M	1991	CP	B3A2	46,XY,t(9;22)[20]	No response	G --> C (71%)	Q252H
27	M	1967	CP	B3A2	46,XY,t(9;22)[20]	No response	A --> G (59%)	M244V
28	M	1979	CP	B3A2	46,XY,t(9;22)[20]	No response	T --> G (51%)	F359V
29	M	1962	AP	B3A2	46,XY,t(9;22)[20]	No response	T --> C (29%)	Y253H
							A --> G (19%)	E459G
30	M	1982	AP	B2A2	46,XY,add(1p),t(9;22)[20]	No response	G --> A (18%)	E255K
							A --> T (13%)	E255V
							T --> C (17%)	M351T
							T --> G (9%)	F359V
31	M	1988	CP	B3A2	46,XY,t(9;22)[20]	No response	T --> C (6%)	M351T
32	M	1958	CP	B3A2	46,XY,t(9;22)[20]	No response	A --> G (38%)	E459G
33	M	1962	CP	B3A2	46,XY,t(9;22)[20]	No response	T --> G (31%)	F359V
34	M	1966	CP	B2A2	46,XY,t(1;9;22)[20]	No response	G --> T (8%)	R473L
							C --> A (9%)	A474E
35	M	1979	CP	B3A2	46,XY,t(9;22)[20]	No response	G --> T (11%)	K262N
36	M	1978	CP	B3A2	46,XY,t(9;22)[20]	Relapsed	G --> A (31%)	G250E
							G --> A (16%)	E255K
37	M	1982	BP	B3A2	47,XY,+8,del(21q),t(9;22)[20]	No response	G --> T (10%)	R473L
							T --> G (11%)	C475G
38	M	1941	CP	B2A2	46,XY,t(9;22)[20]	No response	C --> A (8%)	A474E
							T --> G (8%)	C475G
39	M	2011	CP	B3A2	46,XY,t(9;13;22)[20]	No response	C --> A (13%)	A474E
							T --> G (13%)	C475G
40	F	1956	CP	B3A2	46,XX[7]/46,XX,t(9;22)[13]	No response	C --> A (8%)	A474E
							T --> G (8%)	C475G

41	M	1968	CP	B3A2	46,XY,t(9;22)[20]	No response	G --> T (9%)	R473L
42	F	1987		B3A2	46,XX,t(9;22)[20]	Relapsed after HSCT	C --> A (7%)	A474E
43	M	1943	CP	B3A2	46,XY[16]/45,XY,-7,t(9;22)[6]	Relapsed	T --> C (76%)	Y253H
							C --> T (60%)	T315I
							G --> T (10%)	R473L
44	M	1970	CP	B3A2	46,XY,t(9;22)[20]	No response	C --> T (56%)	T315I
45	M	1988	CP	B3A2	46,XY,t(9;22)[20]	No response	A --> G (9%)	M244V
46	F	1950	CP	B2A2	46,XY,t(9;22)[20]	No response	G --> T (9%)	R473L
47	M	1966	CP	B3A2	46,XY,t(9;22)[20]	No response	T --> C (7%)	Y253H

Table 1: Patients with kinase domain mutation analyzed by next generation sequencing

Materials and Methods

Patients and samples

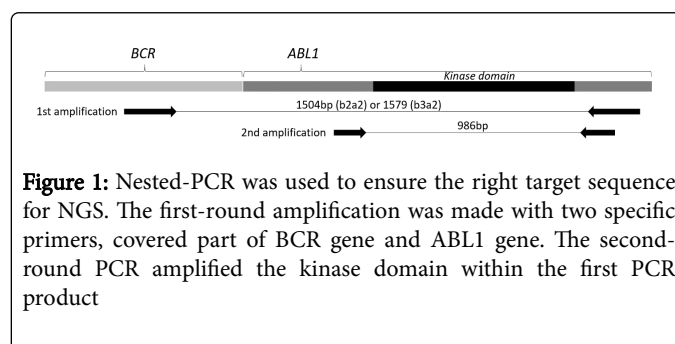
A total of 161 patients (141 treated and 20 un-treated) diagnosed with chronic myeloid leukemia at the National Institute of Hematology and Blood Transfusion (NIHBT), Hanoi, Vietnam, were subjected to this study. Of those, there were 57 females and 104 males, from 6 to 79 years old (average age of 46.9), at time of diagnosis. The cytogenetic and molecular tests for Philadelphia (Ph) chromosome and/or BCR-ABL1 fusion gene are positive. All 141 patients were treated with imatinib (400 mg/day) as front-line therapy for at least 6 months, starting from 2010 to 2016. All those patients either gained no complete cytogenetic response (CCyR) or reported as loss of response at the time of sampling. All patients agreed to participate in this study with written informed consent and the study proposal was approved by the Ethical Committee of the NIHBT.

RNA extraction and cDNA synthesis

Buffy coats were collected from bone marrow aspirate of the patients, followed by total RNA extraction using RNeasy mini kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol. RNA quality and quantity measurements were performed with NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, MA, USA). Reverse transcription from 100ng total RNA was made using Invitrogen SuperScript II Reverse transcriptase (Thermo Fisher Scientific, MA, USA).

BCR-ABL1 kinase domain (KD) amplification

The above-mentioned cDNA product was used for nested-PCR. First round PCR is made with primer pairs to amplify fusion gene BCR-ABL1 and followed by second round PCR to amplify the kinase domain within the ABL gene to ensure the amplification of the right region. This protocol has been described by Sacha, 2003 and adapted by our lab (Figure 1) [15]. The PCR master mix used was KAPA HiFi HotStart ReadyMix (KAPA Biosystems, MA, USA). Final PCR products were run on



1.5% agarose gel to control fragment size and purified using MinElute Gel Extraction kit (QIAGEN, Hilden, Germany), according to manufacturer's protocol. Quality and concentration of purified products were measured using Qubit dsDNA HS Assay kit and Qubit 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA).

DNA library preparation and NGS mutation analysis

A total of 2 ng of each purified PCR product was subjected to perform sequencing library preparation using Nextera XT DNA Library Prep kit (Illumina, CA, USA) according to manufacturer's instruction. In brief, the 986bp DNA fragment was tagged to the sequencing length between 270-300bp, added sequencing adaptor by transposome and followed by barcoding procedure by twelve cycles of PCR amplification. The indices set used to distinguish different samples are combination of i5 and i7 indexing systems from Nextera XT index kit, (Illumina, CA, USA). PCR products were then cleaned up and measured concentration using Qubit 2.0 and KAPA Library Quantification Kit (KAPA Biosystems, MA, USA). The DNA libraries were normalized and purified using coated magnetic beads (using Agencourt AMPureXP, Beckman Coulter, Indianapolis, USA) before pooling to one single tube. At the end, 12.5pM of pooled DNA library was denatured and put to paired-end sequencing, 150 cycles x2, using Illumina MiSeq sequencer and MiSeq Reagent Kit v2 300 cycles (Illumina, CA, USA). For all samples, MiSeq run was set up with QC30 and the number of test sample was calculated in order to achieve minimum of 5,000x coverage.

Sanger sequencing to confirm NGS results

Ten random samples with detected mutation in kinase domain were sent out for confirmation by running on AB3130 Genetic Analyzer Table 2, using BigDye Xterminator kit (Thermo Fisher Scientific, MA, USA). This work was carried out in the molecular biology laboratory of the National Institute of Biotechnology, Hanoi, Vietnam.

Pat. No.	Detected by NGS	Nucleotide substitution (%)	Confirmation by Sanger sequencing
4	Y253H	T --> C (71%)	Confirmed
6	M244V	A --> G (69%)	Confirmed
7	G250E	G --> A (75%)	Confirmed
	M351T	T --> C (20%)	Not detected
9	L248V	C --> G (41%)	Confirmed
	T272N	C --> A (10%)	Not detected
	F317L	C --> A (15%)	Not detected
12	E453K	G --> A (49%)	Confirmed
14	L364I	C --> A (80%)	Confirmed
20	Y253H	T --> C (54%)	Confirmed
	T315I	C --> T (12%)	Not detected
22	Q252H	G --> T (79%)	Confirmed
26	Q252H	G --> C (71%)	Confirmed
43	Y253H	T --> C (76%)	Confirmed
	T315I	C --> T (60%)	Confirmed
	R473L	G --> T (10%)	Not detected

Table 2: List of ten random samples with NGS mutation data confirmed by Sanger sequencing. Only nucleotide substitutions with high ratio were detected by Sanger sequencing, while substitutions equal with 20% and below were not detected.

Bioinformatic analysis

FASTQ data achieved from MiSeq sequencing was analyzed using MiSeq reporter software version 2.5, based on Genetical Analysis Toolkit (Broad Institute, MA, USA), with CLC Genomics Workbench version 9.5 (QIAGEN, Hilden, German) and with Sequence Pilot software (JSI, Ettenheim, Germany) version 4.3.1 for variant calling. The reference sequence used is from NCBI Genbank, accession number NM_005157.5. Aligned sequences, detected variants were then reviewed by Integrative Genomics Viewer software (IGV, Broad Institute). Sanger sequencing data was analyzed using Sequence Analysis software (Thermo Fisher Scientific, MA, USA).

Results and Discussion

In our retrospective study, 20 newly untreated patients, as control group, displayed no mutation by NGS analysis. Of 141 imatinib-treated CML patients, there were 47 cases (33%) carried at least one single mutation within the kinase domain Figure 2A. Findings from previous studies indicate that BCR-ABL1 kinase domain mutations are detected

with a broad range of frequency from 12% to 63% in CML patients, who experienced with imatinib resistance, depending on different cohort [8]. The mutation frequency in our study for the Vietnamese patient group was 33%, which is similar to the Indian study (33%, n=76) [16] and the Malaysian study (32%, n=40) [17], but different in comparison to the mutation frequency published for the Thai (23.9%, n=71) [12], the Chinese (58%, n=127) [18] and the Korean (63%, n=111) [19] populations. Our data again demonstrated that the incidence of mutation varies depending on the different populations.

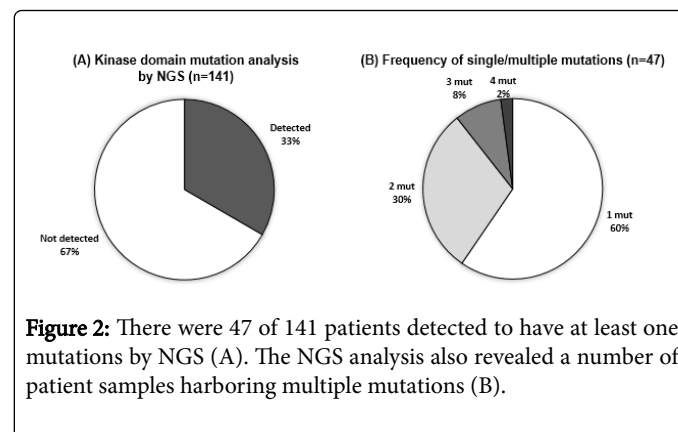


Figure 2: There were 47 of 141 patients detected to have at least one mutations by NGS (A). The NGS analysis also revealed a number of patient samples harboring multiple mutations (B).

Most of cases with imatinib resistance in our study were in chronic phase, which is in line with previous observations [8,11,20]. Of those, 122 cases (86.5%) at chronic phase, 7 cases (5%) at accelerated phase and 12 cases (8.5%) at blast phase. For the group of 47 patients harboring mutation, there were 40 (85.1%) in chronic phase, 4 (8.5%) in accelerated phase and 3 (6.4%) in blast crisis. The common clinical situation of those 47 patients was no response or loss of response to therapy, except one case of minimal response with 50% Ph chromosome and 6 cases of relapse. Nevertheless, there were no significant clinical differences between the group of patients with and without mutation. In fact, the study focused on the investigation of type and ratio of kinase domain mutations. Therefore, resistant patients were mainly selected at early chronic phase for the investigation. In this study, there was a case with a relapse after allogenic stem cell transplantation. The NGS result of this sample showed a new nucleotide substitution (C→A, 7%) of the codon 474. However, further investigation needed to be made to confirm the role of this variation as well as other new variations in this study.

Kinase inhibitor resistant properties in CML patients are often influenced by location and residue of mutations in BCR-ABL1 kinase domain. Different mutated residuals directly or indirectly affect the binding and inhibition abilities of TKI. In our study, mutated residuals were detected along the sequence of kinase domain (Figure 3), including P-loop, SH3, SH2, A-loop and other regions. It has been described in other studies that P-loop is a highly-conserved region of the kinase domain, involved in ATP binding and is a frequent site of mutation. Normally, TKI such as imatinib binds to amino acids in the ABL1 kinase domain, blocks ATP binding site and induces BCR-ABL1 protein into inactive conformation [21]. Mutation occurs within the ATP binding site can prevent imatinib from binding. It is reported that mutations in P-loop are associated with poor prognosis [22].

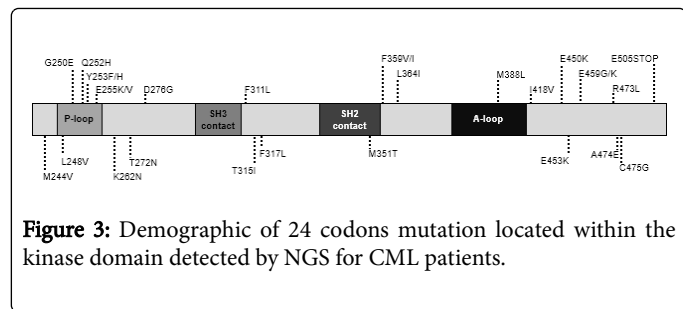


Figure 3: Demographic of 24 codons mutation located within the kinase domain detected by NGS for CML patients.

To date, more than 50 different kinase domain mutations have been described, however there are only 7 of those that currently account for 85% of all mutations [23]. These most frequent appearing mutations are M244V, G250E, Y253F/H, E255K/V, T315I, M351T and F359V. In our study, 28 nucleotide substitutions, that altered 24 amino acids, were found and the high prevalence included most of above observed mutations, including Y253F/H (13.7%), M351T (12.3%), G250E (8.2%), F359V/I (6.3%) and M244V (6.8%). T315I occurred in only 3 of 47 patients (4.1%) which is generally lower in comparison to other studies, as summarized in Soverini's review, 2014 [8]. However, T315I is not always the most dominant variation in all populations, for example, our neighboring Chinese investigation also represents a lower frequency of this mutation compares to others [18]. In addition, unpublished data of our laboratory also showed only 6 of 258 CP-CML cases with T315I-PCR positive. Interestingly, several new amino acid alterations at the far end of C-terminus were detected, including R473L, A474E and C475G with the frequency from 5.5% to 6.8%. These alterations have not been reported elsewhere and needed to have further investigation for confirmation.

Of this study, there were also 19 patients (40%) who carried more than one mutation, including 14 patients with 2 mutations, 4 patients with 3 mutations and 1 patients with 4 mutations Figure 2B. It is already known that TKI-treated CML patients tend to develop multiple mutations against therapy. These, including polyclonal mutation and, mainly, compound mutation [24], could play a crucial role in the progression of the disease. Furthermore, it has been published that patients, whom already harboring mutations, had higher likelihood of relapse associated with development of further mutations compared with patients who did not harbor mutations [14].

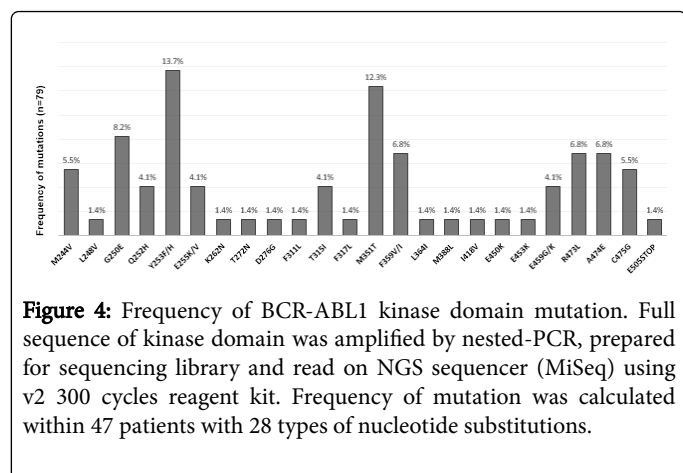


Figure 4: Frequency of BCR-ABL1 kinase domain mutation. Full sequence of kinase domain was amplified by nested-PCR, prepared for sequencing library and read on NGS sequencer (MiSeq) using v2 300 cycles reagent kit. Frequency of mutation was calculated within 47 patients with 28 types of nucleotide substitutions.

To confirm our NGS results as well as the sensitivity of the sequencing, 10 of 47 patient samples, with mutation frequency from 10% to 80%, were picked up for Sanger sequencing. As the result, all

mutations detected by NGS that have frequency higher than 20% were confirmed by Sanger sequencing. However, mutations with abundance lower than 20% were not detected by Sanger analysis Figure 4.

Initially, BCR-ABL1 kinase domain mutation analysis can be conducted by direct Sanger sequencing, which has been largely used and is the standard method for clinical diagnosis [8]. However, this conventional sequencing method is limited in terms of sensitivity and throughput. Newer robust technologies with greater sensitivity are available but most of them are limited by their specificity for a definite and short spectrum of mutations. Next-generation sequencing (NGS) is the only exception [10]. Lately, several NGS/Sanger comparison studies reported that Sanger sequencing had misclassified or underestimated kinase domain mutation status in up to 55% of samples, where mutations with 1-15% abundance were detected by NGS [10,25]. Other publications also demonstrated that NGS systems are able to detect mutation at much earlier time and, therefore, having higher predictive value of emerging drug-resistance mutation [9,26,27]. In our study, all detected mutations by NGS had minimum coverage of 5,000x, which allowed the detection sensitivity to 1% and below.

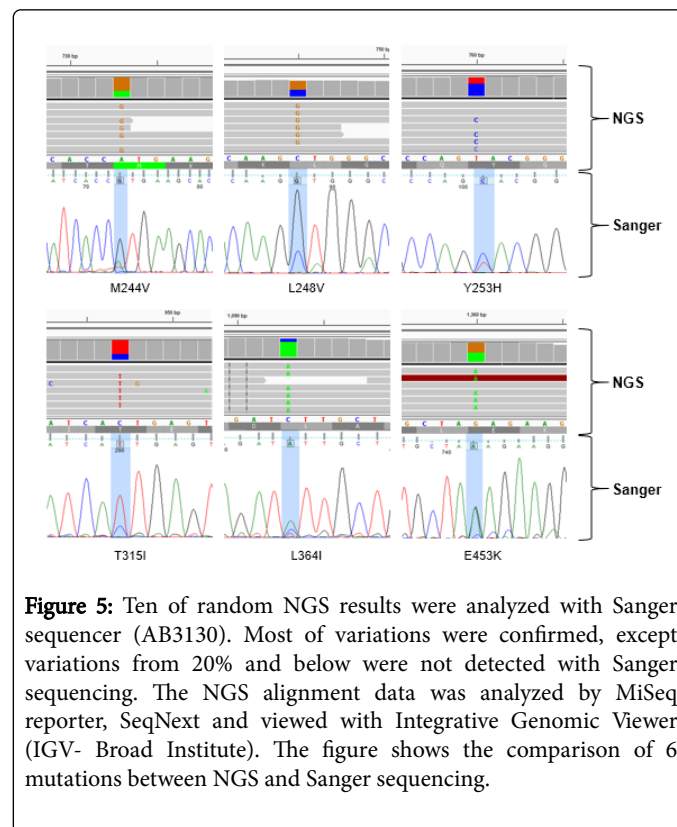


Figure 5: Ten of random NGS results were analyzed with Sanger sequencer (AB3130). Most of variations were confirmed, except variations from 20% and below were not detected with Sanger sequencing. The NGS alignment data was analyzed by MiSeq reporter, SeqNext and viewed with Integrative Genomic Viewer (IGV- Broad Institute). The figure shows the comparison of 6 mutations between NGS and Sanger sequencing.

Furthermore, in 2015, Polakova et al. reported that NGS could have revealed emerging resistant mutants 2-11 months earlier compares to conventional Sanger sequencing method [28]. It is assumed that, kinase domain mutation is time dependent and depends on the pressure exerted by TKI. The mutation can persist at low level for many years before leading to subsequent therapy resistance. These theories are supported by the research work of Parker et al. in 2013 and Vaidya et al. in 2015 [6,7]. Therefore, NGS is suitable for sensitive detection of BCR-ABL1 relevant to TKI choice in imatinib-resistant patients and this is strongly supported by Soverini et al. [29]. This study proved that NGS at the time of imatinib failure reliably identifies

clinically relevant mutations, thus enabling a more effective therapeutic tailoring (Figure 5) [29].

In conclusion, our retrospective study is the first study using NGS method to reveal type and frequency of mutations in BCR-ABL1 kinase domain for a group of Vietnamese CML patients. This data again demonstrated the advantages of next-generation deep sequencing and would represent a missing piece of global puzzle of mutation-driven TKI-resistance in CML.

Conflicts of Interest

The authors herewith confirm no conflict of interests.

Acknowledgments

The authors would like to express sincerest thanks to the Vietnam Academy of Science and Technology (VAST) for its support, grant number: VAST02.05/15-16.

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