Leukemia Research 76 (2019) 87–93

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Research paper

Compound mutations involving T315I and P-loop mutations are the major components of multiple mutations detected in tyrosine kinase inhibitor resistant chronic myeloid leukemia

Ki-Hoon Kang[a], Soo-Hyun Kim[a], Soo-Young Choi[a], Hae-Lyun Yoo[a], Mi-Young Lee[a], Hye-Young Song[a], Kyung-Mi Kee[a], Ji-Hyung Suh[a], Seon-Young Yang[a], Eun-Jung Jang[a], Sung-Eun Lee[b], Dong-Wook Kim[a,b,*]

[a] Leukemia Research Institute, The Catholic University of Korea, Seoul, Republic of Korea
[b] Catholic Hematology Hospital, The Catholic University of Korea, Seoul, Republic of Korea

ARTICLE INFO

Keywords:
Multiple mutations
Compound mutation
Minor mutation
Resistance
Chronic myeloid leukemia

ABSTRACT

To analyze the pattern of multiple mutations detected by Sanger sequencing (SS), we performed subcloning sequencing using 218 samples from 45 patients with tyrosine kinase inhibitor resistant chronic myeloid leukemia. At the first time of multiple mutation detection by SS (baseline), a total of 19 major mutations from 45 samples were detected; these mutations were found in the following order: T315I (68.9%), E255 K (33.3%), Y253H (13.3%), G250E (13.3%), and F317 L (11.1%). Subcloning sequencing of 900 baseline colonies identified 556 different mutant types, and 791 among the 900 were colonies with major mutations (87.9%). The mutations were found in the following order: T315I (36.4%), E255 K (16.2%), Y253H (7.0%), G250E (6.7%), M351 T (6.6%), and E255 V (5.3%). In subcloning sequencing with 4357 colonies of 218 serial samples, 2506 colonies (57.5%) had compound mutations, among which 2238 colonies (89.3%) had at least one major mutation. The median number of mutations in compound mutant colonies was 2 (range, 2–7), and most were double (52.9%) or triple (28.7%) mutations. Additionally, some mutations in allosteric binding sites were detected as low level mutation in 13 patients. With the available retrospective samples before baseline, subcloning sequencing identified low-level mutations of various frequencies (median, 10%) to be major mutations in 20 patients. Thus, compound mutations involving T315I and P-loop mutations were the major components of multiple mutations, and some low-level mutations with potential clinical significance were detected by subcloning sequencing. Hence, more sensitive sequencing assays are needed in patients with multiple mutations.

1. Introduction

BCR-ABL1 kinase domain mutations are a major mechanism of tyrosine kinase inhibitor (TKI) resistance in patients with chronic myeloid leukemia (CML). These mutations interfere the binding of TKIs to the binding site [1–3]. To date, various studies have shown that some of these mutations are strongly related to clinical resistance [2–5], and the sensitivity to different TKIs is dependent on the type of mutation [6–10]. Patients with multiple mutations have poorer prognosis than patients with a single mutation [11,12]. In order to overcome the clinical resistance to imatinib (IM), other TKIs, such as nilotinib (NIL), dasatinib (DAS), bosutinib (BOS), and radotinib (RAD), have been developed. However, these drugs do not affect proteins with the T315I mutation or compound mutations [13–16]. Ponatinib (PON) is sensitive to the majority of mutations, including T315I, but its effect against cancer cells with compound mutations is controversial [10,17]. Moreover, sequential treatment with TKIs has been reported to lead to the emergence of compound mutations in clones, one of the patterns of clonal evolution [18]. In addition, asciminib has been shown to be effective against the majority of mutations, including T315I, found in common TKIs. However, asciminib binds to the allosteric site of the ABL domain and is therefore less sensitive to some mutations, such as A337 V, E355 G, P465S, V468 F, Y469H, I502 L, and P918 L, due to structural changes [19,20]. Furthermore, it is still unclear how many minor clones may be expanded are under treatment with certain TKIs.

In addition to Sanger sequencing (SS), which is the gold standard for

* Corresponding author at: Department of Hematology, Seoul St. Mary's Hospital, Leukemia Research Institute, The Catholic University of Korea, #222 Banpodaero, Seocho-Gu, Seoul, 06591, Republic of Korea.
E-mail address: dwkim@catholic.ac.kr (D.-W. Kim).

https://doi.org/10.1016/j.leukres.2018.10.019
Received 16 March 2018; Received in revised form 16 October 2018; Accepted 19 October 2018
Available online 27 November 2018
0145-2126/

mutation detection, subcloning sequencing, high-performance liquid chromatography (HPLC), and allele specific oligonucleotide-polymerase chain reaction (ASO-PCR) can be used to detect mutations [21–23]. However, SS, HPLC, and ASO-PCR cannot definitively distinguish mutations from compound mutant clones from those of polyclonal mutant clones. These methods also showed limited ability to accurately detect minor clones with clinical significance that may be expanded in the future [24]. Due to these limitations, subcloning sequencing and next-generation sequencing with single clones are now being used as techniques to detect mutations [25–27].

Therefore, in this study, we conducted subcloning sequencing with individual colonies to analyze the compositions of multiple mutations, the patterns of compound mutations, and the patterns of important minor clones.

2. Material and methods

2.1. Patients and samples

Between 2002 and 2015, 735 patients with CML were screened for mutation analysis using SS due to resistance or suboptimal response to various TKIs. Of these, 163 patients had mutations, and 45 patients had multiple mutations. Thus, 218 peripheral blood (PB) samples from 45 patients were serially collected for further SS and subcloning sequencing. In detail, at the first time of multiple mutation detection (baseline) by SS, we performed subcloning sequencing on 45 samples and selected an additional 173 samples (60 retrospective samples from 30 patients and 113 prospective samples from 36 patients) for further analysis. All samples were provided by the Korea Leukemia Bank, and the study was approved by the institutional review board of our institution. Patient consent was obtained in accordance with the Declaration of Helsinki.

2.2. SS analysis

After first detection of multiple mutations, PB mononuclear cells were isolated with Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Nested PCR for the sequenced region (amino acids 218–519) of BCR-ABL1 was carried out with cDNA synthesized from 2 μg total RNA. Briefly, B2A (5′-ttcagaagcttctccctgacat-3′) and A10R1 (5′-tgaggcatctcaggcacgtc-3′) for primary PCR or A4F (5′-ccaaagcgcaacaagcccac-3′) and A10R2 (5′-acagccccacggacgccttg-3′) for secondary PCR were used as primers in 30 μL reaction mixture containing 1 × PCR buffer (30 mM Tris-HCl, 30 mM K^+, and NH_4^+, 2 mM Mg^{2+}) and 2 U LA Taq DNA polymerase (Takara, Shiga, Japan). Sequencing of PCR products was performed in both directions using an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA, USA) after the BigDye1 Terminator v3.1 cycle sequencing reaction.

2.3. Subcloning sequencing

At least 20 colonies were randomly selected and sequenced from most samples, and 4357 colonies from 218 serial samples were analyzed. For subcloning sequencing, the nested PCR-amplified region of BCR-ABL1 in SS was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). Transformed JM109 competent cells (Promega) were plated on LB agar plates containing 100 μg/mL ampicillin. Positive colonies were used for plasmid mini preparation with a Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany) and then sequenced as described for SS [21].

2.4. Definitions and statistics

2.4.1. Definition by Sanger sequencing

Multiple mutations were defined as two or more mutations detected by SS. A major mutation was defined as a predominant mutation detected by SS.

2.4.2. Definition by subcloning sequencing

A single mutant colony was defined as a colony with one missense mutation. A compound mutant colony was defined as a colony with two or more missense mutations. A colony with one or more missense mutations other than the major mutation only identified by subcloning sequencing was defined as a minor mutant colony. A major add-on mutant colony was defined as a colony with a major mutation and a minor mutation (Supplementary Fig. S1 and Table S1). A colony with only insertion (INS) and/or deletion (DEL) was designated as other abnormal colony. All of the above definitions did not include abnormalities such as silent mutations and single nucleotide polymorphism (SNP). A silent mutation is a mutation with a base substitution that results in no change in the translated amino acid. A low-level mutation was defined as a missense mutations representing less than 20% of the total alleles by subcloning sequencing. The time for detection of first multiple mutations was calculated from the time of diagnosis to detection of multiple mutations by SS. Differences in mutant clones were assessed with Fisher's exact tests. Results with *P* values of less than 0.05 were considered significant.

3. Results

3.1. Patient characteristics

A total of 45 patients with a median age of 38 (range, 13–74) years were analyzed; 30 (66.7%) were men, and 15 (33.3%) were women. The numbers of patients with high, intermediate, low, and unknown Sokal risk scores were 18 (40.0%), 7 (15.5%), 4 (8.9%), and 16 (35.6%), respectively. At the first time of multiple mutation detection (baseline), 26, 4, and 15 patients were in chronic phase (CP), accelerated phase (AP), and blast crisis (BC), respectively. TKIs used at baseline were IM (12 patients), NIL (7 patients), DAS (20 patients), and PON (6 patients). The median duration from diagnosis to baseline (the first multiple mutation detection from SS) was 25.8 (range, 2.5–167.4) months. For analysis of the mutation pattern, a total of 4357 colonies from 218 serial samples were analyzed. The median number of total samples contributed for analysis was 4 (range, 1–15) (Table 1).

3.2. Detection of mutation by SS and subcloning sequencing with 45 baseline samples

3.2.1. Detection of mutation by SS

The 19 major mutations were identified by SS at baseline (Supplementary Fig. S2A). These mutations were, in order of decreasing frequency, T315I (31/45; 68.9%), E255 K (15/45; 33.3%), Y253H (6/45; 13.3%), G250E (6/45; 13.3%), F317 L (5/45; 11.1%), E255 V (4/45; 8.9%), V299 L (4/45; 8.9%), M351 T (4/45; 8.9%), M244 V (3/45; 6.7%), Q252H (3/45; 6.7%), F359C (3/45; 6.7%), L248 V (2/45; 4.4%), E355 A (2/45; 4.4%), E459 K (2/45; 4.4%), F311I (1/45; 2.2%), T315 A (1/45; 2.2%), F359I (1/45; 2.2%), F359 V (1/45; 2.2%), and D444 G (1/45; 2.2%). All 19 major mutations detected by SS were specifically resistant to the type of TKIs being administered.

3.2.2. Detection of mutation by subcloning sequencing

Subcloning sequencing of 900 colonies using the same 45 PB samples showed 556 different mutant types. Among the 900 colonies, 791 (87.9%) were major and major add-on mutant colonies, and the proportion of colonies with the 19 major mutations were T315I (328/900; 36.4%), E255 K (146/900; 16.2%), Y253H (63/900; 7.0%), G250E (60/900; 6.7%), M351 T (59/900; 6.6%), E255 V (48/900; 5.3%), V299 L (43/900; 4.8%), M244 V (40/900; 4.4%), F317 L (35/900; 3.9%), F359 V (34/900; 3.8%), Q252H (31/900; 3.4%), E459 K (31/900; 3.4%), F359C (23/900; 2.6%), F311I (20/900; 2.2%), L248 V (18/900; 2.0%), T315 A (18/900; 2.0%), E355 A (17/900; 1.9%), F359I (16/900; 1.8%), and D444 G (9/900; 1.0%) (Supplementary Fig. S2B). In addition, 63 (7%), 43 (4.8%), and 3 (0.3%) colonies were minor mutant,

Table 1
Characteristics of patients who had multiple mutations.

Parameters	Total (n = 45)
Age, median (range)	38 (13–74) yrs
Sex, n (%)	
Male	30 (66.7)
Female	15 (33.3)
Sokal Score, n (%)	
Low	4 (8.9)
Intermediate	7 (15.5)
High	18 (40.0)
NA	16 (35.6)
Disease phase at diagnosis, n (%)	
CP	31 (68.9)
AP	8 (17.8)
BC	6 (13.3)
Disease phase at the first multiple mutation detection, n (%)	
CP	26 (57.8)
AP	4 (8.9)
BC	15 (33.3)
Therapy at the first multiple mutation detection, n (%)	
IM	12 (26.7)
NIL	7 (15.6)
DAS	20 (44.4)
PON	6 (13.3)
Outcome: alive/death*	9/36
Time from diagnosis to the first multiple mutation detection, mo, median (range)	25.8 (2.5-167.4)
Number of analyzing colonies/samples at baseline (45 patients)	900/45
Number of analyzing colonies/samples before baseline (30 patients)	1200/60
Number of analyzing colonies/samples after baseline (36 patients)	2257/113
Number of analyzing colonies/samples with total samples	4357/218
Number of samples in total patients, median (range)†	4 (1-15)

NA, not available; CP, chronic phase; AP, accelerated phase; BC, blast crisis; IM, imatinib; NIL, nilotinib; DAS, dasatinib; BOS, bosutinib; PON, ponatinib. Baseline means the first time of multiple mutation detection by Sanger sequencing. *The median follow-up duration from baseline was 8.6 (range, 0.4–146.4) months. †The median number of samples was 2 (1–5) before baseline and 3 (1–11) after baseline.

wild-type, and other abnormal colonies, respectively, demonstrating higher proportion of the major and major add-on mutant colonies in these samples (Supplementary Table S2A). All types and frequencies of major mutations found using both sequencing methods are presented in Supplementary Table S3.

In a patient (#42), E255 K and T315I were detected by SS at baseline, whereas only T315I was identified by subcloning sequencing. All of the major mutations detected by SS were also identified in the same samples in the other 44 patients by subcloning sequencing.

3.3. The type and frequency of mutations identified in 218 serial samples

Using subcloning sequencing in the 4357 colonies obtained from total 218 samples, a total of 1051 mutations were identified. Of these mutations, missense mutations and silent mutations accounted for 78.4% (824/1051 mutations) and 21.6% (227/1051 mutations), respectively. In addition, 3371 colonies (77.4%) were the major and major add-on mutant colonies, and these mutations were, in order of decreased frequency, T315I (1283/4357; 29.4%), Y253H (379/4357; 8.7%), F317 L (379/4357; 8.7%), E255 K (378/4357; 8.7%), M244 V (248/4357; 5.7%), F359C (226/4357; 5.2%), E255 V (220/4357; 5.0%), and V299 L (220/4357; 5.0%). In a patient (#42) who showed a discordant mutation pattern with two sequencing methods using the baseline sample, E255 K was eventually detected 3 months later by subcloning sequencing using a subsequent sample. Therefore, all major mutations detected by SS were also identified by subcloning sequencing in subsequent samples. Moreover, the proportion of major and major add-on mutant colonies was predominant when compared to the proportion of minor mutant colonies (Supplementary Table S2B). Complete information regarding each colony is detailed in Supplementary Table S4.

3.4. Compound mutant colonies were dominant and mainly consisted of major mutations

Of the 4357 colonies, compound mutant colonies, comprising 57.5% (2506 colonies) of the total colonies were the predominant form in our patients. Additionally, single mutant, wild-type, and other abnormal colonies were found in 33.8% (1474 colonies), 7.8% (339 colonies), and 0.9% (38 colonies) of the total colonies, respectively. Compound mutant colonies comprised more missense mutations than single mutant colonies (80.2% *versus* 62.2%; $P < 0.0001$). Among the 2506 compound mutant colonies, 89.3% (2238 colonies; 14.2% of the major mutant colonies and 75.1% of the major add-on mutant colonies) had at least one major mutation, and 10.7% (268 colonies) were minor mutant colonies. In a comparison of 19 major mutations between compound mutant colonies and single mutant colonies, the proportion of major/major add-on mutant colonies was significantly higher in compound mutant colonies (89.3% *versus* 76.9%; $P < 0.0001$) (Tables 2 and 3). The type and frequency of 19 major mutations in colonies are presented in Fig. 1.

Notably, 997 mutations in 2506 compound mutant colonies were in the following order of decreasing frequency: T315I (876/2506; 35.0%), Y253H (293/2506; 11.7%), F317 L (283/2506; 11.3%), E255 K (229/2506; 9.1%), V299 L (204/2506; 8.1%), M244 V (178/2506; 7.1%), F359C (164/2506; 6.5%), E255 V (166/2506; 6.6%), E459 K (158/2506; 6.3%), and M351 T (129/2506; 5.1%). In contrast, 413 mutations in 1474 single mutant colonies were detected, and these mutations were in the following order of decreasing frequency: T315I (407/1474; 27.6%), E255 K (149/1474; 10.1%), F317 L (96/1474; 6.5%), and Y253H (86/1474; 5.8%). Except L248 V, E255 K, E355 A, and F359 V, the majority of major mutations showed significantly higher frequencies in compound mutant colonies (Supplementary Table S5).

The median number of mutations detected in compound mutant colonies was 2 (range, 2–7); 1325 (52.9%), 719 (28.7%), 303 (12.1%), 122 (4.9%), 31 (1.2%), and 6 (0.2%) colonies had 2, 3, 4, 5, 6, and 7 mutations per colony, respectively (Table 3).

3.5. Minor mutations having potential clinical significance

In the 609 minor mutant colonies, 541 mutations were detected, which were in the following order of decreasing frequency: E355 G (43/609; 7.1%), W235R (10/609; 1.6%), T319 A (10/609; 1.6%), Q300Z (8/609; 1.3%), L266 P (7/609; 1.1%), I314 T (7/609; 1.1%), C330R (7/

Table 2
The components of mutation by subcloning sequencing with 218 samples.

	Single mutant colonies	Compound mutant colonies
Number of mutation in a colony	1	2 (2–7)
Colony (total)	1474 (33.8%)	2506 (57.5%)
Major/Major add-on mutant colony	1133 (76.9%)	2238 (89.3%)
Minor mutant colony	341 (23.1%)	268 (10.7%)
Type of mutations	413	997
Missense mutation	257 (62.2%)	800 (80.2%)
Silent mutation	156 (37.8%)	197 (19.8%)

Other abnormal colonies with INS ± DEL (n = 38) and wild-type colonies (n = 339) were not included.

Table 3
The number of mutation containing in 2506 compound mutant colonies.

The number of mutation in single clone	Major mutant colony number	Major add-on mutant colony number	Minor mutant colony number	Number of colony	Number of colony (%)
Double mutant colony	316	830	179	1325	52.9%
Triple mutant colony	40	619	60	719	28.7%
Quadra mutant colony	0	282	21	303	12.1%
Quintuple mutant colony	0	115	7	122	4.9%
Sextuple mutant colony	0	30	1	31	1.2%
Septuple mutant colony	0	6	0	6	0.2%
Total	356 (14.2%)	1882 (75.1%)	268 (10.7%)	2506	100%

Single mutant colonies (n = 1474), other abnormal colonies with INS ± DEL (n = 38) and wild-type colonies (n = 339) were not included.

609; 1.1%), and W478R (7/609; 1.1%), as shown in Fig. 2 and Supplementary Table S6.

As asciminib is currently available for clinical trials, we focused on allosteric site mutations. Interestingly, A337 V, E355 G, P465S, and Y469H allosteric site mutations were detected in 65 colonies from 24 samples of 13 patients (four A337 V, six E355 G, five P465S, and five Y469 H). The frequencies of allosteric site mutations were in the following order: E355 G (49/65; 75.4%), Y469H (7/65; 10.8%), P465S (6/65; 9.2%), and A337 V (4/65; 6.2%) (Fig. 3). Of the 13 patients, 10 patients had at least one colony with an allosteric site mutation along with one or two major mutations (nine T315I, three Y253H, two Q252H, two F317 L, two F359C, one M244 V, one M351 T, and one F359 V). Furthermore, the allosteric site mutations of the 13 patients were detected at a low level with diverse change throughout the treatment with various TKIs (data not shown).

3.6. Clinical significance of the low-level mutation detected by subcloning sequencing

Among 45 patients, 30 patients had 60 retrospective (before baseline) samples for SS and subcloning sequencing (median, 2 samples per a patient; range, 1–5). The same results were obtained by both methods in 9 patients. In the other 21 patients, subcloning sequencing identified mutations of various frequencies (median, 10%; range, 5–70%). Except one, 20 patients had low-level mutations less than 20% by subcloning sequencing. The patient (# 22) who showed 70% of Q252H by subcloning sequencing demonstrated no mutation by SS because of no amplification. Interestingly, T315I was the most common low-level

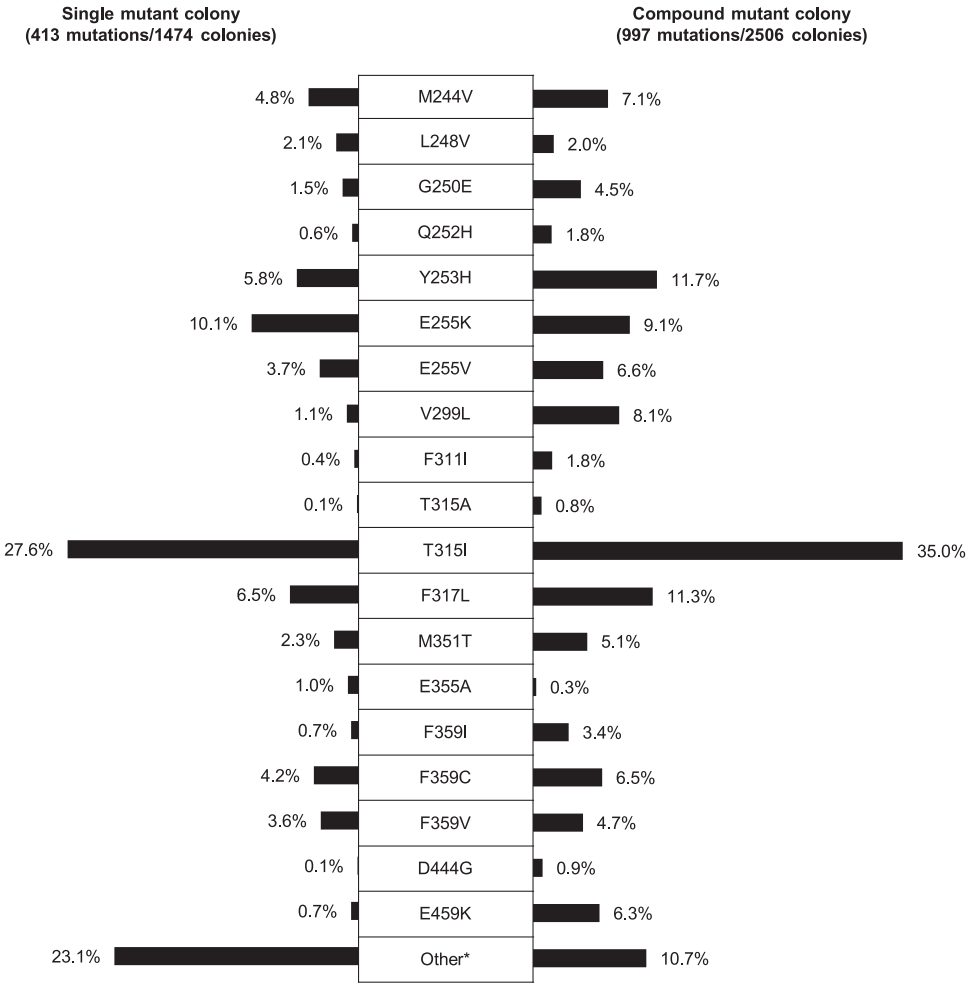

Fig. 1. Comparison of 19 major mutations between single mutant colonies and compound mutant colonies by subcloning sequencing. *Other, it means minor mutant colonies and other abnormal colonies; wild-type colonies were not included. The numbers of different types of 19 major mutations per colony are shown.

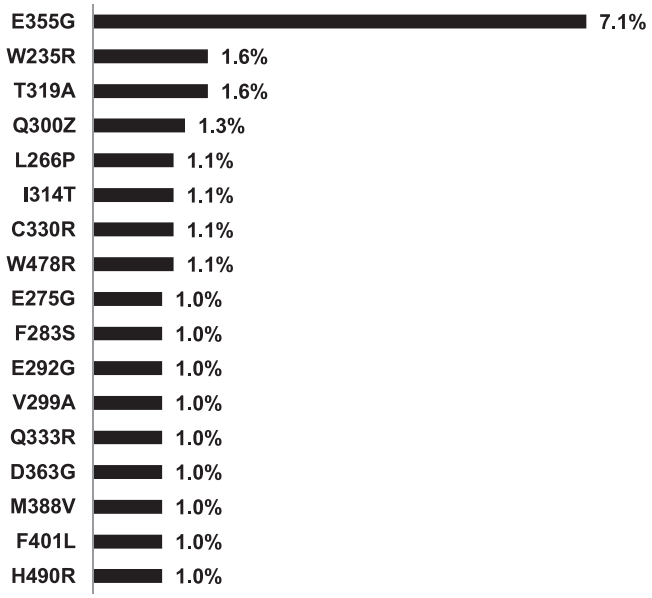

Fig. 2. Type and frequency of minor mutations in 609 minor mutant colonies. Proportions of the top five mutations detected in 609 colonies with only minor mutations by subcloning sequencing.

mutation, and it was detected in 12 patients (Supplementary Table S7). The median time from the first detection of major mutations by subcloning sequencing to baseline was 2.8 (0.6–10.2) months. Among the 12 patients, 8 were treated with DAS, 3 with NIL, and 1 with IM. Meanwhile, 8 of 20 patients who had low-level mutations by subcloning sequencing did not show any mutations by SS. Notably, of the 2 patients (# 23 and # 41) who had samples at the time of CML-CP diagnosis, the T315I mutation (5% as a single mutant colony) was identified in 1 patient (# 23) only by subcloning sequencing; this low-level T315I mutation at diagnosis were eventually detected by SS with subsequent samples (Supplementary Fig. S3).

4. Discussion

Sanger sequencing, the standard technique for mutation detection, is widely used because it reflects clinical outcomes by clonal expansion well, despite its relatively low sensitivity. However, in cases of multiple mutations where two or more mutations are identified by SS, it is impossible to distinguish compound mutations.

Of 45 patients with multiple mutations detected by SS in our study, mutations were detected in 12 patients during first-line TKI treatment and in 33 patients after failure of first-line treatment and during subsequent lines of TKI treatment, reflecting that multiple mutations were due to gradual genetic instability.

In addition, 556 mutations were identified from subcloning sequencing of 900 colonies, whereas only 19 mutations were identified by SS in 45 baseline samples, indicating that there may be various low-level mutations in addition to clinically identifiable major mutations. Moreover, analysis of colonies acquired from the serial samples showed 1051 mutations, which indicated that the complex kinetics of new mutant clones emerged and disappeared as the treatment progressed. This pattern showed continuous progression of genetic instability by initial breakage of genetic stability; additionally, sequential use of TKIs with various sensitivities to different mutations can increase selection pressure to individual clones [25].

Moreover, of the 4357 colonies, 57.5% (2506 colonies) were compound mutant colonies, among which 89.3% at least one major mutation. This finding shows that the majority of compound mutant colonies detected by subcloning sequencing could have clinical significance. Compound mutations have been shown to be more resistant to TKI therapy during IM, NIL, and DAS treatments [10,28]. In our study, the proportions of T315I and P-loop mutations were higher in compound mutant colonies. However, clones with compound mutations are not always the major driver, and therefore, the use of more potent TKIs,

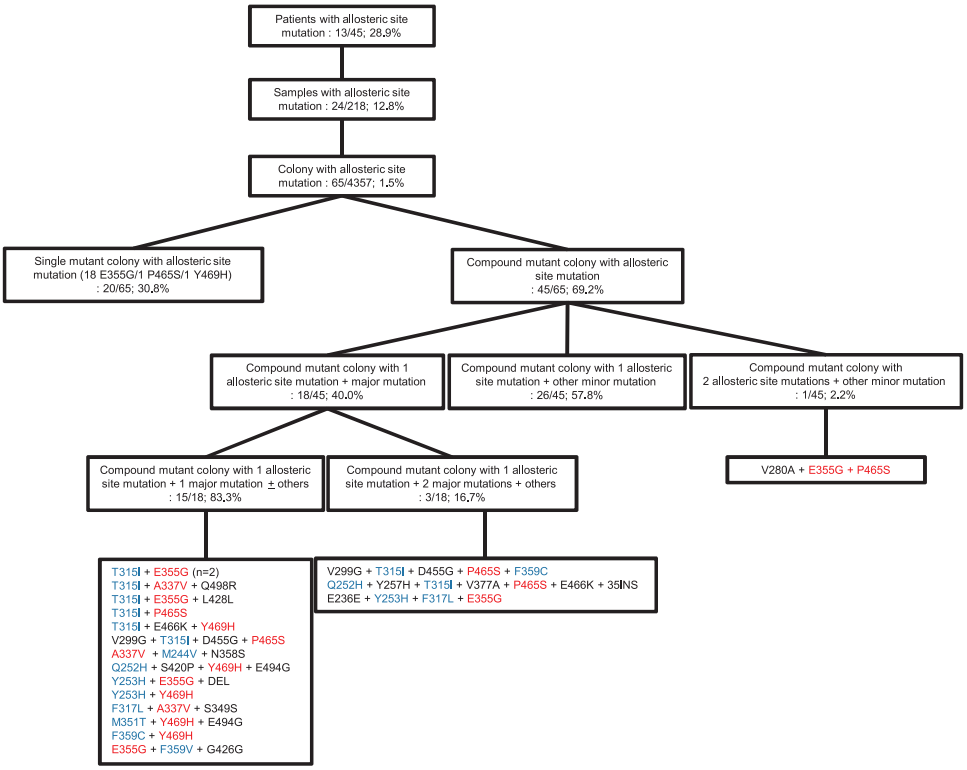

Fig. 3. Characteristics of allosteric site mutations detected by subcloning sequencing. Major mutations and allosteric site mutations are depicted as blue and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

such as PON, on T315I and P-loop mutations can effectively inhibit the kinase activity of the compound mutations [17].

In our study, dynamic changes in the major mutations of each colony were frequently observed with TKI changes or discontinuation, particularly in colonies with compound mutations including T315I (data not shown). This is a common pattern observed when the mutation types comprising the compound mutant clones are exposed to various TKIs and repeat the selection and deselection processes [18,29]. Moreover, this pattern could result from the emergence of the T315I mutation, resulting in disruption of kinase activity; alternatively, clones with mutations with strong transformation activity may become the dominant clone when the selection pressure is lowered by TKI discontinuation. In other words, growth competition between mutant clones can lead to a change in major mutations by varying kinase activity from an add-on mutation within the clone [6,30], and the presence of the T315I mutation appears to play an important role in the selection and deselection processes of compound mutant clones [24,31,32].

To confirm the clinical significance of individual major low-level mutations, we analyzed 60 retrospective samples from 30 patients using subcloning sequencing before the first time of multiple mutation detection by SS. Among the 30 patients, 20 showed various major mutations at a low level (less than 20%). Eventually, all of the low-level mutations had expanded to major mutations that were detectable by SS. In addition, the T315I mutation was identified in 12 of 20 patients. However, the patients had received TKIs other than PON (8 DAS, 3 NIL, and 1 IM) because of the mis-classification by SS. These findings demonstrate that a more sensitive mutation assay with serial samples may be warranted to guide a precise TKI modification after treatment failure. In addition, despite the possibility of artificial compound mutation by PCR-mediated recombination [33], our results could be justified because all individual mutations comprising compound mutations were detected by SS and the compound mutations before baseline were expanded at the time of multiple mutation detection (Supplementary Table S7).

An analysis of 609 colonies comprising only low-level minor mutations showed that the proportion of compound mutant colonies was as low as 44.0%, reflecting that the mutations in the compound mutant colonies did not maintain the growth selection power of TKIs used. Because the allosteric site of ABL1 is important to maintain the structure and/or dynamics at the distant, functional sites, we further analyzed the frequencies of clones with A337 V, E355 G, P465S, and Y469H mutations that had resistance to asciminib [19,20]. Of the 4357 colonies, 65 colonies (1.5%) had mutations that may show resistance to asciminib, and these mutations were present in the serial samples of 13 patients as low-level minor mutations. Interestingly, E355 G was the most common type of mutation (75.4%) in the colonies with allosteric site mutations. As 10 patients (22%) had an allosteric site mutation along with one or two major mutations and the allosteric site mutations remained at a low level with diverse changes under various TKI treatments other than asciminib, we need to focus on the clones in the era of asciminib.

Moreover, as with PON [17], asciminib also successfully suppressed major compound mutant clones that had low sensitivity to NIL and DAS, and compound mutant clones did not appear to be a major driver under asciminib in 2 patients (# 21 and # 36) (Supplementary Fig. S4). As a clone containing the E355G + T315I compound mutation (in patient # 21) also emerged and disappeared after asciminib therapy, it is still unknown whether the compound mutant colony will be expanded with continuing therapy due to the short follow-up period (about 9 months).

Younger age was more likely to have favorable prognostic significance in PON-treated patients with the T315I mutation [34]. The patients of this study were young, which has been reported in a previous study [35]. However, age was not a statistically significant factor for the type of mutations and survival in this cohort, because of the heterogeneous variables in the small number of patients (data not shown).

In conclusion, compound mutant clones were dominant in multiple mutations detected by SS, and the majority of compound mutant clones had at least one major mutation consisting of highly resistant mutations, such as T315I and P-loop mutations. Since SS failed to detect low-level mutations having potential clinical significance and since allosteric site mutations detected in our study may be important for clonal selection in the era of asciminib therapy, mutation assays with higher sensitivity should be applied in case of IM failure, if multiple mutations are detected, and in case of change to another TKI.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

KHK performed the experiments, analyzed the data, and wrote the manuscript. SEL analyzed the data and commented on the manuscript. EJJ provided the clinical information for the samples. SHK and SYC analyzed the data. HLY, MYL, HYS, KMK, JHS, and SYY performed the experiments. DWK designed the experiments, supervised the project, and commented on the manuscript.

Acknowledgments

We thank the Korea Leukemia Bank for providing the CML samples. This work was supported by Korea Leukemia Bank for biomaterial banking and analysis (NRF-2013M3A9B8031236).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2018.10.019.

References

[1] M.E. Gorre, M. Mohammed, K. Ellwood, N. Hsu, R. Paquette, P.N. Rao, C.L. Sawyers, Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification, Science (New York, N.Y.) 293 (2001) 876–880.
[2] N. von Bubnoff, F. Schneller, C. Peschel, J. Duyster, BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study, Lancet (Lond., Engl.) 359 (2002) 487–491.
[3] S. Branford, Z. Rudzki, S. Walsh, I. Parkinson, A. Grigg, J. Szer, K. Taylor, R. Herrmann, J.F. Seymour, C. Arthur, D. Joske, K. Lynch, T. Hughes, Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis, Blood 102 (2003) 276–283.
[4] T. Hughes, G. Saglio, S. Branford, S. Soverini, D.W. Kim, M.C. Muller, G. Martinelli, J. Cortes, L. Beppu, E. Gottardi, D. Kim, P. Erben, Y. Shou, A. Haque, N. Gallagher, J. Radich, A. Hochhaus, Impact of baseline BCR-ABL mutations on response to nilotinib in patients with chronic myeloid leukemia in chronic phase, J. Clin. Oncol. 27 (2009) 4204–4210.
[5] M.C. Muller, J.E. Cortes, D.W. Kim, B.J. Druker, P. Erben, R. Pasquini, S. Branford, T.P. Hughes, J.P. Radich, L. Ploughman, J. Mukhopadhyay, A. Hochhaus, Dasatinib treatment of chronic-phase chronic myeloid leukemia: analysis of responses according to preexisting BCR-ABL mutations, Blood 114 (2009) 4944–4953.
[6] I.J. Griswold, M. MacPartlin, T. Bumm, V.L. Goss, T. O'Hare, K.A. Lee, A.S. Corbin, E.P. Stoffregen, C. Smith, K. Johnson, E.M. Moseson, L.J. Wood, R.D. Polakiewicz, B.J. Druker, M.W. Deininger, Kinase domain mutants of Bcr-Abl exhibit altered transformation potency, kinase activity, and substrate utilization, irrespective of sensitivity to imatinib, Mol. Cell. Biol. 26 (2006) 6082–6093.
[7] T. O'Hare, D.K. Walters, E.P. Stoffregen, T. Jia, P.W. Manley, J. Mestan, S.W. Cowan-Jacob, F.Y. Lee, M.C. Heinrich, M.W. Deininger, B.J. Druker, In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants, Cancer Res. 65 (2005) 4500–4505.
[8] T. O'Hare, C.A. Eide, M.W. Deininger, Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia, Blood 110 (2007) 2242–2249.
[9] S. Redaelli, R. Piazza, R. Rostagno, V. Magistroni, P. Perini, M. Marega, C. Gambacorti-Passerini, F. Boschelli, Activity of bosutinib, dasatinib, and nilotinib against 18 imatinib-resistant BCR/ABL mutants, J. Clin. Oncol. 27 (2009) 469–471.

[10] M.S. Zabriskie, C.A. Eide, S.K. Tantravahi, N.A. Vellore, J. Estrada, F.E. Nicolini, H.J. Khoury, R.A. Larson, M. Konopleva, J.E. Cortes, H. Kantarjian, E.J. Jabbour, S.M. Kornblau, J.H. Lipton, D. Rea, L. Stenke, G. Barbany, T. Lange, J.C. Hernandez-Boluda, G.J. Ossenkoppele, R.D. Press, C. Chuah, S.L. Goldberg, M. Wetzler, F.X. Mahon, G. Etienne, M. Baccarani, S. Soverini, G. Rosti, P. Rousselot, R. Friedman, M. Deininger, K.R. Reynolds, W.L. Heaton, A.M. Eiring, A.D. Pomicter, J.S. Khorashad, T.W. Kelley, R. Baron, B.J. Druker, M.W. Deininger, T. O'Hare, BCR-ABL1 compound mutations combining key kinase domain positions confer clinical resistance to ponatinib in Ph chromosome-positive leukemia, Cancer Cell 26 (2014) 428–442.
[11] A. Quintas-Cardama, H. Kantarjian, S. O'Brien, E. Jabbour, G. Borthakur, F. Ravandi, S. Verstovsek, J. Shan, J. Cortes, Outcome of patients with chronic myeloid leukemia with multiple ABL1 kinase domain mutations receiving tyrosine kinase inhibitor therapy, Haematologica 96 (2011) 918–921.
[12] W.T. Parker, M. Ho, H.S. Scott, T.P. Hughes, S. Branford, Poor response to second-line kinase inhibitors in chronic myeloid leukemia patients with multiple low-level mutations, irrespective of their resistance profile, Blood 119 (2012) 2234–2238.
[13] H.M. Kantarjian, F. Giles, N. Gattermann, K. Bhalla, G. Alimena, F. Palandri, G.J. Ossenkoppele, F.E. Nicolini, S.G. O'Brien, M. Litzow, R. Bhatia, F. Cervantes, A. Haque, Y. Shou, D.J. Resta, A. Weitzman, A. Hochhaus, P. le Coutre, Nilotinib (formerly AMN107), a highly selective BCR-ABL tyrosine kinase inhibitor, is effective in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase following imatinib resistance and intolerance, Blood 110 (2007) 3540–3546.
[14] A. Hochhaus, H.M. Kantarjian, M. Baccarani, J.H. Lipton, J.F. Apperley, B.J. Druker, T. Facon, S.L. Goldberg, F. Cervantes, D. Niederwieser, R.T. Silver, R.M. Stone, T.P. Hughes, M.C. Muller, R. Ezzeddine, A.M. Countouriotis, N.P. Shah, Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy, Blood 109 (2007) 2303–2309.
[15] J.E. Cortes, H.M. Kantarjian, T.H. Brummendorf, D.W. Kim, A.G. Turkina, Z.X. Shen, R. Pasquini, H.J. Khoury, S. Arkin, A. Volkert, N. Besson, R. Abbas, J. Wang, E. Leip, C. Gambacorti-Passerini, Safety and efficacy of bosutinib (SKI-606) in chronic phase Philadelphia chromosome-positive chronic myeloid leukemia patients with resistance or intolerance to imatinib, Blood 118 (2011) 4567–4576.
[16] S.H. Kim, H. Menon, S. Jootar, T. Saikia, J.Y. Kwak, S.K. Sohn, J.S. Park, S.H. Jeong, H.J. Kim, Y.K. Kim, S.J. Oh, H. Kim, D.Y. Zang, J.S. Chung, H.J. Shin, Y.R. Do, J.A. Kim, D.Y. Kim, C.W. Choi, S. Park, H.L. Park, G.Y. Lee, D.J. Cho, J.S. Shin, D.W. Kim, Efficacy and safety of radotinib in chronic phase chronic myeloid leukemia patients with resistance or intolerance to BCR-ABL1 tyrosine kinase inhibitors, Haematologica 99 (2014) 1191–1196.
[17] M.W. Deininger, J.G. Hodgson, N.P. Shah, J.E. Cortes, D.W. Kim, F.E. Nicolini, M. Talpaz, M. Baccarani, M.C. Muller, J. Li, W.T. Parker, S. Lustgarten, T. Clackson, F.G. Haluska, F. Guilhot, H.M. Kantarjian, S. Soverini, A. Hochhaus, T.P. Hughes, V.M. Rivera, S. Branford, Compound mutations in BCR-ABL1 are not major drivers of primary or secondary resistance to ponatinib in CP-CML patients, Blood 127 (2016) 703–712.
[18] N.P. Shah, B.J. Skaggs, S. Branford, T.P. Hughes, J.M. Nicoll, R.L. Paquette, C.L. Sawyers, Sequential ABL kinase inhibitor therapy selects for compound drug-resistant BCR-ABL mutations with altered oncogenic potency, J. Clin. Invest. 117 (2007) 2562–2569.
[19] B.J. Lee, N.P. Shah, Identification and characterization of activating ABL1 1b kinase mutations: impact on sensitivity to ATP-competitive and allosteric ABL1 inhibitors, Leukemia 31 (2017) 1096–1107.
[20] A.A. Wylie, J. Schoepfer, W. Jahnke, S.W. Cowan-Jacob, A. Loo, P. Furet, A.L. Marzinzik, X. Pelle, J. Donovan, W. Zhu, S. Buonamici, A.Q. Hassan, F. Lombardo, V. Iyer, M. Palmer, G. Berellini, S. Dodd, S. Thohan, H. Bitter, S. Branford, D.M. Ross, T.P. Hughes, L. Petruzzelli, K.G. Vanasse, M. Warmuth, F. Hofmann, N.J. Keen, W.R. Sellers, The allosteric inhibitor ABL001 enables dual targeting of BCR-ABL1, Nature 543 (2017) 733–737.
[21] D. Kim, D.W. Kim, B.S. Cho, H.G. Goh, S.H. Kim, W.S. Kim, J. Lee, I.Y. Kweon, S.H. Park, J.H. Yoon, N.D. Kim, H. Chun, Structural modeling of V299L and E459K Bcr-Abl mutation, and sequential therapy of tyrosine kinase inhibitors for the compound mutations, Leuk. Res. 33 (2009) 1260–1265.
[22] T. Ernst, P. Erben, M.C. Muller, P. Paschka, T. Schenk, J. Hoffmann, S. Kreil, P. La Rosee, R. Hehlmann, A. Hochhaus, Dynamics of BCR-ABL mutated clones prior to hematologic or cytogenetic resistance to imatinib, Haematologica 93 (2008) 186–192.
[23] H.Y. Kang, J.Y. Hwang, S.H. Kim, H.G. Goh, M. Kim, D.W. Kim, Comparison of allele specific oligonucleotide-polymerase chain reaction and direct sequencing for high throughput screening of ABL kinase domain mutations in chronic myeloid leukemia resistant to imatinib, Haematologica 91 (2006) 659–662.
[24] S. Soverini, S. Colarossi, A. Gnani, G. Rosti, F. Castagnetti, A. Poerio, I. Iacobucci, M. Amabile, E. Abruzzese, E. Orlandi, F. Radaelli, F. Ciccone, M. Tiribelli, R. di Lorenzo, C. Caracciolo, B. Izzo, F. Pane, G. Saglio, M. Baccarani, G. Martinelli, Contribution of ABL kinase domain mutations to imatinib resistance in different subsets of Philadelphia-positive patients: by the GIMEMA Working Party on Chronic Myeloid Leukemia, Clin. Cancer Res. 12 (2006) 7374–7379.
[25] J.S. Khorashad, T.W. Kelley, P. Szankasi, C.C. Mason, S. Soverini, L.T. Adrian, C.A. Eide, M.S. Zabriskie, T. Lange, J.C. Estrada, A.D. Pomicter, A.M. Eiring, I.L. Kraft, D.J. Anderson, Z. Gu, M. Alikian, A.G. Reid, L. Foroni, D. Marin, B.J. Druker, T. O'Hare, M.W. Deininger, BCR-ABL1 compound mutations in tyrosine kinase inhibitor-resistant CML: frequency and clonal relationships, Blood 121 (2013) 489–498.
[26] K. Machova Polakova, V. Kulvait, A. Benesova, J. Linhartova, H. Klamova, M. Jaruskova, C. de Benedittis, T. Haferlach, M. Baccarani, G. Martinelli, T. Stopka, T. Ernst, A. Hochhaus, A. Kohlmann, S. Soverini, Next-generation deep sequencing improves detection of BCR-ABL1 kinase domain mutations emerging under tyrosine kinase inhibitor treatment of chronic myeloid leukemia patients in chronic phase, J. Cancer Res. Clin. Oncol. 141 (2015) 887–899.
[27] S. Soverini, C. De Benedittis, K.M. Polakova, J. Linhartova, F. Castagnetti, G. Gugliotta, C. Papayannidis, M. Mancini, H. Klamova, M. Salvucci, M. Crugnola, A. Iurlo, F. Albano, D. Russo, G. Rosti, M. Cavo, M. Baccarani, G. Martinelli, Next-generation sequencing for sensitive detection of BCR-ABL1 mutations relevant to tyrosine kinase inhibitor choice in imatinib-resistant patients, Oncotarget 7 (2016) 21982–21990.
[28] H.A. Bradeen, C.A. Eide, T. O'Hare, K.J. Johnson, S.G. Willis, F.Y. Lee, B.J. Druker, M.W. Deininger, Comparison of imatinib mesylate, dasatinib (BMS-354825), and nilotinib (AMN107) in an N-ethyl-N-nitrosourea (ENU)-based mutagenesis screen: high efficacy of drug combinations, Blood 108 (2006) 2332–2338.
[29] T. O'Hare, W.C. Shakespeare, X. Zhu, C.A. Eide, V.M. Rivera, F. Wang, L.T. Adrian, T. Zhou, W.S. Huang, Q. Xu, C.A. Metcalf 3rd, J.W. Tyner, M.M. Loriaux, A.S. Corbin, S. Wardwell, Y. Ning, J.A. Keats, Y. Wang, R. Sundaramoorthi, M. Thomas, D. Zhou, J. Snodgrass, L. Commodore, T.K. Sawyer, D.C. Dalgarno, M.W. Deininger, B.J. Druker, T. Clackson, AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance, Cancer Cell 16 (2009) 401–412.
[30] D.L. Gibbons, S. Pricl, P. Posocco, E. Laurini, M. Fermeglia, H. Sun, M. Talpaz, N. Donato, A. Quintas-Cardama, Molecular dynamics reveal BCR-ABL1 polymutants as a unique mechanism of resistance to PAN-BCR-ABL1 kinase inhibitor therapy, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 3550–3555.
[31] J. Cortes, E. Jabbour, H. Kantarjian, C.C. Yin, J. Shan, S. O'Brien, G. Garcia-Manero, F. Giles, M. Breeden, N. Reeves, W.G. Wierda, D. Jones, Dynamics of BCR-ABL kinase domain mutations in chronic myeloid leukemia after sequential treatment with multiple tyrosine kinase inhibitors, Blood 110 (2007) 4005–4011.
[32] M. Breccia, A.M. Frustaci, L. Cannella, S. Soverini, C. Stefanizzi, V. Federico, S. Grammatico, M. Santopietro, G. Alimena, Sequential development of mutant clones in an imatinib resistant chronic myeloid leukaemia patient following sequential treatment with multiple tyrosine kinase inhibitors: an emerging problem? Cancer Chemother. Pharmacol. 64 (2009) 195–197.
[33] Wendy T. Parker, Stuart R. Phillis, David T.O. Yeung, Timothy P. Hughes, Hamish S. Scott, Susan Branford, Many BCR-ABL1 compound mutations reported in chronic myeloid leukemia patients may actually be artifacts due to PCR-mediated recombination, Blood 124 (1) (2014) 153–155.
[34] Wendy T. Parker, David T.O. Yeung, Alexandra L. Yeoman, Haley K. Altamura, Bronte A. Jamison, Chani R. Field, J. Graeme Hodgson, Stephanie Lustgarten, Victor M. Rivera, Timothy P. Hughes, Susan Branford, The impact of multiple low-level BCR-ABL1 mutations on response to ponatinib, Blood 127 (15) (2016) 1870–1880.
[35] W.Y. Au, P.B. Caguioa, C. Chuah, S.C. Hsu, S. Jootar, D.W. Kim, I.Y. Kweon, W.M. O'Neil, T.K. Saikia, J. Wang, Chronic myeloid leukemia in Asia, Int. J. Hematol. 89 (January (1)) (2009) 14–23.