Molecular analysis of the BCR-ABL1 kinase domain in chronic-phase chronic myelogenous leukemia treated with tyrosine kinase inhibitors in practice: study by the Nagasaki CML Study Group

Key words: Chronic myelogenous leukemia, BCR-ABL1, alternative splicing, mutation, resistance


(1) Department of Hematology, Atomic Bomb Disease and Hibakusha Medicine Unit, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki city, Nagasaki, Japan

(2) Nagasaki CML Study Group, Nagasaki, Japan
Abstract

An appropriate trigger for BCR-ABL1 mutation analysis has not yet been established in unselected cohorts of chronic-phase chronic myelogenous leukemia patients. We examined 92 patients after 12 months of tyrosine kinase inhibitor (TKI) treatment in Nagasaki Prefecture, Japan. Univariate analysis revealed that significant factors associated with not attaining a major molecular response (MMR) were the presence of the minor BCR-ABL1 fusion gene, a low daily dose of TKI, and the emergence of BCR-ABL1 kinase domain mutations conferring resistance to imatinib. Factors associated with the loss of
sustained MMR were a low daily dose of TKI and the emergence of alternatively spliced BCR-ABL1 mRNA with a 35-nucleotide insertion. Taken together, our results suggest that the search for BCR-ABL1 mutations should be initiated if patients have not achieved MMR following 12 months of TKI treatment.

Introduction

The introduction of BCR-ABL1 tyrosine kinase inhibitors (TKIs) has revolutionized the management of patients with chronic myelogenous leukemia (CML) [1-5]. A complete cytogenetic response (CCyR) rate of 60 to 67% and major molecular response (MMR) rate of 22 to 39% at 12 months have been reported for imatinib treatment [1-6]. A 6-year update of the IRIS study was recently presented, which revealed an event-free survival of 86% and overall survival of 88% [7]. Our previous cohort study with various patients (i.e. a group of patients in a practical setting) revealed that imatinib treatment could achieve excellent outcomes for CML patients, at both clinical and molecular levels [6].

The quantification of residual BCR-ABL1 transcripts by quantitative reverse transcription-PCR (QRT-PCR) is a sensitive tool to monitor minimal residual disease. Molecular response is assessed according to the International Scale as the ratio of BCR-ABL1 transcripts to ABL1 transcripts, and is expressed and reported as BCR-ABL1% on a log scale. A BCR-ABL1 expression of <0.1% corresponds to major molecular response (MMR). The IRIS study showed that achieving MMR within 12 months of imatinib therapy was associated with the best progression-free survival. Palandri F, et al. previously
reported that the prognostic value of achieving MMR was greater if the response was stable [8].

An insufficient response to imatinib in CML has been attributed to several causes, of which point mutations in the BCR-ABL1 kinase domain (BCR-ABL1 KD mutations) appear to be the most common, occurring in 30% to 90% of patients who develop resistance to imatinib [9-15]. BCR-ABL1 KD mutations have previously been reported in 21 different amino acids, and were shown to confer differing levels of resistance to imatinib, which was confirmed by differences in the cellular 50% inhibitory concentration (IC50) of TKIs [16]. Several other mechanisms of resistance have been identified, including low plasma concentrations of TKIs, the overexpression of BCR-ABL1 transcripts, drug efflux/influx, and alternative signaling pathway activation [17-21].

Second generation TKIs (2nd-TKIs), such as nilotinib and dasatinib, became widely available in Japan in 2009 [3, 4, 22, 23]. Responses to 2nd-TKIs were found to be rapid and durable, with a higher percentage of patients with chronic-phase CML (CML-CP) surviving at 12 months. Second-TKIs are generally well tolerated, with occurrence of grade 3/4 drug-related adverse events being less and hematological adverse event profiles being more favorable than those of imatinib. Second-TKIs also exhibit increased inhibitory potency against BCR-ABL1 kinase and efficacy in the treatment of patients with many BCR-ABL1 KD mutations that develop from imatinib use [16, 24]; the T315I mutation confers resistance to both imatinib and 2nd-TKIs. Although excellent results have been reported with 2nd-TKIs, most of these were from prospective clinical trials, indicating that the data is from a selected
group of patients. Whether the administration of 2nd-TKIs has improved the outcome of insufficient
responders to imatinib in a practical setting remains unclear, and profiles related to insufficient responses
need to be reevaluated.

To address these issues, we prospectively measured residual BCR-ABL1 transcripts and analyzed the
BCR-ABL1 KD mutation status in 115 CML-CP patients who registered for the mutation analysis study
between March 2010 and March 2012. This study demonstrated the ‘smaller than expected’ impact of
BCR-ABL1 KD mutations, and also highlighted problems associated with a reduced dosage of 2nd-TKIs.

**Patients and Methods**

**Patients and Eligibility Criteria of the Mutation Analysis Study**

A total of 160 patients with CML-CP were confirmed, and 149 patients were either treated or had been
treated with TKI at 11 hospitals in Nagasaki Prefecture, Japan between March 2010 and March 2012.
CML-CP patients who had received TKI during this period and those who had a history of receiving TKI
were eligible for this mutational analysis study. Of the 149 CML-CP patients, informed consent was
obtained from 115 patients for the measurement of BCR-ABL1 fusion transcripts and analysis of
BCR-ABL1 KD mutations (molecular study) (Figure 1). The results of these analyses were notified to
each clinician and the selection of TKIs was left to their judgment. This study was approved by the
Ethical Committees of each participating hospital.
RNA Extraction, Complementary DNA Synthesis

Total leukocytes in the bone marrow and peripheral blood samples were isolated by centrifugation following red blood cell lysis, and total RNA was extracted using TRIzol reagent and the PureLink RNA Micro kit (Invitrogen, CA, USA). cDNA was synthesized using random hexamer primers and SuperScript III Reverse Transcriptase (Invitrogen).

Quantitative Reverse Transcription-Polymerase Chain Reaction Conditions

QRT-PCR for BCR-ABL1 transcripts levels was performed in 411 samples using LightCycler (Roche Diagnostics, Mannheim, Germany) and LightCycler TaqMan Master (Roche Diagnostics). Primers and TaqMan probe sequences published in the EAC network protocol were used for QRT-PCR [25]. When major BCR-ABL1 (ie. b2a2 and b3a2) was not detected, the presence of minor BCR-ABL1 (ie. e1a2) was examined. The amount of the fusion gene in the original sample was calculated by means of a standard curve (created with the BCR-ABL1 fusion gene or ABL1 gene cloned in plasmids) and expressed as the BCR-ABL1/ABL1 ratio.

Direct Sequencing of the ABL1 kinase domain

After quantifying BCR-ABL1 transcripts in 411 samples, a detectable amount of the BCR-ABL1
transcripts was amplified in 273 samples. A total of 264 of the 273 samples (96.7%) were used for the mutation analysis study. The nested PCR strategy was applied for direct sequencing, in which primers were used as previously described [26, 27]. After nested PCR, PCR products were sequenced in both directions with the following primers: ABL-1F, ABL-1R, ABL-2F, and ABL-2R as previously described [28], using the BigDye Terminator v3.1 Cycle Sequencing Kit and ABI Prism 3100xl Genetic Analyzer (Applied Biosystems, CA, USA).

**Categorization of BCR-ABL1 KD Mutations**

BCR-ABL1 KD mutations were categorized as “resistant to imatinib” and “unknown sensitivity” according to the IC$_{50}$ of TKIs summarized in the recommendations from European LeukemiaNet [16]. Twenty-one BCR-ABL1 KD mutations categorized as “resistant to imatinib” were as follows; M244V, L248V, G250E, Q252H, Y253H, Y253F, E255K, E255V, E279K, V299L, F311L, T315I, F317L, M351T, F359V, V379I, L384M, L387M, H396R, H396P, and F486S.

BCR-ABL1 35INS, a retention of 35 intronic nucleotides at the splice junction of exon 8/9, which results in a stop codon after 10 intron-encoded residues, was not included in the BCR-ABL1 KD mutations because BCR-ABL1 35INS was considered to be an alternative spliced variant, and not a point mutation [29-32].
**Definition of Clinical Parameters According to the Response**

The criteria recommended by European LeukemiaNet were used to define responses [33]. CCyR was defined as the absence of Philadelphia chromosome-positive metaphases in the samples. Cytogenetic responses were based on the percentage of Philadelphia chromosome-positive cells among 20 or more cells in metaphase in each bone marrow sample. Fluorescence in situ hybridization on interphase cells was recommended if less than 20 metaphases were evaluable and was performed with BCR-ABL1 extra-signal, dual-color, dual-fusion probes; CCyR was defined when the number of positive cell interphases was less than 2 in 200 (<1%). Major molecular response (MMR) was defined as a ratio BCR-ABL1/ABL1 level <0.042%, which corresponded to a 3-log reduction from the median baseline value calculated in our laboratory, using 30 samples from untreated CML-CP patients. Undetectable BCR-ABL1 transcripts levels were defined as a ratio BCR-ABL1/ABL1 level <0.0042%, which corresponded to the lowest level of detectability by the method (10⁻⁴).

The overall response to TKI was evaluated using criteria proposed by European LeukemiaNet [34]. The trajectory of the molecular response was categorized into four groups using the modified categorization originally described by Palandri F, et al. [8] as follows; QRT-PCR negative, BCR-ABL1 transcripts always undetectable; Stable MMR, BCR-ABL1 transcripts detectable, but always maintained MMR; Unstable MMR, achieved MMR at least once, but could not maintain it; Never MMR, never achieved MMR.
Statistics

Variables were analyzed by Fisher’s exact test to determine significantly associated factors for each group categorized by the trajectory of the molecular response. All tests were 2-sided and values of P<0.05 were considered significant in all analyses. All statistical analyses were performed with Prism Version 5.0 software (GraphPad).

Results

Patient characteristics

Twenty-eight patients were newly diagnosed with CML-CP during the study period (between March 2010 and March 2012). The other 87 patients included in this study had been diagnosed before this period. TKI was not the initial treatment for 19 patients. The characteristics of these patients are summarized in Table 1. Patients included 63 males and 52 females, with a median age at diagnosis of 55 years old (age range: 17–88). The median time from the start of TKI treatment to registration was 5.5 years (range 0.0-11.6). The distribution of Sokal scores at diagnosis was as follows; 47 patients were at low risk, 42 at intermediate risk, and 24 at high risk. Imatinib was administered as an initial treatment in 83 patients, nilotinib in 6, dasatinib in 7, and bosutinib in 1. Two out of 115 patients received TKI as a treatment for molecular relapse following allogeneic hematopoietic stem cell transplantation.
Direct sequencing of BCR-ABL1 KD at diagnosis and within 12 months of the TKI treatment

BCR-ABL1 KD mutations and 35INS were analyzed at diagnosis in 28 untreated CML-CP patients. Although no patient had BCR-ABL1 KD mutations at the time of diagnosis, mutations became detectable within 12 months of the TKI treatment in 3 out of 28 patients (10.7%). The mutations detected and patients were as follows; T315I, UPN-108; T406A, UPN-45; A433T, UPN-61 (Figure 2a).

In the case of UPN-108, the T315I mutation became detectable after 3 months of the dasatinib treatment, at the time the patient achieved MMR. MMR was lost three months later, and the mutation was still detectable. We previously reported the successful treatment of the T315I mutation by imatinib and interferon-α combination therapy [28]; therefore, we added interferon-α to dasatinib. After 5 months of the combination therapy, MMR was re-achieved and the T315I mutation became undetectable.

BCR-ABL1 35INS was detected in 18 out of 28 patients (64.2%) during the course of the treatment by direct sequencing. BCR-ABL1 35INS was detected both before and after the TKI treatment in 8 patients, only at diagnosis in 2 patients, and only after the TKI treatment in 8 patients.

Molecular response after receiving the TKI treatment for longer than 12 months

Ninety-one out of 115 patients were evaluable for their molecular response after receiving at least 12 months of the TKI treatment (Figure 1). These patients included not only 6 newly diagnosed patients who
had been treated with TKI for longer than 12 months, but also 85 patients who had been diagnosed prior to March 2010 and were already taking TKIs. Using the criteria proposed by European LeukemiaNet, 60 out of 91 patients were judged to have achieved the optimal response (i.e. stable optimal response), whereas the remaining 31 patients had not (i.e. unstable optimal response). Based on the trajectory of the molecular response, patients with a stable optimal response were further divided into two groups, stable MMR (29 patients) and QRT-PCR negative (31 patients). Among those with an unstable optimal response, 16 patients that had never achieved MMR were categorized into the “never MMR” group, and 15 patients that achieved MMR at least once were categorized into the “unstable MMR” group.

**BCR-ABL1 KD mutation in the “never MMR” group**

Nine patients in the “never MMR” group were treated with imatinib and 7 patients were treated with 2nd-TKIs. In this group, only 3 patients (33.3%) with imatinib and one patient (14.3%) with 2nd-TKI were taking daily doses of TKIs without any reduction (a standard dose indicated imatinib 400mg/day, nilotinib 600mg/day, and dasatinib 100mg/day). In terms of drug adherence, no significant difference was observed between the 3 groups taking different TKIs (33.3%, 25.0%, and 0.0% in patients treated with imatinib, nilotinib, and dasatinib, respectively). The most frequent reason for dose reduction was hematological toxicity in the 2nd-TKI group, and non-hematological toxicity in the imatinib group (Table 2). BCR-ABL1 KD mutations categorized as resistant to imatinib were detected in 3 out of 16 patients
The mutations detected and patients were as follows: E279K, UPN-24; T315I and E255V, UPN-40; G250E and L387M, UPN-48 (Figure 2b). The BCR-ABL1 KD mutation categorized as unknown sensitivity was not detected. In the case of UPN-24, TKI was switched from imatinib to nilotinib and a QRT-PCR negative status was obtained.

Direct sequencing detected BCR-ABL1 35INS in 12 out of 16 patients (75.0%) in the “never MMR” group. One patient (UPN-24) was found to have both the E279K mutation and BCR-ABL1 35INS. The minor BCR-ABL1 fusion gene was detected in 3 out of 16 patients instead of major BCR-ABL1 fusion gene. These 3 patients had BCR-ABL1 35INS in the transcriptional product of the minor BCR-ABL1 fusion gene, but had no BCR-ABL1 KD mutations.

To determine the effect of long-term exposure of TKI on the occurrence of mutations, we compared the mutation rate of newly diagnosed patients with that of patients taking TKIs for longer than 24 months. In the “never MMR” group, 12 out of 16 patients had been receiving TKI treatment for longer than 24 months. Of these 12 patients, the BCR-ABL1 KD mutation, minor BCR-ABL1 fusion gene, and BCR-ABL1 35INS were detected in 2 patients, 2 patients, and 8 patients, respectively. In 4 newly diagnosed patients, the E279K mutation became detectable in one patient after starting to take imatinib, whereas 35INS was not detected. Whether the length of the TKI treatment (12 to 24 months vs more than 24 months) influenced the frequency of the BCR-ABL1 KD mutation or BCR-ABL 35INS was not clear due to the small number of patients.
BCR-ABL1 KD mutation in the “unstable MMR” group

In the “unstable MMR” group, 12 patients were treated with imatinib and 2 patients were treated with dasatinib. One patient had already stopped taking imatinib before the mutation analysis. In this group, 5 patients (41.7%) with imatinib and one patient (50.0%) with dasatinib were taking daily doses of TKIs without any reduction. In terms of drug adherence, no significant difference was observed between groups taking imatinib and dasatinib. Similar to the “never MMR” group, the most frequent reason for the dose reduction was hematological toxicity in the 2nd-TKI group, and non-hematological toxicity in the imatinib group. BCR-ABL1 KD mutations known to confer resistance to imatinib were not detected in this group, whereas the KD mutation categorized as unknown sensitivity was detected in one patient (R457C mutation in UPN-30) (Figure 2c). BCR-ABL1 35INS was detected in 12 out of 15 patients (80.0%), including one patient (UPN-30) harboring both the R457C mutation and BCR-ABL1 35INS. No significant relationship was observed between the detection of BCR-ABL1 35INS and remission status. The timing of the detection and remission status was as follows; when MMR was achieved, 2 patients; only when MMR was lost, 3 patients; regardless of MMR status, 7 patients.

We compared the mutation rate of newly diagnosed patients with that of patients taking TKIs for longer than 24 months in the “unstable MMR” group. Twelve out of 15 patients had been receiving the TKI treatment for longer than 24 months. Of these 12 patients, BCR-ABL1 35INS was detected in 9 patients,
while no BCR-ABL1 KD mutation was detected. Although no 35INS was detected in 3 newly diagnosed patients, one patient developed the R457C mutation after start taking dasatinib. The attempts to show the impact of the length of the TKI treatment (12 to 24 months vs longer than 24 months) on the frequency of the BCR-ABL1 KD mutation or BCR-ABL 35INS failed because the number of patients was too small.

**BCR-ABL1 KD mutation in the “stable MMR” group**

In the group of patients with a stable MMR, 21 patients were treated with imatinib and 6 patients were treated with 2nd-TKIs. Two patients had already stopped receiving TKI treatment before the mutation analysis study. In this group, 14 patients (66.7%) with imatinib and 3 patients (50.0%) with 2nd-TKIs were taking daily doses of TKIs without any reduction. No significant difference was observed in terms of drug adherence among patients taking 3 different TKIs (66.7%, 50.0%, and 50.0% in patients treated with imatinib, nilotinib, and dasatinib, respectively). BCR-ABL1 KD mutations known to confer resistance to imatinib were not detected in this group, whereas the KD mutation categorized as unknown sensitivity was detected in one patient (Q252R mutation in UPN-76) (Figure 2d). BCR-ABL1 35INS was detected in 10 out of 29 patients (34.5%) with stable MMR. In the case of UPN-76, the patient had BCR-ABL1 35INS, but this disappeared when the Q252R mutation became detectable.

The mutation rate of newly diagnosed patients with that of patients taking TKIs for longer than 24 months was compared to seek for a possible difference, in the “stable MMR” group. Twenty-two out of
29 patients had been receiving TKI treatment for longer than 24 months. Of these 22 patients, BCR-ABL1 35INS was detected in 6 patients, while no BCR-ABL1 KD mutation was detected. In 7 newly diagnosed patients, although no 35INS was detected, one patient developed the Q252R mutation after starting to take dasatinib. The statistical analysis could not show significant effects of the TKI-treatment duration on the mutation rate (12 to 24 months vs longer than 24 months).

**BCR-ABL1 KD mutation in the “QRT-PCR negative” group**

All 31 patients in the QRT-PCR negative group were receiving the TKI treatment during the molecular study. In this group, 25 patients were treated with imatinib and 6 patients were treated with 2nd-TKIs. Sixteen patients (64.0%) with imatinib and 4 patients (66.7%) with 2nd-TKI were taking daily doses of TKIs without any reduction. No significant difference was observed in terms of drug adherence among patients taking 3 different TKIs (64.0%, 75.0%, and 50.0% in patients treated with imatinib, nilotinib, and dasatinib, respectively).

**Factors related to the trajectory of the molecular response after 12 months of the TKI treatment**

To assess significant factors related to the molecular responses, we tested for correlations between factors such as the baseline features of patients, TKI regimen, and results of the KD mutation analysis with the trajectory of the molecular response (the “never MMR”, “unstable MMR”, and “stable MMR”
groups) (Table 3). Three significant factors for not achieving MMR were identified by comparing the “never MMR” group with the other two groups (“unstable MMR” and “stable MMR” groups); the type of BCR-ABL1 fusion gene, low daily dose of TKI, and presence of a BCR-ABL1 KD mutation resistant to imatinib. Furthermore, 2 factors were identified as significant factors for not maintaining stable MMR. By comparing the “stable MMR” group with two other groups, low daily dose of TKI and the presence of 35INS were identified as factors associated with the loss of sustained MMR (Table 3). Patient characteristics, such as sex, median age at the beginning of the TKI treatment, or Sokal risk category were not significant factors for achieving or maintaining MMR. The type of TKI was also not a factor.

To compare the stable optimal response group (“stable MMR” and QRT-PCR negative groups) and unstable optimal response group (“never MMR” and “unstable MMR” groups), we analyzed the following 3 variables; patient characteristics, type of TKI, and daily dose of TKI. The presence of KD mutations, the 35INS, and the type of BCR-ABL1 fusion gene were not included as variables in the analysis because BCR-ABL1 transcripts were absent in the “QRT-PCR negative” group. Two factors negatively correlated with maintaining an optimal response; TKI treatment started at a higher median age (p=0.0480) and a lower daily dose of TKI (p=0.0348). The type of TKI, sex, and Sokal score category did not correlate with the response.

Discussion
We demonstrated that three factors were significantly related to molecular responses in CML-CP patients following TKI treatment in this study. First, adherence to TKI therapy was an important factor for both achieving and maintaining MMR. Second, the presence of BCR-ABL1 KD mutations, especially mutations that confer resistance to imatinib, was a significant predictor for not achieving MMR. Third, the emergence of 35INS, which was detected in 43% of patients, was significantly related to the loss of MMR.

We observed a relatively low optimal stable response rate on a molecular level in this registration study by the Nagasaki CML study group. Thirty-one out of 91 patients (34.1%) lost MMR after 12 months of the treatment. Considering the similar rate of insufficient molecular responses reported in other studies [5, 8], the management of CML-CP patients with an insufficient molecular response is a problem that remains even after the introduction of 2nd-TKIs into practice. These findings prompted us to analyze the clinical and molecular factors useful for predicting molecular responses to TKI. We confirmed the importance of adhering to TKI therapy for an optimal response, which we found to be the only significant factor for both achieving and maintaining MMR. In the case of UPN-48 harboring the G250E and L387M mutations in BCR-ABL1 KD, MMR was not achieved with dasatinib in spite of these mutations being sensitive to dasatinib. This treatment failure may have been due to the standard dose of dasatinib not being tolerated. Some studies, including ours, reported that the amount of TKI administered in practice was often less than the standard dose [17, 18, 35-37]. Therefore, tolerability for a standard dose must be
considered when selecting a TKI. A pharmacological assessment may help in this decision.

Information regarding the BCR-ABL1 KD mutation status is invaluable for the decision algorithm when tailoring therapeutic strategies. A switch to effective TKI resulted in MMR being achieved in two patients (UPN-23 and -108), with the elimination of the mutations. However, in the case receiving the TKI treatment for longer than 12 months, BCR-ABL1 KD mutations categorized as resistance to imatinib were detected in the “never MMR” group only. This finding indicated that sequence analysis of BCR-ABL1 KD should only be considered for this group of patients. In the case of UPN-108, the T315I mutation became detectable after the patient achieved MMR after 3 months of the dasatinib treatment. T315I mutation analysis at diagnosis is often considered important because the presence of T315I mutation at diagnosis could affect the whole treatment strategy. Minami Y, et al. reported that monitoring gene mutations in fractionated hematopoietic stem cells and progenitors at diagnosis may help detect the T315I mutation earlier [38]. Standard methods to detect the T315I mutation and the standard management of patients with this mutation in practice need to be established [28, 39, 40].

In our study, the proportion of patients with BCR-ABL1 KD mutations was only 7% (8 out of 115 patients), which appeared to be less than that of previous studies. Since previous studies have suggested that 2nd-TKIs inhibit the proliferation of CML cells with BCR-ABL1 KD mutations [41], it is possible that the introduction of 2nd-TKIs actually led, at least in part, to the lower frequency of BCR-ABL1 KD mutations in our study.
The presence of BCR-ABL1 35INS significantly correlated with an unstable optimal response in our study. BCR-ABL1 35INS itself was found to not contribute to TKI resistance because the BCR-ABL1 protein derived from the mRNA of BCR-ABL1 35INS lacked the kinase domain necessary for BCR-ABL1 kinase activity [32]. We detected BCR-ABL1 35INS in 34 out of 60 patients (56.7%) by direct sequencing. The emergence of BCR-ABL1 35INS, one of the alternative splicing variants, may reflect the confounding effect of other factors causing TKI resistance rather than directly providing TKI resistance because the precise regulation of RNA splicing is indispensable for maintaining cellular homeostasis [42]. Further assessments regarding BCR-ABL1 35INS in the treatment of CML are needed.

The Sokal scoring system has been utilized to stratify patients by risk in many clinical TKI trials. Although the score was shown to predict the possibility of CCyR and MMR [1, 2], it did not correlate with the trajectory of the molecular response in our study. Marin D, et al. previously reported that adherence to imatinib was the only independent predictor for complete molecular response, while the Sokal score was not [43]. Second-TKIs were also found to improve the outcomes of high-risk patients [3, 4]. Collectively, it is possible that the low adherence to TKIs and introduction of more potent 2nd-TKIs lowered the power of the Sokal score in the statistical analysis performed in our study. A larger analysis may help to confirm the impact of the Sokal score in a practical setting.

The results obtained in our study emphasized the necessity of further searches for clinical and molecular factors that predict clinical responses to TKIs. With more appropriate predicting factors, a new
treatment strategy may be developed in the future to maximize the number of patients achieving an
optimal response.

Authors’ contributions

H.I., T.H., and Y. Miyazaki conceived and designed the study; H.I., H. Tsushima, D.I., T.H., Y.D., S.M.,
K.Y., and Y. Miyazaki collected and analyzed the samples and data; H.I. and Y. Miyazaki performed the
statistical analysis; H.I., H. Tsushima, T.H., and Y. Miyazaki wrote the manuscript and created the figures
and tables; and all authors critically reviewed the manuscript and read and approved the final version of
the manuscript.

Appendix

Members of the Nagasaki CML Study Group are as follows: Y. Kawaguchi (Nagasaki Municipal
Medical Center); S. Kamihira (Nagasaki Municipal Hospital); M. Tomonaga, T. Joh, K. Horio (The
Japanese Red Cross Nagasaki Atomic Bomb Hospital); Y. Takasaki (St. Francis Hospital); Y. Moriuchi,
S. Sato, H. Itonaga (Sasebo Municipal General Hospital); S. Yoshida, E. Matsuo, Y. Moriwaki, J.
Makiyama (National Hospital Organization, Nagasaki Medical Center); D. Ogawa (Nagasaki Prefectural
Shimabara Hospital); H. Soda (Health Insurance, Isahaya General Hospital); R. Yamasaki, H. Nonaka (Japan Labour Health and Welfare Organization, Nagasaki Rosai Hospital); T. Fukushima (University of the Ryukyus); J. Nakashima (Gotoh Central Hospital); and other authors at Nagasaki University.

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Conflict of interest statement

The authors declare no financial or personal conflicts of interest.

References


types of mutations of the Abl gene can be found in chronic myeloid leukemia patients resistant to STI571, and they can pre-exist to the onset of treatment. Blood. 2002;100(3):1014-1018.


domain mutations to imatinib resistance in different subsets of Philadelphia-positive patients: by the

mutation analysis in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors:
recommendations from an expert panel on behalf of European LeukemiaNet. Blood. 2011;118(5):1208-1215.

are associated with both cytogenetic and molecular responses to standard-dose imatinib in chronic

pharmacokinetics and its correlation with response and safety in chronic-phase chronic myeloid


20. Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells:


27. Polakova KM, Lopotova T, Klamova H, Moravecova J. High-resolution melt curve analysis: initial


Figure 1  Patient flow diagram

CML-CP patients between March 2010 and March 2012 (n=160)

Patients included in the molecular analysis (n=115)

Newly diagnosed patients (n=28)  Patients treated with TKI before starting the molecular study (n=87)

Analysis for the trajectory of the molecular response after 12 months of the TKI treatment

Not eligible (n=22)  Not eligible (n=2)

Eligible (n=6)  Eligible (n=85)

12 to 24 months of the TKI treatment (n=6)  More than 24 months of the TKI treatment (n=0)  12 to 24 months of the TKI treatment (n=11)  More than 24 months of the TKI treatment (n=74)
Figure 2  Clinical course of BCR-ABL1 fusion transcripts and the kinase domain mutation

(a) Clinical course of BCR-ABL1 fusion transcripts and the kinase domain mutation in UPN-45, UPN-41, and UPN-168.

(b) Clinical course of BCR-ABL1 fusion transcripts and the kinase domain mutation in UPN-24, UPN-40, and UPN-48.

(c) Clinical course of BCR-ABL1 fusion transcripts and the kinase domain mutation in UPN-30.

(d) Clinical course of BCR-ABL1 fusion transcripts and the kinase domain mutation in UPN-76.

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The trajectory of the molecular response was evaluated in 6 out of 28 patients who were diagnosed with chronic phase chronic myelogenous leukemia (CML-CP) between March 2010 and March 2012, and in 85 out of 87 patients who started tyrosine kinase inhibitor (TKI) treatment before March 2010.

(a) Three patients harbored BCR-ABL1 KD mutations from diagnosis to 12 months of the TKI treatment. The clinical courses of patients harboring BCR-ABL1 KD mutations after over 12 months of the TKI treatment are follows; (b) three patients in the “never MMR” group, (c) one in the “unstable MMR” group, and (d) one in the “stable MMR” group.

Aberrations: IM, indicates imatinib; D, dasatinib; N, nilotinib. Numbers following these abbreviations indicate the dose of each TKI.
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<th>Registraiton of molecular analysis</th>
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<td>AP</td>
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<tr>
<td>BC</td>
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<tr>
<td>Time after the TKI treatment (median), y</td>
<td>0.1 - 11.6 (5.5)</td>
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<tr>
<td>Sokal score at diagnosis, n</td>
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</tr>
<tr>
<td>Low</td>
<td>47</td>
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<tr>
<td>Intermediate</td>
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</tr>
<tr>
<td>High</td>
<td>24</td>
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<tr>
<td>Uncertain or Missing</td>
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<td>Initial treatment</td>
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<tr>
<td>Imatinib</td>
<td>83</td>
</tr>
<tr>
<td>2nd-TKIs</td>
<td>14</td>
</tr>
<tr>
<td>Others</td>
<td>18</td>
</tr>
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<td>Allogeneic hematopoietic stem cell transplantation at any time</td>
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Table 2 Events related to reductions in the TKI dosage

<table>
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<th>Patients with</th>
<th>Patients with</th>
<th>Total, n</th>
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<tbody>
<tr>
<td></td>
<td>Never MMR</td>
<td>Unstable MMR</td>
<td>Stable MMR</td>
<td>QRT-PCR negative</td>
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<td>IM 2nd-TKI</td>
<td>IM 2nd-TKI</td>
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<td>2 1</td>
<td>2 1</td>
<td>3 0</td>
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<td>Non-hematological toxicity, n</td>
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<td>5 0</td>
<td>5 2</td>
<td>6 2</td>
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<td>Economic reasons, n</td>
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<td>0 0</td>
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<tr>
<td>Total, n</td>
<td>6 6</td>
<td>7 1</td>
<td>7 3</td>
<td>9 2</td>
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33
Table 3  Factors evaluated for their influence on the trajectory of the molecular response

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<tr>
<th></th>
<th>Patients with Never MMR</th>
<th>Patients with Unstable MMR</th>
<th>Patients with Stable MMR</th>
<th>Never MMR vs Unstable MMR/Stable MMR</th>
<th>Never MMR/Unstable MMR vs Stable MMR</th>
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<td>15</td>
<td>29</td>
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<tr>
<td>Sex, n</td>
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<td>Male</td>
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<td>7</td>
<td>17</td>
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<td>&lt; 55 years</td>
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<td>≥ 55 years</td>
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<td>Sokal score*, n</td>
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<td>&gt;0.999</td>
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<tr>
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</table>
Table 1

CP, indicates Chronic phase; AP, accelerated phase; BC, blastic crisis; TKI, tyrosine kinase inhibitors (i.e. imatinib, nilotinib, dasatinib, and bosutinib). Second-TKIs include nilotinib, dasatinib, and bosutinib.

Table 2

Non-hematological toxicity was mainly observed in patients treated with imatinib. Of 25 patients with non-hematological toxicity, muscle cramps (n=7, 28.0%), water retention (n=6, 24.0%), and elevations in liver enzymes (n=5, 20.0%) were frequently reported. Hematological toxicity was mainly observed in patients receiving 2nd-TKIs without a stable optimal response. Thrombocytopenia was the most frequent adverse event as hematological toxicity (9 out of 14 patients, 64.3%).

Table 3

* One patient with Never MMR and one patient with Stable MMR were excluded because data for the Sokal score at diagnosis was missing.

†‡ One patient with Unstable MMR and two patients with Stable MMR were excluded because they stopped the TKI treatment before the molecular study.